Phencyclidine-Induced Social Withdrawal Results from Deficient Stimulation of Cannabinoid CB₁ Receptors: Implications for Schizophrenia

Alexandre Seillier^{*,1}, Alex A Martinez¹ and Andrea Giuffrida¹

¹Department of Pharmacology, University of Texas Health Science Center at San Antonio, San Antonio, TX, USA

The neuronal mechanisms underlying social withdrawal, one of the core negative symptoms of schizophrenia, are not well understood. Recent studies suggest an involvement of the endocannabinoid system in the pathophysiology of schizophrenia and, in particular, of negative symptoms. We used biochemical, pharmacological, and behavioral approaches to investigate the role played by the endocannabinoid system in social withdrawal induced by sub-chronic administration of phencyclidine (PCP). Pharmacological enhancement of endocannabinoid levels via systemic administration of URB597, an inhibitor of endocannabinoid degradation, reversed social withdrawal in PCP-treated rats via stimulation of CB₁ receptors, but reduced social interaction in control animals through activation of a cannabinoid/vanilloid-sensitive receptor. In addition, the potent CB agonist CP55,940 reversed PCP-induced social withdrawal in a CB₁-dependent manner, whereas pharmacological blockade of CB₁ receptors by either AM251 or SR141716 reduced the time spent in social interaction in control animals. PCP-induced social withdrawal was accompanied by a decrease of anandamide (AEA) levels in the amygdala and prefrontal cortex, and these deficits were reversed by URB597. As CB₁ receptors are predominantly expressed on GABAergic interneurons containing the anxiogenic peptide cholecystokinin (CCK), we also examined whether the PCP-induced social withdrawal resulted from deficient CB₁-mediated modulation of CCK transmission. The selective CCK2 antagonist LY225910 blocked both PCP- and AM251-induced social withdrawal, but not URB597 effect in control rats. Taken together, these findings indicate that AEA-mediated activation of CB₁ receptors is crucial for social interaction, and that PCP-induced social withdrawal results from deficient endocannabinoid transmission.

Neuropsychopharmacology (2013) **38**, 1816–1824; doi:10.1038/npp.2013.81; published online 1 May 2013

Keywords: cholecystokinin; endocannabinoid; negative symptoms; PCP; social interaction

INTRODUCTION

In Kraepelin's and Bleuler's conceptualizations of schizophrenia, disturbances in social/emotional function are considered the key feature of this pathology and referred to as 'fundamental' over the 'accessory' (or positive) symptoms. In agreement with this description, the negative symptoms of schizophrenia are currently viewed as the illness core of the disease and, together with the cognitive deficits, they are considered the most significant factor contributing to functional impairment (Foussias and Remington, 2010; Milev *et al*, 2005). To date, the pharmacotherapy of negative symptoms has been disappointing, as second-generation antipsychotic medications have not met the expectations, and the development of more effective therapies has been inadequate (Hanson

et al, 2010). Thus, there is a need to understand the pathophysiology of negative symptoms and translate this knowledge into new therapies.

Although cannabis exposure has been associated with a negative impact on the course and expression of psychoses (Sewell *et al*, 2009), recent advances in the neurobiology of the endocannabinoid system have provided an opportunity to revisit the association between cannabinoids and schizophrenia, particularly in the context of the negative symptoms. For instance, polymorphisms of the CB₁ receptor gene *CNR1* have been associated with the hebephrenic type of schizophrenia, which is characterized by predominant negative symptoms, (Chavarria-Siles *et al*, 2008; Ujike *et al*, 2002) and the refractoriness to atypical antipsychotics (Hamdani *et al*, 2008). In addition, the observation that the endocannabinoid anandamide (AEA) is elevated in the cerebrospinal fluid (CSF) of drug-naïve schizophrenics and inversely correlated to negative symptoms (Giuffrida *et al*, 2004) indicates that this endogenous cannabinoid may have a protective role.

Chronic administration of phencyclidine (PCP) in rodents has been widely used to model schizophrenia as it mimics the complex clinical and pathological features of

*Correspondence: Dr A Seillier, Department of Pharmacology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900, USA, Tel: +1 210 567 4211, Fax: +1 210 567 4303, E-mail: seillier@uthscsa.edu

Received 27 November 2012; revised 6 March 2013; accepted 24 March 2013; accepted article preview online 5 April 2013

this disease (Enomoto *et al*, 2007). Also, PCP-treated rats represent the best pharmacological model of social withdrawal (negative symptom) in term of construct, face, and predictive validity (Gururajan *et al*, 2010). We previously showed that systemic administration of URB597, a drug that increases AEA levels by blocking its catabolic enzyme fatty-acid amide hydrolase (FAAH), reverses PCP-induced social withdrawal (Seillier *et al*, 2010), thus strengthening the idea that cannabinoid compounds could attenuate negative symptoms. URB597, however, has been shown to impair social interaction in control rats (Seillier *et al*, 2010). In keeping with these observations, while chronic cannabis consumption ameliorated negative symptoms in schizophrenic patients (Compton *et al*, 2004; Dubertret *et al*, 2006), an amotivational syndrome, strikingly similar to the negative symptoms of schizophrenia, has been described in non-schizophrenic chronic cannabis users (Sewell *et al*, 2009). These data suggest that cannabinoids differentially affect not only the negative and positive symptoms of schizophrenia, but also distinct subject populations (healthy vs schizophrenic).

In this study, we investigated the biochemical and pharmacological mechanisms underlying the diverging effects of URB597 on social interaction in control vs PCP-treated animals, with the intent to elucidate the role played by the endocannabinoid system in the negative symptoms of schizophrenia.

MATERIALS AND METHODS

Subjects

Male Wistar rats (200–225 g; Charles River Laboratories, Wilmington, MA, USA) were housed at 22 ± 1 °C, under a 12-h light-dark cycle with food and water available *ad libitum*, and habituated to the housing conditions for 1 week before the experiments. Animals were treated sub-chronically (twice a day for 7 days) with either vehicle (saline, 1 ml/kg) or PCP (5 mg/kg) via intraperitoneal route (i.p.) and tested 7 days after the last drug injection (Seillier *et al*, 2010). All experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, and approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio.

Social Interaction Paradigm

Social interaction testing was carried out during the light portion of the light-dark cycle, using a procedure previously described (Seillier *et al*, 2010; Supplementary Materials and Methods). Rats received an acute injection of the cannabinoid testing drug 1 h before being placed into an unfamiliar arena, and their behavior was videotaped for 60 min. The total time spent by each rat in investigative sniffing, following, climbing, and aggression was recorded by an experimenter blind to the study.

Pharmacological Studies

To enhance AEA tone, we used the FAAH inhibitor URB597 (0.1, 0.3, or 1.0 mg/kg, i.p.; synthesized by the Southwest

Research Institute, San Antonio) dissolved in Tween-80:polyethylene glycol:physiological saline (0.9%; 5:5:90, respectively; vehicle 1). Doses and time of injection were chosen from previous *in vivo* studies (Seillier *et al*, 2010). The CB agonist CP55,940 (Tocris) was dissolved in vehicle 1 and administered at a dose (0.01 mg/kg, i.p.) selected to have no deleterious effect on social interaction (Genn *et al*, 2004). The CB₁ antagonists AM251 (0.3, 1.0, and 3.0 mg/kg, i.p.; Cayman Chemical) and SR141716 (0.1, 0.3, and 1.0 mg/kg, i.p.; synthesized by the Southwest Research Institute) and the TRPV1 antagonist capsazepine (CPZ; 1, 3, and 10 mg/kg, i.p.; Ascent) were dissolved in vehicle 2. The cholecystokinin (CCK)2 antagonist LY225910 (LY; 0.02, 0.05, and 0.1 mg/kg, i.p.; Tocris) was dissolved in vehicle 1. The dose of antagonist suitable for each pharmacological study (subthreshold dose) was determined through dose–response curves in control animals (Supplementary Figures S1 and S2A).

Biochemical Studies

Immediately after the social interaction test, animals were anesthetized with halothane, their brains rapidly 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 –17 °C and sliced into 1-mm coronal sections using razor blades to dissect out the following brain areas: amygdala, medial prefrontal cortex (mPFC; prelimbic and infralimbic cortices), nucleus accumbens (NAc), and caudate-putamen (CPu). Tissue samples were spiked with 50 pmol of [²H₄]AEA, [²H₄]oleoylethanolamine (OEA), and [²H₅]-2-arachidonyl glycerol (2-AG; internal standards) and processed as previously described (Hardison *et al*, 2006). Briefly, lipids were extracted by adding methanol/chloroform/water (1:2:1, v/v/v), and the chloroform layer was further purified by solid phase extraction using C18 Bond Elut cartridges (100 mg; Varian, USA). Endocannabinoid-containing fractions were analyzed by gas chromatography/chemical ionization mass spectrometry (GC/MS), using an isotope dilution assay.

Western Blots

Tissue samples (amygdala and mPFC; see above) were homogenized in ice-cold lysis buffer containing 50 mM Tris-HCl, 100 mM NaCl, 0.1% Triton X-100, 0.1% SDS, 1 mM Na₃VO₄, 10 mM NaF, 1 mM EDTA, and 1% protease inhibitor cocktail (Sigma Chemical, St. Louis, USA) and centrifuged at 16000 g for 30 min at 4 °C. Equal amount of protein (20 µg) were resolved by SDS–PAGE (10%), transferred onto PVDF membranes (0.2 µm), and incubated for 1 h in 5% fat-free milk in Tris buffer saline + 0.05% Tween-20 (TBS-T buffer) at room temperature. Membranes were then incubated overnight at 4 °C using the following primary antibodies: anti-*N*-arachidonyl phosphatidylethanolamine phospholipase D (NAPE-PLD; 1:200; Cayman Chemical), anti-FAAH (1:500; Cayman Chemical), anti-CB₁ (1:500; kindly provided by Dr Ken Mackie), anti-PKA (1:1000; Cell Signaling), anti-phosphorylated PKA (pPKA; 1:1000; Cell Signaling), and anti-β-actin (1:10000; Sigma Chemical). After three 5-min washes in TBS-T,

membranes were incubated with the appropriate secondary horseradish peroxidase-linked antibodies (1:2000; Santa Cruz) for 60 min at room temperature. Protein bands were visualized using the ECL kit (Amersham, GE Healthcare, Buckinghamshire, England) followed by exposure to X ray. Band immunoreactivity was quantified by densitometry using NIH image software.

Statistical Analysis

Western blot data were analyzed by one-way ANOVA with Treatment (saline, PCP) as between-subject factor. Data from the GC/MS analyses were analyzed by two-way ANOVA with Treatment (saline, PCP) and Drug (vehicle, URB597) as between-subject factors for each brain area investigated. The behavioral data were analyzed by one-way, two-way, or three-way ANOVA, according to the experimental design. The Newman-Keuls test was used for *post hoc* comparisons when required. The level of significance was set at $p < 0.05$.

RESULTS

FAAH Inhibition Reverses PCP-Induced Social Withdrawal

PCP-treated rats spent less time in social interaction, showing a decrease in the number of sniffing and climbing episodes, as previously reported (Seillier *et al*, 2010; Supplementary Table S1). Systemic administration of URB597 (0.1 and 0.3 mg/kg) reversed PCP-induced social withdrawal, with the exception of the highest dose (1 mg/kg), whereas it significantly reduced social interaction in saline-treated rats at all the doses tested (Figure 1a). As pharmacological blockade of FAAH by URB597 enhances AEA efficacy at TRPV1 receptors, which exert neuromodulatory actions opposite to those of CB₁ receptors, we assessed whether pretreatment with either the CB₁ antagonist AM251 (1 mg/kg) or the TRPV1 antagonist CPZ (10 mg/kg) could affect URB597 (0.3 mg/kg) actions on social interaction. AM251 reversed URB597 effect in PCP-, but not in saline-treated rats, whereas CPZ blocked URB597-induced deficit in saline-treated rats, but not its beneficial action in PCP-treated rats (Figure 1b). Neither AM251 nor CPZ reversed PCP-induced social withdrawal (Figure 1b). As previous work has suggested the existence of a cannabinoid-/vanilloid-sensitive receptor (Hajos and Freund, 2002), which is blocked by CPZ but not AM251, we tested whether SR141716, a drug that antagonizes both receptors (Pistis *et al*, 2004; Supplementary Figure S2), could mimic the effect of CPZ. SR141716 (0.1 mg/kg) reversed URB597-induced deficit (Figure 1c), suggesting the involvement of the cannabinoid-/vanilloid-sensitive receptor in the deleterious effect of URB597 in saline-treated rats. In an independent set of experiments, we assessed whether the inability of URB597 to reverse PCP-induced social withdrawal at the dose of 1 mg/kg could be attributed to the recruitment of this receptor, which is different from TRPV1 or CB₁ receptors. As shown in Figure 1d, the PCP-induced social withdrawal was completely prevented by coadministration of URB597 and CPZ, and this beneficial effect was CB₁-dependent, as it was

blocked by AM251. AM251 did not affect the inability of URB597 to reverse PCP-induced social withdrawal in the absence of CPZ (data not shown). Taken together, these data suggest that enhancement of endocannabinoid levels is sufficient to reverse the PCP-induced social interaction deficit.

As URB597 reverses PCP-induced deficit in a CB₁-dependent manner, social withdrawal in PCP-treated rats might result from deficient CB₁ stimulation. As in the case of CB₁ knock-out mice, which shows decreased social interaction (Haller *et al*, 2004; Uriguen *et al*, 2004), pharmacological blockade of CB₁ receptors in saline-treated rats with AM251 produced a dose-dependent social withdrawal (Figure 2a), confirming that CB₁ activation contributes to social interaction. In agreement with this hypothesis, systemic administration of the potent CB agonist CP55,940, at a dose that does not affect social interaction in controls (0.01 mg/kg), reversed social withdrawal in PCP-treated rats in a CB₁-dependent manner (Figure 2b).

Endocannabinoid Transmission is Altered in PCP-Treated Rats Engaged in Social Behavior

As CB₁ receptor expression is not altered in PCP-treated rats (Guidali *et al*, 2011; Seillier *et al*, 2010; Supplementary Figure S3), we investigated whether PCP-induced social withdrawal was accompanied by decreased endocannabinoid mobilization. Endocannabinoid levels are not altered in PCP-treated rats under resting conditions (Seillier *et al*, 2010), and given the fact that they are produced 'on demand', we quantified AEA and 2-AG by MS immediately after the end of the social interaction test. Using this approach, we observed lower AEA levels in either the mPFC (Figure 3a left panel) or amygdala (Figure 3b left panel) of PCP-treated rats vs saline-treated controls. AEA levels in PCP-treated rats engaged in social interaction were similar to those reported in saline-treated rats under resting conditions (Figure 3, dashed line). PCP-treated rats also showed a significant AEA elevation in the NAc (Figure 3c left panel), but no changes in other brain regions (eg, CPu; Figure 3d left panel). Application of URB597 (0.3 mg/kg) enhanced AEA levels throughout the brain areas examined (Figure 3), thus compensating for the AEA deficit observed in the mPFC and amygdala of PCP-treated rats (Figure 3a and b). In particular, a nonlinear regression analysis revealed the existence of a curvilinear relationship between the AEA levels in the amygdala and time spent in social interaction ($R^2 = 0.5571$; Supplementary Figure S4), suggesting an association between intermediate concentrations of AEA and optimal social interaction (as in control animals). On the other hand, lower or higher levels of AEA (as observed in PCP-treated rats or in saline-treated animals receiving URB597, respectively) were associated with a social behavior deficit. In contrast, 2-AG levels (Figure 3 right panels) were incoherent with the behavioral pharmacology described in Figure 1b. Indeed, 2-AG was elevated in both the NAc (Figure 3c) and CPu (Figure 3d) of PCP-treated rats, and a similar trend was also observed in the mPFC (Figure 3a). The deficient AEA production observed in PCP-treated rats did not result from a decrease in the expression of one of the putative AEA-synthesizing enzyme

Figure 1 URB597 reverses phencyclidine (PCP)-induced social withdrawal in a CB₁-dependent manner, but reduced social interaction in control animals in a CB₁-independent manner. (a) URB597 (U; 0.1–1.0 mg/kg, i.p.) affects social interaction in saline- and PCP-treated rats dose-dependently ($F_{3,56} = 40.5$, $p < 0.0001$). (b) Effects of AM251 (1 mg/kg, i.p.) or capsazepine (CPZ; 10 mg/kg, i.p.) on PCP- and URB597-induced changes in social interaction (all two-way interactions $F_{1-2,84} > 5.28$, $p < 0.01$). (c) SRI141716 (SR; 0.1 mg/kg, i.p.) reverses URB597-induced social withdrawal in saline-treated rats (all main effects $F_{1,28} > 6.10$, $p < 0.05$). (d) Effects of CPZ (10 mg/kg, i.p.) and URB597 (1 mg/kg, i.p.) coadministration on PCP-induced social withdrawal and its reversal by AM251 (1 mg/kg, i.p.; $F_{4,35} = 15.5$, $p < 0.0001$). Values are expressed as mean $\pm$ SEM. ($n = 8$ per group) of the time spent in social interaction(s). * $p < 0.05$ compared with saline-treated rats; # $p < 0.05$ compared with URB597 vehicle (V) controls; $^{\square}p < 0.05$ compared to AM251 or CPZ vehicle (VEH) controls; $^{\$}p < 0.05$.

Figure 2 Role of CB₁ receptors in social interaction. (a) AM251 (0.3–3.0 mg/kg, i.p.) decreases social interaction in saline-treated rats dose-dependently ($F_{3,28} = 9.06$, $p < 0.001$). (b) AM251 (1 mg/kg, i.p.) reverses the effect of CP55,940 (0.01 mg/kg, i.p.) on phencyclidine (PCP)-induced social withdrawal ($F_{2,42} = 6.80$, $p < 0.01$). Values are expressed as mean $\pm$ SEM. ($n = 8$ per group) of the time spent in social interaction(s). * $p < 0.05$ compared with the corresponding AM251 vehicle control (VEH/V) or to saline-treated rats; # $p < 0.05$ compared with CP55,940 vehicle controls; $^{\square}p < 0.05$ compared with AM251 vehicle control.

Figure S5). These data are consistent with previous observations showing unaltered AEA levels in PCP-treated rats under resting conditions (Seillier *et al*, 2010), as well as, with the unchanged levels of OEA—another FAAH substrate—in the mPFC and amygdala of PCP-treated animals engaged in social interaction (Supplementary Figure S6). Together, these findings suggest that in this animal model the endocannabinoid system is not dysfunctional, but inadequately recruited.

PKA Activation is Positively Correlated to AEA Levels

In most brain areas, endocannabinoid-dependent plasticity has been generally attributed to 2-AG. In the amygdala, however, endocannabinoid-mediated plastic changes have been shown to involve AEA release through activation of a cyclic adenosine monophosphate-protein kinase A (cAMP-PKA)-dependent pathway (Azad *et al*, 2004). As PCP-treated rats undergoing social interaction showed reduced AEA mobilization in this area, we investigated whether this phenomenon could be linked to decreased PKA phosphorylation (ie, activation). PCP-treated rats showed a higher PKA expression in the amygdala compared with saline-treated controls (Figure 4a), which was negatively correlated with AEA changes (Figure 4b; Pearson's $r = -0.69$, $p < 0.05$). However, pPKA did not differ between

NAPE-PLD, which was instead increased in the mPFC (Supplementary Figure S5), nor from increased expression of the AEA catabolic enzyme FAAH (Supplementary

Figure 3 Endocannabinoid levels in rats undergoing social interaction. Effects of URB597 (U) in saline- and phencyclidine (PCP)-treated rats on anandamide (AEA, left) and 2-arachidonyl glycerol (2-AG; right) levels in the medial prefrontal cortex (mPFC; main effects $F_{1,22} = 22.6$, $p < 0.0001$ and Treatment effect $F_{1,24} = 7.7$, $p < 0.05$ for AEA and 2-AG, respectively; a), amygdala (amy; main effects $F_{1,24} > 132.6$, $p < 0.0001$ for AEA; b), nucleus accumbens (NAc; $F_{1,23} = 10.0$, $p < 0.001$ and $F_{1,24} = 4.3$, $p < 0.05$ for AEA and 2-AG, respectively; c) and caudate-putamen (CPu; $F_{1,22} = 0.0$, NS and $F_{1,23} = 6.8$, $p < 0.05$ for AEA and 2-AG, respectively; d). The dashed line represents AEA or 2-AG levels in saline-treated animals not undergoing behavioral testing. Values are expressed as mean $\pm$ SEM. ($n = 6$ –8 per group) of AEA (pmol/g) or 2-AG (nmol/g) concentrations. * $p < 0.05$ compared with saline-treated rats; # $p < 0.05$ compared with URB597 vehicle (V) controls; + $p < 0.05$ compared with saline-treated vehicle (V) controls.

the two experimental groups (Figure 4a). In the mPFC, the other brain area showing decreased AEA, we found a trend ($p = 0.065$) toward higher PKA levels in PCP- vs saline-treated rats (Figure 4c) and a significant decrease of pPKA in the former group (Figure 4c), which was positively correlated with AEA changes (Figure 4d; Pearson's $r = 0.71$, $p < 0.05$).

PCP-Induced Social Withdrawal Results from Deficient CB₁ Receptor Stimulation

Our observations suggest that PCP-induced social withdrawal is likely due to reduced AEA-induced activation of CB₁ receptors in brain areas relevant to social interaction. To address this hypothesis, we examined whether AM251- and PCP-induced social withdrawal shared a similar molecular mechanism. Anatomical and electrophysiological studies carried out in rodents have shown that CB₁ receptors are primarily located on the presynaptic terminals of GABAergic interneurons expressing the anxiogenic neuropeptide CCK. This expression pattern has been observed in many brain regions, including the mPFC (Marsicano and Lutz, 1999) and amygdala (Ramikie and

Patel, 2011), in which CB₁ stimulation decreases GABA (Antonelli *et al*, 2009) and CCK (Beinfeld and Connolly, 2001) release. CCK has an important role in the neurobiology of anxiety, and administration of CCK2 receptor antagonists to rats can enhance social interaction (Supplementary Figure S1). Given these premises, we hypothesized that the AM251-induced social withdrawal in normal rats might result from the inability of endocannabinoids (possibly AEA) to stimulate CB₁ receptors and consequently reduce CCK2 receptor activation during social interaction. In agreement with this hypothesis, systemic injection of the CCK2 antagonist LY225910, at a dose (0.05 mg/kg) that did not alter social interaction in control rats (Supplementary Figure S1), fully blocked the AM251-induced deficit (Figure 5). Similarly, LY225910 also reversed the PCP-induced social withdrawal, whereas it had no effect on the URB597-induced social withdrawal in saline-treated animals, which is CB₁ independent. These data indicate that CCK2 activation is an important downstream component of CB₁-mediated modulation of social behavior, and foremost that both AM251- and PCP-induced social withdrawal result from a deficit in CB₁ stimulation.

Figure 4 PKA phosphorylation is decreased in the medial prefrontal cortex (mPFC), but not in the amygdala (amy), of PCP-treated rats. Effects of PCP on PKA expression (normalized to β -actin; center panel) and phosphorylation (pPKA; normalized to PKA; right panel) in the (a) amygdala ($F_{1,10}=11.01$, $p<0.01$ and $F_{1,11}=2.04$, NS for PKA and pPKA, respectively) and (c) mPFC ($F_{1,11}=4.19$, $p=0.065$ and $F_{1,10}=16.63$, $p<0.01$ for PKA and pPKA, respectively). Left panels show representative western blot data. Values are expressed as percentage of control (mean $\pm$ SEM; $n=6-7$ per group). * $p<0.05$ compared with saline-treated rats. (b) Correlation analysis of PKA and AEA levels in the amygdala. (d) Correlation analysis of pPKA and AEA levels in the mPFC.

Figure 5 Cholecystinin (CCK)2 receptors modulate AM251- and PCP-induced social withdrawal. Effects of LY225910 (LY; 0.05 mg/kg, i.p.) on PCP-, AM251- (2 mg/kg, i.p.), and URB597- (0.3 mg/kg, i.p.) induced social withdrawal ($F_{3,56}=9.78$, $p<0.0001$). Values are expressed as mean $\pm$ SEM ($n=8$ per group) of the time spent in social interaction(s). * $p<0.05$ compared with saline-treated rats; # $p<0.05$ compared with LY225910 vehicle (VEH) controls.

DISCUSSION

Clinical investigations on the negative symptoms of schizophrenia have been challenged by the lack of understanding of the underlying pathophysiology and the limited availability of adequate animal models. In this study, we showed that social withdrawal, a behavioral correlate of the negative symptoms of the disease, resulted from deficient endocannabinoid-induced activation of CB₁ receptors. PCP-induced social withdrawal was alleviated either directly, by activating CB₁ receptors via the cannabinoid agonist CP55,940, or indirectly by elevating endocannabinoids via pharmacological inhibition of their degrading enzyme. By contrast, administration of the FAAH inhibitor

URB597 to PCP-free rats elevated brain AEA above control levels, leading to social withdrawal through the recruitment of a cannabinoid-/vanilloid-sensitive receptor, different from TRPV1 or CB₁ receptors. Collectively, these results suggest that alterations in endocannabinoid transmission have a role in the pathophysiology of the negative symptoms, or at least in social withdrawal.

According to the 'cannabinoid hypothesis' of schizophrenia, overactivity of the endocannabinoid system contributes to the hyperdopaminergic and hypoglutamatergic transmission underlying the positive and negative symptoms, respectively. As a corollary to this hypothesis, pharmacological blockade of CB₁ receptor via the CB₁ antagonist SR141716 (Rimonabant) was assumed to improve schizophrenic symptoms in humans. Instead, the outcome of this clinical trial was disappointing, as no symptom improvement was obtained over placebo controls (Meltzer *et al*, 2004). On the other hand, as already observed in previous studies showing an association between cannabis intake and reduced negative symptomatology (Compton *et al*, 2004; Dubertret *et al*, 2006), administration of the cannabinoid agonist dronabinol (synthetic Δ -9-tetrahydrocannabinol) ameliorated symptoms in a small group of treatment-resistant schizophrenics (Schwarcz *et al*, 2009). The possibility that overactive CB₁ receptors might account for the emergence of schizophrenic symptoms has also been challenged. Indeed, although initial postmortem studies showed increased CB₁ binding in cortical areas of schizophrenic patients (Dean *et al*, 2001; Zavitsanou *et al*, 2004), more recent measurements of CB₁ mRNA or protein have not confirmed this putative upregulation (Dalton *et al*, 2011; Koethe *et al*, 2007; Uriguen *et al*, 2009), and found instead decreased CB₁ density in the dorsolateral prefrontal cortex (Eggan *et al*, 2008). Moreover, CB₁ abnormalities have been related to specific schizophrenia subtypes, as suggested by the association of some polymorphisms of the CB₁ receptor

gene *CNR1* with the hebephrenic type of schizophrenia (Chavarria-Siles *et al*, 2008; Ujike *et al*, 2002). On the same line, Dalton *et al* (2011) showed that only paranoid schizophrenics had higher CB₁ levels in the dorsolateral prefrontal cortex, whereas the disorganized and residual subgroups had lower CB₁ densities, as reported by Eggan *et al* (2008). Finally, a recent imaging study has shown that CB₁ receptor binding is positively correlated with the severity of positive symptoms, whereas patients with reduced CB₁ binding had more pronounced negative symptomatology (Wong *et al*, 2010).

Our data support the idea that deficient, rather than overactive, endocannabinoid transmission may contribute to the expression of negative symptoms, or at least social withdrawal. Indeed, we found that the 'on demand' production of AEA occurring during social interaction (Trezza *et al*, 2012) is significantly reduced in PCP-treated rats in brain areas relevant to social behavior, such as the mPFC and amygdala. The resulting deficient CB₁ activation cannot be attributed to decreased CB₁ expression, nor to disrupted CB₁ function/coupling, as neither one are affected in our animal model (Seillier *et al*, 2010). Nevertheless, disrupted CB₁ expression/function has been reported in other models of schizophrenia (Guidali *et al*, 2011). Also, omega-3 fatty-acid deficiency, which is associated with the negative symptoms of schizophrenia (Sethom *et al*, 2010), has been shown to impair social exploration in mice by reducing CB₁ receptor function (Lafourcade *et al*, 2011). These observations provide converging evidence that CB₁ receptors contribute to the pathophysiology of schizophrenia. In our model, AEA levels were not altered under resting conditions (Seillier *et al*, 2010), and the lack of changes in the expression/function of CB₁ receptor and metabolic enzymes indicate that the endocannabinoid system of PCP-treated rats is not dysfunctional (Seillier *et al*, 2010), but inadequately recruited, possibly as a consequence of altered upstream events (eg, attenuation of PKA signaling). A similar scenario has been described, for example, after cocaine exposure, which causes a reduction of the ability of mGluR5 receptors to mobilize endocannabinoids (Fourgeaud *et al*, 2004). Interestingly, PKA has been shown to activate *N*-acyltransferase, an enzyme involved in the biosynthesis of the AEA precursor *N*-arachidonyl-phosphatidyl-ethanolamine (Cadas *et al*, 1996), suggesting that reduced PKA phosphorylation can lead to decrease AEA synthesis.

In addition to the association between reduced endocannabinoid transmission and social withdrawal in PCP-treated rats, our study indicates that stimulation of CB₁ receptors can reverse the behavioral deficit in these animals. In agreement with our data, a recent study showed that self-administration of the cannabinoid agonist WIN55,212-2 attenuated PCP-induced deficits in sociability (Spano *et al*, 2010), strengthening the idea that CB₁ stimulation reduces the severity of the negative symptoms. Although these findings are in line with the negative correlation between CSF AEA levels and negative symptoms observed in schizophrenic patients (Giuffrida *et al*, 2004), they contrast with the observation that cannabis use precipitates psychotic symptoms in vulnerable subjects (Sewell *et al*, 2009). This discrepancy, however, may be attributed to unique neurobiological mechanisms underlying the different

categories of schizophrenic symptoms (including cognitive deficits), which can recruit the endocannabinoid system in different ways. For example, although social withdrawal in PCP-treated rats is associated with reduced CB₁ activation, working memory deficits in the same animals have been linked to increased activity at CB₁ receptors (Seillier *et al*, 2010). In addition, alterations of endocannabinoid transmission in control animals via URB597, AM251, or chronic WIN55,212-2 (Spano *et al*, 2010) produced deleterious effects similar to those observed in PCP-treated rats, suggesting that cannabinoid drugs trigger different behavioral responses in distinct experimental groups, as in the case of schizophrenics *vs* healthy subjects (Rentzsch *et al*, 2011). Finally, chronic cannabis consumption has been shown to attenuate negative symptoms in the former group (Compton *et al*, 2004; Dubertret *et al*, 2006), but to induce an 'amotivational syndrome' in the latter (Sewell *et al*, 2009). This syndrome, which is reminiscent of the negative symptomatology, might result from CB₁ desensitization or decreased endocannabinoid mobilization due to chronic cannabis exposure (Di Marzo *et al*, 2000).

Reduced amygdala–prefrontal functional connectivity has been associated to emotional abnormalities in schizophrenia (Hoptman *et al*, 2010), which arise from increased neuronal excitation over inhibition within specific micro-circuitries (Lisman, 2011). This neuronal disinhibition may result from functional alterations of perisomatic GABAergic interneurons (primarily basket cells) containing either the calcium-binding protein parvalbumin (PV) or the neuropeptide CCK. Specifically, Curley and Lewis (2012) have suggested an increased ratio of CCK⁺ to PV⁺ cells-mediated activity, which may lead to a CCK-mediated enhancement of neuronal excitability via facilitation of glutamatergic transmission (Deng *et al*, 2010) and depression of GABA release from CCK⁺ interneurons (Lee and Soltesz, 2010). This increased activity of cortical excitatory neurons can impair social behavior, which in turn can be rescued by elevating PV⁺ cell excitability (Yizhar *et al*, 2011). As CB₁ receptors are predominantly expressed on CCK⁺ terminals (Marsicano and Lutz, 1999; Ramikie and Patel, 2011), and CB₁ activation produces a selective inhibition of these cells (Hentges *et al*, 2005), we postulated that endocannabinoid-induced inhibition of CCK⁺ interneurons is lost/reduced in PCP-treated rats, thus resulting in social withdrawal (Supplementary Figure S7). In support of this hypothesis, our study and other reports have shown increased AEA (Trezza *et al*, 2012) and decreased CCK levels (Panksepp *et al*, 2004) during social interaction in normal rats. In PCP-treated rats, which are characterized by deficient AEA mobilization, endocannabinoid elevation back to control levels, or direct activation of CB₁ receptors or blockade of CCK2 receptors, reverses the social behavior deficit. By contrast, when AEA concentration is increased above control levels, as in the case of saline-treated animals receiving URB597, AEA may lose its selective inhibitory action on CCK⁺ interneurons and target the cannabinoid-/vanilloid-sensitive receptor on excitatory terminals (Pistis *et al*, 2004). A similar phenomenon has been reported in the hypothalamus of mice where blockade of AEA reuptake did allow this endocannabinoid to reach glutamatergic inputs (Hentges *et al*, 2005).

In conclusion, we propose that the negative symptoms of schizophrenia are associated with a deficiency in CB₁ receptor function. This observation may reconcile several controversial findings and provide a new frame to understand the cannabis self-medication hypothesis for negative symptoms.

ACKNOWLEDGEMENTS

This work was supported by NARSAD and NIMH RO1MH91130-01A1 (to AG).

DISCLOSURE

The authors declare no conflict of interest.

REFERENCES

- Antonelli T, Tomasini MC, Mazza R, Fuxe K, Gaetani S, Cuomo V *et al* (2009). Cannabinoid CB₁ and cholecystokinin CCK₂ receptors modulate, in an opposing way, electrically evoked [3H]GABA efflux from rat cerebral cortex cell cultures: possible relevance for cortical GABA transmission and anxiety. *J Pharmacol Exp Ther* 329: 708–717.
- Azad SC, Monory K, Marsicano G, Cravatt BF, Lutz B, Zieglansberger W *et al* (2004). Circuitry for associative plasticity in the amygdala involves endocannabinoid signaling. *J Neurosci* 24: 9953–9961.
- Beinfeld MC, Connolly K (2001). Activation of CB₁ cannabinoid receptors in rat hippocampal slices inhibits potassium-evoked cholecystokinin release, a possible mechanism contributing to the spatial memory defects produced by cannabinoids. *Neurosci Lett* 301: 69–71.
- Cadas H, Gaillet S, Beltramo M, Venance L, Piomelli D (1996). Biosynthesis of an endogenous cannabinoid precursor in neurons and its control by calcium and cAMP. *J Neurosci* 16: 3934–3942.
- Chavarria-Siles I, Contreras-Rojas J, Hare E, Walss-Bass C, Quezada P, Dassori A *et al* (2008). Cannabinoid receptor 1 gene (CNR1) and susceptibility to a quantitative phenotype for hebephrenic schizophrenia. *Am J Med Genet B Neuropsychiatr Genet* 147: 279–284.
- Compton MT, Furman AC, Kaslow NJ (2004). Lower negative symptom scores among cannabis-dependent patients with schizophrenia-spectrum disorders: preliminary evidence from an African American first-episode sample. *Schizophr Res* 71: 61–64.
- Curley AA, Lewis DA (2012). Cortical basket cell dysfunction in schizophrenia. *J Physiol* 590: 715–724.
- Dalton VS, Long LE, Weickert CS, Zavitsanou K (2011). Paranoid schizophrenia is characterized by increased CB₁ receptor binding in the dorsolateral prefrontal cortex. *Neuropsychopharmacology* 36: 1620–1630.
- Dean B, Sundram S, Bradbury R, Scarr E, Copolov D (2001). Studies on [³H]CP-55940 binding in the human central nervous system: regional specific changes in density of cannabinoid-1 receptors associated with schizophrenia and cannabis use. *Neuroscience* 103: 9–15.
- Deng PY, Xiao Z, Jha A, Ramonet D, Matsui T, Leitges M *et al* (2010). Cholecystokinin facilitates glutamate release by increasing the number of readily releasable vesicles and releasing probability. *J Neurosci* 30: 5136–5148.
- Di Marzo V, Berrendero F, Bisogno T, Gonzalez S, Cavaliere P, Romero J *et al* (2000). Enhancement of anandamide formation in the limbic forebrain and reduction of endocannabinoid contents in the striatum of delta9-tetrahydrocannabinol-tolerant rats. *J Neurochem* 74: 1627–1635.
- Dubertret C, Bidard I, Ades J, Gorwood P (2006). Lifetime positive symptoms in patients with schizophrenia and cannabis abuse are partially explained by co-morbid addiction. *Schizophr Res* 86: 284–290.
- Eggen SM, Hashimoto T, Lewis DA (2008). Reduced cortical cannabinoid 1 receptor messenger RNA and protein expression in schizophrenia. *Arch Gen Psychiatry* 65: 772–784.
- Enomoto T, Noda Y, Nabeshima T (2007). Phencyclidine and genetic animal models of schizophrenia developed in relation to the glutamate hypothesis. *Methods Find Exp Clin Pharmacol* 29: 291–301.
- Fourgeaud L, Mato S, Bouchet D, Hemar A, Worley PF, Manzoni OJ (2004). A single in vivo exposure to cocaine abolishes endocannabinoid-mediated long-term depression in the nucleus accumbens. *J Neurosci* 24: 6939–6945.
- Foussias G, Remington G (2010). Negative symptoms in schizophrenia: avolition and Occam's razor. *Schizophr Bull* 36: 359–369.
- Genn RF, Tucci S, Marco EM, Viveros MP, File SE (2004). Unconditioned and conditioned anxiogenic effects of the cannabinoid receptor agonist CP 55,940 in the social interaction test. *Pharmacol Biochem Behav* 77: 567–573.
- Giuffrida A, Leweke FM, Gerth CW, Schreiber D, Koethe D, Faulhaber J *et al* (2004). Cerebrospinal anandamide levels are elevated in acute schizophrenia and are inversely correlated with psychotic symptoms. *Neuropsychopharmacology* 29: 2108–2114.
- Guidali C, Vigano D, Petrosino S, Zamberletti E, Realini N, Binelli G *et al* (2011). Cannabinoid CB₁ receptor antagonism prevents neurochemical and behavioural deficits induced by chronic phencyclidine. *Int J Neuropsychopharmacol* 14: 17–28.
- Gururajan A, Taylor DA, Malone DT (2010). Current pharmacological models of social withdrawal in rats: relevance to schizophrenia. *Behav Pharmacol* 21: 690–709.
- Hajos N, Freund TF (2002). Pharmacological separation of cannabinoid sensitive receptors on hippocampal excitatory and inhibitory fibers. *Neuropharmacology* 43: 503–510.
- Haller J, Varga B, Ledent C, Barna I, Freund TF (2004). Context-dependent effects of CB₁ cannabinoid gene disruption on anxiety-like and social behaviour in mice. *Eur J Neurosci* 19: 1906–1912.
- Hamdani N, Tabeze J-P, Ramoz N, Ades J, Hamon M, Sarfati Y *et al* (2008). The CNR1 gene as a pharmacogenetic factor for antipsychotics rather than a susceptibility gene for schizophrenia. *Eur Neuropsychopharmacol* 18: 34–40.
- Hanson E, Healey K, Wolf D, Kohler C (2010). Assessment of pharmacotherapy for negative symptoms of schizophrenia. *Curr Psychiatry Rep* 12: 563–571.
- Hardison S, Weintraub ST, Giuffrida A (2006). Quantification of endocannabinoids in rat biological samples by GC/MS: technical and theoretical considerations. *Prostaglandins Other Lipid Mediat* 81: 106–112.
- Hentges ST, Low MJ, Williams JT (2005). Differential regulation of synaptic inputs by constitutively released endocannabinoids and exogenous cannabinoids. *J Neurosci* 25: 9746–9751.
- Hoptman MJ, D'Angelo D, Catalano D, Mauro CJ, Shezhad ZE, Kelly AMC *et al* (2010). Amygdalofrontal functional disconnection and aggression in schizophrenia. *Schizophr Bull* 36: 1020–1028.
- Koethe D, Llenos IC, Dulay JR, Hoyer C, Torrey EF, Leweke FM *et al* (2007). Expression of CB₁ cannabinoid receptor in the anterior cingulate cortex in schizophrenia, bipolar disorder, and major depression. *J Neural Transm* 114: 1055–1063.
- Lafourcade M, Larrieu T, Mato S, Duffaud A, Sepers M, Matias I *et al* (2011). Nutritional omega-3 deficiency abolishes endocannabinoid-mediated neuronal functions. *Nat Neurosci* 14: 345–350.

- Lee SH, Soltesz I (2010). Requirement for CB₁ but not GABA_B receptors in the cholecystokinin mediated inhibition of GABA release from cholecystokinin expressing basket cells. *J Physiol* 589: 891–902.
- Lisman J (2011). Excitation, inhibition, local oscillations, or large-scale loops: what causes the symptoms of schizophrenia? *Curr Opin Neurobiol* 22: 1–8.
- Marsicano G, Lutz B (1999). Expression of the cannabinoid receptor CB₁ in distinct neuronal subpopulations in the adult mouse forebrain. *Eur J Neurosci* 11: 4213–4225.
- Meltzer HY, Arvanitis L, Bauer D, Rein W (2004). Placebo-controlled evaluation of four novel compounds for the treatment of schizophrenia and schizoaffective disorder. *Am J Psychiatry* 161: 975–984.
- Milev P, Ho BC, Arndt S, Andreasen NC (2005). Predictive values of neurocognition and negative symptoms on functional outcome in schizophrenia: a longitudinal first-episode study with 7-year follow-up. *Am J Psychiatry* 162: 495–506.
- Panksepp J, Burgdorf J, Beinfeld MC, Kroes RA, Moskal JR (2004). Regional brain cholecystokinin changes as a function of friendly and aggressive social interactions in rats. *Brain Res* 1025: 75–84.
- Pistis M, Perra S, Pillolla G, Melis M, Gessa GL, Muntoni AL (2004). Cannabinoids modulate neuronal firing in the rat basolateral amygdala: evidence for CB₁- and non-CB₁-mediated actions. *Neuropharmacology* 46: 115–125.
- Ramkise TS, Patel S (2011). Endocannabinoid signaling in the amygdala: anatomy, synaptic signaling, behavior, and adaptations to stress. *Neuroscience* 204: 38–52.
- Rentsch J, Buntebart E, Stadelmeier A, Gallinat J, Jockers-Scherubl MC (2011). Differential effects of chronic cannabis use on preattentive cognitive functioning in abstinent schizophrenic patients and healthy subjects. *Schizophr Res* 130: 222–227.
- Schwarcz G, Karajgi B, McCarthy R (2009). Synthetic delta-9-tetrahydrocannabinol (dronabinol) can improve the symptoms of schizophrenia. *J Clin Psychopharmacol* 29: 255–258.
- Seillier A, Advani T, Cassano T, Hensler JG, Giuffrida A (2010). Inhibition of fatty-acid amide hydrolase and CB₁ receptor antagonism differentially affect behavioural responses in normal and PCP-treated rats. *Int J Neuropsychopharmacol* 13: 373–386.
- Sethom MM, Fares S, Bouaziz N, Melki W, Jemma R, Keki M *et al* (2010). Polyunsaturated fatty acids deficits are associated with psychotic state and negative symptoms in patients with schizophrenia. *Prostaglandins Leukot Essent Fatty Acids* 83: 131–136.
- Sewell RA, Ranganathan M, D'Souza DC (2009). Cannabinoids and psychosis. *Int Rev Psychiatry* 21: 152–162.
- Spano MS, Fadda P, Frau R, Fattore L, Fratta W (2010). Cannabinoid self-administration attenuates PCP-induced schizophrenia-like symptoms in adult rats. *Eur Neuropsychopharmacol* 20: 25–36.
- Trezza V, Damsteegt R, Manduca A, Petrosino S, Van Kerkhof LWM, Pasterkamp RJ *et al* (2012). Endocannabinoids in amygdala and nucleus accumbens mediate social play reward in adolescent rats. *J Neurosci* 32: 14899–14908.
- Ujike H, Takaki M, Nakata K, Tanaka Y, Takeda T, Kodama M *et al* (2002). CNR1, central cannabinoid receptor gene, associated with susceptibility to hebephrenic schizophrenia. *Mol Psychiatry* 7: 515–518.
- Uriguen L, Garcia-Fuster MJ, Callado LF, Morentin B, La Harpe R, Casado V *et al* (2009). Immunodensity and mRNA expression of A_{2A} adenosine, D₂ dopamine, and CB₁ cannabinoid receptors in postmortem frontal cortex of subjects with schizophrenia: effect of antipsychotic treatment. *Psychopharmacology (Berl)* 206: 313–324.
- Uriguen L, Perez-Rial S, Ledent C, Palomo T, Manzanares J (2004). Impaired action of anxiolytic drugs in mice deficient in cannabinoid CB₁ receptors. *Neuropharmacology* 46: 966–973.
- Wong DF, Kuwabara H, Horti AG, Raymond V, Brasic J, Guevara M *et al* (2010). Quantification of cerebral cannabinoid receptors subtype 1 (CB₁) in healthy subjects and schizophrenia by the novel PET radioligand [¹¹C]OMAR. *Neuroimage* 52: 1505–1513.
- Yizhar O, Fenno LE, Prigge M, Schneider F, Davidson TJ, O'shea DJ *et al* (2011). Neocortical excitation/inhibition balance in information processing and social dysfunction. *Nature* 477: 171–178.
- Zavitsanou K, Garrick T, Huang XF (2004). Selective antagonist [³H]SR141716A binding to cannabinoid CB₁ receptors is increased in the anterior cingulate cortex in schizophrenia. *Prog Neuropsychopharmacol Biol Psychiatry* 28: 355–360.

Supplementary Information accompanies the paper on the Neuropsychopharmacology website (<http://www.nature.com/npp>)