Journal of
Cerebral Blood Flow
& Metabolism

11C-DPA-713 has much greater specific binding to translocator protein 18 kDa (TSPO) in human brain than does 11C-(R)-PK11195

Journal:	*Journal of Cerebral Blood Flow and Metabolism*
Manuscript ID	JCBFM-0587-16-ORIG.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Kobayashi, Masato; Kanazawa University, School of Health Sciences Jiang, Teresa ; National Institute of Mental Health Telu, Sanjay ; National Institute of Mental Health Zoghbi, Sami; NIMH, MIB Gunn, Roger; Imanova Limited, Burlington Danes Building Rabiner, EUGENII; Imanova Limited, Clinical Applications Owen, David; Imperial College London, Guo, Qi; Integrated Sciences & Technology, Global Pharmaceutial R&D Pike, Victor; National Institute of Mental Health Innis, Robert; NIMH, MIB Fujita, Masahiro; National Institute of Mental Health, PET Radioligand Project Team, the Biomarkers Consortium ; the Biomarkers Consortium PET Radioligand Project Team
Keywords:	Positron Emission Tomography, Brain Imaging, Inflammation, Kinetic modelling, Microglia, Receptor imaging

^{11}C-DPA-713 has much greater specific binding to translocator protein 18 kDa (TSPO) in human brain than does ^{11}C-(*R*)-PK11195~~Comparison of four ^{11}C-labeled radioligands to quantify translocator protein 18 kDa (TSPO) in human brain: DPA-713, ER176, PBR28, and (*R*)-PK11195~~

Masato Kobayashi*[1], Teresa Jiang*[1], Sanjay Telu[1], Sami S. Zoghbi[1], Roger N. Gunn[2,3], Eugenii A. Rabiner[2], David R. Owen[3], Qi Guo[2], Victor W. Pike[1], Robert B. Innis[1], ~~and~~ Masahiro Fujita[1], and the Biomarkers Consortium PET Radioligand Project Team

[1] *Molecular Imaging Branch, National Institute of Mental Health, National Institute of Health, Bethesda, Maryland, USA*

[2] *Imanova Ltd, London, UK.*

[3] *Division of Brain Sciences, Department of Medicine, Imperial College, London, UK.*

* These authors contributed equally to this work.

For submission to *J. Cereb. Blood Flow Metab.* as an Original Article, August 2016

Running Headline: Higher specific binding of DPA713 than PK111195~~Comparison of four ^{11}C-radioligands for TSPO~~

Word count: ~~4299~~ 3991 words (<6,000)

Correspondence:
Masahiro Fujita, MD, PhD
Staff Scientist
National Institute of Mental Health
Bldg. 10, Rm. B1D43, 10 Center Drive, MSC-1026,
Bethesda, MD 20892-1026, USA
Phone: +1-301-451-8898
Fax: +1-301-480-3610
E-mail: fujitam@mail.nih.gov

Funding

This study was funded by the Intramural Research Program of the National Institute of Mental Health, NIH (project numbers ZIAMH002852 and ZIAMH002793 under clinical protocols NCT02147392 and NCT02147392) and as a public-private partnership supported by the NIMH and the Foundation for the NIH Biomarkers Consortium (www.biomarkersconsortium.org).

ABSTRACT

Positron emission tomography (PET) radioligands for translocator protein 18 kDa (TSPO) are widely used to measure neuroinflammation, but controversy exists whether~~regarding whether the magnitude of superiority~~ second generation radioligands ~~are~~ are superior to~~have actually better than~~to the prototypical agent ^{11}C-(*R*)-PK 11195 in human imaging. This study sought to quantitatively measure the "signal to background~~noise~~" ratio (assessed as binding potential (BP_{ND})) of ^{11}C-(*R*)-PK11195 compared to one of the most promising second generation radioligands, ^{11}C-DPA-713. Healthy subjects had dynamic PET scans and arterial blood measurements of radioligand after injection of either ^{11}C-(*R*)-PK11195 (16 subjects) or ^{11}C-DPA-713 (22 subjects). To measure the amount of specific binding, a subset of these subjects was scanned after administration of the TSPO blocking drug XBD173 (30 – 90 mg PO). ^{11}C-DPA-713 showed a significant sensitivity to genotype~~, whe~~ in brain, whereas ^{11}C-(*R*)-PK11195 did not. Lassen occupancy plot~~Lassen/occupancy plot~~ analysis revealed that the specific binding of ^{11}C-DPA-713 was much greater than that of ^{11}C-(*R*)-PK11195. The BP_{ND} in high-affinity binders ~~(HABs)~~ was about ~~7~~ten-fold higher ~~3~~4.8 for ^{11}C-DPA-713 (7.3) than ~~, but only 0.75~~ for ^{11}C-(*R*)-PK11195 (0.75). ~~U~~Although the high specific binding of ^{11}C-DPA-713 suggests it is an ideal ligand to measure TSPO, we also found that its distribution volume increased over time, consistent with the accumulation of radiometabolites in brain. ~~sing published data from studies of two other prominent TSPO radioligands, the rank order of increasing sensitivity of these four radioligands (based on BP_{ND}) in HABs was: ^{11}C-(*R*)-PK11195 (0.8) < ^{11}C-PBR28 (1.2) < ^{11}C-DPA-713 (4.8) ≈ ^{11}C-ER176 (4.2).~~

Keywords: 18 kDa translocator protein (TSPO); positron emission tomography; rs6971 polymorphism; XBD173, metabolite-corrected arterial input

INTRODUCTION

Translocator protein 18 kDa (TSPO) is highly expressed in inflammatory cells, including activated microglia, macrophages, and reactive astrocytes[1]. ^{11}C-PK 11195 was the first PET radioligand developed to image TSPO[2], and its higher affinity enantiomer, ^{11}C-(*R)*-PK11195[3], has successfully imaged areas of inflammation in brain[4] and in the periphery[5]. Several second generation ^{11}C-labeled radioligands have been developed to increase the specific (i.e., receptor-bound) component of brain uptake; these include ^{11}C-DAA1106[6], ^{11}C-PBR28[7], and ^{11}C-DPA-713[8]. These second generation radioligands are widely thought to have greater "signal to background~~noise~~" ratio than ^{11}C-(*R)*-PK11195, based largely on full-blockade studies in monkeys. As a measure of signal to background~~noise~~, we used binding potential (BP_{ND}), which is the ratio at equilibrium of specific to nondisplaceable uptake, and found that the BP_{ND} of ^{11}C-PBR28[9] in brain of rhesus macaques was 70-fold higher than that of ^{11}C-(*R*)-PK11195[10]. However, comparable blocking studies for ^{11}C-(*R*)-PK11195 have not been performed in human subjects. Instead, the BP_{ND} value for ^{11}C-(*R*)-PK11195 in human brain has been indirectly estimated with a pseudo-reference region[11] but never directly measured.

Imaging studies conducted with the second-generation radioligand ^{11}C-PBR28 found that the subject's genotype affected the affinity of radioligand binding to TSPO[12]. Specifically, the single nucleotide polymorphism (SNP) rs6971 generates a co-dominantly expressed genotype: homozygous high affinity binder (HAB), homozygous low affinity binder (LAB), and heterozygous mixed affinity binder (MAB)[13]. All second-generation radioligands tested to date are sensitive, more or less, to this polymorphism, with an in vitro affinity ratio of HAB to LAB of about 4:1 for DPA-713 and 50:1 for PBR28[14]. However, the sensitivity of ^{11}C-(*R*)-PK11195 to this genotype is unclear. Although in vitro receptor binding studies found that ^{11}C-(*R*)-PK11195

is insensitive to the genotype[14], in vivo imaging results ~~have been mixed~~are inconsistent among organs. ~~In addition, w~~Whole-body imaging studies suggest that the brain is not sensitive to this genotype but that peripheral organs like lung and heart are[10]. However, these results were not based on ~~planar whole body images~~full quantification using radiometabolite-corrected arterial input function; ~~the increased sensitivity of tomographic imaging~~full quantification might be able to distinguish whether the brain is also sensitive to polymorphism rs6971.

This study sought to directly measure in humans the "signal to backgroundnoise ratio" (assessed as BP_{ND}) of ^{11}C-(*R*)-PK11195 using the receptor blocking drug XBD173[15, 16] in humans, and to compare it with one of the most promising second generation radioligands, ^{11}C-DPA-713, which showed superiority to ^{11}C-(*R*)-PK11195 in rodent model of neuroinflammation [17] and superiority to a widely used second generation radioligand ^{11}C-PBR28 in patients with epilepsy [18]. We compared these two radioligands with regard to their sensitivity to polymorphism rs6971 and to their ability to absolutely quantify TSPO in brain as distribution volume (V_T~~). We further compared these results with those published for two other prominent ^{11}C-labeled radioligands, namely ^{11}C-PBR28 and because this radioligand has been widely used during recent years^{11}C-ER176~~ [19-21].

MATERIALS AND METHODS

Radiopharmaceutical Preparation

^{11}C-DPA-713 was synthesized as described previously[22] with high radiochemical purity (>99%) and a specific activity of 104 ± 57 GBq/μmol at the time of injection under our Investigational New Drug Application (IND) #116,950. ^{11}C-(*R*)-PK 11195 was synthesized as

described previously[23] with high radiochemical purity (>98%) and specific activity at time of injection of 104 ± 71 GBq/μmol under IND #101,908.

Subjects

This study was approved by the Institutional Review Board of the National Institutes of Health, and informed consent was obtained from all subjects. Twenty-two healthy volunteers (11 males, 11 females, 32 ± 9 years old) had ^{11}C-DPA-713 brain PET scans. The distribution of gender and affinity type was 9M/5F HABs, 1M/4F MABs, and 1M/2F LABs. Sixteen healthy volunteers (7 males, 9 females, 32 ± 10 years old) had ^{11}C-(*R*)-PK11195 scans. The distribution of gender and affinity type was 1M/4F HABs, 4M/4F MABs, and 2M/1F LABs. All subjects were free of current medical or psychiatric illnesses based on medical history, physical examination, electrocardiogram, and laboratory blood and urine tests, which included a complete blood count, serum chemistries, thyroid function test, urinalysis, and urine drug screening. TSPO affinity type was determined by in vitro receptor binding to TSPO on leucocyte membranes or genetic analysis as previously described[24].

Measuring ^{11}C-DPA-713 and ^{11}C-(*R*)-PK11195 in Arterial Blood

To determine arterial input function for brain PET scans, blood samples (1 mL each) were drawn from the radial artery at 15-second intervals until 2.5 minutes, followed by samples (3–11 mL) at 3, 4, 6, 8, 10, 15, 20, 30, 40, 50, 60, 75, and 90 minutes. The concentration of parent radioligand was measured using high-performance liquid chromatography (HPLC) after separating plasma from whole blood within a few minutes of blood sampling, as previously described[25]. The activity of whole blood was also measured.

Plasma concentration of the parent compound and whole blood activity were fitted to a triexponential function with weighting to minimize the relative distances of the measured values from the fitted values (relative weighting). For one subject who had ^{11}C-DPA-713 scans with and without XBD173, biexponential fitting was performed because triexponential fitting did not converge. The plasma free fraction (f_P) was measured by ultrafiltration, as previously described[26].

Scan Procedures

Most PET scans were performed on an Advance Tomograph (GE Healthcare, UK) except ~~or~~ three pairs of baseline scans and the those after XBD173 administration using ^{11}C-DPA-713 where a High-Resolution Research Tomograph (HRRT) scanner (CTI, Knoxville, TN, USA) was used. ~~To compare baseline scans and the scans after XBD173 administration, the same scanner was always used and the two scans~~All pairs of baseline and XBD173 blocked scans were performed on the same day. After injection of ^{11}C-DPA-713 or ^{11}C-(*R*)-PK11195 over one minute, dynamic three-dimensional emission scans were acquired for 90 minutes. The dynamic data were separated into 27 frames of increasing duration from 30 seconds to five minutes. One transmission scan using ^{68}Ge or ^{137}Cs rod was acquired before radioligand injection. The position of the transmission scan was corrected for motion before applying attenuation correction. PET images were reconstructed with filtered back projection (Hanning filter with 4.0 cutoff) or ordered subset expectation maximization (2 iterations and 30 subsets) for Advance Tomograph and HRRT, respectively. After the image reconstruction, to match spatial resolution, HRRT data were smoothed with 6.5 mm full-width-half-maximum using a Gaussian filter.

XBD173 Administration

To induce partial blockade of radioligand binding to TSPO, XBD173 (401.5 g/mol) was administered orally ~105 minutes before injection of the PET ligand[15]. XBD173 was administered in six HABs who had ^{11}C-DPA-713 scans and all 16 subjects who had ^{11}C-(*R*)-PK11195 scans. Dosing occurred as follows: for ^{11}C-DPA-713 scans, 30 mg for three subjects and 90 mg for three subjects; for ^{11}C-(*R*)-PK11195 scans, 45 mg for six subjects and 90 mg for 10 subjects. We began with a dose of 45 mg for ^{11}C-(*R*)-PK11195, and our goal was to achieve >50% receptor occupancy for accurate estimate of V_{ND}. Because ^{11}C-(*R*)-PK11195 scans showed variable receptor occupancy due to low levels of specific binding, we subsequently increased the dose to 90 mg for ^{11}C-(*R*)-PK11195. However, for ^{11}C-DPA-713, which was studied later and showed greater BP_{ND}, even the lowest dose of 30 mg provided measurement of 7162-100% receptor occupancy.

Brain Image Analysis

Image and kinetic analyses for brain PET images were performed using PMOD (version 3.6). All time frames of dynamic PET images were realigned for motion correction. After coregistering PET and a sagittal MR image of 1-mm contiguous slices obtained using a 3.0-T Achieva device (Philips Health Care, Andover, MA, USA), the images were spatially normalized to the MRI template in Montreal Neurological Institute (MNI) space. The PET data in the following volumes of interest (VOI) were obtained in the MNI space: frontal (432 cm^3), occipital (172), parietal (247), temporal (251), medial temporal (36), and cingulate (28) cortices, thalamus (17), caudate (16), putamen (17), cerebellum (195), brainstem (6.5), and white matter (8.3).

Calculating Distribution Volume with Radiom~~M~~etabolite-Corrected Arterial Input Function

Distribution volume (V_T) is an index of receptor density and equals the ratio at equilibrium of the concentration of radioligand in tissue to that in plasma. The concentration of radioligand in tissue represents the sum of specific binding (receptor-bound) and nondisplaceable uptake (nonspecifically bound and free radioligand in tissue water)[27].

Regional V_T values were calculated using an unconstrained two-tissue compartment model and Logan plot[28], using the radiometabolite-corrected plasma input function. For the two-compartment model, brain data for each frame were weighted by assuming that the standard deviation/mean of the data was proportional to the inverse square root of noise equivalent counts; radioactivity in whole blood was used to correct brain data for the vascular component, assumed to be 5% of total brain volume. The delay between the arrival of ^{11}C-DPA-713 or ^{11}C-(*R*)-PK11195 in the radial artery and brain was estimated by fitting the whole brain. For Logan plot, weighting, delay, and consideration of the vascular component were not included as in ~~typical~~ the standard Logan plot analyses. The one-compartment model was not considered because previous studies had reported the superiority of the two-compartment model for both ^{11}C-DPA-713 [7] and ^{11}C-(*R*)-PK11195[8, 29]. Because almost all brain regions express TSPO, we did not apply a reference region method in the kinetic analysis.

Time-Stability Analysis

To determine the minimal scan length for reliable measurement and to indirectly assess whether ^{11}C-DPA-713 or ^{11}C-(*R*)-PK11195 radiometabolites enter the brain, the time-stability of V_T was examined by increasingly truncating the 90-minute scan by five-minute increments to the

shortest length of 0 to 40 minutes in baseline scans. ~~The time stability of V_T was also examined in six ^{11}C-PBR28 scans after administration of XBD173 using previously published data[15].~~

Estimating Nondisplaceable Distribution Volume

A modified Lassen plot was used to estimate the specific and nondisplaceable components of ^{11}C-DPA-713 and ^{11}C-(*R*)-PK11195 in brain in two ways: 1) with ~~the~~ Lassen occupancy plot using the difference in V_T at baseline and after partial blockade with XBD173, and 2) with the polymorphism plot (^{11}C-DPA-713 only), using the difference in V_T between two affinity types[30 31]. In general, the Lassen plot enables estimation of nondisplaceable uptake (V_{ND}) as *x*-intercept by measuring the linear relationship of total uptake (V_T) as *x*-axis and specific binding (V_S) as *y*-axis in several brain regions. Here, V_S can be substituted with proportional variables:

1) Lassen occupancy plot: V_T at baseline ($V_T^{Baseline}$) minus V_T after partial blockade (V_T^{Block}) for occupancy plot, under the assumption that V_{ND} and the percentage occupancy are the same across all regions.

$$V_T^{Baseline} - V_T^{Block} = Occ \cdot (V_T^{Baseline} - V_{ND}) \quad (1)$$

Equation (1) indicates that the slope and *x*-intercept correspond to receptor occupancy and V_{ND}, respectively. To improve identifiability of V_{ND} for ^{11}C-DPA-713 and ^{11}C-(*R*)-PK11195, the regression was performed by constraining V_{ND} to be equal among HABs or MABs.

2) Polymorphism plot: Group mean V_T for HABs (V_T^{HAB}) minus group mean V_T for MABs (V_T^{MAB}) for polymorphism plot, under the assumption that V_{ND} is uniform across all regions. Similar to the occupancy plot, this assumption leads to the following:

$$V_T^{HAB} - V_T^{MAB} = \Delta \cdot (V_T^{HAB} - V_{ND}) \quad (2)$$

Equation (2) estimates both genetic effect (the slope) and a population average for V_{ND} (the *x*-intercept). The same plot was performed using data from the other pairs of affinity types, i.e., HABs vs. LABs and MABs vs. LABs.

Statistical Analysis

The identifiability of V_T by the two compartment model was expressed as a percentage and equaled the ratio of the standard error (SE) of V_T divided by the value of V_T itself. A lower percentage indicates better identifiability. Repeated measures two-way analysis of variance was used to compare V_T among different affinity groups by using region as the repeating parameter within subjects. A value of $P < 0.05$ was considered significant. Linear regression for the modified Lassen occupancy and polymorphism plots was performed using Prism 5 (GraphPad Software, Inc. La Jolla, CA, USA). The other statistical analyses were performed with SPSS (version 22 for Windows; SPSS Inc. Chicago, IL, USA). Group data are expressed as mean ± SD.

RESULTS

Pharmacologic Effects

No adverse or clinically detectable pharmacologic effects were observed with either ^{11}C-DPA-713 (28 scans) or ^{11}C-(*R*)-PK11195 (32 scans), or in any of the 22 subjects (six and 16 subjects who had ^{11}C-DPA-713 and ^{11}C-(*R*)-PK11195 scans, respectively) who received XBD173. Supplemental Table 1 lists the administered doses of the PET ligands. No significant changes in heart rate, blood pressure, respiratory rate, or ECGs were observed during the PET scan, nor in blood or urine test results repeated after the scan.

Kinetic Analysis

The brain time-activity curves of ^{11}C- DPA-713 clearly showed its sensitivity to polymorphism rs6971, whereas ^{11}C-(*R*)-PK11195 showed no sensitivity (Fig. 1). For HABs and MABs, ^{11}C- DPA-713 showed greater peak radioactivity uptake and slower washout than ^{11}C-(*R*)-PK11195, indicating the presence of greater specific binding of this second-generation radioligand.

Three major findings were obtained during the kinetic analysis of brain and plasma data. First, the Logan plot gave excellent fitting for all datasets (Supplemental Fig. 1) and identified V_T well for both radioligands, but an unconstrained two-tissue compartment model failed to identify V_T well in some scans. For ^{11}C- DPA-713 the two-tissue compartment model poorly identified V_T (%SE = ≥ 10) or failed to converge in three to nine out of the 12 regions in five of 28 scans. For ^{11}C-(*R*)-PK11195 scans the two-tissue compartment model poorly identified V_T (%SE ≥ 10%) in three to seven regions in three of 32 scans. Because the Logan plot can underestimate V_T when the data are noisy, we compared it to the two-tissue compartment model in the datasets where the compartment model showed good identifiability of < 10% SE. In fact, the Logan plot minimally underestimated V_T values: -2.8±11.8% for ^{11}C-DPA-713 and -0.3±6.0% for ^{11}C-(*R*)-PK11195. Thus, because the Logan plot identified V_T well in all regions and did not underestimate V_T, we used this non-compartmental approach to compare the two radioligands.

The second major finding was that ^{11}C-DPA-713 showed significant sensitivity to genotype, whereas ^{11}C-(*R*)-PK11195 did not. Statistical analysis confirmed that V_T for HABs, MABs, and LABs differed significantly for ^{11}C-DPA-713 (P=0.001, F=10.95, df=2, 19), but not for ^{11}C-(*R*)-PK11195 (P=0.37, F=1.06, df=2, 13) (Table 1). For example, the whole brain V_T of

^{11}C- DPA-713 in HABs (3.6 ± 0.6 mL · cm^{-3}) was about 1.9-fold higher than that in MABs (1.9 ± 0.2 mL · cm^{-3}).

The third major finding was that V_T for both radioligands was unstable over time – i.e., V_T increased with increasing scan duration, consistent with the accumulation of radiometabolites in brain. The most sensitive condition for assessing the presence of radiometabolites is when there is little or low levels of specific receptor binding in brain by the parent radioligand to dilute the impact of radiometabolites. For this reason, we compared the time-stability of the radioligands in LABs and also in HABs after administration of XBD173 (Fig. 2 and Table 2). During the last 40 minutes of the scan of LABs, V_T of whole brain increased by 25% for ^{11}C-DPA-713 and, similarly, by 26% for ^{11}C-(*R*)-PK11195. Scans of HABs after XBD173 administration showed 13% increase for ^{11}C- DPA-713 and a larger 27% increase for ^{11}C-(*R*)-PK11195. Given that ^{11}C- DPA-713 has greater specific binding than ^{11}C-(*R*)-PK11195, this apparent effect of radiometabolites was diminished for ^{11}C- DPA-713 in HABs (Fig. 2A). That is, during the same time interval, V_T of whole brain increased by only 8% for ^{11}C-DPA-713 but by 26% for ^{11}C-(*R*)-PK11195.

Estimating Specific and Nondisplaceable Uptake

For ^{11}C-DPA-713, BP_{ND} was estimated in two ways: by receptor blockade in HABs and by the so-called polymorphism plot, which compares uptake between two affinity types[15, 31]. After receiving XBD173, all six HABs showed marked blocking effects, both in plasma and in brain (Fig. 3). In plasma, XBD173 significantly increased the concentration of ^{11}C-DPA-713, consistent with its blocking the distribution of the radioligand to peripheral organs[23]. In brain, receptor blockade increased peak radioactivity uptake (secondary to higher plasma

concentrations), increased washout, and decreased total uptake over the 90-minute scan. The Lassen occupancy plot ~~Lassen plot~~ of baseline and blocked scans showed an excellent linear regression and determined V_{ND} as 0.44~~61~~ mL · cm^{-3} with a 95% confidence interval of 0.34 – 0.53~~78~~ and receptor occupancy (i.e., slope) of 91~~6~~ ± 12~~8~~% (Fig. 4A). Based on the estimated V_{ND} (0.44~~61~~), the ratio of specific to nondisplaceable uptake (i.e., BP_{ND}) for whole brain was 7.3~~4.8~~ ± 2.2~~1~~, 3~~2~~.4~~2~~ ± 0.3, 1.8~~0~~ ±0.2 in HABs, MABs, and LABs, respectively.

The polymorphism plot, which was the second method of estimating the BP_{ND} of ^{11}C-DPA-713, showed excellent fit for all three combinations of the genotypes (Fig. 5). V_{ND} and its 95% confidence interval (in parentheses) was 1.21 (0.87–1.56) for HABs – MABs, 0.88 (0.32 – 1.44) for HABs - LABs, and 0.99 (0.76 – 1.23) for MABs – LABs. The average (1.03) of these three V_{ND} values from the polymorphism plot was 2~~1~~.3~~7~~ times that obtained via the Lassen occupancy plot~~Lassen/occupancy plot~~ (0.44~~61~~). BP_{ND} of whole brain was 2.59±1.32 for HABs, 0.88±0.36 for MABs, and 0.18±0.41 for LABs. Largely because of the higher values of V_{ND} from the polymorphism plot, the BP_{ND} values from the genetic analysis were about one third~~half~~ those obtained via the Lassen occupancy plot~~Lassen/occupancy plot~~ (Table 1). The cause for the different V_{ND} by Lassen occupancy and polymorphism plots is unclear but possibilities are the small sample size and intersubject variability in V_{ND} including possible differences among different affinity types.

For ^{11}C-(*R*)-PK11195, BP_{ND} was estimated with only receptor blockade because ^{11}C-(*R*)-PK11195 showed no significant sensitivity to genotype (Table 1). ^{11}C-(*R*)-PK11195 showed relatively small blocking effects from XBD173, both in plasma and brain (Fig. 3), consistent with ^{11}C-DPA-713 having greater specific binding in brain and periphery. The Lassen occupancy plot~~Lassen plot~~ of baseline and blocked scans determined V_{ND} well as 0.42 (95% C.I. 0.31 - 0.53)

and 0.39 mL · cm^{-3} (95% C.I. 0.28 - 0.51) for HABs and MABs, respectively (Fig. 4B and C). However, this good identifiability of V_{ND} was achieved only by constraining V_{ND} to be equal among HABs (Fig. 4B) or MABs (Fig. 4C) because of noisy data due to low specific binding in baseline scans. Receptor occupancy was 31% ± 29% in HABs and 23% ± 18% in MABs. Using V_{ND} values from these Lassen occupancy plots~~Lassen plots~~ (0.42 for HABs and 0.39 for MABs), we calculated that the ratio of specific to nondisplaceable BP_{ND} for whole brain was 0.75 ± 0.32 in five HABs and 0.89 ± 0.40 in MABs. Because XBD173 binding is affected by the rs6971 SNP[32], the three LABs who received XBD173 showed no measurable binding blockade. By using the average V_{ND} in HABs and MABs, BP_{ND} in whole brain in LABs was 0.48 ± 0.44.

Because only free ligand enters the brain, V_T/f_P theoretically reflects receptor binding more accurately than V_T, although the additional measurement of f_P may decrease precision. XBD173 might have slightly altered f_P for both ^{11}C-DPA-713 and ^{11}C-(*R*)-PK11195 (Supplemental Table 2). Therefore, Lassen occupancy plots~~Lassen plots~~ were performed using V_T/f_P. For ^{11}C-DPA-713, both occupancy (Supplemental Fig. 2A) and polymorphism (Supplemental Fig. 3) plots showed a linear regression with high R^2 and narrow confidence intervals for *x*-intercept, which were similar to the plots obtained using V_T. For occupancy plot, BP_{ND} was similar regardless of whether V_T/f_P or V_T was used: 7.4~~4.6~~ for HABs using V_T/f_P (Supplemental Table 3) versus 7.3~~4.8~~ using V_T (Table 2~~1~~). The polymorphism plot using V_T/f_P yielded a BP_{ND} of 3.9 for HABs versus 2.6 using V_T. For ^{11}C-(*R*)-PK11195, the occupancy plot obtained using V_T/f_P provided a good linear regression with high R^2 and a narrow confidence interval for HABs (Supplemental Fig. 2B) but not for MABs (Supplemental Fig. 2C). One MAB showed a paradoxical 55% increase in V_T/f_P after binding blockade, but this may have been due to f_P measurement errors; occupancy plot was therefore performed after eliminating this subject

(Supplemental Fig. 2D). The occupancy plots for HABs (Supplemental Fig. 2B) and MABs (Supplemental Fig. 2D) yielded V_{ND}/f_P of 54 and 44 for HABs, and MABs, respectively. BP_{ND} for HABs was 1.3 (Supplemental Table 3), which was greater than the 0.75 obtained using V_T (Table 1).

DISCUSSION

^{11}C-DPA-713 was markedly superior to ^{11}C-(*R*)-PK11195 for quantifying TSPO in healthy human brain. With regard to perhaps the single most important comparison, the BP_{ND} ("signal to ~~noise~~background ratio") of HABs for ^{11}C-DPA-713 was about ten~~six~~ times greater than that for ^{11}C-(*R*)-PK11195. Comparing the accumulation of radiometabolites in brain was more complicated. In LABs (who are most sensitive to radiometabolites in brain), both radioligands had similar time instability of V_T during the last 40 minutes of the scan. However, in HABs and MABs (which comprise roughly 95% of our subjects screened to date), ^{11}C-DPA-713 showed better time stability of V_T than ^{11}C-(*R*)-PK11195. This differential effect of genotype was expected, as ^{11}C-DPA-713 has much greater specific binding of parent radioligand, which dilute the effects of radiometabolites.

Sensitivity to genotype

^{11}C-DPA-713 showed a clear sensitivity to genotype. Specifically, V_T (mL · cm^{-3}) was highest in HABs (3.619), then MABs (1.943), then LABs (1.21), and V_{ND} was estimated by occupancy plot to be 0.4461. In contrast, no statistically significant differences between the three genotypes were observed with ^{11}C-(*R*)-PK11195 indicating that in vivo ^{11}C-(*R*)-PK11195 binding in brain is insensitive to genotype. However, the observed insensitivity must be

interpreted with caution. ~~Nevertheless~~First, the sample sizes in this study may have been too small to detect a minor sensitivity. Second, because t~~T~~he "signal to background~~noise~~" ratio of ^{11}C-(*R*)-PK11195 was quite small (BP_{ND} = 0.75) and the brain uptake was low peaking at only 1.5 SUV, ~~may~~ the PET data may have lacked the sensitivity to detect an existing genotype effect. In fact, the noise associated with ^{11}C-(*R*)-PK11195 was evident in other measurements. For example, the scatter associated with the occupancy plot was greater for ^{11}C-(*R*)-PK11195 than for ^{11}C-DPA-713 (Fig. 4). ~~Nevertheless, in vivo ^{11}C-(*R*)-PK11195, at least in brain, may be entirely insensitive to genotype.~~

~~A complication is that sensitivity to TSPO genotype may depend on the organ where it is expressed. For example, whole body imaging found~~In this study, we found no sensitivity to genotype of ^{11}C-(*R*)-PK 11195 in brain, but we previously reported ~~that ^{11}C-(*R*)-PK 11195 showed no measurable~~ substantial sensitivity of this radioligand ~~in the brain but clear sensitivity~~ in several peripheral organs, including ~~heart,~~ lung, spleen, and kidney[10]. Although genetic sensitivity may vary among organs, a more parsimonious interpretation is that the radioligand's high non-specific uptake (i.e., low specific binding) in brain has obscured a sensitivity that actually exists and would be detected in a much larger sample size. That is, only organs with a very strong specific signal, like lung, spleen, and kidney, are able to detect the genetic effect in small sample sizes. ~~Taken together, this suggests that much remains unknown regarding the genetic sensitivity of ligand binding to TSPO.~~

Instability of receptor measurements over time

Distribution volume (V_T) reflects a product two variables: the density of receptors and the affinity of the radioligand for the receptors. As these two variables are independent of time (i.e.,

are expected not to change during the course of 90-minute scan), V_T should also not be dependent on time. However, because of noise in the PET and plasma data, a minimal number of time points are required to identify V_T, which should then be stable using the remainder of the data. However, we found that V_T of both ^{11}C-(*R*)-PK11195 and ^{11}C-DPA-713 increased with increased scan duration, showing that the input of parent radioligand itself could not explain the brain uptake. For example, V_T in LABs of both radioligands increased by about 25% during the last 40 min of the scan (Fig. 2). One common cause of time stability is the accumulation radiometabolites in brain, which would not be explained by an input function of only the parent radioligand. Thus, comparison of V_T between individuals or groups might be secondary to differences in the metabolism of the radioligand and not the density of receptors in brain. Thus, caution should be used when either of these radioligands is used to measure TSPO in LABs. The very high specific binding of ^{11}C-DPA-713 obviates this concern for HABs and MABs, as potential radiometabolites are such a small percentage of total brain uptake. For example, V_T of ^{11}C-DPA-713 in HABs increased by only 8% during the last 40 minutes of the scan.

~~***Comparison with ^{11}C-PBR28other TSPO radioligands***~~

~~Because of the apparent insensitivity of ^{11}C-(*R*)-PK11195 to the genotype, we have developed a close analog, ^{11}C-ER176, which has higher affinity for TSPO and lower lipophilicity, and which shows almost no sensitivity to genotype as assessed via in vitro binding to human brain homogenates[28]. However, PET imaging studies in healthy subjects found that ^{11}C-ER176 showed marked sensitivity in vivo[29]. The cause of the discrepant genetic sensitivity between in vitro and in vivo conditions is unknown, but may involve in vivo protein-protein interactions that are disrupted by tissue homogenization or dilution of post-mortem tissue. Such discrepancies between in vitro and in vivo binding are well known for G-protein coupled receptors, where~~

attachment of the receptor to its G-protein increases the affinity of agonists[30]. A similar interaction may occur for TSPO with any of the three proteins that combine with TSPO to form the so-called "permeability transition pore"[31, 32]. The disappointing in vivo result obtained with ^{11}C-ER176 underscores that those who seek to develop improved radioligands for TSPO cannot depend on in vitro screening to reflect in vivo sensitivity to polymorphism rs6971.

Another complication is that sensitivity to TSPO genotype may depend on the organ where it is expressed. For example, whole body imaging found that ^{11}C-(*R*)-PK-11195 showed no measurable sensitivity in the brain but clear sensitivity in several peripheral organs, including heart, lung, spleen, and kidney[10]. Taken together, this suggests that much remains unknown regarding the genetic sensitivity of ligand binding to TSPO.

An additional question is whether ^{11}C-ER176 is so sensitive to genotype that LABs should be excluded from clinical studies. We think not for two reasons. First, although our sample sizes were small, the V_T of LABs was identified almost as well by the unconstrained two-compartment model as the other genotypes. For whole brain, the identifiability (SE) was 0.9% in HABs, 3.4% in MABs, and 1.2% in LABs. Second, the BP_{ND} values of ^{11}C-ER176 in LABs, based on V_{ND} measured in HABs after blockade, was 1.4 (Table 2). This value is quite good and about the same as the BP_{ND} value of 1.2 in HABs obtained using ^{11}C-PBR28[15]. However, additional studies in LABs should be performed to further assess the reliability of ^{11}C-ER176 in this genotype. In addition, LAB subjects are, in our experience, quite rare and comprised only 5% of all 488 subjects screened to date at our institution (unpublished data).

The current results provided us with opportunity to compare four three ^{11}C-labeled TSPO radioligands, ^{11}C-DPA-713, ^{11}C-(*R*)-PK1119, and ^{11}C-PBR28[15] that have been assessed in similar ways in healthy subjects — namely, via kinetic imaging with an arterial input function

where specific and nondisplaceable uptake was measured using both receptor blockade and the polymorphism plot. Thus, we were able to compare results from three radioligands that were evaluated at National Institute of Mental Health (^{11}C-DPA-713, ^{11}C-(*R*)-PK11195, and ^{11}C-ER176[29]) to published results ofTo include ^{11}C-PBR28[15] in the comparison, we performed with additional time stability analysis performed for ^{11}C-PBR28. In all of these studies, XBD173 administered to genetically homogeneous HABs reliably estimated V_{ND} based on the Lassen occupancy plot. Several comparisons can be made. First, of the four three radioligands, only ^{11}C-(*R*)-PK11195 lacked in vivo sensitivity to polymorphism rs6971 in brain (Table 2), with the caveats noted above. Second, in LABs (who have low or little specific binding) and HABs after partially blocking specific binding with XBD173, all three radioligand showed gradual increase in V_T with longer duration of the data, which is consistent with radiometabolites entering the brainwas stably estimated over time only for ^{11}C-ER176 and not for ^{11}C-DPA-713, ^{11}C-(*R*)-PK11195, or ^{11}C-PBR28; this suggests that ^{11}C-ER176 generates the least amount of brain-penetrant radiometabolites as a percentage of total radioactivity in brain. Third, the value of BP_{ND} was highest for ^{11}C-DPA-713, followed by ^{11}C-ER176, ^{11}C-PBR28, and ^{11}C-(*R*)-PK11195 (Table 2). Taken together, these studies in healthy subjects suggest that, among these four three radioligands, TSPO in brain is most robustly quantified by ^{11}C-DPA-713ER176.

CONCLUSION

^{11}C-DPA-713 was markedly superior to ^{11}C-(*R*)-PK11195 for quantifying TSPO in healthy human brain. The BP_{ND} value ("signal to backgroundnoise") of ^{11}C-DPA-713 was tensix times that of ^{11}C-(*R*)-PK11195 in HABs. The very low specific binding of ^{11}C-(*R*)-PK11195 in brain (BP_{ND} = 0.75) may explain why its sensitivity to genotype has only been detected in

peripheral organs, like lung, that have much higher density of TSPO than that in brain [10]. Although the high specific binding of ^{11}C-DPA-713 suggests it is an ideal ligand to measure TSPO, we also found that its distribution volume increased over time, consistent with the accumulation of radiometabolites in brain. Although this putative radiometabolite is a small percentage of brain uptake in HABs and MABS, it is a significant problem for LABs, who may need to be excluded from clinical studies. ~~^{11}C-DPA-713 was markedly superior to another widely used radioligand ^{11}C-PBR28. The BP_{ND} value of ^{11}C-DPA-713 was six times that of ^{11}C-PBR28 in HABs. However, when broadening the comparison to two other TSPO radioligands (^{11}C-PBR28 and ^{11}C-ER176), ^{11}C-ER176 is our recommended PET radioligand, as it has a high "signal to backgroundnoise" ratio close to that of ^{11}C-DPA-713 as well as stable values of V_T during the scan, consistent with no radiometabolites accumulating in brain.~~

ACKNOWLEDGEMENTS

We thank Mohammad B. Haskali, Talakad G. Lohith, Stal Shrestha, Maria D. Ferraris Araneta, Denise Rallis-Frutos, Emily Page, Jeih-San Liow, Kimberly J. Jenko, Aneta Kowalski, Emily Fennell, Sanche Mabins, and the staff of the PET Department for successfully completing the studies, Ioline Henter for providing excellent editorial assistance, Katharine Henry for assisting with data analysis.

Author Contributions Statement

Masato Kobayashi, Teresa Jiang, Sanjay Telu, Sami S. Zoghbi, Roger Gunn, Ilan Rabiner, David R. Owen, Qi Guo, Victor W. Pike, Robert B. Innis, ~~and~~ Masahiro Fujita, and the Biomarkers

Consortium PET Radioligand Project Team all: 1) substantially contributed to the conception and design of the study, the acquisition of data, or the analysis and interpretation of data; 2) drafted the article or revised it critically for important intellectual content; and 3) gave final approval for this version of the manuscript.

DISCLOSURE:

This study was funded by the IRP-NIMH-NIH: project numbers ZIAMH002852 and ZIAMH002793 under clinical protocols NCT02147392 and NCT02147392. R.N.Gunn and E.A. Rabiner are employees of Imanova Ltd.

Supplementary material for this paper can be found at http://jcbfm.sagepub.com/content/by/supplemental-data.

"Supplementary information is available at the *Journal of Cerebral Blood Flow & Metabolism* website – www.nature.com/jcbfm"

REFERENCES

1. Papadopoulos V, Baraldi M, Guilarte TR, Knudsen TB, Lacapere JJ, Lindemann P *et al.* Translocator protein (18kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. *Trends Pharmacol Sci* 2006; 27(8)**:** 402-9.

2. Camsonne R, Crouzel C, Comar D, Maziere M, Prenant C, Sastre J *et al.* Synthesis of N-[^{11}C]methyl, N-(methyl-1-propyl), (chloro-2-phenyl)-1-isoquinoline carboxamide-3 (PK11195): a new ligand for peripheral benzodiazepine receptors. *J Labelled Compd Radiopharm* 1984; 21**:** 985-991.

3. Shah F, Hume SP, Pike VW, Ashworth S, McDermott J. Synthesis of the enantiomers of [N-methyl-11C]PK 11195 and comparison of their behaviours as radioligands for PK binding sites in rats. *Nucl Med Biol* 1994; 21(4)**:** 573-81.

4. Cagnin A, Kassiou M, Meikle SR, Banati RB. Positron emission tomography imaging of neuroinflammation. *Neurotherapeutics* 2007; 4**:** 443-452.

5. van der Laken CJ, Elzinga EH, Kropholler MA, Molthoff CF, van der Heijden JW, Maruyama K *et al.* Noninvasive imaging of macrophages in rheumatoid synovitis using ^{11}C-(*R*)-PK11195 and positron emission tomography. *Arthritis Rheum* 2008; 58**:** 3350-3355.

6. Zhang MR, Kida T, Noguchi J, Furutsuka K, Maeda J, Suhara T *et al.* [^{11}C]DAA1106: radiosynthesis and in vivo binding to peripheral benzodiazepine receptors in mouse brain. *Nucl. Med. Biol.* 2003; 30(5)**:** 513-9.

7. Briard E, Zoghbi SS, Imaizumi M, Gourley JP, Shetty HU, Hong J *et al.* Synthesis and evaluation in monkey of two sensitive ^{11}C-labeled aryloxyanilide ligands for imaging brain peripheral benzodiazepine receptors in vivo. *J Med Chem* 2008; 51**:** 17-30.

8. Endres CJ, Pomper MG, James M, Uzuner O, Hammoud DA, Watkins CC *et al.* Initial evaluation of 11C-DPA-713, a novel TSPO PET ligand, in humans. *J Nucl Med* 2009; 50(8)**:** 1276-82.

9. Imaizumi M, Briard E, Zoghbi SS, Gourley JP, Hong J, Fujimura Y *et al.* Brain and whole-body imaging in nonhuman primates of [^{11}C]PBR28, a promising PET radioligand for peripheral benzodiazepine receptors. *Neuroimage* 2008; 39**:** 1289-98.

10. Kreisl WC, Fujita M, Fujimura Y, Kimura N, Jenko KJ, Kannan P *et al.* Comparison of [^{11}C]-(*R*)-PK 11195 and [^{11}C]PBR28, two radioligands for translocator protein (18 kDa) in human and monkey: implications for positron emission tomographic imaging of this inflammation biomarker. *NeuroImage* 2010; 49**:** 2924-2932.

11. Kropholler MA, Boellaard R, Schuitemaker A, Folkersma H, van Berckel BN, Lammertsma AA. Evaluation of reference tissue models for the analysis of [^{11}C](*R*)-PK11195 studies. *J. Cereb. Blood Flow Metab.* 2006; 26(11)**:** 1431-41.

12. Kreisl WC, Jenko KJ, Hines CS, Lyoo CH, Corona W, Morse C *et al.* A genetic polymorphism for translocator protein 18 kDa affects both in vitro and in vivo radioligand binding in human brain to this putative biomarker of neuroinflammation. *J Cereb Blood Flow Metab* 2013; 33**:** 53-58.

13. Owen DR, Howell OW, Tang SP, Wells LA, Bennacef I, Bergstrom M *et al.* Two binding sites for [3H]PBR28 in human brain: implications for TSPO PET imaging of neuroinflammation. *J Cereb Blood Flow Metab* 2010; 30**:** 1608-18.

14. Owen DR, Gunn RN, Rabiner EA, Bennacef I, Fujita M, Kreisl WC *et al.* Mixed-affinity binding in humans with 18-kDa translocator protein ligands. *J Nucl Med* 2011; 52(1)**:** 24-32.

15. Owen DR, Guo Q, Kalk NJ, Colasanti A, Kalogiannopoulou D, Dimber R *et al.* Determination of [^{11}C]PBR28 binding potential in vivo: a first human TSPO blocking study. *J Cereb Blood Flow Metab* 2014; 34**:** 989-994.

16. Rupprecht R, Rammes G, Eser D, Baghai TC, Schule C, Nothdurfter C *et al.* Translocator protein (18 kD) as target for anxiolytics without benzodiazepine-like side effects. *Science* 2009; 325**:** 490-3.

17. Doorduin J, Klein HC, Dierckx RA, James M, Kassiou M, de Vries EF. [11C]-DPA-713 and [18F]-DPA-714 as new PET tracers for TSPO: a comparison with [11C]-(R)-PK11195 in a rat model of herpes encephalitis. *Mol Imaging Biol* 2009; 11(6)**:** 386-98.

18. Gershen LD, Zanotti-Fregonara P, Dustin IH, Liow JS, Hirvonen J, Kreisl WC *et al.* Neuroinflammation in Temporal Lobe Epilepsy Measured Using Positron Emission Tomographic Imaging of Translocator Protein. *JAMA Neurol* 2015; 72(8)**:** 882-8.

19. Kreisl WC, Lyoo CH, McGwier M, Snow J, Jenko KJ, Kimura N *et al.* In vivo radioligand binding to translocator protein correlates with severity of Alzheimer's disease. *Brain* 2013; 136(Pt 7)**:** 2228-38.

20. Sandiego CM, Gallezot JD, Pittman B, Nabulsi N, Lim K, Lin SF *et al.* Imaging robust microglial activation after lipopolysaccharide administration in humans with PET. *Proc Natl Acad Sci U S A* 2015; 112(40)**:** 12468-73.

21. Herranz E, Gianni C, Louapre C, Treaba CA, Govindarajan ST, Ouellette R *et al.* Neuroinflammatory component of gray matter pathology in multiple sclerosis. *Ann Neurol* 2016.

22. James ML, Fulton RR, Henderson DJ, Eberl S, Meikle SR, Thomson S *et al.* Synthesis and in vivo evaluation of a novel peripheral benzodiazepine receptor PET radioligand. *Bioorg Med Chem* 2005; 13(22)**:** 6188-94.

23. Kreisl WC, Fujita M, Fujimura Y, Kimura N, Jenko KJ, Kannan P *et al.* Comparison of [^{11}C]-(*R*)-PK 11195 and [^{11}C]PBR28, two radioligands for translocator protein (18 kDa) in human and monkey: Implications for positron emission tomographic imaging of this inflammation biomarker. *Neuroimage* 2010; 49**:** 2924-32.

24. Kreisl WC, Jenko KJ, Hines CS, Lyoo CH, Corona W, Morse CL *et al.* A genetic polymorphism for translocator protein 18 kDa affects both in vitro and in vivo radioligand binding in human brain to this putative biomarker of neuroinflammation. *J Cereb Blood Flow Metab* 2013; 33(1)**:** 53-8.

25. Zoghbi SS, Shetty HU, Ichise M, Fujita M, Imaizumi M, Liow JS *et al.* PET imaging of the dopamine transporter with ^{18}F-FECNT: a polar radiometabolite confounds brain radioligand measurements. *J. Nucl. Med.* 2006; 47(3)**:** 520-527.

26. Gandelman MS, Baldwin RM, Zoghbi SS, Zea-Ponce Y, Innis RB. Evaluation of ultrfiltration for the free fraction determination of single photon emission computed tomography (SPECT) tracers: ß-CIT, IBF, and iomazenil. *J. Pharm. Sci.* 1994; 83**:** 1014-1019.

27. Innis RB, Cunningham VJ, Delforge J, Fujita M, Gjedde A, Gunn RN *et al.* Consensus nomenclature for in vivo imaging of reversibly binding radioligands. *J Cereb Blood Flow Metab* 2007; 27**:** 1533-9.

28. Logan J, Fowler JS, Volkow ND, Wolf AP, Dewey SL, Schlyer DJ *et al.* Graphical analysis of reversible radioligand binding from time-activity measurements applied to [N-^{11}C-methyl]-(-)cocaine PET studies in human subjects. *J. Cereb. Blood Flow Metab.* 1990; 10**:** 740-747.

29. Kropholler MA, Boellaard R, Schuitemaker A, van Berckel BN, Luurtsema G, Windhorst AD *et al.* Development of a tracer kinetic plasma input model for (*R*)-[^{11}C]PK11195 brain studies. *J. Cereb. Blood Flow Metab.* 2005; 25(7)**:** 842-51.

30. Owen DR, Guo Q, Kalk NJ, Colasanti A, Kalogiannopoulou D, Dimber R *et al.* Determination of [(11)C]PBR28 binding potential in vivo: a first human TSPO blocking study. *J Cereb Blood Flow Metab* 2014; 34(6)**:** 989-94.

31. Cunningham VJ, Rabiner EA, Slifstein M, Laruelle M, Gunn RN. Measuring drug occupancy in the absence of a reference region: the Lassen plot re-visited. *J Cereb Blood Flow Metab* 2010; 30**:** 46-50.

32. Owen DR, Lewis AJ, Reynolds R, Rupprecht R, Eser D, Wilkins MR *et al.* Variation in binding affinity of the novel anxiolytic XBD173 for the 18 kDa translocator protein in human brain. *Synapse* 2011; 65**:** 257-9.

FIGURE LEGENDS

Fig. 1. Mean brain radioactivity concentrations of ^{11}C-DPA713 (A) and ^{11}C-(*R*)-PK11195 (B) in each binder group.

HABs = high-affinity binders, MABs = mixed-affinity binders, LABs = low-affinity binders.

Error bars denote SD.

Fig. 2. Time-stability analysis for kinetic analysis of ^{11}C-DPA713 (A) and ^{11}C-(*R*)-PK11195 (B). Total distribution volume (V_T) obtained from baseline scans for HABs, MABs, and LABs are plotted as a function of duration of image acquisition. V_T was calculated for whole brain using a Logan plot. V_T values are normalized as percentage of terminal value attained from 90 minutes of imaging. The data represent mean ± SD of all three subjects.

HABs = high-affinity binders, MABs = mixed-affinity binders, LABs = low-affinity binders.

Fig. 3. Data from a representative ^{11}C-DPA713 subject in whole brain (A) and arterial plasma (B) at baseline (○) and after blockade (●). Data from a representative ^{11}C-(*R*)-PK11195 subject in brain (C) and arterial plasma (D) at baseline (○) and after blockade (●). Blockade was done with 90 mg XBD173. Time courses of parent concentrations in arterial plasma were triexponentially fitted.

Fig. 4. Lassen occupancy plot~~Lassen plot~~ to determine nondisplaceable uptake (V_{ND}) of ^{11}C-DPA713 in HABs (n = 6; A) and that of ^{11}C-(*R*)-PK11195 in HABs (n = 5; B) and MABs (n = 8; C). Each point represents a brain region in an individual subject.

HABs = high-affinity binders, MABs = mixed-affinity binders.

Fig. 5. Polymorphism plot to determine a population average of nondisplaceable distribution uptake (V_{ND}) of ^{11}C-DPA713 in 14 HABs, five MABs, and three LABs. The plots were performed for three different combinations of the affinity types. The plots were performed using average V_T values in each VOI of the same affinity type.

Table 1. Parameters of ^{11}C-DPA713 and ^{11}C-(*R*)-PK11195 in whole brain

Parameters	^{11}C-DPA713	^{11}C-(*R*)-PK11195
V_T^{HAB} (mL/cm^3)	3.611 ± 0.560.56	0.74 ± 0.14
V_T^{MAB} (mL/cm^3)	1.94 ± 0.17	0.74 ± 0.16
V_T^{LAB} (mL/cm^3)	1.21 ± 0.15	0.60 ± 0.18
V_{ND} (mL/cm^3)	0.44.61 (0.344-0.5378)	0.42 (0.31-0.53)
	4.8 ± 2.1	0.75 ± 0.32

HABs = high-affinity binders, MABs = mixed-affinity binders, LABs = low-affinity binders. Data are mean ± SD.

Nondisplaceable uptake (V_{ND}) was calculated from the occupancy plots of HABs.

Table 2. Comparison of four ^{11}C-radioligands to image translocator protein (TSPO)

Ligand	V_T HABs mL*cm^{-3}	V_{ND}	BP_{ND} HABs (n)	BP_{ND} MABs (n)	BP_{ND} LABs (n)	Time Stability* of V_T LABs (n)	Time Stability* of V_T HABs after blockade (n)
^{11}C-DPA-713	3.6	0.44	7.3 (14)	3.4 (5)	1.8 (3)	25% (3)	13% (6)
^{11}C-(*R*)-PK11195	0.7	0.42	0.8 (5)	0.9 (8)	0.5 (3)	26% (3)	27% (5)
~~^{11}C-PBR28[15]~~	~~4.3~~	~~1.98[30]~~	~~1.2 (16)~~	~~0.5 (10)~~	~~Not reliably measured~~	~~Not reliably measured[33]~~	~~25% (6)~~

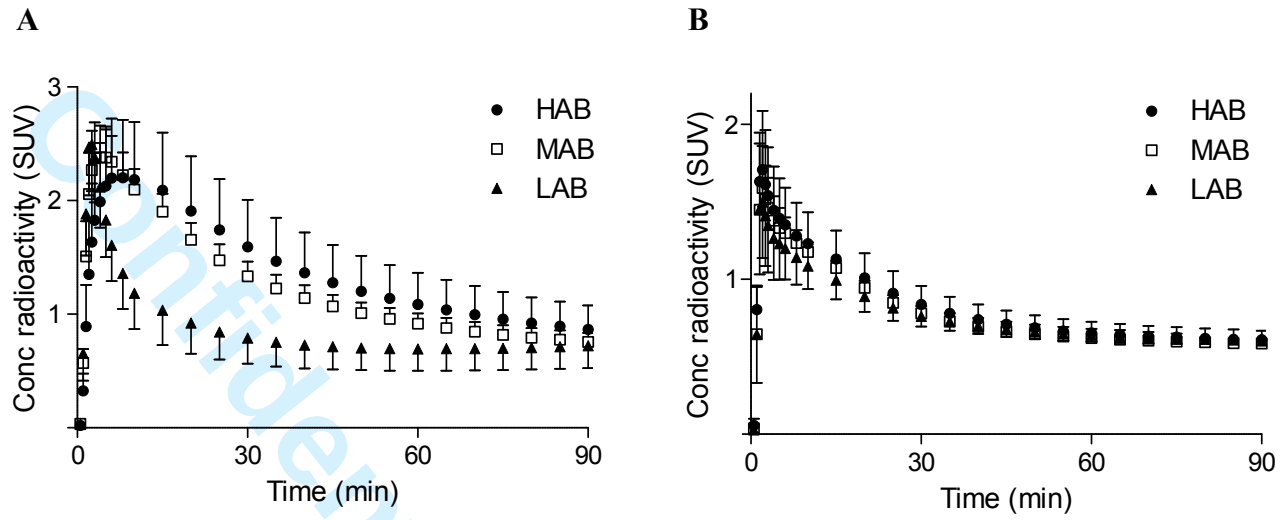
~~Ligand~~	~~V_T HABs mL*cm^{-3}~~	~~BP_{ND} HABs~~	~~BP_{ND} MABs~~	~~BP_{ND} LABs~~	~~Time Stability* of V_T LABs (n)~~	~~Time Stability* of V_T HABs after blockade (n)~~
~~^{11}C-DPA-713~~	~~3.6~~	~~7.24.8~~	~~3.42.2~~	~~1.80~~	~~25% (3)~~	~~13% (6)~~
~~^{11}C-(*R*)-PK11195~~	~~0.7~~	~~0.8~~	~~0.9~~	~~0.5~~	~~26% (3)~~	~~27% (5)~~

^{11}C-ER176 [29]	3.3	4.2	3.4	1.4	< 5% (2)	6% (3)
^{11}C-PBR28 [15]	4.3	1.2	0.5	Not reliably measured	Not reliably measured [33]	25% (6)

SNP = single nucleotide polymorphism rs6971, HABs = high-affinity binders, MABs = mixed-affinity binders, LABs = low-affinity binders; V_T = total distribution volume

* % increase of V_T in last 40 minutes.

Figures 1.

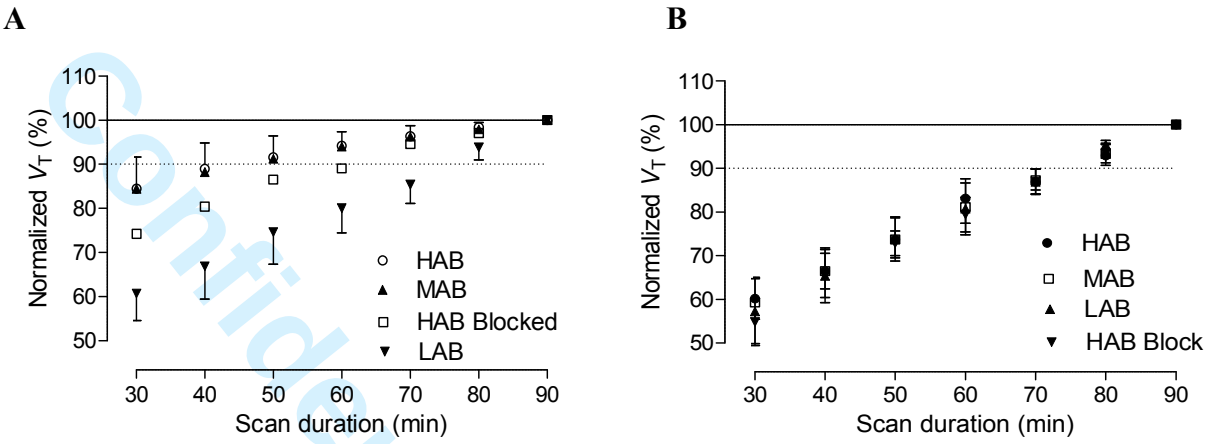


Mean brain radioactivity concentrations of ^{11}C-DPA713 (A) and ^{11}C-(*R*)-PK11195 (B) in each binder group.

HABs = high-affinity binders, MABs = mixed-affinity binders, LABs = low-affinity binders.

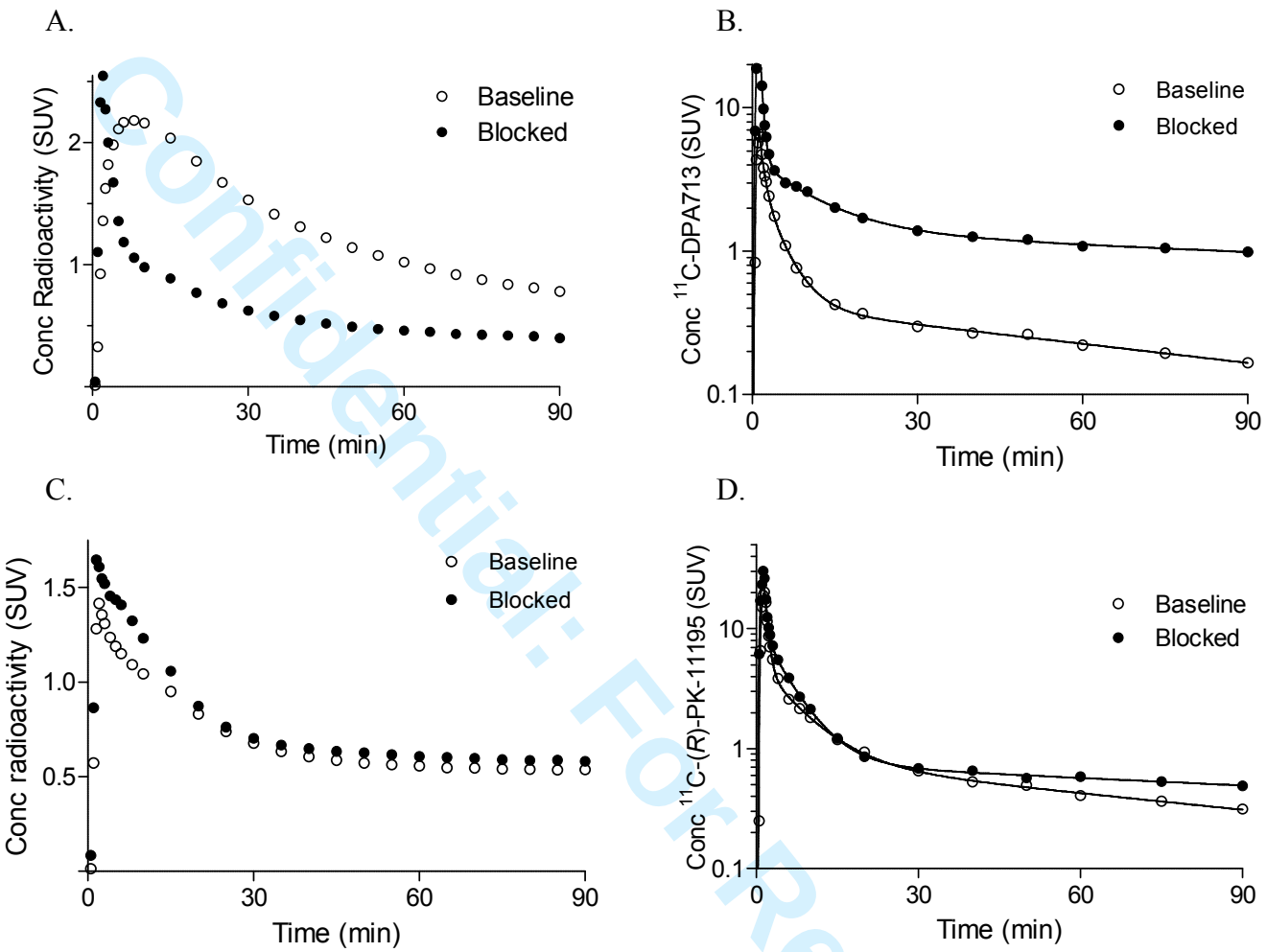
Error bars denote SD.

Figure 2.



Time–stability analysis for kinetic method of ^{11}C-DPA713 (A) and ^{11}C-(*R*)-PK11195 (B). Total distribution volume (V_T) obtained from baseline scans for HABs, MABs, and LABs are plotted as a function of duration of image acquisition. V_T was calculated for whole brain using a Logan plot. V_T values are normalized as percentage of terminal value attained from 90 minutes of imaging. The data represents mean ± SD of all three subjects.

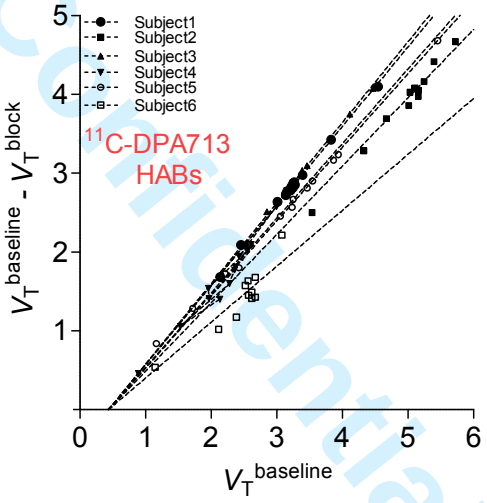
Figure 3.



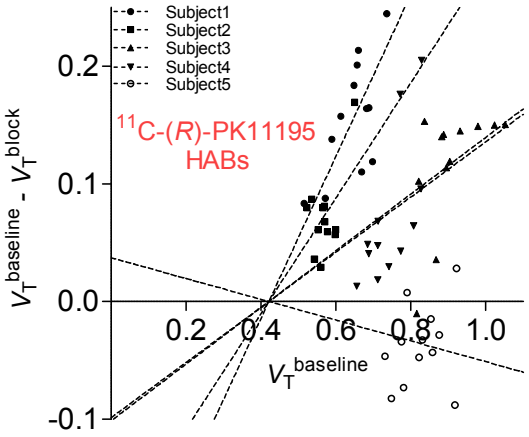
Data of a representative ^{11}C-DPA713 subject in whole brain (A) and arterial plasma (B) at baseline (○) and after blockade (●). Data of a representative ^{11}C-(*R*)-PK11195 subject in brain (C) and arterial plasma (D) at baseline (○) and after blockade (●). Blockade was done with 90 mg XBD173. Time courses of parent concentration in arterial plasma were triexponentially fitted.

Figure 4.

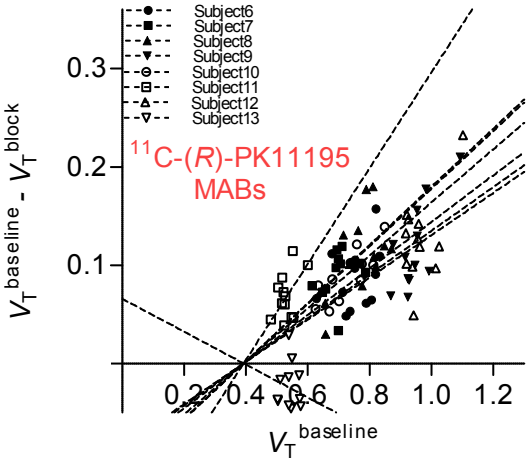
A



B

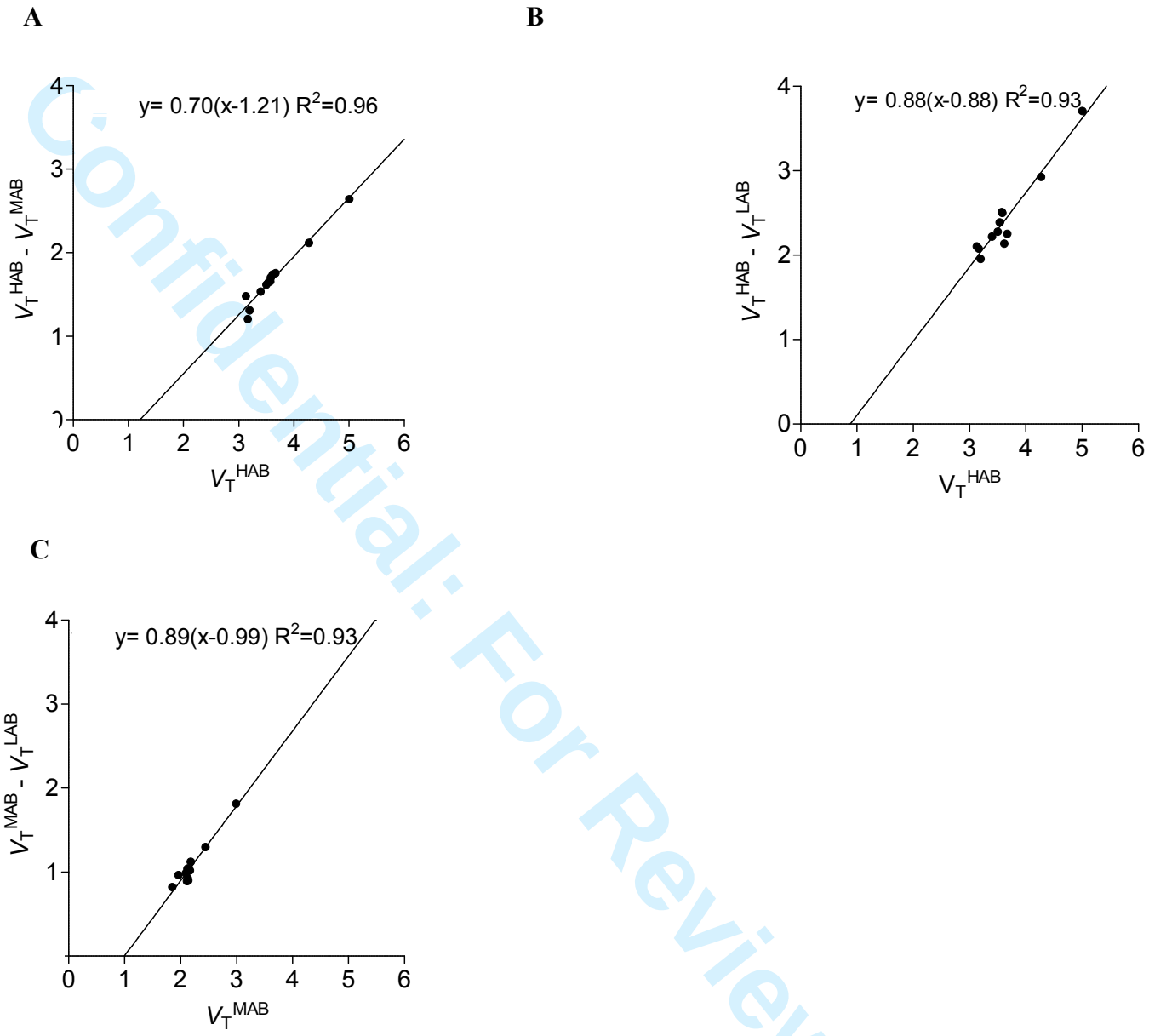


C



Lassen occupancy plot to determine V_{ND} of ^{11}C-DPA713 in HABs (n = 6; A) and that of ^{11}C-(*R*)-PK11195 in HABs (n = 5; B) and MABs (n = 8; C). Each point represents a brain region in an individual subject.

Figure 5.



Polymorphism plot to determine a population average of nondisplaceable distribution volume (V_{ND}) of ^{11}C-DPA713 in 14 HABs, 5 MABs, and 3 LABs. The plots were performed for three different combinations of the affinity types. The plots were performed using average V_T values in each VOI of the same affinity type.

December 14, 2016

Drs. Ulrich Dirnagl / Martin Lauritzen
Editors-in-Chief
Journal of Cerebral Blood Flow & Metabolism

Re: JCBFM-0587-16-ORIG

Dear Drs. Ulrich Dirnagl / Martin Lauritzen,

Enclosed please find our revised manuscript, which now has a revised title "^{11}C-DPA-713 has much greater specific binding to translocator protein 18 kDa (TSPO) in human brain than does ^{11}C-(*R*)-PK11195." We have incorporated the Reviewers' helpful suggestions, and believe this has improved the paper's overall quality. We present our responses item by item in the pages that follow.

The change of the title reflects the changes we made in response to the comments by both reviewers. We have removed comparisons with ^{11}C-PBR28 and ^{11}C-ER176.

The page numbers below are based on the revised manuscript where the changes are highlighted.

To respond to comment #10 of Referee 2, we performed additional analyses and found an error in Lassen occupancy plot for subject 6 in Fig. 4A. We apologize for the error and have corrected. The main conclusions of this study remain the same, i.e., specific binding of ^{11}C-DPA-713 (BP_{ND} = 7.3) was much greater than that of ^{11}C-(*R)*-PK11195 (0.75).

The revised manuscript has been seen and approved by all authors. If I can provide you with any further information, please contact me. We look forward to hearing from your office.

Sincerely,

Masahiro Fujita, MD, PhD
Molecular Imaging Branch
National Institute of Mental Health
Bldg. 10, Rm. B1D43, 10 Center Dr, MSC-1026
Bethesda, MD 20892-1026, USA
Phone: 301-451-8898
Fax: 301-480-3610
E-mail: fujitam@mail.nih.gov

Referee : 1

Comments to the Corresponding Author
This manuscript by Kobayashi et al. addresses two very important issues with regards to in vivo imaging of neuroinflammation with TSPO radioligands, that is the sensitivity of the outcome measures to the rs6971 polymorphism and the signal-to-noise ratio of [11C]DPA-713 and of the reference radioligand (R)[11C]PK11195. …
The authors include in the manuscript also the comparison with published results using [11C]PBR28 and to results that are in press using the novel TSPO radioligand [11C]ER176. …
However, this reviewer is not comfortable with the title of the manuscript and the way the Discussion is structured. This reviewer believes that the title should include only the radioligands that have been examined in this work, since the evaluation of [11C]ER176 has been presented in another manuscript that is in press. This manuscript in press has not been submitted to the reviewer, so it is difficult to follow the presentation and discussion of the findings regarding [11C]ER176. In addition, the manuscript becomes more confusing with the digression about [11C]ER176. If the purpose of the work was to compare [11C]DPA-713 and [11]PK11195, then the manuscript should focus on that comparison. Then of course a question is why the authors compare [11C]DPA-713 with [11C]PK11195 instead of comparing directly [11C]ER176 with [11C]PK11195 if [11C]ER176 was intended to be a leading candidate? The suggestion of this reviewer is to re-write the discussion focusing on the major comparison.

Our response: We take reviewer's comment and have revised the manuscript to focus on the comparison between ^{11}C-DPA-713 and (*R*)-PK11195. The title of the manuscript has been changed to

"^{11}C-DPA-713 has much greater specific binding to translocator protein 18 kDa (TSPO) in human brain than does ^{11}C-(*R*)-PK11195."

In Discussion and Table 2, we removed the comparison with ^{11}C-PBR28 and ^{11}C-ER176.

Specific comments.
1. Introduction, page 3, line 27. Did you really measure BPnd in study 9 and 10?
Our response: We measured BP_{ND} in the monkey studies cited as references 9 and 10. Unlike human studies where full receptor blockade is difficult because of possible pharmacological effects, in these monkey studies, we administered the blocking agent to cause full receptor blockade. BP_{ND} was calculated as $(V_T^{baseline} - V_T^{blocked}) / V_T^{blocked}$. To indicate BP_{ND} was measured by full blockade, "full" has been added to the sentence below, which is just prior to the sentence citing references 9 and 10.

"These second generation radioligands are widely thought to have greater "signal to background" ratio than ^{11}C-(*R)*-PK11195, based largely on full-blockade studies in monkeys."

2. Introduction, page 4, line 3. What do you mean by "in vivo imaging results have been mixed"?

Our response: We agree that the meaning of this sentence was not clear. We have rewritten as

"Although in vitro receptor binding studies found that ^{11}C-(*R*)-PK11195 is insensitive to the genotype[14], in vivo imaging results are inconsistent among organs."

The subsequent sentence,

"Whole-body imaging studies suggest that the brain is not sensitive to this genotype but that peripheral organs like lung and heart are[10]."

provides an explanation for the inconsistency among organs.

3. Introduction, page 4, line 8. "Planar whole body". This is not correct, whole body PET images are not planar, they are tomographic. What do you really mean here?

Our response: We agree that this sentence was not correct. We have rewritten as shown below, which we originally meant to state.

"However, these results were not based on full quantification using radiometabolite-corrected arterial input function; full quantification might be able to distinguish whether the brain is also sensitive to polymorphism rs6971."

4. Introduction, page 4, line 20. [11C]DPA-713 is presented as "one of the most promising second generation radioligands..". On which basis did the author make this statement?

Our response: We have revised this sentence and added basis to state "one of the most promising second generation radioligands".

In the revised sentence, underlined portion has been added:
one of the most promising second generation radioligands, ^{11}C-DPA-713, which showed superiority to ^{11}C-(*R*)-PK11195 in rodent model of neuroinflammation [17] and superiority to a widely used second generation radioligand ^{11}C-PBR28 in patients with epilepsy [18].

5. Scan procedures, page 6, line 36-37. Please, specify the n. of order and subsets for the OSEM reconstruction using the HRRT and the cut-off of the filter used for FBP using the GE Advance. Please specify also if the OSEM reconstruction of the HRRT data also included PSF modelling. Finally, the authors must specify how many PET measurements were performed using the HRRT and how many were performed using the GE Advance and for which radioligand. These info are missing but are important for the interpretation of the results. The resolution of the GE Advance is lower than the resolution of the HRRT

and the data from both systems are reconstructed using different algorithms, which makes the data acquired on the two systems not direclty comparable, unless some standardization of the reconstruction is made. Such standardization is not mentioned in the manuscript, so more clarification is needed.

Our response:
The requested parameters for OSEM,

"2 iterations and 30 subsets"

and for FBP

"Hanning filter with 4.0 cutoff"

have been added (p. 7). PSF modeling was not included.

The revised manuscript provides the requested details of the scanner usage:

"Most PET scans were performed on an Advance Tomograph (GE Healthcare, UK) except or three pairs of baseline scans and the those after XBD173 administration using ^{11}C-DPA-713 where a High-Resolution Research Tomograph (HRRT) scanner (CTI, Knoxville, TN, USA) was used." (p. 6)

The selection of the scanners was made based on the availability of the scanners.

To match spatial resolution of Advance Tomograph and HRRT,

"After the image reconstruction, to match spatial resolution, HRRT data were smoothed with 6.5 mm full-width-half-maximum using a Gaussian filter." (p. 7)

Because large VOIs were used in the analysis and HRRT data were smoothed, it is unlikely that the difference of the scanner affected the results of this study. The revised manuscript provides volumes of the VOIs as follows.

"The PET data in the following volumes of interest (VOI) were obtained in the MNI space: frontal (432 cm^3), occipital (172), parietal (247), temporal (251), medial temporal (36), and cingulate (28) cortices, thalamus (17), caudate (16), putamen (17), cerebellum (195), brainstem (6.5), and white matter (8.3)." (p. 8)."

6. Estimation of nondisplaceable binding. Vnd has been estimated with the Lassen´s plot by constraining Vnd to be the same value across all subjects. It would be important to show also the estimation of VND without such constrain to evaluate whether the mean value obtained with such approach is comparable with Vnd obtained with the other two methods. From the estimation of Vnd it seems that most of the total binding of [11C]DPA713 in HABs (~80%) is specific and this is higher than the proportion of specific binding for [11C]PBR28 (~50%). The authors present this indirectly in table 2 when presenting the

estimates of BPnd, but it should be also commented separately. In fact, many groups have selected [11C]PBR28 as TSPO radioligand of choice to measure neuroinflammation in pathological conditions, such as Alzheimer´s disease, schizophrenia or Parkinson´s disease, but based on the data presented in this manuscript [11C]DPA713 might be preferable in situations in which as small difference is expected between groups, since a larger proportion of VT is specific binding. This message should be conveyed to the PET research community.

Our response:

As for the first half of the comment 6, i.e., performing Lassen occupancy plot in each subject, we did not include this analysis because the x-intercept, V_{ND}, was not well determined in several data sets (in the original manuscript, RESULTS, Estimating Nondisplaceable Distribution Volume, 1) Lassen occupancy plot). The causes of the poor identifiability are variability of data in each subject for both radioligands, particularly for ^{11}C-(*R*)-PK11195 and low receptor occupancy detected by ^{11}C-(*R*)-PK11195.

By performing Lassen occupancy plot for each subject, we obtained the following V_{ND} values (numbers in parentheses are 95% confidence interval).

^{11}C-DPA-713:
Subject 1: 0.38 (0.28-0.47), Subject 2: 0.90 (0.62-1.15), Subject 3: 0.37 (0.23-0.50), Subject 4: 0.48 (0.22-0.68), Subject 5: 0.31 (0.21-0.41), Subject 6: 0.66 (-0.16-1.11)
The average V_{ND} from these individual fitting is 0.52, which is close to 0.44 obtained by fitting data of all subjects and reported in the revised manuscript.

^{11}C-(*R*)-PK11195:
HABs:
Subject 1: 0.36 (-0.82-0.49), Subject 2: 0.45 (-27.1-0.51), Subject 3: 0.60 (-infinity-0.77), Subject 4: 0.64 (0.26-0.70), Subject 5: 1.07 (0.88-+infinity)
MABs:
Subject 1: 0.27 (-infinity-0.59), Subject 2: 0.13 (-infinity-0.51), Subject 3: 0.44 (-13.6-0.61), Subject 4: 0.73 (0.34-0.82), Subject 5: 0.43 (0.13-0.53), Subject 6: 0.33 (-infinity-0.45), Subject 7: 0.66 (-infinity-0.83), Subject 8: 0.07 (-infinity-0.51)
The average V_{ND} of ^{11}C-(*R*)-PK11195 from these individual fitting was 0.62 and 0.38 in HABs and MABs, respectively. V_{ND} obtained by fitting data of all of HABs or MABs together was 0.42 (HABs) and 0.39 (MABs), which are reported in the manuscript.

Because fitting in each subject showed large 95% confidence intervals particularly for ^{11}C-(*R*)-PK11195, in the manuscript, we report the results by only the constrained fitting where data of each group were fitted together.

By following comments by both Referee 1 and 2, we have removed comparisons with ^{11}C-PBR28.

Referee : 2

The data provided is novel and as such, the paper is a valuable and thorough contribution to the literature on PET-neuroimaging of TSPO... I have, however, some suggestions for further explorations and clarifications in the manuscript.

1. Title: The title is somewhat misleading in that it suggests that the paper presents novel data from four radioligands. In fact, it is only in the discussion that the two other radioligands are mentioned. The title should be changed to reflect that.

Our response: We take reviewer's comment and have revised the title of the manuscript to

"^{11}C-DPA-713 has much greater specific binding to translocator protein 18 kDa (TSPO) in human brain than does ^{11}C-(*R*)-PK11195."

2. Abstract: The abstract states that "…controversy exists re. whether second generation…". I disagree. I don't think that anyone reasonably insightful in TSPO neuroimaging believes that PK11195 is second to none.

Our response: Although many people suspect ^{11}C-(*R*)-PK11195 is not a good radioligand, it is still widely used. A possible reason is that it is not clear in human how much better second generation TSPO ligands are than^{11}C-(*R*)-PK11195. Nearly all comparisons between ^{11}C-(*R*)-PK11195 and second generation radioligands have been performed in rodent models. By considering these, we have rewritten this sentence as shown below.

Revised sentence:
controversy exists regarding the magnitude of superiority second generation radioligands have to the prototypical agent ^{11}C-(*R)*-PK 11195 in human imaging.

3. Abstract: The authors repeatedly use the term "signal to noise" when they mean "signal to background". The non-displaceable binding cannot be considered noise, so please substitute the term throughout the manuscript.

Our response: We have changed "signal to noise" to "signal to background" throughout the manuscript.

4. Abstract: "Lassen-occupancy". Later, the authors refer to the plots as "Lassen" only. Admittedly, there is a slight confusion about the term. A reasonable compromise could be to substitute with "Lassen occupancy plot", throughout the manuscript.

Our response: We have changed to "Lassen occupancy plot" throughout the manuscript.

5. Abstract: "Using published data…" should be replaced with "Compared to published data…"

Our response: By considering the major comment of Reviewer 1 and Comment 1 of Reviewer 2, we have deleted the specified sentence.

6. P.5. Were any of the volunteers scanned with PK11195 included in previous publications? If so, please add information.

Our response: None of volunteers scanned with PK 11195 was included in previous publications.

7. P.5. Are there any known sex differences in brain TSPO binding? Did you observe any? And how about age?

Our response: We agree that it is important to investigate the effect of sex and age in TSPO. However, the sample size of the current study is small. In a separate study with a much larger sample size where all scans were performed using ^{11}C-PBR28, we are investigating these effects.

8. P.5. How quickly after blood sampling were the samples centrifuged? The radiotracers tend to bind to white blood cells in a time-dependent manner. Please add information.

Our response: As the reviewer pointed out, because white blood cells express TSPO, if the blood samples are left too long, the radioligand may bind to TSPO in white blood cells. We centrifuged all samples within a few minutes after blood sampling. We have revised this section and added the information on the interval between sampling and centrifuging.

9. P.8, first paragraph. What is the meaning of the sentence "…were not included as in typical Logan plot analysis"?

Our response: What we meant is the following. The original publication of Logan plot (*J. Cereb. Blood Flow Metab.* 1990; 10**:** 740-747) does not include weighting, delay and the vascular component, and nearly all subsequent applications of the Logan plot did not include these parameters. Therefore, we applied Logan plot is the same way.

We changed "typical" to "standard," which may be less confusing.

10. P.8, P.11 and P. 12. Re. radiometabolites – I propose that you assess this issue more directly by computing the VND based on the different time frames used for computing VT. This should provide you with a more direct and quantifiable measure of radiometabolite contributions.

Our response: We take reviewer's point that radiometabolites that do not bind to TSPO should in theory contribute to V_{ND} but not to V_S. However, because of the noise of the data and the narrow range of TSPO density across brain regions, there was uncertainty in determining V_{ND} even by

using the full length data. To respond to this reviewer's comment, we performed additional analysis by using 50 and 70 min data and compared the results with those of the full length 90 min data, which are used in the manuscript. We performed this analysis only for ^{11}C-(*R*)-PK11195 that had the lowest levels of BP_{ND}, and hence is expected to have the lowest levels of certainty in V_{ND}. The results are shown in the tables below.

Because of the uncertainty of V_{ND} indicated by the 95% confidence intervals, these results are not easily interpretable. Although investigating time stability of V_{ND} is an interesting thought, we have decided not to include in the manuscript.

Please note that unlike V_{ND}, V_T values were well identified in both baseline and blocked scans as stated in RESULTS Kinetic Analysis, major findings #1 (page 11).

^{11}C-(*R*)-PK11195	Baseline V_T^{HAB}	Blocked V_T^{HAB}	V_{ND}	95% C.I.
50 min	0.55	0.50	0.36	0.25-0.47
70 min	0.65	0.60	0.40	0.32-0.49
90 min	0.76	0.68	0.42	0.31-0.53

^{11}C-(*R*)-PK11195	Baseline V_T^{MAB}	Blocked V_T^{MAB}	V_{ND}	95% C.I.
50 min	0.56	0.51	0.37	0.29-0.45
70 min	0.67	0.60	0.37	0.26-0.48
90 min	0.76	0.68	0.39	0.28-0.51

Although we do not report time stability of V_{ND} because of the uncertainty, we do agree the importance of time stability of distribution volume. Therefore, we have added a new subsection titled "***Instability of receptor measurements over time***" in DISCUSSION (pp. 17 – 18).

When we examined the time stability of V_{ND}, we found an error in Lassen occupancy plot for subject 6 in Fig. 4A. We apologize for the error and have corrected. The main conclusions of this study remain the same, i.e., specific binding of ^{11}C-DPA-713 was much greater than that of ^{11}C-(*R*)-PK11195.

In Fig. 4A, for subject 6, $V_T^{baseline}$ was correct but $V_T^{baseline}$ - $V_T^{blocked}$ was wrong. In the original manuscript, subject 6 showed markedly lower receptor occupancy of 62% than the other subject (≥90%). After the correction, receptor occupancy of subject 6 became 71%, which is close to ≥87% occupancy in the other subjects. After the correction, 95% confidence interval of V_{ND} improved from 0.44 – 0.78 to 0.34 – 0.53. V_{ND} has been corrected from 0.61 to 0.44. By using the correct V_{ND}, BP_{ND} of ^{11}C-DPA-713 was 7.3, 3.4, and 1.8 in HABs, MABs, and LABs, respectively. Please note that subject 6 is one of the three subjects who received a low dose of 30 mg XBD173.

The corresponding changes have been made in Supple Fig. 2A, page 7 subsection **XBD173 Administration** (corrected from 62 to 71%) and page 14 subsection **Estimating Specific and Nondisplaceable Uptake** (receptor occupancy has been corrected to 91 ± 12%).

11. P 12. Why is VND determined by blocking experiments almost half the value determined from the genomic plot? To me it suggests that the assumption of equal VND in HAB, MAB and LAB is wrong. Please discuss.

Our response: The cause of the different V_{ND} by the two methods is unclear. Because we have only speculations, we added one sentence shown below in RESULTS, at the end of the results of polymorphism plot (page 14).

The added sentence:
The cause for the different V_{ND} by Lassen occupancy and polymorphism plots is unclear but possibilities are the small sample size and intersubject variability in V_{ND} including possible differences among different affinity types.

12. P. 15, first paragraph. In the past, it was thought that it was an advantage that PK11195 showed so little sensitivity to genotype. Now it has become clear that this apparent lack of sensitivity simply arises from the low brain signal of the radioligand. I think the paragraph should be rephrased to reflect that. Similarly, below (p. 16) the authors mention that genotype matters for PK11195 in organs without blood-brain barrier. This is likely to be because of the limited blood-brain barrier permeability of PK11195, and that binding in organs other than the brain is sufficiently high to separate the signals from people with different genotypes.

Our response:
By considering the reviewer's comment, we have rearranged the first paragraph of Sensitivity to genotype as shown below.

Revised section:
In contrast, no statistically significant differences between the three genotypes were observed with ^{11}C-(*R)*-PK11195 indicating that in vivo ^{11}C-(*R)*-PK11195 binding in brain is insensitive to genotype. However, the observed insensitivity must be interpreted with caution. First, the sample sizes in this study may have been too small to detect a minor sensitivity. Second, because the "signal to background" ratio of ^{11}C-(*R)*-PK11195 was quite small (BP_{ND} = 0.75) and the brain uptake was low peaking at only 1.5 SUV, the PET data may have lacked the sensitivity to detect an existing genotype effect. In fact, the noise associated with ^{11}C-(*R)*-PK11195 was evident in other measurements. For example, the scatter associated with the occupancy plot was greater for ^{11}C-(*R)*-PK11195 than for ^{11}C-DPA-713 (Fig. 4).

We also added the following paragraph to provide additional considerations.
Added paragraph:
In this study, we found no sensitivity to genotype of ^{11}C-(*R*)-PK 11195 in brain, but we previously reported substantial sensitivity of this radioligand in several peripheral organs, including lung, spleen, and kidney[10]. Although genetic sensitivity may vary among organs, a more parsimonious interpretation is that the radioligand's high non-specific uptake (i.e., low specific binding) in brain has obscured a sensitivity that actually exists and would be detected in

a much larger sample size. That is, only organs with a very strong specific signal, like lung, spleen, and kidney, are able to detect the genetic effect in small sample sizes.

13. P. 15, second paragraph- p16 until the last paragraph. The discussion re. ER176, in vitro vs in vivo, seems out of place given that there is no experimental data on ER176 included in the manuscript. These paragraphs should be considerably reduced, also given what is mentioned in comment 12.

Our response: We have deleted these paragraphs.

14. Table 1. Please show BPND for all genotypes and add sample sizes.

15. Table 2. Please add a column with VND values. Once you have computed the time-dependent VND, you can add that information instead.

Our response for comments #14 and #15:
Because the original version of Table 2 had BP_{ND} of all genotypes, we kept these and added a column of V_{ND} as the reviewer requested. We also added samples sizes in Tables 2.

16. Figure 3C. The legend says "representative data" but wasn't it often hard to see any blocking? How come that the plasma 11C-PK11195 TAC is so much higher than DPA713 in the blocked condition, relative to the unblocked, towards the end of the measurements? Was that seen for all data sets? If so, it may suggest that the in vivo affinity to TSPO is higher for PK11195 than for DPA713, at least in the periphery.

Our response: ^{11}C-DPA-713 showed clear differences between baseline and blocked scans in both brain (A) and blood (B) while ^{11}C-(*R*)-PK11195 showed only small differences in brain (C) and blood (D). If specific binding in peripheral organs is blocked, parent concentration in plasma increase as we observed in the current human study using ^{11}C-DPA-713 and in a previous monkey study on ^{11}C-PBR28 (Imaizumi et al., NeuroImage. 2008 Feb 1;39(3):1289-98.). We don't see evidence that ^{11}C-(*R*)-PK11195 has greater affinity in peripheral TSPO.

From the NeuroImage paper, please compare panels C and D and also note the different scales:

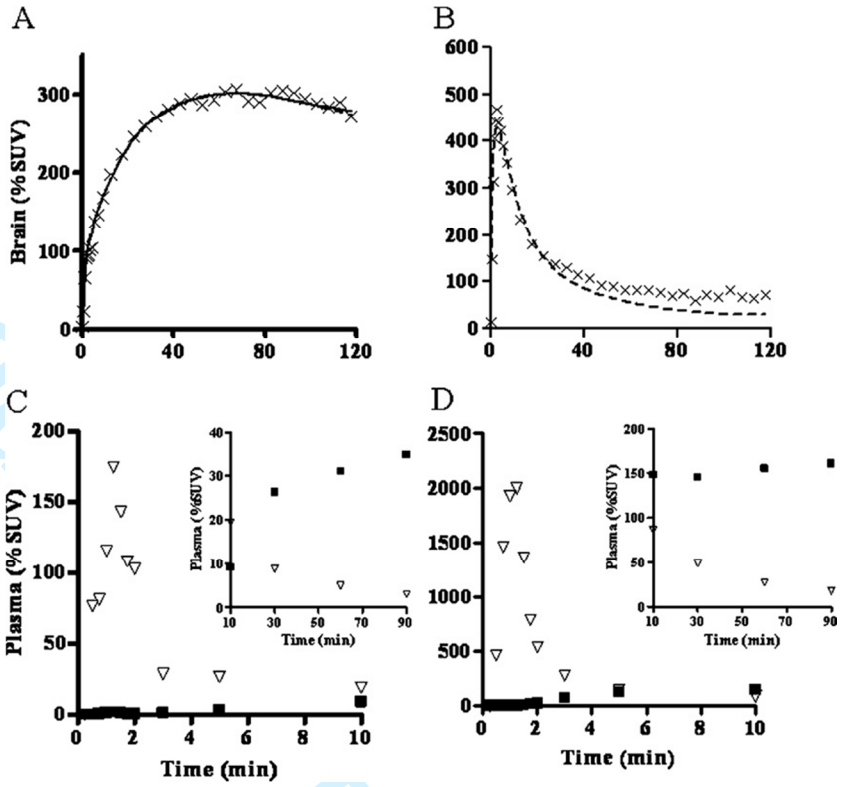


Fig. 2. Representative [^{11}C]PBR28 brain time–activity curves, compartmental fittings and radioligand concentrations in plasma at baseline (A and C; scan #6, Table 2) and after receptor blockade (B and D; scan #7, Table 2). The aryloxyanilide derivative DAA1106 (3 mg/kg IV) was injected with [^{11}C]PBR28 in the blocking experiment. In the brain curves (A and B), the dashed and solid line is the one- and two-tissue compartment fitting, respectively. For the plasma curves (C and D), the inset graphs show the lower plasma activities after 10 min. Symbols: (×) putamen, (▽) plasma parent, ( ) major radiometabolite.

17. Figure 4. Please add the radioligand name and the genotype to the figures, to improve readability.

Our response: The radioligand name and the genotype have been added to Fig. 4.

18. Figure 5. Were the plots based on averaged regional VT values? If so, please add the information to the legend.

Our response: The plots were based on average regional V_T values. The following sentence has been added to the Legend "The plots were performed using average V_T values in each VOI of the same affinity type." The corresponding change has been made in the Legend of **Supplemental Figure 3.**