INTRODUCTION
Alcohol use disorder (AUD) is a chronic relapsing disorder characterized by the inability to stop drinking despite one’s awareness of negative consequences. Repeated exposure to high doses of alcohol has been associated with neurotoxicity that can result in cognitive deficits [1]. There is increased recognition that alcohol-induced neuroinflammation contributes to its neurotoxicity [2]. However, the mechanisms underlying the neurotoxicity from high doses of alcohol are poorly understood.

Microglia (about 10–15% of the brain’s cells) are the resident innate immune cells in the brain and are activated in response to cellular stressors [3, 4]. Once activated, microglia release pro-inflammatory cytokines and chemokines, glutamate, adenosine triphosphate (ATP), and reactive oxygen species [5, 6], all of which contribute to the inflammatory process. In animal models, alcohol can activate microglia and induce the production of inflammatory mediators [7]. Administration of alcohol at doses that mimic binge drinking in humans was shown to activate microglia and increase the release of pro-inflammatory cytokines and chemokines in rodents [2, 8]. 18-kDa translocator protein (TSPO) is expressed in active microglia and is considered to be a marker of neuroinflammation [9, 10] (although TSPO is also expressed in resting microglia, astrocytes, and neurons [11]). A preclinical autoradiography study with the neuroinflammation ligand [3H]PK-11195 that binds to TSPO, found that 4 days of binge drinking in rats increased [3H]PK-11195 binding in hippocampus and entorhinal cortex [12]. Furthermore, knockdown of TSPO in neurons of male adult drosophilae increased their sensitivity to alcohol’s sedative effects and blocked tolerance development to repeated alcohol exposures, identifying TSPO as a modulator of alcohol’s effects [13].

In humans, alcohol abuse induces inflammation in the brain and body [2], affecting immunity and increasing susceptibility to certain infectious diseases [14]. Moreover, there is evidence that alcohol abuse increases systemic markers of inflammation such as C-reactive protein and cytokines [15]. Similarly, studies using postmortem brain tissue from patients with AUD showed increases in cytokines (monocyte chemoattractant protein 1 [MCP-1]) and in markers of microglial activation [2, 16]. Gene expression studies on postmortem brains have also reported increases in genes involved with inflammation in AUD [17]. In contrast, brain imaging studies using PET and the TSPO ligand...
[11C]PBR28 have reported decreased binding in AUD participants compared to controls [18, 19]. One study showed a 10% reduction in PBR28 binding in the brain of 15 subjects with moderate AUD compared to 15 controls [18], although this effect was no longer significant with the removal of the one patient with 24 days of abstinence. Another study found a 20% reduction in PBR28 binding only in the hippocampus in 9 AUD subjects compared to 20 controls that was driven by 3 AUD high-affinity binders (TSPO rs6971 homozygous), whereas there were no group differences in medium-affinity binders (rs6971 heterozygous) [19]. Although both studies seem to indicate decreases in PBR28 binding in AUD, the effect sizes are small and restricted to some of the AUD patients, but not others.

Here, we further characterize the effects of heavy alcohol exposure on neuroinflammation as assessed with PET and [11C] PBR28. In order to control for heterogeneity among AUD patients, we conducted parallel studies in humans and in a rodent model of alcohol dependence. Based on animal findings with the TSPO ligand [11C]PK-11195 [12], we initially hypothesized higher [11C] PBR28 binding in AUD patients and in alcohol-dependent compared to -nondependent rats, reflecting neuroinflammation after chronic alcohol consumption. However, given the two recent studies showing the opposite to the expected finding of lower [11C]PBR28 binding in AUD participants versus controls [18, 19], we expected to replicate these findings in our clinical and preclinical data set. Additionally, TSPO has a cholesterol-binding domain in its fifth transmembrane loop and cholesterol binding leads to structural changes in TSPO shifting the equilibrium toward the translocator monomer [20]. Because alcohol can modify plasma cholesterol levels [21, 22], we also explored the association between cholesterol levels and [11C]PBR28 binding in humans and hypothesized an inverse relation between them. Finally, because TSPO deletion mutations in rodents and the rs6971 TSPO polymorphism in humans alters adrenocorticotropic hormone (ACTH)-induced plasma cortisol concentrations [23], we also explored the relationship between plasma ACTH and cortisol levels and whole brain [11C]PBR28 binding.

MATERIALS AND METHODS

Human study Participants. Nineteen patients with AUD and 17 HC completed the study. Groups were matched for age, gender, body mass index (BMI), and TSPO genotype (see Table 1 for demographics and clinical characteristics). Participants were medically screened to exclude ferromagnetic implants, major medical problems, chronic use of psychoactive medications, neurological problems, or head trauma; and current diagnosis of a substance use disorder (other than alcohol abuse and/or dependence in the AUD group or nicotine dependence for either group), past history of drug abuse or other psychiatric disorders that needed treatment as assessed by the Structured Clinical Interview for the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) [24]. Women were by the Structured Clinical Interview for the Diagnostic and other psychiatric disorders that needed treatment as assessed. All participants underwent brain imaging with [11C]PBR28 PET and MRL. [11C]PBR28 was produced as described by Fujita et al. [30]. Mean (±SD) injection dose (ID) of [11C]PBR28 was 18.8 ± 0.7 mCi (range, 15.5–19.4 mCi), which was injected intravenously over a 1 min period. Mean molar activity (A_m) of [11C]PBR28 at the time of injection did not differ between groups (mean AUD = 2.4 ± 1.1 Ci/μmol, mean HC = 2.9 ± 1.5 Ci/μmol, p > 0.05) at the time of injection. Prior to tracer injection, a transmission scan was obtained using cesium—137 to correct for attenuation.

PET study and image data acquisition. All participants underwent brain imaging with [11C]PBR28 PET and MRL. [11C]PBR28 was produced as described by Fujita et al. [30]. Mean (±SD) injection dose (ID) of [11C]PBR28 was 18.8 ± 0.7 mCi (range, 15.5–19.4 mCi), which was injected intravenously over a 1 min period. Mean molar activity (A_m) of [11C]PBR28 at the time of injection did not differ between groups (mean AUD = 2.4 ± 1.1 Ci/μmol, mean HC = 2.9 ± 1.5 Ci/μmol, p > 0.05) at the time of injection. Prior to tracer injection, a transmission scan was obtained using cesium—137 to correct for attenuation.

Dynamic [11C]PBR28 PET scans were performed in list mode using a high resolution research tomograph (HRRT; n = 24) (Siemens, Knoxville, TN, USA) or a GE Advance (n = 12) (GE Healthcare, Waukesha, WI, USA). There were no effects of scanner type on whole brain [11C]PBR28 binding in the overall sample (ANCOVA corrected for genotype: F = 0.1, p = 0.3), and scanner type did not differ between groups (χ² = 0.22, p = 0.6). The time of injection ranged from 10:03 a.m. to 2:34 p.m. There were no group differences in injection time (F = 1.4, p = 0.2) and no effect of injection time on whole brain [11C]PBR28 binding in the overall sample (Pearson’s correlation corrected for genotype r = 0.1, p = 0.5, ns). We therefore did not include scanner type or injection time as covariates in our analyses. [11C]PBR28 PET raw data were

| Table 1. Demographics and clinical characteristics of study participants |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Characteristic | AUD (n = 19) | HC (n = 17) | p value |
|----------------|---------------|---------------|--------|
| Age, years | 47.6 ± 10.1 | 47.5 ± 10.9 | 0.9 |
| BMI | 26.6 ± 4.2 | 27.8 ± 3.3 | 0.3 |
| Gender | 5 female | 8 female | 0.3 |
| WASI full IQ score | 89.9 ± 16.5 | 103.2 ± 18.4 | 0.03 |
| rs6971 genotype | 11 high | 11 high | 0.7 |
| Smoking | 8 medium | 6 medium | 0.0001 |
| TLFB average drinks/day | 8.9 ± 4.9 | 0.1 ± 0.2 | <0.0001 |
| TLFB drinking days/week | 5.9 ± 1.5 | 0.4 ± 0.7 | <0.0001 |
| Alcohol drinking years | 29.5 ± 13.5 | 16.2 ± 17.6 | 0.015 |
| LDH (kg) | 1439 ± 1331 | 34 ± 494 | <0.0001 |
| Abstinence (days) | 2.8 ± 2.4 | 4081 ± 1053 | 0.01 |
| ADS | 13.5 ± 7.6 | 0.1 ± 0.3 | <0.0001 |
| STAI trait | 38.9 ± 11.8 | 26.7 ± 6.2 | 0.001 |

P values in bold are considered statistically significant. ADS, addiction severity index; AUD, alcohol use disorder; BMI, body mass index; HC, healthy controls; LDH, lifetime drinking history; STAI, state–trait anxiety inventory; TSPO, translocator protein in humans; AD, alcohol dependence; AUD, alcohol use disorder; STAI, state–trait anxiety inventory.
reconstructed with a 3D-ordered subset expectation maximization (OSEM) algorithm to generate 27 frames of data for each subject. During the 90-min scan session, 23 time points of arterial blood samples were collected from the radial artery. Both whole blood and the corresponding plasma samples were counted and 16 plasma samples were analyzed with radiolabelled plasma exposure was used to quantify the fraction of intact [11C]PBR28 and the free fraction (f_p) of [11C]PBR28 in plasma [30]. There were no differences in plasma protein binding (f_p) between AUD and HC (mean AUD = 0.016 ± 0.005, mean HC = 0.018 ± 0.004, t = 0.88, p = 0.4).

**MRI acquisition.** For structural magnetic resonance imaging (MRI), T1-weighted 3D magnetization prepared rapid acquisition gradient echo (MPRAGE; TR/TE = 2200/4.25 ms, 1-mm isotropic resolution) pulse sequences was acquired with a 3T Prisma Scanner (Siemens Medical Systems) [31] to provide anatomical coregistration for [11C]PBR28 images and to control for potential brain atrophy in AUD participants.

**Image preprocessing and regions of interest.** Analysis of functional neuroimages (AFNI) and functional software library (FSL) tools were used for spatial normalization and coregistration to the Montreal Neurological Institute (MNI) space [31]. Briefly, T1-weighted MR images were aligned along the AC-PC line. Average [11C]PBR28 images were coregistered to the realigned MRI images. The corresponding spatial transformation was applied into the [11C]PBR28 dynamic images. The FreeSurfer image analysis suite (v 5.3.0; http://surfer.nmr.mgh.harvard.edu) was used to delineate regions of interest (ROIs); whole brain, cortical gray matter, white matter, hippocampus, and thalamus) in the subject’s anatomical space to generate time–activity curves for [11C]PBR28 within these ROIs.

**[11C]PBR28.** Standardized uptake value (SUV) calculated by body weight and injection dose for each subject was used for normalization of both blood input function and image data. A three-exponential function and a sigmoidal function were used to build up the models of plasma and parent [11C]PBR28 fraction, respectively. The PMOD-PKIN tool (v 3.8 PMOD Technologies, Zurich, Switzerland) was used to generate the total distribution volume (V_T) using a 2-tissue compartment model (2TCM), based on time–activity curves of the ROIs and the blood input function model. Spatially normalized dynamic [11C]PBR28 images were transformed to V_T parametric images using Logan analysis with the PXMOD tool (v 3.8) (PMOD Technologies Ltd., Zurich, Switzerland).

**Statistical analysis.** Group comparisons on the ROI measures were performed with SPSS version 20. Multivariate analyses were performed with group (AUD/HC) and genotype (rs6971 heterozygous or medium-allele) as between-group variables, and ROIs as the dependent variables, using Roy’s largest root, and age as a covariate. Effect sizes are reported as partial eta-squared (η²). Post hoc multivariate and univariate tests on differences between AUD and HC were performed for medium-affinity and high-affinity binders separately. We tested associations between whole brain [11C]PBR28 and plasma cholesterol, cortisol, and ACTH. Additionally, group differences in gray matter volume (GMV) were explored using voxel-based morphometry (Supplemental Material 1), which revealed decreased GMV only in the bilateral temporal cortex in AUD. The average of responses to obtain alcohol performed by dependent and nondependent rats was compared with an unpaired Student’s t test. The results were reported as mean ± standard error of the mean. Significance threshold for all tests was set at p < 0.05.

Correlational analyses between whole brain [11C]PBR28 V_T and cholesterol, cortisol, and ACTH were performed in groups combined, and separately for AUD and HC, using partial correlations in SPSS 22, with rs6971 as covariate. We also explored Pearson’s correlations for each genotype separately.

**Rodent study**
*Rats.* Adult male Wistar rats (n = 20) were purchased from Charles River (Kingston, New York, USA) and weighed 250–300 g at the beginning of the study. Rats were group housed (2–3 per cage) in standard plastic cages lined with woodchip bedding and maintained under a reverse 12 h/12 h light/dark cycle (lights on at 8 p.m.) at 21 ± 2 °C with ad libitum access to food and water. All procedures were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the National Institute on Drug Abuse and National Institute of Mental Health Intramural Research Program Animal Care and Use Committees.

**Operant alcohol self-administration and alcohol vapor exposure.** Rats were trained to lever press for access to alcohol or water in standard operant chambers (Med Associates, St. Albans, VT, USA), as previously reported [32]. To habituate the rats to alcohol's taste, they were given free access to alcohol (10%, v/v) and water for 1 day in their home cages. They were subsequently subjected to an overnight session in operant chambers with access to one lever (right lever) that delivered water (100 μl) with food freely available. After 1 day off, rats were subjected to a 2-h session followed by a 1-h session (the next day), with one lever that delivered alcohol (right lever). The subsequent sessions lasted 30 min, and two levers were available (left lever: water; right lever: alcohol). The operant sessions were conducted on a fixed ratio 1 schedule of reinforcement (i.e., each lever press resulted in fluid delivery). Upon stable levels of responding to alcohol, the rats were separated into two groups: (1) exposed to chronic, intermittent alcohol vapor to induce dependence (dependent rats); (2) exposed to air (nondependent rats). Cycles of alcohol intoxication and withdrawal occurred daily for 6 weeks. Over a 24-h period, the alcohol vapor was ON for 14 h (from 6 p.m. to 8 a.m.) consecutively, and operant alcohol self-administration (twice per week) occurred during the 10-h period without alcohol vapor between 6 and 8 h into withdrawal. In this model, rats exhibit reliable signs of alcohol dependence, including a negative emotional-like state and somatic symptoms during withdrawal (for review, see refs. [33, 34]). During vapor exposure, the target blood alcohol levels were 150–250 mg/dl that was maintained for at least 4 weeks.

**PET study.** The rats were removed from vapor around 6 a.m. and transported by car with temperature controlled (21 ± 2 °C) from the Baltimore to the Bethesda Campus in less than 2 h. Alcohol-dependent (n = 10; weight: 547 ± 40 g) and nondependent rats (n = 10; weight: 573 ± 60 g) were scanned in pairs under anesthesia (2–5% isoflurane). One dependent rat was removed from the study due to radiotracer administration failure. Each rat was scanned between 11 a.m. and 4 p.m. (i.e., 5–10 h into withdrawal). A pair of rats (one dependent and one nondependent) was placed side by side in prone position in a small animal PET scanner (microPET Focus-220, Siemens). Following a 10-min transmission scan using Co-57 as the source, [11C]PBR28 (mean ID, 459 ± 151 μCi; A_In ≥ 0.34 Ci/μmol) was administered intravenously. Data acquisition was started simultaneously, and continued for 60 min in list mode. Thirty minutes after the end of the [11C]PBR28 scan, a 30-min [18F]FDG (100–400 μCi) scan was obtained for anatomical coregistration with the [11C]PBR28 images.

**Image analysis.** The Wistar rat brain template [35] was used for coregistration and for defining ROIs (whole brain, gray matter, white matter, hippocampus, and thalamus). For each animal, their [18F]FDG brain images were used as template to coregister their [11C]PBR28 images using PMOD. The coregistration parameters were calibrated on a set of 20 rat brains.
RESULTS

Human clinical characteristics

Table 1 provides a summary on demographics and clinical characteristics. AUD patients drank an average of 8.9 ± 4.9 alcoholic drinks per day in the 90 days prior to the study, with an average of 5.9 ± 1.5 drinking days per week. HC drank 0.1 ± 0.2 drinks per day on an average of 0.4 ± 0.7 days per week. AUD patients had higher trait anxiety scores than HC (p = 0.001).

HC had higher IQ scores compared to AUD (t = 2.3, p = 0.03) and there were ten smokers in the AUD group, but none in the HC group (χ² = 12.4, p < 0.0001; see Table 1). Since smoking is associated with lower TSPO binding in brain [36], we also assessed whether there were differences in the percentage of smokers between TSPO genotypes. The high-binder group tended to have a higher percentage of smokers (7 smokers and 15 nonsmokers) than the middle-binder group (3 smokers and 11 nonsmokers) for the AUD and HC pooled together but the difference was not significant (χ² = 0.46, p = 0.50). Similarly, for the AUD group, the high-binder group tended to have a higher percentage of smokers (seven smokers and four nonsmokers) than the middle-binder group (three smokers and five nonsmokers), which was also not significant (χ² = 1.27, p = 0.26).

C-reactive protein and cholesterol levels were within the normal range in both groups and there were no group differences (see Supplementary Table 1). ACTH, cortisol, and liver AST were higher in AUD compared to HC (all p < 0.05).

Human PET [11C]PBR28. Group mean images and time–activity curves for [11C]PBR28 SUV in the whole brain are shown in Fig. 1.

There was a significant main effect of TSPO genotype rs6971 on [11C]PBR28 Vₜ (G = 0.97, Fₜₕ = 5.2, p = 0.002, η² = 0.49); high-affinity binders had higher Vₜ in all ROIs (all p < 0.0001). There was no main group effect (G = 0.18, Fₜₕ = 1.0, p = 0.46, η² = 0.15), no interaction effect of rs6971 × group (G = 0.33, Fₜₕ = 1.8, p = 0.16, η² = 0.25), and no effect of age (G = 0.30, Fₜₕ = 1.6, p = 0.19, η² = 0.23) on [11C]PBR28 Vₜ. However, when separating the analyses by genotype, there was a significant group effect in medium-affinity binders only (G = 6.36, Fₜₕ = 8.9, p = 0.006, η² = 0.86) with univariate tests showing lower [11C]PBR28 Vₜ in AUD compared to HC in whole brain (Fₜₕ = 6.5, p = 0.027), gray matter (Fₜₕ = 6.9, p = 0.023), white matter (Fₜₕ = 4.8, p = 0.05), hippocampus (Fₜₕ = 6.4, p = 0.028), and thalamus (Fₜₕ = 6.7, p = 0.025) (Fig. 2).

Correlations with cholesterol, cortisol, and ACTH. Cholesterol in plasma correlated negatively with whole brain [11C]PBR28 Vₜ in groups pooled together (rₜ = −0.39, p = 0.02; corrected for rs6971), largely driven by a negative correlation in AUD patients (rₜ = −0.53, p = 0.02) but not in controls (rₜ = −0.46, p = 0.45, ns). The correlation was significant in medium-affinity binders only (rₜ = −0.60, p = 0.02) (Fig. 3). (Note that in medium-binder HC the correlation was also significant rₜ = −0.85, p = 0.03, but not in HC high binders).

Cortisol in plasma showed a trend of a negative correlation with whole brain [11C]PBR28 Vₜ in groups pooled together (rₜ = −0.34, p = 0.06; corrected for rs6971), but there were no associations between cortisol and [11C]PBR28 binding in AUD or HC separately. Separate analyses by genotype revealed that in medium-affinity binders whole brain [11C]PBR28 Vₜ was negatively associated with cortisol levels (rₜ = −0.74, p = 0.002); however, since for medium binders both Vₜ and cortisol were lower in AUD than controls, this correlation might have been driven by the group differences. The correlation between ACTH levels and brain [11C]PBR28 Vₜ were not significant either for the pooled or the separate analyses.

Cortisol and cholesterol plasma levels were positively correlated at trend level (r = 0.31, p = 0.08). Supplementary Tables 2 and 3 provide zero-order correlations and linear regression models on the effects of AUD diagnosis, rs6971, age, BMI, smoking status, cholesterol, cortisol, and ACTH with brain [11C]PBR28 Vₜ, showing that in addition to rs6971, cholesterol (and cortisol at trend level) was an independent predictor of [11C]PBR28 Vₜ.

PET [11C]PBR28 in rodents

Alcohol-dependent rats exhibited escalated responding for alcohol compared with nondependent rats (dependent: 55.6 ± 2.4 alcohol deliveries in 30 min; nondependent: 35.4 ± 3.3 alcohol deliveries in 30 min; p < 0.05). Note that the dependent rats received more than 4 weeks of blood alcohol levels ranging from 150 to 250 mg/dl during the 14 h of alcohol vapor exposure every day. The dependent and nondependent rats were submitted to 30-min oral self-administration sessions twice a week for 6 weeks. During the self-administration sessions, the average blood alcohol levels of nondependent rats were estimated to be less than 50 mg/dl, whereas the average blood alcohol levels of dependent rats were estimated to be around 100 mg/dl.

There were no significant differences in the time–activity curves for [11C]PBR28 SUV in the brain of alcohol-dependent and nondependent rats (Supplementary Figure 2).

Multivariate analyses on the comparison of the SUV measure in all ROIs showed a trend effect for higher values in alcohol-dependent rats compared to nondependent rats, largely driven by a correlation in medium-affinity binders (rs6971), largely driven by a negative correlation in AUD patients (rₜ = −0.53, p = 0.02) but not in controls (rₜ = −0.46, p = 0.45, ns). The correlation was significant in medium-affinity binders only (rₜ = −0.60, p = 0.02) (Fig. 3). (Note that in medium-binder HC the correlation was also significant rₜ = −0.85, p = 0.03, but not in HC high binders).

Fig. 1 Average [11C]PBR28 whole brain time–activity curves in AUD and HC for high- and medium-affinity binders. Bars represent standard deviation. SUV standard uptake value.
dependent than nondependent rats ($\Theta = 0.97$, $F_{5,13} = 2.5$, $p = 0.083$, $\eta^2 = 0.49$). However, the univariate analyses showed no differences between alcohol-dependent and nondependent rats in whole brain, gray matter, white matter, hippocampus, or thalamus ($p > 0.3$; Fig. 4).

**DISCUSSION**

We found no AUD group differences on $[^{11}C]PBR28$ binding in our clinical study and no group differences in $[^{11}C]PBR28$ uptake in the brain of alcohol-dependent versus nondependent rats. However, when separately analyzing by TSPO genotype, we found lower $[^{11}C]PBR28$ binding in the brain of AUD patients than in HC for medium-affinity binders only. We also corroborated our hypothesis of an inverse association between $[^{11}C]PBR28$ binding and plasma cholesterol levels that was significant in groups pooled together, due to significance in medium-affinity binders.

These findings contrast with those from previous preclinical in vitro autoradiography studies that showed higher binding of the TSPO tracer $[^{11}C]PK-11195$ and of the TSPO ligand $[^{11}C]$DAA1106 in rats 3–7 days after intrastriatal injection on alcohol [37]; and those from postmortem brain studies that showed increases in the expression of genes involved in neuroinflammation in alcoholics compared to controls [17]. Indeed, when we initially designed this study, we had expected to find elevated $[^{11}C]PBR28$ binding in the brain of AUD compared to HC, consistent with chronic alcohol-induced neuroinflammation. However, after two recent clinical PET studies showed lower $[^{11}C]PBR28$ binding in AUD compared to controls, which suggested that neuroinflammation in AUD might not be associated with microglial activation [18, 19], we changed our hypothesis to predict that we would replicate $[^{11}C]PBR28$ decreases in the brain of AUD and in rodents chronically exposed to alcohol.
Fig. 3 Cholesterol correlated negatively with whole brain $[^{11}C]PBR28$ in groups pooled together (correction for genotype (cholesterol: $f_{16} = -0.4$, $p = 0.02$); largely driven by a negative correlation in AUD participants ($f_{16} = -0.5$, $p = 0.02$), and by a significant correlation in medium-affinity binders ($f_{16} = -0.6$, $p = 0.02$).

Although we found no group differences between AUD and HC in $[^{11}C]PBR28$ brain binding when analyses were pooled across genotypes, separate analyses by genotype revealed lower $[^{11}C]$PBR28 binding in AUD in medium-affinity binders but no differences in high-affinity binders. Furthermore, we did not observe differences in radiotracer uptake between alcohol-dependent and non-dependent rats. It is important to mention that both human high-affinity binders and all rats have an alanine in their TSPO-binding site (these groups show similar results), whereas human medium-affinity binders express equal amounts of TSPO-binding sites that have either an alanine or a threonine residue. These findings inspire multiple interpretations. One explanation for decreased $[^{11}C]PBR28$ binding in AUD proposed previously [18, 19] could be a loss of microglia or astrocytes in AUD. A previous postmortem study found lower microglia and astrocytes in AUD in various brain regions, including the hippocampus [38]. However, this interpretation would not explain why we did not find group differences in brain $[^{11}C]PBR28$ binding in the high-affinity binders nor in our preclinical model of alcohol dependence. Second, TSPO expression may be decreased due to potential loss of mitochondrial density with chronic alcohol exposure [19] as has been reported to occur in preclinical models of alcoholism [39]. Furthermore, suppressed neurogenesis in AUD may contribute to the group differences as TSPO is also expressed by neural stem cells [40]. Although this again would not explain the lack of an effect in the high-affinity binders nor in our preclinical model.

Finally, our most plausible explanation is that an endogenous TSPO ligand is expressed more in AUD participants than controls, competing with radiotracer binding in vivo but not in vitro. Supporting this interpretation are preclinical findings that have reported increases in the brain concentration of the diazepam-binding inhibitor (DBI), which is an endogenous TSPO ligand [41] and its mRNA with chronic alcohol exposure [42, 43] as well as clinical studies showing increases in DBI in the cerebrospinal fluid of alcoholics compared to that of controls [44, 45].

We found the first evidence of an inverse correlation between cholesterol and $[^{11}C]PBR28$ in all groups pooled together (HC + AUD), which was driven by a significant correlation in the AUD group, and medium-affinity binders (Supplementary Table 2). Given that TSPO has a cholesterol-binding domain that affects the conformation of TSPO if cholesterol is bound [20], endogenous concentrations of cholesterol in blood and brain could affect binding of $[^{11}C]PBR28$ to TSPO, potentially influencing our results. Moreover, because the TSPO rs6971 polymorphism leads to an amino acid substitution located at the site of the TSPO cholesterol-binding domain [23]. This could lead to differential sensitivity to cholesterol’s effects on TSPO and could explain why the inverse association with cholesterol was observed in medium-affinity binders but not in high binders. Although we did not see group differences in AUD and HC in cholesterol levels, cholesterol levels could have contributed to the lower $[^{11}C]PBR28$ binding observed in AUD. Indeed, abnormalities in cholesterol levels have been associated with cognitive impairments in alcoholism [8]. Moreover, in our multiple regression model, we see cholesterol as an independent predictor of $[^{11}C]PBR28$ binding along with TSPO genotype rs6971. Thus, future studies should take cholesterol levels into account when measuring $[^{11}C]PBR28$ binding in the brain.

We also observed a significant inverse association between $[^{11}C]$PBR28 binding in brain and levels of cortisol in plasma of medium-affinity binders only. This is consistent with findings that TSPO deletion in rodents [46] and its corresponding rs6971 polymorphism in humans has recently been associated with diurnal variation (morning versus evening in cortisol levels in saliva in patients with bipolar disorder and AUD [47]. In our sample, all blood draws for plasma cortisol, cholesterol, and ACTH analyses were drawn in the morning, thus investigating effects of rs6971 on diurnal variance in cortisol was not possible; and tracer injection time (ranging from 10 a.m. to 2:30 p.m.) did not correlate with whole brain $[^{11}C]$PBR28 binding in groups pooled together. TSPO rs6971 has furthermore been shown to alter ACTH-induced plasma corticosteroid concentrations [23]. ACTH levels were not correlated with $[^{11}C]PBR28$ binding in the brain. In flies, TSPO mRNA expression was reported to be significantly lower in females versus males [13]. Nevertheless, in our studies, we did not find evidence for gender differences in $[^{11}C]PBR28$ binding in brain. Benzodiazepines bind to TSPO [48], but we did not see differences in $[^{11}C]PBR28$ binding...
smoking status, which is relevant since human smokers were recently shown to have lower brain binding of the TSPO ligand $[^{11}]$CDA1106 than nonsmoking controls in vivo [36]. However, here we showed no effect of smoking on $[^{11}]$CIPBR28 binding (Supplementary Tables 1 and 2), which could reflect lower number of cigarettes smoked per day in our participants (11 ± 8) versus that of Brody et al. (14 ± 4 SD) and or differences in radiotracers or other sample characteristics.

In summary, we found evidence for lower $[^{11}]$CIPBR28 binding in the brain of AUD patients with a medium-affinity binding genotype and no differences in an animal model of alcohol dependence. This could reflect disrupted microglia activation during alcoholism and/or competition with an alcoholism-associated increase of endogenous TSPO ligand(s) observed in those with a medium-affinity binder genotype.

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**ADDITIONAL INFORMATION**
The online version of this article (https://doi.org/10.1038/s41386-018-0085-x) contains supplementary material, which is available to authorized users.

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