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ORIGINAL ARTICLE

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

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Second-generation radioligands for translocator protein (TSPO), an inflammation marker, are confounded by the codominant rs6971 polymorphism that affects binding affinity. The resulting three groups are homozygous for high-affinity state (HH), homozygous for low-affinity state (LL), or heterozygous (HL). We tested if *in vitro* binding to leukocytes distinguished TSPO genotypes and if genotype could affect clinical studies using the TSPO radioligand [^{11}C]PBR28. *In vitro* binding to leukocytes and [^{11}C]PBR28 brain imaging were performed in 27 human subjects with known TSPO genotype. Specific [^{3}H]PBR28 binding was measured in prefrontal cortex of 45 schizophrenia patients and 47 controls. Leukocyte binding to PBR28 predicted genotype in all subjects. Brain uptake was ~40% higher in HH than HL subjects. Specific [^{3}H]PBR28 binding in LL controls was negligible, while HH controls had ~80% higher binding than HL controls. After excluding LL subjects, specific binding was 16% greater in schizophrenia patients than controls. This difference was insignificant by itself ($P = 0.085$), but was significant after correcting for TSPO genotype ($P = 0.011$). Our results show that TSPO genotype influences PBR28 binding *in vitro* and *in vivo*. Correcting for this genotype increased statistical power in our postmortem study and is recommended for *in vivo* positron emission tomography studies.

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INTRODUCTION

Translocator protein 18 kDa (TSPO) is a proposed biomarker for neuroinflammation. Positron emission tomography (PET) can measure the density of this biomarker using radioligands that bind to TSPO.[1] The prototypical radioligand [^{11}C](*R*)-PK 11195 has been used to detect increased TSPO density in diverse disorders characterized by neuroinflammation, including Alzheimer's disease, stroke, multiple sclerosis, rheumatoid arthritis, and frontotemporal dementia.[2–6] However, [^{11}C](*R*)-PK 11195 has several disadvantages, including low specific-to-nonspecific binding.[7] These limitations prompted the development of radioligands with greater specific signal, including [^{11}C]PBR28, [^{18}F]PBR06, [^{11}C]DAA1106, [^{11}C]DPA713, [^{18}F]PBR111, [^{18}F]FEPPA, and [^{11}C]AC-5216.[8–14]

Unfortunately, quantification of binding for all tested second-generation TSPO radioligands is confounded by the expression of two different forms of TSPO, coded by the rs6971 single-nucleotide polymorphism (SNP).[15] This SNP in exon 4 of the TSPO gene causes a nonconservative alanine-to-threonine substitution in position 147. Among persons of European ancestry, the predominant form (alanine) has a prevalence of ~70% and high-affinity binding; the polymorphism (threonine) has a prevalence of ~30% and low-affinity binding. The prevalence of three resulting combinations is: 49% for homozygous high-affinity (HH); 9% for homozygous low-affinity (LL); and 42% for heterozygotes (HL) (http://hapmap.ncbi.nlm.nih.gov/cgi-perl/snp_details_phase3?name=rs6971&source=hapmap28_B36&tmpl=snp_details_phase3).

The existence of these two forms of TSPO was first suggested by PET scans using [^{11}C]PBR28, which has about 50-fold differential affinity in HH compared with LL subjects.[7,16] Furthermore, we now know that all second-generation TSPO radioligands have differential affinity, ranging from fourfold to 50-fold.[17] A recent study by Mizrahi *et al*[18] using [^{18}F]FEPPA was the first to demonstrate the association of TSPO genotype and variability in PET imaging. To decrease variability in our PET studies, we routinely exclude LL subjects (about 9% of the population) based on low-affinity binding to peripheral leukocytes. Nevertheless, our PET subjects are a mixture of HH and HL genotypes, which adds variability to measurements. Furthermore, PET studies using other second-generation radioligands include all three genotypes (HH, HL, and LL).

The differential affinity of TSPO confounds the clinical application of TSPO radioligands because it adds variance to the

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measurements. However, the degree to which TSPO genotype influences *in vivo* binding measured by PET is unknown. This study sought to assess the utility of *in vitro* receptor binding to distinguish these three groups (HH, LL, and HL) and to extrapolate its impact on the sensitivity of future clinical PET studies. More specifically, we sought to determine: (1) if TSPO genotype correlates with *in vitro* PBR28 binding using peripheral leukocytes and *in vivo* using brain PET imaging; (2) if differential affinity exists for PBR28 both in controls and in disease states (in this case, schizophrenia); and (3) if correcting for genotype improves the sensitivity of PBR28 to detect group differences in TSPO density measured in postmortem brain from schizophrenia subjects and healthy controls. Finally, we estimated the nondisplaceable uptake of [^{11}C]PBR28 to further characterize the potential clinical utility of this radioligand. Since HH and HL subjects are expected to have different contributions of specific binding, we used the measured total brain binding with PET (*in vivo* study) and the specific binding in postmortem brain tissue (*in vitro* study) to estimate nondisplaceable uptake. Because LL subjects are excluded from our PET scans, we used only HH and HL subjects for leukocyte binding and PET imaging studies in live subjects. For the postmortem study conducted to assess between-group differences, all three genotypes were included.

MATERIALS AND METHODS

This study was approved by the NIMH Combined Neurosciences Institutional Review Board and all volunteers gave their informed consent before inclusion in the study.

In Vitro Study

In Vitro Leukocyte Assay. Leukocytes (white blood cells) were collected from 32 healthy volunteers (27 were used in the *in vivo* study) and 9 patients with Alzheimer's disease. Leukocytes were isolated and homogenized as previously described.[7]

To determine whether subjects were high- or mixed-affinity binders, heterologous receptor binding assays were performed using the racemic radioligand [^{3}H]PK 11195 (3.2 GBq/μmol; Perkin-Elmer Life and Analytical Sciences, Boston, MA, USA), with cold PBR28 as the displacer. In brief, 100 μL [^{3}H]PK 11195 ($\sim$0.9 nmol/L) was added to each assay tube, followed by 100 μL of 12 cold PBR28 concentrations (0.01 to 3,000 nmol/L), 50 mmol/L HEPES (pH = 7.4) buffer (to determine total binding), or 10 μmol/L PBR28 (to determine nonspecific binding). In all, 100 μL leukocyte homogenate suspension (20 μg/mL protein) was added to each tube and incubated for 30 minutes in a shaking water bath at 23°C. Samples were filtered with a Brandel cell harvester (Gaithersburg, MD, USA) through Whatman GF/A filter paper (pretreated with 0.5% polyethyleneimine), followed by three washes of 1 mL ice-cold 50 mmol/L HEPES buffer (pH = 7.4; 4°C). Radioactivity was measured with liquid scintillation counting for five minutes using 4 mL of Ultima-Gold (Perkin-Elmer, Chicago, IL, USA).

Data for heterologous binding assays were analyzed using nonlinear regression curve-fitting software provided by GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). A mean K_D of 4.7 nmol/L for [^{3}H]11195 was used as the dissociation constant to calculate K_i.[7] One- and two-site fitting were compared to determine which equation fits best. Two-site fitting was the preferred model when the P value for null hypothesis (the one-site fitting) was <0.05. The HH were identified as subjects whose equation fit best to one-site fitting with a $K_i < 1$ nmol/L. HL were identified as subjects whose data best fit the two-site equation.

Genetic Analysis. Genomic DNA from 49 subjects was included to genotype the SNP, rs6971, within the TSPO gene on chromosome 22q13.2. This technique integrates a polymerase chain reaction (PCR)-based assay with laser scanning technology to excite fluorescent dyes present in the specially designed TaqMan probes (Applied Biosystems,-Carlsbad, CA, USA). One probe perfectly matched to the common (G) allele labeled with VIC, and the second probe matched to the variant (A) allele labeled with 6-carboxyfluorescein (FAM). Genomic DNA was extracted from blood using the AutoGen automated systems and AutoGenFlex STAR Whole Blood Extraction kit, catalog # AGKT-WB-640 (Holliston, MA, USA). In all, 6 μL of human genomic DNA, at 2.5 ng/μL, from each subject, was applied to 48 wells of a 96-well PCR plate and allowed to dry at 50°C for $\sim$3 hours. A working master mix was prepared that contained: 0.25 μL of TaqMan genotyping probe mix (40 $\times$), 2.25 μL of TaqMan Genotyping Master Mix (Applied Biosystems), and 2.5 μL of water. Ultimately, a total of 5 μL of this mix was added to the dry DNA samples along with 10 μL of mineral oil to avoid evaporation during PCR cycling. The context sequence [VIC/FAM] was as follows:

CCCCTACCTGGCCTGGCTGGCCTTC[A/G]CGACCACACTCAACTACTGCGTATG

Among 11 control samples genotyped, 4 of 4 A/A, 4 of 4 G/A, and 3 of 3 G/G samples produced the expected genotypes.

Postmortem Brain Samples. Postmortem samples of dorsolateral prefrontal cortex (DLPFC) tissue from 45 patients with schizophrenia and 47 healthy controls were collected as previously described.[19] Informed consent was obtained from family members according to established guidelines. Medical, psychiatric, and substance use history, smoking status, and demographic information were collected by telephone interview with next-of-kin within 1 week of donation (Supplementary Table 1).

For individuals with schizophrenia, each case was reviewed by two board-certified psychiatrists to establish DSM-IV Axis I lifetime psychiatric diagnoses, using psychiatric record reviews and/or family informant interviews.[19] Normal controls had no history of significant psychological problems or care, psychiatric admissions, lifetime history of substance abuse or dependence, or acute substance intoxication. Toxicology testing was conducted on every case to screen for ethanol and illicit drugs. For individuals with schizophrenia, additional testing was performed by National Medical Services (Willow Grove, PN, USA) to assess antipsychotic medication use at the time of death. Whenever possible, use of antipsychotic medications was culled from available medical records and converted to CPZ (chlorpromazine) equivalent (CPZE) doses in milligrams.

Gray matter tissue from the crown of the middle frontal gyrus was obtained from the coronal slab midway between the frontal pole and the most anterior extent of the genu of the corpus callosum. Dorsolateral prefrontal cortex corresponding to Brodmann's areas 9 and 46 was dissected on dry ice using a hand-held dental drill and immediately stored at -80°C.

All brain samples had been genotyped for multiple loci, including the rs6971 SNP, using Human 1 M duo v3 chip via standard procedures (Illumina, San Diego, CA, USA).

In Vitro [^{3}H]PBR28 Receptor Binding Assay. Tissue was homogenized in buffer (20 mmol/L HEPES, 5 mmol/L $MgCl_2$, 1 mmol/L EDTA, pH 7.4) with a Teflon pestle using a Glas-Col Homogenizing System and centrifuged at 25,000 g for 25 minutes at 4°C. The pellet was resuspended, aliquotted, and stored at -80°C. Protein concentration was determined using the Micro BCA Protein Assay Kit (Thermo-Scientific, Rockford, IL, USA).

To determine the specific binding of PBR28, a two-point binding assay was performed in triplicate. In all, 100 μL [^{3}H]PBR28 (specific activity 2.3 GBq/μmol; Amersham, UK) was added to each assay tube, followed by 100 μL of buffer (to determine total binding), or 10 μmol/L cold PBR28 (to determine nonspecific binding). In all, 100 μL DLPFC homogenate suspension ($\sim$20 μg/mL) was added last to initiate the incubation. Specific binding was calculated by subtracting nonspecific binding from total binding.

Positron Emission Tomography Imaging with [^{11}C]PBR28

Twenty-seven healthy volunteers (45.7 $\pm$ 17.9 years) underwent [^{11}C]PBR28 PET imaging. Supplementary Table 2 shows demographic information for each subject.

[^{11}C]PBR28 was synthesized as described in Investigational New Drug Application #76 441, a copy of which is available at: http://pdsp.med.unc.edu/snidd/. At the time of injection, [^{11}C]PBR28 had high radiochemical purity (>99%) and high-specific activity (122 $\pm$ 67 GBq/μmol). The injected dose of [^{11}C]PBR28 was 658 $\pm$ 62 MBq.

The procedures for scan acquisition and image analysis are similar to those previously described for [^{11}C]PBR28;[16] these are described in detail in the Supplementary Material. Total distribution volume (V_T, mL/cm^3) was calculated for each brain region. We followed proposed consensus nomenclature for reversibly binding radioligands,[20] where V_T is the sum of both specific and nondisplaceable uptake, which equals the ratio of radioligand concentration in the brain to that in arterial plasma at equilibrium and is proportional to receptor density.

Estimation of Nondisplaceable Uptake of [^{11}C]PBR28

We used the postmortem results to estimate the relative expression of H and L forms in HL subjects, and then used total uptake (V_T) measured in HH and HL subjects from the PET data to estimate specific (V_S) and nondisplaceable uptake (V_{ND}) using the following equations.

$$V_T = V_S + V_{ND} \quad (1)$$

$$V_{S(HL)} = xV_{S(HH)} \quad (2)$$

Where x was determined by dividing the specific binding of [^{3}H]PBR28 in postmortem tissue in HL subjects by that in HH subjects. By combining equations (1 and 2), and assuming V_{ND} is uniform across subjects:

$$V_{T(HH)} = V_{S(HH)} + V_{ND} \quad (3)$$

$$V_{T(HL)} = xV_{S(HH)} + V_{ND} \quad (4)$$

Using the measured values of V_T from the PET data of HH and HL subjects, we then solved for V_{ND} to estimate the nonspecific uptake of [^{11}C]PBR28.

Statistical Analysis

Statistical analysis was performed using SPSS Statistics 17.0. Differences in specific binding of [^{3}H]PBR28 (*in vitro* study) and distribution volume (V_T) of [^{11}C]PBR28 (*in vivo* study) were compared between HH and HL groups by independent sample *t*-tests, and among all three genotypes by one-way analysis of variance (ANOVA). Differences in the specific binding of [^{3}H]PBR28 between schizophrenia patients and controls was compared using factorial ANOVA with genotype as a fixed factor. Associations between [^{3}H]PBR28 binding and demographic variables were evaluated using Pearson correlation analysis. Observed *P* values from *t*-tests were corrected for multiple comparisons using the false discovery rate,[21] with *P* value threshold for significance adjusted to $= (n/7) \times 0.05$, where n is the rank of the observed *P* value for each of the seven brain regions tested. Effects of demographic variables were evaluated with factorial ANOVA.

Data are given as mean ± s.d.

RESULTS

In Vitro Leukocyte Assay Results Predicted Translocator Protein Genotype

To determine if the HH and HL genotypes of TSPO correlated with *in vitro* PBR28 binding, leukocytes were collected from 32 healthy volunteers and 9 patients with Alzheimer's disease. In 19 of these subjects, the displacement curve best fit a one-site model, suggesting that these subjects express TSPO with high affinity for PBR28 (HH; Figure 1). The average K_i for these subjects was 0.52 ± 0.21 nmol/L. For the remaining 22 subjects, the displacement curve best fit a two-site model, suggesting that these subjects expressed two variants of TSPO (HL), one with high affinity for PBR28 (K_i of 0.22 ± 0.13 nmol/L) and one with low affinity for PBR28 (K_i of 24.0 ± 17.5 nmol/L). All subjects were genotyped for the Ala147Thr polymorphism, and one- and two-site fits correctly identified the genotype in all subjects. That is, all 22 subjects with a two-site fit of binding to leukocytes were heterozygous, and all of the 19 HH subjects with a one-site fit were homozygous, for the G-allele.

The Ala147Thr Polymorphism Reduced [^{11}C]PBR28 Binding *In Vivo*

To determine if TSPO genotype correlated with *in vivo* PBR28 binding, 27 healthy volunteers from the *in vitro* study (9 HH and 18 HL) also underwent PET imaging with [^{11}C]PBR28. The HH subjects had ~40% greater [^{11}C]PBR28 binding than HL subjects in all regions measured ($P < 0.02$; Table 1). The greater brain uptake in HH versus HL subjects was not due to differential peripheral factors of metabolism or plasma protein binding of the radioligand. Brain uptake was measured as V_T, which corrected for differences in metabolism by normalizing brain uptake to exposure from only the parent radioligand, separated from radiometabolites. Even though the modeling corrects for differences in the arterial input function, the time-activity curves for parent radioligand were very similar for both HH and HL groups (Supplementary Figure 1A). In contrast, the brain time-activity curves were different, with HH subjects showing slower washout than HL subjects (Supplementary Figure 1B). In addition, the plasma-free fraction (f_P) of [^{11}C]PBR28 was not statistically different between the HH and HL groups (4.6% versus 3.8%, $P = 0.19$, overall mean = 4.1 ± 1.4%).

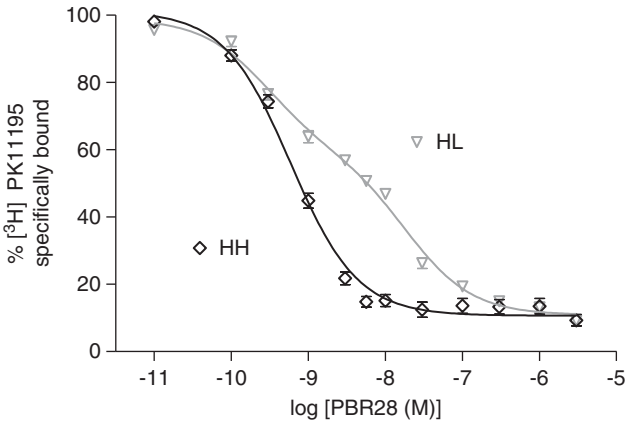

Figure 1. Representative displacement by PBR28 of [^{3}H]PK 11195 binding to leukocyte membranes. PBR28 showed a one-site displacement in high-affinity state (HH) subjects ($n = 17$) but a two-site displacement in heterozygous (HL) subjects ($n = 17$). One- and two-site fitting was determined by GraphPad Prism 5.0 and was performed without knowledge of genotype. Symbols represent mean ± s.d., which was sometimes smaller than the symbol itself.

Table 1. Total [^{11}C]PBR28 binding (V_T, mL/cm^3)

Region	*HH (n = 9)*	*HL (n = 18)*	P *value*	*Adjusted* P *value threshold (FDR corrected)*
Medial temporal cortex	4.6 (1.5)	3.0 (0.8)	0.001	0.007
Cingulate cortex	4.5 (0.6)	3.0 (0.8)	0.003	0.014
Temporal cortex	4.6 (1.5)	3.2 (0.9)	0.007	0.021
Whole brain	4.5 (1.4)	3.2 (0.9)	0.008	0.029
Frontal cortex	4.5 (1.4)	3.3 (0.9)	0.008	0.036
Parietal cortex	4.4 (1.3)	3.3 (0.9)	0.014	0.043
Occipital cortex	4.5 (1.4)	3.3 (0.9)	0.018	0.050

HH, high-affinity binder; HL, mixed-affinity binder; FDR, false discovery rate. Data are given as mean with s.d. below in parenthesis.

Translocator Protein Showed Differential Affinity in Brain Tissue from Both Control and Schizophrenia Subjects

To simulate the effect of genotype on a potential clinical PET study of TSPO, we measured [^{3}H]PBR28 binding in postmortem samples of DLPFC from 45 patients with schizophrenia and 47 control subjects, all with known TSPO genotype. Among the controls,

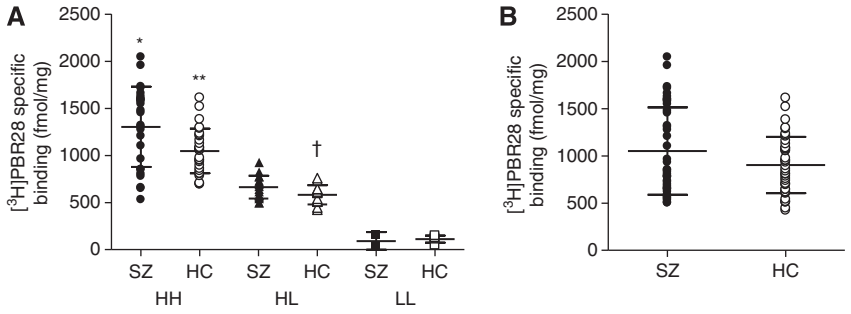

Figure 2. Specific [^{3}H]PBR28 binding in dorsolateral prefrontal cortex (DLPFC) from schizophrenia patients (SZ) and healthy controls (HC) with (**A**) and without (**B**) stratification based on genotype. (**A**) Using a one-way analysis of variance (ANOVA), specific binding in the high-affinity state (HH) group was statistically significant as follows: $^*P<0.001$ versus heterozygous (HL) and low-affinity state (LL) SZ, and $P=0.007$ versus HH HC. $^{**}P<0.001$ versus HL and LL HC, $^{\dagger}P<0.001$ versus LL HC. (**B**) The distribution of specific binding for HH and HL subjects shown in this panel would mimic positron emission tomography (PET) imaging results in living patients, after our typical exclusion of LL subjects. One-way ANOVA resulted in nonsignificant differences between HC and SZ groups ($P=0.085$). When genotype was added to the statistical model as a fixed factor; however, SZ patients had significantly greater specific binding than HC subjects ($P=0.011$).

specific binding was higher in HH subjects (1,047 ± 237 fmol/mg) than HL (582 ± 101 fmol/mg) and LL subjects (111 ± 38 fmol/mg) ($P<0.001$; Figure 2A). Although LL subjects had completely separate specific binding values, HH and HL subjects had overlapping values, showing that binding was affected by factors other than genotype. Specific binding in HL controls was, on average, 56% of that in HH controls. Individuals with schizophrenia had a similar distribution of binding among the three genotypes (Figure 2A). Specific binding was greater in HH patients (1,303 ± 426 fmol/mg) than in HL (664 ± 121 fmol/mg) and LL patients (91 ± 95 fmol/mg, $P<0.001$). No interaction was noted between specific [^{3}H]PBR28 binding and age at death, gender, race, height, weight, or body mass index.

Notably, HH schizophrenia patients had greater specific [^{3}H]PBR28 binding than HH controls (Figure 2A; $P=0.007$); however, HL schizophrenia patients did not have significantly higher binding than HL controls ($P=0.060$), suggesting a blunted association between schizophrenia and TSPO density in the HL subjects. Moreover, when HH and HL subjects were grouped together, brain tissue from individuals with schizophrenia showed 16% greater specific binding of [^{3}H]PBR28 than brain tissue from healthy controls (Figure 2B). By itself, this difference was initially not statistically significant ($P=0.085$). However, when binding affinity status was included as a covariate in the statistical model, this difference reached significance ($P=0.011$).

Schizophrenia patients were older at age of death (55.3 ± 14.0 versus 41.6 ± 13.7 years), which could explain the difference in [^{3}H]PBR28 binding between patient and controls. A significant correlation was noted in HH schizophrenia patients between [^{3}H]PBR28 binding and age of death ($r=0.44$, $P=0.023$); however, no correlation was seen in HH controls or in HL subjects of either group. In addition, a stronger correlation was seen between [^{3}H]PBR28 binding and duration of illness among HH patients ($r=0.54$, $P=0.006$), suggesting that age at death is a confounding factor related to the length of the course of schizophrenia.

Because the difference in [^{3}H]PBR28 binding could also be explained by premortem antipsychotic medication use in schizophrenia patients, we investigated the possible association between [^{3}H]PBR28 binding and antipsychotic use. We found that specific binding was the same in schizophrenia patients taking antipsychotics at the time of death (1,028 ± 445 fmol/mg, $n=34$) as those not taking antipsychotics (1,136 ± 545 fmol/mg, $n=9$), even after correcting for TSPO genotype ($P=0.687$). Specific [^{3}H]PBR28 binding did not correlate with median, lifetime, or last CPZE dose in HH or HL patients ($P>0.21$). When the statistical model included TSPO genotype and antipsychotic medication use as covariates, schizophrenia patients still had significantly greater specific [^{3}H]PBR28 binding ($P=0.015$).

Schizophrenia patients had lower measures of brain pH (6.2 ± 0.2 versus 6.5 ± 0.3), shorter brain storage time before analysis (8.1 ± 2.8 versus 10.1 ± 3.2 years), and greater prevalence of premortem nicotine use (79.1% versus 31.0%) than controls (Supplementary Table 1); however, neither brain pH nor brain storage time significantly correlated with specific [^{3}H]PBR28 binding in either controls or schizophrenia patients ($P>0.1$). In addition, nicotine users did not have different specific binding than nonusers ($P=0.312$, LL subjects excluded).

Estimation of Nondisplaceable Uptake of [^{11}C]PBR28

We used the postmortem results to estimate the relative expression of H and L forms in HL subjects, and then used total uptake (V_T) in HH and HL subjects to estimate specific (V_S) and nondisplaceable uptake (V_{ND}).

$$V_T = V_S + V_{ND} \quad (1)$$

We assumed that binding to the L form is negligible (2%) compared with that to the H form and that V_{ND} is uniform across all subjects. The postmortem study showed that specific binding in HL subjects was 56% of that in HH subjects (Figure 2).

$$V_{S(HL)} = 0.56 V_{S(HH)} \quad (2)$$

V_T for whole brain in HH subjects, as measured by PET, was 1.4 times that in HL subjects (Table 1).

$$V_{T(HH)} = 4.5\text{mL/cm}^3 = V_{S(HH)} + V_{ND} \quad (3)$$

$$V_{T(HL)} = 3.2\text{mL/cm}^3 = 0.56 V_{S(HH)} + V_{ND} \quad (4)$$

The solution of all four equations predicts that $V_{ND} = 1.6\ \text{mL/cm}^3$.

DISCUSSION

The results of this study demonstrate that both *in vitro* and *in vivo* binding of PBR28 correlated with the Ala147Thr polymorphism in the TSPO gene. *In vitro* displacement assays with peripheral leukocytes accurately predicted TSPO genotype in all subjects. Thus, it appears that either leukocyte binding or genotyping can be used to accurately separate TSPO genotypes. *In vivo*, brain uptake of [^{11}C]PBR28 was on average 40% higher in HH than in HL subjects, but with significant overlap. In addition, TSPO binding in postmortem brain from individuals with schizophrenia was 16% higher than in control brain, an effect that was statistically significant only after correcting for TSPO genotype. Our results strongly suggest that clinical studies with [^{11}C]PBR28 will have

increased statistical power and require smaller sample sizes if they incorporate the genotype of the subjects. Thus, we recommend measuring the Ala147Thr polymorphism in all future studies using [^{11}C]PBR28, as well as in other second-generation TSPO radioligands.

The results of this study are particularly important because they demonstrate that the Ala147Thr polymorphism is associated with reduced binding affinity to PBR28 *in vitro* as well as total radioligand binding *in vivo*. This indicates that PET data from clinical studies using [^{11}C]PBR28 are influenced by the presence or absence of this TSPO polymorphism, underscoring the importance of determining genotype in studies using this radioligand. Based on our results, displacement binding assay results are equivalent to those from genotype analysis using PCR, allowing flexibility for investigators interested in using [^{11}C]PBR28.

It is important to note that although we have treated this Ala147Thr polymorphism as a nuisance variable that adds noise to PET imaging, it might also have clinical significance. Translocator protein in the outer mitochondrial membrane transports cholesterol to CYP11A1 on the inner mitochondrial membrane to synthesize pregnenolone, a precursor for steroids. Thus, increased expression of TSPO in macrophages and activated microglia appear critical for the increased production of steroids by these inflammatory cells. We are aware of only one prior study on the effect of this Ala147Thr polymorphism on steroid production, in which Costa *et al*[22] found that Thr147 homozygous or heterozygous volunteers had less pregnenolone in peripheral lymphocytes than Ala147 homozygous volunteers. Therefore, it is possible that HL and LL individuals have a reduced ability to respond to host cell injury via TSPO-mediated pathways.

Translocator Protein Binding in Schizophrenia

The finding that schizophrenic brain has greater specific binding of [^{3}H]PBR28 than control brain was not a working hypothesis of this study; however, this result is consistent with the hypothesis that inflammatory processes are associated with schizophrenia. These results are particularly important given conflicting results seen in prior studies on schizophrenia. While some studies reported no significant differences in microglial cell density or TSPO binding sites between patients with schizophrenia and controls,[23–25] others found elevated microglial density in DLPFC, temporal gyrus, temporal cortex, and frontal cortex of schizophrenia patients.[26–28] Still others reported decreased microglical activation or TSPO specific binding in schizophrenia patients compared with controls.[29,30] A study using [^{11}C](*R*)-PK 11195 found a 16% increase in total gray matter binding in schizophrenia patients;[31] however, all 10 patients included were taking antipsychotic medication and the effect of antipsychotics on microglial activation is unknown. In our study, specific [^{3}H]PBR28 binding was not greater in schizophrenic DLPFC taking antipsychotics at time of death than in those not taking antipsychotics, when differences in TSPO genotype were taken into account. Moreover, specific binding was greater for schizophrenia patients than controls even after accounting for antipsychotic use in the statistical model. While we cannot conclude definitively that antipsychotic use does not influence TSPO density, the results from our study show no evidence of such an association.

Using Genotype with Other Translocator Protein Radioligands

While this study was specific to PBR28, all other second-generation TSPO radioligands examined to date have differential affinity for the target protein.[17] Thus, the Ala147Thr polymorphism may confound results using any of these second-generation TSPO radioligands by increasing variance or by the differential inclusion of the three genotypes in experimental and control groups. In fact, a recent report demonstrated that TSPO genotype was associated with differences in brain uptake of [^{18}F]FEPPA in human subjects.[18] Therefore, we recommend genotyping subjects before prospective clinical studies using any second-generation TSPO radioligands. Furthermore, the original and prototypical ligand [^{11}C]*(R)*-PK 11195 may also have differential affinity, with conflicting results reported using *in vitro*[32] and *in vivo*[7] methods. Thus, additional *in vitro* and *in vivo* studies are necessary to determine the utility of genotype in studies using [^{11}C](*R*)-PK 11195.

One of the most important attributes of a PET radioligand is its ratio of specific to nondisplaceable brain uptake, the latter of which is the sum of nonspecific binding and free radioligand in tissue water. For targets like TSPO, which are widely distributed in brain, nondisplaceable uptake of the cognate radioligand can only be measured after pharmacological blockade by nonradioactive ligand. Such blockade is often impossible to perform in humans because the drug is not available or because of pharmacological effects. Our postmortem and *in vivo* results provide a relatively unique opportunity to estimate nondisplaceable uptake without pharmacological blockade. We used the postmortem results to estimate the relative expression of H and L forms in HL subjects, and then used total uptake (V_T) in HH and HL subjects to estimate specific (V_S) and nondisplaceable uptake (V_{ND}).

Our estimation of V_{ND} yielded two interesting results. First, the postmortem study showed that specific binding in HL subjects was 56% (and not 50%) of that in HH subjects (Figure 2). These results suggest that 56% of TSPO in heterozygotes is in the H form, not the 50% that would be expected if HL subjects were to express the H and L versions of the TSPO gene in equal amounts. Such a disparity could occur by differential expression of the two genes or differential turnover of two TSPO proteins. Second, our calculations predict that V_{ND} of [^{11}C]PBR28 is 1.6 mL/cm^3. Thus, the ratio of specific to nondisplaceable uptake of [^{11}C]PBR28 in HH subjects is 1.8/1 ($=(4.5–1.6)/1.6$). This estimated value of V_{ND} and V_{ND}/f_P in human subjects (1.6 and 39.0 mL/cm^3, respectively) is very similar to that measured in monkey brain (1.6 and 28.6 mL/cm^3, respectively) after pharmacological blockade.[33] Although specific binding often differs between species, nonspecific binding (after correcting for plasma protein binding) is often thought to be similar across species, because of the similar chemical composition of brain tissue.

These calculations show that *in vitro* binding and *in vivo* brain uptake in a situation of altered receptor binding (i.e., affected by genotype) can be used to calculate nondisplaceable uptake in human brain. Similar measurements could be made for other TSPO radioligands to compare their relative ratios of specific to nondisplaceable uptake. This method is preferred over simply using V_T of LL subjects as an estimation of V_{ND}. Prior studies using [^{11}C]PBR28 have shown that kinetic modeling using either the one- and two-tissue compartment models provides poor fitting with measured brain PET data in LL subjects.[16] Therefore, V_T of LL subjects cannot be accurately calculated using kinetic modeling and cannot be used to estimate V_{ND} of [^{11}C]PBR28. Our method of calculating V_{ND} is admittedly speculative and requires the assumption that (1) V_{ND} is uniform among subjects, (2) the ratio of specific binding between HH and HL subjects is the same *in vitro* and *in vivo*, and (3) specific binding of PBR28 to the L version of TSPO is negligible. However, these are reasonable assumptions given (1) the similar chemical composition of brain tissue among human subjects, (2) TSPO density is not expected to change disproportionately between HH and HL subjects postmortem, and (3) results from our postmortem study show dramatically lower specific binding in LL subjects than HH subjects.

In conclusion, the Ala147Thr TSPO polymorphism was associated with reduced *in vivo* binding with [^{11}C]PBR28. The leukocyte binding assay accurately predicted the Ala147Thr polymorphism and may be used as an alternative to genotyping. Controlling for this polymorphism will not only provide more accurate

quantitation of TSPO density but also increase statistical power and reduce the necessary sample size of clinical studies using [^{11}C]PBR28. Patients with schizophrenia had greater TSPO binding in DLPFC. Whether this truly reflects a neuroinflammatory process in schizophrenia is unknown, but could be explored with TSPO PET imaging in live subjects after correcting for the Ala147Thr polymorphism.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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