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Title: PCP-based mice models of schizophrenia: differential behavioral, neurochemical and cellular effects of acute and subchronic treatments

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Abstract

Rational: N-methyl-D-aspartate receptor (NMDA-R) hypofunction has been proposed to account for the pathophysiology of schizophrenia. Thus, NMDA-R blockade has been used to model schizophrenia in experimental animals. Acute and repeated treatments have been successfully tested; however, long-term exposure to NMDA-R antagonists more likely resembles the core symptoms of the illness.

Objectives: to explore whether schizophrenia-related behaviors are differentially induced by acute and subchronic PCP treatment in mice, and to examine the neurobiological bases of these differences.

Results: Subchronic PCP induced a sensitization of acute locomotor effects. Spontaneous alternation in a T-maze and novel object recognition performance were impaired after subchronic but not acute PCP, suggesting a deficit in working memory. On the contrary, reversal learning and immobility in the tail suspension test were unaffected. Subchronic PCP significantly reduced basal dopamine but not serotonin output in mPFC, and markedly decreased the expression of tyrosine hydroxylase in the ventral tegmental area. Finally, acute and subchronic PCP treatments evoked a different pattern of *c-fos* expression. At 1h post-treatment, acute PCP increased *c-fos* expression in many cortical regions, striatum, thalamus, hippocampus and dorsal raphe. However, the increased *c-fos* expression produced by subchronic PCP was restricted to the retrosplenial cortex, thalamus, hippocampus and supramammillary nucleus. Four days after the last PCP injection, *c-fos* expression was still increased in the hippocampus of subchronic PCP-treated mice.

Conclusions: Acute and subchronic PCP administration differently affects neuronal activity in brain regions relevant to schizophrenia, which could account for their different behavioral effects.

Keywords: behavior, c-fos, dopamine, phencyclidine, glutamate, NMDA, serotonin, schizophrenia, reversal learning, working memory.

Introduction

Compelling evidence supports a deficient glutamate-mediated excitatory neurotransmission through NMDA glutamate receptors (NMDA-R) in schizophrenia (Coyle et al. 2003; Coyle 2006; Moghaddam and Javitt 2012). NMDA-R hypofunction hypothesis stemmed from the clinical observation that sub-anesthetic doses of non-competitive NMDA-R antagonists such as phencyclidine (PCP) and ketamine induced psychotic reactions in healthy individuals and exacerbated the symptomatology in schizophrenic patients (Coyle, 2006; Javitt and Zukin 1991; Krystal et al. 1994; Luby et al. 1959). Thereafter, NMDA-R blockade in rodents was proposed as an animal model of the disease (Kantrowitz and Javitt 2010; Krystal et al. 2003, Neill et al. 2010; Jones et al. 2011). Rodent models of schizophrenia are very useful in preclinical neuroscience research and in antipsychotic drug development. However, they have obvious limitations due to the unknown etiology of the disease, the high inter-individual symptom variability and the inability to reproduce subjective symptoms of the illness. Therefore, current animal models of schizophrenia are intended to assess specific endophenotypes instead of modeling the disease as a whole (Amann et al. 2010; Kaffman and Krystal 2012; Robbins 2012).The identification of rodent endophenotypes represents a critical step to elucidate the neurobiological basis of the deficits and the development of new treatment strategies.

Non-competitive NMDA-R antagonists such as PCP, ketamine and dizolcipine (MK-801) have been very effective in triggering schizophrenia-like positive symptoms, negative symptoms and cognitive deficits in rodents (Bondi et al. 2012; Frohlich and Van Horn 2014; Neill et al. 2010). However, behavioral manifestations induced by NMDA-R antagonists seem particularly dependent on pharmacological and procedural features, which difficult the clarification of their neural underlying mechanisms. Hence, there is evidence for drug specific disturbances (Dix et al, 2010; Seillier and Giuffrida 2009). As an example, withdrawal from subchronic PCP but not MK-801 caused a delay-dependent impairment of working memory, reduced social interaction and

enhanced d-amphetamine-induced motor activity (Seillier and Giuffrida 2009), suggesting that other mechanisms apart from NMDA-R blockade may contribute to behavioral effects of NMDA-R antagonists.

Once restricted to a certain drug, other factors such as the age of the subjects, the strain, the dose, and the duration of the treatments do also account. Age appears to be a key factor, since differential behavioral effects have been observed after NMDA-R antagonist administration to young or adult subjects, possibly due to differential brain NMDA-R subunit composition along the lifetime and the distinct biophysical, pharmacological and signaling properties of each receptor subtype (Paoletti et al. 2013). Strain differences have also been described for PCP-induced hyperlocomotion and immobility responses in the forced swim test (Mouri et al. 2012; Van den Buuse 2010). Moreover, the effects of non-competitive NMDA-R antagonists are clearly dose-dependent (Hiyoshi et al. 2014; Van den Buuse 2010). Finally, treatment duration also impacts on the behavioral phenotype caused by NMDA-R blockade, and several studies suggested that chronic treatments produce more robust and long-lasting effects than acute drug treatments (Egerton et al. 2008; Jentsch and Roth 1999; Neill et al. 2014; Spielewoy and Markou 2003; Thomson et al. 2011). In this context of high pharmacological and methodological variability using NMDA-R antagonists, standardization studies are a pre-requisite to further explore the cellular, and network elements involved in their behavioral effects. The aim of the present study was to evaluate the possible differential behavioral effects of acute and subchronic PCP treatment in adult C57BL6/J mice, to further explore their underlying neurobiological bases using neurochemical and histological techniques.

Materials and methods

Subjects

We used 8-12 week-old male C57BL/6J mice (Charles River, France). Animals were group-housed five per cage and kept in a controlled environment (12 h light–dark cycle and 22±2°C room temperature) with food and water provided *ad-libitum*. Mice

underwent the reversal learning task were individually housed and food-restricted as described below. Animal experiments followed the Principles of laboratory animal care and the Guidelines for care and use of mammals in neuroscience and behavioral Research (National Research Council 2003). All experimental procedures were in strict accordance with European Union regulations (2010/63 UE, 22nd September 2010), and were approved by the Institutional Animal Care and Use Committee.

Drugs and treatments

Phencyclidine hydrochloride (Sigma-Aldrich, UK) was dissolved in physiological saline, pH adjusted to 6.5-7.0 and administered by subcutaneous route (s.c.). The volume of injection was 10 ml/kg. Doses are expressed as free base.

Mice were administered PCP or saline for 10 days (once daily on days 1–5, 8–12) as previously described Hashimoto et al. (2005). This treatment regime has been previously shown to induce long-lasting memory deficits in the NOR in mice, that are reversed by antipsychotic drugs (Tanibuchi et al. 2009). Three experimental groups were used: subchronic, acute and control. Mice on the subchronic group received PCP (10 mg/kg, s.c., 10 days). Mice in the acute group received saline (10 ml/kg, s.c., 9 days) and PCP on the last day of treatment (10 mg/kg, s.c., day 12). Control mice received saline (10 ml/kg, s.c., 10 days).

All efforts were made to ensure that the experimenter was blind to treatment conditions during scoring of the behavioral tests.

Locomotor activity measurements

Distance moved (cm) in an open-field was used as a measure of locomotor activity. The open-field (30 x 30 cm) was made of black plastic and dimly illuminated (30-40 lux). Locomotor activity measurements were performed on days 1 and 12, and started 10 min after drug administration. Data were collected in 5-min bins by the videotrack system software (Viewpoint, France) during 90 min.

Spontaneous alternation and Novel object recognition in a T-maze

On withdrawal day 4 (WD4), mice were habituated for 10 min to the T-maze (Panlab, Barcelona) and spontaneous alternation was measured. The spontaneous alternation task is used to assess spatial working memory in rodents and is based on the innate tendency of rodents to explore a prior unexplored arm of a maze. Alternation was defined as successive entries into the three arms of the maze, on the overlapping triplet sets. The percentage alternation was calculated as the ratio of actual to possible alternations as previously reported (Hiramatsu et al. 1997). Twenty-four hours later (withdrawal day 5, WD5), mice were put back in the maze for 10 min where two identical objects were presented (T1), one in each lateral arm end, and the time the animals spent exploring each object was recorded. Three min later (T2), mice were put again in the maze for 10 min, where one of the familiar objects was replaced by a novel object (in a counterbalanced way), and the total time spent exploring each of the two objects (novel and familiar) was computed. Object exploration was defined as the orientation of the nose to the object at a distance < 1 cm. A recognition index (RI) was calculated as the percentage of the time exploring the novel object divided by the total time exploring the two objects (novel and familiar) as described [RI = N/(F+N) $*$ 100]. RI > 50 % is considered to reflect greater memory retention for the familiar object.

Tail suspension test

On withdrawal day 7 (WD7) mice were tested in the tail suspension test (TST). TST was conducted essentially as described by Steru et al. (1985). Briefly, mice were suspended 30 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail and were observed on a monitor through a video camera system (Smart, Panlab). The time mice spent immobile (s) was recorded during 6 min.

Reversal learning paradigm

A different set of animals was used for the reversal learning testing. Prior to the beginning of training, mice were handled daily for three days, individually housed and put on a food-restriction schedule (to attain an 80% of free-feeding body weight). Water was available *ad-libitum*. The procedure was conducted in a T-maze (Panlab,

Barcelona) and consisted of different phases (see a schematic representation of the training and testing protocol in the Results section). *Pre-training:* all mice were initially familiarized with the testing apparatus during a 10-min day session. The next day, mice were adapted to consume food rewards (Grain-based rodent tablets 5TUM, TestDiet, Zurich) in the maze. Initially, 10 food pellets were placed in the maze and mice were trained in 10-min day sessions till criterion of ≥ 8 pellets eaten. Once criterion achieved, the food pellets were placed at the end of the goal-arms and the same criterion was applied. *Discrimination*: after habituation, mice were trained to turn right or left in the T-maze in order to get a food reward. Animals received one 10-trials session per day and were trained to a criterion of ≥ 8 correct choices out of 10 in two consecutive days. Once the criterion on discrimination was reached, the pharmacological treatment started. *Treatment*: during the treatment with PCP (subchronic: 10 mg/kg, s.c., 10 days; acute: 10 ml/kg, s.c., 9 days plus 10 mg/kg, s.c., on day 12) or saline (10 ml/kg, s.c., 10 days) mice were individually housed but no food-restriction was applied, thus they had free access to food and water *ad-libitum*. *Re-discrimination:* on withdrawal day 1 (WD1), mice were put back to food-restriction conditions. Three days later, (WD4) re-training on discrimination was performed with the same criteria as before. *Reversal:* in this phase, the rewarded egocentric response was reversed and mice received the food-pellet after turning on the opposite direction as in discrimination and re-discrimination training. Animals received one 10-trials session per day and were trained to a criterion of ≥ 8 correct choices out of 10 in two consecutive days. Note that discrimination, re-discrimination and reversal phases were performed in a drug-free state. The main measures of the animals' ability to learn the discriminations/reversals were: (i) the number of total trials (TT) to criterion, and (ii) the number of incorrect trials (IT, errors) to criterion.

Microdialysis experiments

The effects of acute and subchronic PCP treatments on the extracellular serotonin (5-HT) and dopamine (DA) levels in the medial PFC (mPFC) were measured by *in vivo*

microdialysis as previously described (Castañé et al. 2008). On day 11 of treatment, one concentric dialysis probe equipped with a Cuprophan membrane (2-mm long) was implanted in the mPFC at coordinates (in mm): AP+2.2; ML-0.2; DV-3.4 (Franklin and Paxinos, 1997), of anaesthetized mice (inhaled isofluorane carried in medical oxygen induced at 2.5% and maintained at 1.5 % concentrations at a rate of 0.6-0.7 L/min). Microdialysis experiments were performed 20-24 h after surgery on day 12 (the last day of treatment). For serotonin determinations, the aCSF was pumped at 2.0 µl/min (WPI model sp220i) and dialysates were collected every 30 min. Serotonin concentration in dialyzed samples was analyzed by HPLC with amperometric detection (Hewlett Packard 1049) at +0.6 V. For dopamine determinations, the aCSF containing 10 µM nomifensine was pumped at 1.65 µl/min (WPI model sp220i) and dialysates were collected every 20 min. In this case, brain dialysates were collected on micro vials containing 5 µl of 10 mM perchloric acid and dopamine concentration in dialyzed samples was analyzed by HPLC with amperometric detection (Hewlett Packard 1049) at +0.6 V. Following an initial 100-min stabilization period, four baseline samples were collected before the systemic drug administration, and then, successive dialysate samples were collected. Baseline serotonin and dopamine levels were calculated as the average of the four pre-drug samples. At the end of sample collection, brains were removed and sectioned to ensure proper probe placement after cresyl violet staining.

In-situ hybridization and immunohistochemistry studies

Immediate and late effects of PCP treatments on *c-fos* and tyrosine hydroxylase gene expression were examined by *in-situ* hybridization. Mice were sacrificed by cervical dislocation 1h or 96h (WD4) after the end of treatments. Animals processed at 96h received a saline injection 1h before the sacrifice. The brains were rapidly removed, frozen on dry ice, and stored at –30 ºC. Brain tissue sections, 14 mm thick, were cut using a microtome-cryostat (HM500 OM; Microm, Walldorf, Germany), thaw-mounted onto APTS (3-aminopropyltriethoxysilane, Sigma, St Louis, MO, USA)-coated slides and kept at – 30 ºC. *c-fos* oligonucleotide probe was complementary to bases 131-178

(GenBank ID:NM_022197). Tyrosine hydroxylase oligonucleotide probe was complementary to bases 1435-1474 (GenBank ID:NM_009377). Labeling of the probes ([^{33}P]-dATP (>2500 Ci/mmol); PerkinElmer NEN Radiochemicals, Boston, MA, USA), tissue sectioning and *in-situ* hybridization procedures were carried out as described (Kargieman et al. 2007; Santana et al. 2011). Hybridized sections were exposed to Biomax MR film (Kodak, Sigma-Aldrich, Madrid, Spain) for 7 days with intensifying screens. Relative optical densities (R.O.D.) were measured with the AIS computerized image analysis system (AIS, Imaging Research, St Catherines, Ontario, Canada). Individual values of optical densities were calculated as the mean of 2-4 sections per mice (n = 3 mice/group). *NeuN immunohistochemistry*: VTA neuronal integrity was evaluated by comparing number of VTA NeuN-positive cells in saline and subchronic PCP-treated animals sacrificed at 96h. 14μm-thick frozen brain tissue sections were processed for immunohistochemical visualization of NeuN by standard streptavidin-biotin-peroxidase methodology using Anti-NeuN antibody (MAB377, Millipore, Madrid, Spain).

Statistical analysis

Data from spontaneous alternation, novel object recognition, tail suspension test, reversal learning, basal 5-HT and DA concentrations, TH and *c-fos* expression, and NeuN inmunolocalization were analyzed by a one-way ANOVA (treatment as between factor of variation). Data from the locomotor activity and microdialysis experiments were analyzed by a two-way ANOVA with repeated measures (treatment as a between factor and time/fraction as within factor of variation). Significant main effects in the ANOVAs were further explored by *post-hoc* Newman-Keuls comparisons to establish simple effects. Data are given as mean ± SEM. Statistical significance was set at the 95% confidence level.

Results

Locomotor activity effects induced by acute and subchronic PCP treatments

Subchronic PCP administration induced a sensitization of acute locomotor effects (Fig.1). Two-way ANOVA with repeated measures on day 1 showed a significant main effect of time [$F_{(17, 272)} = 12.30$; $p < 0.001$], treatment [$F_{(2,16)} = 18.09$; $p < 0.001$] and interaction between time and treatment [$F_{(34, 272)} = 8.96$; $p < 0.001$]. Subsequent *post-hoc* Newman-Keuls comparisons showed a significant increase of the distance moved in the subchronic PCP group compared to the control group ($p < 0.01$). Two-way ANOVA with repeated measures on day 12 showed a significant main effect of time [$F_{(17, 323)} = 4.46$; $p<0.001$], treatment [$F_{(2,19)} = 26.28$; $p < 0.001$] and interaction between time and treatment [$F_{(34, 323)} = 3.41$; $p<0.001$]. P*ost-hoc* Newman-Keuls comparisons showed a significant increase of distance moved in the subchronic and acute groups compared to the control group ($p < 0.01$, $p < 0.05$ respectively) and a significant difference between the subchronic and acute groups ($p < 0.01$) (Fig.1a). Similarly, two-way ANOVA with repeated measures on the accumulated distance data showed a significant main effect of day [$F_{(1,16)} = 19.42$; $p < 0.001$], treatment [$F_{(2,16)} = 33.95$; $p<0.001$] and interaction between day and treatment [$F_{(2,16)} = 7.43$; $p < 0.01$]. *Post-hoc* Newman-Keuls comparisons showed a significant increase of total distance moved in the subchronic group compared to the control group ($p < 0.01$) on day 1, and a significant increase of total distance moved in the acute and subchronic groups compared to the control group ($p < 0.05$, $p < 0.01$, respectively) on day 12. Notably, on day 12 the total distance moved in the subchronic treated mice was significantly higher than in acute-treated mice ($p < 0.01$) (Fig.1b).

Effects of acute and subchronic PCP treatments on working memory

Subchronic but not acute PCP treatment induced working memory deficits in the spontaneous alternation (Fig.2a) and novel object recognition (Fig.2b) paradigms. One-way ANOVA for the percentage of alternations in a T-maze showed a significant effect of treatment [$F_{(2,18)} = 5.91$; $p < 0.05$]. *Post-hoc* Newman-Keuls comparisons showed a significant decrease of the percentage of alternations in the subchronic-treated mice compared to control ($p < 0.05$) and acute-treated ($p < 0.05$) mice (Fig.2a). One-way

ANOVA showed non-significant differences on the total number of arms visits in the three groups of mice (mean ± SEM (counts) were: control, 49 ± 5; acute, 50 ± 4; subchronic, 57 ± 4) [$F_{(2,20)=}$1.361; n.s.]. One-way ANOVA of the recognition index data showed a significant effect of treatment [$F_{(2,26)}$ = 8.26; $p < 0.01$]. *Post-hoc* Newman-Keuls comparisons showed a significant decrease of the recognition index in the subchronic-treated mice compared to control ($p < 0.01$) and acute-treated ($p < 0.01$) mice (Fig.2b). The three groups of mice spent the same time exploring the objects on T1 and T2. Thus, one-way ANOVA for T1 data showed non-significant differences on the total time of exploration (mean ± SEM (s) were: control, 31 ± 3; acute, 38 ± 5; subchronic, 35 ± 6) [$F_{(2,28)}$=0.530 ; n.s]. One-way ANOVA for T2 data showed non-significant differences on the total time of exploration (mean ± SEM (s) were: control, 33 ± 4; acute, 39 ± 5; subchronic, 34 ± 4) [$F_{(2,28)}$=0.596 ; n.s].

Effects of acute and subchronic PCP treatments in the tail suspension test

Acute and subchronic PCP-treated mice showed higher immobility times than control mice in the tail suspension test. However, immobility differences among groups did not reach statistical significance ([$F_{(2,28)}$ = 2.61; n.s.], n.s., one-way ANOVA) (Fig. 3).

Effects of acute and subchronic PCP treatments on the reversal of an egocentric response discrimination

Before treatment, the three groups of mice did not differ in the total number of trials [$F_{(2,26)}$ = 0.54; n.s.] and incorrect trials [$F_{(2,26)}$ = 0.47; n.s.] to reach performance criterion on an egocentric response discrimination (Fig.4b). On the re-discrimination phase, one-way ANOVA showed a significant effect of treatment on the total number of trials [$F_{(2,26)}$ = 4.58; $p < 0.05$] and incorrect trials [$F_{(2,26)}$ = 4.14; $p < 0.05$] to reach performance criterion. *Post-hoc* Newman-Keuls comparisons showed a significant difference between acute and control/subchronic groups ($p < 0.05$, in all cases) (Fig.4c). Non-significant differences between groups were observed during the reversal of the egocentric response discrimination. Thus, the three groups of mice did not differ in the

total number of trials [$F_{(2,26)} = 0.79$; n.s.] and incorrect trials [$F_{(2,26)} = 1.06$; n.s.] to reach criterion (Fig. 4d).

Effects of acute and subchronic PCP treatments on mPFC serotonin and dopamine output

Basal extracellular concentrations of 5-HT in dialysate samples of mPFC were: control, 1.1 ± 0.2; acute, 1.5 ± 0.4; subchronic, 1.8 ± 0.5 expressed as fmol/60 µl. Non-significant differences between treatments were found in basal 5-HT concentrations [$F_{(2,9)} = 0.80$; n.s.] (Fig. 5a). The systemic administration of PCP (10 mg/kg, s.c.) increased the extracellular levels of 5-HT in mPFC similarly in the acute and subchronic groups (Fig. 5c). Thus, two-way repeated measures ANOVA showed a significant effect of treatment [$F_{(2,16)} = 6.08$; $p < 0.05$], fraction [$F_{(9,144)} = 10.55$; $p < 0.01$] and treatment x fraction interaction [$F_{(18,144)} = 3.32$; $p < 0.01$]. *Post-hoc* Newman-Keuls test revealed significant differences between the acute and subchronic groups *versus* the control group ($p < 0.05$).

Basal extracellular concentrations of DA in dialysate samples of mPFC were: control, 7.0 ± 0.4; acute, 5.7 ± 0.5; subchronic, 3.9 ± 0.3 expressed as fmol/30 µl. One-way ANOVA showed a significant effect of treatment on basal DA concentrations [$F_{(2,12)} = 12.90$; $p < 0.01$]. Basal DA concentrations in mPFC in the subchronic group were significantly lower than in the control and acute groups ($p < 0.01$, $p < 0.05$, respectively, *post-hoc* Newman-Keuls test) (Fig. 5b). The systemic administration of PCP (10 mg/kg, s.c.) increased the extracellular levels of DA in mPFC only in the subchronic group (Fig. 5d), indicating a sensitization of DA release after subchronic PCP, as observed with locomotor activity. Thus, two-way repeated measures ANOVA showed a significant effect of treatment [$F_{(2,12)} = 8.16$; $p < 0.01$], fraction [$F_{(11,132)} = 4.25$; $p < 0.01$] and treatment x fraction interaction [$F_{(22,132)} = 4.15$; $p < 0.01$]. *Post-hoc* Newman-Keuls test showed a significant difference between the subchronic group *versus* the control group ($p < 0.01$) and the acute group ($p < 0.05$).

Effects of acute and subchronic PCP treatments on tyrosine hydroxylase (TH) mRNA expression in the VTA

TH mRNA expression in the VTA was markedly attenuated after subchronic (but not acute) PCP treatment. One-way ANOVA showed a significant effect of treatment [$F_{(2,8)}$ = 17.94; $p < 0.01$], with a significant decrease of TH mRNA expression in the subchronic group compared to the control ($p < 0.01$) and the acute ($p < 0.05$) groups (*post-hoc* Newman-Keuls test) (Fig. 6a, b).

NeuN immunohistochemistry in the VTA

The number of NeuN positive cells in the VTA of control and subchronic PCP-treated mice was similar. Thus, one-way ANOVA showed non-significant effects of treatment [$F_{(1,6)}$ = 2.42; n.s.], (Fig. 6c).

c-fos *mRNA expression after acute and subchronic PCP treatments*

Acute and subchronic PCP treatments induced a different pattern of *c-fos* expression in mice brains at both 1h and 96h post-treatment. At 1h, *post-hoc* Newman-Keuls test after significant main effect of the ANOVA showed that acute PCP treatment significantly increased *c-fos* expression (from anterior to posterior AP) in the medial prefrontal cortex ($p <0.05$), cingulate cortex ($p < 0.01$), dorsolateral striatum ($p < 0.05$), dorsomedial striatum ($p < 0.05$), piriform cortex ($p < 0.01$), ventral striatum ($p < 0.01$), retrosplenial cortex ($p < 0.01$), thalamus ($p < 0.01$), posterior hippocampus ($p < 0.01$), dorsal raphe ($p < 0.01$), and ectorhinal/perirhinal cortices ($p < 0.01$). In contrast, *c-fos* expression induced by subchronic PCP treatment was restricted to the restrosplenial cortex ($p < 0.05$), thalamus ($p < 0.01$), posterior hippocampus ($p < 0.05$) and the supramammilary nucleus ($p < 0.01$) (Fig. 7a).

At 96h post-treatment, we observed differential changes (mainly decreases) in *c-fos* expression due to the two PCP regimens. Acute PCP group significantly decreased *c-fos* mRNA expression in the dorsal raphe ($p < 0.01$). In contrast, subchronically PCP-treated mice showed significantly decreased *c-fos* mRNA in the cingulate cortex ($p <$

0.01), dorsomedial striatum ($p < 0.01$), piriform cortex ($p < 0.05$), dorsal raphe ($p < 0.01$), but an increased *c-fos* mRNA in the posterior hippocampus ($p < 0.05$) (Fig. 8).

Discussion

The present study investigated the behavioral manifestations produced by acute and subchronic PCP treatment in adult C57BL/6J mice, the long-term behavioral consequences of the two treatment regimens and some of their neurobiological substrates. Acute and subchronic PCP induced differential behavioral, neurochemical and cellular effects. In particular, subchronic PCP treatment evoked a sensitization of acute locomotor effects and working memory deficits. Moreover, subchronic PCP selectively disrupted dopaminergic neurotransmission in mPFC and produced a differential pattern *c-fos* expression, likely reflecting a differential involvement of some brain networks in both experimental conditions. Although the effects of subchronic PCP administration on behavioral variables have been previously reported (see Introduction), the present study shows a comprehensive view of PCP effects using behavioral, neurochemical and histological techniques. We are currently assessing the effects of the same treatment regime on brain oscillations in various areas of the behaving mice, in order to correlate behavioral changes with the activity of selected neuronal populations.

Acute PCP treatment increased the locomotor activity of adult mice, an effect potentiated by the subchronic treatment. Behavioral sensitization is typically observed with dopaminergic drugs such as amphetamine and cocaine (Featherstone et al. 2007; Pierce and Kalivas 1997; Post and Rose 1976), and has also been observed in C57BL/6J and C57BL/6N mice after subchronic/chronic PCP treatment (Mouri et al. 2012; Xu and Domino 1994). The involvement of dopaminergic neurotransmission in the motor circuit of the basal ganglia is extensively documented (Alexander and Crutcher 1990; Gerfen 2000; Groenewegen 2003). An increased striatal DA release may partly mediate the hyperlocomotion induced by non-competitive NMDA-R antagonists, since PCP hyperlocomotor effects in mice can be antagonized by administration of DA receptor antagonists (Chartoff et al. 2005; Nagai et al. 2003). However, glutamatergic

mechanisms have also been described to contribute to NMDA-R mediated locomotor effects in dopamine-deficient mice (Chartoff et al. 2005) and after neurotoxic lesions of dopaminergic neurons (Lapin and Rogawski 1995). Likewise, the application of muscimol ($GABA_A$ agonist) in the anterior nucleus of the thalamus fully prevented the hyperlocomotion induced by the NMDA-R antagonist MK-801 (López-Hill and Scorza 2012), thus counteracting the increased thalamic activity produced by NMDA-R blockade (Santana et al. 2011; Vaisanen et al. 2004). Hence, a recent report indicates that PCP mainly inhibits GABAergic neurons of the reticular nucleus of the thalamus, which provides feed-forward inhibition to the rest of thalamic nuclei (Troyano-Rodríguez et al. 2014). This effect breaks the physiological balance between excitatory and inhibitory transmission in the thalamus and increases thalamic excitatory inputs to the neocortex. Moreover, other anatomical data support a glutamatergic contribution, since basal ganglia receive dense glutamatergic inputs predominantly from prefrontal cortical areas, as well as from the hippocampus, periventricular thalamus, and amygdala (Carlsson and Carlsson 1990; Graybiel 1990; Phillipson and Griffiths 1985; Post and Rose 1976). Therefore, differential dopamine-glutamate interactions in the basal ganglia may contribute to acute PCP locomotor effects and as well as PCP-induced behavioral sensitization.

We then explored whether acute and subchronic PCP treatment differentially activated *c-fos* expression in mice brain, as a surrogate marker of neuronal activity (Dragunow and Faull 1989; Konkle and Bielajew 2004; Panagis et al. 1997; Sager et al. 1988). Acute PCP induced a similar *c-fos* expression in mice than we previously described in rats using the same methodology (Kargieman et al. 2007; Santana et al. 2011). Thus, at 1h post-treatment acute PCP (10 mg/kg) produced a significant increase of *c-fos* mRNA in many cortical areas such as mPFC, cingulate, retrosplenial, pyriform and ectorhinal/perirhinal cortices. Moreover, thalamic nuclei, dorsal raphe and to a lesser extent the posterior hippocampus also exhibited increased *c-fos* expression. After subchronic PCP most cortical areas activated by the acute PCP challenge returned to

control values. The retrosplenial cortex, thalamus and posterior hippocampus showed a significantly lower *c-fos* expression compared to the acute PCP group, yet still higher than controls. Moreover, the supramammillary nucleus (SuM) showed a very significant increase in *c*-fos expression after subchronic –but not acute- PCP. Interestingly, inactivation of the SuM has been shown to normalize both the hippocampal gamma waves and the hyperactivity induced by acute NMDA-R antagonists like MK-801 and ketamine in rats (Ma and Leung 2000, 2007). In our hands, the same subchronic PCP treatment regime also evoked a sustained hippocampal gamma oscillatory activity (Castañé, Lladó-Pelfort et al. unpublished observations). Thus, the activation of SuM produced by subchronic PCP may also contribute to PCP-induced locomotor sensitization in mice.

In this study, differential long-term consequences of both acute and subchronic PCP treatment in mice were also examined. For this purpose, animals in withdrawal were exposed to several behavioral paradigms to evaluate cognitive function such as working memory and reversal learning, and depressive-like responses. We adapted the novel object recognition test paradigm to evaluate working memory function in mice by performing the assay in a T-maze and using an inter-trial interval (ITI) of low duration (3 minutes). Under these conditions, we observed that mice of the subchronic PCP group showed working memory deficits by means of decreased spontaneous alternation in the T-maze and decreased recognition index in the NOR test. The NOR paradigm has been previously used to demonstrate short- (1h ITI) and long-term (24h ITI) memory deficits after repeated PCP treatments in mice (Tanibuchi et al. 2009). However, this is the first time showing that working memory deficits of subchronic PCP-treated mice can be observable using this paradigm. Several neurotransmitters have been related to working memory, in particular dopamine (Landau et al. 2009; Vijayraghavan et al. 2007; Williams and Goldman-Rakic 1995). Early work in rats and monkeys demonstrated that elevating or depleting DA in the PFC impaired spatial working memory performance (Simon, 1981; Bubser and Schmidt 1990; Murphy et al. 1996). In

agreement, we have shown a dopaminergic neurotransmission dysregulation in mPFC of subchronically PCP-treated mice. Concretely, we observed a decreased basal DA output in mPFC in microdyalisis experiments associated with a decreased tyrosine hydroxylase mRNA expression in the VTA. These changes were not due to neuronal loss in the VTA, since NeuN positive cells were similar in control and subchronic PCP groups. Moreover, the last PCP administration in the subchronic group produced a greater DA release in the mPFC, further suggesting that dopaminergic neurotransmission is particularly affected by the repeated PCP exposure.

On the contrary, subchronic PCP treatment in mice had no observable effects on either discrimination learning or reversal of an egocentric response task. Reversal learning performance is dependent on prefrontal serotonin transmission (Boulougouris et al. 2008; Clarke et al. 2004, 2005). In accordance with the lack of effect of PCP on reversal learning, we observed comparable basal extracellular 5-HT levels in mPFC in the three groups of mice. To date, only another study has investigated the influence of subchronic PCP treatment in mice in a touch-screen based visual discrimination and reversal with no significant effects being observed (Brigman et al. 2009). However, studies performed in rats have led to impaired reversal learning abilities after subchronic PCP treatments (Jentsch and Taylor 2001), specially using operant settings (Idris et al. 2010; Mc Lean et al. 2009, 2010, 2011). Recently, Fellini et al (2014) re-evaluated the effects of acute and sub-chronic PCP administration on reversal of a double visual discrimination task in rats, and found that acute but not sub-chronic PCP impaired reversal performance. Altogether, these data suggest that not all reversal learning tasks can be considered equivalent. The nature of stimuli used, the duration treatment/withdrawal and other procedural particularities (pre-training, operant/not operant task, re-discrimination) may engage different cognitive processes. Therefore, special attention should be put on designing and evaluating reversal learning abilities in rodents in order to clarify the impact of PCP treatments.

In order to investigate differential effects of acute and subchronic PCP on negative symptoms, we performed the tail suspension test on WD7. Both acute and subchronic PCP-treated mice showed increased immobility scores, however these effects did not reached statistically significance. Recently, Mouri and collaborators (2012) have described strain differences specially affecting chronic PCP-induced immobility responses in the forced swimming test. These authors described that immobility responses are less intense and durable in mice with C57BL/6J background compared to ddY or C57BL/6N, lasting no more than three days after finishing the chronic PCP treatment. Previous and present findings suggest that depressive-like effects induced by PCP treatments are not enduring, and mice with a C57BL/6J genetic background should not be used to reveal depressive-like responses of PCP treatments in the tail suspension and forced swimming tests at very long times.

Finally, we determined the expression of *c-fos* mRNA at WD4 to investigate residual (drug-free) brain activity changes due to acute and subchronic PCP treatments that may account for the behavioral effects. Basal *c-fos* mRNA expression was higher in the posterior hippocampus, and lower in the cingulate and pyriform cortices, dorsomedial striatum and dorsal raphe in the subchronic group of mice. Acute PCP also decreased basal *c-fos* expression in the dorsal raphe at WD4, suggesting a residual effect on brain activity in this area due to both PCP regimens that may relevant to depressive-like responses.

In summary, the present study shows that acute and subchronic PCP administration differently affects neuronal activity in brain regions relevant to schizophrenia, which could account for their different behavioral effects. In particular, subchronic –but not acute- PCP evoked a locomotor sensitization and induced working memory deficits which may be partly accounted for by activity changes in several brain areas, including a reduced basal dopaminergic function and an increased activity of thalamocortical networks, among others.

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Figure captions

Figure 1. Locomotor activity measurements (90 min) were performed on day 1 (D1) and day 12 (D12) and started 10 min after drug administration. Data are expressed as mean ± SEM (n = 6-8 mice/group) of distance moved (cm) in 5 min intervals (a) or total distance moved in 90 min (b). A single administration of PCP (10 mg/kg, s.c.) increased locomotor activity as shown in the subchronic group on D1and the acute group on D12. Repeated PCP administration (10 mg/kg, s.c., 10 days) produced a sensitization of acute locomotor effects (D12). * $p < 0.05$, ** $p < 0.01$ vs control; # $p < 0.05$, ## $p < 0.01$ vs acute (*post-hoc* Newman-Keuls).

Figure 2. Mean ± SEM of percentage of alternation in a T-maze (a) and recognition index (RI) (b). Spontaneous alternation was evaluated on withdrawal day 4 (WD4). Mice of the subchronic PCP group exhibited a significant decrease of percentage of alternations (n = 6-8/group). Novel object recognition (NOR) test was performed in withdrawal day 5 (WD5). A 10 min (Trial 1)-3 min (Inter trial interval)-10 min (Trial 2) procedure was used to evaluate working memory in the NOR. Mice of the subchronic group showed RI values consistent with no memory retention of the familiar object. * $p < 0.05$, ** $p < 0.01$ vs control; # $p < 0.05$, ## $p < 0.01$ vs acute (*post-hoc* Newman-Keuls) (n =8-11 mice/group).

Figure 3. Mean ± SEM of immobility time (s) in the tail suspension test (n = 8-12/group). The test was performed at withdrawal day 7 (WD7).

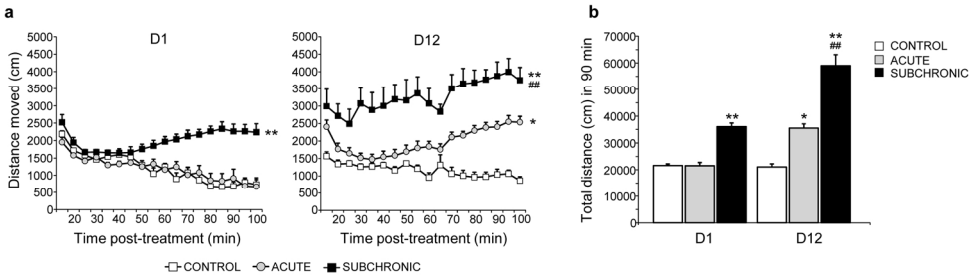
Figure 4. Schematic representation of the behavioral training and testing protocol (a). Data are expressed as mean ± SEM of total trials to criterion (TT), and incorrect trials to criterion (IT) in the discrimination phase (b), re-discrimination phase (c), and reversal phase (d). * $p < 0.05$ vs control; # $p < 0.05$ vs subchronic (*post-hoc* Newman-Keuls) (n =5-12 mice/group).

Figure 5. Basal extracellular levels of 5-HT (a) and DA (b) in mPFC of mice of the three groups of treatment. Data are expressed as mean ± SEM of four basal samples. Effects of the systemic acute and subchronic PCP administration (10 mg/kg, s.c.) on dialysate 5-HT (c) and DA (d) concentrations in the mPFC of mice. Data are expressed as percentages of pretreatment values and are given as mean ± SEM (5–9 mice/group). * $p < 0.05$, ** $p < 0.01$ vs control; # $p < 0.05$ vs acute (*post-hoc* Newman-Keuls).

Figure 6. Tyrosine hydroxylase mRNA expression in the ventral tegmental area (VTA). (a) Bar graphs showing mean ± SEM of optical density (arbitrary units) in the three experimental groups of mice (n=3-4 mice/group). (b) Representative autoradiograms showing the expression of tyrosine hydroxylase in coronal sections of VTA (AP in mm: -3.08). (c) Mean ± SEM of NeuN positive cells/μm^2 in the VTA (n=3-4 mice/group).

Figure 7. *c-fos* mRNA expression in mice brain coronal sections after acute and subchronic PCP treatments. Mice were sacrificed 1h post-treatment. (a) Representative photographs of c-fos expression at different AP coordinates (from Bregma in mm): 1.18, -1.58 and -3.08 in the different experimental groups. (b) Bar graphs showing mean ± SEM of optical density (arbitrary units) in the three experimental groups of mice (n=3 mice/group). * $p < 0.05$, ** $p < 0.01$ vs control; # $p < 0.05$, ## $p < 0.01$ vs acute (*post-hoc* Newman-Keuls).

Figure 8. *c-fos* mRNA expression in mice brain coronal sections after acute and subchronic PCP treatments. Mice were sacrificed at 96h post-treatment. Data are expresses as mean ± SEM of optical density (arbitrary units) in the three experimental groups of mice (n=3/group). * $p < 0.05$, ** $p < 0.01$ vs control; # $p < 0.05$ vs acute (*post-hoc* Newman-Keuls).



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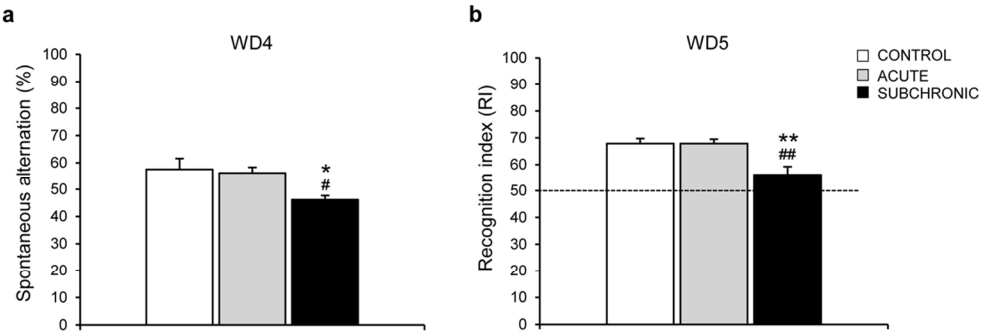

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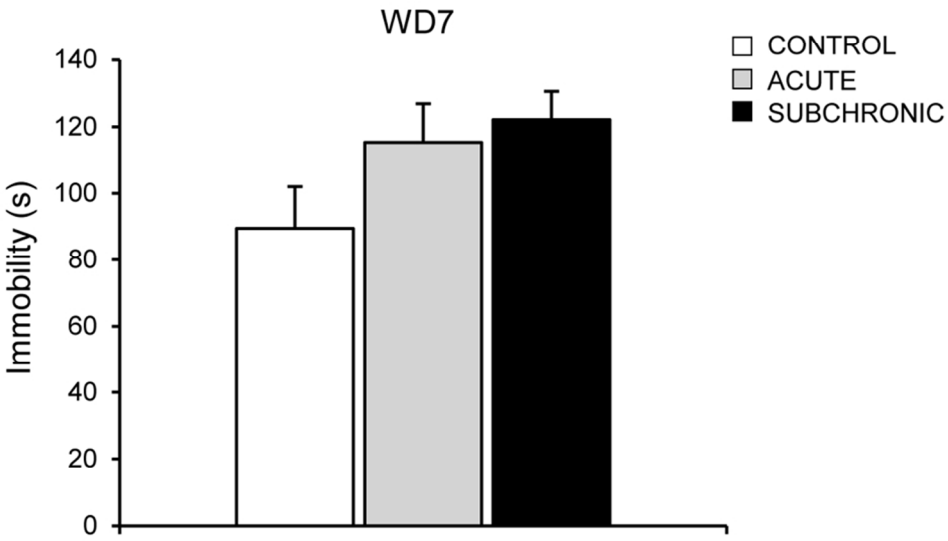

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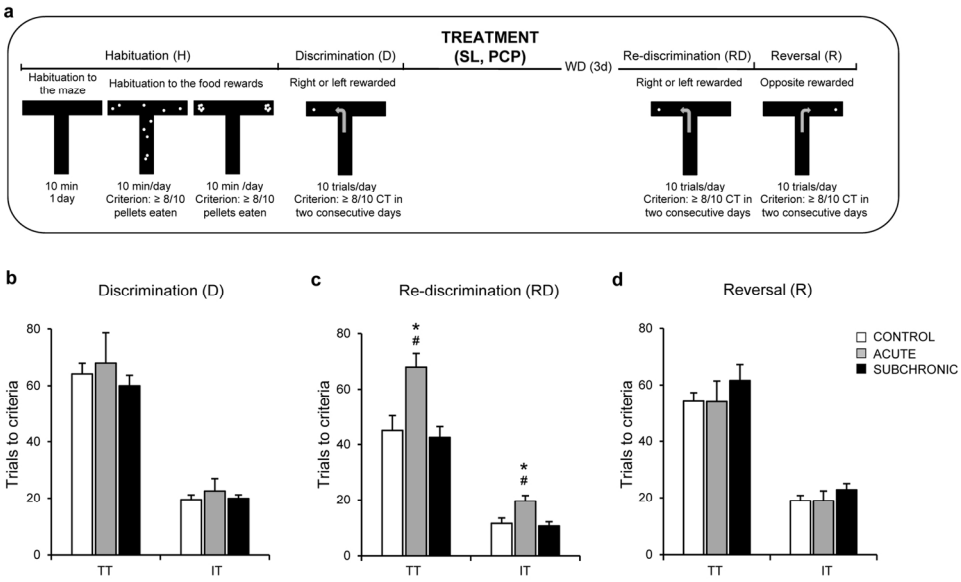

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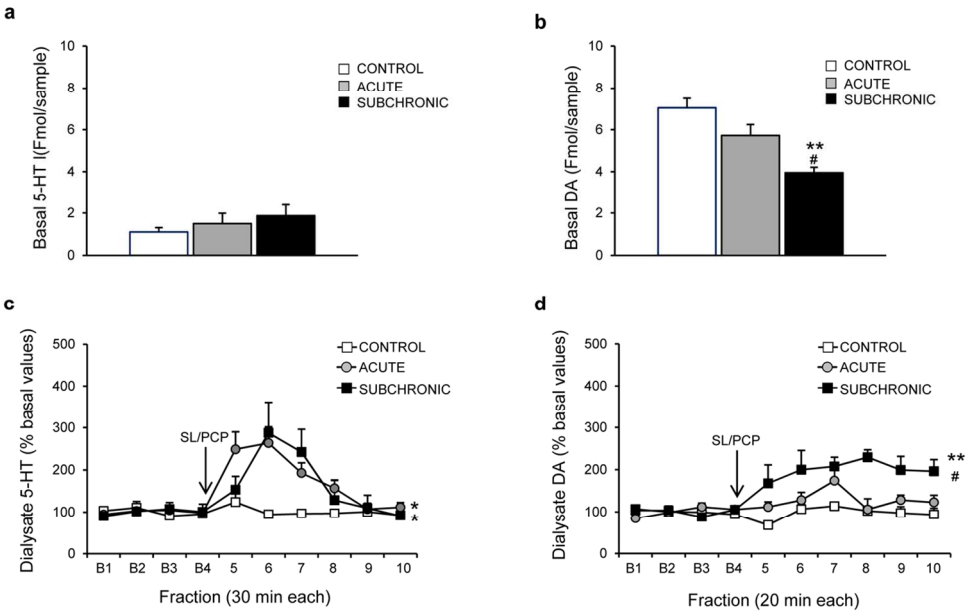

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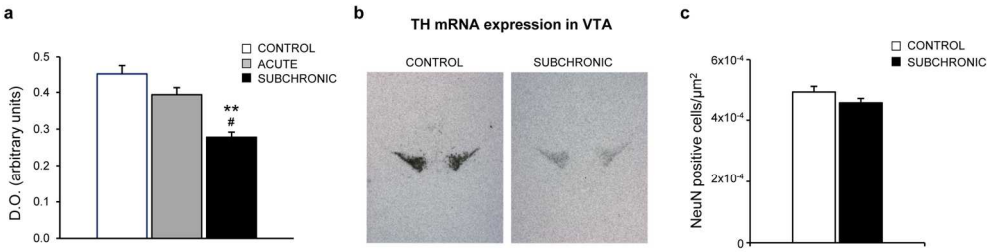

Figure 6. Tyrosine hydroxylase mRNA expression in the ventral tegmental area (VTA). (a) Bar graphs showing mean ± SEM of optical density (arbitrary units) in the three experimental groups of mice (n=3-4 mice/group). (b) Representative autoradiograms showing the expression of tyrosine hydroxylase in coronal sections of VTA (AP in mm: -3.08). (c) Mean ± SEM of NeuN positive cells/µm2 in the VTA (n=3-4 mice/group).
170x46mm (300 x 300 DPI)

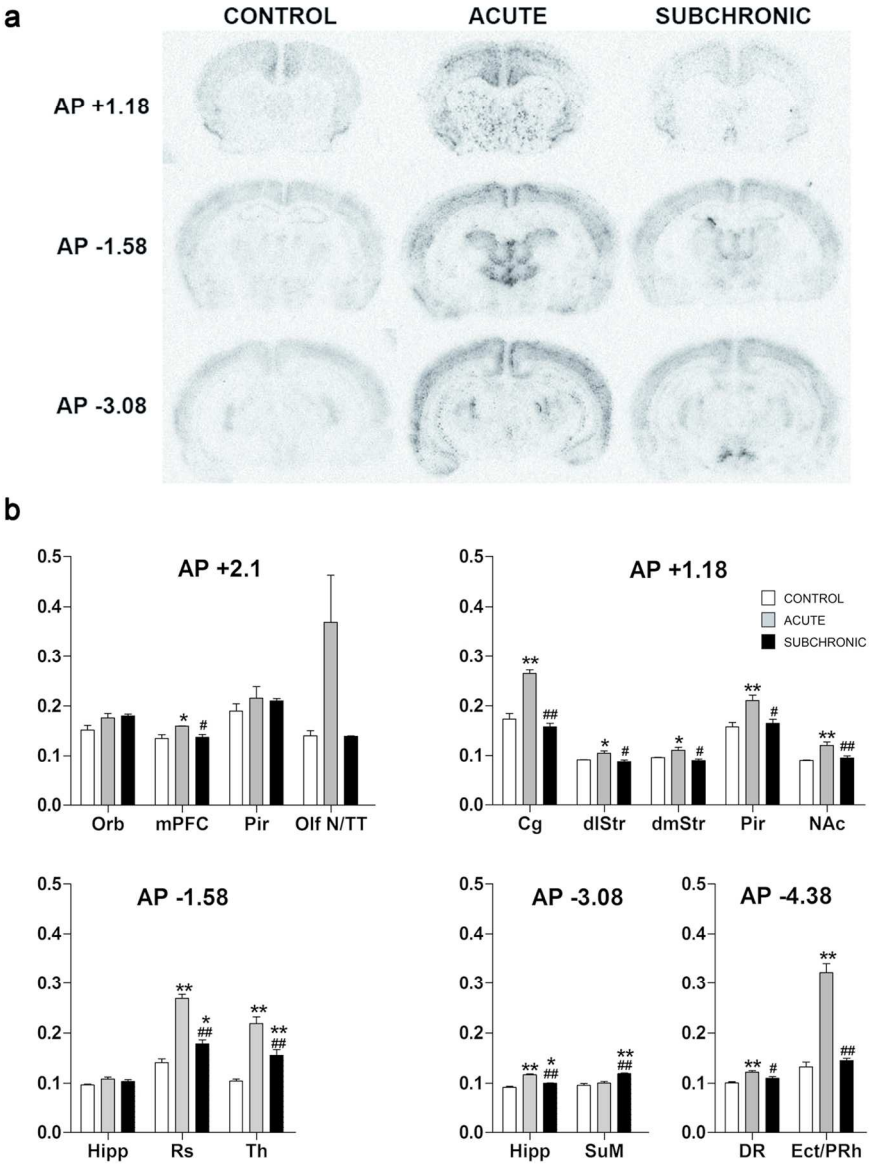

Figure 7. c-fos mRNA expression in mice brain coronal sections after acute and subchronic PCP treatments. Mice were sacrificed 1h post-treatment. (a) Representative photographs of c-fos expression at different AP coordinates (from Bregma in mm): 1.18, -1.58 and -3.08 in the different experimental groups. (b) Bar graphs showing mean ± SEM of optical density (arbitrary units) in the three experimental groups of mice (n=3 mice/group). * $p < 0.05$, ** $p < 0.01$ vs control; # $p < 0.05$, ## $p < 0.01$ vs acute (post-hoc Newman-Keuls).
124x160mm (300 x 300 DPI)

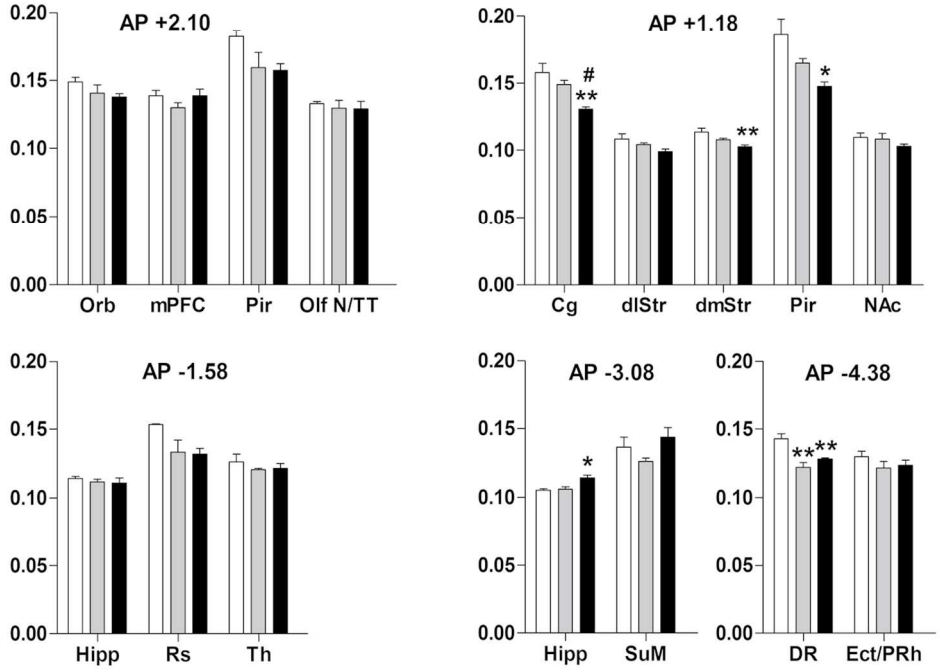

Figure 8. c-fos mRNA expression in mice brain coronal sections after acute and subchronic PCP treatments. Mice were sacrificed at 96h post-treatment. Data are expresses as mean ± SEM of optical density (arbitrary units) in the three experimental groups of mice (n=3/group). * $p < 0.05$, ** $p < 0.01$ vs control; # $p < 0.05$ vs acute (post-hoc Newman-Keuls).
124x95mm (300 x 300 DPI)