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ARTICLE

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Inhibition of fatty-acid amide hydrolase and CB_1 receptor antagonism differentially affect behavioural responses in normal and PCP-treated rats

Alexandre Seillier[1], Tushar Advani[1], Tomasso Cassano[2], Julie G. Hensler[1] and Andrea Giuffrida[1]

[1] *Department of Pharmacology, UTHSCSA, San Antonio, TX, USA*
[2] *Department of Biomedical Sciences, University of Foggia, Foggia, Italy*

Abstract

The 'cannabinoid hypothesis' of schizophrenia tulates that over-activity of the endocannabinoid system might contribute to the aetiology of schizophrenia. In keeping with this hypothesis, increased expression of CB_1 receptors, elevation of the endocannabinoid anandamide (AEA) and cannabinoid-induced cognitive changes have been reported in animal models of schizophrenia and psychotic patients. In this study we measured brain endocannabinoid levels and [^{35}S]GTPγS binding stimulated by the CB receptor agonist CP55,940 in rats undergoing withdrawal from subchronic administration of phencyclidine (PCP), a well-established pharmacological model of schizophrenia. We also investigated whether systemic application of the fatty-acid amide hydrolase (FAAH) inhibitor URB597 or CB_1 receptor blockade by AM251 affected the following PCP-induced behavioural deficits reminiscent of schizophrenia-like symptoms: (1) working-memory impairment (cognitive deficit), (2) social withdrawal (negative symptom), and (3) hyperactivity in response to *d*-amphetamine challenge (positive symptoms). PCP-treated rats showed increased endocannabinoid levels in the nucleus accumbens and ventral tegmental area, whereas CB_1 receptor expression and CP55,940-stimulated [^{35}S]GTPγS binding were unaltered. URB597 reversed the PCP-induced social withdrawal but caused social withdrawal and working-memory deficits in saline-treated rats that were comparable to those observed after PCP treatment. Administration of AM251 ameliorated the working-memory deficit in PCP-treated rats, but impaired working memory in saline-injected controls. Taken together, these results suggest that FAAH inhibition may improve negative symptoms in PCP-treated rats but produce deleterious effects in untreated animals, possibly by disturbing endocannabinoid tone. A similar pattern (beneficial for schizophrenia-related cognitive deficits, but detrimental under normal conditions) accompanies CB_1 receptor blockade.

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Introduction

The 'cannabinoid hypothesis' of schizophrenia was originally based on the observation that the psychotomimetic ingredient of marijuana, delta-9-tetrahydrocannabinol can exacerbate psychosis (D'Souza *et al*. 2005), produce perceptual alterations and/or psychotic symptoms in healthy subjects similar to those observed in schizophrenia patients (D'Souza *et al*. 2004; Emrich *et al*. 1997), and be a risk factor for the development of schizophrenia (Arseneault *et al*. 2002). Several lines of evidence indicate that over-activity of the endocannabinoid system and alteration of CB_1 receptor function may play a role in the pathophysiology of schizophrenia (D'Souza, 2007; Pryor, 2000). First, elevation of the endocannabinoid anandamide (AEA) has been reported in the blood (De Marchi *et al*. 2003) and cerebrospinal fluid (CSF)

Address for correspondence: A. Seillier, Ph.D., Department of Pharmacology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX, 78229-3900, USA.
Tel.: (210) 567 4211 *Fax*: (210) 567 4303
Email: seillier@uthscsa.edu

(Giuffrida *et al*. 2004; Leweke *et al*. 1999, 2007) of schizophrenia patients. Second, t-mortem studies have shown increased CB_1 receptor binding in the anterior (Zavitsanou *et al*. 2004) and posterior (Newell *et al*. 2006) cingulate cortex and prefrontal cortex (PFC) (Dean *et al*. 2001), two brain regions implicated in schizophrenia. Third, a triplet repeat $(AAT)_n$ polymorphism for the CB_1 receptor gene has been associated with the hebephrenic subtype of schizophrenia (Ujike *et al*. 2002). Recently, studies carried out in rat models of schizophrenia have provided additional support for the involvement of the endocannabinoid system in this pathology (Malone *et al*. 2007; Vigano *et al*. 2008). Using the social isolation rearing model, Malone and co-workers (2007) showed decreased CB_1 receptor expression in the caudate putamen (CPu) and amygdala, whereas Vigano and colleagues (2008) observed an increase in the ventral tegmental area (VTA) and amygdala of phencyclidine (PCP)-treated rats, as well as an elevation of the endocannabinoid 2-arachidonylglycerol (2-AG) in PFC.

Other studies suggest that an overactive endocannabinoid system may represent a compensatory adjustment to the disease and possibly have a protective role in schizophrenia. Indeed, the AEA elevation observed in the CSF of drug-naive schizophrenia patients has been negatively correlated to psychotic symptoms (Giuffrida *et al*. 2004; Leweke *et al*. 2007).

To elucidate the role of the endocannabinoid system in psychoses, we investigated possible changes in endocannabinoid transmission in rats undergoing withdrawal from subchronic PCP, a well-established pharmacological model of schizophrenia (Enomoto *et al*. 2007; Jentsch & Roth, 1999; Seillier & Giuffrida, 2009). Given the ability of the dopaminergic system to modulate endocannabinoid transmission (Giuffrida *et al*. 1999), changes in dopamine (DA) levels were also investigated. We also assessed whether increasing AEA tone [via systemic administration of the fatty-acid amide hydrolase (FAAH) inhibitor URB597], or blocking CB_1 receptors (via the CB_1 antagonist AM251) affected the following PCP-induced behavioural deficits: (1) impairment of working memory in a variable-delayed alternation task in a T-maze, (2) social withdrawal, and (3) motor hyperactivity in response to *d*-amphetamine challenge. As AEA has been shown to have a protective effect in schizophrenia (Giuffrida *et al*. 2004), we hypothesize that URB597-induced AEA elevation should ameliorate the behavioural deficits in PCP-treated rats. By contrast, if AEA is not protective, blockade of CB_1 receptors should improve PCP-induced deficits.

Materials and methods

Drugs

PCP, *d*-amphetamine, GDP (disodium salt), Tween-80 and polyethylene glycol (PEG) were purchased from Sigma/RBI (USA). [^{35}S]GTPγS (1250 Ci/mmol) and [^{3}H]CP55,940 (139.6 Ci/mmol) from PerkinElmer (USA); CP55,940, AM251, JTE907 and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) from Tocris (USA); GTPγS (tetralithium salt) from Roche/Boehringer–Mannheim (USA); methanol, chloroform, water, hexane from Honewell/Burdick & Jackson (USA); 2-AG and URB597 from Cayman Chemicals (USA); BSTFA from Supelco (USA) and saline solution from Hospira Inc. (USA).

Animals and drug treatment

All experiments were carried out according to the NIH Guide for the Care and Use of Laboratory Animals and approved by the IACUC of the University of Texas Health Science Center at San Antonio. Male Wistar rats (200–225 g; Charles River Laboratories, USA) were housed at 22 ± 1 °C, under a 12-h light/dark cycle (lights on 07:00 hours) with food and water available *ad libitum*. Animals were habituated to the housing conditions for 1 wk and behavioural testing was carried out during the light period of the cycle.

Animals were treated subchronically with either saline (1 ml/kg) or PCP (5 mg/kg) according to Jentsch *et al*. (1997), by intraperitoneal (i.p.) route, twice a day for 7 d, and sacrificed 5 d [post-treatment day (PTD)5] or 10 d (PTD10) after the last drug injection (Fig. 1), two time-points corresponding to the beginning and the end of the behavioural battery (Seillier & Giuffrida, 2009), respectively.

Endocannabinoid measurements

Animals ($n = 24$) were anaesthetized with halothane, their CSF rapidly collected from the cisterna magna using a 27-gauge hypodermic needle. Rats were decapitated and their brains collected, frozen in 2-methylbutane (-45 °C), and stored at -80 °C until use. Frozen brains were placed on a stainless-steel mould (Roboz; Rockville, USA) kept at -15 °C and sliced into 1-mm coronal sections using razor blades to dissect out the following brain areas: medial PFC (prelimbic and infralimbic cortices), anterior cingulate cortex (aCgCx), CPu, nucleus accumbens (NAc), globus pallidus, thalamus, amygdala, entorhinal cortex, dorsal and ventral hippocampus, VTA and substantia nigra. CSF and tissue samples were spiked

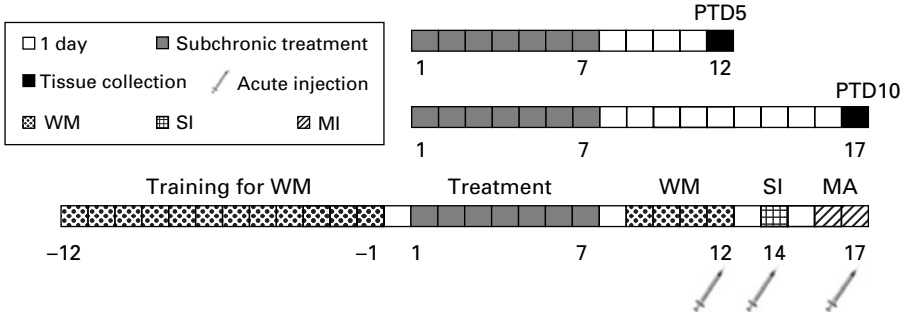

Fig. 1. Treatment schedule. For the endocannabinoid measurements (first two panels), animals were treated subchronically (from days 1 to 7, twice a day i.p.) with either saline (1 ml/kg) or phencyclidine (PCP) (5 mg/kg) and killed either 5 d (day 12) or 10 d (day 17) after the last drug administration. For the behavioural measurements (bottom panel), animals were trained for the working-memory (WM) test for 12 d, treated subchronically (from days 1 to 7, twice a day i.p.) with either saline (1 ml/kg) or PCP (5 mg/kg), and tested for WM and social interaction (SI) on days 12 and 14, respectively. Motor activity (MA) was assessed in response to a novel environment and a mild stressor (saline) on day 16, and after *d*-amphetamine injection (1 mg/kg i.p.) on day 17. The behavioural measurements were separated by 1-d intervals. On days 12, 14 and 17, rats received an acute injection of either vehicle (1 ml/kg), URB597 (0.3 mg/kg) or AM251 (1 mg/kg) 1 h before the beginning of the behavioural assessment (or before the habituation period for the MA test).

with 50 pmol of [$^2H_4$]AEA and [$^2H_5$]-2-arachidonyl glycerol (internal standards) and processed as previously described (Hardison *et al*. 2006). Lipids were extracted by adding methanol/chloroform/water (1:2:1, v/v/v). In the case of tissue samples, the chloroform layer was further purified by solid phase extraction (SPE) using C_{18} Bond Elut cartridges (100 mg; Varian, USA) as previously described (Hardison *et al*. 2006). No SPE was applied to CSF samples. Endocannabinoid-containing fractions were analysed by gas chromatography/chemical ionization mass spectrometry (GC/MS) using an isotope dilution assay as previously described (Hardison *et al*. 2006).

Measurements of DA and its metabolites

Tissue samples dissected from the same brain areas reported above were homogenized in 0.1 m perchloric acid (100 μl/10 mg of tissue) and centrifuged at 10000 ***g*** for 8 min at 4 °C. Supernatants were analysed by high-performance liquid chromatography (HPLC) to quantify DA and relative metabolites, DOPAC and homovanillic acid (HVA). Analyses were carried out by microbore HPLC using a SphereClone 150-mm × 2-mm column (3-μm packing) with a BAS Unijet cell (Bioanalytical Systems Inc., USA) and a 6-mm diameter glassy carbon electrode at $+650$ mV, connected to an electrochemical detector (INTRO; Antec Leyden, The Netherlands). The mobile phase consisted of 85 mM sodium acetate, 0.34 mM EDTA, 15 mM sodium chloride, 0.81 mM octanesulphonic acid (sodium salt), 5% methanol (v/v) (pH 4.85). The flow rate was 220 μl/min and the total runtime 15 min.

Autoradiography

Rat brains ($n=32$) were rapidly removed at PTD5 or PTD10 (Fig. 1), frozen on dry ice and stored at -80 °C until use. Coronal sections (20 μm) were cut at -16 °C in a cryostat at the level of the medial PFC (from Bregma 3.2 to 2.2 mm), CPu (1.6 to 1.0 mm), dorsal hippocampus (-3.14 to -3.6 mm) and VTA (-5.2 to -5.3 mm) according to the Paxinos atlas of the rat brain (Paxinos & Watson, 1998). Sections were thaw-mounted onto gelatin-coated glass slides, desiccated at 4 °C for 18 h under vacuum and stored at -80 °C until use.

Autoradiography of CP55,940-stimulated [^{35}S]-GTPγS binding in brain sections was performed as previously described (Sim-Selley & Martin, 2002) with slight modifications. Slide-mounted sections were equilibrated in Tris–HCl buffer (50 mM, pH 7.4), supplemented with 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, 0.2 mM dithiothreitol and 0.5% bovine serum albumin (BSA) for 10 min at 25 °C. Sections were pre-incubated in Tris–HCl buffer containing GDP (2 mM) and DPCPX (1 μM), an adenosine A_1 receptor antagonist, for 15 min at 25 °C, and then incubated in the same buffer plus 40 pM [^{35}S]GTPγS either in the absence or presence of CP55,940 (1 nm to 10 μM), for 2 h at 25 °C. Basal [^{35}S]GTPγS binding was defined in the absence of CP55,940. Non-specific [^{35}S]GTPγS binding was defined in the absence of CP55,940 and in the presence of 10 μM GTPγS. The incubation was stopped by two washes (2 min each) in ice-cold 50 mM Tris–HCl, followed by a rinse in ice-cold deionized water (30 s). Sections were dried on a slide

warmer and exposed to Kodak Biomax MR film for 48 h.

Autoradiography of [^{3}H]CP55,940 binding to CB_1 receptors was performed as previously described (Andersson *et al*. 2005; Fattore *et al*. 2007) with slight modifications to determine the duration of post-incubation wash times to obtain the highest percent of specific radioligand binding. Slide-mounted sections were pre-incubated in Tris–HCl buffer (50 mM, pH 7.4), as above and then incubated in 4 nm [^{3}H]CP55,940 for 2 h in assay buffer containing 0.5% BSA. Non-specific binding was determined by incubating adjacent sections with the CB_1 antagonist AM251 (1 μM). Slides were rinsed for 1 h in ice-cold Tris–HCl containing 0.3% BSA, followed by three washes in cold Tris–HCl (1 h each) and a brief dip in cold deionized water. Sections were dried and exposed to Kodak Biomax MR film (Amersham) for 3–6 wk.

Digitized autoradiograms were analysed using the NIH Image version 1.47. [^{3}H]CP55,940 binding was quantified using simultaneously exposed [^{3}H]-labelled standards (ART-123; American Radiochemicals, USA) which had been calibrated according to Geary and colleagues (1983, 1985). The amount of ligand bound was determined by converting optical density measurements to femtomoles per milligram of protein. Specific binding was calculated by subtracting non-specific binding from total binding on adjacent sections. For CP55,940-stimulated [^{35}S]GTPγS binding, standard curves were fitted to pixel data obtained from the [^{14}C]-labelled standards and tissue equivalent values (nCi/g) provided by American Radiochemicals, which were used to transform regional densitometric values into relative radioactivity measures. [^{35}S]GTPγS non-specific binding was subtracted from basal values and from binding in the presence of CP55,940.

Behavioural testing and drug treatment

Rats ($n = 48$) were tested 36 h after the last drug (or vehicle) injection using a previously published behavioural battery (Seillier & Giuffrida, 2009; see Fig. 1 for time schedule): (1) variable-delayed alternation task in a T-maze; (2) social interaction; (3) motor activity in response to a (*a*) novel environment, (*b*) mild stressor, or (*c*) *d*-amphetamine challenge. The behavioural measurements were separated from each other by 1-d intervals. In each test (i.e. day 12 for working memory, day 14 for social interaction, and day 17 for motor activity) animals received an acute i.p. injection of either vehicle (Tween-80/PEG/saline, 5/5/90, 1 ml/kg), URB597 (0.3 mg/kg), or AM251 (1 mg/kg), 1 h before the behavioural test (or just before the habituation period in the motor activity test). The dose and timing of URB597 and AM251 were chosen from previous *in-vivo* studies (Fegley *et al*. 2005; Laviolette & Grace, 2006; Morgese *et al*. 2007; Solinas *et al*. 2006).

Working memory

Working memory was measured using a discrete paired-trial delayed alternation paradigm in a T-maze (modified from Aultman & Moghaddam, 2001). The arms of the T-maze were made of black acrylic ($15 \times 30 \times 40$ cm, w/h/l). The main alley was 65 cm long. Movable guillotine doors separated (*a*) the main alley from the start box, (*b*) each side arm from two goal boxes at the end of the arms, both baited with food (Honey Nut Cheerios). Each side arm also had a door to open/close the entrance. A holding cage adjacent to the T-maze was used to host the animals in the inter-trial intervals (2-min), during which the maze was wiped clean with 10% alcohol. All experiments were carried out without food restriction.

A 12-d-long training for the delayed alternation task preceded the drug treatment. Details on the training are provided elsewhere (Seillier & Giuffrida, 2009). At the end of drug treatment, animals were tested for 3 d on the *discrete alternation task* (no delay) and for 1 d on the *delayed alternation task* (PTD5) consisting of a *forced run* (access to only one arm), and a *choice run* (access to both arms; only the arm opposite to the one visited during the forced run was baited). Animals had to enter the baited arm to succeed. The animals had 10 s to consume food; during this time, the maze was wiped clean to remove any olfactory cues. An intra-trial delay of either 0 (no delay) or 60 s separated each forced run from a choice run. The 60-s delay was selected from previous data (Jentsch *et al*. 1997; Seillier & Giuffrida, 2009). One hour before the delayed alternation task, rats received an acute administration of either vehicle, URB597 or AM251. Criterion for the *discrete alternation task* was defined as a success rate of 80%.

Social interaction

Social withdrawal was tested using the social interaction test (adapted from Sams-Dodd, 1998*a*), 7 d after the last drug injection (Fig. 1). As previously described (Seillier & Giuffrida, 2009), animals were tested in pairs (two unfamiliar rats receiving the same treatment and housed in different home cages) and matched up according to their body weights. Rats

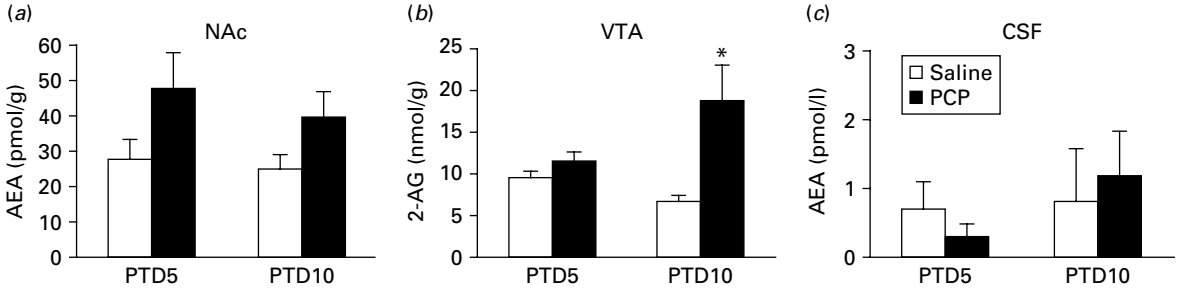

Fig. 2. Effects of subchronic phencyclidine (PCP) on (*a*) anandamide (AEA) levels in the nucleus accumbens (NAc) and (*c*) CSF, and (*b*) 2-AG levels in the ventral tegmental area (VTA) at PTD5 and PTD10 ($n=6$ for each experimental group). * $p<0.05$ compared to saline control. ANOVA revealed a significant main effect of treatment ($F_{1,17}=5.08$, $p<0.05$) for AEA in NAc. Values are expressed as mean ± S.E.M. of pmol/g (AEA), nmol/g (2-AG) or pmol/l (CSF).

received an acute administration of either vehicle, URB597 or AM251 1 h before the test, and were then placed simultaneously into an unfamiliar arena (made of black acrylic; dimensions: $100\times100\times40$ cm) and their behaviour recorded for 60 min. A score of 1 point was assigned to each of the following behavioural occurrences: (1) investigative sniffing (sniffing the conspecific's snout or other parts of the body); (2) following (rat moves towards and follows the conspecific); (3) climbing over or under (climbing over the conspecific's back or pushing the head and forepart of the body beneath the conspecific).

Motor activity

Various parameters (e.g. distance travelled, number of rearings, time spent in the centre of the box, motor speed) were recorded using the ActiMot Activity Measuring System version 6.07 (TSE Systems GmbH, Germany).

Nine days after the last drug injection (Fig. 1), animals were placed in the Actimot boxes for 60 min to assess their motor activity in response to a novel environment. All rats then received a saline injection (1 ml/kg i.p.) as a mild stressor and were monitored for additional 120 min. The following day, animals were returned to the Actimot boxes immediately after receiving an acute administration of either vehicle, URB597 or AM251. After 60 min, they received a *d*-amphetamine injection (1 mg/kg i.p.) and their motor activity was measured for 120 min.

Statistical analyses

Neurochemical and autoradiography data were analysed by two-way ANOVA with treatment (saline, PCP) and time (PTD5 or PTD10) as between-subject factors. Dose–response curves for CP55,940-stimulated [^{35}S]GTPγS binding were fitted by nonlinear regression using the KaleidaGraph software (version 4.0.1, Synergy Software, USA) according to the model:

$$E=E_{\max}/(1\pm \mathrm{EC}_{50}/[A])^{n},$$

where E is the response at the CP55,940 concentration $[A]$, $E_{\max}$ is the maximal response, EC_{50} is drug concentration that yields a half-maximal response, and n is the slope factor. The effect of AM251 or JTE907 on CP55,940-stimulated [^{35}S]GTPγS binding was analysed by one-way ANOVA, followed by Newman–Keuls test.

Data from the *discrete alternation test* were analysed by two-way ANOVA with treatment (saline, PCP) as between-subject factor and time (day) as repeated measure factor. The *delayed alternation* data, as well as social interaction and motor activity, were analysed by three-way ANOVA with treatment (saline, PCP) and drug (vehicle, URB597, AM251) as between-subject factors and delay (0, 60 s) or time (10-min intervals) as within-subject factor. The Newman–Keuls test was used for *post-hoc* comparisons when required. The level of significance was set at $p<0.05$.

Results

Endocannabinoid measurements

PCP-treated rats ($n=24$) showed a significant increase in AEA levels in the NAc (Fig. 2*a*; main effect of treatment: $F_{1,17}=5.08$, $p<0.05$). A similar trend although not significant was observed in the medial PFC (Table 1). We also observed a significant 2-AG elevation in the VTA at PTD10 (Fig. 2*b*, $p<0.01$; treatment × time interaction: $F_{1,18}=4.35$, $p=0.05$), whereas there was no change of either AEA or 2-AG levels

Table 1. Effect of subchronic phencyclidine (PCP) on anandamide levels

	PTD5		PTD10	
	Vehicle	PCP	Vehicle	PCP
mPFC	29.78 ± 12.49	47.21 ± 14.88	27.81 ± 5.97	45.61 ± 10.43
CgCx	22.08 ± 6.11	26.96 ± 6.14	33.79 ± 4.84	34.83 ± 5.06
CPu	44.02 ± 15.47	48.28 ± 8.62	45.13 ± 4.58	51.88 ± 8.20
GP	45.43 ± 11.29	47.68 ± 8.05	48.92 ± 7.76	53.13 ± 7.46
Tha	28.29 ± 3.17	32.94 ± 4.51	32.11 ± 5.45	43.79 ± 8.13
Amy	54.27 ± 24.61	45.41 ± 10.20	46.29 ± 5.87	50.20 ± 7.79
EC	56.74 ± 9.10	89.48 ± 12.57	53.75 ± 15.37	53.13 ± 9.65
dHip	41.84 ± 9.16	46.22 ± 5.15	52.32 ± 6.71	45.20 ± 7.33
vHip	50.27 ± 15.89	60.97 ± 11.63	51.57 ± 6.58	48.97 ± 11.11
VTA	60.04 ± 20.80	65.46 ± 18.39	76.51 ± 14.53	56.41 ± 9.10
SN	43.93 ± 14.15	50.98 ± 9.64	53.58 ± 9.03	58.68 ± 9.82

mPFC, Medial prefrontal cortex; CgCx, anterior cingulate cortex; CPu, caudate putamen; GP, globus pallidus; Tha, thalamus; Amy, amygdala; EC, entorhinal cortex; dHip, dorsal hippocampus; vHip, ventral hippocampus; VTA, ventral tegmental area; SN, substantia nigra.
Each value represents the mean ± S.E.M. of anandamide levels expressed in pmol/g.

in other brain areas (Tables 1 and 2) nor in the CSF (Fig. 2*c*) at any of the time-points examined.

DA measurements

DA and its metabolites were measured in the same brain areas collected from the rats used for endocannabinoid quantifications (contralateral hemisphere). DA levels were unchanged throughout the brain with the exception of the amygdala at PTD10 (saline: 206.9 ± 52.9; PCP: 435.2 ± 112.4 pg/mg of wet tissue, $p < 0.05$; treatment × time interaction: $F_{1,17} = 4.59$, $p < 0.05$). HVA levels were significantly elevated in the NAc at PTD5 (saline: 400.4 ± 45.2; PCP: 563.1 ± 70.1 pg/mg of wet tissue, $p < 0.05$; treatment × time interaction: $F_{1,20} = 5.90$, $p < 0.05$) but returned to control level at PTD10. A similar, but not significant trend was observed in the CPu (data not shown). Brain DOPAC did not change at any time-point (data not shown). Finally, we observed a trend towards an increase of DA turnover (DOPAC/DA ratio) at PTD10 in the PFC (saline: 0.80 ± 0.32; PCP: 1.74 ± 0.39, $p = 0.08$; treatment × time interaction: $F_{1,16} = 6.33$, $p < 0.05$).

CB_1 receptor function and expression

In the NAc of naive rats, CP55,940 (1 nm to 10 μM) stimulated [^{35}S]GTPγS binding in a concentration-dependent manner (E_{max}: 175 ± 2.9% above basal; EC_{50}: 317.5 ± 54.5 nm). Based on these findings, we used 1 μM CP55,940 in all subsequent experiments.

Table 2. Effect of subchronic phencyclidine (PCP) on 2-AG levels

	PTD5		PTD10	
	Vehicle	PCP	Vehicle	PCP
CSF	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
mPFC	4.48 ± 0.85	4.24 ± 0.73	5.21 ± 0.60	6.19 ± 0.79
CgCx	2.96 ± 0.64	3.14 ± 0.80	3.82 ± 0.59	3.88 ± 0.98
CPu	5.36 ± 0.45	5.77 ± 0.52	4.93 ± 0.35	6.11 ± 0.35
NAc	7.57 ± 0.99	7.22 ± 0.89	7.29 ± 0.61	8.62 ± 1.61
GP	5.74 ± 0.70	6.24 ± 1.64	8.63 ± 1.35	9.34 ± 1.60
Tha	5.22 ± 0.85	5.25 ± 1.19	7.09 ± 1.29	6.98 ± 0.93
Amy	8.50 ± 1.47	10.90 ± 2.25	9.74 ± 1.74	10.70 ± 0.90
EC	11.64 ± 1.19	14.34 ± 1.34	12.70 ± 2.15	15.10 ± 2.12
dHip	6.64 ± 0.74	6.39 ± 0.49	6.89 ± 0.49	6.92 ± 0.47
vHip	12.19 ± 2.13	8.57 ± 0.91	10.67 ± 1.31	10.13 ± 1.00
SN	12.61 ± 2.66	12.05 ± 1.54	12.05 ± 2.66	14.24 ± 1.18

CSF, Cerebrospinal fluid; mPFC, medial prefrontal cortex; CgCx, anterior cingulate cortex; CPu, caudate putamen; NAc, nucleus accumbens; GP, globus pallidus; Tha, thalamus; Amy, amygdala; EC, entorhinal cortex; dHip, dorsal hippocampus; vHip, ventral hippocampus; SN, substantia nigra.
Each value represents the mean ± S.E.M. of 2-AG levels expressed in nmol/l (CSF) or nmol/g (brain areas).

The specificity of CP55,940-stimulated [^{35}S]GTPγS binding was assessed in the presence of the CB_1 receptor antagonist AM251 (100 nM) or the CB_2

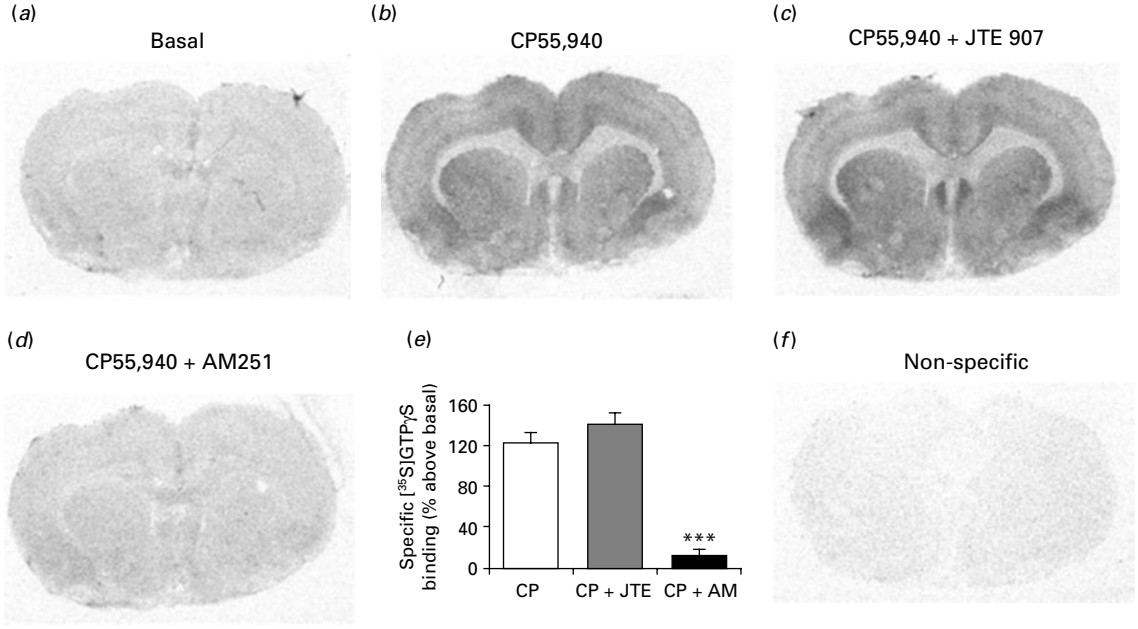

Fig. 3. Autoradiograms of [^{35}S]GTPγS binding in caudate putamen (Bregma 1.6 to 1.0 mm) coronal sections from naive rats incubated in [^{35}S]GTPγS (40 pM). (*a*) Basal [^{35}S]GTPγS binding in the absence of the CB agonist CP55,940 (CP). CP-stimulated [^{35}S]GTPγS binding (*b*) was not affected by the CB_2 antagonist JTE 907 [JTE, 100 nM (*c*)], but was inhibited by the CB_1 antagonist AM251 [AM, 100 nM (*d*)]. (*e*) Quantitative analysis of [^{35}S]GTPγS binding in the presence of CP alone (□), CP ± JTE (▩) or CP ± AM (■). Values are mean ± S.E.M. of specific [^{35}S]GTPγS binding expressed as percentage above basal. (*f*) Non-specific binding in the presence of 10 μM GTPγS. *** $p<0.001$ compared to CP alone.

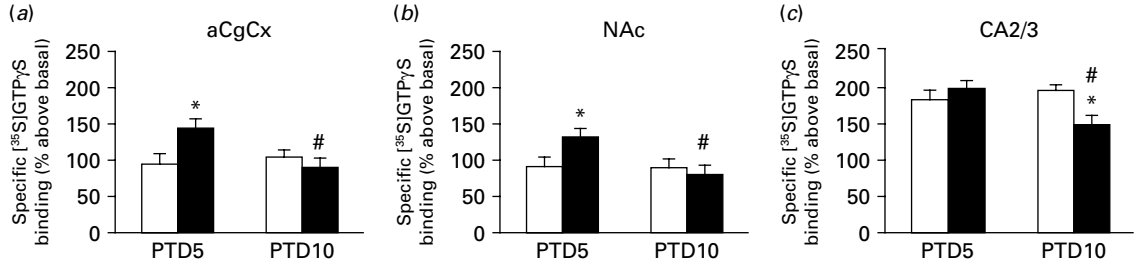

Fig. 4. CP55,940-stimulated [^{35}S]GTPγS binding in saline- (□) and PCP- (■) treated rats in (*a*) the anterior cingulate cortex (aCgCx), (*b*) nucleus accumbens (NAc) and (*c*) hippocampus CA2/3. Values are mean ± S.E.M. of specific [^{35}S]GTPγS binding expressed as percent above basal ($n=8$ for each experimental group). * $p<0.05$ compared to saline controls; # $p<0.05$ compared to PCP-treated rats at PTD5.

antagonist JTE907 (100 nM). AM251 markedly reduced CP55,940-stimulated [^{35}S]GTPγS binding, whereas JTE907 had no effect (Fig. 3), thus indicating that, in the brain, CP55,940 is mainly acting at CB_1.

The effect of subchronic PCP on the functional coupling of CB_1 receptors to G proteins was studied in an independent group of rats ($n=32$). CP55,940-stimulated [^{35}S]GTPγS binding was increased in aCgCx (Fig. 4*a*, $p<0.05$; treatment × time interaction: $F_{1,27}=6.78$, $p<0.05$) and NAc (Fig. 4*b*, $p<0.05$; treatment × time interaction: $F_{1,26}=5.02$, $p<0.05$) of PCP-treated rats at PTD5, but returned to control levels at PTD10. On the other hand, we observed decreased binding in the CA2/3 region of the hippocampus at PTD10 (Fig. 4*c*, $p<0.05$; treatment × time interaction: $F_{1,26}=7.31$, $p<0.05$). In PCP-treated rats, basal [^{35}S]GTPγS binding was reduced in aCgCx (saline: 203.0 ± 13.9; PCP: 160.1 ± 15.8, $p<0.05$) at PTD5. A similar trend although not significant was observed in NAc at the same time-point

Table 3. Effects subchronic phencyclidine (PCP) on CP55,940-stimulated [^{35}S]GTPγS binding

	PTD5		PTD10	
	Vehicle	PCP	Vehicle	PCP
mPFC	157.1 ± 22.2	185.4 ± 24.8	131.1 ± 26.3	136.3 ± 26.5
CPu	130.9 ± 16.0	160.0 ± 7.7	116.0 ± 8.0	105.6 ± 13.5
CA1	155.5 ± 20.4	165.1 ± 19.5	170.0 ± 9.2	138.5 ± 15.0
DG	143.4 ± 23.1	143.4 ± 13.6	133.7 ± 7.7	127.1 ± 14.7
VTA	22.6 ± 3.9	33.4 ± 7.3	37.8 ± 7.0	25.2 ± 6.3

mPFC, Medial prefrontal cortex; CPu, caudate putamen; CA1, hippocampus CA1; DG, dentate gyrus; VTA, ventral tegmental area.
Each value represents the mean ± S.E.M. of CP55,940-stimulated [^{35}S]GTPγS binding (% above basal).

Table 4. Effects subchronic phencyclidine (PCP) on [^{3}H]CP55,940 binding

	PTD5		PTD10	
	Vehicle	PCP	Vehicle	PCP
mPFC	647.0 ± 48.1	671.8 ± 18.4	648.4 ± 22.0	648.5 ± 24.8
CgCx	668.0 ± 13.7	672.1 ± 23.4	623.6 ± 19.3	612.7 ± 31.3
CPu	769.4 ± 26.2	786.4 ± 26.3	731.5 ± 19.0	721.4 ± 24.4
NAc	676.6 ± 12.4	700.0 ± 26.3	653.3 ± 20.4	661.7 ± 27.9
CA1	906.3 ± 25.9	879.1 ± 21.4	897.3 ± 26.4	826.1 ± 34.2
CA2/3	929.5 ± 30.4	891.6 ± 34.5	892.8 ± 29.7	828.5 ± 32.7
DG	887.6 ± 26.3	870.5 ± 36.9	899.8 ± 23.4	820.0 ± 28.7
VTA	834.1 ± 56.6	912.5 ± 29.4	923.5 ± 53.7	843.9 ± 36.1

mPFC, Medial prefrontal cortex; CgCx, anterior cingulate cortex; CPu, caudate putamen; NAc, nucleus accumbens; CA1 & CA2/3, hippocampus CA1 & CA2/3; DG, dentate gyrus; VTA, ventral tegmental area.
Each value represents the mean ± S.E.M. of [^{3}H]CP55,940 binding.

(saline: 205.1 ± 8.4; PCP: 180.9 ± 10.7, $p = 0.08$), whereas we found a trend towards an increase in the CA2/3 region of the hippocampus at PTD10 (saline: 173.8 ± 7.1; PCP: 197.0 ± 10.7, $p = 0.07$). The absolute values of CP55,940-stimulated [^{35}S]GTPγS binding were not different between saline- and PCP-treated rats in these brain areas (aCgCx at PTD5, saline: 390.4 ± 24.3; PCP: 386.8 ± 32.0. NAc at PTD5, saline: 398.1 ± 26.2; PCP: 417.8 ± 31.5. CA2/3 at PTD10, saline: 506.7 ± 18.3; PCP: 483.8 ± 24.8). No changes were found in the other areas studied (Table 3). The CB_1 receptor expression, measured by [^{3}H]CP55,940 binding autoradiography, was unaltered in the whole brain at both time-points (Table 4).

Behavioural studies

Performance in the alternation task (days 9–11) was >80% criterion and improved over the 3 d testing ($F_{2,74} = 11.50$, $p < 0.0001$, data not shown). Subchronic PCP did not affect this behavioural measure before the exposure to URB597 or AM251. The percentage of correct responses (Fig. 5*a*, PTD5) decreased as a function of the delay duration ($F_{1,37} = 112.11$, $p < 0.0001$) in all treatment groups. As expected, the impairment at 60-s delay was more severe in PCP- than in saline-treated rats ($p < 0.05$). The PCP-induced deficit was attenuated by AM251 ($p < 0.05$), but not URB597 (Fig. 5*a*). Interestingly, both URB597 and AM251 impaired this task in saline-treated animals at 60-s delay ($p < 0.05$ and $p < 0.01$, respectively) in a way similar to PCP, whereas no deficits were observed without delay. A pattern similar to that of URB597 was obtained with the cannabinoid transport blocker OMDM-2 (5 mg/kg, same regimen as URB597, data not shown), a drug that also elevates brain AEA levels (de Lago *et al*. 2005).

PCP-treated rats spent significantly less time in social interaction (Fig. 5*b*, $p < 0.05$), showing a decrease in the number of sniffing ($p < 0.05$) and climbing ($p < 0.05$) episodes (data not shown). The PCP-induced social withdrawal was reversed by URB597 ($p < 0.01$). However, this drug reduced social interaction in saline-treated controls ($p < 0.05$). A similar effect was obtained with OMDM-2 (5 mg/kg, data not shown). AM251 did not affect this behavioural measure in either saline- or PCP-treated animals. However, pre-treatment with AM251 blocked URB597 effect in PCP-treated rats (time spent in interaction: URB597, 1108 ± 11 s; URB597 + AM251, 845 ± 44 s; $p < 0.001$), but not in saline-treated rats (URB597, 868 ± 31 s; URB597 + AM251, 755 ± 64 s; $p > 0.05$); three-way ANOVA followed by Newman–Keuls *post-hoc* ($n = 8$ for each experimental group; time spent in interaction by control rats: 1115 ± 53 s). At this dose, URB597 was equally effective in increasing AEA levels in the CPu, an area known to express FAAH (Maccarrone *et al*. 2008), in either saline- and PCP-treated rats (78% and 86% increase over control, respectively; $n = 8$, $p < 0.05$ each; two-way ANOVA followed by Newman–Keuls *post-hoc*).

PCP-treated rats did not differ from saline-treated controls in distance travelled in response to *d*-amphetamine (Fig. 5*c*) nor in any of the other motor parameters measured (data not shown). No significant

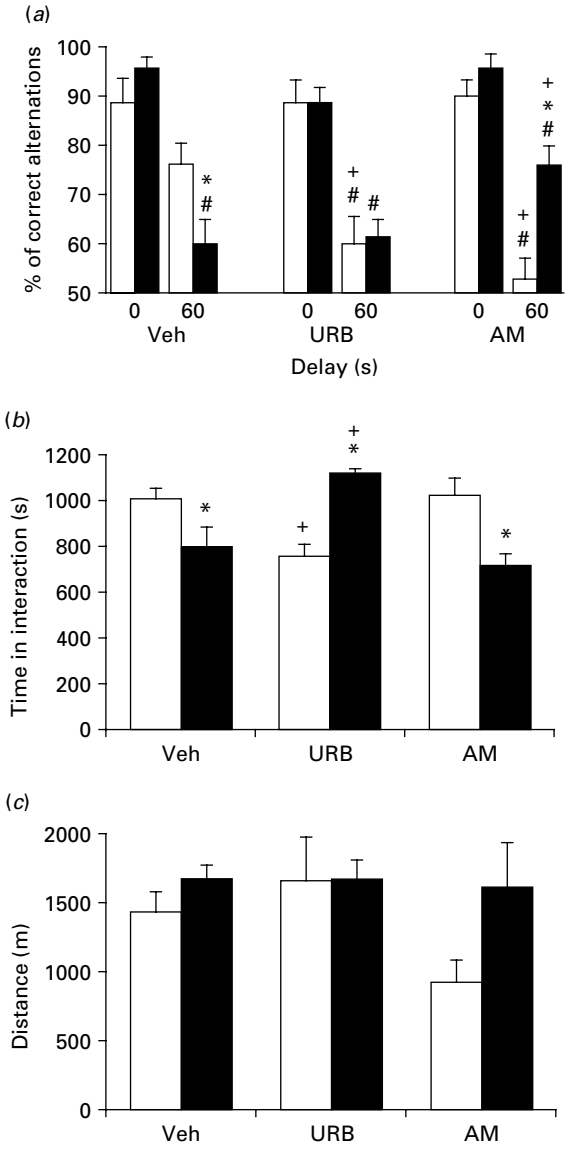

Fig. 5. Effects of URB597 (URB) and AM251 (AM) in saline- (□) and PCP- (■) treated rats on (*a*) the delayed-alternation task, (*b*) social interaction, and (*c*) motor activity following *d*-amphetamine injection. Values are expressed as mean ± S.E.M. ($n = 8$ for each experimental group). ANOVA revealed a significant interaction between treatment, delay and drug ($F_{2,37} = 5.10$, $p < 0.05$) for the delayed-alternation task and between treatment and drug ($F_{2,42} = 18.76$, $p < 0.0001$) for the social interaction test. * $p < 0.05$ compared to saline control; # $p < 0.05$ compared to delay 0; + $p < 0.05$ compared to vehicle (Veh) control. See text for details.

changes were observed after AM251 or URB597 (Fig. 5*c*). Although not significantly, AM251 tended to reduce motor velocity ($F_{1,41} = 3.38$, $p = 0.07$), percent time spent in hyperactivity ($F_{1,41} = 3.33$, $p = 0.08$) and in movement ($F_{1,41} = 3.25$, $p = 0.08$) in saline-treated rats (data not shown).

Discussion

Our study shows a localized enhancement of endocannabinoid levels in rats subchronically treated with PCP. Specifically, we observed increased 2-AG in the VTA 10 d after withdrawal from PCP and a slight elevation of AEA in NAc throughout the experiment. Although we found altered CB_1 receptor-stimulated [^{35}S]GTPγS binding in areas relevant to schizophrenia following PCP treatment, basal [^{35}S]GTPγS binding was also affected in the same regions. CB_1 receptor-binding sites remained unchanged. PCP-induced working-memory deficit and social withdrawal were reversed by the CB_1 antagonist AM251 and the FAAH inhibitor URB597 in a CB_1-dependent fashion, respectively. Paradoxically, these drugs produced some behavioural impairment in saline-treated rats, suggesting that alterations of endocannabinoid transmission in untreated rats may produce detrimental effects similar to those observed after subchronic PCP.

To date, disturbances of endocannabinoid transmission have been reported in only two animal models of schizophrenia (Malone *et al*. 2007; Vigano *et al*. 2008). Malone *et al*. (2007) observed CB_1 receptor down-regulation in the CPu and amygdala, and FAAH up-regulation in the CPu and NAc of rats undergoing social isolation. On the other hand, Vigano and colleagues (2008) reported increased CB_1 expression in the VTA and amygdala, and elevated 2-AG in the PFC of rats exposed to chronic intermittent PCP treatment. They also found altered CB_1 receptor-stimulated [^{35}S]GTPγS binding in the PFC, hippocampus, substantia nigra, cerebellum and globus pallidus. The discrepancies between these and our data may be attributed to differences in drug regimen (intermittent *vs*. repeated PCP; 5–10 d *vs*. 72-h withdrawal) and age of subjects (juvenile *vs*. adult rats). In addition, Vigano *et al*. measured endocannabinoid levels immediately after the behavioural tasks, whereas our measurements were carried out in a separate group of rats that did not undergo behavioural testing and probably reflect endocannabinoid basal values. This difference in the experimental design may be crucial as endocannabinoids are produced 'on demand' in response to neuronal activity (Piomelli, 2003). The mechanisms and source(s) responsible for 2-AG increase in the VTA remain undetermined and may be linked to parallel increases of DA levels and turnover in the amygdala and PFC, respectively. Indeed, there are

close anatomical and functional interactions between the PFC, amygdala and VTA (Floresco & Tse, 2007; Stevenson & Gratton, 2003; Stevenson *et al*. 2003) and electrophysiological data show that DA neurons in the VTA can release 2-AG after PFC stimulation (Melis *et al*. 2004).

On the other hand, AEA elevation in NAc may reflect an increase of dopaminergic transmission in this brain area, as indicated by the elevated HVA 5 d after PCP withdrawal, which in turn could lead to AEA increase via stimulation of DA D_2 receptors (Giuffrida *et al*. 1999).

In previous studies, we found elevated AEA in the CSF of drug-naive schizophrenia patients (Giuffrida *et al*. 2004; Leweke *et al*. 1999, 2007). By contrast, PCP-treated rats showed no changes in CSF AEA. This discrepancy may depend on differences in the neurochemistry of schizophrenia and corresponding animal models. For example, first-episode schizophrenia patients have increased striatal DA neurotransmission (Laruelle *et al*. 1996, 2003) and the AEA elevation observed in these patients may be a consequence of striatal hyperdopaminergia (Giuffrida *et al*. 1999). By contrast, we found no significant changes of striatal DA in PCP-treated rats.

Our data showed no changes in CB_1 receptor expression but increased receptor-stimulated [^{35}S]GTPγS binding in aCgCx and NAc and a reduction in the CA2/3 fields of the hippocampus of PCP-treated rats. The result in aCgCx is in line with a previous report showing increased CB_1 receptor binding in this area in schizophrenia patients (Zavitsanou *et al*. 2004). However, the changes in CB_1 receptor function observed in our study have to be viewed cautiously as basal [^{35}S]GTPγS binding was also altered in these regions.

As previously reported (Jentsch *et al*. 1997; Marquis *et al*. 2007; Seillier & Giuffrida, 2009), withdrawal from subchronic PCP caused a delay-dependent impairment of working memory. In this study, we showed that AM251 reversed the PCP-induced working-memory deficit, but worsened this behavioural measure in saline-treated rats. CB_1 receptors are abundant in the PFC (Egerton *et al*. 2006), a brain region implicated in the delay-dependence of working-memory tasks (Delatour & Gisquet-Verrier, 1999), and modulate neural transmission in this area (Egerton *et al*. 2006). In agreement with the hypo-frontality hypothesis of schizophrenia (Manoach, 2003) and the observation that chronic PCP decreases PFC activity (Morris *et al*. 2005), inhibition of CB_1 receptors might facilitate glutamatergic transmission in the PFC by reversing the CB_1-mediated suppression of excitatory post-synaptic currents (Auclair *et al*. 2000). Interestingly, Vigano *et al*. (2008) showed increased 2-AG in the PFC of PCP-treated rats after performing in the novel object recognition test, a PFC-dependent task. Therefore, CB_1 receptor blockade might limit the effect of excessive 2-AG and consequently alleviate the observed behavioural impairment. In addition, since steady dopaminergic activity is important for optimal PFC-dependent cognition (Murphy *et al*. 1996) and given the fact that PFC stimulation reduces DA neuron firing in the VTA via 2-AG (Melis *et al*. 2004), blockade of CB_1 receptors in PCP-treated rats may counteract the suppressive effect of 2-AG in the VTA, thus restoring normal DA transmission in the PFC. By contrast, in saline-treated rats, AM251 may increase DA in the PFC above physiological levels and possibly produce a cognitive deficit.

Our study supports the current view that stimulation of the cannabinoid system impairs working memory in a variety of behavioural paradigms (for review, see Egerton *et al*. 2006). In keeping with these observations, systemic administration of URB597 or OMDM-2, two drugs that elevate endocannabinoid tone in the brain (de Lago *et al*. 2005; Fegley *et al*. 2005), caused a working-memory deficit in saline-treated rats. These compounds, however, did not enhance the PCP-induced deficit, possibly because of a ceiling effect.

As expected (Lee *et al*. 2005; Qiao *et al*. 2001; Seillier & Giuffrida, 2009), we observed reduced social interaction after withdrawal from subchronic PCP. This deficit was reversed by URB597 and OMDM-2. Surprisingly, these compounds decreased social interaction in saline-treated rats. Hypo-locomotion or increased anxiety are unlikely to account for the observed effects of URB597 since this drug: (1) did not affect any of the motor parameters analysed in this and other studies (Hill *et al*. 2007; Jayamanne *et al*. 2006); (2) decreases (rather than increases) anxiety at this dosage (Hill *et al*. 2007; Moise *et al*. 2008). In addition, there is general agreement that PCP-induced social withdrawal in rats does not reflect increased anxiety (Enomoto *et al*. 2007; Lee *et al*. 2005; Sams-Dodd, 1998*b*) and is not reversed by the anxiolytic agent chlordiazpoxide (Snigdha & Neill, 2008). The diverging URB597-induced effects on social interaction observed in saline- *vs*. PCP-treated rats may be attributed to differences in endocannabinoid levels between these two experimental groups when engaged in behavioural tasks (despite the similar basal levels found under resting conditions). In particular, URB597 might increase endocannabinoid tone in PCP-treated rats to a level sufficient to restore normal

function, but elevate brain endocannabinoids in saline-treated animals above physiological levels, leading to a behavioural deficit. This hypothesis is currently under investigation. As previously reported (Trezza & Vanderschuren, 2007), we did not observed any disruptive effect of AM251 on social interaction in either saline- or PCP-treated rats. However, this compound reversed the beneficial effect of URB597 in PCP-treated rats, but not URB597-induced social withdrawal in saline-treated rats. Preliminary data in our laboratory suggest that the later effect is blocked by the TRPV1 antagonist capsazepine (A. Seillier *et al*., unpublished observations). Thus, additional research is needed to understand the molecular mechanisms underlying the divergent effects of URB597 in these experimental groups.

In agreement with other studies (Collins *et al*. 2006; Egerton *et al*. 2008), we did not find sensitization of amphetamine-induced motor responses in PCP-treated rats. As previously reported (Corbille *et al*. 2007; Hill *et al*. 2007; Thiemann *et al*. 2008*a*; Trezza & Vanderschuren, 2007), URB597 or AM251 did not affect spontaneous motor activity when administered alone. In addition, our data confirmed the report from Thiemann *et al*. (2008*a*) showing lack of effect of AM251 on amphetamine-induced hyperactivity. On the other hand, these results differed from behavioural experiments carried out with the CB_1 antagonist SR141716A which enhances motor responses following amphetamine challenge (Masserano *et al*. 1999; Thiemann *et al*. 2008*b*), suggesting that the SR141716A effect might depend on the blockade of other cannabinoid-like receptors (Thiemann *et al*. 2008*a*). In agreement with this hypothesis, genetic ablation of CB_1 receptors does not affect either spontaneous or amphetamine-induced motor activity (Houchi *et al*. 2005; Thiemann *et al*. 2008*b*).

In conclusion, subchronic PCP did not cause any remarkable changes in endocannabinoid transmission, with the exception of altering AEA and 2-AG levels in NAc and VTA, respectively. Nevertheless, pharmacological manipulation of the endocannabinoid system had dramatic consequences on behavioural measures relevant to schizophrenia, thus indicating a role for this system in psychoses. Indeed, URB597-induced endocannabinoid enhancement, precipitated psychotic-like behaviours in normal rats, but ameliorated PCP-induced social withdrawal, thus supporting the observation that endocannabinoids' elevation may have a protective effect in schizophrenia (Giuffrida *et al*. 2004; Leweke *et al*. 2007). Similarly, CB_1 antagonism had a detrimental effect on cognition under normal conditions, but ameliorated PCP-induced deficit. Further studies are necessary to elucidate the pharmacological and molecular mechanisms responsible for these divergent effects.

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Statement of Interest

None.

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