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Evaluation of the sensitivity of the novel $\alpha 4\beta 2^*$ nicotinic acetylcholine receptor PET radioligand ^{18}F -(-)-NCFHEB to increases in synaptic acetylcholine levels in rhesus monkeys

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Abstract

Objective— ^{18}F -(-)-NCFHEB (also known as ^{18}F -(-)-Flubatine) is a new radioligand to image $\alpha 4\beta 2^*$ nicotinic acetylcholine receptors *in vivo* with positron emission tomography (PET), with faster kinetics than previous radioligands such as ^{18}F -2-F-A85380. The goal of this study was to assess the sensitivity of ^{18}F -(-)-NCFHEB-PET to increases in synaptic acetylcholine concentration induced by acetylcholinesterase inhibitors.

Methods—Two rhesus monkeys were scanned four times each on a Focus 220 scanner: first at baseline, then during two bolus plus infusions of physostigmine (0.06–0.28 mg/kg), and finally following a bolus injection of donepezil (0.25 mg/kg). The arterial input function and the plasma free fraction f_p were measured. ^{18}F -(-)-NCFHEB volume of distribution V_T was estimated using the multilinear analysis MA1 and then normalized by plasma free fraction f_p .

Results— ^{18}F -(-)-NCFHEB f_p was 0.89 ± 0.04 . At baseline, ^{18}F -(-)-NCFHEB V_T/f_p ranged from 7.9 ± 1.3 mL plasma/cm³ tissue in the cerebellum to 34.3 ± 8.4 mL plasma/cm³ tissue in the thalamus. Physostigmine induced a dose-dependent reduction of ^{18}F -(-)-NCFHEB V_T/f_p of $34 \pm 9\%$ in the putamen, $32 \pm 8\%$ in the thalamus, $25 \pm 8\%$ in the cortex, and $23 \pm 10\%$ in the hippocampus. With donepezil, ^{18}F -(-)-NCFHEB V_T/f_p was reduced by $24 \pm 2\%$, $14 \pm 3\%$ and $14 \pm 5\%$, $10 \pm 6\%$ in the same regions.

Conclusion— ^{18}F -(-)-NCFHEB can be used to detect changes in synaptic acetylcholine concentration and is a promising tracer to study acetylcholine dynamics with shorter scan durations than previous radioligands.

Keywords

^{18}F -(-)-NCFHEB; ^{18}F -(-)-Flubatine; nicotinic acetylcholine receptors; acetylcholine; rhesus monkeys; brain; physostigmine; donepezil

Several radioligands have been developed to study $\alpha 4\beta 2^*$ nicotinic acetylcholine receptors ($\alpha 4\beta 2^*$ -nAChRs) with positron emission tomography (PET) and with single photon emission computed tomography (SPECT). The first generation of radioligands usable in humans were based on the A-85380 scaffold (Sullivan et al., 1996): ^{123}I -5-I-A85380 for SPECT (Vaupel et al., 1998) and ^{18}F -2-F-A85380 for PET (Koren et al., 1998). Both ^{123}I -5-I-A85380 and ^{18}F -2-F-A85380 are limited by slow kinetics and long experimental protocols are needed with these tracers to perform displacement studies. Importantly, ^{123}I -5-I-A85380 was shown to be sensitive to increases in acetylcholine concentration following administration of an acetylcholinesterase inhibitor, physostigmine, in both baboons (Fujita et al., 2003) and in human subjects (Esterlis et al., 2013), suggesting its usefulness to study cholinergic compromise. Similar sensitivity to increases in acetylcholine was also shown for $\alpha 4\beta 2^*$ -nAChRs PET radioligands in pre-clinical models (Ding et al., 2000; Hillmer et al., 2013; Valette et al., 2005), but the findings were not tested or confirmed in humans.

In the past few years, several other radioligands have been developed in order to find a more suitable tracer allowing shorter studies. To date, two such radioligands have been tested in humans: ^{18}F -(-)-NCFHEB (also known as ^{18}F -(-)-Flubatine) (Sabri et al., 2011) and ^{18}F -AZAN (Wong et al., 2013). Both tracers were reported to have faster kinetics than previous compounds and to allow quantification of $\alpha 4\beta 2^*$ -nAChRs in humans with scan durations of less than two hours. The goal of the present study was to investigate whether ^{18}F -(-)-NCFHEB is also suitable to measure increases in acetylcholine concentration following administration of acetylcholinesterase inhibitors.

MATERIAL AND METHODS

Animals and Study Plan

Two rhesus monkeys were included in the study (two males, age = 6 and 5 years, body weight = 14.1 and 7.2 kg). All experiments were conducted in accordance with the Yale University Institutional Animal Care and Use Committee guidelines. Both animals underwent a baseline scan, and three blocking scans: two with physostigmine (0.15 ± 0.09 mg/kg; range=0.06–0.28 mg/kg), one with donepezil (0.25 mg/kg).

Radiochemistry

The synthesis of ^{18}F -(-)-NCFHEB was published elsewhere (Bois et al., 2013). Briefly, the synthetic route used a semi-automated microfluidic procedure derived from recently published methods (Fischer et al., 2013; Patt et al., 2013) by means of an radiolabeling of an ammonium salt on a Advion Nanotek unit (Advion, Ithaca, NY) followed by manual deprotection of the Boc protective group in acidic conditions to give the final product after semi-preparative HPLC purification and saline formulation for injection. Specific activity at the end of synthesis was 106 ± 36 MBq/nmol.

PET imaging

Animals were initially anesthetized with ketamine hydrochloride (10 mg/kg, IM), transported to the PET facility, intubated, and maintained on oxygen and 1.5–3% isoflurane throughout the study. For all studies, including baseline scans, the peripheral antimuscarinic agent glycopyrrolate (4 µg/kg, IM) was given to reduce secretions. A 2-h interval was allowed between ketamine administration and injection of the radiotracer or drugs. Vital signs, including respiration rate, blood pressure, heart rate, and temperature, were monitored continuously and recorded every 15 min, and temperature was kept constant at 37°C with heated water blankets. Arterial blood sampling was performed using a line inserted in a radial or femoral artery. PET scans were performed on the Focus 220 PET scanner (Siemens/CTI, Knoxville, TN, USA), which has an intrinsic resolution of 1.4 mm at the center of the field of view. A transmission scan was acquired for image reconstruction.

The radiotracer and all blocking drugs were injected using a syringe pump (Harvard PHD 22/2000, Harvard Apparatus, Holliston, Massachusetts, United States).

Physostigmine and donepezil were obtained from Sigma Aldrich (St. Louis, Missouri, United States). For physostigmine (n=4) studies, total doses of 0.06 (n=1), 0.14 (n=2), and 0.28 (n=1) mg/kg of physostigmine were administered using a bolus-plus-infusion protocol beginning 10 min before tracer injection. The bolus phase lasted 10 min, with doses of 20 (n=2) and 40 (n=2) µg/kg, respectively, and was followed by an infusion, maintained throughout the scan (2 hours), with rates of 60 (n=2) and 120 (n=2) µg/kg/hr, though for one physostigmine study the infusion was stopped after 8 min due to side effects (see results). Donepezil was administered 23±6 min before tracer injection as a 1-minute bolus (0.25 mg/kg).

¹⁸F-(–)-NCFHEB (36.9±0.8 MBq) was injected as a 3-minute bolus and list-mode data were acquired for 120 minutes. The injected mass was 10±4 ng/kg (max 17 ng/kg). Dynamic scan data were binned into a sequence of 33 frames (6 × 30 sec; 3 × 1 min; 2 × 2 min; 22 × 5 min), and each frame was reconstructed with the appropriate corrections (attenuation, normalization, scatter, randoms, and deadtime), using a FORE/FBP algorithm and a Shepp filter with a cut-off frequency of 0.15 times the sampling frequency (reconstructed image resolution approximately 3.2 mm).

Arterial Input Function Measurement

Sequential discrete arterial blood samples were taken at 0.75, 1.5, 2.25, 3, 3.75, 4.5, 5.25, 6, 8, 10, 15, 20, 25, 30, 40, 50, 60, 75, 90, 105 and 120 min postinjection. Plasma was separated from blood cells by centrifugation (2930g for 5 min at 4°C; Allegra X-22R Centrifuge, Beckman Coulter, Fullerton, CA, USA). Whole blood and plasma samples were counted in a cross-calibrated well counter (Wizard 1480, Perkin Elmer, Waltham, MA, USA).

In order to measure the ligand metabolism in plasma, seven selected plasma samples (3, 8, 15, 30, 60, 90 and 120 min postinjection; 0.5 to 2 mL) were mixed with urea at a final concentration of 8 M, and filtered through 1.0 µm Whatman 13 mm GD/X syringe filter (GE Florham Park, NJ USA). The filtrate was then analyzed by reverse-phase HPLC using a

modified column-switching system (Hilton et al., 2000). Up to 3 mL of filtrate was loaded on the automatic HPLC system (Shimadzu, Kyoto, Japan), and a mobile phase of 1:99 (v:v) acetonitrile:water was eluted through the self-packed capture column with solid phase extraction C18 sorbent (Strata-X, Phenomenex, Torrance, CA, USA) at a flow rate 2 mL/min. Then the content of the capture column was back-flushed onto a Phenomenex Gemini-NX C18 analytical column (250×4.6mm, 5 μm) (Phenomenex, Torrance, CA, USA), with a mobile phase consisting of 80:20 20mM ammonium bicarbonate: acetonitrile at a flow rate of 1.75 mL/min for the analytical column. The output of the HPLC column was connected to a fraction collector (CF-1 Fraction Collector, Spectrum Chromatography, Houston, TX, USA). Fractions were collected every two minutes and counted in a cross-calibrated well counter.

To correct for the loss of activity during the filtration step, and especially the sample time effect on that loss, the ratio of the radioactivity in filtrate and plasma was obtained and fitted to a curve with a constant asymptotic value ($f_F(t) = f_F^\infty - \Delta f_F e^{-\alpha t}$). All three parameters f_F^0 , f_F^∞ and α were fitted with positivity constraints. The fraction of radioactivity that went through the filter at $t=0$ ($f_F(0) = f_F^\infty - \Delta f_F$) was $95 \pm 2\%$, and the asymptotic value f_F^∞ was $100 \pm 2\%$ (i.e., the activity loss was moderate (5%), but greater for early samples and negligible for latter samples). The unchanged fraction in the filtrate was fitted to an integrated gamma function:

$$f_H(t) = a \left(1 - b \left(\int_0^{d \times t} e^{-u} u^{c-1} du / \int_0^\infty e^{-u} u^{c-1} du \right) \right) e^{-\beta t}$$

All five parameters a , b , c , d and β were fitted, with positivity constraints on all parameters, and additional constraints on parameter b ($b < 1$) and c ($c < 10$). The unchanged fraction in plasma was then computed as $f_H(t) \times f_F(t)/f_F(0)$, since $f_H(t)$ is the ratio of the parent concentration in the filtrate $[P]_F$ to total (i.e., parent plus metabolites) radioactivity concentration in the filtrate $[T]_F$, $f_F(t)$ is the ratio of total radioactivity concentrations in filtrate $[T]_F$ and plasma $[T]_P$, and $f_F(0)$ is ratio of the parent concentrations in filtrate $[P]_F$ and plasma $[P]_P$ (assuming radiochemical purity after synthesis is 100%) and thus $f_H(t) \times f_F(t)/f_F(0)$ is equal to $([P]_F/[T]_F) \times ([T]_F/[T]_P)/([P]_F/[P]_P) = [P]_P/[T]_P$. Finally the arterial input function was computed as the product of the plasma radioactivity concentration and the unchanged fraction.

To estimate ^{18}F -(-)-NCFHEB plasma free fraction, about 740 kBq of ^{18}F -(-)-NCFHEB was added (<30 μL) to a blood sample withdrawn before injection to produce a blood standard. After mixing and centrifugation, plasma water was separated from plasma proteins using ultrafiltration tubes (Centrifree UF device number 4104, Millipore, Billerica, MA, USA) and centrifugation (1228g for 20 min; IEC Medilite centrifuge, Thermo Fisher Scientific, Waltham, MA, USA). Plasma and water samples were counted in triplicate and free fraction (f_p) was estimated as the ratio of the mean concentration in filtered and unfiltered plasma.

The plasma clearance of ^{18}F -(-)-NCFHEB was computed as follow: first, the concentration of unchanged ^{18}F -(-)-NCFHEB in plasma was extrapolated to infinity using a mono-exponential fit of measured curve from 80-min post-injection; then the clearance of ^{18}F -(-)-NCFHEB was computed as the ratio of the injected dose divided by the area under the curve (AUC) of the (extrapolated) concentration of unchanged ^{18}F -(-)-NCFHEB in plasma.

Drug concentration in plasma

Plasma samples were obtained at 1, 5, 10, 15, 30, 60, 90 and 120 minutes after the start of the administration of the acetylcholinesterase inhibitors in order to measure the plasma concentration of physostigmine and donepezil, and the area under the curve (AUC) was used to compare plasma exposures and changes in PET binding measures.

Magnetic Resonance Imaging

MR images were previously acquired for each rhesus monkey on a Siemens 3.0T Trio scanner, using an extremity coil. T1-weighted images were acquired in the coronal plane with a spin echo sequence (TE = 3.34, TR = 2530, flip angle = 7° , section thickness = 0.50 mm, field-of-view = 140 mm, image matrix = $256 \times 256 \times 176$ pixels, matrix size = $0.547 \times 0.547 \times 0.500$ mm). Non-brain tissue was removed and the image cropped to $176 \times 176 \times 176$ pixels using MEDx software (Medical Numerics Inc., Germantown, MD, USA) before co-registration with PET images.

Regional TAC Computation

Regional time-activity curves (TACs) were computed using a template of regions, defined on a template brain (a representative MR image of a brain, from an animal not included in the study) for the cerebellum (5.9 cm^3), caudate (0.43 cm^3), putamen (0.38 cm^3), brainstem (1.9 cm^3), hippocampus (0.32 cm^3), occipital (5.2 cm^3), frontal (2.5 cm^3) and temporal (3.2 cm^3) cortices, and the thalamus (0.25 cm^3). To apply the ROIs to the PET images, two coregistration transforms were estimated. First, a non-linear transformation was used (bioimagesuite program; version 2.5; <http://www.bioimagesuite.org/>) between the template MRI and the MR image of each animal. This nonlinear coregistration algorithm is based on the method proposed by (Rueckert et al., 1999) with modifications (Papademetris et al., 2001). Then, the MR and PET images of each monkey were coregistered using a rigid body registration using an automatically-selected PET sum image (Sandiego et al., 2013). Finally, the ROI voxel coordinates were computed in native PET space using the two aforementioned coregistrations and the mean ROI value for each frame was computed by trilinear interpolation. Quality of the coregistrations and final placement of the ROIs on the PET images was visually inspected.

Kinetic Modeling

Based on the results from previous studies (Bois et al., 2013; Gallezot et al., 2013), the volume of distribution V_T (Innis et al., 2007) was estimated using the multilinear analysis MA1 (Ichise et al., 2002), with a starting time for the fits (t^*) of 30 min post-injection. The volumes of distribution were normalized by the plasma free f_p , and denoted V_T/f_p , since the

test-retest variability of V_T/f_P was found to be slightly better than that of V_T (Bois et al., 2013; Gallezot et al., 2013).

RESULTS

Side-effects of physostigmine and donepezil

In one animal, both physostigmine and donepezil had an effect on the respiratory rate (RR). In the unaffected animal (animal 1), RR tended to increase during the first half of the isoflurane anesthesia, then reached a plateau (for about the last 90-min of the PET scan) (Figure 1A). In the affected animal (animal 2), the same pattern was seen at baseline, but administration of physostigmine at 0.14 mg/kg reversed that trend and the respiratory rate started decreasing before reaching a plateau. In one study, a dose of 0.28 mg/kg physostigmine was attempted; RR dropped quickly from 33 breaths/min (BPM) to 6 BPM 30 min after the start of the physostigmine infusion. At this point, the infusion was stopped (0.06 mg/kg was effectively administered), and RR quickly rose to pre-infusion level (Figure 1B). In the donepezil study in the same animal, RR also quickly dropped from 26 to 9 BPM at 5-min after the start of the bolus of donepezil, and then quickly rose back to a plateau of 20 BPM (stabilizing 30 min after the start of the bolus of donepezil). Drug effects on other physiological measures are summarized in Table I. Since the other physiological parameters varied more slowly than RR, the effects on these physiological parameters were summarized by computing the percent change of the average value of each physiological parameter after the start of drug administration with respect to the average value in the hour preceding the drug administration. For baseline studies, the time of tracer administration was used to define the pre-drug and post-drug time-intervals.

Arterial input function

$^{18}\text{F}(-)\text{-NCFHEB } f_P$ was high, at 0.89 ± 0.04 ($n=8$). $^{18}\text{F}(-)\text{-NCFHEB}$ metabolism in plasma was moderately fast, with only $58 \pm 5\%$ of total plasma radioactivity being attributed to unchanged $^{18}\text{F}(-)\text{-NCFHEB}$ at 15 min post-injection and only $15 \pm 3\%$ at 120 min post-injection (Figure 2A). $^{18}\text{F}(-)\text{-NCFHEB}$ plasma clearance was 0.28 ± 0.11 L/min at baseline. Neither physostigmine nor donepezil changed $^{18}\text{F}(-)\text{-NCFHEB}$ plasma clearance: the relative changes were $3 \pm 22\%$ and $-1 \pm 22\%$, respectively.

$^{18}\text{F}(-)\text{-NCFHEB}$ volumes of distribution

Sample time-activity for one monkey at baseline are shown in Figure 2B. At baseline, the normalized volume of distribution V_T/f_P ranged from 7.9 ± 1.3 mL/cm³ in the cerebellum to 34.3 ± 8.4 mL/cm³ in the thalamus. Physostigmine administration resulted in a dose-dependent decrease of $^{18}\text{F}(-)\text{-NCFHEB } V_T/f_P$ in both animals (Figure 3). In an average across all doses, the largest percentage reductions of V_T/f_P were observed in the putamen ($34 \pm 9\%$) and caudate ($33 \pm 10\%$), followed by the thalamus ($32 \pm 8\%$), the brainstem ($30 \pm 8\%$), the cortex ($25 \pm 8\%$), the hippocampus ($23 \pm 10\%$) and finally the cerebellum ($22 \pm 13\%$). At the same dose (0.14 mg/kg), larger reductions were observed in animal 2 ($37 \pm 4\%$ versus $17 \pm 4\%$ in animal 1), which was consistent with the higher plasma concentration of physostigmine measured in animal 2 (the AUC was 0.037 ng/ $\mu\text{L} \cdot \text{min}$ for animal 2 versus 0.011 ng/ $\mu\text{L} \cdot \text{min}$ for animal 1). Donepezil (0.25 mg/kg) administration also

resulted in a decrease of $^{18}\text{F}(-)\text{-NCFHEB } V_T/f_P$ in both animals (Table II). The regional rank order of the effect of donepezil was similar to that of physostigmine (putamen > caudate > thalamus~brainstem > cortex > hippocampus) except for the cerebellum in which the donepezil effect was comparatively larger. In animal 1, the plasma concentration of donepezil was larger than in animal 2 ($\text{AUC} = 0.43 \text{ ng}/\mu\text{l} \cdot \text{hr}$ versus $0.24 \text{ ng}/\mu\text{l} \cdot \text{hr}$), and slightly larger reductions of V_T/f_P were observed in animal 1 than in animal 2 ($-18 \pm 4\%$ versus $-14 \pm 7\%$).

Binding potentials BP_{ND}

Using blocking studies with nicotine, the non-displaceable volume of distribution V_{ND} of $^{18}\text{F}(-)\text{-NCFHEB}$ was estimated to be about $5.9 \pm 0.9 \text{ mL}/\text{cm}^3$ (Bois et al., 2013). Based on this value, $^{18}\text{F}(-)\text{-NCFHEB}$ binding potential BP_{ND} would be 4.2 in the thalamus, 0.98–1.1 in the basal ganglia, 0.68–1.3 in the cortex and 0.19 in the cerebellum. Using these BP_{ND} estimates, the percent reduction of $^{18}\text{F}(-)\text{-NCFHEB } V_T$ induced by physostigmine can be extrapolated to estimate the percent reduction of $^{18}\text{F}(-)\text{-NCFHEB } BP_{ND}$: this reduction was 43–86% in the basal ganglia, 21–85% in the cortex, and 23–47% in the thalamus. Similarly, the percent reduction of $^{18}\text{F}(-)\text{-NCFHEB } BP_{ND}$ induced by donepezil was estimated as 42–49% in the basal ganglia, 17–54% in the cortex, and 14–20% in the thalamus.

DISCUSSION

In the present study, we show that the novel $\alpha 4\beta 2^*$ -nAChR PET radioligand $^{18}\text{F}(-)\text{-NCFHEB}$ (also known as $^{18}\text{F}(-)\text{-Flubatine}$) is sensitive to pharmacologically induced increases in synaptic concentrations of acetylcholine. We showed that with physostigmine ($0.06\text{--}0.28 \text{ mg}/\text{kg}$), $^{18}\text{F}(-)\text{-NCFHEB } V_T/f_P$ was reduced by 18%–38% in thalamus, 22–43% in basal ganglia, and 15–33% in cortex, and with donepezil ($0.25 \text{ mg}/\text{kg}$) $^{18}\text{F}(-)\text{-NCFHEB } V_T/f_P$ was reduced by 14% in the thalamus, 17–20% in the basal ganglia, 9–14% in the cortex.

Previous studies had shown that some $\alpha 4\beta 2^*$ -nAChR radioligands used for PET and SPECT imaging were sensitive to increases in synaptic concentrations of acetylcholine induced by physostigmine: ^{18}F -norcloro-fluoro-epibatidine in anesthetized non-human primates (Ding et al., 2000), ^{123}I -5-I-A85380 in anesthetized non-human primates (Fujita et al., 2003) and humans (Esterlis et al., 2013), ^{18}F -2-F-A85380 in anesthetized non-human primates (Valette et al., 2005) and ^{18}F -Nifene in vitro (Easwaramoorthy et al., 2007) and in anesthetized rats (Hillmer et al., 2013). The reductions of $^{18}\text{F}(-)\text{-NCFHEB } V_T/f_P$ observed in this study with physostigmine are of the same order of magnitude as in previous studies in non-human primates. Specifically, with ^{123}I -5-I-A85380, the radioactivity concentration in the thalamus of anesthetized baboons decreased by 14–17% following the injection of a 5-min bolus of physostigmine ($0.067\text{--}0.2 \text{ mg}/\text{kg}$). The authors did not calculate volume of distribution; however, based on the fact that there were transient increases in plasma concentration of the radiotracer after administration of physostigmine, percent change in the volume of distribution was likely higher than the reported changes in radioactivity concentrations (Fujita et al., 2003). With ^{18}F -2-F-A85380, a 160-min infusion of physostigmine ($0.1\text{--}0.4$

mg/kg) in baboons produced a 33–40% reduction in putamen V_T and a 27% reduction in cortical V_T (Valette et al., 2005). With ^{18}F -norchloro-fluoro-epibatidine, administration of 0.03 mg/kg of physostigmine in baboons produced a 24% reduction of the putamen to cerebellum ratio, and 14–19% reduction of the cortical to cerebellum ratio (Ding et al., 2000).

Moreover, the regional heterogeneity of the effect of physostigmine seen in this study was also observed in previous studies (Ding et al., 2000; Esterlis et al., 2013; Valette et al., 2005), and the higher reduction of V_T/f_P in the basal ganglia than in the cortex is similar to what was observed in other anesthetized non-human primates studies (Ding et al., 2000; Valette et al., 2005).

In contrast, in humans, a one-hour infusion of physostigmine (1–1.5 mg) resulted in a higher reduction of ^{123}I -5-I-A85380 V_T in cortex ($29 \pm 17\%$) than in the basal ganglia ($19 \pm 15\%$) and thalamus ($19 \pm 15\%$) (Esterlis et al., 2013). This different rank order may be due to difference in tracer, species, and anesthesia effect in the non-human primates studies. In particular all non-human primates studies were carried out after induction of the anesthesia with ketamine, which is known to affect acetylcholine release (Kim et al., 1999). Isoflurane, which was used in most of these non-human primates studies to maintain anesthesia, also affects acetylcholine release (Shichino et al., 1998).

It is difficult to predict the direction and amplitude of changes that may be observed in the absence of anesthesia in human studies. Isoflurane decreased the (baseline in the context of this study) acetylcholine concentration measured with microdialysis (Shichino et al., 1998), which could potentially increase baseline VTs and BPNDs. On the other hand ketamine, which was used to induce anesthesia in this study, tends to increase acetylcholine concentration measured by microdialysis (Kim et al., 1999). Moreover, both anesthetic agents also affect nicotinic receptors, independent of their effect on acetylcholine concentration (Violet et al., 1997; Yamakura et al., 2000). Thus, while the combined effect of these actions is difficult to predict, we expect findings in humans to be in line with the previous SPECT study (Esterlis et al., 2013), based on the similarity of the two tracers' binding potentials in non-human primates.

Our group had previously calculated nonspecific volume of distribution V_{ND} of ^{18}F -(-)-NCFHEB in rhesus monkey by administration of a nicotine block (Bois et al., 2013). Here, we used that V_{ND} value to estimate change in binding potential BP_{ND} . The percent reduction of ^{18}F -(-)-NCFHEB BP_{ND} induced by physostigmine was estimated to be 43–86% in the basal ganglia, 21–85% in the cortex, and 23–47% in the thalamus. One of the limitations of the current study is that, though there is evidence of specific binding of ^{18}F -(-)-NCFHEB in the cerebellum in blockade studies with nicotine (Bois et al., 2013), the cerebellum BP_{ND} estimate is only 0.19, and thus the percent reduction of ^{18}F -(-)-NCFHEB BP_{ND} was greater than 100% in some studies (range 63–226%), indicating that part of the effect measured could be due to noise, or other physiological effects due to the high doses of physostigmine. For comparison, in the human ^{123}I -5-I-A85380 study that administered a lower dose of physostigmine (1–1.5 mg), the binding potential BP_F was reduced by about 19% in the basal

ganglia, 29% in the cortex, 36% in the cerebellum, and 19% in the thalamus (Esterlis et al., 2013).

The adverse effects of physostigmine seen in this study are not likely to be due to direct effect on the nicotinic receptors. *In vitro*, high concentrations of physostigmine are needed to inhibit the binding of radioligands at the acetylcholine binding site on $\alpha 4\beta 2^*$ -nAChRs ($K_i > 25 \mu\text{M}$) (Mukhin et al., 2000); these concentrations are ~1000 higher than the concentrations needed to inhibit AChE ($K_i \sim 30 \text{ nM}$) (Triggle et al., 1998).

To confirm the results obtained with physostigmine, we also examined the effects of a second acetylcholinesterase inhibitor, donepezil, on ^{18}F -(-)-NCFHEB binding. This second inhibitor has been shown in microdialysis studies to lead to increases in acetylcholine which are less robust than physostigmine at the peak, but long lasting (Tsukada et al., 2004; Tsukada et al., 2000), in part due to the long plasma half life of donepezil after IV administration (63 min in rhesus monkey vs. 15–31 min in rats, dogs and humans for physostigmine) (Shiraishi et al., 2005; Triggle et al., 1998). A bolus injection of donepezil (0.25 mg/kg) produced a 75% increase in acetylcholine concentration at the peak and a 50% increase at two hours after injection (Tsukada et al., 2004). For comparison, a bolus injection of physostigmine (0.05 mg/kg) produced a 175% increase in acetylcholine concentration at the peak but a less than 25% increase at two hours post injection (Tsukada et al., 2000). In this study physostigmine was administered using a bolus plus infusion protocol to achieve a more long-lasting increase in acetylcholine concentration, and donepezil was administered using a simple bolus injection, at the highest dose previously used in the literature. Donepezil (0.25 mg/kg) resulted in reductions of ^{18}F -(-)-NCFHEB V_T/f_p of 17–20% in the basal ganglia, 9–14% in the cortex and 14% in the thalamus, which were less robust than the reductions from physostigmine.

During this study, the injected activity was limited to 37 MBq, an amount of activity that was estimated to be sufficient with a ^{18}F -labelled tracer for PET imaging on the Focus 220, and for arterial-input function measurement, due to concerns about the tracer dose limit, which tends to be low for high affinity tracers such as ^{18}F -(-)-NCFHEB, and potential toxicity, which is a potential issue with most nicotinic ligands. With this limitation, the maximal injected mass was 17 ng/kg, well below the No Observable Effect Level (NOEL) dose in rats (6.2 $\mu\text{g/kg}$) (Smits et al., 2014). The tracer dose limit is not precisely known since no self-saturation studies have been done in rhesus monkeys yet, but the plasma concentration of unchanged and free ^{18}F -(-)-NCFHEB during the last hour of each scan was less than 10 pM for all scans (average = $5.7 \pm 1.7 \text{ pM}$), which based on the K_i measured *in vitro* for the $\alpha 4\beta 2$ subtype (0.11 nM) (Deuther-Conrad et al., 2004), would lead to an occupancy of less than 10%.

In vitro, ^{18}F -(-)-NCFHEB has about a 20 fold higher affinity for the $\alpha 4\beta 2$ subtype of nicotinic receptors than for the $\alpha 3\beta 4$ (Deuther-Conrad et al., 2004). This selectivity is lower than that of previous tracers (e.g., ^{18}F -2-F-A-85380). However, the *in vivo* selectivity of ^{18}F -(-)-NCFHEB is likely to be higher in most brain regions: indeed, for *in vivo* imaging at tracer dose, one needs to compare the ratio of B_{max}/K_D for both receptor subtypes. The relative distribution of the $\alpha 4\beta 2$ and $\alpha 3\beta 4$ subtypes has been studied quantitatively in non-

human primates or humans to the best of our knowledge, but in rats, the concentration of the $\alpha 4\beta 2$ subtype is (at least twice) higher than the concentration of the $\alpha 3\beta 4$ subtype in all brain regions except in a few midbrain nuclei and in the medulla (Perry et al., 2002). Thus in most regions, ^{18}F -(-)-NCFHEB binding is expected to be more than 40 times more selective for $\alpha 4\beta 2$ than for $\alpha 3\beta 4$. In the areas of the midbrain and medulla with a majority of $\alpha 3\beta 4$ receptors, the binding potential of ^{18}F -(-)-NCFHEB for these receptors is expected to be low, i.e. 0.2, based on a maximal concentration of 3 pmol/g (Perry et al., 2002), a K_D of 2.58 nM (Deuther-Conrad et al., 2004), and a tissue free fraction f_{ND} of 0.15, even without taking account the strong partial volume effect which would lower the binding potential of these small nuclei.

Taken together these studies demonstrate that we now have a reliable paradigm to measure increases in synaptic acetylcholine in vivo. This has been performed in multiple species (rats, nonhuman primates, human subjects), with several different radiotracers (^{123}I -5-IA-85380, ^{18}F -(-)-NCFHEB, ^{18}F -Nifene), and now with two different acetylcholinesterase inhibitors (physostigmine and donepezil). The consistency across studies, species, radiotracers and pharmacological agents supports developing this paradigm with ^{18}F -(-)-NCFHEB in human subjects. This paradigm will be useful to investigate dysfunctional cholinergic signaling in psychiatric populations.

CONCLUSION

^{18}F -(-)-NCFHEB can be used to detect increases in synaptic acetylcholine concentration. This finding, combined with previous reports that ^{18}F -(-)-NCFHEB has fast kinetics in humans, indicates that ^{18}F -(-)-NCFHEB is a promising tracer to probe acetylcholine neurotransmission in human subjects.

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Figure 1.

Effect of drug administration on respiratory rate. **(A)** In animal A1, there was no visible drug effects on the respiratory rate, as the evolution of the respiratory rate during the anesthesia was similar during the baseline study and the studies with drugs: slow increase during the first three hours, and no changes in that trend around the start of the drug administration (time 0). **(B)** In animal A2, the evolution of the respiratory rate during baseline scans was similar to that of animal A1, but physostigmine and donepezil produced reductions in respiratory rate.

Figure 2.

(A) Representative curve of the unchanged fraction of ^{18}F -(+)-NCFHEB in arterial plasma.

(B) Representative time-activity curves of ^{18}F -(+)-NCFHEB at baseline in selected brain regions: thalamus (solid circles), frontal cortex (triangles), putamen (squares) and cerebellum (open circles). The solid line shows the metabolite-corrected arterial input function.

Figure 3.

Effect of physostigmine on ¹⁸F-(+)-NCFHEB volumes of distribution: for each animal (**A**: animal 1, **B**: animal 2), physostigmine induced a dose-dependent reduction of ¹⁸F-(+)-NCFHEB V_T/f_P estimates.

Table I

Drug effects on physiological recordings

Animal	Study	RR	HR	pCO2	BP	
					systolic	diastolic
A1	Baseline	13%	-1%	-1%	14%	13%
	P 0.14	22%	7%	23%	-5%	-8%
	P 0.28	7%	13%	27%	6%	1%
	D	15%	1%	-4%	5%	-6%
A2	Baseline	7%	-5%	-4%	-11%	-19%
	P 0.14	-30%	3%	11%	7%	13%
	P 0.06*	-35%†	7%	7%	18%	12%
	D	-31%	3%	6%	17%	22%

All changes are reported as the percent difference between the average recording after the drug administration (or during PET scan for baseline studies) divided by the average recording in the hour preceding the drug administration (or PET scan for baseline studies). Abbreviations used: RR=respiratory rate, HR=heart rate, BP=blood pressure, P=physostigmine, D=donepezil.

* Infusion was interrupted, target dose was 0.28 mg/kg.

† Using data during the physostigmine infusion only, and not the part of the PET scan after the interruption of the infusion.

Table II

Reduction of ^{18}F -(-)-NCFHEB V_T/f_P induced by donepezil (0.25 mg/kg) and physostigmine (0.15±0.08 mg/kg).

ROI	Reduction of V_T/f_P	
	Physostigmine (n=4)	Donepezil (n=2)
Cerebellum	-22±13%	-23±2%
Brainstem	-30±8%	-14±2%
Caudate	-33±10%	-21±1%
Putamen	-34±9%	-24±2%
Hippocampus	-23±10%	-10±6%
Occipital	-25±9%	-16±8%
Frontal	-25±9%	-11±1%
Temporal	-25±8%	-14±6%
Thalamus	-32±9%	-14±3%