

Non-Steady-State Measurement of *in vivo* Radioligand Binding with Positron Emission Tomography: Specificity Analysis and Comparison with *in vitro* Binding

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We previously have developed a non-steady-state method for *in vivo* measurement of radioligand-receptor binding in brain using positron emission tomography (PET) and ¹⁸F-spiperone (¹⁸F-SP). This method has proven to be highly sensitive to the detection of decreases in the apparent number of available specific binding sites. The purposes of this investigation are to demonstrate the specificity of this PET assay and compare findings to *in vitro* binding assays. Three to six studies were performed in each of five male baboons. Each animal was pretreated with either ketanserin [serotonergic (S₂)], eticlopride [dopaminergic (D₂)], or unlabeled SP to compete with ¹⁸F-SP for specific binding sites. Sequential PET scans and arterial-blood samples were collected for 3 hr after intravenous injection of ¹⁸F-SP. Data were analyzed with a three-compartment model that considered the accumulation of radiolabeled metabolites in arterial blood. Five baboons were killed, and radioligand-receptor binding *in vitro* was measured by homogenate techniques. There was no detectable *in vitro* or *in vivo* specific binding of SP in cerebellum. The specific binding of SP in striatal tissue *in vitro* was approximately 74% to D₂ sites and 26% to S₂ sites, whereas ketanserin displaced all specific binding in frontal cortex. In close agreement, specific binding measured *in vivo* with PET revealed that 68% of apparent striatal binding could be blocked by pretreatment with eticlopride, and 34% by ketanserin. The small apparent difference between receptor binding *in vitro* and *in vivo* may result from the relatively poor resolution of PET. No differences in the number of binding sites in left and right brain regions were detected by either PET or *in vitro* assays. These results provide operational validation for this PET assay of radioligand binding *in vivo* and demonstrate the lack of right/left differences in striatal SP binding.

We have developed a non-steady-state method for measurement of radioligand binding *in vivo* using positron emission tomography (PET) and ¹⁸F-spiperone (¹⁸F-SP). A three-compartment model that represents the *in vivo* behavior of an intravenously injected radioligand forms the basis of this assay technique (Mintun et al., 1984; Perlmutter et al., 1986, 1989). This method has been used to demonstrate an increase in radioligand binding in a patient with untreated parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; Perlmutter et al., 1987).

Validation of this or any other PET-based measurement of *in vivo* radioligand binding (Farde et al., 1986, 1989; Maziere et al., 1986; Wong et al., 1986a,b; Eckernas et al., 1987; Logan et al., 1987; Coenen et al., 1988; Frost et al., 1989; Huang et al., 1989; Swart et al., 1990) has been a difficult task because of the lack of an appropriate "gold standard." Although there is a vast accumulation of data from experiments that employed *in vitro* techniques, these results depend heavily upon assay conditions (Seeman et al., 1984), which clearly differ from the *in vivo* milieu (Bennett and Wooten, 1986; Perlmutter and Raichle, 1986). Nevertheless, some workers have compared *in vitro* binding measurements with those derived from *in vivo* methods (e.g., Farde et al., 1986; Wong et al., 1986b). This approach can yield interesting information but has limitations, because such comparisons depend upon several crucial assumptions that must be reviewed cautiously.

An alternate approach is to determine the ability of the *in vivo* PET assay to detect predictable alterations in radioligand binding. In this regard, we previously have demonstrated the sensitivity of our non-steady-state method to a decrease in the apparent maximum number of available specific binding sites (Perlmutter et al., 1989). That study, however, did not demonstrate the specificity of the *in vivo* PET assay. Therefore, the purposes of this study are to evaluate the specificity of our PET method and to compare it to that measured with *in vitro* techniques using the same ligands and animals. We also have investigated potential left/right-brain differences in receptor binding as detected with either assay technique. These results provide a basis for interpreting studies of baboons with unilateral brain lesions.

Materials and Methods

Positron emission tomography. All PET studies were performed with the PETT VI system in the high-resolution mode (Ter-Pogossian et al., 1982; Yamamoto et al., 1982). Data were recorded simultaneously for

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seven tomographic "slices" with a center-to-center separation of 14.4 mm. In-plane (i.e., transverse) reconstructed resolution was about 12 mm in the center of the field of view, and axial resolution was about 14 mm at the center.

Radioligand. No-carrier-added ^{18}F -SP was prepared as described previously (Perlmutter et al., 1989). Specific activities determined by HPLC ranged from 1000 to 5000 Ci/mmol at the time of injection. The chemical purity of ^{18}F -SP determined by HPLC showed, in the worst case, a total mass of less than 2 μg SP and less than 2 μg nitro-SP. The nitro-SP has at least 100-fold lower affinity for SP binding sites than SP (Pogun et al., 1982).

Experimental procedures. Three to six separate PET studies were done on five different male baboons weighing from 8 to 12 kg. We performed at least two control experiments (i.e., without pretreatment) on each animal. The controls were interspersed among the pretreatment studies for each animal. For each study, the animal was fasted overnight but permitted free access to water until 2 hr prior to the experiment. The animal initially was anesthetized with ketamine (10–15 mg/kg, i.m.), injected with atropine (0.2 mg, i.m.) to decrease secretions, paralyzed with gallamine (2–4 mg/kg, i.v.), intubated with a soft-cuffed endotracheal tube, and ventilated through a heat and moisture exchanger (Breathaid, BA*1530, Terumo Corporation, Tokyo, Japan) with 70% nitrous oxide and 30% oxygen throughout the study. A 20-gauge plastic catheter was inserted after local anesthesia with lidocaine (1%) into a femoral artery to permit arterial-blood sampling, and a similar catheter was placed into an arm vein for administration of drugs and radiotracers. Blood pressure and pulse were monitored with an intraarterial pressure transducer. Body temperature was monitored with a rectal probe (Tele-Thermometer, YSI, Yellow Springs, OH) and maintained between 35.5°C and 37.5°C with a heating blanket. Frequent arterial blood gas measurements confirmed that pCO_2 and pO_2 were constant throughout each procedure.

Unlabeled SP (0.2–1 mg/kg), ketanserin (10–25 mg/kg), (–)-sulpiride (5–10 mg/kg), and eticlopride (0.37–4.0 mg/kg) were dissolved in 5 ml of 0.9% saline or lactate-buffered saline. All compounds were from Research Biochemicals Inc. (Natick, MA) and were injected intravenously with careful monitoring of blood pressure and pulse. Unlabeled SP was injected intravenously over 5–10 min within 30 min of induction of anesthesia and 3 hr prior to administration of ^{18}F -SP. Ketanserin was administered 1 hr prior to ^{18}F -SP; (–)-sulpiride and eticlopride were injected 2 hr prior to ^{18}F -SP.

The head of the baboon was positioned and secured within the scanner using a modified stereotaxic head holder with mouth and ear bars. A lateral skull radiograph with the position of the PET slices marked by radiopaque wires provided a permanent record of the animal's position within the scanner (Perlmutter et al., 1989). Both the modified stereotaxic head holder and the lateral skull radiograph permitted precise repositioning of the animals for repeated studies. Attenuation characteristics of the head were determined prior to each study by obtaining a transmission scan with a ring source of $^{68}\text{Ge}/^{68}\text{Ga}$.

For each experiment, regional cerebral blood flow (CBF) was measured using a 40-sec PET scan after the intravenous injection of 25–40 mCi H_2^{15}O (Herscovitch et al., 1983; Raichle et al., 1983; Videen et al., 1987). Regional cerebral blood volume (CBV) was determined following the forced inhalation of 50–85 mCi C^{15}O and a 5-min scan (Martin et al., 1987; Videen et al., 1987). CBF and CBV were measured just prior to injection of ^{18}F -SP.

At least 3.5 hr after induction of anesthesia, 1.9–16.4 mCi of no-carrier-added ^{18}F -SP (Perlmutter et al., 1989) was injected intravenously over 15 sec. Sequential PET scans were started immediately with the injection and continued repeatedly for 3 hr. Scan durations were 60–120 sec initially; longer scans (up to 10 min) were required at the end of the study to maintain coincidence counts above 100,000 per slice. The midpoint (weighted for radioactive decay of ^{18}F) of each scan was recorded as the time elapsed from the start of radioligand injection.

Arterial-blood samples (0.3 ml) were collected every 3–5 sec for the first 60–120 sec after administration of ^{18}F -SP and then with decreasing frequency during the remainder of the study (Perlmutter et al., 1989). These samples were counted in an NaI(Tl) well-type scintillation counter that was cross-calibrated with PETT VI (Mintun et al., 1984). Eleven arterial-blood samples also were analyzed to determine the percentage of total radioactivity present as ^{18}F -SP and as radiolabeled metabolites (Perlmutter et al., 1989). Each sample was assayed in duplicate, and the mean values for each time point were fit empirically by an equation (Perlmutter et al., 1989). This equation and the measurements of total

^{18}F radioactivity were used to calculate the arterial-blood counts due to ^{18}F -SP at any given time.

The free fraction of ^{18}F -SP in blood, f_1 , was determined for each study using a microfiltration technique (Perlmutter et al., 1989). These determinations were performed in duplicate or triplicate.

Studies on the same animal were performed at least 3 weeks apart to permit sufficient time for recovery. These experiments were approved by the Animal Studies Committee of the Washington University School of Medicine.

Anatomical placement of regions of interest (ROIs; all 1.1 cm \times 1.1 cm in plane), including midline cerebellum, left and right striata, and frontal cortex, was found using proportional measurements and comparisons with a stereotaxic atlas of the baboon brain (Davis and Huffman, 1968; Perlmutter et al., 1989). This permitted identification of a set of ROIs for a control study (i.e., without pretreatment of unlabeled ligand) for each animal. Once this set of ROIs was established, an automated routine extracted regional measurements from identically placed regions on all sequential PET images collected after injection of ^{18}F -SP, as well as from the CBF and CBV images. The same set of ROIs for each animal was used for all subsequent studies in that same animal.

PET data analysis. A three-compartment model describing the *in vivo* behavior of ^{18}F -SP was used to analyze the data from striatal regions, and a two-compartment model (no specific binding of ^{18}F -SP assumed in cerebellum) was used to analyze cerebellar data (Mintun et al., 1984; Perlmutter et al., 1986, 1989). The details of implementation and assumptions of this method have been described (Mintun et al., 1984; Perlmutter et al., 1986, 1987, 1989). Briefly, this method utilizes the following direct measurements: time-dependent regional brain radioactivity, time-dependent concentrations of radioligand and its metabolites in arterial blood, free fraction of radioligand in blood (f_1), and regional CBF and CBV. We then estimate the free fraction of radioligand, f_2 (i.e., the amount free divided by the sum of the free and non-specifically bound radioligand), in cerebellum and assume that it is the same in striatum. Then, for striatal regions, we estimate the local permeability-surface-area product (PS) for ^{18}F -SP, the apparent combined forward-rate constant (k_1' ; i.e., the product of the association rate constant of radioligand for specific binding sites and the apparent maximum number of available specific binding sites), and the dissociation rate constant, k_{-1} , of radioligand-receptor complex (Perlmutter et al., 1986, 1989). Parameter estimation was performed as previously described (Perlmutter et al., 1986).

Additionally, time-dependent radioactivity measurements for frontal, striatal, and cerebellar regions were compared after decay correction and normalization to the mCi of ^{18}F -SP injected.

In vitro analysis. Animals were killed by injection of pentobarbital (100 mg/kg, i.v.); the brains were quickly removed and dissected, and samples were placed on dry ice and stored at -70°C until further analysis. Membranes were prepared as previously described (Todd and Bauer, 1988) and assayed for ^3H -spiperone (specific activity, 23–25 Ci/mmol; New England Nuclear) binding in the presence and absence of various unlabeled compounds. Samples (40–100 μg protein) were assayed in triplicate in a total volume of 1 ml that contained 120 mM NaCl, 50 mM Tris (pH, 7.4), and 0.1–10.0 nM ^3H -spiperone \pm 1 μM unlabeled competitor. All incubations were for 15 min at 37°C . Samples were diluted with cold buffer, rapidly collected onto glass-fiber filters, and washed twice more with cold buffer using a modified Brandel cell harvester. Preliminary studies demonstrated that under these conditions ^3H -spiperone binding displaceable by eticlopride or by ketanserin was saturable, reversible, and maximal by 10–12 min of incubation. The inclusion of 1–2 mM CaCl_2 or 1–4 mM MgCl_2 did not affect binding.

The equilibrium binding variables K_d and B_{max} were determined from membrane binding data using the nonlinear curve-fitting program LIGAND (Munson and Rodbard, 1980). A constant percent error model of variance was assumed. Left/right regional brain values were compared by two-tailed *t* tests using pooled data.

Results

Measurements of regional CBF, CBV, and the free fraction of ^{18}F -SP in arterial blood (f_1), as well as the estimates of the free fraction in brain tissue (f_2) and regional PS for ^{18}F -SP, are shown in Tables 1 and 2. No consistent changes in these variables were found after pretreatment with ketanserin, eticlopride, or unlabeled

Table 1. Regional hemodynamics in baboons measured with PET

Dose	Subject	n	Striatal CBF		Striatal CBV	
			Left (SD)	Right (SD)	Left (SD)	Right (SD)
None	A	2	0.011 (0.004)	0.012 (0.005)	0.033 (0.001)	0.033 (0.001)
	B	3	0.025 (0.010)	0.023 (0.010)	0.038 (0.003)	0.035 (0.003)
	C	3	0.015 (0.005)	0.015 (0.005)	0.038 (0.007)	0.037 (0.008)
	D	3	0.016 (0.006)	0.016 (0.006)	0.041 (0.014)	0.048 (0.019)
	E	2	0.016 (0.001)	0.015 (0.001)	0.040 (0.006)	0.044 (0.004)
Ketanserin						
10	B	1	0.011	0.012	0.040	0.045
14	C	1	0.019	0.019	0.074	0.071
25	A	1	0.018	0.018	0.047	0.041
Eticlopride						
0.4	D	1	0.010	0.010	0.034	0.036
2	D	1	0.015	0.014	0.050	0.049
4	E	1	0.012	0.012	0.021	0.021
SP						
0.2	B	1	0.011	0.011	0.027	0.027
0.2	C	1	0.015	0.015	0.053	0.052
0.2	D	1	0.013	0.012	0.047	0.047

Dose refers to intravenous dose (mg/kg) of unlabeled ligand administered prior to ^{18}F -SP. Subject refers to the identity of each baboon (A-E). Regional CBF (ml/sec/cc) was measured using H_2^{15}O and a 40-sec PET scan. Regional CBV (ml/cc) was measured with C^{15}O and a 5-min PET scan.

beled SP. Individual changes in these values reflect biological variability among subjects, biological variability over time within the same subject, and limitations of the reproducibility of the PET methodology. Measurements of the fraction of radiolabeled metabolites of ^{18}F -SP in arterial blood did not change consistently after pretreatment with any of the unlabeled ligands.

Estimates of relevant binding variables, including the apparent combined forward-rate constant, k_1' (i.e., the product of the association rate constant and the apparent maximum number of specific binding sites), and the dissociation rate constant, k_{-1} , are shown in Table 3. We found no significant right/left differences by paired t tests in the combined forward-rate constants

Table 2. Effect of receptor ligands on free fraction and PS values of ^{18}F -SP

Dose	Subject	n	f_1 (SD)	f_2 (SD)	Striatal PS	
					Left (SD)	Right (SD)
None	A	2	0.040 (0.010)	0.010 (0.001)	0.083 (0.003)	0.082 (0.004)
	B	3	0.044 (0.007)	0.012 (0.0005)	0.060 (0.012)	0.058 (0.009)
	C	3	0.035 (0.002)	0.009 (0.002)	0.067 (0.023)	0.069 (0.022)
	D	3	0.066 (0.014)	0.015 (0.004)	0.040 (0.009)	0.040 (0.010)
	E	2	0.042 (0.017)	0.012 (0.004)	0.053 (0.018)	0.050 (0.022)
Ketanserin						
10	B	1	0.049	0.017	0.030	0.032
14	C	1	0.039	0.013	0.048	0.050
25	A	1	0.040	0.012	0.072	0.069
Eticlopride						
0.4	D	1	0.062	0.014	0.097	0.096
2	D	1	0.081	0.028	0.031	0.028
4	E	1	0.039	0.012	0.068	0.066
SP						
0.2	B	1	0.027	0.008	0.080	0.094
0.2	C	1	0.042	0.010	0.075	0.073
0.2	D	1	0.072	0.020	0.032	0.029

Dose refers to intravenous dose (mg/kg) of unlabeled ligand administered prior to ^{18}F -SP. Subject refers to the identity of each baboon (A-E). f_1 refers to the free fraction of ^{18}F -SP in arterial blood measured by microfiltration techniques. f_2 indicates the free fraction of ^{18}F -SP in brain tissue (i.e., the amount free divided by the sum of the nonspecifically bound and free). This is estimated from the PET and arterial-blood data using a two-compartment model applied to the cerebellum. PS is the permeability-surface-area product of the blood-brain barrier for ^{18}F -SP (cc/sec for a 1 cc tissue volume), calculated as described in Materials and Methods.

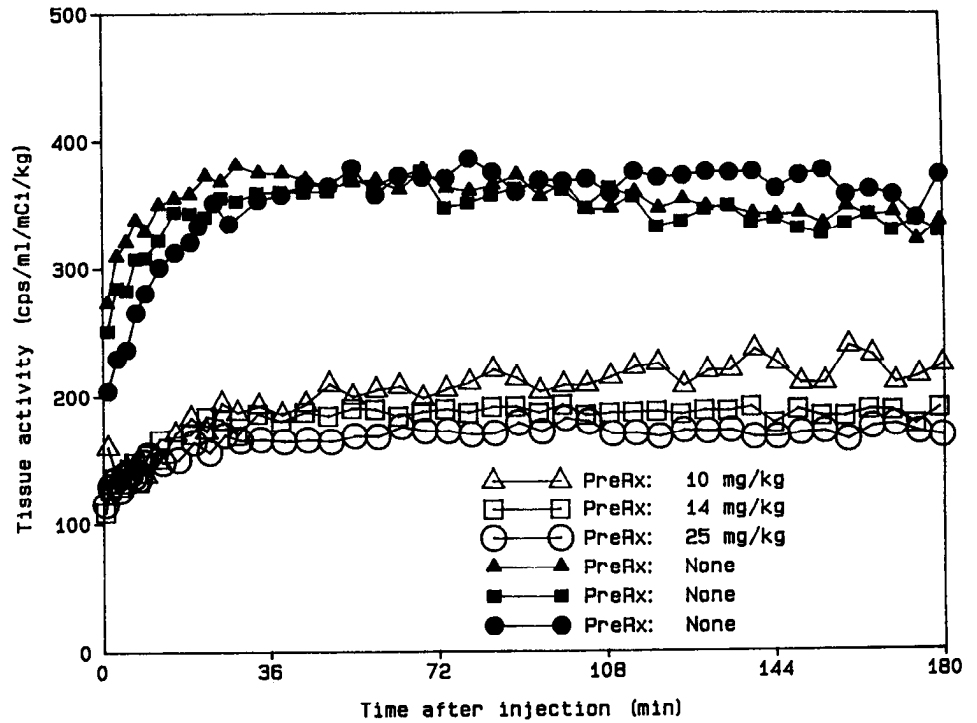


Figure 1. Effects of ketanserin on striatal uptake. Decay-corrected measurements of radioactivity in counts per sec/ml/[mCi injected]/[kg body weight] (cps/ml/mCi/kg) are shown for the left striatum of baboons after the intravenous injection of ¹⁸F-SP. Three “PreRx” studies (open symbols) were preceded by ketanserin (10–25 mg/kg, i.v.) to block S₂ receptors. The other three PET studies (solid symbols) were not preceded by ketanserin administration and served as controls. These data demonstrate that doses of ketanserin greater than 10 mg/kg did not block additional S₂ binding sites in striatum, because the tissue-activity curves were not shifted downward substantially.

Table 3. Kinetic analysis of ¹⁸F-SP binding *in vivo*

Dose	Subject	n	Left striatum		Right striatum		
			k ₁ '	k ₋₁	k ₁ '	k ₋₁	
None	A	2	0.111 ± 0.020 ^a	0.00022 ± 0.00004	0.107 ± 0.019	0.00020 ± 0.00003	
	B	3	0.068 ± 0.010	0.00017 ± 0.00002	0.066 ± 0.008	0.00014 ± 0.00002	
	C	3	0.194 ± 0.066	0.00038 ± 0.00016	0.184 ± 0.088	0.00036 ± 0.00013	
	D	3	0.073 ± 0.002	0.00024 ± 0.00009	0.070 ± 0.006	0.00028 ± 0.00016	
	E	2	0.097 ± 0.058	0.00015 ± 0.00003	0.075 ± 0.031	0.00013 ± 0.00000	
Ketanserin	10	B	1	0.040 (59%) ^b	0.00014 (82%)	0.038 (58%)	0.00013 (93%)
	14	C	1	0.097 (50%)	0.00032 (84%)	0.085 (46%)	0.00026 (72%)
	25	A	1	0.096 (86%)	0.00020 (83%)	0.108 (101%)	0.00022 (110%)
	Mean			65%	83%	68%	92%
Eticlopride	0.4	D	1	0.031 (42%)	0.00028 (117%)	0.030 (43%)	0.00028 (100%)
	2	D	1	0.023 (32%)	0.00066 (275%)	0.019 (27%)	0.00060 (214%)
	4	E	1	0.031 (32%)	0.00052 (347%)	0.028 (37%)	0.00045 (346%)
	Mean ^c			32%	311%	32%	280%
SP	0.2	B	1	0.006 (9%)	0.00003 (18%)	0.012 (18%)	0.00013 (93%)
	0.2	C	1	0.069 (36%)	0.00054 (142%)	0.073 (40%)	0.00057 (158%)
	0.2	D	1	0.012 (16%)	0.00032 (133%)	0.013 (18%)	0.00038 (136%)
	Mean			20%	98%	25%	129%

Dose refers to intravenous dose (mg/kg) of unlabeled ligand administered prior to ¹⁸F-SP. Subject refers to the identity of each baboon (A–E). Binding values are per cc of brain tissue, expressed as sec⁻¹. k₁' denotes the combined forward-rate constant (the product of the apparent maximum number of specific binding sites and the association rate constant of ¹⁸F-SP for those specific sites). k₋₁ is the dissociation rate constant of radioligand-receptor complex. Both of these turnover rate constants are estimated from the PET and arterial-blood data using a three-compartment model as described in Materials and Methods.

^a Control values are expressed as sec⁻¹ ± SD.

^b Percentages are the percent of the mean control values for that subject.

^c Mean of the values after pretreatment with eticlopride includes only the 2 mg/kg and 4 mg/kg values.

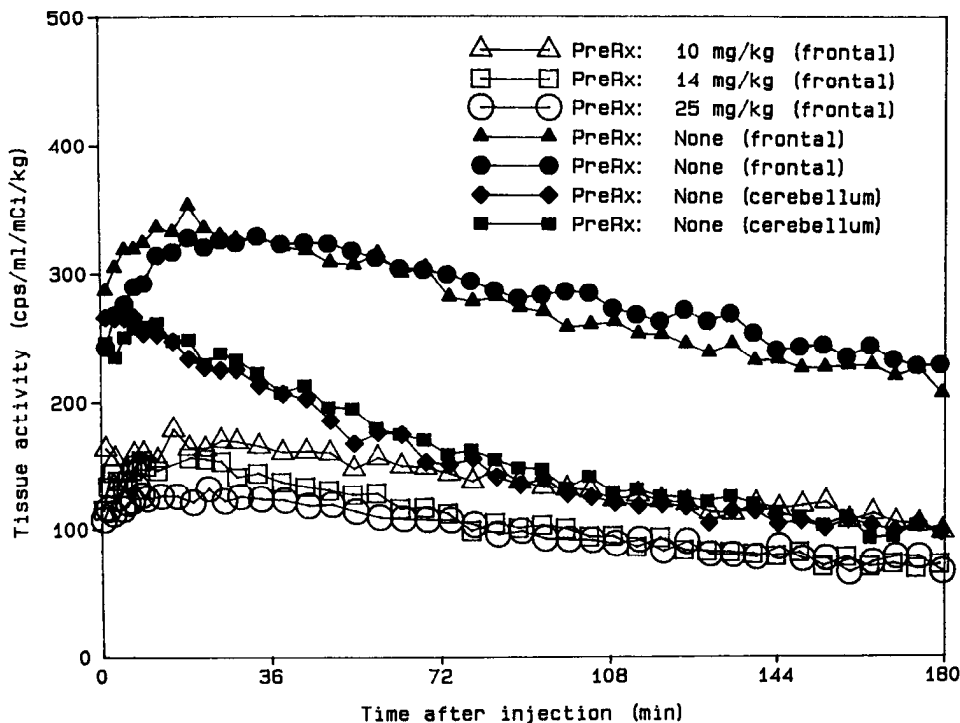


Figure 2. Effects of ketanserin on frontal cortex uptake. Decay-corrected measurements of radioactivity in counts per sec/ml/[mCi injected]/[kg body weight] (cps/ml/mCi/kg) are shown for frontal cortex and cerebellum of baboons after the intravenous injection of ^{18}F -SP. Three "PreRx" studies (open symbols) were preceded by ketanserin (10–25 mg/kg, i.v.) to block S_2 receptors. The other two PET studies (solid symbols) were not preceded by ketanserin administration and served as controls. There were no substantial differences among the frontal tissue-activity curves obtained after pretreatment with the three different doses of ketanserin and the control cerebellar tissue-activity curves. These data demonstrate that doses greater than 10 mg/kg of ketanserin block all observable specific S_2 binding sites in frontal cortex.

($p = 0.31$) or the dissociation rate constants ($p = 0.57$). Because of the variability across subjects, the effects of pretreatment are best demonstrated by comparisons of individual values to the mean control values for the same subject.

Pretreatment with the S_2 ligand ketanserin blocked about 35% (left) and 32% (right) of the apparent specific striatal binding of ^{18}F -SP *in vivo*. The lack of substantial changes in the striatal tissue-activity curves for increasing doses of ketanserin demonstrate that the doses employed gave maximum S_2 receptor-blocking effects (Fig. 1). Furthermore, these doses caused the tissue-activity curves for the S_2 -rich frontal cortex to appear similar to the cerebellar curves (Fig. 2). This strongly supports the notion that the doses of ketanserin that were employed were sufficient to block all S_2 specific binding *in vivo* in brain tissues.

Pretreatment with the D_2 ligand eticlopride (2 or 4 mg/kg) blocked 68% (left) and 73% (right) of the apparent specific binding of ^{18}F -SP *in vivo* in the striata. The higher dose of eticlopride, 4 mg/kg, produced no additional blocking compared to a dose of 2 mg/kg. In contrast, 0.37 mg/kg eticlopride did not block all apparent specific binding sites for ^{18}F -SP (Fig. 3, Table 3).

Pretreatment with unlabeled SP (0.2 mg/kg) decreased the estimated combined forward-rate constant of ^{18}F -SP more than either ketanserin or eticlopride (Table 3). Higher doses of SP reduce specific binding to a greater extent but, in doing so, do not permit estimation of the combined forward-rate constant due to the low amount of specific binding compared to non-specifically bound ^{18}F -SP. For example, pretreatment with unlabeled SP (1 mg/kg) reduced the striatal tissue-activity curve to the level of a cerebellar tissue-activity curve, presumably by blocking all specific binding sites in striatum. However, the combined forward-rate constant could not be estimated with confidence.

Sulpiride at an intravenous dose of 5, 8, and 9.6 mg/kg did not cause appreciable change in striatal tissue-activity curves compared to those of controls. Higher intravenous doses of the

ligand caused cardiovascular instability and could not be used safely in baboons.

Interestingly, none of the unlabeled ligands altered the uptake of radioactivity in cerebellum, as demonstrated by a lack of change in tissue-activity curves after pretreatment with eticlopride, ketanserin, or unlabeled spiperone (Fig. 4). These *in vivo* results are consistent with the lack of specific binding sites in baboon brain as determined *in vitro* (Table 4).

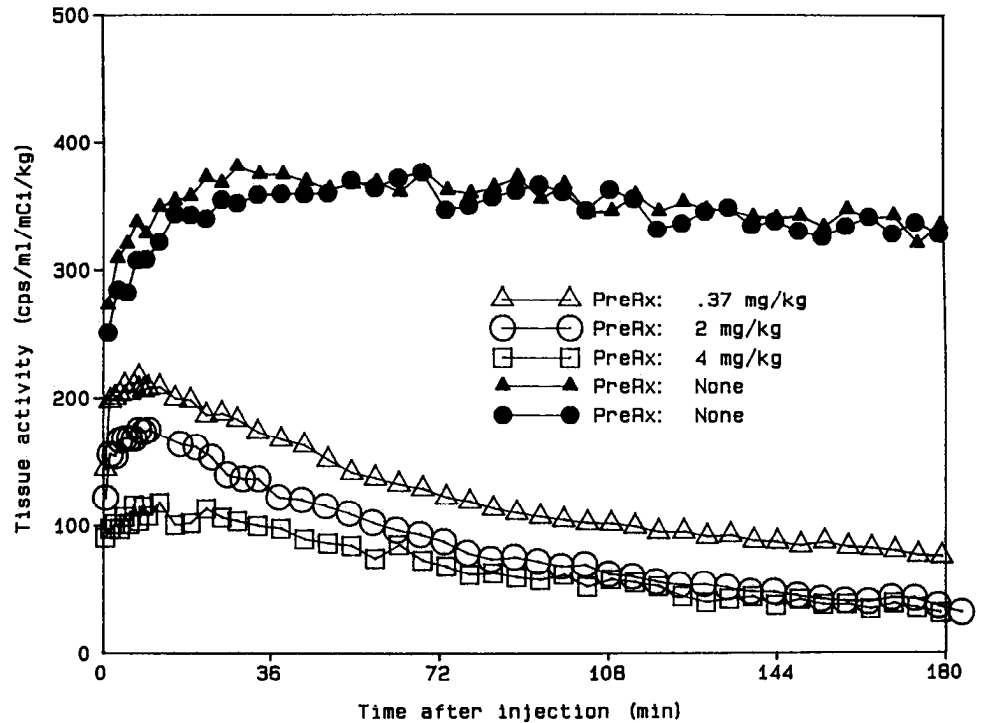
The results of *in vitro* binding studies closely agreed with the results of *in vivo* experiments. As shown in Table 4, binding to D_2 receptors accounted for about 74% of ^3H -SP specific binding in striatum (averaging the B_{max} data for left and right caudate and putamen). Binding by the radioligand to S_2 receptors accounted for the remainder. No specific ^3H -SP binding in cerebellum was detected. Additionally, there were no significant left/right differences in K_d or B_{max} for ^3H -SP binding to either D_2 or S_2 receptors in these tissues. Given the relatively small number of subjects and the variance in measurements (Table 4), there was a 75–90% chance to detect at least a 50% difference in B_{max} but only a 50–80% chance to detect at least a 100% difference in K_d .

A summary of nonspecific binding by ^3H -SP *in vitro* is presented in Table 5. There were no significant differences in nonspecific binding to membranes across brain regions. There were also no significant left/right regional differences in nonspecific binding of ^3H -SP.

Discussion

Results of this investigation further validate our non-steady-state technique for measurement of radioligand binding *in vivo* with PET and ^{18}F -SP. We now have demonstrated the specificity of this *in vivo* assay and compared these results to those of an *in vitro* binding assay. Furthermore, we also have evaluated the adequacy of the baboon's cerebellum as a region of brain devoid of specific binding sites. This type of operational validation is

Figure 3. Effects of eticlopride on striatal uptake. Decay-corrected measurements of radioactivity in counts per sec/ml/[mCi injected]/[kg body weight] (*cps/ml/mCi/kg*) are shown for left striatum of baboons after the intravenous injection of ^{18}F -SP. Three "PreRx" studies (open symbols) were preceded by eticlopride (0.37–4 mg/kg, i.v.) to block D_2 receptors. Two PET studies (solid symbols) were not preceded by eticlopride administration and served as controls. Pretreatment with 0.37 mg/kg did not appear to maximally block specific binding sites in striatum, whereas 2 and 4 mg/kg of the D_2 ligand were not substantially different from each other. The "tails" of the tissue-activity curves reflect binding to receptors to a greater degree than the "fronts" of the curves due to clearance of free and nonspecifically bound ^{18}F -SP.



necessary because of the lack of a "gold standard" for *in vivo* binding and requires complete understanding of the methodological issues that impact interpretation of our findings.

The specificity of this PET measurement depends not only on the receptor specificity of ^{18}F -SP *in vivo*, but also upon the resolution of the PET image. *In vitro* binding studies demonstrated that about 75% of the specific binding of ^3H -SP in striatum was to D_2 sites, whereas only about 68% of the specific binding *in vivo* of ^{18}F -SP was blocked by pretreatment with eticlopride. Although the difference may not be statistically significant, it could reflect that the PET measurement of radioactivity from striatum is "contaminated" by inclusion of spillover from nearby temporal cortex, an area that contains S_2

binding sites for ^{18}F -SP that are unaffected by unlabeled eticlopride. This volume averaging (Mazziotta et al., 1981) may contribute, in part, to the higher apparent S_2 binding in striatum. Fortunately, such volume averaging from nearby tissues will become less important as the resolution of PET scanners continues to improve (Spinks et al., 1988; Litton et al., 1990). An alternate explanation that may contribute to the potential difference between the results from PET and *in vitro* assays is that *in vivo* access to specific binding sites is different from that under *in vitro* assay conditions. Pharmacodynamic factors such as drug-induced alterations by ketanserin or eticlopride in radioligand delivery to the striatum are unlikely to cause a change in the measured apparent combined forward-rate constant. This is be-

Table 4. Specific binding *in vitro* of ^3H -SP to different brain regions

Displacer	Caudate		Putamen		Cerebellum	
	K_d (pM)	B_{\max} (pmol/mg)	K_d (pM)	B_{\max} (pmol/mg)	K_d (pM)	B_{\max} (pmol/mg)
Eticlopride						
Left	54.9 ± 11.7	0.18 ± 0.03	46.5 ± 9.5	0.13 ± 0.02	—	ND
Right	58.0 ± 12.6	0.18 ± 0.02	68.2 ± 16.9	0.11 ± 0.02	—	ND
<i>p</i> (<i>t</i> test)	NS	NS	NS	NS		
Ketanserin						
Left	117.5 ± 54.5	0.08 ± 0.03	159.4 ± 153.0	0.05 ± 0.02	—	ND
Right	15.3 ± 6.8	0.06 ± 0.02	60.9 ± 53.1	0.02 ± 0.02	—	ND
<i>p</i> (<i>t</i> test)	NS	NS	NS	NS		
SP						
Left	113.4 ± 32.5	0.18 ± 0.03	105.3 ± 31.7	0.16 ± 0.03	—	ND
Right	268.0 ± 48.9	0.24 ± 0.03	243.4 ± 55.9	0.20 ± 0.04	—	ND
<i>p</i> (<i>t</i> test)	NS	NS	NS	NS		

All values are means ± SEM for membranes from five baboons assayed two to four times for specific ^3H -SP binding as described in Materials and Methods. ND, not detected; NS, not significant. All *t* tests compared values for tissues from the right and left sides of the brain.

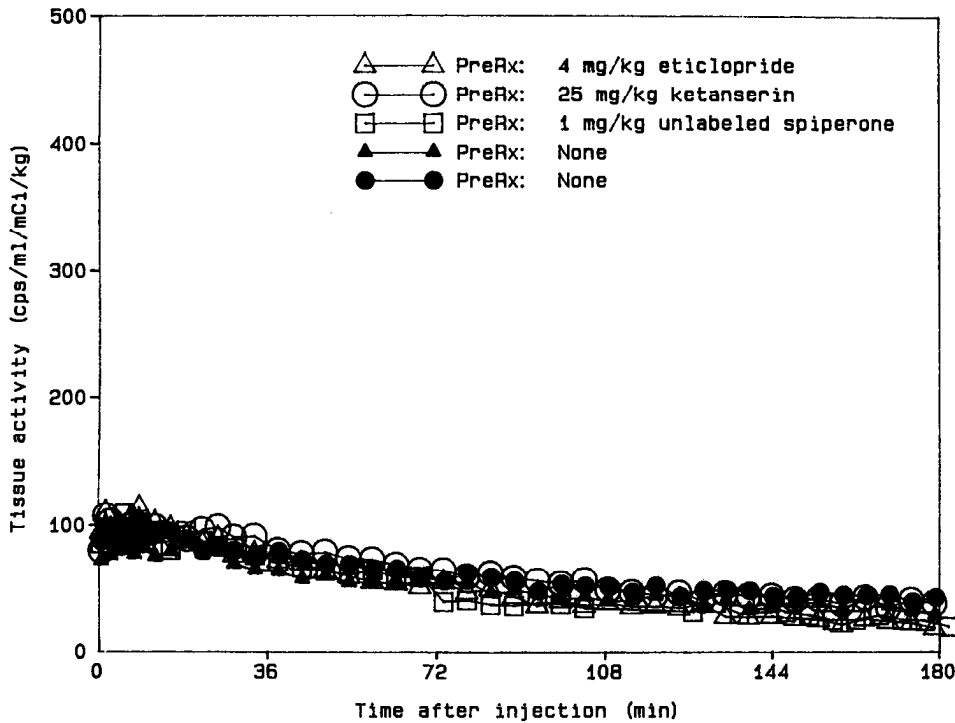


Figure 4. Effects of unlabeled ligands on cerebellar uptake. Decay-corrected measurements of radioactivity in counts per sec/ml/[mCi injected]/[kg body weight] (cps/ml/mCi/kg) are shown for the cerebellum of baboons after the intravenous injection of ^{18}F -SP. Three "PreRx" studies (open symbols) were preceded by intravenous injection of maximal receptor-blocking doses of either eticlopride, ketanserin, or unlabeled SP. The other two PET studies (solid symbols) were not preceded by administration of unlabeled ligands and served as controls. There were no substantial differences among the five tissue-activity curves, which demonstrates that doses of ligands which maximally block available specific binding sites in striatum (Figs. 1, 3; Table 3) do not block specific binding sites in cerebellum.

cause our method measures or estimates changes in variables such as regional CBF or regional PS for ^{18}F -SP and incorporates them into the calculation of the apparent combined forward-rate constant. Additionally, the arterial blood content of ^{18}F -SP did not change with the various pretreatment conditions (i.e., metabolism of the radioligand did not change), nor did the free fraction of ^{18}F -SP in blood change. The relatively close values for specificity between the *in vivo* and *in vitro* methods are noteworthy.

Another important technical issue of the *in vivo* PET assay is the degree that S_2 or D_2 receptors are blocked competitively by the pretreatment doses of ketanserin or eticlopride. We have shown that the dose of ketanserin that we used caused the tissue-activity curve for the frontal cortex (a region that we found by *in vitro* assay to contain only S_2 binding sites for ^{18}F -SP) nearly to overlap the cerebellar curve. Because the cerebellum lacks S_2 receptors, this result suggests that the dose of the S_2 ligand that we used blocked all specific binding *in vivo* by ^{18}F -SP in frontal cortex. Because the frontal cortex has greater S_2 -specific binding than the striatum, it is reasonable to assume, though not absolutely proven, that all striatal S_2 sites were also blocked. Furthermore, higher doses of ketanserin did not change substantially the striatal or frontal cortex tissue-activity curves for ^{18}F -SP (Figs. 1, 2). Pretreatment with eticlopride (0.37 mg/kg) did not appear to block all specific striatal binding sites, as demonstrated by the modest lowering of the tissue-activity curves (Fig. 3) and the intermediate decrease in the magnitude of the apparent combined forward-rate constant for ^{18}F -SP (Table 3). The next-higher dose (2 mg/kg) appeared to block maximally D_2 -specific binding of ^{18}F -SP in striatum, as evidenced by the lack of substantial change in either the striatal tissue-activity curve (Fig. 3) or the estimated combined forward-rate constant for ^{18}F -SP (Table 3) after pretreatment with 4 mg/kg of the unlabeled ligand. Doses of eticlopride greater than 4 mg/kg were not tolerated by the animals.

As previously discussed (Perlmutter et al., 1989), we have

chosen to report our values of the combined forward-rate constant as the "apparent forward-rate constant" because of the possible nonequilibrium conditions for the unlabeled ligand (i.e., eticlopride, ketanserin, or SP) during the course of the ^{18}F -SP study. Maximal binding by ketanserin almost certainly has occurred prior to the injection of ^{18}F -SP. This is because of the relatively fast kinetics of intravenously administered ketanserin and eticlopride, as revealed by preliminary data with the radiolabeled ligands (Baron et al., 1985; Kohler et al., 1986; Moerlein and Perlmutter, 1990).

It should also be noted that the "apparent combined forward-rate constant" and the dissociation rate constant estimated with this PET technique reflect averaged composite values for all specific binding within the ROI. Our data demonstrate that baboon striatum contains S_2 - and D_2 -specific binding sites for ^{18}F -SP. The degree to which these two different sites contribute to the binding values that we estimate depends in part upon their availability. After high-dose eticlopride pretreatment, D_2 sites contribute less (probably none at the higher doses in this study), and the estimated binding constants therefore reflect the characteristics and number of available S_2 sites. The individual contributions from D_2 and S_2 sites are not discernable unless high doses of specific antagonists are used to block binding sites. This could produce undesirable clinical effects in humans and

Table 5. Nonspecific binding *in vitro* of ^3H -SP to different brain regions

	Caudate	Putamen	Cerebellum
Left	0.291 \pm 0.031	0.312 \pm 0.030	0.325 \pm 0.064
Right	0.390 \pm 0.095	0.346 \pm 0.046	0.203 \pm 0.017
<i>p</i> (<i>t</i> test)	NS	NS	NS

Units are fmol bound/nM free/mg protein. All values are means \pm SEM for tissues from five baboons assayed two to four times for ^3H -SP binding not displaceable by $1\ \mu\text{M}$ SP. These values are the n_1 parameter from LIGAND analyses corrected for the amount of protein assayed.

would not be reasonable. More specific radioligands would be a better approach.

Our analyses assume only one type of dopamine D₂ receptor. Recently, evidence for multiple subtypes of D₂ receptors has appeared (Todd et al., 1989; O'Malley et al., 1990; Sokoloff et al., 1990). All of these subtypes bind SP and eticlopride with high affinity and, if present in baboon brain, would have been measured as D₂ sites in this study. Current pharmacological tools cannot distinguish easily among these receptor subtypes. Messenger RNAs for three individual subtypes can be detected with reverse transcription and DNA amplification (O'Malley et al., 1990; Sokoloff et al., 1990). Preliminary studies demonstrate the presence of mRNAs for D2A₄₁₅, D2A₄₄₄, and D₃ receptor subtypes in baboon basal ganglia (R. D. Todd, unpublished observations). How the PET-derived variables k_1' and k_{-1} vary with D₂ receptor subtypes is unknown. More specific radioligands for PET could help clarify these issues.

It is important to note that the apparent combined forward-rate constant changed in a consistent manner with pretreatment of the primates with the various unlabeled ligands. Other estimated variables, such as the regional PS for ¹⁸F-SP and the free fraction in brain tissue, f_2 , did not change consistently. The dissociation rate constant, k_{-1} , also appears to have increased after pretreatment, though not as consistently as k_1' . Interpretation of the change in k_{-1} is more difficult because these estimates are not as reliable as those of k_1' . As shown previously (Perlmutter et al., 1989) and confirmed here, the individual estimates (for each parameter estimation) of k_1' have an average coefficient of variation of 9.9% ($\pm 4.5\%$), significantly lower ($p < 0.000001$; two-tailed, paired *t* test) than the average coefficient of variation for k_{-1} ($14.2\% \pm 5.0\%$).

Others have suggested that the cerebellum is not devoid of specific binding sites (Logan et al., 1987; Swart et al., 1990). Our results do not agree with these conclusions. We found no consistent change in the cerebellar tissue-activity curves after pretreatment with eticlopride, ketanserin, or unlabeled SP. If specific sites were present, large doses of these unlabeled ligands would have diminished the uptake of ¹⁸F-SP. Furthermore, there was no consistent alteration in f_2 estimated from the cerebellar data. Part of this discrepancy with other previous reports may stem from species variability; the rat may have specific binding sites for SP in the cerebellum (Swart et al., 1990) that are not present in primates. On the other hand, Logan et al. (1987) also studied baboons using PET VI in the high-resolution mode and found that pretreatment with (+)-butaclamol prior to injection of ¹⁸F-SP modestly lowered the cerebellar tissue uptake of radioactivity. It is possible that this finding was due to slight misalignment of the animal within the scanner that caused receptor-rich noncerebellar tissue to be included in the "cerebellar" region. Partial volume averaging from nearby occipital lobe, a region that we found with *in vitro* analysis to contain S₂ and D₂ sites (data not shown), may have contributed to the "apparent specific binding" in cerebellum. We have diminished the effects from nearby occipital lobe by also using the high-resolution mode of PET VI as well as by careful placement of the animal within the scanner, as previously described (Perlmutter et al., 1989). Finally, our *in vitro* data also support our contention based on PET data that cerebellum in baboon contains negligible specific binding sites for SP.

Although all of these lines of evidence strongly support the idea that there is a lack of specific binding of ¹⁸F-SP in cere-

bellum, they do not directly address the issue of a regionally constant f_2 . In this study, we found that nonspecific binding *in vitro* was invariant across different brain regions, which suggests that f_2 is regionally invariant. Nevertheless, it is important to realize that the notions of nonspecific binding for *in vitro* and *in vivo* methods may be different. Nonspecific binding as measured *in vitro* is the residual quantity of radioligand remaining in tissue, either homogenate or slices, after the tissue has been exposed to high concentrations of specific antagonists; that is, it is the amount of nondisplaceable radioligand in this tissue, which may include radioligand entrapped in filters, and so on. This is to be distinguished from the displaceable fraction that reflects specific binding. Nonspecific binding *in vivo* superficially appears similar. Its definition is that amount of radioligand in a compartment that is not free and not bound to specific binding sites as defined in a compartmental model developed to reflect the *in vivo* behavior of the radioligand (Perlmutter et al., 1986). Operationally in a brain devoid of specific binding sites, the value of the free fraction acts to modify the movement of radioligand from the extravascular space back to the intravascular compartment (Perlmutter et al., 1986). Although nonspecific binding for *in vitro* and *in vivo* studies appears similar biologically, operationally it may be different depending upon conditions of the assay. Therefore, these *in vitro* data do not prove that an *in vivo* f_2 estimated for ¹⁸F-SP in the cerebellum is identical to that in the striatum.

We did not assume that PS for ¹⁸F-SP is the same for the cerebellum and the striatum, and values of PS were estimated for each region. Recent data suggest that pial vessels in cerebellum have greater intravascular volume than those in the cerebrum (Holash et al., 1990). If the PET ROIs included pial vessels within infolded regions of the cerebellum, then the assumption of a regionally constant PS would be inaccurate, because PS estimates are dependent upon regional CBV (Mintun et al., 1984).

An additional finding of this study (within the limits of statistical power as described in Results) was the lack of left/right differences in specific binding sites in striatum. This was true for both *in vivo* and *in vitro* assays and suggests that unilateral lesion studies can use the unperturbed side of a subject as an internal control. Furthermore, these results indicate that side-to-side randomization can be used for control of surgical techniques, without correcting for laterality differences.

This study provides additional operational validation of the non-steady-state measurement of *in vivo* radioligand binding with PET. Not only has the PET assay performed in a predictable and consistent fashion, but it also gives results that compare closely with those from *in vitro* binding studies. There was considerable intersubject variability in the estimated apparent combined forward-rate constant (Table 3), but much less intrasubject variability. Previous studies have demonstrated the sensitivity of this *in vivo* PET technique to a decrease in the number of apparently available specific binding sites for ¹⁸F-SP (Perlmutter et al., 1989) and to an apparent upregulation of specific binding for ¹⁸F-SP in a patient with untreated MPTP-induced parkinsonism. Therefore, further investigation with this non-invasive methodology is warranted. Its major limitations continue to be the long duration necessary for each PET study (3–4 hr) and the fact that it estimates the apparent combined forward-rate constant for ¹⁸F-SP rather than the maximum number of specific binding sites (B_{max}) itself.

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