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Quantification of endocannabinoids in postmortem brain of schizophrenic subjects

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ABSTRACT

Numerous studies have implicated the endocannabinoid system in the pathophysiology of schizophrenia. Endocannabinoids have been measured in blood and cerebrospinal fluid in schizophrenic patients but, to the date, there are no published reports dealing with measurements of endocannabinoid levels in schizophrenics' brain tissue. In the present study, postmortem brain samples from 19 subjects diagnosed with schizophrenia (DSM-IV) and 19 matched controls were studied. In specific brain regions, levels of four endocannabinoids (2-arachidonoylglycerol (2-AG), arachidonoylethanolamine (anandamide, AEA), dihomo-γ-linolenoylethanolamine (LEA), and docosahexaenoylethanolamine (DHEA)) and two cannabimimetic compounds (palmitoyl-ethanolamine (PEA) and oleoyl-ethanolamine (OEA)) were measured using quantitative liquid chromatography with triple quadrupole mass spectrometric detection. Suffering from schizophrenia significantly affects the brain levels of 2-AG ($p < 0.001$), AEA ($p < 0.0001$), DHEA ($p < 0.0001$), LEA ($p < 0.01$) and PEA ($p < 0.05$). In schizophrenic subjects, the three studied brain regions (cerebellum: 130 ± 18%; $p = 0.16$; hippocampus: 168 ± 28%, $p < 0.01$; prefrontal cortex: 237 ± 45%, $p < 0.05$) showed higher 2-AG levels when compared to matched controls. Conversely, AEA levels were lower in all brain regions of schizophrenic subjects (cerebellum: 66 ± 7%, $p < 0.01$; hippocampus: 66 ± 7%, $p < 0.01$; prefrontal cortex: 75 ± 10%, $p = 0.07$). Statistically significant lower levels of DHEA were also found in cerebellum (60 ± 6%, $p < 0.001$) and hippocampus (68 ± 7%, $p < 0.05$) of schizophrenic subjects. PEA (71 ± 6%, $p < 0.05$) and LEA (72 ± 6%, $p < 0.05$) levels were also found to be lower in cerebellum. No significant differences were found in OEA levels.
Our results evidence specific alterations in the levels of some endocannabinoids in different brain regions of schizophrenic subjects. Furthermore, these data evidence the involvement of the endocannabinoid system in the pathophysiology of schizophrenia.

1. Introduction

Schizophrenia is a chronic mental disorder that affects about 1% of the population worldwide. However, after more than a century of studying this disease, its cause remains unknown. Until recently, the hypotheses concerning the biological basis of schizophrenia have been mainly focused on the role of the neurotransmitter dopamine. Nevertheless, – and given the lack of findings associated with the dopaminergic system in the brain of schizophrenic patients – the biological explanation of this disorder based on other potential neurotransmitter substrates has been an area of recent investigation (Insel, 2010).

The endocannabinoid system constitutes a newly discovered system of neuromodulation. It comprises the cannabinoid receptors, along with their endogenous ligands, enzymes involved both in the biosynthesis and the degradation of these ligands, and putative membrane transport proteins (Pertwee et al., 2010). Endocannabinoids (ECs) derive from membrane phospholipids and share some pharmacologic properties with Δ^9-THC. To date, anandamide (N-arachidonoylethanolamine, AEA) and 2-arachidonoylglycerol (2-AG) are the most thoroughly studied ECs. They are produced on demand and released in the extracellular environment, where they can bind and activate CB1 and CB2 cannabinoid receptors (Alexander and Kendall, 2009; Pertwee et al., 2010).

There is a growing body of evidence suggesting that alterations in the endocannabinoid system may be involved in the pathophysiology of schizophrenia (Fernandez-Espejo et al., 2009). More specifically, previous studies have reported altered EC levels in cerebrospinal fluid (Leweke et al., 1999; Giuffrida et al., 2004) and blood (De Marchi et

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al., 2003) in schizophrenic patients. Additionally, different research groups have shown specific changes in the density of CB1 receptors in postmortem brains of subjects with schizophrenia (Dean et al., 2001; Zavitsanou et al., 2004; Newell et al., 2006; Dalton et al., 2011). Neuroimaging studies have also reported an elevated mean binding to CB1 receptors in most brain regions of subjects with schizophrenia in comparison to controls (Wong et al., 2010). However, it has been suggested that some of these differences may be a consequence of the antipsychotic treatment (Urigüen et al., 2009).

Within this context, the aim of the present study was to quantify EC levels in three different postmortem brain regions of schizophrenic subjects. Moreover, we elucidated how the fact of having undergone antipsychotic treatment affects these levels.

2. Materials and methods

2.1. Postmortem human brain samples

Human brain samples were obtained from autopsies performed in the Basque Institute of Legal Medicine (Bilbao) in compliance with policies of research and ethical boards for postmortem brain studies. After a retrospective search for antemortem medical information, 19 brains of subjects diagnosed with schizophrenia – according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) (American Psychiatric Association, 1994) – were matched to 19 brains of control subjects in a paired design. The criteria to choose control subjects were the absence of neuropsychiatric disorders and the absence of drug abuse. A blood toxicological screening was performed in all subjects to determine the presence of antipsychotics, other drugs and ethanol. A positive toxicological test for *Cannabis* was considered an exclusion criterion. According to the absence or presence of antipsychotic drugs in the toxicological screening, schizophrenic subjects were divided into two groups, namely, antipsychotic-free (n = 11) and antipsychotic-treated (n = 8). Demographic characteristics did not differ significantly between controls, antipsychotic-free and antipsychotic-treated schizophrenic subjects (Table 1).

Specimens of cerebellum (CB), hippocampus (HC) and prefrontal cortex (PFC) (Brodmann's area 9), were dissected at autopsy (0.5–1 g tissue) following standard procedures (Rajkowska and Goldman-Rakic, 1995). They were immediately stored at −80 °C until assays were carried out. Brain pH values were obtained at autopsy and the RNA integrity number (RIN) was also assayed as previously reported (García-Sevilla et al., 2010). The group averages for the above mentioned parameters are shown in Table 1. A full description of demographics of the definitive pairs of antipsychotic-free schizophrenics, antipsychotic-treated schizophrenics and their individually matched controls are shown in Tables S1 and S2, respectively.

2.2. Materials and drugs

Arachidonoylethanolamine (anandamide, AEA), arachidonoylethanolamine-d8 (AEA-d8), 2-arachidonoyl glycerol (2-AG), 2-arachidonoyl glycerol-d8 (2-AG-d8), dihomo-linolenoyl ethanolamine (LEA), docosahexaenoyl ethanolamine (DHEA), palmitoyl ethanolamine (PEA), and oleoyl ethanolamine (OEA) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Acetonitrile (ACN) and methanol (MeOH) were obtained from J.T. Baker (Deventer, The Netherlands). Chloroform and formic acid were purchased from Riedel-de Haën (Seelze, Germany). All solvents and chemicals were of HPLC grade or higher. Water was purified by a Milli-Q Gradient system (Millipore, Milford, MA, USA).

2.3. Quantification of ECs by liquid chromatography coupled with triple quadrupole mass spectrometry (LC/MS/MS)

EC levels were measured by a LC/MS/MS method, which was previously validated for EC determination in human postmortem brain tissue (Lehtonen et al., 2011). The method was selective, accurate and precise for concentrations within a range of 0.4–70 nM for N-acylethanolamines (NAEs: i.e., AEA, DHEA, LEA, PEA, and OEA) and 40–11,000 nM for 2-AG.

2.4. Data analysis

GraphPad Prism™ version 5.0 (GraphPad Software, San Diego, CA, USA), InVivoStat statistical software (Clark et al., 2012) and Excel (Microsoft Co., Redmond, WA, USA) programs were used to carry out statistical analyses. A two-way analysis of variance (ANOVA) was used to evaluate separately the effects of schizophrenia and brain region and the potential interaction between both of them in EC levels. The Fisher Least Significant Difference (LSD) test was used in post hoc analyses to evaluate the differences between schizophrenic subjects and controls in each brain region. Pearson's coefficient for simple correlation was calculated in order to test possible associations between EC levels and age, postmortem interval or storage time. The ratios between 2-AG and NAEs levels were calculated for the three studied brain regions and were compared between schizophrenia and control groups by unpaired two-tailed Student's *t*-test. For each brain region, EC levels of control, antipsychotic-free and antipsychotic-treated groups were compared using one-way ANOVA followed by a Dunnett's multiple comparison post hoc test to compare each schizophrenic group with the control group. The level of significance was chosen at $p = 0.05$. All data are presented as scattergrams of the data points. Horizontal solid lines represent the average of individual values.

3. Results

3.1. Endocannabinoid levels in postmortem human brain of schizophrenic subjects and matched controls

The LC/MS/MS method used for the present study was previously validated (Lehtonen et al., 2011) and was able to quantify the two most common ECs, 2-AG and AEA, and four other NAEs (i.e., DHEA, LEA, PEA and OEA) in the CB, HC and PFC of human brain samples. No significant correlations were found (data not shown) between any of the EC levels and the demographic variables of age, postmortem interval and storage time.

Table 1
Demographic characteristics of postmortem brain samples.

Schizophrenic subjects (n = 19) and matched controls (n = 19)						
Group	Gender (M/F)	Age (years)	PMI (h)	pH	RIN	Storage (months)
Schizophrenic subjects	15M/4F	46 ± 3	15 ± 1	6.3 ± 0.05	6.2 ± 0.2	53 ± 6
Sch AP-F (n = 11)	9M/2F	45 ± 4	15 ± 1	6.3 ± 0.07	6.1 ± 0.2	50 ± 8
Sch AP-T (n = 8)	6M/2F	49 ± 5	16 ± 2	6.2 ± 0.08	6.2 ± 0.5	59 ± 9
Control group	15M/4F	45 ± 3	17 ± 1	6.4 ± 0.08	6.4 ± 0.3	59 ± 8

Group values are means ± SEM. Abbreviations: AP-F (antipsychotic free), AP-T (antipsychotic treated), F (female), M (Male), RIN (RNA integrity number), PMI (postmortem interval).