Validation of an Extracerebral Reference Region Approach for the Quantification of Brain Nicotinic Acetylcholine Receptors in Squirrel Monkeys with PET and 2-^{18}F-Fluoro-A-85380

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The aim of the present study was to explore the applicability of an extracerebral reference region for the quantification of cerebral receptors with PET. **Methods:** Male squirrel monkeys underwent quantitative PET studies of cerebral nicotinic acetylcholine receptors (nAChRs) with 2-^{18}F-fluoro-A-85380 (2-FA). Data from dynamic PET scans were analyzed with various compartment- and non–compartment-based models, including a simplified reference tissue model (SRTM). Nondisplaceable volume-of-distribution (VDnd) values were determined in regions of interest after the blockade of 2-FA–specific binding by nicotine infusion. Binding potential values, estimated with the cerebellum and muscle as reference regions, were compared and the reproducibility of measurements was determined. **Results:** One- and 2-tissue-compartment modeling and linear graphic analysis provided similar total volume-of-distribution (VD_T) values for each studied region. VD_T values were high in the thalamus, intermediate in the cortex and midbrain, and low in the cerebellum and muscle, consistent with the distribution pattern of nAChR containing α_4 and β_2 receptor subunits ($\alpha_4\beta_2$*). The administration of nicotine at 2 mg/kg/d via an osmotic pump resulted in a nearly complete saturation of 2-FA–specific binding and led to very small changes in volumes of distribution in the cerebellum and muscle (−9% ± 4% [mean ± SEM] and 0% ± 6%, respectively), suggesting limited specific binding of the radioligand in these areas. VD_T measured in muscle in 15 monkeys was reasonably constant (3.0 ± 0.2, with a coefficient of variation of 8%). VDnd in studied brain regions exceeded VD_T in muscles by a factor of 1.3. With this factor and with muscle as a reference region, BP* values calculated for studied brain regions with the SRTM were in good agreement with those obtained with the cerebellum as a reference region. Significant correlations were observed between BP* values estimated with these 2 approaches. The reproducibilities of BP* measurements obtained with the 2 methods were comparable, with coefficients of variation of less than 11% and 13% for the thalamus and the cortex, respectively. **Conclusion:** These results suggest that the accurate quantification of nAChRs can be performed with 2-FA and a reference region outside the brain, providing a novel approach for the quantification of brain receptors when no suitable cerebral reference region is available.

Key Words: PET; nonhuman primates; radioligand; in vivo binding; nicotinic acetylcholine receptors

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Current methods for the quantification of cerebral receptors by PET use either a blood input function (invasive approach) or a reference tissue approach (noninvasive approach) (*1*). However, the use of an arterial input function is not always practical because of the invasiveness of this approach and the limited quantity of blood that can be drawn in small animals. Additionally, the reference tissue approach is also not always feasible because reference tissue either may not be available or may not be useful because of insufficient size, radioactive spillover from adjacent areas, or a pathologic condition changing the brain receptor expression in the reference region. To develop an alternate approach, we evaluated the potential of using an extracerebral reference tissue approach for receptor quantification. For this purpose, we performed a series of PET studies in squirrel monkeys with 2-^{18}F-fluoro-3-(2(*S*)-azetidinylmethoxy)pyridine (2-^{18}F-fluoro-A-85380; 2-FA), a PET radioligand suitable for the quantification of $\alpha_4\beta_2$* nicotinic acetylcholine receptors (nAChRs) in vivo (*2–7*).

The volume of distribution (VD) of a specific binding compartment (VDsb), defined as the ratio of the concentration of specifically bound radioligand in tissue to the concentration of free radioligand in blood plasma at equilibrium, is often used for receptor quantification in vivo. At concentrations

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of radioligand (F) lower than the dissociation constant (K_d), VDsb can be expressed as follows:

$$VD_{sb} = \frac{SB}{F} = \frac{B_{max}}{K_d}. \quad \text{Eq. 1}$$

In this equation, SB is specific binding of radioligand and B_{max} is the density of receptors available for radioligand binding. If receptor affinity and the percentage of occupancy by an endogenous neurotransmitter are the same, then VDsb can be used to evaluate the densities of receptors. VDsb can be obtained as the difference between the total VD (VD_T) and the nondisplaceable VD (VDnd). VDnd can be measured while receptors are saturated with a dose of unlabeled ligand or by measurement of VD_T in a reference region, if available. These VD values can also be used to calculate the binding potential (BP*). BP*, defined as the ratio of VDsb to VDnd (*8*), is a measure of the density of receptors available for radioligand binding and also characterizes the specificity of radioligand binding in vivo; it is expressed as follows:

$$BP^* = \frac{VD_T - VDnd}{VDnd} = \frac{VD_T}{VDnd} - 1. \quad \text{Eq. 2}$$

Unlike VDsb, BP* is not sensitive to possible errors from assaying the radioligand concentration in blood plasma (i.e., estimation of free, unbound radioligand). In addition, BP* may be calculated without blood sampling when a reference region is available.

Here, we explored the use of muscle as an extracerebral reference region for the quantification of central receptors. Ideally, a reference region should not express the receptor under investigation and should allow an estimation of VDnd in the target region in the brain. However, it is likely that VDnd in brain tissue will not be equal to VD_T in muscle (VDmsl). In this situation, if the ratio of VDnd in brain tissue to VDmsl is constant, the term α, reflecting the relationship between the 2 values, can be introduced, as follows:

$$VDnd = \alpha VDmsl. \quad \text{Eq. 3}$$

Equation 2 can be rewritten by substituting αVDmsl for VDnd (Eq. 3), as follows:

$$BP^* = \frac{VD_T - \alpha VDmsl}{\alpha VDmsl} = \frac{VD_T}{\alpha VDmsl} - 1. \quad \text{Eq. 4}$$

Consistent with Equation 4, if α is known and constant, BP* can be calculated on the basis of VD_T in the brain and VDmsl. With Equation 2, BP* calculated with muscle as a reference region (BPmsl) can be expressed as follows:

$$BPmsl = \frac{VD_T}{VDmsl} - 1. \quad \text{Eq. 5}$$

By substituting Equation 5 for VD_T in Equation 4, BP* can be expressed as follows:

$$BP^* = \frac{BPmsl + 1}{\alpha} - 1. \quad \text{Eq. 6}$$

To explore the possibility of using muscle as an extracerebral reference region for the quantification of cerebral receptors, we first evaluated VDmsl and VD_T values in various brain areas. The variability of VDmsl values was correlated with 2-FA metabolism. To determine VDnd values in various brain areas and to assess the specific binding of 2-FA in muscle and the cerebellum, blocking and displacement studies with nicotine were conducted. In blocking studies, PET measurements were obtained in monkeys chronically exposed to nicotine. In displacement studies, a bolus infusion paradigm was used, with nicotine being infused intravenously during PET once the equilibrium of the radiotracer was reached. The BP* values obtained with muscle and the cerebellum as reference regions were then compared. The reproducibility of BP* measurements was assessed by performing 2 or 3 PET scans on the same animal. Finally, 2 additional series of PET experiments were performed to investigate the possible influences of the injected mass of 2-FA and of the scanning duration on the observed BP* values.

MATERIALS AND METHODS

Radiochemistry

^{18}F-Fluoride was produced by use of an RDS111 negative-ion cyclotron, and 2-FA was synthesized by use of a modified semiautomated method (*9*). The final product was formulated as a sterile and pyrogen-free isotonic solution. The radiochemical purity of the product was greater than 98%, and the specific activity was in the range of 120–1,100 GBq/μmol (mean ± SD, 400 ± 200 GBq/μmol).

PET and MRI

The adult male squirrel monkeys (*Saimiri sciureus*; 730–1,100 g) used in the present study were maintained in facilities fully accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC), and all experimentation was conducted in accordance with the guidelines of the Institutional Care and Use Committee of the Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, and the *Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research* (*10*).

Overall, 43 PET scans were obtained for 20 animals. Data were acquired with a Siemens Exact ECAT HR+ tomograph (Siemens Medical Solutions; 63 slices, center-to-center spacing of 2.4 mm, in-plane reconstructed resolution, full width at half maximum of 4.7 mm at the center of the field of view, and reconstructed axial spatial resolution of 4.2 mm in 3-dimensional mode). Before each radioligand administration, transmission scans were obtained with 3 rotating ^{68}Ge–^{68}Ga sources and were used to correct for photon attenuation. PET images were reconstructed from the raw data with a standard filtered-backprojection algorithm and a ramp filter.

Because the size of the PET scanner field of view could accommodate 2 squirrel monkey heads, most PET studies were performed with simultaneous acquisition of data from 2 animals. To

avoid potential complexity in attenuation and scatter corrections, animals were positioned head to head in the scanner in the same z-axis. Monkeys were initially anesthetized with alfadolone and alfaxolone acetate (1.5 mg/kg; Saffan; Arnolds Veterinary Products), given intramuscularly. Anesthesia was then maintained with 1%–2.5% isoflurane. An individually molded thermoplastic face mask was secured to a custom-made monkey head holder attached to a backboard.

Anatomic MRI brain images were acquired with a 3.0-T Siemens Magnetom Allegra MRI unit (Siemens Medical Solutions). Each MRI scan was performed on a single animal with a continuous intravenous infusion of alfadolone and alfaxolone acetate (8–11 mg/kg/h) to maintain anesthesia.

Vital signs, including heart rate, electrocardiogram (during PET studies only), respiration rate, CO_2 levels, and oxygen saturation in the blood (always maintained above 95%), were continuously monitored during the studies.

A total of 38 PET studies with a bolus administration of 2-FA (34 ± 18 MBq/kg injected intravenously in approximately 1 mL of saline over 20 s, followed by a 1-mL saline flush) were performed; 6 of these studies were designed to saturate the receptors. In control studies, the mass of the administered radioligand ranged from 0.03 to 0.32 nmol/kg (average, 0.13 ± 0.06 nmol/kg). For receptor saturation studies, a known amount of nonradiolabeled 2-FA was added to the radioactive compound to achieve a mass in the range of 0.6–6.1 nmol/kg. The acquisition of dynamic PET scans started with the injection of 2-FA and continued for 5 h in most of the experiments. Five PET experiments were performed with 7 h of scanning time. Five additional PET studies were performed with 8 h of scanning time and with a bolus-plus-infusion administration of 2-FA.

Coregistration and Placement of Regions of Interest (ROIs)

ROIs were drawn on the T1-weighted MR images (voxel size, 0.57 × 0.57 × 1.22 mm) of one monkey by referring to a stereotactic atlas (*11*). To reduce spillover effects, ROIs for the thalamus and the cerebellum (the regions with the highest and the lowest radioactivity accumulations, respectively) were reduced in size by factors of 1.8 and 8, respectively, and placed in the middle of each structure. ROIs for the muscles were placed on the back of the neck, in the area of the semispinalis cervicis, splenius capitis, and obliquus capitis muscles.

The ROI volumes (number of voxels) and the number of planes were 0.15 mL (378 voxels) and 4 planes, 0.12 mL (304 voxels) and 4 planes, 0.28 mL (705 voxels) and 6 planes, 0.13 mL (316 voxels) and 3 planes, and 0.4 mL (1,012 voxels) and 6 planes for the thalamus, cerebellum, temporal cortex, midbrain, and muscle, respectively. This standard ROI template was copied to the MR images for each monkey, and the positions of the ROIs were adjusted, if necessary, without changing the sizes of the ROIs. The final ROIs for each monkey were copied to the coregistered PET images. For coregistration, dynamic PET images for each monkey were resliced to the MRI voxel size (0.57 × 0.57 × 1.22 mm), and average PET images were manually aligned with the corresponding T1-weighted MR images by use of the fusion mode of PMOD v. 2.75 software (PMOD Technologies Ltd.). The obtained transformation parameters were applied to the respective resliced dynamic PET images.

Arterial Plasma Analysis

Arterial blood samples for the measurement of nonmetabolized 2-FA were taken from either saphenous or tail arteries by repeated puncture. Twelve to 20 blood samples (0.1–0.3 mL each) were collected at predetermined intervals that progressed from 2 min to 60 min over the duration of the study. Immediately after collection, samples were dispensed into heparin–lithium fluoride–coated tubes and placed on ice. The plasma was separated by centrifugation for 5 min at 3,000*g*, and radioactivity was measured with a Cobra γ-counter (Packard Instruments). Separation of nonmetabolized 2-FA from the radioactive metabolites was accomplished by solid-phase extraction as described elsewhere (*12*). The radioactivity of the parent compound fraction, corrected for decay and protein binding, was used as an input function in PET data analysis.

The binding of 2-FA to plasma proteins was determined with 9 plasma samples obtained from 9 monkeys. The unbound (free) radioligand was separated by ultrafiltration with a Centricon YM-10 filtration device (Millipore Corp.).

Analysis of PET Data

To obtain standardized uptake values (SUVs), (*13*) radioactivity concentrations (kBq/cm^3) were normalized to the injected dose (kBq) per gram of body weight: SUV = tissue radioactivity/dose radioactivity. One- and 2-tissue-compartment kinetic models (1TCM and 2TCM, respectively) were applied for the calculation of VD_T values in target and reference regions. Nonlinear least-squares fitting of unweighted time–activity curves was performed with the Marquardt algorithm (*14*) and an arterial plasma input function. The contribution of fractional blood volume in the brain was fixed at 5% for all brain ROIs (*15*) and 3% for muscle ROIs (*16*). In addition, 2-FA VD values were quantified by linear graphic analysis (Logan analysis) (*17*). A 4-parameter reference tissue model (*18,19*) and a simplified reference tissue model (*1*) were used for BP* calculations with either the cerebellum or muscle as a reference region.

Blocking and Displacement Studies with Nicotine

Blocking and displacement studies were performed to determine VDnd values and to assess for 2-FA–specific binding sites in the cerebellum and muscle. PET studies were performed at baseline and during continual exposure to nicotine at 2 mg/kg/d (n = 4). For nicotine exposure, 2 ALZET osmotic pumps (model 2004; DURECT Corp.; delivering 0.25 μL/h for 4 wk), each delivering nicotine at 1 mg/kg/d, were implanted under the skin on the back of each animal under ketamine–isoflurane anesthesia. The pumps were implanted 1 wk apart. PET experiments were conducted 1–2 wk after implantation of the second pump.

For the displacement studies (n = 5), 2-FA–specific binding was displaced by an intravenous nicotine infusion. A bolus infusion paradigm (K_{bolus} = 380 min) was used, with 2 mL of radioligand solution being administered over 5 min, followed by continuous infusion over the rest of the study (8 h) at a constant rate of 0.316 mL/h. The average rate (mean ± SD) of 2-FA continuous infusion was 0.04 ± 0.01 nmol/kg/h. At 5.5 h after the beginning of 2-FA administration, displacement of the specifically bound radioligand was initiated by the intravenous administration of nicotine at a rate of 0.24 mg/kg/h for the first 15 min, followed by 0.12 mg/kg/h for the rest of the PET study. Nicotine doses were always given as the free base.

Parametric BP* Maps

Parametric BP* maps were constructed by the method of Gunn et al. (*20*) with PMOD v. 2.75 software. Two types of maps were constructed, one with muscle and another with the cerebellum as a reference region. In both cases, time–activity curves for the