Supplementary Online Content

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This supplementary material has been provided by the authors to give readers additional information about their work.

eAppendix. Methods and Results

I. eMethods.

Participants

Twenty four of the 27 non-cannabis-using controls have been included in our previous cohorts[1,2], whereas 13 of the 24 long-term cannabis users have been reported in a glutamate/ [^{18}F]FEPPA V_T correlational study[3]; however, group differences in [^{18}F]FEPPA V_T between cannabis users and non-cannabis-using controls is unique to the present study as results have not been previously reported. Three controls and three cannabis users were excluded from all analyses due to having the low-affinity binder genotype that did not allow [^{18}F]FEPPA PET quantification (n=3), or due to technical/methodological problems (n=3). Of the three with technical/methodological problems, one was a cannabis user and two were non-cannabis-using controls; the cannabis user (HAB) had excessive motion and missing image PET data as the subject exited the scanner on two occasions (35 minutes post-injection for 22 minutes, and 75 minutes post-injection for 15 minutes), one control (MAB) had excessive motion (>1 cm) and issues with blood acquisition, and the other control (HAB) had issues with quantification of blood/plasma ratios (blood/plasma ratios did not fit bi-exponential interpolation, resulting in extremely high K1), precluding reliable quantification.

PET and structural MRI data acquisition and analysis

All [^{18}F]FEPPA scans were performed using a high-resolution CPS-HRRT PET scanner (Siemens Molecular Imaging, Knoxville, TN, USA). To minimize head motion, each participant was custom-fitted with a thermoplastic mask prior to the start of the scan. Arterial blood was collected for the first 22.5 minutes at a rate of 2.5 mL/min after radioligand injection using an automatic blood sampling system (Model PBS-101, Veenstra Instrument, Joure, Netherland). Manual samples were taken at -5, 2.5, 7, 12, 15, 20, 30, 45, 60, 90, and 120 min relative to time of injection. Dispersion and metabolite-corrected plasma input function was generated as previously described.[4] Proton density (PD)-weighted brain MR images required for the delineation of each region of interest (ROI) were obtained for each subject using a 3T MR-750 scanner (General Electric Medical Systems, Milwaukee, WI, USA), except for four controls in which a 1.5T General Electric Signa scanner was used (General Electric Medical Systems). Time-activity curves were extracted for the dorsolateral prefrontal cortex (DLPFC), medial prefrontal cortex (mPFC), anterior cingulate cortex (ACC), cerebellum, temporal cortex (temporal), and gray matter (GM) using a validated in-house imaging pipeline[5]. Total distribution volumes (V_T) in the above ROIs were derived from the time-activity curve and plasma input function using a two-tissue compartment model, which has been validated for [^{18}F]FEPPA quantification[4]. To assess whether there was a difference in [^{18}F]FEPPA V_T between cannabis users and non-cannabis-using controls, we also sampled the dorsal caudate, orbitofrontal cortex (OFC), thalamus, ventral striatum, dorsal putamen, ventrolateral prefrontal cortex (VLPFC), insula, inferior parietal cortex, occipital cortex, and hippocampus.

The prioritized regions (DLPFC, mPFC, temporal cortex, anterior cingulate cortex, cerebellum and gray matter) were selected as primary ROIs based on the known effects of cannabis on cortical regions. A number of studies in cannabis users have reported structural abnormalities with long-term cannabis use including reductions in cortical volumes[6-8], alterations in cortical thickness[9], and reduced cortical gyrification[10,11]. Alterations in brain activation patterns across cortical regions have also been reported in cannabis users[12-15]. Importantly, a CB1 [^{18}F]FMPEP-d_2 PET study in long-term cannabis users showed a downregulation of brain cannabinoid CB1 receptors, an effect that was selective to cortical brain regions and correlated with years of cannabis smoking[*16*]. Furthermore, the above regions are relatively large and stable permitting reliable [^{18}F]FEPPA V_T quantification. The prefrontal subregions (DLPFC, mPFC, VLPFC, OFC) were defined based on their cytoarchitectural differences from adjacent cortex, which was mapped onto the external morphology of the cortex[17-19]. All other ROIs were derived from the anatomical label atlas of Talairach transformed to the standard ICBM/MNI 152 Brain, which is included in the WFU toolbox for SPM[20], and "trimmed" as we previously described in Rusjan et al. 2006[5]. The ventral striatum and dorsal putamen subdivisions follow the guidelines in Mawlawi et al. 2001[21]. These ROIs were previously used by our group[1,2] and others at our center[22,23].

Blood serum levels of cytokines and high-sensitivity C-reactive protein

Serum samples were isolated from whole-blood samples acquired at the time of the [^{18}F]FEPPA PET scan (prior to radiotracer injection). Whole blood was collected in red-top serum collection tubes (BD Vacutainer® 367815) and allowed 40 minutes to clot before centrifugation (3000 RCF for 5 minutes at 4°C). Serum samples were stored at -80°C until analysis. Samples were transported on dry ice to Dr. Cynthia Weickert's lab in Australia. Eight cytokines (IFNγ, TNFα, IL-1β, IL-2, IL-6, IL-8, IL-10 and IL-12) from the Human High Sensitivity T-Cell panel (HST-CYTOMAG60SK, Merck Millipore, Billerica, MA, USA) were analyzed. Serum samples were thawed at 4°C and were centrifuged at 1400g to remove any aggregate protein that may potentially obstruct the measurements. The supernatant was then transferred to a fresh tube and was diluted 1:2 in assay buffer. A 9-pont standard curve with serial dilutions of 1:4 was generated using reconstituted stock standard supplied by the manufacturer. A low and a high concentration quality control (QC) supplied by the manufacturer for each analyte were also used to determine assay accuracy. Samples were run across two separate plates. A pooled internal control (IC) sample was run in duplicate on each plate. The coefficient of variance in ICs across both plates was 3.3% for intra-plate duplicates and 2.7% for inter-plate averages across all analytes. The coefficient of variance of the QCs across analytes on both plates was 5.2% for low range QCs and 3.5% for high range QCs. Data was generated using the Millipore Analyst Software (Merck Millipore, Billerica, MA, USA), which was calculated average values against a 5-parameter logistic standard curve corrected by background readings. The average minimum detectable value across both plates was 0.03 pg/mL for IFNγ, 0.03 pg/mL for IL-10, 0.02 pg/mL for IL-12, 0.01 pg/mL for IL-1β, 0.03 pg/mL for IL-2, 0.01 pg/mL for IL-6, 0.02 pg/mL for IL-8, and 0.03 pg/mL for TNFα (see table below).

HsCRP was measured in serum using high-sensitivity ELISA according to the manufacturer's instructions. (IBL-international, Hamburg, Germany). Ten microliters of serum was diluted 1:1000 for each sample. Samples were run across two plates and both were run back to back by the same investigator, who was blind to the diagnosis. A five-point standard curve was generated using 10, 5, 1, 0.4 and 0 µg/mL calibrators that were prepared by the manufacturer. Sample reads ranged from 0.01 mg/L to 13.34 mg/L. An IC sample was run in duplicate on both plates and had an intra-plate coefficient of variance of 2.2% and 4.9% for each plate, respectively.

Analyte	Calibration range (pg/mL)	Kit specified sensitivity (pg/mL)	Achieved sensitivity (pg/mL)
IFNγ	0-2500	0.47	0.03
IL-10	0-6000	0.51	0.03
IL-12	0-2000	0.16	0.02
IL-1β	0-2000	0.14	0.01
IL-2	0-2000	0.18	0.03
IL-6	0-6000	0.11	0.01
IL-8	0-1250	0.12	0.02
TNFα	0-1750	0.16	0.03
CRP*	0-10	0.02	0.01
*CRP values are in µg/mL			

Blood serum levels of THC, OH-THC, COOH-THC, CBD metabolites

Briefly, 1 mL serum specimens were spiked with deuterated (D3) internal standards (Cerilliant) and then solid phase extracted using the Bond Elut Certify II (200 mg) cartridges (Agilent Technologies). The extracts were evaporated under nitrogen and BSTFA +1% TMCS-derivatized prior to the GC-MS analysis using a ThermoFisher platform: ISQ-LT single quadrupole mass spectrometer with Trace 1310 gas chromatograph fitted with a 20 m x 0.18 mm x 0.18 µm TG-5ms GC Column. The analyte quantification was in SIM mode using the target ions as follows (retention times also indicated): THC 386 Da (6.32 min); OH-THC 371 Da (7.30 min); COOH-THC 371 Da (7.82 min); CBD-390 Da (3.78 min). Calibration with internal standardization was performed with linear regression curve fits with 1/X weighting. Unknowns were quantified against a standard curve ranging from 0.2 to 200 ng/mL for THC (LOD 0.5 ng/mL), OH-THC (LOD 1 ng/mL) and COOH-THC (LOD 1 ng/mL) and from 0.1 to 200 ng/mL for CBD (LOD 0.2 ng/mL). Result validation was based on 3-level quality control (ACQ Science). CBD levels were not detected in blood of cannabis users in our setting, except for two cannabis users- one with 0.8 ng/mL and the other below the limit of quantification (LOQ=0.2 ng/mL). Despite having a positive urine drug screen for cannabis,

one of the cannabis users did not have detectable levels of THC metabolites in serum; notably, removing this individual did not affect the main results (main effect of group on [^{18}F]FEPPA V_T: $F_{(1,47)}$=7.2, p=0.01; gray matter as a whole: $F_{(1,47)}$=6.8, p=0.01).

Statistical Analysis

To assess whether there was a difference in [^{18}F]FEPPA V_T between cannabis users and non-cannabis-using controls, we ran a separate linear mixed model analysis including all gray matter regions sampled. ROIs included were: dorsal caudate, orbitofrontal cortex (OFC), thalamus, ventral striatum, dorsal putamen, ACC, mPFC, DLPFC, cerebellum, ventrolateral prefrontal cortex (VLPFC), insula, temporal cortex, inferior parietal cortex, occipital cortex, and hippocampus.

II. eResults.

[^{18}F]FEPPA V_T across multiple brain regions between long-term cannabis users and non-cannabis-using controls

Whole brain analysis revealed higher TSPO levels in cannabis users (mean: 12.8 mL/cm^3; 95% CI: 11.4 to 14.2 mL/cm^3) compared to non-cannabis-using controls (mean: 10.5 mL/cm^3; 95% CI: 9.2 to 11.8 mL/cm^3) across all gray matter regions sampled (main group effect: $F_{(1,48)}$=6.0, p=0.02; ROI effect: $F_{(1,700)}$=58.0, p<0.001; 22.2% higher). Results remained unchanged after controlling for tobacco (main group effect: $F_{(1,47)}$=6.8, p=0.01), and sex (main group effect: $F_{(1,47)}$=11.0, p=0.002).

Effect of sex on [^{18}F]FEPPA V_T

There was a significant effect of sex on [^{18}F]FEPPA V_T (main sex effect: $F_{(1,47)}$=7.5, p=0.009), such that females had higher TSPO levels than males. A post-hoc analysis in each group separately revealed that this effect was primarily driven by the cannabis user group ($F_{(1,21)}$=12.1, p=0.002) rather than the non-cannabis-using control group ($F_{(1,24)}$=0.3, p=0.6).

eTable 1. Associations Between [^{18}F]FEPPA V_T and Peripheral Cytokine Serum Levels (pg/mL) in Long-Term Cannabis Users, Adjusted for rs6971 TSPO Genotype

[^{18}F]FEPPA V_T	IFNγ		IL10		IL12*		IL1b	
	r	**p**	**r**	**p**	**r**	**p**	**r**	**p**
DLPFC	-.20	.49	-.35	.20	.02	.95	.06	.83
mPFC	-.22	.42	-.36	.19	-.01	.97	.03	.90
Temporal	-.19	.49	-.41	.14	-.02	.95	-.003	.99
ACC	-.24	.39	-.45	.09	-.06	.84	-.03	.92
Cerebellum	-.11	.69	-.22	.43	.10	.74	.12	.67
GM	-.17	.55	-.33	.23	.05	.85	.06	.83

[^{18}F]FEPPA V_T	IL2		IL6		IL8		TNFα	
	r	**p**	**r**	**p**	**r**	**p**	**r**	**p**
DLPFC	-.01	.96	-.06	.83	-.20	.47	-.22	.44
mPFC	-.05	.86	.10	.73	-.27	.34	-.26	.36
Temporal	-.05	.85	-.05	.85	-.24	.39	-.15	.61
ACC	-.09	.75	.12	.67	-.31	.26	-.33	.23
Cerebellum	.07	.79	-.13	.65	-.06	.82	-.12	.68
GM	.02	.95	-.11	.69	-.17	.54	-.15	.60

Abbreviations: ACC, anterior cingulate cortex; DLPFC, dorsolateral prefrontal cortex; GM, gray matter; IL, interleukin; IFNγ, interferon gamma; mPFC, medial prefrontal cortex; temporal, temporal cortex; TNFα, tumor necrosis factor alpha; TSPO, translocator protein 18kDa; V_T, total distribution volume.
*results are presented after removing significant outlier.

eTable 2. Associations Between [^{18}F]FEPPA V_T and High-Sensitivity CRP Blood Serum Levels (µg/mL) in Long-Term Cannabis Users (n=15, Removing Cannabis User With High CRP Levels), Adjusted for rs6971 TSPO Genotype

[^{18}F]FEPPA V_T	High-sensitivity CRP*	
	r	**p**
DLPFC	.57	.03
mPFC	.55	.04
Temporal	.57	.03
ACC	.54	.05
Cerebellum	.61	.02
GM	.59	.03

Abbreviations: ACC, anterior cingulate cortex; CRP, C-reactive protein; DLPFC, dorsolateral prefrontal cortex; GM, gray matter; mPFC, medial prefrontal cortex; temporal, temporal cortex; TSPO, translocator protein 18kDa; V_T, total distribution volume.
*results are presented after removing significant outlier.

eTable 3. Associations Between [^{18}F]FEPPA V_T and THC, COOH-THC and OH-THC Blood Serum Levels (ng/mL) in Long-Term Cannabis Users, Adjusted for rs6971 TSPO Genotype

[^{18}F]FEPPA V_T	THC		OH-THC		COOH-THC	
	r	**p**	**r**	**p**	**r**	**p**
DLPFC	-.40	.20	-.35	.29	-.62	.03
mPFC	-.56	.06	-.49	.13	-.73	.007
Temporal	-.39	.22	-.40	.22	-.65	.02
ACC	-.43	.17	-.33	.32	-.65	.02
Cerebellum	-.31	.33	-.23	.50	-.45	.14
GM	-.35	.26	-.33	.33	-.57	.06

Abbreviations: ACC, anterior cingulate cortex; COOH-THC, 11-Nor-9-carboxy-THC; DLPFC, dorsolateral prefrontal cortex; GM, gray matter; mPFC, medial prefrontal cortex; OH-THC, 11-Hydroxy-THC; temporal, temporal cortex; THC, tetrahydrocannabinol; TSPO, translocator protein 18kDa; V_T, total distribution volume.

eTable 4. Association Between [^{18}F]FEPPA V_T and Chronic Stress and Anxiety as Measured by TICS and BAI, Respectively, in Long-Term Cannabis Users, Adjusted for rs6971 TSPO Genotype

[^{18}F]FEPPAV_T	Stress score (Total TICS)		Anxiety score (BAI)	
	r	**p**	**r**	**p**
DLPFC	.51	.02	.44	.05
mPFC	.51	.01	.43	.05
Temporal	.54	.009	.40	.07
ACC	.63	.002	.44	.04
Cerebellum	.40	.06	.37	.10
GM	.50	.02	.45	.04

Abbreviations: ACC, anterior cingulate cortex; BAI, Beck Anxiety Inventory; DLPFC, dorsolateral prefrontal cortex; GM, gray matter; mPFC, medial prefrontal cortex; temporal, temporal cortex; TICS, Trier Inventory for Chronic Stress.

eTable 5. Association Between [^{18}F]FEPPA V_T and Chronic Stress and Anxiety as Measured by TICS and BAI, Respectively, in Non-Cannabis-Using Controls, Adjusted for rs6971 TSPO Genotype

[^{18}F]FEPPAV_T	Stress score (Total TICS)		Anxiety score (BAI)	
	r	**p**	**r**	**p**
DLPFC	.10	.75	.04	.89
mPFC	.11	.70	.04	.90
Temporal	.06	.85	-.004	.99
ACC	.06	.85	-.01	.96
Cerebellum	.06	.83	-.02	.96
GM	.13	.65	.04	.91

Abbreviations: ACC, anterior cingulate cortex; BAI, Beck Anxiety Inventory; DLPFC, dorsolateral prefrontal cortex; GM, gray matter; mPFC, medial prefrontal cortex; temporal, temporal cortex; TICS, Trier Inventory for Chronic Stress.

eTable 6. Association Between [^{18}F]FEPPA V_T and Estimated Lifetime and Past-Year Cannabis Use (Grams) in Long-Term Cannabis Users, Adjusted for rs6971 TSPO Genotype

[^{18}F]FEPPA V_T	Estimated lifetime cannabis use		Estimated lifetime cannabis use (after controlling for sex)		Past-year cannabis use	
	r	**p**	**r**	**p**	**r**	**p**
DLPFC	-.51	.01	.36	.10	-.12	.59
mPFC	-.51	.01	-.37	.09	-.19	.40
Temporal	-.48	.02	-.34	.13	-.07	.76
ACC	-.37	.08	-.24	.28	-.19	.38
Cerebellum	-.51	.01	-.37	.09	-.11	.62
GM	-.52	.01	-.38	.08	-.15	.50

Abbreviations: ACC, anterior cingulate cortex; DLPFC, dorsolateral prefrontal cortex; GM, gray matter; mPFC, medial prefrontal cortex; temporal, temporal cortex; TSPO, translocator protein 18kDa.

eTable 7. Association Between [^{18}F]FEPPA V_T and Cannabis Craving and Severity of Dependence as Measured by MCQ and SDS Scores, Respectively, in Long-Term Cannabis Users, Adjusted for rs6971 TSPO Genotype

[^{18}F]FEPPA V_T	Cannabis craving score (MCQ)		Severity of dependence score (SDS)	
	r	**p**	**r**	**p**
DLPFC	.05	.83	-.12	.57
mPFC	-.12	.59	-.15	.50
Temporal	.06	.79	-.15	.49
ACC	-.05	.82	-.06	.77
Cerebellum	-.09	.67	-.33	.12
GM	-.03	.89	-.19	.39

Abbreviations: ACC, anterior cingulate cortex; DLPFC, dorsolateral prefrontal cortex; GM, gray matter; MCQ, Marijuana Craving Questionnaire; mPFC, medial prefrontal cortex; SDS, Severity of Dependence Scale; temporal, temporal cortex; TICS, Trier Inventory for Chronic Stress; TSPO, translocator protein 18kDa.

eReferences

1. Hafizi S, Da Silva T, Gerritsen C, et al. Imaging Microglial Activation in Individuals at Clinical High Risk for Psychosis: an In Vivo PET Study with [(18) F] FEPPA. *Neuropsychopharmacology: official publication of the American College of Neuropsychopharmacology.* 2017.
2. Hafizi S, Tseng H-H, Rao N, et al. Imaging microglial activation in untreated first-episode psychosis: a PET study with [18F] FEPPA. *American Journal of Psychiatry.* 2016;174(2):118-124.
3. Shakory S, Watts JJ, Hafizi S, et al. Hippocampal glutamate metabolites and glial activation in clinical high risk and first episode psychosis. *Neuropsychopharmacology.* 2018;43(11):2249.
4. Rusjan PM, Wilson AA, Bloomfield PM, et al. Quantitation of translocator protein binding in human brain with the novel radioligand [18F]-FEPPA and positron emission tomography. *Journal of Cerebral Blood Flow & Metabolism.* 2011;31(8):1807-1816.
5. Rusjan P, Mamo D, Ginovart N, et al. An automated method for the extraction of regional data from PET images. *Psychiatry Research: Neuroimaging.* 2006;147(1):79-89.
6. Churchwell JC, Lopez-Larson M, Yurgelun-Todd DA. Altered frontal cortical volume and decision making in adolescent cannabis users. *Frontiers in Psychology.* 2010;1.
7. Price JS, McQueeny T, Shollenbarger S, Browning EL, Wieser J, Lisdahl KM. Effects of marijuana use on prefrontal and parietal volumes and cognition in emerging adults. *Psychopharmacology.* 2015;232(16):2939-2950.
8. Wilson W, Mathew R, Turkington T, Hawk T, Coleman RE, Provenzale J. Brain morphological changes and early marijuana use: a magnetic resonance and positron emission tomography study. *Journal of addictive diseases.* 2000;19(1):1-22.
9. Lopez-Larson MP, Bogorodzki P, Rogowska J, et al. Altered prefrontal and insular cortical thickness in adolescent marijuana users. *Behavioural brain research.* 2011;220(1):164-172.
10. Mata I, Perez-Iglesias R, Roiz-Santiañez R, et al. Gyrification brain abnormalities associated with adolescence and early-adulthood cannabis use. *Brain research.* 2010;1317:297-304.
11. Shollenbarger SG, Price J, Wieser J, Lisdahl K. Impact of cannabis use on prefrontal and parietal cortex gyrification and surface area in adolescents and emerging adults. *Developmental cognitive neuroscience.* 2015;16:46-53.
12. Becker B, Wagner D, Gouzoulis-Mayfrank E, Spuentrup E, Daumann J. The impact of early-onset cannabis use on functional brain correlates of working memory. *Progress in Neuro-Psychopharmacology and Biological Psychiatry.* 2010;34(6):837-845.
13. Padula CB, Schweinsburg AD, Tapert SF. Spatial working memory performance and fMRI activation interaction in abstinent adolescent marijuana users. *Psychology of Addictive Behaviors.* 2007;21(4):478.
14. Schweinsburg AD, Nagel BJ, Schweinsburg BC, Park A, Theilmann RJ, Tapert SF. Abstinent adolescent marijuana users show altered fMRI response during spatial working memory. *Psychiatry Research: Neuroimaging.* 2008;163(1):40-51.
15. Tapert SF, Schweinsburg AD, Drummond SP, et al. Functional MRI of inhibitory processing in abstinent adolescent marijuana users. *Psychopharmacology.* 2007;194(2):173-183.
16. Hirvonen J, Goodwin R, Li C-T, et al. Reversible and regionally selective downregulation of brain cannabinoid CB1 receptors in chronic daily cannabis smokers. *Molecular psychiatry.* 2012;17(6):642-649.
17. Rajkowska G, Goldman-Rakic PS. Cytoarchitectonic definition of prefrontal areas in the normal human cortex: I. Remapping of areas 9 and 46 using quantitative criteria. *Cerebral Cortex.* 1995;5(4):307-322.
18. Rajkowska G, Goldman-Rakic PS. Cytoarchitectonic definition of prefrontal areas in the normal human cortex: II. Variability in locations of areas 9 and 46 and relationship to the Talairach Coordinate System. *Cerebral cortex.* 1995;5(4):323-337.
19. Uylings HB, Sanz-Arigita EJ, de Vos K, Pool CW, Evers P, Rajkowska G. 3-D Cytoarchitectonic parcellation of human orbitofrontal cortex: Correlation with postmortem MRI. *Psychiatry Research: Neuroimaging.* 2010;183(1):1-20.
20. Maldjian JA, Laurienti PJ, Kraft RA, Burdette JH. An automated method for neuroanatomic and cytoarchitectonic atlas-based interrogation of fMRI data sets. *Neuroimage.* 2003;19(3):1233-1239.
21. Mawlawi O, Martinez D, Slifstein M, et al. Imaging human mesolimbic dopamine transmission with positron emission tomography: I. Accuracy and precision of D2 receptor parameter measurements in ventral striatum. *Journal of Cerebral Blood Flow & Metabolism.* 2001;21(9):1034-1057.

22. Attwells S, Setiawan E, Wilson AA, et al. Inflammation in the neurocircuitry of obsessive-compulsive disorder. *JAMA psychiatry.* 2017;74(8):833-840.
23. Setiawan E, Wilson AA, Mizrahi R, et al. Role of translocator protein density, a marker of neuroinflammation, in the brain during major depressive episodes. *JAMA psychiatry.* 2015;72(3):268-275.