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Binding of a tritiated inverse agonist to cannabinoid CB_1 receptors is increased in patients with schizophrenia

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Abstract

This study sought to determine whether cannabinoid-1 (CB_1) receptor binding was altered in the postmortem dorsolateral prefrontal cortex (DLPFC) of individuals with schizophrenia (schizophrenia; n=47) compared to controls (n=43). The CB_1 receptor inverse agonist radioligand [^{3}H]MePPEP was used to measure specific binding to CB_1 receptors. The specific binding of [^{3}H]MePPEP to CB_1 receptors was 20% higher in patients with schizophrenia than in controls. Power analyses suggested that 53 subjects per group would be needed to detect a similar difference in vivo with positron emission tomography (PET) and the structurally related inverse agonist radioligand [^{18}F]FMPEP-d_2 (80% statistical power, $p<0.05$).

Keywords

cannabinoid; CB_1; schizophrenia; dorsolateral prefrontal cortex; [^{3}H]MePPEP

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Contributors KJ designed the study; wrote the protocol; managed literature searches; collected, analyzed, and interpreted the data; and drafted and revised the manuscript. JH designed the study; wrote the protocol; managed literature searches; conducted the statistical analysis; interpreted the data; and drafted and revised the manuscript. IDH managed literature searches; and drafted and critically revised the manuscript. KBA designed the study; wrote the protocol; and collected and analyzed data. SZ designed the study; wrote the protocol; helped interpret the data; and critically revised the manuscript. TMH dissected brain tissues; and drafted and critically reviewed the manuscript. AD-S organized and provided demographic data on brain tissues; managed literature searches and data interpretation; and drafted and critically revised the manuscript. RBI designed the study; wrote the protocol; analyzed and interpreted the data; and critically revised the manuscript. JEK provided the tissues; provided the genotyping and demographic data; and critically revised the manuscript. All authors contributed to and have approved the final manuscript.

1. Introduction

The cannabinoid CB_1 receptor binds endogenous and exogenous cannabinoids in the brain and modulates various neurotransmitters (Howlett et al., 2002). CB_1 receptors may be altered in schizophrenia, but the direction of this alteration remains unclear. Three of five studies that used in vitro radiography methods found significantly increased CB_1 receptor binding in patients with schizophrenia compared with controls; an additional study found no difference in CB_1 levels in the DLPFC of subjects with non-paranoid schizophrenia but did find elevated levels (22%) of CB_1 binding in individuals with paranoid schizophrenia compared to controls (Dalton et al., 2011;Dean et al., 2001;Deng et al., 2007;Newell et al., 2006;Zavitsanou et al., 2004). In contrast, three of four postmortem studies using immunodetection methods found lower CB_1 receptor density (B_{max}) in patients with schizophrenia compared to healthy controls (Eggan et al., 2008;Eggan et al., 2010;Koethe et al., 2007;Uriguen et al., 2009). The discrepancies in results could be attributed to methodology.

This study sought to determine whether CB_1 receptor binding is altered in the postmortem DLPFC of a large cohort of individuals with schizophrenia and controls using in vitro receptor binding assays, and to use these data to determine the sample size needed to conduct in vivo positron emission tomography (PET) imaging studies. Receptor binding data were also analyzed to assess the potential influence of a common functional variation (*rs2023239*) in the gene coding for CB_1 receptors (*CNR1*), which was previously shown to be associated with higher CB_1 receptor binding in vitro (Hutchison et al. 2008)

2. Materials and Methods

2.1. Human Postmortem Brain Samples

2.1.1 Subjects—Postmortem DLPFC tissue samples from patients with schizophrenia and healthy controls were collected as previously described (Lipska et al., 2006); n=8 schizophrenic and 9 controls for the homologous assay; n=47 schizophrenic and 43 controls for the two-point assay). Tissue samples from the homologous binding assay were not included in the two-point assay cohort. 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 one week of donation (see 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 (Lipska et al., 2006). 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) to assess antipsychotic medication use at time of death. Whenever possible, use of antipsychotic medications was culled from available medical records and converted to chlorpromazine (CPZ) equivalent (CPZE) doses in milligrams.

2.1.2 Dissection—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. DLPFC 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.

2.2. In vitro [^{3}H]MePPEP Receptor Binding Assay

Tissue was homogenized in buffer (20 mM HEPES, 5 mM $MgCl_2$, 1 mM 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 re-suspended, aliquotted, and stored at −80 °C. Protein concentration was determined using the Bradford Protein Assay (Bio-Rad, Hercules, CA).

To determine affinity (K_D) and B_{max} (n=9 controls, n=8 schizophrenic) of MePPEP for the CB_1 receptor, a homologous binding assay was performed in triplicate. One hundred μL [^{3}H]MePPEP (specific activity 83 Ci/mmol; ~ 0.13 nM, diluted in buffer with 0.5% w/v BSA; Amersham GE Healthcare, UK) was added to each assay tube, followed by 100 μL of 10 cold MePPEP (Pharmacore, High Point, NC) concentrations (0.01 nM- 100 nM), buffer (to determine total binding), or 1 μM Rimonibant (Eli Lilly, Indianapolis, IN) (to determine non-specific binding). Eight hundred μL DLPFC homogenate suspension (20 μg/ mL) was added and incubated for 90 minutes in a shaking water bath at 23°C. Samples were filtered with a Brandel cell harvester (Gaithersburg, MD) through Whatman GF/A filter paper, followed by three washes of 3 mL ice-cold 50 mM Tris-HCl 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).

To determine CB_1 receptor binding, total and non-specific binding was determined (n=43 controls, n=47 schizophrenic). Specific binding was calculated by subtracting non-specific binding from total binding.

2.3. Genotyping of CNR1 single nucleotide polymorphism (SNP)

Genotyping was performed with Illumina Human 1M duo v3 chip via standard procedures (Illumina, Inc. San Diego, CA). The target *CNR1* gene SNP (*rs2023239*) was not directly determined, and was therefore imputed by Impute2 software (Howie et al., 2009). Data from Hapmap 3 r2 (http://hapmap.ncbi.nlm.nih.gov/cgi-perl/gbrowse/hapmap3r2_B36/) plus the Human genome 1000 project (http://www.1000genomes.org/) were used as reference, including CEU and YRI populations. The Human genome build 36 map (http://www.ncbi.nlm.nih.gov/projects/genome/assembly/grc/human/) was used.

2.4. Statistical Analysis of Two-Point Assay Data

Statistical analyses were calculated using SPSS software (version 19, SPSS Inc., Chicago, IL). We used linear regression with group status and confounding variables as independent variables to examine group differences in [^{3}H]MePPEP specific binding, and Pearson's Chi-square test, *t*-test, and Pearson's correlation coefficient to examine differences between groups in clinical and demographic variables, *CNR1* gene variation, and to test for correlations between radioligand binding and these variables. The potential influence of the *rs2023239* SNP was assessed by including C allele carrier status as a dichotomous independent variable in a regression model.

Effect size was calculated as the group difference in specific binding divided by standard deviation (SD) in the control group. This effect size was used to estimate how many subjects would be needed to observe a similar effect in vivo using PET and the radioligand [^{18}F]FMPEP-d_2 (K_b=0.19 nM) (Donohue et al., 2008;Terry et al., 2010). For this power analysis, mean and SD of prefrontal cortical [^{18}F]FMPEP-d_2 distribution volume (V_T) were assumed to be 18.7 and 5.2, respectively (actual values from 39 healthy male subjects scanned to date in our lab). V_T reflects specific binding and was calculated from brain and plasma radioactivity using compartmental modeling (Terry et al., 2010). Sample sizes for two-tailed *t*-tests were calculated using freely available software (Dupont and Plummer, 1990), and assumed α=0.05 (two-tailed) and β=0.20 (power of 80%).

3. Results

3.1 [^{3}H]MePPEP Binding to DLPFC

K_D and B_{max} were first determined via homologous binding assays in a small cohort of DLPFC. The K_D of MePPEP was 0.31±0.11 nM in controls and 0.31±0.14 nM in patients with schizophrenia. The B_{max} was 0.10±0.08 pmol/mg protein in controls and 0.08 ± 0.04 pmol/mg protein in subjects with schizophrenia. Although K_D and B_{max} were similar between controls and patients with schizophrenia in this preliminary analysis, large variation (%COV>35%) may have prevented the detection of biologically meaningful effect sizes, suggesting that a significantly larger sample size might be necessary to identify differences in B_{max} between the two groups.

In the subsequent analysis of a larger cohort, specific binding—determined by two-point assays—of [^{3}H]MePPEP to CB_1 receptors was about 20% higher in patients with schizophrenia (0.566±0.208 pmol/mg protein) than in controls (0.471±0.173 pmol/mg protein) (Figure 1). The linear regression model showed a statistically significant effect of group on CB_1 receptor binding ($t=-1.99$, $p=0.050$), but none of the confounding variables were statistically significant. When confounding variables were omitted from the statistical model, the group difference was statistically more significant ($t=-2.35$, $p=0.021$). Among patients with schizophrenia, no statistically significant correlations were observed between specific [^{3}H]MePPEP binding and any of the potentially confounding variables (Table 2). In hindsight, the lack of even a trend-level difference between K_D and B_{max} may have been caused by the relatively small effect of disease status (i.e., schizophrenia) and the large variation of specific binding.

3.2 Power Analysis

Power analysis suggested that 53 subjects per group would be needed to detect a difference of this magnitude in vivo with 80% statistical power and $p<0.05$ using PET and [^{18}F]FMPEP-d_2.

3.3 Genotype of CNR1 SNP

Among patients with schizophrenia and controls, 45% and 49% carried the *rs2023239C* allele, respectively ($\chi^2=0.16$; $p=0.69$). C allele carrier status did not significantly predict CB_1 receptor binding when included in a regression model with group status and potential confounding variables ($F=0.63$, $p=0.43$). Among both healthy controls and patients with schizophrenia, C allele carriers had higher specific binding than non-carriers (6%, n=38 for healthy controls and 8%, n=42 for patients with schizophrenia), but this difference was not statistically significant.

4. Discussion

This study found that the specific binding of [^{3}H]MePPEP to CB_1 was 20% higher in the postmortem DLPFC of patients with schizophrenia than in healthy controls. These results are consistent with most published findings that used in vitro radiography methods (Dalton et al., 2011;Dean et al., 2001;Newell et al., 2006;Zavitsanou et al., 2004). Similarly, the lone in-vivo PET study in humans investigating this issue found increased CB_1 receptor binding in individuals with schizophrenia, although this increase was only statistically significant in the pons (Wong et al., 2010). Our results suggest that this observed lack of statistical significance may be due to the small sample size of that cohort. Conversely, our results are not consistent with published findings using immunodetection (Eggan et al., 2008;Eggan et al., 2010;Koethe et al., 2007;Uriguen et al., 2009). Several factors may have led to the

disparate results obtained via the different methods, including condition of the protein, location of the receptor, or specificity of the antibody or radioligand for the receptor.

Although medication effects cannot be discounted as a possible factor in binding in schizophrenia, no association was detected between binding and antipsychotic use at time of death, as measured by either toxicology or CPZE estimates.

Another issue of interest is whether the in vitro results obtained at 23°C could reasonably be used to estimate the sample size of an in vivo PET study, which would, of course, measure binding at 37°C. First, we performed our assays at 23°C because most published studies of CB_1 receptor binding have used this temperature. This allowed our results to be more accurately compared to prior publications. Second, we expect that our primary finding of elevated CB_1 receptor binding at 23°C will be replicated if performed at 37°C. The effect of temperature on MePPEP binding to CB_1 is, to our knowledge, unknown; however, for most G-protein coupled receptors, K_D increases as temperature increases. Unless temperature disproportionately affects K_D in patients compared to controls, we expect that our result at 23°C would be replicated at 37°C and, therefore, that these results are appropriate for the power calculation of a future PET study.

Power analysis suggested that 53 subjects per group would be needed to detect a similar difference in vivo using PET and [^{18}F]FMPEP-d_2. While this large sample size may seem initially discouraging, a clinical imaging study comparing patients with schizophrenia and healthy controls would have several advantages over in vitro studies. Specifically, in vivo imaging would measure multiple brain regions simultaneously and could include patients in different phases of the illness. It would also obviate many of the confounding factors inevitably associated with in vitro methods.

Finally, genetic variation at *rs2023239* in the *CNR1* gene has been associated with substance abuse, and increased CB_1 receptor binding has been found in post-mortem samples from patients with alcohol dependence who carry the C allele of this locus (Hutchison et al., 2008). The present study found that this SNP had no main effect on specific binding. The study therefore failed to replicate the previous findings obtained in patients with alcohol dependence; however, we did find a non-significant trend towards increased binding in C allele carriers from both groups compared with non-carriers.

Taken together, the results presented here support previous findings of increased CB_1 specific binding in patients with schizophrenia, and lend further credence to the hypothesis that the cannabinoid system is altered in the brain of individuals with schizophrenia.

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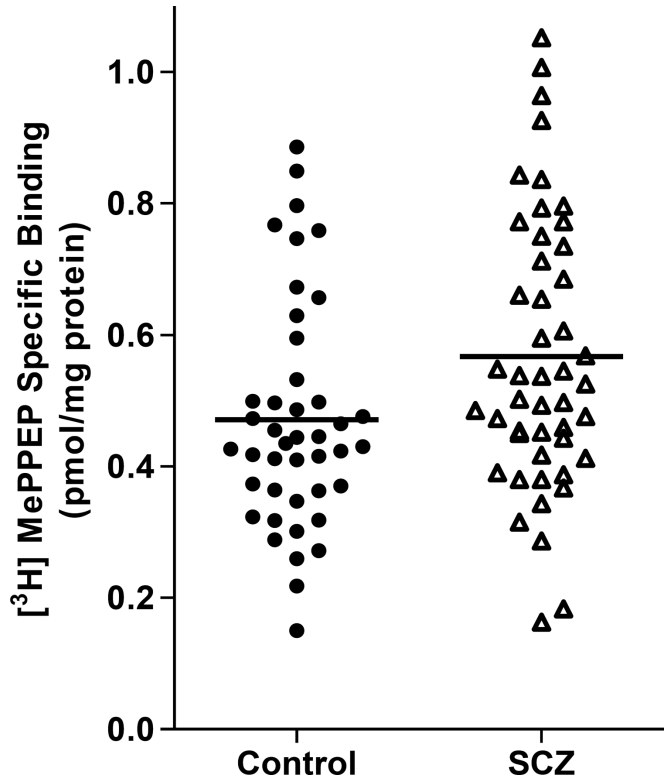

Fi1 1.

Cannabinoid CB_1 specific binding of [^{3}H]MePPEP (pmol/mg protein) in postmortem dorsolateral prefrontal cortex (DLPFC) of controls and patients with schizophrenia (SCZ). CB_1 binding potential was 20% higher in patients with SCZ; analyses were controlled for categorical and continuous variables.

Table 1

Summary of subject demographics.

	Controls (n = 43)	Schizophrenia (n = 47)	p
[^{3}H]MePPEP specific binding * (pmol/mg protein)	0.471 ± 0.173	0.566 ± 0.209	0.050
Gender			
Male	28	23	
Female	15	24	0.122
Race			
African American	27	27	
Caucasian	15	19	
Hispanic	1	1	
Age at Death (years) *	43 ± 15	55 ± 15	<0.001
Age of Onset of Illness	NA	23.1 ± 7.3	NA
Duration of Illness (years)	NA	31.5 ± 11.8	NA
pH *	6.5 ± 0.3	6.2 ± 0.2	<0.001
Postmortem Interval (hours)	36.7 ± 13.3	42.0 ± 26.0	0.219
Body Mass Index	30.9 ± 6.59	29.4 ± 9.10	0.374
Brain Weight (g) *	1370 ± 175	1228 ± 143	0.017
Time Frozen (years) *	10.2 ± 3.60	8.11 ± 2.87	0.005
Manner of Death			
Natural	33	35	
Accidental	10	6	
Suicide	0	4	
Undetermined	0	2	
Habitual Smoker at TOD *	11	38	<0.001
Comorbid Alcohol Abuse/ Dependence	0%	14	
Comorbid Substance Abuse/ Dependence	0	8	
THC or Metabolites in Toxicology	0	1	
Nicotine *	8/35	18/27	0.028
Cotinine *	10/33	21/21	0.010
Chlorpromazine Equivalents			
Last Dose (mg qd)		776 ± 642	
Average Daily Dose (mg qd)		619 ± 426	
Lifetime Dose (mg)		6227139 ± 4474756	

Individuals with SCZ were older at time of death, had smaller brains, were more likely to smoke cigarettes, and their samples had shorter freezer times and lower pHs. When information about smoking status at the time of death was missing, habitual smoking was verified through toxicological analyses of nicotine and cotinine levels in blood or brain tissue. Not all subjects were tested for nicotine or cotinine, and values are therefore expressed as a ratio of total tested.

* $p < 0.05$;

** $p<0.001$.

Table 2

Correlations between CB_1 receptor binding in the dorsolateral prefrontal cortex (DLPFC) of individuals with schizophrenia (SCZ) and controls.

	SCZ		Controls	
Variable	**R**	**p**	**R**	**p**
pH	−0.034	0.818	0.024	0.879
PMI	0.163	0.273	0.023	0.884
BMI	−0.036	0.818	0.243	0.121
Age of Onset	−0.01	0.989	N.A.	N.A.
Duration of Illness	0.11	0.499	N.A.	N.A.
Age at Death	0.035	0.817	−0.273	0.076
Brain Weight	0.204	0.169	0.114	0.467
Freezer Storage Time	0	0.998	−0.121	0.465
Age at First Hospitalization	0.02	0.915	N.A.	N.A.
Median CPZE	−0.15	0.341	N.A.	N.A.
Cumulative Lifetime CPZE	−0.3	0.051	N.A.	N.A.
Last Known CPZE	−0.25	0.147	N.A.	N.A.
	t	p	t	p
Smoking Status	0.94	0.352	0.422	0.675
Gender	0.108	0.915	0.154	0.879
Comorbid Alcohol Abuse/ Dependence	0.28	0.780	N.A.	N.A.
Comorbid Substance Abuse/ Dependence	0.47	0.642	N.A.	N.A.
Antipsychotic drugs in brain at autopsy	−0.46	0.646	N.A.	N.A.
Nicotine in brain at autopsy	−0.23	0.823	0.70	0.489
Cotinine in brain at autopsy	−1.30	0.200	0.56	0.581
Gender	0.108	0.915	0.154	0.879

BMI: body mass index; CPZE: chlorpromazine equivalents; PMI: postmortem interval