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Imaging of cerebral $\alpha_4\beta_2^*$ nicotinic acetylcholine receptors with (–)-[^{18}F]Flubatine PET: Implementation of bolus plus constant infusion and sensitivity to acetylcholine in human brain*

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Abstract

The positron emission tomography (PET) radioligand (–)-[^{18}F]flubatine is specific to $\alpha_4\beta_2^*$ nicotinic acetylcholine receptors (nAChRs) and has promise for future investigation of the acetylcholine system in neuropathologies such as Alzheimer's disease, schizophrenia, and substance use disorders. The two goals of this work were to develop a simplified method for $\alpha_4\beta_2^*$ nAChR quantification with bolus plus constant infusion (B/I) (–)-[^{18}F]flubatine administration, and to assess the radioligand's sensitivity to acetylcholine fluctuations in humans. Healthy human subjects were imaged following either bolus injection ($n = 8$) or B/I ($n = 4$) administration of (–)-[^{18}F]flubatine. The metabolite-corrected input function in arterial blood was measured. Free-fraction corrected distribution volumes (V_T/f_p) were estimated with modeling and graphical analysis techniques. Next, sensitivity to acetylcholine was assessed in two ways: 1. A bolus injection paradigm with two scans ($n = 6$), baseline (scan 1) and physostigmine challenge (scan 2; 1.5 mg over 60 min beginning 5 min prior to radiotracer injection); 2. A single scan B/I paradigm ($n = 7$) lasting up to 240 min with 1.5 mg physostigmine administered over 60 min beginning at 125 min of radiotracer infusion. Changes in V_T/f_p were measured. Baseline V_T/f_p values were $33.8 \pm 3.3 \text{ mL/cm}^3$ in thalamus, $12.9 \pm 1.6 \text{ mL/cm}^3$ in cerebellum, and ranged from 9.8 to 12.5

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Appendix A. Supplementary data

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mL/cm³ in other gray matter regions. The B/I paradigm with equilibrium analysis at 120 min yielded comparable V_T/f_p values with compartment modeling analysis of bolus data in extrathalamic gray matter regions (regional means <4% different). Changes in V_T/f_p following physostigmine administration were small and most pronounced in cortical regions, ranging from 0.8 to 4.6% in the two-scan paradigm and 2.8 to 6.5% with the B/I paradigm. These results demonstrate the use of B/I administration for accurate quantification of (–)-[¹⁸F]flubatine V_T/f_p in 120 min, and suggest possible sensitivity of (–)-[¹⁸F]flubatine binding to physostigmine-induced changes in acetylcholine levels.

Introduction

The acetylcholine system plays a critical role in modulating signaling processes of other neurotransmitter systems in the brain (Picciotto et al., 2012) and influences neurocognitive processes such as learning, memory, and motor function (Hurst et al., 2013). In addition, the acetylcholine system is implicated in neuropsychiatric disorders including schizophrenia, depression, substance abuse disorders, and Alzheimer's disease (Dani and Bertrand, 2007). PET imaging of the most abundant nicotinic acetylcholine receptor (nAChR) subtype in the brain, $\alpha_4\beta_2^*$ nAChRs, is a vital tool to interrogate the acetylcholine system *in vivo*. Studies using $\alpha_4\beta_2^*$ nAChR specific radioligands from the A-85380 family, primarily 2-[¹⁸F]FA-85380 PET (Gallezot et al., 2005) and 5-[¹²³I]IA-85380 SPECT (Vaupel et al., 1998), have led to important clinical findings in mood disorders (Saricicek et al., 2012; Hannestad et al., 2013), substance use disorders (Cosgrove et al., 2009; Esterlis et al., 2010; Brody et al., 2006; Brody et al., 2014), Alzheimer's disease (Kendziorra et al., 2011; Ellis et al., 2008; Mitsis et al., 2009), Parkinson's disease (Kas et al., 2009; Fujita et al., 2006), and schizophrenia (D'Souza et al., 2012; Esterlis et al., 2014).

Widespread extension of these imaging techniques to further investigate $\alpha_4\beta_2^*$ nAChR specific pathology has been limited by two technical factors. First, the kinetic properties of 2-[¹⁸F]FA-85380 and 5-[¹²³I]IA-85380 are slow and require lengthy imaging protocols of at least 5 h, presenting major logistical barriers to frequent imaging sessions. This has prompted the development of second-generation $\alpha_4\beta_2^*$ nAChR specific radioligands, including [¹⁸F]nifene (Hillmer et al., 2012), [¹⁸F]AZAN (Wong et al., 2013), and (–)-[¹⁸F]flubatine (Sabri et al., 2015), with faster kinetic properties and shorter scanning protocols. A second limitation is that conventional PET outcome measures of distribution volumes or binding potentials have restricted interpretation to ligand-receptor binding properties at equilibrium. The *in vivo* interrogation of changes in neurotransmitter levels, using molecular imaging to indirectly observe competition between radiotracer and neurotransmitter at the binding site, can provide alternative PET measurements of neurotransmitter function. Such experiments are structured around the theoretical framework known as the occupancy model (Laruelle, 2000; Finnema et al., 2015), which has been implemented for human use with the dopamine system (Martinez et al., 2003; Shotbolt et al., 2012) and extended to examine the acetylcholine system in humans with 5-[¹²³I]IA-85380 SPECT (Esterlis et al., 2013). However, the use of recently developed $\alpha_4\beta_2^*$ nAChR PET radioligands to measure acetylcholine fluctuations has been limited to preclinical

experiments (Gallezot et al., 2014; Hillmer et al., 2013), and has yet to be extended to human studies.

Previously, our group demonstrated that the second generation $\alpha_4\beta_2^*$ nAChR radioligand (–)-[¹⁸F]flubatine (full name, (–)-[¹⁸F]norchloro-fluoro-homoepibatidine; also known as (–)-[¹⁸F]NCFHEB (Brust et al., 2008)) exhibited dose-dependent sensitivity to changes in acetylcholine levels induced by physostigmine (Gallezot et al., 2014) in nonhuman primates. Physostigmine is an acetylcholinesterase inhibitor that inhibits hydrolysis of extracellular acetylcholine, thereby increasing acetylcholine levels. The central aim of this work was to develop and validate a practical imaging paradigm to examine physostigmine-induced acetylcholine fluctuations in humans with (–)-[¹⁸F]flubatine. This aim was accomplished in two parts. The first goal was to characterize and validate (–)-[¹⁸F]flubatine quantification of $\alpha_4\beta_2^*$ nAChRs with bolus plus constant infusion (B/I) radiotracer administration (Carson et al., 1993), an experimental approach previously implemented for 2-[¹⁸F]FA-85380 PET (Kimes et al., 2008) and 5-[¹²³I]IA-85380 SPECT (Staley et al., 2005). The B/I paradigm establishes true equilibrium between radioligand in the plasma and at the receptor site, enabling simple quantification of radioligand uptake in tissue while permitting assay of subsequent neurotransmitter perturbation within a single scan. The second goal of this work was to assess the sensitivity of (–)-[¹⁸F]flubatine to acetylcholine increases induced by physostigmine administration. This was done both with a two-scan bolus paradigm and with a single B/I PET scan to compare the sensitivities of each method to physostigmine-induced extracellular acetylcholine changes.

Materials and methods

Human subjects

Sixteen healthy volunteers (9 M, 7 F; 21–49 years of age; 83.7 ± 26.1 kg) were recruited from the local community. Written informed consent was obtained from all subjects. Upon intake, subjects were screened for the following criteria: No current or history of major medical illness; no psychotropic drug use in the previous six months; no DSM IV Axis I diagnosis for neurological or psychiatric disorders; not pregnant or breastfeeding. Negative pregnancy tests were confirmed on the day of the PET scan. A subset of subjects who smoked tobacco was intentionally included to validate the imaging paradigms for both smokers and healthy non-smokers. Four subjects reported smoking for at least 12 years and currently consumed 0.75–1.5 packs of cigarettes/day, with Fagerström test for Nicotine Dependence (FTND (Heatherton et al., 1991)) scores ranging from 3 to 8. These subjects refrained from smoking for 7–9 days prior to scanning consistent with our previous studies (Cosgrove et al., 2009), confirmed by CO levels of 2.75 ± 2.76 ppm and urine cotinine levels <100 ng/mL on scan days. Study approval and oversight were administered by the Human Investigation Committee, Yale University School of Medicine, and Yale-New Haven Hospital Radiation Safety Committee, in accordance with the Protection of Human Subjects of Research and Ethical Principle and Guidelines.

Study overview

This study consisted of four distinct sets of scans. First, to assess the kinetic properties of (–)-[¹⁸F]flubatine, eight PET scans (6 M (2 smokers); 2 F) with (–)-[¹⁸F]flubatine administered *via* bolus injection were acquired for 150 min and analyzed with standard modeling methods. Second, to assess the suitability of bolus plus constant infusion (B/I) experimental design and quantification, four (–)-[¹⁸F]flubatine PET scans (3 M; 1 F) with B/I radiotracer administration were acquired for up to 240 min and analyzed with both kinetic analysis and equilibrium methods. Third, to assess (–)-[¹⁸F]flubatine sensitivity to acetylcholine levels, six subjects (5 M (2 smokers); 1 F) received two bolus injection (–)-[¹⁸F]flubatine PET scans on separate days. The first scan was made at baseline, while the second scan administered 1.5 mg physostigmine over 60 min beginning 5 min prior to radiotracer injection. Fourth, the sensitivity of (–)-[¹⁸F]flubatine binding to acetylcholine levels with a single B/I scan was assessed. This was done in seven subjects (4 M (2 smokers); 3 F), administering (–)-[¹⁸F]flubatine as a B/I over 210–240 min, with 1.5 mg physostigmine administered over 60 min beginning at 125 min of radiotracer infusion.

Radiochemical production of (–)-[¹⁸F]Flubatine

Radiochemical production of (–)-[¹⁸F]flubatine was conducted following previously published methods (Bois et al., 2015). In brief, cyclotron-produced [¹⁸F]fluoride underwent nucleophilic substitution with the butoxycarbonyl-protected trimethylammonium precursor followed by deprotection in acid. The solution was then purified with high-pressure liquid chromatography methods prior to final formulation and quality control measurements. The total synthesis time was 114 ± 18 min ($n = 25$), yielding chemical purities >98% and radiochemical purities >99% for the radiotracer. Specific activities at end of synthesis were 337 ± 522 GBq/ μ mol (range: 51–2347 GBq/ μ mol).

PET data acquisition

PET data were acquired with a high-resolution research tomograph (HRRT; Siemens/CTI, Knoxville, TN). This scanner has a spatial resolution of 2–3 mm (full-width at half-maximum) at the center of the field of view. For blood sampling, a catheter was placed in the radial artery at the wrist. For (–)-[¹⁸F]flubatine administration, a second catheter was placed in a vein in the antecubital area. Before scanning, an optical motion-tracking tool (Vicra, NDI Systems, Waterloo, Canada) was placed on the subject's head.

Prior to all PET scanning procedures, a transmission scan for attenuation correction was obtained over 6 min with a rotating ¹³⁷Cs point source. Acquisition of list-mode PET data began simultaneously with initial administration of (–)-[¹⁸F]flubatine. For bolus studies, the entire 181 ± 77 MBq (–)-[¹⁸F]flubatine dose was administered as a slow bolus over 1 min, and PET data were acquired for a total of 150 min. For B/I studies, a fraction of the total administered 244 ± 58 MBq (–)-[¹⁸F]flubatine dose corresponding to the K_{bol} was given as a slow bolus over the first minute of scanning, with the rest of the dose infused at a constant rate for the remainder of the study. Infusion studies were conducted for 210–240 min total, broken into two sessions from 0 to 60 min and from 90 min to the end of the study; subjects left the scanner bed during the 30 min break between sessions. A ¹³⁷Cs transmission scan

was then repeated after all emission data was acquired for attenuation and to aid in frame-by-frame motion correction.

The design of infusion studies, specified by the magnitude of the injected bolus in units of infusion duration (K_{bol} ; Carson et al., 1993), was determined based on the results of the bolus studies. Because (–)-[^{18}F]flubatine exhibits a wide range of kinetic properties across different brain regions (Sabri et al., 2015), the infusion schedule was only optimized for regions with similar brain kinetics. We elected to determine the optimal K_{bol} for cortical gray matter, as these regions are vital for interrogating acetylcholine's influence over memory and cognition. The resulting K_{bol} value used for these infusion studies was 360 min. For a 240 min infusion protocol, this corresponds to 60% of the total administered mass injected as a bolus ($360/(360 + 240)$) and 40% administered as a constant infusion.

Input function and free fraction measurements

The (–)-[^{18}F]flubatine input function was measured in the arterial plasma. Continuous arterial sampling was acquired over the first 7 min of scanning at a rate of 4 mL/min. Discrete arterial samples were acquired at 3, 8, 15, 30, 45, and 60 min after the start of (–)-[^{18}F]flubatine administration, followed by additional samples every 30 min until the end of scanning.

For each sample, the radioactivity concentration was measured in both whole blood and plasma with a cross-calibrated gamma counter (Wizard 1480/2480, Perkin Elmer, Waltham, MA). To measure the parent fraction in discrete blood samples, column-switching HPLC analysis was used (Hilton et al., 2000). Plasma samples were treated with urea to a final concentration of 8 M, loaded onto a hand-packed sorbent capture column (Strata-X, Phenomenex, Torrance, CA), and eluted with a mobile phase of 1% acetonitrile and 99% water (v/v) at 2 mL/min over 4 min. Activity on the capture column was then backflushed onto a Phenomenex Gemini-NX C18 analytic column ($4.6 \times 250 \text{ mm}^2$, 5 μm), eluted with a mobile phase of 20% acetonitrile and 80% 20 mM ammonium bicarbonate (v/v) at a flow rate of 1.75 mL/min as previously described (Gallezot et al., 2014). Under these conditions, parent (–)-[^{18}F]flubatine eluted at ~11 min. The eluent was collected at 2 min intervals with a fraction collector (CF-1 Fraction Collector, Spectrum Chromatography, Houston, TX) and the fractions were counted with an automatic gamma counter. The parent fraction was calculated as the ratio of radioactivity eluted at (–)-flubatine's retention time to the total eluted radioactivity. The course of parent (–)-[^{18}F]flubatine during each scan was uniquely parameterized with an inverted gamma function. The final parent input function was calculated as the product of measured radioactivity concentration in plasma and the parameterized parent (–)-[^{18}F]flubatine function.

The plasma free fraction (f_p) was measured with ultrafiltration techniques. Arterial blood samples were acquired immediately prior to radiotracer injection and spiked with (–)-[^{18}F]flubatine. Plasma samples were extracted in triplicate, with 300 μL aliquots loaded onto ultrafiltration cartridges (Millipore Centrifree, Billerica, MA, USA) and centrifuged at 1228g for 20 min. The f_p was measured as the ratio of radioactivity in the ultrafiltrate to the total radioactivity in the plasma sample.

Magnetic resonance data acquisition

Magnetic resonance (MR) images of the brain were acquired for anatomical localization of (–)-[¹⁸F]flubatine uptake. MR data were acquired with a 3 T Trio Scanner (Siemens Medical Systems, Erlangen, Germany). The weighted gradient-echo (MPRAGE) sequence included TE = 3.3 ms; TI = 1100 ms; TF = 2500 ms; FA = 7°, resulting in 1 mm³ isotropic resolution.

Image data processing

List-mode PET data were divided into time frames of 6 × 0.5 min, 3 × 1 min, 2 × 2 min, followed by 5 min frames for the duration of the scan, and reconstructed with the ordered-subsets expectation maximization-based MOLAR algorithm (Carson et al., 2003), which includes corrections for attenuation, normalization, randoms, scatter, dead time, and subject motion. PET data were then transformed into native MR space by registering the summation of PET data over the first 10 min of acquisition to each subject's individual T1-weighted MR image with a mutual information algorithm constrained to six parameters (FLIRT, FSL 3.2; Analysis Group; FMRIB, Oxford, UK). The native T1-weighted MR image was next co-registered to the AAL (Anatomical Automatic Labeling for SPM2) MR template using a nonlinear transformation algorithm from BioImage Suite software (version 2.5; <http://www.bioimagesuite.com>). Regions of interest (ROIs) were delineated by transforming template coordinates of each region back to native PET space. Identified ROIs included the gray matter regions of frontal cortex, occipital cortex, parietal cortex, temporal cortex, cingulate cortex, caudate, putamen, hippocampus, thalamus, cerebellum, and the white matter regions of centrum semiovale and corpus callosum.

(–)-[¹⁸F]Flubatine kinetic analysis

The primary outcome measure in this study was the free fraction corrected total distribution volume (V_T/f_p), which is the ratio between total (–)-[¹⁸F]flubatine radioactivity in tissue and free parent (–)-[¹⁸F]flubatine present in arterial plasma at equilibrium (Innis et al., 2007). Regional (–)-[¹⁸F]flubatine V_T/f_p was calculated with four different analysis methods: the one-tissue (1TCM) and two-tissue (2TCM) compartment models (see Gunn et al., 2001), the multilinear analysis method (MA1; Ichise et al., 2002), and equilibrium analysis with arterial blood data (EQ) (Carson et al., 1993; Lassen, 1992).

For baseline (no physostigmine challenge) experiments, the first set of analyses evaluated PET data from 8 bolus scans with non-equilibrium analysis methods (1TCM, 2TCM, MA1) to calculate (–)-[¹⁸F]flubatine V_T/f_p . The second analysis examined PET data from 4 B/I scans, including two smokers, lasting 210–240 min to assess the suitability of this protocol for accurate (–)-[¹⁸F]flubatine V_T/f_p quantification. The rates of change for (–)-[¹⁸F]flubatine in tissue and arterial plasma were calculated as % change/hour to assess the quality of radiotracer equilibrium. Next, all analysis methods (1TCM, 2TCM, MA1, EQ) were evaluated using scan durations of both abridged to 150 min (matching the duration of bolus studies) and the full 210–240 min to assess stability of V_T/f_p estimation in shorter infusion protocols. For EQ analysis, V_T/f_p was estimated at the time of each plasma sample as the ratio of PET concentration (C_{PET}) divided by the metabolite-corrected input function concentration (C_p) corrected by the free fraction ($V_T/f_p = (C_{PET}/C_p)/f_p$). C_{PET} averaged PET data over 2 frames both preceding and following the blood sample (20 min total). The

quality of equilibrium at each timepoint was assessed by evaluating %change/hour. V_T/f_p estimates using EQ were compared with those of MA1 to examine the suitability of equilibrium analysis methods for (–)-[^{18}F]flubatine quantification.

(–)-[^{18}F]Flubatine physostigmine challenge

A major goal of this work was to assess the sensitivity of (–)-[^{18}F]flubatine to increases in acetylcholine levels induced by physostigmine. (–)-[^{18}F]Flubatine PET data following physostigmine administration were acquired and evaluated with two schemes, similar to previous work (Marenco et al., 2004).

The first experimental design consisted of a two-scan paradigm, conducted with 6 subjects. A baseline PET scan, with bolus injection of (–)-[^{18}F]flubatine, was first acquired for 150 min. Within the following month (next day for $n = 4$ of 6 subjects), a second (–)-[^{18}F]flubatine PET scan of 150 min was acquired, with 1.5 mg physostigmine administered IV over 60 min beginning 5 min prior to bolus injection of radiotracer. Glycopyrrolate, a muscarinic acetylcholine receptor antagonist that does not cross the blood brain barrier, was given to minimize potential nausea symptoms from physostigmine. Glycopyrrolate was administered as 200 μg over 1 min (IV) 10 min prior to radiotracer administration. Next, 5 min before radiotracer administration, 0.48 mg physostigmine was given as a slow bolus over 10 min, followed by 1.02 mg given as a constant infusion over the next 50 min, as conducted previously (Esterlis et al., 2013). V_T/f_p values in gray matter regions were calculated for each scan with MA1 as described above. Finally the change in V_T/f_p was calculated as $V_T/f_p = [V_T/f_p(\text{Baseline}) - V_T/f_p(\text{Physo})] / V_T/f_p(\text{Baseline})$.

The second experimental design consisted of a single day bolus plus constant infusion (B/I) paradigm, conducted with seven subjects, two of whom were smokers. (–)-[^{18}F]Flubatine was given with a K_{bol} of 360 min as described above. PET data acquisition was conducted for 240 min total, broken into two sessions from 0 to 60 min and from 90 to 240 min. At 120 min, 200 μg glycopyrrolate was given IV over 1 min. At 125 min, physostigmine was administered IV, with 0.48 mg given as a slow bolus over 10 min, followed by 1.02 mg given as an infusion over the next 50 min, terminating physostigmine administration at 185 min. V_T/f_p values in gray matter regions were calculated with EQ, using the 120 min timepoint as baseline V_T/f_p . Post-drug V_T/f_p was estimated at the time of each plasma sample. The change in V_T/f_p was calculated as $V_T/f_p = [V_T/f_p(\text{Baseline}) - V_T/f_p(\text{Physo})] / V_T/f_p(\text{Baseline})$. Cohen's d effect sizes (Cohen, 1988) of V_T/f_p were calculated for both methods in all regions.

Results

(–)-[^{18}F]Flubatine in the blood

HPLC analysis of arterial plasma samples revealed high and stable (–)-[^{18}F]flubatine parent fractions of $88.7 \pm 4.1\%$ at 90 min post-injection and $82.9 \pm 6.4\%$ at 210 min post-injection, shown in Fig. 1. There were no observed differences in parent fraction between bolus and B/I experimental designs. The measured (–)-[^{18}F]flubatine free fraction (f_p) was $86.4 \pm 1.8\%$.

Bolus studies

Compartment modeling analysis of (–)-[¹⁸F]flubatine data yielded visually good fit quality with 2TCM, shown in Fig. 2A, with the exception of one scan with noisy PET data due to low administered (–)-[¹⁸F]flubatine dose (59.6 MBq). Analysis of bolus (–)-[¹⁸F]flubatine injection data with 2TCM yielded high V_T/f_p values in thalamus (23.1 ± 3.6 mL/cm³). All other gray matter regions exhibited relatively homogenous V_T/f_p values ranging from 9.7 to 12.4 mL/cm³, shown in Table 1. The 1TCM (shown in Fig. 2B) underestimated 2TCM V_T/f_p by $5.0 \pm 3.2\%$ on average in gray matter (Supplementary Fig. 1A). The corrected Akaike Information Criterion (cAIC; Hurvich and Tsai, 1989) favored the 2TCM in all gray matter regions except the thalamus. Analysis of the bolus data with MA1 ($t^* = 90$ min) showed excellent agreement with the 2TCM, underestimating V_T/f_p by $0.8 \pm 4.6\%$ on average in gray matter regions (Supplementary Fig. 1B).

The time course of (–)-[¹⁸F]flubatine concentration in the white matter regions of centrum semiovale and corpus callosum exhibited different kinetic properties from extrathalamic gray matter regions, with much more prolonged increases in radioligand uptake followed by slower washout (see Fig. 2). In white matter, the cAIC again favored the 2TCM, with 1TCM slightly underestimating V_T/f_p values (see Table 1). The measured V_T/f_p values of 12.4 ± 1.2 mL/cm³ in centrum semiovale were higher than those of many gray matter regions, while the corpus callosum had the lowest observed V_T/f_p values of 6.8 ± 1.5 mL/cm³.

Infusion studies

Simulations of B/I data based on (–)-[¹⁸F]flubatine's kinetic properties in extrathalamic gray matter regions from bolus studies yielded an optimal K_{bol} of 360 min. Sample PET data acquired with this design are shown in Fig. 3. This protocol established stable (–)-[¹⁸F]flubatine concentrations in extrathalamic gray matter regions after 120 min, with an average change in tissue concentrations of $+4.7 \pm 3.1\%$ per hour. (–)-[¹⁸F]flubatine concentrations in arterial plasma were similarly constant, with a change of $-0.5 \pm 6.0\%$ per hour. This good equilibrium yielded stable estimations of extrathalamic gray matter V_T/f_p from equilibrium analysis methods at 120 min, changing at $+5.6 \pm 5.8\%$ per hour (range of $+4.4\%$ per hour in occipital cortex to $+9.9\%$ per hour in hippocampus). The rate of change for V_T/f_p remained small at 210 min and 240 min after radioligand infusion, at $+3.2 \pm 6.4\%$ per hour ($n = 4$) and $+1.8 \pm 4.4\%$ per hour ($n = 3$), respectively. Importantly, smokers did not noticeably differ from nonsmokers in the rate of change in (–)-[¹⁸F]flubatine concentrations, both in arterial plasma and tissue.

Since 150 min of data were collected in bolus studies, B/I data were truncated to 150 min and analyzed with non-equilibrium methods to directly compare the experimental designs. Values of V_T/f_p calculated from the B/I data with MA1 agreed within $3.1 \pm 3.0\%$ of 2TCM values on average. In extrathalamic gray matter regions, V_T/f_p values from B/I data yielded slightly higher but comparable V_T/f_p values to those of bolus experiments, as indicated in Table 2. There were no significant differences in injected specific activity or administered cold mass between bolus (SA: 140 ± 91 GBq/μmol; mass: 0.35 ± 0.11 μg/kg) and B/I studies (SA: 645 ± 702 GBq/μmol; mass: 0.22 ± 0.16 μg/kg). The rate of (–)-[¹⁸F]flubatine transfer from arterial plasma to tissue, K_1 , was similar between bolus and B/I schemes based on

2TCM analyses (Table 2). The use of EQ V_T/f_p estimation methods provided good agreement with the results of MA1 analyses. At 120 min, EQ yielded an average difference of $3.7 \pm 2.8\%$ compared to MA1 (Fig. 4A), while EQ at 210 min improved to an average difference of $2.7 \pm 2.0\%$ (Fig. 4B).

The B/I scheme was not optimized for the thalamus or white matter regions. In the thalamus, the rate of V_T/f_p change was $12.0 \pm 6.2\%$ per hour at 210 min post-infusion, resulting in a bias of $19.8 \pm 8.8\%$ with EQ compared to MA1. Similarly, equilibrium was not established in the centrum semiovale after 210 min infusion (V_T/f_p change of $13.8 \pm 1.9\%$ per hour), biasing V_T/f_p quantification by $10.3 \pm 2.3\%$ with EQ compared to MA1. In contrast, in the corpus callosum the change in V_T/f_p was $5.2 \pm 4.8\%$, similar to rates observed in gray matter regions, providing good agreement in V_T/f_p estimates between EQ and MA1.

(-)-[¹⁸F]Flubatine physostigmine challenge

For the two-scan bolus injection experimental scheme, there were no significant differences in injected specific activity or administered cold mass between baseline (SA: 122 ± 22 GBq/ μ mol; mass: 0.35 ± 0.12 μ g/kg) and physostigmine challenge studies (SA: 105 ± 100 GBq/ μ mol; mass: 0.34 ± 0.12 μ g/kg). One subject reported moderate nausea symptoms following physostigmine administration. On average, physostigmine administration yielded small

V_T/f_p with the two bolus scan experimental scheme. These effect sizes were medium in cortical regions, ranging from $d = 0.50$ to 0.88 , corresponding to V_T/f_p ranging from $4.6 \pm 5.2\%$ to $2.3 \pm 4.6\%$. Small effect sizes were observed outside cortex. These data are summarized in Table 3, with individual subject responses illustrated in Supplementary Fig. 2A. Physostigmine administration did not change f_p , with an average f_p of $0.7 \pm 1.4\%$ (baseline $f_p = 86.1 \pm 1.4\%$; post physostigmine $f_p = 86.7 \pm 0.7\%$).

(-)-[¹⁸F]Flubatine V_T/f_p decreased following physostigmine administration in the B/I experimental scheme, shown in Fig. 5A. A maximal decrease was observed in all regions at 210 min following initial radio-tracer administration (85 min after the beginning of physostigmine injection). This effect was most pronounced in cortical regions. The regional pattern of V_T/f_p magnitudes was comparable across both methods; greatest in cortical regions, while smaller magnitudes were observed in cerebellum, basal ganglia, and hippocampus. With the B/I approach, the frontal cortex exhibited the greatest (-)-[¹⁸F]flubatine V_T/f_p of $6.5 \pm 4.9\%$, corresponding to an effect size of 1.5 , while the smallest change of $0.0 \pm 5.3\%$ was observed in hippocampus, as summarized in Table 3, with individual subject results illustrated in Supplementary Fig. 2B. For comparison, in baseline B/I studies the average V_T/f_p across all regions at 210 min was $1.5 \pm 1.4\%$. Large effect sizes ($d > 1.0$) were observed in cerebellum, cingulate cortex, frontal cortex, occipital cortex, parietal cortex, and temporal cortex. A visual comparison of (-)-[¹⁸F]flubatine V_T/f_p images pre- and post-physostigmine administration is shown in Fig. 5B.

Discussion

This work develops a simple, logistically feasible PET imaging method to examine changes in acetylcholine levels with the $\alpha_4\beta_2^*$ nAChR specific radioligand (-)-[¹⁸F]flubatine. To accomplish this goal, we first validated the use of bolus plus constant infusion (-)-

[^{18}F]flubatine administration in a single scan. The data here indicate that quantification of (–)-[^{18}F]flubatine V_T/f_p with equilibrium analysis after 120 min of radioligand infusion agrees well with compartment modeling of bolus data in all extrathalamic gray matter regions. Subsequent scans administering 1.5 mg physostigmine after 125 min (–)-[^{18}F]flubatine infusion revealed decreases in V_T/f_p ranging from 0.0 to 6.5% with regional effect sizes as high as 1.5. Thus, these data preliminarily indicate that (–)-[^{18}F]flubatine is sensitive to acetylcholine fluctuations induced by physostigmine.

(–)-[^{18}F]Flubatine is a second-generation $\alpha_4\beta_2^*$ nAChR specific PET radioligand with suitable kinetic properties for accurate quantification of these receptors. First-in-human characterizations of (–)-[^{18}F]flubatine in both arterial plasma (Patt et al., 2014) and tissue with thorough kinetic analysis with compartment modeling (Sabri et al., 2015) were recently reported. Our present analysis of this radiotracer confirms many of these findings, including similar behavior in arterial blood, comparable V_T and f_p values, and a similar V_T rank order across gray matter regions. In addition, of the baseline bolus injection studies the two smokers imaged exhibited the highest rank order V_T/f_p values in most regions, consistent with previous reports of elevated $\alpha_4\beta_2^*$ nAChR binding in abstinent smokers (Cosgrove et al., 2009). The authors of the initial (–)-[^{18}F]flubatine characterization recommended the use of V_T without correcting the arterial input function for metabolites since these values were high and stable in healthy controls. However, it must be stressed that a lack of differences in parent fraction or f_p should be established in different subject populations or specific experimental settings to use this outcome measure with validity. For example, differences in 5-[^{123}I]IA-85380 parent fraction and f_p were reported between nonsmokers and abstinent smokers (Cosgrove et al., 2009), and between men and women (Cosgrove et al., 2007). Moreover, we observed variation (range, 78–92%) in parent fraction between subjects, demonstrating the importance of correcting the (–)-[^{18}F]flubatine input function for metabolites. Thus, we used V_T/f_p as the primary outcome measure for this first validation of physostigmine challenge in humans. The accurate quantification of f_p is a critical matter because V_T/f_p improved test-retest reproducibility over V_T in rhesus monkeys (Bois et al., 2015). Notably, f_p values in the presence of physostigmine did not differ from baseline values. Future test-retest studies in humans are necessary to determine whether correction for f_p and (–)-[^{18}F]flubatine metabolism offer improved quantification reliability.

The use of a B/I (–)-[^{18}F]flubatine administration scheme accurately quantified V_T and V_T/f_p as evaluated by comparing with the values measured following bolus radioligand injection. A B/I scheme is desirable because it can allow for shorter subject time in the PET camera and the measurement of both baseline and acetylcholine perturbation in a single scanning session using a single radiosynthesis (Carson, 2000). An important caveat to the presented B/I implementation with (–)-[^{18}F]flubatine was the necessity to optimize the radioligand infusion schedule (K_{bol}) for only a subset of brain regions. Owing to the much greater $\alpha_4\beta_2^*$ nAChR density in thalamus (Paterson and Nordberg, 2000), (–)-[^{18}F]flubatine kinetics are much slower in this region. To minimize the duration of radioligand infusion required for (–)-[^{18}F]flubatine equilibrium and measurement, we elected to forgo accurate quantification in the thalamus since EQ resulted in a bias of ~20%. This decision was made both for the pragmatic reason of measuring V_T/f_p in as many brain regions as possible, and

also because acetylcholine neurotransmission in cortical regions plays a critical role in learning, memory, and cognition (Hurst et al., 2013). Our K_{bol} of 360 min reliably provided (–)-[^{18}F]flubatine equilibrium within 120 min, as the rate of V_T/f_p change at this time was $+5.6 \pm 5.8\%$ per hour. Critically, this rate of change did not noticeably differ between smokers and nonsmokers, validating the use of this experimental protocol for quantifying $\alpha_4\beta_2^*$ nAChRs in smokers. Using the $K_{bol} = 360$ min design, the rate of (–)-[^{18}F]flubatine transfer from arterial plasma to tissue (K_1) (Innis et al., 2007), were nearly identical to those observed in bolus studies. Thus if measurement of K_1 is not desired, PET data for the initial 90 min prior to radioligand equilibrium are not required, which would further reduce PET scan time and subject discomfort. Bolus injection protocols are still the current desired method for standard scans measuring V_T/f_p , owing to the inability to accurately measure the thalamus with B/I. Yet, the use of venous blood sampling to measure parent radioligand in plasma is a further possible improvement to subject comfort afforded by implementing a B/I scheme, which would make this protocol the preferred method for V_T/f_p measurements. Future studies will examine the possibility of using venous sampling with B/I administration of (–)-[^{18}F]flubatine.

The use of a reference region devoid of specific radioligand uptake to estimate specific binding with BP_{ND} can provide an alternative approach to avoiding arterial blood sampling. Because $\alpha_4\beta_2^*$ nAChRs are ubiquitous in gray matter, previous reports with second-generation $\alpha_4\beta_2^*$ nAChR PET radioligands have used the white matter region of corpus callosum to estimate BP_{ND} (Wong et al., 2013; Sabri et al., 2015), despite indication of radioligand sensitivity to nicotine in this region (Wong et al., 2013). In our hands, (–)-[^{18}F]flubatine uptake in the white matter region of corpus callosum exhibited the lowest observed V_T/f_p values of 6.8 ± 1.5 mL/cm³ and was amenable to accurate quantification with equilibrium techniques in the context of a B/I scheme. In contrast, the centrum semiovale exhibited higher V_T/f_p values (calculated with kinetic analysis techniques) than some cortical regions, and further exhibited prolonged uptake compared to gray matter regions such that with the B/I scheme suitable equilibrium was not reached for accurate quantification. This variation in (–)-[^{18}F]flubatine kinetics in white matter regions raises the possibility that (–)-[^{18}F]flubatine nondisplaceable kinetics may be regionally non-uniform throughout the brain. Future displacement studies will therefore be vital to examine the extent of specific binding in white matter and uniformity of nondisplaceable (–)-[^{18}F]flubatine kinetics throughout the brain, which are critical considerations for accurately implementing reference region methods (Lammertsma et al., 1996). Thus we presently report V_T and V_T/f_p to minimize the assumptions in our reported measurements of (–)-[^{18}F]flubatine uptake.

To assess the sensitivity of (–)-[^{18}F]flubatine to fluctuations in acetylcholine levels, we measured V_T/f_p both before and after administration of the acetylcholinesterase inhibitor physostigmine. These measurements of physostigmine-induced changes in (–)-[^{18}F]flubatine binding (V_T/f_p) were made both with a two-scan bolus scheme and a B/I scheme to identify the best protocol for this important experiment. (–)-[^{18}F]Flubatine V_T/f_p in cortex decreased following physostigmine in four of the six two-scan bolus experiments and six of the seven B/I experiments (Supplementary Fig. 2). The average regional V_T/f_p

values ranged from 0.8 to 4.6% yielding effect sizes of 0.15–0.88 in the two-scan bolus scheme, compared to V_T/f_p values of 0.0–6.5% resulting in effect sizes of 0.0–1.54 with the B/I scheme. Larger population standard deviations in V_T/f_p were also observed with the two-scan bolus (3.6–6.2%) scheme relative to the B/I scheme (2.5–5.6%). The subjects scanned with the B/I scheme had a greater weight average and population variance (88.0 ± 30 kg) compared to the two-bolus scheme (81 ± 18 kg), thus we conclude the greater and less variable B/I V_T/f_p values to result from methodological factors inherent in the experimental design rather than differences in subject population. This reduced variation in displacement measurement is consistent with previous reports of increased effect sizes for B/I studies compared to two scan bolus studies, that were observed following nicotine displacement of [^{11}C]raclopride (Marenco et al., 2004). In addition, the B/I scheme has the practical advantages of completing the measurement in a single scan on a single day, thus avoiding potential day-to-day physiological and methodological variations. Owing to the ~110 min half-life of ^{18}F , a two-scan bolus scheme requires two separate days of successful scanning for completion. The single scan B/I scheme is therefore further desirable due to its reduced subject burden and diminished time and expense, which together with its improved sensitivity to physostigmine makes it the preferable method for future experiments measuring acetylcholine fluctuations.

The administration of 1.5 mg physostigmine (average 0.018 mg/kg) resulted in small V_T/f_p values of 2.8–6.5%. The low physostigmine dose, necessary due to peripheral side effects such as nausea and accelerated heart rate, likely induced only small increases in extracellular acetylcholine. Previous studies administering much larger physostigmine doses of 0.15 ± 0.08 mg/kg to nonhuman primates induced dose dependent ($-$)-[^{18}F]flubatine V_T/f_p values of 15–43%²⁷, giving an upper limit to the expected changes in V_T/f_p with the 1.5 mg dose. Moreover, a human study with 5-[^{123}I]IA-85380 administering a similar physostigmine dose (Esterlis et al., 2013) reported larger changes of $18\% \pm 11\%$ in cortex ($5.5\% \pm 4.3\%$ here), $14\% \pm 11\%$ in striatum ($3.0\% \pm 5.1\%$ here), and $17\% \pm 10\%$ in cerebellum ($2.8\% \pm 2.5\%$ here). Notably, the regional V_T/f_p rank orders are consistent between this previous report and our present findings. The differences in magnitude of reported V_T/f_p may be due to a number of factors, such as the need to correct for f_p after physostigmine with 5-[^{123}I]IA-85380 that was not necessary for ($-$)-[^{18}F]flubatine. Electrophysiology experiments with ($-$)-flubatine on human $\alpha_4\beta_2$ nAChRs expressed in *Xenopus* oocytes indicated that while ($-$)-flubatine is a partial agonist for $\alpha_4\beta_2$ nAChRs, it also has antagonist activity limiting its agonist efficacy (see Supplementary Materials; Supplementary Fig. 4). Interestingly, ($-$)-flubatine is also an efficacious α_7 partial agonist with $\text{EC}_{50} = 1 \mu\text{M}$ for this low affinity ($K_i = 119 \pm 69$ nM; Deuther-Conrad et al., 2004) site (data not shown). The antagonist properties of ($-$)-[^{18}F]flubatine for $\alpha_4\beta_2$ nAChRs versus the agonist properties of 5-[^{123}I]IA-85380 (Horti et al., 1999) may contribute to ($-$)-[^{18}F]flubatine having less sensitivity to competition with the endogenous agonist acetylcholine at the $\alpha_4\beta_2^*$ nAChR site. One limitation of V_T/f_p as an outcome measure is that it includes a component of nonspecific uptake (V_{ND}/f_p) in the outcome measure, thereby underestimating the change in specifically bound radiotracer displacement. Nonetheless, use of the presented ($-$)-[^{18}F]flubatine B/I paradigm is likely sensitive to

changes in acetylcholine levels, and future studies with a larger sample size could detect differences in acetylcholine transmission between controls and patient groups.

The presence of physostigmine both decreased $(-)-[^{18}\text{F}]$ flubatine concentrations in tissue while increasing its concentration in arterial plasma. This observation is consistent with previous 5- $[^{123}\text{I}]$ IA-85380 SPECT studies in nonhuman primates (Fujita et al., 2003) and humans (Esterlis et al., 2013). The increase in $(-)-[^{18}\text{F}]$ flubatine plasma concentrations is likely due to radiotracer displacement at peripheral sites. Given the lack of a reference region devoid of specific $(-)-[^{18}\text{F}]$ flubatine binding in humans, future physostigmine challenge studies should continue to use blood-based measurements of distribution volumes or specific radioligand binding in lieu of measurements exclusively based on radioligand concentration in tissue. In bolus studies, f_p was not affected by physostigmine, suggesting that V_T measurements may be sufficient for detecting physostigmine-induced effects if the outcome measure is strictly V_T .

The timing of V_T/f_p (Physo) measurement in the B/I paradigm importantly influences observation of acetylcholine-induced changes to $(-)-[^{18}\text{F}]$ flubatine uptake (Endres and Carson, 1998). Thus, dynamic measurements of V_T/f_p were made with equilibrium analysis to identify the time of maximal V_T/f_p . Although the population average of $V_T/f_p(\text{Physo})$ was minimal at the end of scanning (240 min; Fig. 5A), the population average of V_T/f_p , considered the primary outcome measure for physostigmine challenge, was greatest at 210 min post-injection (see Supplementary Fig. 3). At 240 min, V_T/f_p slightly decreased from 210 min (for example, from 6.5% to 5.5% in frontal cortex), suggesting that our scanning protocol observed the maximal $(-)-[^{18}\text{F}]$ flubatine displacement following physostigmine administration. This timing is consistent with previous microdialysis studies in the frontal cortex of rhesus monkeys, which found maximal acetylcholine concentrations at 15 min after the bolus administration of 0.05 mg/kg physostigmine, which then returned to baseline levels after 2 h (Tsukada et al., 2000). Thus given the infusion of the physostigmine dose over an hour (125–185 min after radioligand injection), the 210 min timepoint is a reasonable time to expect maximal changes in $(-)-[^{18}\text{F}]$ flubatine uptake from baseline.

The reported changes in $(-)-[^{18}\text{F}]$ flubatine uptake following physostigmine administration most likely resulted from increased extracellular acetylcholine competing with the radiotracer at the $\alpha_4\beta_2^*$ nAChR site. This conclusion is based on a large body of literature reporting small changes in radioligand binding following physostigmine administration with 5- $[^{123}\text{I}]$ IA-85380 in rhesus monkeys (Fujita et al., 2003) and humans (Esterlis et al., 2013), 2- $[^{18}\text{F}]$ FA-85380 in baboons (Valette et al., 2005), $[^{18}\text{F}]$ nifene in rats (Hillmer et al., 2013), and importantly a dose-dependent effect with $(-)-[^{18}\text{F}]$ flubatine in rhesus monkeys (Gallezot et al., 2014). Physostigmine does not directly compete with 5- $[^{123}\text{I}]$ IA-85380 (Mukhin et al., 2000) or $[^{18}\text{F}]$ nifene (Easwaramoorthy et al., 2007) in vitro, making it unlikely that physostigmine directly displaced $(-)-[^{18}\text{F}]$ flubatine at the $\alpha_4\beta_2^*$ nAChR site. However, changes in receptor-radioligand affinity due to physostigmine or increased acetylcholine may confound interpretation of this measure. Indeed, evidence for a low-affinity physostigmine binding site on the $\alpha_4\beta_2^*$ nAChR has been reported (Zwart et al., 2005). While further in vitro work may be needed to systematically examine these potential

influences, we expect them to be quite small compared to competition of high-affinity acetylcholine with (–)-[¹⁸F]flubatine at the $\alpha_4\beta_2^*$ nAChR. We thus infer that the observed decreases in V_T/f_p are likely most attributable to increased extracellular acetylcholine.

In conclusion, we report here the validation of a paradigm of bolus plus constant infusion radioligand administration that allows for accurate measurement of (–)-[¹⁸F]flubatine V_T and V_T/f_p after 120 min of PET data acquisition. Subsequent administration of physostigmine while maintaining radioligand infusion provided evidence of decreased V_T/f_p in cortical regions. Thus, this paradigm may allow for measurements of fluctuating acetylcholine levels with a single PET scanning procedure and straightforward analysis methods. Future studies should confirm these findings for extension to models of neuro-psychiatric disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
(-)-[¹⁸F]Flubatine parent fraction averaged across all baseline scans (n = 12). Error bars indicate population standard deviations.

Fig. 2.

Time activity curves from bolus injection (-)-[¹⁸F]flubatine with compartment model fits from a representative subject. A) Optimal fits with the 2-tissue compartment model (2TCM). B) Optimal fits with the 1-tissue compartment model (1TCM). Regions shown include cerebellum (△), thalamus (■), frontal cortex (◆), the white-matter region of centrum semiovale (○), and arterial plasma (×).

Fig. 3.
 (-)-[¹⁸F]Flubatine time activity curves with a bolus plus constant infusion radiotracer administration from a representative subject. Regions shown include cerebellum (▲), putamen (■), frontal cortex (◆), temporal cortex (▼), corpus callosum (◇), and arterial plasma (×).

(-)-[¹⁸F]Flubatine B/I Equilibrium Analysis

Fig. 4.

Comparison of (-)-[¹⁸F]flubatine V_T/f_P quantification with bolus ($n = 8$) and bolus-constant-infusion ($n = 4$) paradigms in extrathalamic gray matter regions of frontal cortex, occipital cortex, parietal cortex, temporal cortex, cingulate cortex, caudate, putamen, hippocampus, and cerebellum. A) Comparison of quantification in a B/I experiment between MA1 ($t^* = 90$ min) and equilibrium analyses after 120 min scanning (EQ_{120}). B) Comparison of quantification in a B/I experiment between MA1 ($t^* = 90$ min) and equilibrium analyses after 210 min scanning (EQ_{210}).

Fig. 5. Physostigmine-induced changes to (-)-[¹⁸F]flubatine V_t/f_p in a single B/I scan. A) Population average V_t/f_p value for the duration of PET scanning. Physostigmine (1.5 mg) was administered at 125 min, denoted by the dashed line. Regions shown include cerebellum (▲), striatum (●), frontal cortex (◆), and parent (-)-[¹⁸F]flubatine in arterial plasma (×), shown in blue to denote the different axis scales shown on the right. B) Voxel-wise (-)-[¹⁸F]flubatine V_t/f_p images at baseline (110–130 min) and post-physostigmine (200–220 min).

Table 1

Regional average (—)-[¹⁸F]flubatine V_T and V_T/f_P estimates with bolus data (n = 8).

	<u>2TCM</u>		<u>1TCM</u>		<u>MA1 (t* = 90 min)</u>	
	V_T	V_T/f_P	V_T	V_T/f_P	V_T	V_T/f_P
Caudate	8.6 ± 1.5	9.9 ± 1.7	7.8 ± 0.9	9.1 ± 1.0	8.3 ± 1.1	9.7 ± 1.3
Cerebellum	10.8 ± 2.2	12.4 ± 2.6	10.0 ± 1.6	11.6 ± 1.9	10.5 ± 2.1	12.3 ± 2.5
Cingulate	8.8 ± 1.3	10.2 ± 1.5	8.8 ± 1.2	10.2 ± 1.3	9.0 ± 1.3	10.5 ± 1.5
Frontal	9.3 ± 1.4	10.9 ± 1.6	9.0 ± 1.1	10.5 ± 1.3	9.1 ± 1.3	10.6 ± 1.5
Hippocampus	8.4 ± 1.1	9.8 ± 1.2	8.2 ± 1.0	9.5 ± 1.0	8.7 ± 1.3	10.2 ± 1.4
Occipital	8.8 ± 1.7	10.3 ± 1.9	8.7 ± 1.5	10.2 ± 1.7	8.8 ± 1.6	10.3 ± 1.8
Parietal	8.8 ± 1.3	10.3 ± 1.4	8.8 ± 1.2	10.2 ± 1.4	8.8 ± 1.3	10.3 ± 1.5
Putamen	10.0 ± 2.0	11.6 ± 2.3	9.4 ± 1.2	11.0 ± 1.3	10.0 ± 1.6	11.7 ± 1.8
Temporal	8.3 ± 1.4	9.7 ± 1.6	8.5 ± 1.2	9.9 ± 1.3	8.4 ± 1.3	9.8 ± 1.5
Thalamus	19.9 ± 3.0	23.1 ± 3.6	18.8 ± 2.1	21.9 ± 2.4	19.2 ± 3.0	22.4 ± 3.5
<i>Centrum semiovale</i>	<i>11.2 ± 1.3</i>	<i>13.0 ± 1.5</i>	<i>9.7 ± 1.1</i>	<i>11.3 ± 1.2</i>	<i>10.7 ± 1.1</i>	<i>12.4 ± 1.2</i>
<i>Corpus callosum</i>	<i>6.4 ± 1.2</i>	<i>6.9 ± 1.4</i>	<i>6.3 ± 1.0</i>	<i>7.3 ± 1.2</i>	<i>6.5 ± 1.3</i>	<i>6.8 ± 1.5</i>

All units are mL/cm³. Values given are mean ± population standard deviation.

Italics are used to distinguish the white matter regions of centrum semiovale and corpus callosum.

Table 2

Comparisons of K_1 and V_T/f_P values between bolus (n = 8) and B/I (n = 4) protocols, and comparisons of modeling analyses with equilibrium analyses.

	K_1 (mL·min ⁻¹ ·cm ⁻³)		V_T/f_P (mL/cm ³)			
	Bolus; 2TCM	B/I; 2TCM	Bolus; MA1	B/I; MA1	B/I; EQ _{120 min}	B/I; EQ _{210 min}
Caudate	0.24 ± 0.03	0.25 ± 0.05	9.7 ± 1.3	11.6 ± 2.7	11.2 ± 1.9	11.1 ± 2.0
Cerebellum	0.28 ± 0.02	0.29 ± 0.05	12.3 ± 2.5	12.8 ± 2.2	12.9 ± 1.6	12.3 ± 1.7
Cingulate	0.30 ± 0.04	0.29 ± 0.04	10.5 ± 1.5	11.3 ± 1.7	11.2 ± 1.2	11.0 ± 1.3
Frontal	0.33 ± 0.02	0.33 ± 0.05	10.6 ± 1.5	11.0 ± 1.5	11.1 ± 1.2	10.9 ± 1.2
Hippocampus	0.25 ± 0.03	0.25 ± 0.03	10.2 ± 1.4	11.5 ± 1.1	10.9 ± 0.5	11.2 ± 0.9
Occipital	0.32 ± 0.04	0.32 ± 0.05	10.3 ± 1.8	9.8 ± 1.6	9.9 ± 1.2	9.8 ± 1.3
Parietal	0.31 ± 0.05	0.31 ± 0.04	10.3 ± 1.5	10.8 ± 1.9	10.8 ± 1.6	10.6 ± 1.5
Putamen	0.30 ± 0.02	0.31 ± 0.05	11.7 ± 1.8	12.4 ± 2.0	11.9 ± 1.2	11.9 ± 1.3
Temporal	0.28 ± 0.04	0.27 ± 0.05	9.8 ± 1.5	10.1 ± 1.6	10.1 ± 1.3	9.9 ± 1.3
Thalamus	0.31 ± 0.02	0.32 ± 0.05	22.4 ± 3.5	33.8 ± 3.3	27.1 ± 2.5 ^a	28.6 ± 3.0 ^a
<i>Centrum semiovale</i>	<i>0.14 ± 0.05</i>	<i>0.18 ± 0.05</i>	<i>12.4 ± 1.2</i>	<i>12.6 ± 2.1</i>	<i>10.1 ± 1.2^a</i>	<i>11.2 ± 1.4^a</i>
<i>Corpus callosum</i>	<i>0.12 ± 0.03</i>	<i>0.14 ± 0.04</i>	<i>6.8 ± 1.5</i>	<i>8.6 ± 2.3</i>	7.2 ± 0.8	6.9 ± 0.6

Values given are mean ± population standard deviation.

Italics are used to distinguish the white matter regions of centrum semiovale and corpus callosum.

^aIndicates inaccurate estimates due to lack of equilibrium

Table 3

Physostigmine induced changes in (—)-[¹⁸F]flubatine V_T/f_P and corresponding effect sizes (Cohen's d) with bolus (n = 6) and B/I (n = 7) designs.

	Bolus		B/I	
	V_T/f_P	Cohen's d	V_T/f_P	Cohen's d
Caudate	0.8 ± 5.7%	0.15	2.7 ± 5.6%	0.48
Cerebellum	1.1 ± 4.0%	0.28	2.8 ± 2.5%	1.12
Cingulate	1.3 ± 3.6%	0.36	4.8 ± 4.4%	1.09
Frontal	2.2 ± 3.6%	0.61	6.5 ± 5.0%	1.30
Hippocampus	1.1 ± 6.2%	0.18	0.0 ± 5.3%	0.00
Occipital	3.7 ± 7.0%	0.53	4.8 ± 4.4%	1.09
Parietal	4.6 ± 5.2%	0.88	6.3 ± 4.1%	1.54
Putamen	4.0 ± 4.9%	0.82	3.2 ± 4.6%	0.70
Temporal	2.3 ± 4.6%	0.50	5.1 ± 3.4%	1.50
Thalamus	1.9 ± 5.5%	0.35	N/A	N/A

V_T/f_P values indicate mean ± population standard deviation.