

Involvement of the endocannabinoid system in phencyclidine-induced cognitive deficits modelling schizophrenia



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Abstract

Recent advances in the neurobiology of cannabinoids have renewed interest in the association between cannabis and schizophrenia. Our studies showed that chronic-intermittent phencyclidine (PCP) treatment of rats, an animal model of schizophrenia-like cognitive deficit, impaired recognition memory in the novel object recognition (NOR) test and induced alterations in CB₁ receptor functionality and in endocannabinoid levels mainly in the prefrontal cortex. In this region, we observed a significant reduction in GTPγS binding (−41%) accompanied by an increase in the levels of the endocannabinoid 2-AG (+38%) in PCP-treated rats, suggesting that a maladaptation of the endocannabinoid system might contribute to the glutamatergic-related cognitive symptoms encountered in schizophrenia disorders. Moreover, we evaluated the ability of the main psychoactive ingredient of marijuana, Δ⁹-tetrahydrocannabinol (THC), to modulate the cognitive dysfunctions and neuroadaptations in the endocannabinoid system induced by PCP. Chronic THC co-treatment worsened PCP-induced cognitive impairment, without inducing any effect per se, and in parallel, it provoked a severe reduction in the levels of the other endocannabinoid, AEA, vs. either vehicle (−73%) or PCP (−64%), whereas it reversed the PCP-induced increase in 2-AG levels. These results point to the involvement of the endocannabinoid system in this pharmacological model of cognitive dysfunction, with a potentially different role of AEA and 2-AG in schizophrenia-like behaviours and suggest that prolonged cannabis use might aggravate cognitive performances induced by chronic PCP by throwing off-balance the endocannabinoid system.

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Introduction

Cannabis sativa is one of the most frequently abused substances among schizophrenia patients (Jablensky et al., 1992). Although some researchers have interpreted this phenomenon as a method of self-therapy for counterbalancing negative symptoms of schizophrenia or side-effects of antipsychotic treatment (Dixon et al., 1991; Krystal et al., 1999), recent meta-analyses of epidemiological studies seem to suggest that cannabis use could be a risk factor or contributory

cause for developing psychoses (Degenhardt and Hall, 2006; Hall and Degenhardt, 2006; Henquet et al., 2005; Semple et al., 2005). However, the presence of a relationship between the endocannabinoid system and schizophrenia is corroborated by several observations. First, CB₁ receptors are mainly distributed in areas of the human brain implicated in schizophrenia, including prefrontal cortex, anterior cingulate cortex, basal ganglia, and hippocampus (Herkenham et al., 1990); post-mortem studies have shown alterations in CB₁ receptor densities in the prefrontal and anterior/posterior cingulate cortices of schizophrenia patients compared to normal controls (Dean et al., 2001; Newell et al., 2006; Ujike and Morita, 2004; Zavitsanos et al., 2004). Anandamide levels are abnormally elevated in the cerebrospinal fluid and plasma of

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schizophrenia patients (De Marchi et al., 2003; Giuffrida et al., 2004; Leweke et al., 1999, 2007) and appear to be inversely correlated with psychotic symptoms, suggesting a compensatory adaptation to the disease state (De Marchi et al., 2003; Giuffrida et al., 2004). Last, an association between CB₁ receptor gene polymorphisms and schizophrenia has been reported (Leroy et al., 2001).

In light of this multifaceted background it is of great interest to investigate the effects of the pharmacological modulation of the cannabinoid system in different experimental animal models of schizophrenia reproducing positive, negative or cognitive symptoms of the disease. Generally, CB₁ receptor agonists reduce the hyperlocomotion induced by amphetamine, quinpirole and cocaine (Gorriti et al., 1999; Marcellino et al., 2008; Moreira and Guimarães, 2005; Przegaliński et al., 2005), although not always by acting via CB₁ receptors; CB₁ antagonists can reduce, increase or not affect the hyperlocomotion induced by different dopaminergic agents (Corbillé et al., 2007; Ferrer et al., 2007; Madsen et al., 2006; Masserano et al., 1999; Poncelet et al., 1999). Moreover, CB₁ gene deletion reduces the hyperlocomotion induced by both dopaminergic agents and phencyclidine (PCP) (Corbillé et al., 2007; Haller et al., 2005). Regarding sensory motor information gating processes, generally CB₁ receptor agonists disrupt pre-pulse inhibition (PPI) (Bortolato et al., 2005; Hajós et al., 2008; Martin et al., 2003; Schneider and Koch, 2005), whereas cannabidiol and the CB₁ receptor antagonist, SR141716A, reverse MK801- or PCP-induced PPI deficit (Ballmaier et al., 2007; Long et al., 2006; but see also Martin et al., 2003). In contrast, to the best of our knowledge there are no data exploring the ability of cannabinoid compounds to alter the cognitive deficit associated with schizophrenia. Here we studied the role of the endocannabinoid system and its pharmacological modulation in a model based on chronic injection of low doses of PCP (Cochran et al., 2003; Pratt et al., 2008), which produces a specific pattern of hypometabolism within the rat prefrontal cortex, thereby reproducing the so-called 'hypofrontality' that is observed in PCP drug abusers (Wu et al., 1991) and schizophrenia patients (Andreasen et al., 1992; Tamminga et al., 1992; Wolkin et al., 1992). We chose a glutamatergic model of schizophrenia due to the well documented relationship between glutamate and cannabinoid systems. Several studies have reported that CB₁ agonists can reduce glutamatergic synaptic transmission in several brain regions involved in the regulation of gating functions (Auclair et al., 2000; Azad et al., 2003; Fujiwara and Egashira, 2004; Misner and Sullivan,

1999; Robbe et al., 2001) and the activation of CB₁ receptors is involved in mGluR5 receptor mechanisms of PCP (Kinney et al., 2005). First, we verified the cognitive impairment induced by chronic intermittent PCP exposure by using the Novel Object Recognition (NOR) test. The NOR is a non-rewarded, ethologically relevant, relatively simple test based on the natural propensity of rats to explore novel objects (Bevins and Besheer, 2006). It has been listed under the TURNS initiative [Treatment Units for Research on Neurocognition and Schizophrenia (www.TURNS.ucla.edu)] as relevant for studying learning and memory deficits in schizophrenia and it is widely used for testing cognitive deficits in schizophrenia (Fujita et al., 2008; Grayson et al., 2007; Hashimoto et al., 2005). Then, we evaluated whether chronic PCP treatment per se alters the endocannabinoid system in terms of CB₁ receptor density and efficiency and endocannabinoid levels. Finally, a chronic treatment with Δ^9 -tetrahydrocannabinol (THC) was combined with chronic PCP to evaluate its influence on both the cognitive impairment induced by PCP and endocannabinoid system functionality.

Material and method

Animals

Juvenile Lister-Hooded male rats (Harlan, Udine, Italy) weighing 126–150 g at the time of arrival were used. Rats were grouped 3–4 per cage and housed on a 12 h light/dark cycle (lights on 08:00 hours) with food/water available ad libitum. All experiments were conducted during the light phase and carried out in strict accordance with the guidelines released by the Italian Ministry of Health (D.L. 116/92 and D.L. 111/94-B), and the European Community directives regulating animal research (86/609/EEC). All efforts were made to minimize the number of animals used and their suffering.

Drugs

Phencyclidine hydrochloride (PCP, Sigma-Aldrich, Dorset, UK), was dissolved in saline. THC, a generous gift from GW Pharmaceutical (Salisbury, UK), was dissolved in ethanol, cremophor and saline (1:1:18). Clozapine (Sigma, Milan, Italy) was dissolved in a minimum volume of acetic acid, made up to volume with distilled water and pH adjusted to 6 with 0.1 m NaOH. The dose and timing of clozapine was chosen on the basis of previous studies (Grayson et al., 2007; Hashimoto et al., 2005; McLean et al., 2008).

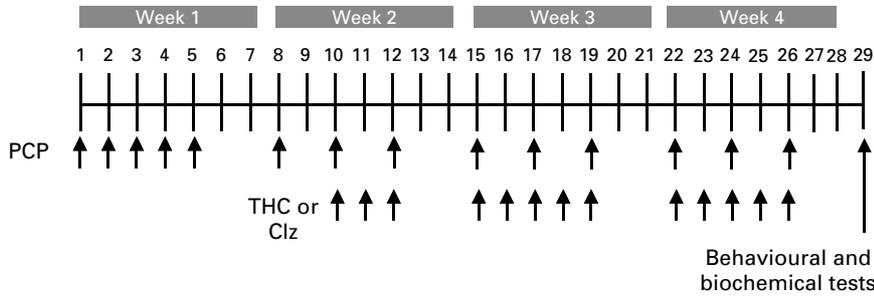


Figure 1. Treatment schedule. A total of 40 male Lister Hooded rats were randomly assigned to the different groups. Clz, Clozapine; PCP, phencyclidine; THC, Δ^9 -tetrahydrocannabinol.

Treatment regimes

The rats were allowed to acclimate in their new environment for 1 wk before the start of the treatment.

Chronic-intermittent PCP exposure. Intraperitoneal (i.p.) injections of PCP (2.58 mg/kg) or saline (1 ml/kg) once daily according to the procedure of Cochran et al. (2003) were performed at the time-interval indicated in Figure 1 (scheme of treatment).

PCP+THC chronic treatment. Chronic treatment with THC (0.5 mg/kg i.p.) or vehicle was combined with PCP according to the scheme of treatment in Figure 1.

As positive control in object recognition test, chronic treatment with clozapine (5 mg/kg i.p.) or vehicle was combined with PCP according to the scheme of treatment in Figure 1.

Behavioural tests

NOR test

Behavioural testing started 72 h after the end of chronic treatment (day 29). The experimental apparatus used for the object recognition test was an open-field box (in cm, 60 wide \times 60 deep \times 60 high) made of Plexiglas, placed in a dimly lit room. On the day of testing, the animals were habituated in a quiet laboratory for a 1-h period, before experimental procedures began. Animals performed each test individually. A 10-min habituation session preceded the experimental trials. In the object recognition task, each rat was placed into the box and exposed to two identical objects for a period of 10 min (familiarization phase). The rats were then returned to their home cage for a 1-h inter-trial interval, the entire box was cleaned, both objects removed and one replaced with an identical familiar copy and one with a novel object. Following this interval, rats were returned to explore

the familiar and novel objects in the test box for a 3-min test phase.

Object exploration was defined as the rats sniffing, licking or touching the objects with forepaws whilst sniffing but not by leaning against, turning around, standing or sitting on the objects. The exploration time of each object in each trial was recorded manually using two stopwatches and the following factors were calculated: E_1 = the total exploration time of both objects in the familiarization trial ($E_{F1} + E_{F2}$), E_2 = the total exploration time of both objects in the test trial ($E_T + E_N$). The discrimination index (DI) represents the difference in exploration time expressed as a proportion of the total time spent exploring the two objects during the test trial ($DI = (E_N - E_F) / (E_N + E_F) \times 100$). The behaviour was also recorded by using a video camera mounted above the experimental apparatus. Tapes were analysed offline by a trained observer who was unaware of the treatment condition. In an attempt to avoid the presence of olfactory trails, sawdust was stirred and the objects were thoroughly cleaned with 0.1% acetic acid after each rat.

For locomotor activity, data were acquired and analysed by the ANYMZE software (Ugo Basile, Varese, Italy) using a video camera mounted above the arena. Using the software, the experimenter drew the floor of the apparatus on the computer screen and divided it into four equal squares. The total number of line crossings between squares was recorded. These supplementary analyses are particularly important to examine when the experimental treatment alters object recognition (Bevins and Besheer, 2006).

Biochemical assays

Autoradiographic-binding studies

Seventy-two hours after the last injection of PCP and/or THC, adult rats were decapitated and brains were rapidly removed and frozen in liquid nitrogen and stored at -80°C until processing. Coronal sections

(20 μm) were cut on a cryostat and thaw-mounted on gelatin-coated slides. The sections were briefly dried at 30 °C and stored at –80 °C until they were processed for autoradiographic-binding studies.

[³H]CP-55,940 receptor autoradiographic binding

The [³H]CP-55,940 receptor autoradiographic binding was performed as previously described (Rubino et al., 1997) and here briefly summarized. Slides were brought to room temperature, then incubated for 2.5 h at 37 °C with 10 nM [³H]CP-55,940 (PerkinElmer Life Sciences, Milan, Italy) in binding buffer [50 mM Tris–HCl (pH 7.4), 5% BSA]. Adjacent cerebral sections were incubated in parallel with 10 μM CP-55,940 to assess non-specific binding. Sections were washed for 1 h at 4 °C in 50 mM Tris–HCl (pH 7.4), 1% BSA buffer and again for 3 h in the same conditions. They were then dipped in 50 mM Tris–HCl buffer (pH 7.4, 5 min) to remove excess BSA, dipped briefly in distilled water, and dried under a cool air stream. Autoradiograms were generated by exposing the dried sections for 7 d to Hyperfilm ³H (GE Healthcare, Milan, Italy).

CP-55,940-stimulated [³⁵S]GTP γ S binding in autoradiography

This was determined as previously described (Rubino et al., 2000a), with slight modifications. Briefly, slides were incubated in assay buffer [50 mM Tris–HCl, 3 mM MgCl₂, 0.2 mM EGTA, 100 mM NaCl, 10 mU/adenosine deaminase, 0.1% BSA (pH 7.4)] at 25 °C for 10 min then in 3 mM GDP in assay buffer at 25 °C for 15 min. They were then transferred to assay buffer containing 3 mM GDP and 0.04 nM [³⁵S]GTP γ S with (stimulated) or without (basal) 5 μM CP-55,940 and incubated at 25 °C for 2 h. Slides were rinsed twice in 50 mM cold Tris buffer and once in deionized water, dried, and exposed to βmax film (GE Healthcare) for 48 h.

Image analysis

The intensity of the autoradiographic films was assessed by measuring the grey levels with an image analysis system consisting of a scanner connected to a PC running Microsoft Windows. The images were analysed using Image-Pro Plus 5.0 (Media Cybernetics, Silver Spring, MD, USA) as previously described. Each area of both sides of the brain was traced with the mouse cursor using the Paxinos and Watson (2005) atlas as reference, and light transmittance was determined as the grey level. The grey level of densitometric measurements calculated

after subtraction of the film background density was established within the linear range, determined using tritium standards (³H Microscales, Amersham Pharmacia Biotech, Milan, Italy) for receptor-binding studies. For GTP γ S autoradiography, ³⁵S standards were prepared in the laboratory and agonist-stimulated activity was calculated by subtracting the optical density in basal sections (GDP only) from that of agonist-stimulated sections and results are expressed as percent stimulation over basal activity, as previously described (Sim et al., 1996).

Endocannabinoid levels

Lipid extraction and endocannabinoid measurement

Tissue extraction

Tissues were homogenized in 5 vol chloroform/methanol/Tris–HCl 50 mM (2:1:1) containing 20 pmol of d8-AEA, and d5-2-AG. Deuterated standards were synthesized from d8 arachidonic acid and ethanolamine or glycerol, as described, respectively, in Devane et al. (1992) and Bisogno et al. (1997). Homogenates were centrifuged at 13 000 g for 16 min (4 °C), the aqueous phase plus debris was collected and extracted again twice with 1 vol chloroform. The organic phases from the three extractions were pooled and the organic solvents evaporated in a rotating evaporator. Lyophilized samples were then stored frozen at –80 °C under nitrogen atmosphere until analysed.

Analysis of endocannabinoid content

Lyophilized extracts were re-suspended in chloroform/methanol 99:1 by volume. The solutions were then purified by open bed chromatography on silica as described in Bisogno et al. (1997). Fractions eluted with chloroform/methanol 9:1 by volume [containing AEA, 2-AG, and palmitoylethanolamide (PEA)] were collected, the excess solvent was evaporated with a rotating evaporator, and aliquots were analysed by isotope dilution-liquid chromatography/atmospheric pressure chemical ionization/mass spectrometry (LC–APCI–MS) carried out under conditions described previously (Marsicano et al., 2002), allowing the separation of 2-AG and AEA. Mass spectrometric (MS) detection was carried out in the selected ion monitoring mode using m/z values of 356 and 348 (molecular ions +1 for deuterated and undeuterated AEA) and 384.35 and 379.35 (molecular ions +1 for deuterated and undeuterated 2-AG). The area ratios between signals of deuterated and undeuterated AEA varied

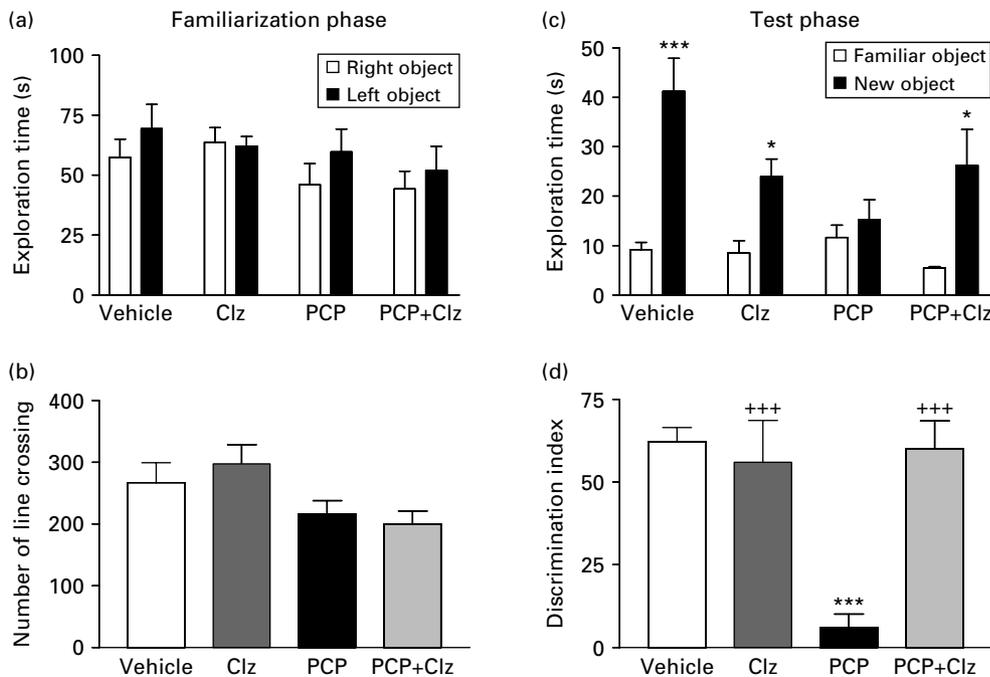


Figure 2. Effect of chronic phencyclidine (PCP) (2.58 mg/kg i.p.) on the novel object recognition (NOR) test. Panels (a) and (b) represent the exploration time of identical objects (a) and the number of line crossing (b) during the familiarization trial. Panels (c) and (d) represent the exploration time of the familiar vs. novel objects (c) and the discrimination index (d) during the test phase. The effect of chronic clozapine (Clz, 5 mg/kg i.p.) is reported as positive control. The NOR test was performed 72 h after the last drugs injection. Data are expressed as the mean \pm S.E.M. of at least four animals per group. (c) * $p < 0.05$, *** $p < 0.001$ vs. familiar object (Student's t test). (d) *** $p < 0.001$ vs. vehicle; +++ $p < 0.001$ vs. PCP (one-way ANOVA followed by Bonferroni post-hoc comparison).

linearly with varying amounts of undeuterated AEA (30 fmol to 100 pmol). The same applied to the area ratios between signals of deuterated and undeuterated 2-AG in the 100 pmol to 20 nmol interval. AEA and 2-AG levels in unknown samples were therefore calculated on the basis of their area ratios with the internal deuterated standard signal areas. For 2-AG, the areas of the peaks corresponding to 1(3)- and 2-isomers were added together. The amounts of endocannabinoids were expressed as picomoles/milligrams of lipid extracted.

Statistical analysis

Data are expressed as mean \pm S.E.M. Student's t test was performed to compare the effect of treatment on the time spent exploring the two familiar and novel objects vs. familiar objects. Analyses of the locomotor activity and DI data were performed using a one-way ANOVA followed by post-hoc Bonferroni comparison. Biochemical data were analysed using Student's t test or one-way ANOVA followed by post-hoc Bonferroni comparison. p values < 0.05 were considered statistically significant.

Results

Chronic-intermittent PCP exposure

NOR test

In the NOR test, repeated PCP administration caused a significant cognitive deficit 72 h after the last PCP injection (Figure 2). Vehicle- and PCP-treated rats spent equivalent times exploring the identical objects (left and right) in the familiarization phase (Figure 2a). During the test phase rats treated subchronically with vehicle clearly differentiated the familiar and novel objects as they spent significantly ($p < 0.001$) longer exploring the novel object compared to the familiar object (Figure 2c). In contrast, the ability to discriminate familiar and novel objects was abolished by chronic PCP treatment. There was no significant difference in exploration of the novel and familiar objects (Figure 2c) and there was a significant reduction (-90%) in the DI ($F_{3,16} = 17.01$, $p < 0.0001$, $p < 0.001$ for post-hoc comparison) (Figure 2d). Statistical analysis revealed no significant effect of PCP treatment on locomotor activity (Figure 2b), as shown by the number of line crossings.

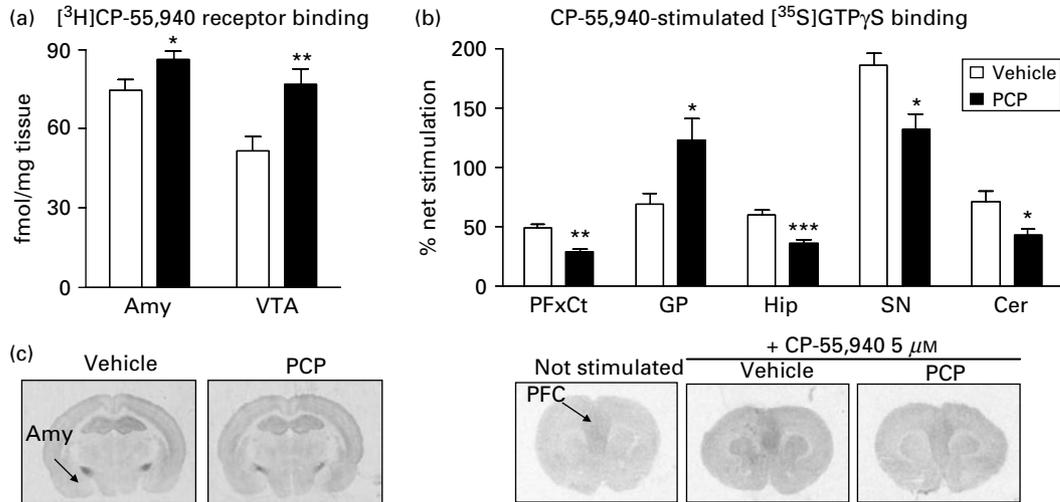


Figure 3. Effect of chronic phencyclidine (PCP) treatment on (a) CB_1 receptor binding and (b) net CP-55,940-stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding. (c) Autoradiograms of representative coronal sections of the amygdala and prefrontal cortex showing alteration in CB_1 receptor binding and net CP-55,940-stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding, respectively. Bars indicate the mean \pm s.e.m. of at least four animals. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. controls (Student's t test). Amy, amygdala; VTA, ventral tegmental area; PFC, prefrontal cortex; GP, globus pallidus; Hip, hippocampus; SN, substantia nigra; Cer, cerebellum.

Chronic treatment with clozapine, an atypical antipsychotic drug, significantly reduced the PCP-induced impairment in NOR producing a significant increase in time spent exploring the novel object compared with the familiar object ($p < 0.05$) and restoring the DI ($F_{3,16} = 17.01$, $p < 0.0001$, $p < 0.001$ for post-hoc comparison) respectively to PCP (Figure 2c, d). Clozapine per se failed to reveal any significant treatment effect.

CB₁ receptor functionality

In order to investigate the potential involvement of the cannabinoid system in the cognitive impairment produced by PCP, we measured the CB_1 receptor levels and efficiency in several brain regions of vehicle- and PCP-treated rats. In the $[^3\text{H}]\text{CP-55,940}$ receptor-binding study, among all the studied brain areas expressing CB_1 receptors, PCP exposure induced a slight but significant enhancement in CB_1 receptor density in the amygdala (+15%, $p < 0.05$) and a more sustained increase in the ventral tegmental area (+48%, $p < 0.01$) when compared to the control group (Figure 3a). CP-55,940-stimulated $\text{GTP}\gamma\text{S}$ binding revealed a different alteration than that observed in the receptor-binding assay (Figure 3b). In fact, prolonged exposure to PCP induced significant reduction in the percentage of CB_1 net stimulation in the prefrontal cortex (−41%, $p < 0.0001$), hippocampus (−40%, $p < 0.0001$), substantia nigra (−29%, $p < 0.01$) and

cerebellum (−39%, $p < 0.01$) and an increase in the globus pallidus (+77%, $p < 0.05$) when compared to controls. Basal $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding was not affected by PCP treatment in any of the cerebral areas studied (data not shown). Representative autoradiograms containing the amygdala and prefrontal cortex are shown in Figure 3c.

Endocannabinoid levels

Due to the presence of alterations in CB_1 receptor efficiency in brain areas involved in cognitive functions that might be related to the impairment in NOR (prefrontal cortex and hippocampus), we evaluated endocannabinoid levels in these regions (Figure 4). In the prefrontal cortex of PCP-treated rats, AEA levels were reduced by about 24% without reaching any statistical significance, whereas 2-AG levels were significantly increased (+38%, $p < 0.05$). Neither AEA nor 2-AG levels were altered in the hippocampus.

Effect of chronic THC treatment in rats treated with PCP

NOR test

The effect of chronic treatment with a low dose of THC on cognitive impairment produced by PCP is shown in Figure 5. All groups of rats spent equivalent time exploring the identical objects (left and right) in the familiarization phase (Figure 5a) without any changes

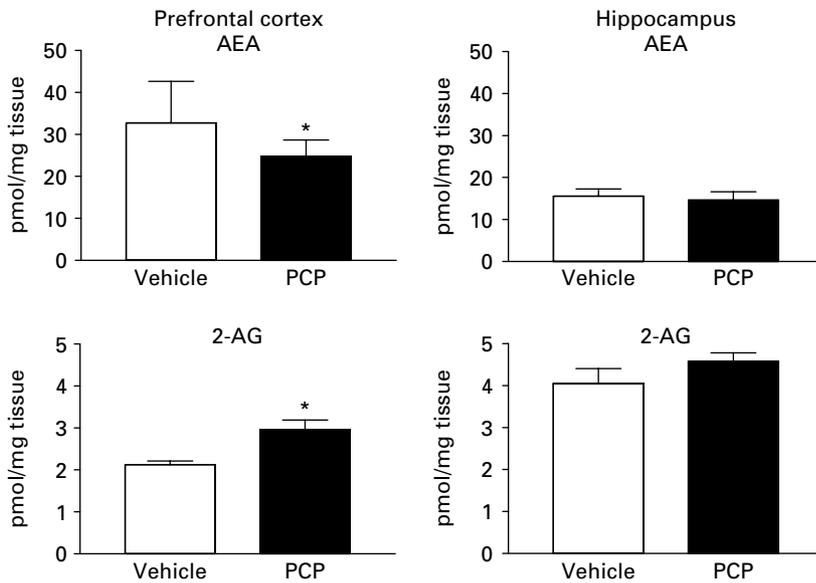


Figure 4. Effect of chronic phencyclidine (PCP) treatment on endocannabinoid (AEA and 2-AG) content in the prefrontal cortex and hippocampus. Data are expressed as the mean \pm S.E.M at least four animals per group. * $p < 0.05$ vs. vehicle (Student's t test).

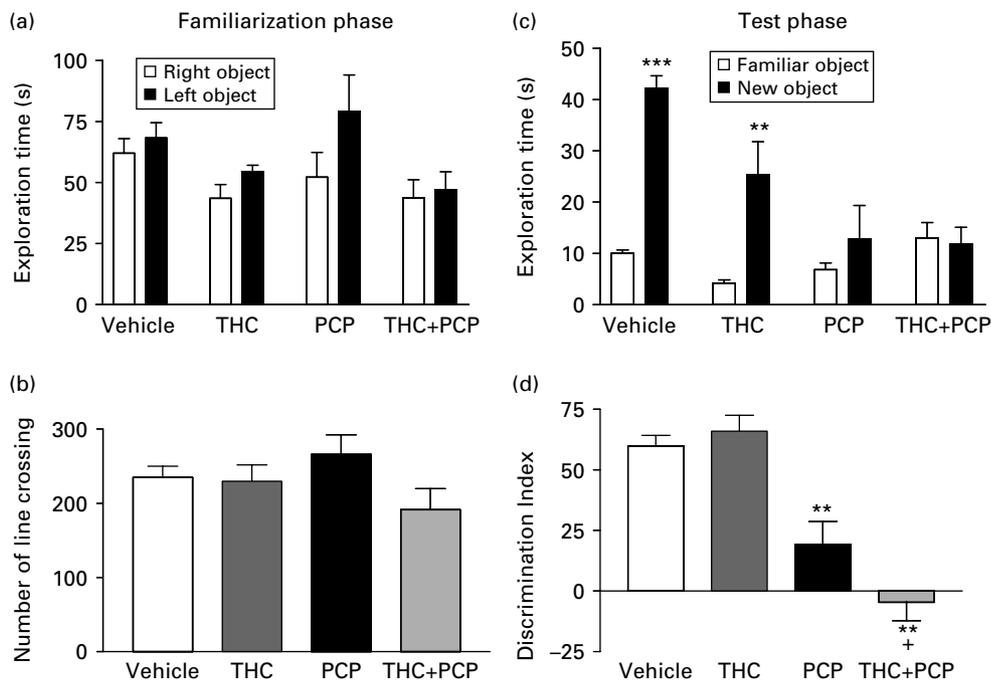


Figure 5. Effect of chronic Δ^9 -tetrahydrocannabinol (THC) treatment (0.5 mg/kg i.p.) on phencyclidine (PCP)-induced deficit in the novel object recognition (NOR) test. Panels (a) and (b) represent the exploration time of identical objects (a) and the number of line crossing (b) during the familiarization trial. Panels (c) and (d) represent the exploration time of the familiar vs. novel objects (c) and the discrimination index (d) during the test phase. The NOR test was performed 72 h after the last drugs injection. Data are expressed as the mean \pm S.E.M at least six animals per group. (c) ** $p < 0.01$, *** $p < 0.001$ vs. familiar object (Student's t test). (d) ** $p < 0.01$ vs. vehicle; + $p < 0.05$ vs. PCP (one-way ANOVA followed by Bonferroni post-hoc comparison).

in locomotion (Figure 5b). In the test phase, rats treated with THC alone differentiated the familiar and novel objects in a similar manner to controls as shown

by the significant increase ($p < 0.01$) in exploration time of the new object compared with the familiar one (Figure 5c). As expected PCP alone significantly

Table 1. Effect of chronic PCP and THC co-treatment on CB₁ receptor binding (left) and net CP-55,940-stimulated [³⁵S]GTPγS binding (right). Data are expressed as the mean ± s.e.m. of at least four animals

	[³ H]CP-55,940 receptorbinding (fmol/mg of tissue)				CP-55,940-stimulated GTPγS (% of net stimulation)			
	Vehicle	THC	PCP	THC + PCP	Vehicle	THC	PCP	THC + PCP
PFC	90.9 ± 5.1	92.17 ± 4.8	95.93 ± 4.8	103.5 ± 9.5	49 ± 3.5	↓30.8 ± 4.3**	↓29 ± 2.3***	↓15.3 ± 3.2***+†
NAc	90.89 ± 5.3	89.97 ± 5.4	94.96 ± 6.5	91.08 ± 13.9	29.4 ± 4.8	21.3 ± 3.8	24.2 ± 3.8	16.8 ± 3.1
CPu	173 ± 7.8	182.8 ± 6.8	176.5 ± 5.6	191.8 ± 12.9	30.6 ± 4.9	27.3 ± 7.9	27.8 ± 4.2	21.2 ± 4.8
GP	312.4 ± 16.8	355.9 ± 18	312.1 ± 14.1	312.1 ± 22	69.6 ± 9	65.2 ± 14.9	↑123.3 ± 18.3*	112.3 ± 18.8
Hypo	64.7 ± 3.5	60.63 ± 3.9	70.97 ± 3.9	60.97 ± 3.7	23.8 ± 5.5	22.1 ± 5.3	35.4 ± 5.4	38.5 ± 8.5
Thal	65.49 ± 2.7	67.25 ± 3.1	68.86 ± 2.6	60.8 ± 3.5	20 ± 4.7	19.8 ± 4.3	19.5 ± 4.9	22.6 ± 6.4
Amy	74.41 ± 4	85.46 ± 3.7	↑86.12 ± 3.4*	73.2 ± 4.1	31.9 ± 4.9	48.6 ± 11.8	29.1 ± 4.7	22.6 ± 6.4
Hip	148.3 ± 4.9	153.2 ± 3.6	157.4 ± 3.7	149.3 ± 3.9	60.1 ± 4.2	47.2 ± 5.8	↓35.9 ± 3.3***	↓42.2 ± 2.8*
SN	444.1 ± 26.7	466.5 ± 27.9	440.8 ± 20.6	448.1 ± 18.4	186.4 ± 10.1	199.7 ± 17.34	↓132.5 ± 12.9**	↑179 ± 13.3+
VTA	51.58 ± 5.2	64.29 ± 4.1	↑76.62 ± 6.1**	64.4 ± 3.8	27.4 ± 8.4	12.7 ± 3	16 ± 6.2	26.8 ± 1.6
Cer	255.1 ± 8.8	↓227.5 ± 8.9*++	266.3 ± 6.6	↓214.7 ± 8.1***++	71.2 ± 9	↓45.3 ± 6.6*	↓43.1 ± 5.1*	↓43.8 ± 8.8*

PFC, prefrontal cortex; NAc, nucleus accumbens; CPu, caudate putamen; GP, globus pallidus; Hypo, hypothalamus;

Thal, thalamus; Amy, amygdala; Hip, hippocampus; SN, substantia nigra; VTA, ventral tegmental area; Cer, cerebellum.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. vehicle; + $p < 0.05$, ++ $p < 0.01$, vs. PCP; † $p < 0.05$ vs. THC (Bonferroni post-hoc test).

reduced the DI ($F_{3,26} = 18.40$, $p < 0.0001$, $p < 0.001$ for post-hoc comparison). The association THC + PCP worsened this situation. In fact, during the test phase the co-treated rats spent the same time in exploring new and familiar objects (Figure 5c) and showed a significant impairment in recognition memory vs. controls (-177% , $F_{3,26} = 18.40$, $p < 0.0001$, $p < 0.001$ for post-hoc comparison) which significantly fell when compared to PCP alone (-128% , $F_{3,26} = 18.40$, $p < 0.0001$, $p < 0.05$ for post-hoc comparison) (Figure 5d). Thus, we demonstrated that prolonged exposure of THC worsens the PCP-altered recognition memory.

CB₁ receptor functionality

Table 1 summarizes the results of [³H]CP-55,940 receptor and CP-55,940-stimulated GTPγS autoradiographic-binding assays performed in rats treated with PCP alone and in association with THC. First, in the group of rats receiving THC alone residual alterations in CB₁ receptor binding and functionality were still present 72 h after the last injection. More precisely CB₁ receptor binding was reduced in the cerebellum and a significant reduction in GTPγS binding was present in the prefrontal cortex (-37% , $F_{3,12} = 17.45$, $p < 0.0001$, $p < 0.01$ for post-hoc comparison) and cerebellum (-36% , $F_{3,12} = 3.581$, $p < 0.05$, $p < 0.05$ for post-hoc comparison). The picture of the PCP-treated group was the same as described above (Figure 3), but when THC was combined with PCP the desensitization of

CB₁ receptors in the prefrontal cortex was much more pronounced than that induced by PCP alone, reaching a 69% decrease in respect to vehicle group ($F_{3,12} = 17.45$, $p < 0.0001$, $p < 0.001$ for post-hoc comparison) and a 47% reduction when compared to PCP or THC alone ($F_{3,12} = 17.45$, $p < 0.0001$, $p < 0.05$ for post-hoc comparison) (Table 1). In contrast, in the substantia nigra the THC co-treatment reversed the impairment in CB₁ functionality produced by PCP. In fact, the CP-55,940-stimulated GTPγS was the same as in the control group. In the other brain regions, the CB₁ receptor system did not reveal differences between PCP alone or THC + PCP.

Endocannabinoid levels

The effect of THC co-treatment on PCP-induced alterations in endocannabinoid content in the prefrontal cortex is shown in Figure 6. Chronic THC alone produced a significant reduction in AEA levels (-56% , $F_{3,10} = 4.854$, $p < 0.05$, $p < 0.05$ for post-hoc comparison) in respect to controls, whereas no changes in 2-AG levels were observed. As previously described, PCP alone did not significantly affect AEA levels but produced a significant increase in 2-AG ($F_{3,12} = 5.752$, $p < 0.05$, $p < 0.05$ for post-hoc comparison). The co-treatment provoked a further reduction in AEA levels vs. either vehicle (-73% , $F_{3,10} = 4.854$, $p < 0.05$, $p < 0.01$ for post-hoc comparison) or PCP (-64% , $F_{3,10} = 4.854$, $p < 0.05$, $p < 0.05$ for post-hoc comparison),

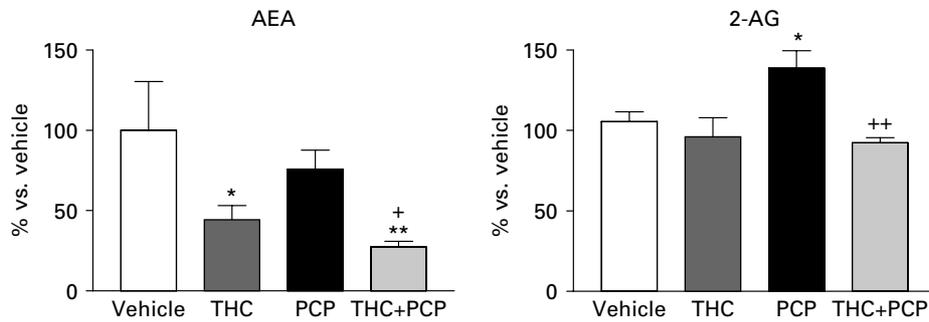


Figure 6. Effect of chronic phencyclidine (PCP) and Δ^9 -tetrahydrocannabinol (THC) co-treatment on endocannabinoid (AEA and 2-AG) content in the prefrontal cortex. Data are expressed as the mean \pm S.E.M at least four animals per group. * $p < 0.05$, ** $p < 0.01$ vs. vehicle; + $p < 0.05$, ++ $p < 0.01$ vs. PCP (one-way ANOVA followed by Bonferroni post-hoc comparison).

whereas it reversed the PCP-induced increase in 2-AG levels.

Discussion

Schizophrenia is associated with substantial cognitive impairment that is severe and does not respond well to the available treatments. Therefore, the development and validation of animal models of cognitive deficit are crucial in clarifying the underlying neuropathology and discovering new pharmacological treatments for such deficits. It is well known that repeated PCP injections induce enduring cognitive deficits with particular relevance to schizophrenia (Abdul-Monim et al., 2006, 2007; Amitai et al., 2007; Grayson et al., 2007; Hashimoto et al., 2005; Idris et al., 2005; Jentsch et al., 1997a,b; Rodefer et al., 2005). In the present study we have shown that chronic-intermittent PCP injections produce a robust cognitive deficit in male Lister Hooded rats that persisted 72 h after the last PCP injection. In the familiarization phase of the NOR test, there was no difference in the level of motivation, curiosity and interest among all the groups studied as demonstrated by the lack of difference in the total amount of time spent exploring two identical objects. During the test phase, chronic PCP reduced the ability to discriminate between novel and familiar objects, thus showing an impairment associated with recognition memory deficit. As previously shown, this deficit was improved by concomitant chronic administration of clozapine (Grayson et al., 2007; Hashimoto et al., 2005).

However, the major aim of the present work was to verify whether the endocannabinoid system is involved in this PCP model of cognitive dysfunction. First, we explored the levels and functional coupling of CB₁ receptors in PCP-treated rats vs. controls. We observed a significant and localized increase in

receptor-binding sites in the amygdala and ventral tegmental area whereas alterations in the CB₁ receptor efficiency were widely distributed through the brain. GTP γ S binding was altered in several brain areas implicated in schizophrenia; in particular, it was reduced in the prefrontal cortex and hippocampus, increased in the globus pallidus and decreased in the substantia nigra and cerebellum. To the best of our knowledge this is the first study reporting changes in cannabinoid receptor density and efficiency in an animal model of schizophrenia. Previous studies have demonstrated varying data on CB₁ receptor binding in post-mortem schizophrenia patients revealing different results depending on the specific brain region examined; no changes in CB₁ receptor density were reported in the hippocampus and caudate putamen, whereas an increase of CB₁ receptor binding was found in cortical areas in schizophrenia patients (Dean et al., 2001; Deng et al., 2007; Koethe et al., 2007; Newell et al., 2006; Zavitsanou et al., 2004). The discrepancy of binding studies from animal and human assays could be due to the unavoidable heterogeneity of humans such as the interference of concurrent drug therapies and the lack of systematic comparison with other mental disorders in which endocannabinoid signalling might also be dysregulated.

In addition, among all the brain areas showing alterations in CB₁ receptor functionality, the reduction observed here in the prefrontal cortex and hippocampus appears very interesting due to the important role of these regions in mediating cognitive functions altered in NOR (Barker et al., 2007; Dere et al., 2007). For this reason, in these cerebral areas we also assessed the levels of the two main endocannabinoids, AEA and 2-AG. We found in the prefrontal cortex of PCP-treated rats a significant elevation of 2-AG levels and a 25% reduction in AEA (not reaching statistical significance). In contrast, no significant difference in

the level of either endocannabinoid was found in the hippocampus. Despite several suggestions that the endocannabinoid system might play a role in the pathogenesis of schizophrenia, thus far no study has addressed the question of whether or not the endogenous ligands are altered in the brain of animals modelling schizophrenia. Previous biochemical analysis in humans has focused on the impact of schizophrenia on serum and CSF levels of anandamide (De Marchi et al., 2003; Giuffrida et al., 2004; Leweke et al., 1999, 2007; Potvin et al., 2008). These studies agree by suggesting that anandamide signalling may be overactive in schizophrenia patients and that CSF anandamide levels are negatively correlated with psychotic symptoms. In parallel, Pryor (2000) proposed a chronic over-release of 2-AG by platelets as a causal factor of the cognitive deficit associated with negative schizophrenia symptoms, whilst more recently, Potvin et al. (2008) after measuring plasma levels in patients, did not find changes in baseline 2-AG between schizophrenia patients and controls. Our experimental data indicate that in the prefrontal cortex of PCP-treated animals there is a dysregulation of the endocannabinoid system characterized by a significant up-regulation of 2-AG levels coupled with a significant reduction in CB₁-stimulated GTP γ S binding. The prefrontal cortex is hypoactive in schizophrenia patients as shown by the reduced regional cerebral blood flow (Sato et al., 1993; Weinberger et al., 1986) and by the decreased glucose utilization observed in schizophrenia patients (Andreasen et al., 1992; Tamminga et al., 1992; Wolkin et al., 1992) as well as in PCP abusers (Wu et al., 1991). The protocol of Cochran and collaborators (2003) used in the present study well reproduces the 'hypofrontality' as evidenced by the decrease in the rates of glucose utilization, reduction in markers of GABAergic interneurons (parvalbumin mRNA expression) and the deficit in executive functions (Egerton et al., 2005, 2008; for review see Pratt et al., 2008). Our data indicate that the endocannabinoid system is also involved in this dysregulation and could play a role in the observed cognitive impairments. In fact, the integrity of the prefrontal cortex is essential in cognitive processes and glutamate is indeed central to the activity of this brain structure as most of the afferents and efferents from the cortical area are glutamatergic in nature (Moghaddam, 2002). It has been reported that PCP administration, by blocking NMDA receptors present in GABAergic chandelier cells, reduces GABA release onto pyramidal neurons. The resultant disinhibition increases pyramidal neuron activity thereby enhancing glutamate release (for review see McGuire et al.,

2008; Pratt et al., 2008). This mechanism may occur locally at the prefrontal cortex and/or at regions with ascending glutamatergic projections to this region. The increase in glutamate may then lead to an excessive stimulation of glutamate receptors, including AMPA and kainate receptors which might cause cognitive dysfunction (Moghaddam and Adams, 1998; Moghaddam et al., 1997) and be responsible for the elevation of 2-AG levels observed here, and found previously to follow prefrontal cortex neuron stimulation (Melis et al., 2004).

Several studies have reported that CB₁ agonists can reduce glutamatergic synaptic transmission in several brain regions including the prefrontal cortex (Auclair et al., 2000). Interestingly, the bulk of evidence identifies the endocannabinoid 2-AG as the most probable retrograde mediator at glutamatergic synapses (Gerdeman et al., 2002; Robbe et al., 2002; Sjöström et al., 2003; Straiker and Mackie, 2005). In particular, 2-AG is synthesized in the post-synaptic terminal of glutamatergic synapses following activation of metabotropic mGluR5 glutamate receptors (Jung et al., 2005) and is thought to suppress glutamate release through activation of CB₁ receptors located in pre-synaptic glutamatergic axon terminals (Katona et al., 2006). In light of these premises, we can speculate that the increased level of 2-AG observed in the prefrontal cortex of PCP-treated rats represents an adaptive reaction aimed at reducing glutamatergic transmission that, due to the plasticity of the endocannabinoid system, also causes CB₁ receptor internalization and desensitization, as observed with several other types of G-protein-coupled receptors.

Next, our interest was to evaluate the ability of the main psychoactive ingredient of marijuana, THC, to modulate the cognitive dysfunctions and neuroadaptations in the endocannabinoid system induced by PCP. Low doses of chronic THC co-treatment worsened PCP-induced cognitive impairment without any effect per se after a 72 h washout period. The behavioural effect of prolonged THC co-exposure was in agreement with human studies supporting the negative influence of cannabis on the expression and course of schizophrenia cognitive impairment (Coulston et al., 2007; D'Souza et al., 2005; Linszen et al., 1994). In CB₁ receptor-binding assays, chronic THC decreased per se GTP γ S coupling in the prefrontal cortex and cerebellum in accordance with several animal studies showing desensitization after in-vivo repeated treatment with THC (for review see Breivogel et al., 1999; Rubino et al., 2000b; Sim-Selley and Martin, 2002). Furthermore, in this cerebral area, prolonged THC exposure per se produced a robust

decrease in AEA content. This result disagrees with a previous report of Di Marzo and collaborators (2000) where no changes in AEA levels in the cerebral cortex were found after chronic THC treatment. Several reasons can explain this discrepancy, including the dose (0.5 mg/kg vs. 10 mg/kg), treatment regimen (3 wk vs. 1 wk), withdrawal time (72 h vs. 2 h) and selected cortical area (prefrontal cortex vs. cortex) used in the two studies. Furthermore, a recent study has shown that, similar to our present observations, the local injection of a different CB₁ receptor agonist, WIN55,212-2, reduces the release of AEA and enhances that of 2-AG, although from a different brain region, i.e. the hypothalamus (Béquet et al., 2007). More interestingly, the PCP+THC treatment produced a new scenario in the prefrontal cortex consisting of a further and more intense CB₁ receptor desensitization, an additional reduction in AEA levels, this time achieving statistical significance, and a return to control values of 2-AG tissue concentrations. Taken together, THC co-exposure induces a new scenario in which other elements of the endocannabinoid system appear. The suppression of PCP-induced compensatory increase of 2-AG, together with the worsened CB₁ efficiency are likely to contribute to further enhance glutamatergic neurotransmission, thus worsening the cognitive performance. At the same time, the strong decrease in AEA levels might contribute to the dysfunction of the dopaminergic system. In fact, it has been suggested that AEA is implicated in the intrinsic regulation of dopamine release, by acting retrogradely on CB₁ receptors located in a subset of dopaminergic presynaptic terminals to limit dopamine release. Consequently, the strong decrease we observed in AEA content in the prefrontal cortex might produce a robust increase in dopamine release as observed in schizophrenia patients (McGuire et al., 2008) and this might be associated with the worsened behavioural performance, in accord with previous literature highlighting an antipsychotic-like role of AEA (Beltramo et al., 2000; Giuffrida et al., 2004). Under this perspective, it is possible to speculate that AEA and 2-AG might subserve different physiological mechanisms and might play different roles in the pathophysiology of schizophrenia. It is unsurprising that the endocannabinoids play dissimilar functions in the brain since several other studies have pointed out the different sensitivity of these two endogenous mediators to physiological or pathological processes in rats (Giuffrida et al., 1999; González et al., 2000; Kirkham et al., 2002; Valenti et al., 2004; Viganò et al., 2003, 2004; Rubino et al., 2008). There are several possible reasons for this different sensitivity of AEA

and 2-AG, e.g. to circadian rhythms or various pathological conditions, not least the presence of different enzymes regulating their synthesis and degradation (Di Marzo et al., 2001). Furthermore, it has been suggested that under certain conditions, elevation of AEA levels might decrease the biosynthesis of 2-AG via activation of TRPV₁ receptors (Maccarrone et al., 2008; Di Marzo and Cristino, 2008), and this might explain why also in this study the two compounds underwent opposite changes following the same type of stimulus.

To sum up, our study is the first to demonstrate the occurrence and, possibly, the role of an apparent maladaptation of the endocannabinoid system in a cognitive schizophrenia-like model, and to show that the aspects of cognitive performance are worsened after prolonged THC injections. These findings would be consistent with the theory that marijuana may be a risk factor for development or worsening of the schizophrenia disorder. If cannabinoid dysregulation is considered as a potential cause of cognitive symptomatology or a setting event for the disorder, then it may be possible to design therapeutic interventions to address this issue by using the wide range of ligands that directly or indirectly act on CB₁ receptors and on the enzymes that synthesize or inactivate the endocannabinoids (Di Marzo and Petrosino, 2007).

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Statement of Interest

None.

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