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Distinct neuronal activation patterns are associated with PCP-induced social withdrawal and its reversal by the endocannabinoid-enhancing drug URB597

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Abstract

The fatty acid amide hydrolase inhibitor, URB597, an endocannabinoid enhancing drug, reverses social withdrawal in the sub-chronic PCP rat model of schizophrenia, but reduces social interaction (SI) in controls. To identify the anatomical substrates associated with PCP-induced social withdrawal and the contrasting effects of URB597 on SI in PCP- versus saline-treated rats, we analyzed SI-induced c-Fos expression in 28 brain areas relevant to schizophrenia and/or social behavior following vehicle or URB597 administration. In saline-treated rats, SI was accompanied by changes in c-Fos expression in the infralimbic and orbitofrontal cortices, dorsomedial caudate putamen, ventrolateral nucleus of the septum, dorsolateral periaqueductal gray (dIPAG) and central amygdala. Except for the dIPAG, these changes were not observed in PCP-treated rats or in saline-treated rats receiving URB597. In the dorsomedial part of the bed nucleus of the stria terminalis (dmBNST), SI-induced c-Fos expression was observed only in PCP-treated rats. Interestingly, URB597 in PCP-treated rats restored a similar c-Fos expression pattern as observed in saline-treated rats: activation of the orbitofrontal cortex, inhibition of the central amygdala and suppression of activation of the dmBNST. These data suggest that orbitofrontal cortex, central amygdala and dmBNST play a critical role in the reversal of PCP-induced social withdrawal by URB597.

Keywords

amygdala; anandamide; FAAH; c-Fos; neuronal activation; orbitofrontal cortex; phencyclidine; schizophrenia; social interaction

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AUTHOR CONTRIBUTIONS

JM and AS designed the study.

AS performed the pharmacological and behavioral experiments.

JM performed histological experiments and analyses and wrote the manuscript.

AS performed the statistical analyses, supervised the project, data discussion and reviewed the manuscript.

AG supervised the project, data discussion and reviewed the manuscript.

1. INTRODUCTION

Schizophrenia is a debilitating psychiatric disorder affecting 1% of world population (Tandon et al., 2008). The associated cognitive (e.g. attention and memory deficits), positive (e.g. delusions and hallucinations), and negative symptoms (e.g. anhedonia and social withdrawal) significantly impair patients' personal and professional lives. In particular, negative symptoms often lead to homelessness and are thought to be the primary driver for suicide in schizophrenics (Balhara and Verma, 2012). Antipsychotic pharmacotherapy has shown some success in alleviating positive symptoms; however, treatment options for negative and cognitive symptoms remain very limited. Consequently, there is an urgent need to decipher the underlying causes of these symptoms and develop new pharmacological strategies.

Human studies indicate that a dysfunction of the endocannabinoid system might play a role in the pathophysiology of this mental disorder. Postmortem studies in schizophrenic patients have shown altered expression levels of cannabinoid CB1 receptors in several brain areas, including the dorsolateral prefrontal cortex (Dean et al., 2001, Eggen et al., 2008, Uriguen et al., 2009, Eggen et al., 2010, Dalton et al., 2011) and cingulate cortex (Zavitsanou et al., 2004, Newell et al., 2006). Abnormal endocannabinoid transmission has been also associated to negative symptoms. For example, in drug naïve schizophrenics, increased cerebrospinal fluid levels of the endocannabinoid anandamide (AEA) (Leweke et al., 1999, Giuffrida et al., 2004, Leweke et al., 2007, Koethe et al., 2009) were inversely correlated to the severity of negative symptoms (Giuffrida et al., 2004), suggesting that increased AEA production may be beneficial in schizophrenia.

Using the sub-chronic phencyclidine (PCP) rat model of schizophrenia (Jentsch et al., 1997, Seillier and Giuffrida, 2009), we previously showed that PCP-induced social withdrawal, a behavioral correlate of negative symptoms, results from deficient stimulation of CB1 receptors and is reversed by systemic administration of URB597, an inhibitor of the AEA catabolic enzyme fatty acid amide hydrolase (FAAH) (Seillier et al., 2010, Seillier et al., 2013). Despite its beneficial effects in PCP rats, URB597 impaired social interaction (SI) in control rats (Seillier et al., 2010, Seillier et al., 2013). These opposite behavioral effects observed in the two experimental groups have been attributed to URB597 ability to increase endocannabinoid tone in PCP-treated rats to a level sufficient to restore SI, whereas in saline-treated rats URB597 administration produced AEA supra-physiological concentrations, leading to social deficit via activation of a TRPV1-like receptor (Seillier et al., 2010, Seillier et al., 2013). The different endocannabinoid mobilization in saline- vs. PCP-treated rats following SI and/or URB597 administration could lead to the recruitment of different brain circuitries. To identify these circuitries and further study the effects of URB597 on the neurobiological mechanisms of SI, we carried out an extensive analysis of c-Fos expression, a commonly used marker of neuronal activity (Curran and Morgan, 1995, Harris, 1998, Farivar et al., 2004), in 28 cortical, limbic and subcortical regions relevant to schizophrenia and social behavior with the intent to investigate the brain activation patterns associated with: (a) social withdrawal in PCP-treated rats and its reversal by URB597; (b) the SI deficit induced by URB597 in control rats.

2. MATERIAL AND METHODS

2.1. Animals

Adult male Wistar rats (200-225g) were purchased from Charles River Laboratories (Wilmington, MA, USA). Rats were housed two per cage and kept in a controlled environment (12-h light/dark cycle, +22°C) with water and food *ad libitum*. Rats were habituated to the housing conditions for 1 week before the experiments and sub-chronically treated with either saline (1 mL/kg, i.p.) or PCP (5 mg/kg, i.p., Sigma, St Louis, MO, USA) twice a day for 7 days followed by a 7-day washout period before behavioral assessment. A timetable of the pharmacological treatment is reported in Fig. 1. This treatment produces a phenotype reminiscent of schizophrenia symptoms consisting of working memory deficits, hyperlocomotor activity in response to *d*-amphetamine challenge and social withdrawal (Seillier and Giuffrida, 2009). All experiments were designed to minimize the number of animals used and their discomfort according to the ethical guidelines of the National Institute of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio.

2.2. Social interaction test

Seven days after the last PCP (or saline) injection, rats received an acute injection of URB597 (0.3 mg/kg, i.p., synthesized by the Southwest Research Institute, San Antonio, USA) or vehicle (Tween80:PEG:saline, 5:5:90, 1 mL/kg, i.p.) 60 min before the behavioral assessment (Fig. 1). Treatment groups (n=8 rats per group) were randomized using the Latin square method.

For the SI test, two rats matched for body weight and receiving the same pharmacological treatment were placed in an unfamiliar open arena (100 × 100 × 40 cm) under dim light (5 lux) for 60 min and their behavior videotaped. Additional rats placed alone in the arena (arena exploration only) were used as controls for SI (n=8 rats per group). Consequently, our experimental design comprises 8 experimental groups: saline-SI-vehicle, PCP-SI-vehicle, saline-SI-URB597, PCP-SI-URB597, saline-exploration-vehicle, PCP-exploration-vehicle, saline-exploration-URB597, PCP-exploration-URB597 (n=8 rats per group).

An experimenter blind to treatments measured SI as total time (sec) spent by each rat performing the following behaviors: sniffing, following and climbing over or under the conspecific (for details see (Seillier and Giuffrida, 2009)).

2.3. Tissue preparation

Immediately after the 60 min period of behavioral test (arena exploration or SI), rats (n=4 rats per group) were anesthetized with sodium pentobarbital (100 µL / 100 g, i.p.) and 15 min later they were transcardiacally perfused with heparinized (50 IU heparin /mL) phosphate buffered solution (0.1 M PBS, pH 7.6) followed by cold (4°C) 0.1 M PBS containing 4% paraformaldehyde (PFA, Sigma, St Louis, MO, USA) for 10 min. Brains were rapidly removed, post-fixed for 1 h at 4°C in 4% PFA and cryoprotected overnight in a

series of 0.1 M PBS / sucrose baths (10%, 20% and 30%), frozen in isopentane at -40°C and stored at -80°C until further use.

2.4. Immunohistochemistry

Coronal sections ($14\text{ }\mu\text{m}$) were cut in a microtome at -17°C and collected on Superfrost-plus slides (Thermo Fisher, Bartlesville, OK, USA). After Hemalun–Phloxine (HP) staining, anatomical areas (Fig. 2) were identified using the Paxinos and Watson rat brain atlas (Paxinos and Watson, 1998). To avoid counting the same neuron twice, three to six non-consecutive coronal sections of each brain region of interest (ROI) were processed for c-Fos immunohistochemistry. Sections were thawed and rinsed in 0.1 M PBS for 5 min and placed in a 10 mM citrate bath for 20 min at 100°C . Endogenous peroxidase activity was inhibited by adding 3% H_2O_2 in methanol for 15 min. Sections were rinsed in 0.1 M PBS for 15 min and incubated with a rabbit polyclonal anti-c-Fos antibody (1:500, Santa Cruz, Dallas, TX, USA) overnight at 4°C and processed with the Dako streptavidin-biotin-peroxidase kit according to the manufacturer's instructions (Dako, Carpinteria, CA, USA). Binding was revealed using 3,30-diaminobenzidine (DAB). Negative controls were obtained by omitting the primary or secondary antibodies during the immunohistochemical procedure.

2.5. Analysis of c-Fos-IR neuron density

Slides were visualized and imaged with a fully motorized Zeiss M2 microscope equipped with a digital camera (Zeiss Axiocam, Thornwood, NY, USA). The Neurolucida software (MBF Bioscience, Williston, VT, USA) was used to navigate through the sections and draw the ROI referring to geometrical and anatomical landmarks as shown in Fig. 2. c-Fos immunoreactive (c-Fos-IR) neurons were manually counted in each brain hemisphere using the “Cell counter” tool of Image J (NIH, Bethesda, MD, USA). Data was expressed as density of c-Fos-IR neurons (number of nuclei per mm^2). For each ROI, c-Fos expression was measured bilaterally in three to six brain sections ($n=6\text{--}12$ measures total) of each rat ($n=4$ rats per group). The values obtained for each ROI were filtered for outliers by the Carling's method (median rule) using the recommended ideal fourths and the constant k_2 adjusted for sample size [$k_2 = (17.63n - 23.64)/(7.74n - 3.71)$] (Carling, 2000) and then averaged.

2.6. Statistical analysis

Behavioral data were analyzed by two-way analysis of variance (ANOVA) with Treatment (saline, PCP) and Drug (vehicle, URB597) as between factors. For the immunohistochemistry data, c-Fos density was analyzed by three-way ANOVA with Treatment (saline, PCP), Drug (vehicle, URB597) and Behavior (arena exploration, SI) as between factors. Differences between groups were assessed by post-hoc comparisons using the Newman-Keuls test. Statistical significance was set at $p < 0.05$.

3. RESULTS

3.1. Social behavior following PCP treatment and FAAH inhibition

As previously shown (Seillier and Giuffrida, 2009, Seillier et al., 2010, Seillier et al., 2013), PCP-treated rats spent less time interacting with each other than saline-treated controls (Fig.

3). Systemic administration of URB597 (0.3 mg/kg, i.p.) reversed PCP-induced social withdrawal. The same drug, however, produced social deficit in saline-treated rats. In the arena exploration group, no behavioral difference between groups (PCP treatment or URB597 injection) was noticed by the observer (data not shown).

3.2. Neuronal activation patterns associated with arena exploration and SI following PCP treatment and FAAH inhibition

For clarity, the results are described step by step, even though all data were analyzed using three-way ANOVA followed by Newman-Keuls post hoc test. First, we reported the c-Fos expression levels in rats exploring the arena alone (Section 3.2.1.), followed by c-Fos data in saline- and PCP-treated rats after SI (Section 3.2.2.). Finally, we described the effects of FAAH inhibition on c-Fos expression following SI in both experimental groups (Section 3.2.3.).

3.2.1. c-Fos expression in rats exploring the arena alone—In animals exploring the arena alone, we found no difference in c-Fos expression between groups (saline-vehicle, PCP-vehicle, saline-URB597, PCP-URB597). However, the three-way ANOVA revealed a Drug effect in the ventral BNST ($F_{1,23}=5.61$, $p<0.05$; Table 1), suggesting that URB597 significantly decreased c-Fos expression in this area irrespective of Behavior or Treatment.

3.2.2. c-Fos expression following SI

3.2.2.1. Saline-treated rats: The SI test was associated with c-Fos expression changes in a limited number of brain areas. Specifically, c-Fos increased in the infralimbic cortex, orbitofrontal cortex, VLS, and dorsomedial caudate putamen (CPu) (Fig. 4, Fig. 5 and Table 1). By contrast, the posterior central amygdala (CeA) showed a decreasing trend ($p=0.051$) in c-Fos nuclei density (Fig. 5 and Table 1). In addition, the three-way ANOVA revealed a Treatment effect in the basolateral amygdala (BLA) ($F_{1,23}=4.53$, $p<0.05$; Fig. 4 and Table 1) and VTA ($F_{1,24}=4.52$, $p<0.05$; Fig. 4 and Table 1), indicating that PCP-treated animals had a lower c-Fos expression in these brain areas, irrespective of Behavior or Drug. No significant changes in c-Fos expression were observed in the other ROI (Fig. 4 and Table 1), except for the dorsolateral periaqueductal gray (PAG) where the ANOVA revealed a Behavior effect ($F_{1,22}=16.53$, $p<0.001$).

3.2.2.2. PCP-treated rats: PCP treatment prevented the neuronal activation induced by social behavior in the infralimbic cortex, orbitofrontal cortex, and dorsomedial CPu (Fig. 4, Fig. 5 and Table 1), as well as the decrease in neuronal activation observed in the posterior CeA (Fig. 5 and Table 1). In addition, the c-Fos induction reported in the VLS of saline-treated rats was not observed in PCP-treated animals (Fig. 4 and Table 1). Finally, we found c-Fos activation in the dorsal part of the BNST and a similar trend in the anterior CeA in PCP-treated rats, whereas these brain areas showed no changes in c-Fos after SI in saline-treated rats (Fig. 4 and Table 1). PCP treatment did not affect c-Fos expression in the other ROI analyzed (Table 1), with the exception of the basolateral amygdala (BLA; $F_{1,23}=4.53$, $p<0.05$; Fig. 4 and Table 1) and the VTA ($F_{1,24}=4.52$, $p<0.05$; Fig. 4 and Table 1).

3.2.3. Effect of FAAH inhibition on c-Fos expression following SI

3.2.3.1. Saline-treated rats: After URB597 injection, saline-treated animals presented a c-Fos pattern similar to that observed in PCP-treated rats. Namely, URB597 prevented activation of the infralimbic and orbitofrontal cortex (Fig. 5 and Table 1), and of the dorsomedial CPu (Fig. 4 and Table 1), as well as the decrease in neuronal activation observed in the posterior CeA. In addition, the c-Fos activation reported in the VLS of saline-treated rats was not observed after URB597 administration (Fig. 4 and Table 1). URB597 also decreased neuronal activation in the cingulate cortex 2 of saline-treated rats undergoing SI (Fig. 4 and Table 1), but had no effect on c-Fos expression in the other ROI analyzed (Table 1), with the exception of the ventral BNST as mentioned previously.

3.2.3.2. PCP-treated rats: URB597 restored c-Fos activation in the orbitofrontal cortex (Fig. 5 and Table 1) and prevented neuronal activation of the posterior CeA (Fig. 5 and Table 1) in PCP-treated rats. URB597 also reversed the activation of the dorsal part of the BNST following SI (Fig. 4 and Table 1). In PCP-treated animals, URB597 decreased activation of the cingulate cortex 2 compared to saline-treated rats undergoing SI (Fig. 4 and Table 1). The pattern of c-Fos expression observed in PCP-treated animals was not affected by URB597 treatment in all the other ROI analyzed, with the exception of the ventral BNST as mentioned previously.

4. DISCUSSION

In this study we showed that rats undergoing SI present c-Fos activation changes in a discrete number of brain areas (infralimbic and orbitofrontal cortices, dorsomedial CPu, and posterior CeA). This pattern of activation is disrupted in PCP-treated animals as well as in saline-treated rats receiving URB597, which both display social withdrawal (Seillier et al., 2010, Seillier et al., 2013). More importantly, systemic administration of URB597 reversed PCP-induced social withdrawal and the associated changes in c-Fos activation/inactivation observed in the orbitofrontal cortex, dmBNST, and posterior CeA, thereby confirming their involvement in SI.

4.1. Neuronal correlates of SI in control animals

The assessment of the neuronal correlates of SI by behaviorally induced c-Fos expression requires careful choice of experimental controls. In particular, the use of rats in their home cages as control animals may lead to false positives resulting from motor activity and/or sensory (e.g. olfactory, visual) and cognitive exploratory activities. To avoid these confounders, we used rats placed alone in an arena, which served as control animals for exploratory activity. This experimental design allows identifying brain areas that are mainly engaged by SI rather than motor activity, stress, or sensory and exploratory activities. In the animals undergoing arena exploration, PCP and URB597 had little or no effect on c-Fos expression in all the ROI investigated, which is consistent with the lack of effect of sub-chronic PCP treatment or URB597 on locomotion (Seillier and Giuffrida, 2009, Seillier et al., 2010, Enkel et al., 2013). When compared to rats undergoing arena exploration, saline-treated rats engaged in SI showed changes in c-Fos activation in brain areas that are part of the reward / motivational network (i.e. infralimbic cortex, orbitofrontal cortex, dorsomedial

CPu, VLS, posterior CeA, dorsolateral PAG) (Goodson, 2005, Goodson and Kabelik, 2009, O'Connell and Hofmann, 2011).

These observations differ from previous studies assessing brain activation following SI. For example, Salchner and colleagues reported that SI enhanced c-Fos expression in 28 out of 32 brain areas of young and aged rats (Salchner et al., 2004). The discrepancy between the limited brain activation observed in our study versus the broad activation reported by Salchner et al. may be attributable to several methodological differences. First, these authors compared c-Fos activation patterns between rats undergoing SI versus controls left in their home cages, rather than exploring an unfamiliar arena as in our experimental design (see above). Second, the SI test was performed under more stressful conditions (bright light, 150 lux) than our protocol (dim light, 5 lux), which might affect the overall experiment since stress recruits different brain circuits than those traditionally involved in social behavior (Matsuda et al., 1996). In this regard, it is noteworthy that we found no changes in saline-treated rats in brain areas known for their involvement in stress, such as the BNST, hypothalamus, BLA or prelimbic cortex (LeDoux, 2003, Choi et al., 2007, Herman et al., 2008, Walker et al., 2009, Itoi and Sugimoto, 2010, Orsini et al., 2011, Tan et al., 2011, Haufler et al., 2013).

Another study comparing c-Fos expression in rats undergoing SI versus exploring a familiar arena (controls) found no differences in 39 ROI investigated (Thompson et al., 2009). However, the study of Thompson et al. was designed to assess the pro-social effects of 3,4-methylenedioxy-methamphetamine (MDMA) and the baseline SI observed in their control animals was very low and similar to the baseline reported in our socially withdrawn animals (*i.e.* after subchronic PCP). Thus, it is plausible that such low sociability may have impacted the c-Fos readout.

Studies on social behavior, such as social defeat and bonding in prairie voles, or play in juvenile rats, postulate the existence of a social behavior network consisting of reciprocal connections between VLS, extended medial amygdala (*i.e.*, central and medial amygdala and BNST), preoptic area, anterior hypothalamus, ventromedial hypothalamus, and PAG-central gray (Young et al., 2008, O'Connell and Hofmann, 2011, Young et al., 2011). In particular, PFC, VLS, nucleus accumbens, medial amygdala, and VTA have been generally involved in mediating SI (Salchner et al., 2004, Young et al., 2008, O'Connell and Hofmann, 2011, Young et al., 2011, Varlinskaya et al., 2013). Our data do show changes in activation of the amygdala, VLS and PAG following SI, but do not match perfectly the aforementioned circuitry, as we did not find any change in c-Fos expression in the PFC, anterior cingulate, medial amygdala, or hypothalamus. These discrepancies may be due to the fact that SI in adult male rats engage different brain areas than social behaviors relevant to aggression, bonding or play.

4.2. Neuronal correlates of social withdrawal induced by PCP treatment and FAAH inhibition

Sub-chronic PCP treatment and acute FAAH inhibition by URB597 in saline-treated rats produced social withdrawal. As previously shown by our group, URB597 produced divergent effects on SI, as it increased the time spent in SI in PCP-treated rats, but induced

social withdrawal in saline-treated rats (Seillier et al., 2010, Seillier et al., 2013). Interestingly, the brain areas normally engaged by SI in saline-treated rats were not activated in these two experimental groups. Specifically, PCP treatment or acute URB597 administration prevented the SI-induced c-Fos expression in the infralimbic and orbitofrontal cortices and in the dorsomedial CPu observed in saline-treated rats. An opposite pattern was found in the CeA, where both PCP and URB597 prevented the decrease in c-Fos density seen in saline-treated rats undergoing SI. Overall, the level of SI observed in PCP- and URB597-treated rats paralleled the SI-induced changes of c-Fos activation in the orbitofrontal cortex and the CeA, hence suggesting their involvement in social behavior. In addition, following SI, sub-chronic PCP treatment induced c-Fos expression in the dorsomedial BNST, a brain area, which was not engaged in saline-treated rats. Optogenetic and neuroimaging studies have shown that the BNST is an important relay to the CeA and OFC through the nucleus accumbens, and that it plays a central role in anxiety and dysfunctional reward (Jennings et al., 2013, Lalumière, 2014, Kruger et al., 2015). Thus, the increased c-Fos expression observed in the CeA of PCP-treated rats following SI may result from alterations of neuronal activity in the BNST induced by PCP administration.

The infralimbic cortex, the orbitofrontal cortex and the dorsomedial CPu are key structures for decision-making, motivation, learning and habit formation (Bechara et al., 2000, Quirk and Beer, 2006, Yin et al., 2006, Balleine et al., 2007, Balleine et al., 2011, Schoenbaum et al., 2011). Although PCP-treated rats display social cognition deficits (such as deficient social novelty preference), these deficits are unlikely to contribute to social withdrawal (Seillier and Giuffrida, 2016). Consequently, PCP-induced social withdrawal may reflect lack of motivation to interact with conspecifics (*i.e.* social anhedonia). In agreement with the latter hypothesis, we found that PCP treatment reduces c-Fos expression in the VTA, a brain area known for its role in reward and motivation. The VTA has bidirectional connections with the BNST (Jennings et al., 2013). Activation of BNST glutamatergic projections has been shown to promote anxiety, while activation of its GABAergic projections has anxiolytic effects, presumably by disinhibiting the VTA (Jennings et al., 2013). Since c-Fos expression in the VTA is decreased in PCP-treated rats, we could speculate that this treatment leads to activation of BNST GABAergic afferents to the VTA. This is in agreement with the observation that anxiety does not contribute to social withdrawal in our model, as PCP-treated rats are less anxious than control animals in the elevated plus maze test (Seillier and Giuffrida, 2011) and open field test (Seillier and Giuffrida, 2012). However, we did not observe changes in c-Fos density in the nucleus accumbens, the dopaminergic output of the VTA (Lisman and Grace, 2005), nor in the dorsolateral PAG, another brain area involved in reward and motivational processes (Goodson, 2005, Goodson and Kabelik, 2009, O'Connell and Hofmann, 2011).

On the other hand, the changes in c-Fos expression observed in the CeA, BNST and orbitofrontal cortex of PCP-treated rats may reflect alterations of other dimensions of social behavior regulated by these brain areas (Bamshad et al., 1997, Jasnow et al., 2004, Northcutt and Lonstein, 2009, Bosch and Neumann, 2010, Bychowski et al., 2013, Lukas et al., 2013).

4.3. Neuronal correlates of the beneficial effect of FAAH inhibition on SI in PCP-treated rats

Our data highlight three brain areas that might play a critical role in URB597-induced reversal of social withdrawal in PCP-treated rats: the orbitofrontal cortex, the posterior CeA, and the dorsomedial BNST. The first two areas have reciprocal c-Fos activation patterns that parallel the behavior observed in saline- and PCP-treated rats (increased and decreased SI, respectively; Fig. 5 and Table 1). In the dorsomedial BNST, URB597 prevented the abnormal neuronal activity observed in PCP-treated rats undergoing SI (Fig. 4 and Table 1).

The orbitofrontal cortex and the posterior CeA are strongly connected. Although the orbitofrontal cortex activates the CeA through a direct pathway (Cavada et al., 2000, Barbas et al., 2003), an indirect pathway inactivates the same area by sending excitatory efferents to GABAergic interneurons in the intercalated nuclei (Royer et al., 1999, Ghashghaei and Barbas, 2002), which exert inhibitory influence on the CeA output to autonomic nuclei (Royer et al., 1999, Saha et al., 2000, Barbas et al., 2003, Rempel-Clower, 2007). Based on the c-Fos expression patterns observed in our study, we hypothesize that SI requires activation of the orbitofrontal cortex and of the inhibitory pathway to the CeA. Such circuitry could involve excitatory cortical projections to the lateral / capsular part of the CeA, where oxytocin receptor activation has been shown to activate a CeA inhibitory circuitry and to facilitate social behavior (Huber et al., 2005). Accordingly, previous observations carried out in the PCP model showed that oxytocin injection in the CeA can reverse PCP-induced social deficits (Lee et al., 2005), which suggests a critical role of the CeA in PCP-induced SI deficits. Thus, lack of activation in the orbitofrontal cortex may result in disinhibition of the CeA and deficient social behavior. These pathways likely involve endocannabinoid neurotransmission since CB1 receptor expression is present in these brain areas (Herkenham et al., 1990, Herkenham et al., 1991, Herkenham, 1992, Mailleux and Vanderhaeghen, 1992, Tsou et al., 1998, Hajos and Freund, 2002), and, as previously mentioned, URB597 administration disrupts or restores SI-induced orbitofrontal activation and CeA inactivation in saline- and PCP-treated rats, respectively.

The paradoxical effects of URB597 on SI in PCP- and saline-treated rats have been attributed to URB597-induced elevation of AEA levels in the brain, which compensates for the deficient endocannabinoid mobilization and CB1 activation reported in PCP-treated rats, but produces supra-physiological AEA concentrations in saline-treated animals leading to activation of a TRPV1-like receptor (Seillier et al., 2013). It is not known whether these differences in AEA levels are due to a differential recruitment of AEA production enzymes in PCP- and saline-treated rats. Since AEA production is activity-dependent, differences in neuronal activity in specific brain areas (as assessed by c-Fos expression) may explain regional differences in AEA production. However, the increased (or decreased) c-Fos expression in a specific area following URB597 administration does not necessarily reflect parallel changes in AEA tone, and further investigations are necessary to determine whether the observed changes in c-Fos activation patterns result from alterations of endocannabinoid signaling in the brain areas identified in our study.

Finally, the BNST is an important output of the CeA, from which it primarily receives inhibitory GABAergic afferents (Dong et al., 2001, Jankord and Herman, 2008). As such, the lack of inhibition of the CeA during SI in PCP-treated rats could drive the disinhibition

of the BNST. Interestingly, the infralimbic cortex also exerts inhibitory influences on this area (Hurley et al., 1991, Vertes, 2004, Jalabert et al., 2009) as well as on the CeA (Quirk and Beer, 2006, Quirk et al., 2006). Consequently, the lack of activation observed in the infralimbic cortex of PCP-treated rats undergoing SI may also contribute to the disinhibition observed in these two structures. On the other hand, URB597 administration may reverse social deficits in PCP-treated rats by increasing endocannabinoid mobilization and normalizing CB1 stimulation within this circuitry. It is noteworthy that URB597 does not induce c-Fos expression changes in the BNST of saline-treated rats, which indicate that this brain area may not be involved in URB597 deleterious effect on SI.

Social withdrawal has been shown to correlate with increased c-Fos expression in the CeA and BNST (Marvel et al., 2004). In addition, inhibition of the dorsal vagal complex normalized c-Fos expression in these areas and reversed LPS-induced social withdrawal (Marvel et al., 2004). These findings parallel the increased c-Fos expression observed in the CeA and BNST of PCP-treated rats (which is correlated with social withdrawal), as well as the normalization of c-Fos expression in these areas after acute URB597 administration (which also normalized normalization of social behavior) (Seillier et al., 2013). Together, these findings strengthen the hypothesis of a link between modulation of neuronal activity in these key brain areas and social withdrawal.

Overall, we can hypothesize that PCP-treatment disrupts a social interaction circuitry that primarily includes the orbitofrontal cortex, CeA and BNST, and that is modulated by few specific regions, such as the infralimbic cortex. The proper functioning of this circuitry may require appropriate endocannabinoid tone, as URB597 reverses social deficits in PCP-treated rats by normalizing CB1 stimulation in this circuitry.

5. CONCLUSIONS

The present data highlight a restricted number of brain regions that may be involved in SI and social withdrawal (*i.e.* infralimbic and orbitofrontal cortices, dorsomedial CPu, VLS, CeA, dorsal BNST, and dorsolateral PAG). Among these brain areas, the orbitofrontal cortex, CeA and dorsal BNST might be part of a circuit that mediates the endocannabinoid-induced reversal of social withdrawal in PCP rats. Several studies have shown alterations of the orbitofrontal cortex in schizophrenic patients (Hoptman et al., 2005, Larquet et al., 2010, Jackowski et al., 2012, Nenadic et al., 2014, Strauss et al., 2014) and have linked such dysfunction to negative symptoms (Sanfilipo et al., 2000, Gur et al., 2004, Lacerda et al., 2007, Kerns et al., 2008, Stip et al., 2009). Thus, our results suggest that modulation of endocannabinoid tone in the orbitofrontal cortex could be a promising approach to treat behavioral deficits relative to the negative symptoms of schizophrenia.

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Abbreviations

AEA	anandamide
BNST	bed nucleus of the stria terminalis
BLA	basolateral amygdala
CeA	central amygdala
CPu	caudate putamen
FAAH	fatty acid amide hydrolase
MDT	mediodorsal thalamus
PAG	periaqueductal gray
PCP	phencyclidine
ROI	region of interest
SI	social interaction
VLS	ventrolateral septum

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Highlights

1. URB597 restored SI in PCP rats but induced social withdrawal in control animals
2. Neuronal activity changes in IL, OFC, VLS, dmCPu, and CeA paralleled SI in controls
3. PCP treatment affected neuronal activity in IL, OFC, VLS, dmCPu, CeA, and BNST
4. URB597 affected neuronal activity in IL, OFC, VLS, dmCPu, CeA, and Cg2 of controls
5. URB597 normalized neuronal activity in the OFC, CeA, BNST, and Cg2 of PCP rats

Figure 1.

Treatment and experiment schedule. Animals were sub-chronically treated with either saline (1 mL/kg, i.p.) or PCP (5 mg/kg, i.p.) twice a day for 7 days followed by a 7-day washout period before behavioral assessment (on day 14). Sixty minutes before the task, animals received an acute injection of URB597 (0.3 mg/kg, i.p.) or vehicle (1 mL/kg, i.p.). Social interaction was assessed over a sixty minutes period of time. Rats placed in the arena alone were used as a control (exploration). Immediately after the behavioral assessment, rats were anesthetized and sacrificed fifteen minutes later to process their brains for c-Fos immunohistochemistry.

Figure 2.

Schematic diagrams showing the brain regions of interest (ROI). Diagrams are adapted from Paxinos and Watson's Rat Brain Atlas (1998). The grey shaded areas indicate the delineation of the areas in which c-Fos nuclei were counted. Abbreviations: IL infralimbic cortex; PrL prelimbic cortex; Cg1 cingulate cortex 1; OFC orbitofrontal cortex; Cg2 cingulate cortex 2; dlCPu dorsolateral CPu; dmCPu dorsomedial CPu; VLS, ventrolateral septum; AcbSh nucleus accumbens shell; AcbC nucleus accumbens core; dmBNST dorsomedial bed nucleus of the stria terminalis; vmBNST ventromedial bed nucleus of the stria terminalis; PVN paraventricular nucleus of the hypothalamus; AH anterior hypothalamic area; aCeA anterior central nucleus of the amygdala; pCeA posterior central nucleus of the amygdala; MeA medial nucleus of the amygdala; BLA basolateral nucleus of the amygdala; CA1 cornu ammonis 1 of the hippocampus; CA3 cornu ammonis 3 of the hippocampus; DG dentate gyrus of the hippocampus; MDT mediodorsal thalamus; vIENT ventrolateral entorhinal cortex; cmENT centromedial entorhinal cortex; VTA ventral tegmental area; SN substantia nigra; dIPAG dorsolateral periaqueductal gray; vIPAG ventrolateral periaqueductal gray.

Figure 3.

Effect of URB597 on social interaction in saline- and PCP-treated rats. Values are expressed as mean $\pm$ S.E.M. Two-way ANOVA followed by a Student Newman–Keuls post hoc test, $n=8$ for each experimental group, * $p<0.05$, saline vs. PCP groups; # $p<0.05$, vehicle vs. URB597 groups.

Figure 4.

Effect of systemic URB597 on c-Fos density after social interaction in saline- and PCP-treated rats. Values are mean $\pm$ SEM (number of c-Fos-IR nuclei/mm²). Three-way ANOVA followed by a Student Newman–Keuls post hoc test. $n=6-12$ slices per brain region, $n=4$ animals per group. * $p<0.05$, vs. saline-vehicle group (arena exploration only), # $p<0.05$, vs. saline-vehicle group (social interaction). Abbreviations: IL infralimbic cortex; Cg2 cingulate cortex 2; dmCPu dorsomedial CPu; VLS, ventrolateral septum; dmBNST dorsomedial bed nucleus of the stria terminalis; aCeA anterior central nucleus of the amygdala; BLA basolateral nucleus of the amygdala; VTA ventral tegmental area; dlPAG dorsolateral periaqueductal gray.

Figure 5.

Effect of systemic URB597 (b, d, f, h) on c-Fos nuclei densities in the OFC (A) and the posterior CeA (B) after arena exploration (a-d) and social interaction (e-h) in saline- (a-b, e-f) and PCP- (c-d, g-h) treated rats. Diagrams are adapted from Paxinos and Watson's Rat Brain Atlas (1998). Shaded areas delineate the brain regions where c-Fos nuclei were counted. Insets show representative photomicrographs of c-Fos-IR in ROI. Scale bar: 100µm. Values are mean $\pm$ SEM (number of c-Fos-IR nuclei/mm²). Three-way ANOVA followed by Student Newman-Keuls post hoc test (n=6-12 slices per brain region; n=4 animals per group). * $p < 0.05$, vs. saline-arena exploration-vehicle group, # $p < 0.05$, vs. saline-social interaction-vehicle group. Abbreviations: OFC orbitofrontal cortex; pCeA posterior central nucleus of the amygdala.

Table 1

Effect of systemic FAAH inhibition on the density of c-Fos-IR nuclei in different brain regions after arena exploration or social interaction in saline- and PCP-treated rats.

	ARENA EXPLORATION				SOCIAL INTERACTION			
	Vehicle		URB597		Vehicle		URB597	
Brain region	Saline	PCP	Saline	PCP	Saline	PCP	Saline	PCP
<i>Bregma +3.2mm</i>								
PrL	98 ± 11	111 ± 22	100 ± 9	79 ± 10	128 ± 18	107 ± 14	110 ± 8	104 ± 31
Cg1	78 ± 16	74 ± 21	49 ± 12	42 ± 7	90 ± 8	71 ± 10	71 ± 8	84 ± 22
IL	96 ± 10	106 ± 17	104 ± 4	95 ± 7 [#]	160 ± 8 [*]	107 ± 4 [#]	109 ± 20 [#]	114 ± 21 [#]
OFC	108 ± 8	103 ± 11	110 ± 2	109 ± 5	172 ± 18 [*]	134 ± 13 [#]	129 ± 12 [#]	175 ± 17 [*]
<i>Bregma +1.6mm</i>								
Cg2	159 ± 14	146 ± 11	133 ± 10 [#]	121 ± 8	176 ± 20	138 ± 4	116 ± 8 [#]	105 ± 15 [#]
dmCPu	78 ± 11 [#]	83 ± 3 [#]	81 ± 16 [#]	85 ± 13 [#]	152 ± 12 [*]	109 ± 18 [#]	81 ± 16 [#]	79 ± 10 [#]
dlCPu	71 ± 12	79 ± 8	79 ± 22	77 ± 11	96 ± 10	75 ± 19	72 ± 11	72 ± 9
AcbSh	167 ± 21	153 ± 11	127 ± 15	108 ± 5	166 ± 17	137 ± 24	146 ± 9	138 ± 16
AcbC	69 ± 17	72 ± 5	67 ± 13	54 ± 12	80 ± 13	72 ± 9	73 ± 8	73 ± 8
VLS	239 ± 36	286 ± 25	234 ± 31 [#]	283 ± 18	362 ± 37 [*]	267 ± 25	315 ± 15	229 ± 19 [#]
<i>Bregma -0.3mm</i>								
dmBNST	112 ± 19	116 ± 8	103 ± 21	104 ± 26	100 ± 21 ^{*#}	204 ± 17	106 ± 14	109 ± 17
vmBNST	155 ± 27	160 ± 28	120 ± 2	119 ± 17	169 ± 24	194 ± 15	151 ± 21	136 ± 18
<i>Bregma -1.8mm</i>								
PVN	137 ± 30	160 ± 30	156 ± 5	141 ± 31	196 ± 2	189 ± 18	185 ± 34	143 ± 11
AH	187 ± 3	197 ± 17	168 ± 22	167 ± 8	174 ± 9	185 ± 11	177 ± 11	203 ± 4
aCeA	159 ± 33	146 ± 16	145 ± 10	142 ± 16	115 ± 7 [#]	205 ± 18	165 ± 10	150 ± 8
<i>Bregma -2.8mm</i>								
CA1	82 ± 12	84 ± 11	76 ± 7	74 ± 7	80 ± 14	83 ± 1	84 ± 10	58 ± 18
CA3	36 ± 3	38 ± 4	26 ± 5	31 ± 5	27 ± 4	32 ± 5	40 ± 7	21 ± 6
DG	66 ± 11	63 ± 4	65 ± 10	48 ± 8	66 ± 3	80 ± 5	75 ± 10	55 ± 11
MDT	245 ± 38	248 ± 32	279 ± 20	180 ± 53	178 ± 27	226 ± 19	221 ± 21	163 ± 17
BLA	128 ± 15	97 ± 18	136 ± 15	118 ± 25	148 ± 19	133 ± 12	141 ± 12	104 ± 14
pCeA	136 ± 13	149 ± 8	138 ± 10	126 ± 12	98 ± 6 [§]	153 ± 11 [#]	150 ± 15 [#]	97 ± 6 [*]
MeA	158 ± 23	130 ± 38	138 ± 36	149 ± 31	144 ± 13	159 ± 13	154 ± 17	149 ± 19
<i>Bregma -5.2mm</i>								
VTA	218 ± 36	198 ± 13	271 ± 25	230 ± 30	264 ± 33	223 ± 4	284 ± 6	240 ± 24
SN	106 ± 10	99 ± 16	131 ± 17	95 ± 4	130 ± 11	100 ± 9	91 ± 13	95 ± 8
vlENT	159 ± 6	106 ± 15	158 ± 22	196 ± 37	204 ± 39	204 ± 40	216 ± 49	120 ± 13
<i>Bregma -7.8mm</i>								

	ARENA EXPLORATION				SOCIAL INTERACTION			
	Vehicle		URB597		Vehicle		URB597	
Brain region	Saline	PCP	Saline	PCP	Saline	PCP	Saline	PCP
IENT	200 ± 8	207 ± 30	156 ± 3	160 ± 12	151 ± nd	217 ± nd	261 ± nd	233 ± 26
dIPAG	216 ± 26	212 ± 22	247 ± 21	216 ± 29	309 ± 17	290 ± 44	311 ± 17	291 ± 17
vIPAG	273 ± 35	265 ± 19	305 ± 25	263 ± 41	272 ± 2	261 ± 49	305 ± 41	319 ± 53

Values are mean ± SEM (number of c-Fos-IR nuclei/mm²). n=6-12 slices per brain region, n=4 animals per group.

* p<0.05, vs. saline-vehicle group (arena exploration only),

p<0.05, vs. saline-vehicle group (social interaction),

\$ p=0.051.

nd, not detectable due to technical difficulties.

Abbreviations: PrL prelimbic cortex; Cg1 cingular cortex 1; IL, infralimbic cortex; OFC, orbitofrontal cortex; Cg2 cingular cortex 2; dmCPu dorsomedial caudate putamen; dlCPu dorsolateral caudate putamen; AcbSh nucleus accumbens shell; AcbC nucleus accumbens core; VLS, ventrolateral septum; dmBNST dorsomedial stria terminalis; vmBNST ventromedial stria terminalis; PVN paraventricular nucleus of the hypothalamus; AH anterior hypothalamic nucleus; aCeA, anterior nucleus of the central amygdala; CA1 cornu ammonis 1 of the hippocampus; CA3 cornu ammonis 3 of the hippocampus; DG dentate gyrus of the hippocampus; MDT mediodorsal thalamus; BLA, basolateral amygdala; pCeA, posterior nucleus of the central amygdala; MeA medial nucleus of the amygdala; VTA, ventral tegmental area; SN substantia nigra; vIENT ventrolateral entorhinal cortex; IENT lateral entorhinal cortex; dIPAG dorsolateral periacqueductal gray; vIPAG ventrolateral periacqueductal gray.