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Neural basis of the potentiated inhibition of repeated haloperidol and clozapine treatment on the phencyclidine-induced hyperlocomotion

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Abstract

Clinical observations suggest that antipsychotic effect starts early and increases progressively over time. This time course of antipsychotic effect can be captured in a rat phencyclidine (PCP)-induced hyperlocomotion model, as repeated antipsychotic treatment progressively increases its inhibition of the repeated PCP-induced hyperlocomotion. Although the neural basis of acute antipsychotic action has been studied extensively, the system that mediates the potentiated effect of repeated antipsychotic treatment has not been elucidated. In the present study, we investigated the neuroanatomical basis of the potentiated action of haloperidol (HAL) and clozapine (CLZ) treatment in the repeated PCP-induced hyperlocomotion. Once daily for five consecutive days, adult Sprague-Dawley male rats were first injected with HAL (0.05 mg/kg, sc), CLZ (10.0 mg/kg, sc) or saline, followed by an injection of PCP (3.2 mg/kg, sc) or saline 30 min later, and motor activity was measured for 90 min after the PCP injection. C-Fos immunoreactivity was assessed either after the acute (day 1) or repeated (day 5) drug tests. Behaviorally, repeated HAL or CLZ treatment progressively increased the inhibition of PCP-induced hyperlocomotion throughout the five days of drug testing. Neuroanatomically, both acute and repeated treatment of HAL significantly increased PCP-induced c-Fos expression in the nucleus accumbens shell (NAs) and the ventral tegmental area (VTA), but reduced it in the central amygdaloid nucleus (CeA). Acute and repeated CLZ treatment significantly increased PCP-induced c-Fos expression in the ventral part of lateral septal nucleus (LSv) and VTA, but reduced it in the medial prefrontal cortex (mPFC). More importantly, the effects of HAL and CLZ in these brain areas underwent a time-dependent reduction from day 1 to day 5. These findings suggest that repeated HAL achieves its potentiated inhibition of the PCP-induced hyperlocomotion by acting on the NAs, CeA and VTA, while CLZ does so by acting on the mPFC, LSv and VTA.

Keywords: Antipsychotic sensitization, Clozapine, c-Fos, Haloperidol, PCP-induced hyperlocomotion

Abbreviations: CeA, central amygdaloid nucleus; CLZ, clozapine; DLS, dorsolateral striatum; HAL, haloperidol; LS, lateral septal nucleus; LSv, ventral part of lateral septal nucleus; MeA, medial amygdaloid nucleus; mPFC, medial prefrontal cortex; NA, nucleus accumbens; NAc, nucleus accumbens core; NAs, nucleus accumbens shell; PCP, phencyclidine; VTA, ventral tegmental area

1. Introduction

A growing number of clinical studies suggest that antipsychotic action starts early and increases in magnitude with repeated treatment (Agid et al., 2003, 2006; Emsley et al., 2006; Glick et al., 2006; Kapur et al., 2005; Leucht et al., 2005; Raedler et al., 2007). This observation is strongly supported by the finding that improvement of psychotic symptoms occurs within the first week of treatment and shows a progressive increase over the subsequent weeks. For example, Agid et al. (2003) examined 42 double-blind, comparator-controlled studies (> 7000 patients) using a meta-analysis technique. They found that psychotic symptoms improved within the first week of treatment and showed a progressive improvement over subsequent weeks, with the overall pattern of improvement approximating an exponential curve. Leucht et al. (2005) analyzed a large homogeneous database of original patient data from 7 randomized, double-blind

studies of the efficacy of amisulpride in patients with schizophrenia spectrum disorders and found the same results. More improvement occurs in the first few days than in any later period of equal duration (Leucht et al., 2005). Subsequent studies show that the onset occurs within the first day, contemporaneous with the blockade of dopamine receptors (Kapur et al., 2005), and early nonimprovement (< 20% reduction in Brief Psychiatric Rating Scale total score at 1 week) predicts nonresponse at 4 weeks (Correll et al., 2003).

Animal models play an important role in delineating the time course of antipsychotic action and related behavioral mechanisms (Abekawa et al., 2007; Sun et al., 2009). Among many animal models of antipsychotic drugs, the phencyclidine (PCP)-induced hyperlocomotion model seems especially useful for this purpose. First, the PCP model is sensitive to antipsychotic action. Antipsychotic drugs, both typical (haloperidol), and atypical (clozapine, olanzapine, risperidone, aripiprazole) *acutely*

inhibit hyperlocomotion induced by PCP in rodents. Atypical drugs are less likely to cause extrapyramidal motor syndromes (EPS) and sustained prolactin elevation in comparison to typicals. They also show a preferential inhibition on the PCP-induced hyperlocomotion over the amphetamine-induced hyperlocomotion, whereas typicals do not seem to possess this preference. Thus, the PCP model is useful in distinguishing different classes of antipsychotics (Gleason and Shannon, 1997; Maurel-Remy et al., 1995; Millan et al., 1999). Second, repeated treatment of antipsychotic drugs progressively potentiates the inhibition of the PCP-induced hyperlocomotion across multiple drug testing sessions and prolonged this action within sessions (Sun et al., 2009), a time course of behavioral changes closely mimicking clinical effects. This property is not shared by antidepressants or anxiolytics (Sun et al., 2009). Therefore, it appears that the repeated PCP-induced hyperlocomotion model could serve as a valid model for the investigation of the neurobiological and behavioral mechanisms of action of repeated antipsychotic treatments.

c-Fos, a protein product of immediate-early gene *c-fos* has been used as a molecular biomarker for identifying neuronal activation after a variety of behavioral, environmental, and pharmacological stimuli. In the field of antipsychotic drugs, the ability of antipsychotic drugs to induce c-Fos expression in the forebrain regions has been closely linked to the action sites of antipsychotic activity and liability for producing extrapyramidal side effects (Robertson and Fibiger, 1992; Robertson et al., 1994). Acute administration of typical antipsychotic haloperidol (HAL) and atypical drug clozapine (CLZ) produces a different induction pattern of c-Fos expression in the forebrain, with acute HAL increasing Fos-positive neurons in the dorsolateral striatum (DLSt), nucleus accumbens shell and core (NAs and NAc), and lateral septal nucleus (LS) and acute CLZ producing such effects in the NAs, medial prefrontal cortex (mPFC) (Robertson and Fibiger, 1992; Robertson et al., 1994). Interestingly, psychotomimetic drugs of abuse such as PCP, amphetamine and MK-801 also increase c-Fos expression in these brain areas (De Leonibus et al., 2002; Habara et al., 2001; Rotllant et al., 2010; Sato et al., 1997; Zuo et al., 2009). Based on these observations, we postulated that by examining how antipsychotic treatment alters c-Fos expression induced by PCP in the above mentioned brain regions at different time points of treatment, we may be able to identify the neuroanatomical bases of *acute* and *potentiated* inhibition of HAL or CLZ treatment on PCP-induced hyperlocomotion. In the present study, we used the c-Fos immunohistochemistry technique, together with a pharmacological tool (i.e. PCP) and behavioral observations (i.e. motor activity), to delineate the neural circuitries upon which HAL and CLZ act to achieve their acute and potentiated inhibitory effects on the PCP-induced hyperlocomotion. Because we considered the c-Fos effect in the context of antipsychotic activity (i.e. PCP-induced hyperlocomotion), our approach ensured that the brain regions identified are behaviorally and neurochemically relevant to the specific action of HAL and CLZ.

2. Methods

2.1. Animals

Male Sprague-Dawley rats (220–250 g upon arrival, Charles River, Portage, MI) were housed in pairs in transparent polycarbonate cages (48.3 cm × 26.7 cm × 20.3 cm) under 12-hr light/dark cycle (lights on from 6:30 am to 6:30 pm), with food and water available ad libitum. All animals were maintained in our colony with a controlled temperature (21 ± 1 °C) and a relative humidity of 55–60%. Animals were allowed at least one week of habituation to the animal facility before being used in experiments. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Nebraska-Lincoln.

2.2. Drugs

The injection solution of haloperidol (HAL, 5.0 mg/ml ampoules, Sico Pharmaceuticals, Inc., Irvine, CA) was obtained by mixing drugs with sterile water. Phencyclidine hydrochloride (PCP, a gift from NIDA Drug Supply Program) and clozapine (CLZ, a gift from NIMH Drug Supply Program) were dissolved in 0.9% saline and 1.0% glacial acetic acid in distilled water, respectively. We chose haloperidol (0.05 mg/kg, sc) and clozapine (10.0 mg/kg, sc) because they are clinically relevant doses based on the dopamine D₂ receptor occupancy data (50–75% occupancy) (Kapur et al., 2003) and animal behavioral assessment of antipsychotic activity (Li et al., 2010, 2011). The dose for PCP was 3.2 mg/kg, which has been shown to induce a robust hyperlocomotion effect without causing severe stereotypy (Gleason and Shannon, 1997; Kalinichev et al., 2008; Sun et al., 2009). Haloperidol, clozapine and phencyclidine were administered subcutaneously.

2.3. Locomotor activity apparatus

Sixteen activity boxes were housed in a quiet room. The boxes were 48.3 cm × 26.7 cm × 20.3 cm transparent polycarbonate cages, which were similar to the home cages but were each equipped with a row of 6 photocell beams (7.8 cm between two adjacent photobeams) placed 3.2 cm above the floor of the cage. A computer detected the disruption of the photocell beams and recorded the number of beam breaks. All experiments were run during the light cycle from 9 am to 4 pm.

2.4. Experimental procedure

Thirty-two rats were randomly assigned to one of four groups ($n = 8/\text{group}$): vehicle (water) + vehicle (saline, SAL), vehicle (water) + PCP, haloperidol (0.05 mg/kg) + PCP, clozapine (10.0 mg/kg) + PCP. After two days of habituation to the testing room and the testing boxes (30 min/day for 2 days), the drug test days started. On the first drug day, rats were brought to the testing room, and injected with vehicle (sterile water), haloperidol (0.05 mg/kg), or clozapine (10.0 mg/kg). They were then immediately placed in locomotor activity boxes for 30 min. At the end of the 30-min testing, rats were taken out and injected with either 0.9% saline (sc) or PCP (3.2 mg/kg, sc) and placed back in the boxes for another 90 min. Locomotor activity (number of photobeam breaks) was measured in 5 min intervals throughout the entire 120-min testing session. Half of rats from each group (4/group) were randomly selected and sacrificed immediately after the end of the 90-min testing for acute c-Fos assay. The remaining rats were repeatedly tested for another 4 days (a total of 5 tests) and sacrificed at the end of the last drug test day.

2.5. c-Fos immunohistochemistry

Immediately after the locomotor activity test, all rats were anesthetized with sodium pentobarbital (100.0 mg/kg) and then perfused with 4% paraformaldehyde. Brains were post-fixed and cryoprotected in 30% sucrose, and coronal sections (40 µm) were cut on a cryostat. Procedures for Fos immunohistochemistry followed the protocol by Zhao and Li (2010). Briefly, sections were incubated with a rabbit polyclonal anti-c-Fos (Ab-5, PC38) antibody raised against residues 4–17 of human c-Fos (1:20000, Calbiochem, CA, USA) for 48 h at 4 °C. Sections were then incubated with a biotinylated goat anti-rabbit secondary antibody (1:200, Vector Laboratories, Burlingame, CA, USA) in PBS containing 1% NGS for 2 h at RT. They were processed with avidin-biotin horseradish peroxidase complex (1:200, Vectastain Elite ABC Kit, Vector Laboratories). The immunoreaction was visualized with peroxidase substrate (DAB Substrate Kit for Peroxidase, Vector Laboratories). After staining, sections were mounted on gelatin-coated slides, air-dried, dehydrated and coverslipped. As a control, the primary antibody was substi-

tuted with normal rabbit serum. No corresponding nucleus or cytoplasm was immunostained in the control.

2.6. Fos-immunoreactive (Fos-I) cell counting

Photomicrographs were captured with a digital camera (INFINITY lite, Canada) equipped with an Olympus CX41RF microscope (Japan) using $\times 10$ objective lens. Fos-I cells characterized by clearly stained nuclei was counted bilaterally in one section with comparable anatomical levels across the treatment groups. The brain regions analyzed included the neural sites that were either implicated in the action of PCP and/or in the regulation of locomotor activity [e.g., the medial prefrontal cortex (mPFC), nucleus accumbens shell (NAS), nucleus accumbens core (NAc), dorsolateral striatum (DLSt), ventral part of lateral septal nucleus (LSv), medial amygdaloid nucleus (MeA), central amygdaloid nucleus (CeA), and ventral tegmental area (VTA)]. The levels of brain slices were: Bregma 3.00 mm for mPFC, 1.92 mm for NAs, NAc and DLSt, 1.44 mm for LSv, -2.92 mm for MeA and CeA, -6.24 mm for VTA according to Paxinos and Watson (2007) (Figure 1). With the help of ImageJ software (developed at the US National Institutes of Health), cell counts were made within a $680 \times 510 \mu\text{m}^2$ unit area of each interest region by an experimenter blind to the treatment condition. The images were thresholded and then analyzed. In a given area from distinct treatments, the images were thresholded to the same value by means of eliminating background and noise staining to ensure that the Fos-I cells were selected. The number of Fos-I nuclei of a given brain region from bilateral sites per rat was averaged. The values from four rats of each treatment group were averaged to obtain the final mean \pm SEM.

2.7. Statistical analysis

Data for locomotor activity and the number of c-Fos immunoreactive cells were expressed as mean \pm SEM and analyzed using a two-way analysis of variance (ANOVA) with treatment conditions (VEH + VEH, VEH + PCP, HAL + PCP, CLZ + PCP) \times test days (Day1, Day5) as between-subject factors, followed by post-hoc Tukey tests to detect two-group difference. Locomotor activity data from each daily test were analyzed using a factorial repeated measures ANOVA with the between-subjects factor being the treatment conditions ("Treatment", e.g. vehicle, HAL or CLZ in combination with vehicle or PCP), and the within-subjects factor being the 5-min time block ("Block", e.g. block for 90 min after PCP injection). A one-way ANOVA was used to test two-group difference where appropriate. The independent-samples *T* test was used to compare the acute and repeated effect of different treatment groups. A conventional two-tailed level of significance at the 0.05 level was required. Percent inhibition was calculated by subtracting the motor activity of the HAL/CLZ + PCP group (T) from the motor activity of the VEH + PCP group (P), dividing by the motor activity of the VEH + PCP group, and multiplying by 100[(P - T)/P \times 100].

3. Results

3.1. Repeated HAL or CLZ treatment potentiated the inhibition on the PCP-induced hyperlocomotion

On day 1, PCP significantly increased the mean motor activity during the 90-min testing period compared to the vehicle treatment [Figure 2, $F_{(3, 12)} = 25.14$, $p < 0.001$, post-hoc Tukey tests, $p < 0.001$ vs. vehicle]. This psychomotor stimulating effect was significantly attenuated by pretreatment with HAL ($p = 0.008$) and CLZ ($p = 0.042$). However, in comparison to the vehicle controls, rats treated with HAL + PCP and CLZ + PCP still had significantly higher motor activity ($p = 0.004$ versus HAL, $p < 0.001$ versus CLZ), suggesting that the attenuation at the tested doses was not complete.

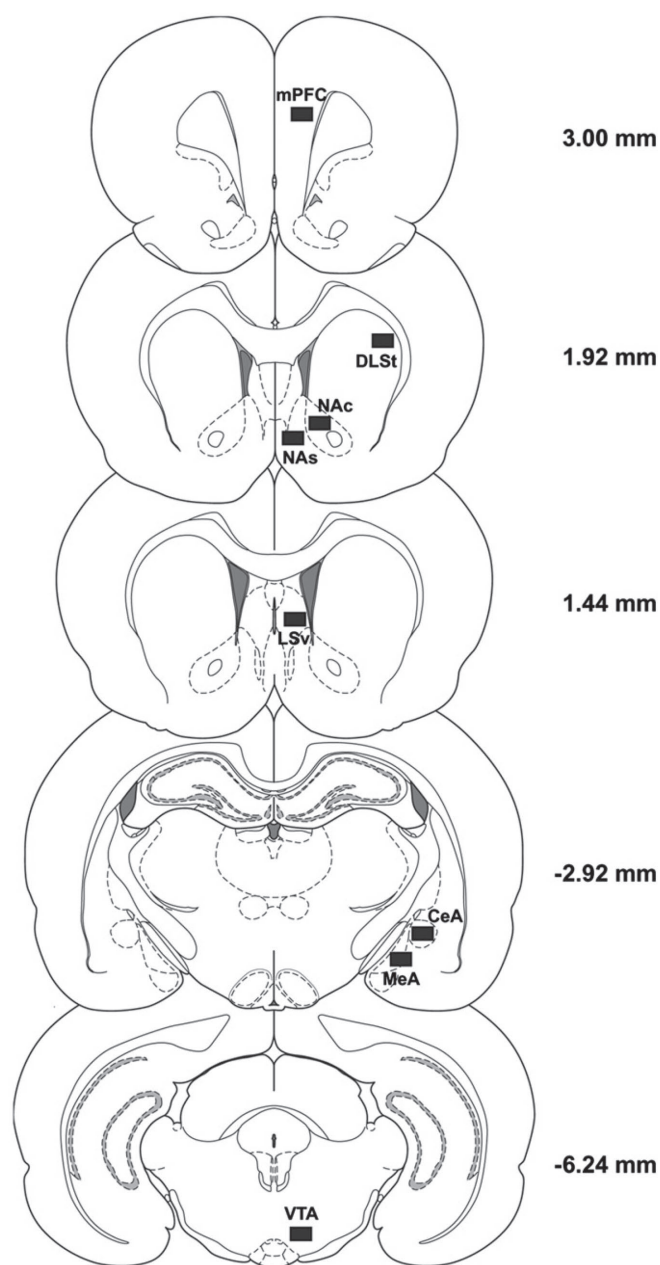


Figure 1. Schematic representation of the brain regions (black boxed areas) sampled for c-Fos immunoreactivity assessment. Distance from Bregma in the rostrocaudal planes is indicated. Drawings were modified from Paxinos and Watson (2007).

Across the 5 drug test days, repeated PCP treatment tended to progressively increase locomotor activity. In contrast, repeated HAL and CLZ pretreatment progressively enhanced their efficacy in inhibiting PCP-induced hyperlocomotion. A two-way repeated measures ANOVA revealed a main effect of "Treatment" [$F_{(3, 12)} = 90.31$, $p < 0.001$], no main effect of "Day" [$F_{(4, 48)} = 1.53$, $p = 0.21$], but a significant interaction between "Treatment" and "Day" [$F_{(12, 48)} = 1.89$, $p = 0.046$]. The enhanced inhibition by repeated HAL and CLZ treatment was also revealed by the percent inhibition measure. According to this measure, HAL reduced PCP-induced hyperlocomotion by 45% on day 1, but 77% on day 5. Similarly, CLZ reduced PCP-induced hyperlocomotion by 29% on day 1, but up to 49% on day 5.

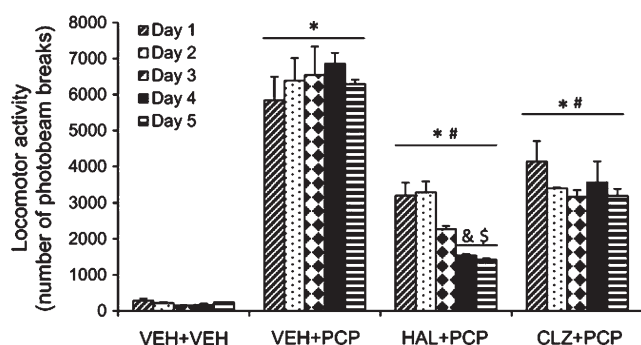


Figure 2. Effects of repeated haloperidol or clozapine treatment on PCP-induced hyperlocomotion over the five test days. Bars represent means \pm SEM. * $p < 0.05$ versus VEH + VEH control; # $p < 0.05$ versus VEH + PCP group; &, \$ $p < 0.05$ between Day 1, Day 2 and Day 4, Day 5.

3.2. Repeated HAL or CLZ treatment prolonged the time course of the inhibitory action on PCP-induced hyperlocomotion within session

Figure 3 shows the time course (measured in 5-min blocks for the 90-min period after PCP administration) of the effects of HAL or CLZ pretreatment on PCP-induced hyperlocomotion on the first and last days of drug testing. The two-way repeated

measures ANOVA revealed a main effect of "Treatment" [$F_{(3, 12)} = 25.14, p < 0.001$ for day 1; $F_{(3, 12)} = 18.55, p < 0.001$ for day 5], a main effect of "Time" [$F_{(17, 204)} = 4.63, p < 0.001$ for day 1; $F_{(17, 204)} = 8.52, p < 0.001$ for day 5], and a significant interaction between "Treatment" and "Time" [$F_{(51, 204)} = 2.32, p < 0.001$ for day 1; $F_{(51, 204)} = 5.38, p < 0.001$ for day 5]. With repeated treatment, HAL and CLZ prolonged their inhibition of PCP-induced hyperlocomotion. For example, on day 1, the significant inhibitory effect of HAL and CLZ started at the 15-min testing point after the PCP injection, and lasted for the remainder of the test session (Figure 3a, all $ps < 0.05$ in comparison to the vehicle + PCP group). On day 5, the significant inhibition advanced to the 10-min point (Figure 3b, all $ps < 0.05$).

3.3. Effects of acute HAL or CLZ treatment on PCP-induced c-Fos immunoreactivity

A one-way ANOVA revealed a significant effect in the mPFC [$F_{(3, 12)} = 27.64, p < 0.001$], NAs [$F_{(3, 12)} = 59.14, p < 0.001$], NAc [$F_{(3, 12)} = 14.03, p < 0.001$], DLSt [$F_{(3, 12)} = 104.18, p < 0.001$], LSv [$F_{(3, 12)} = 24.14, p < 0.001$], CeA [$F_{(3, 12)} = 77.04, p < 0.001$], VTA [$F_{(3, 12)} = 250.05, p < 0.001$]. In comparison to the vehicle treatment, acute PCP treatment significantly increased the number of c-Fos positive cells in the mPFC ($p < 0.001$), NAs ($p < 0.001$), NAc ($p = 0.016$), LSv ($p = 0.021$), CeA ($p < 0.001$) and VTA ($p < 0.001$), while having no detectable effect in the DLSt and MeA (Figure 4a). Acute HAL pretreatment further increased c-Fos expression induced by PCP in the NAs, NAc and VTA (all $ps < 0.05$),

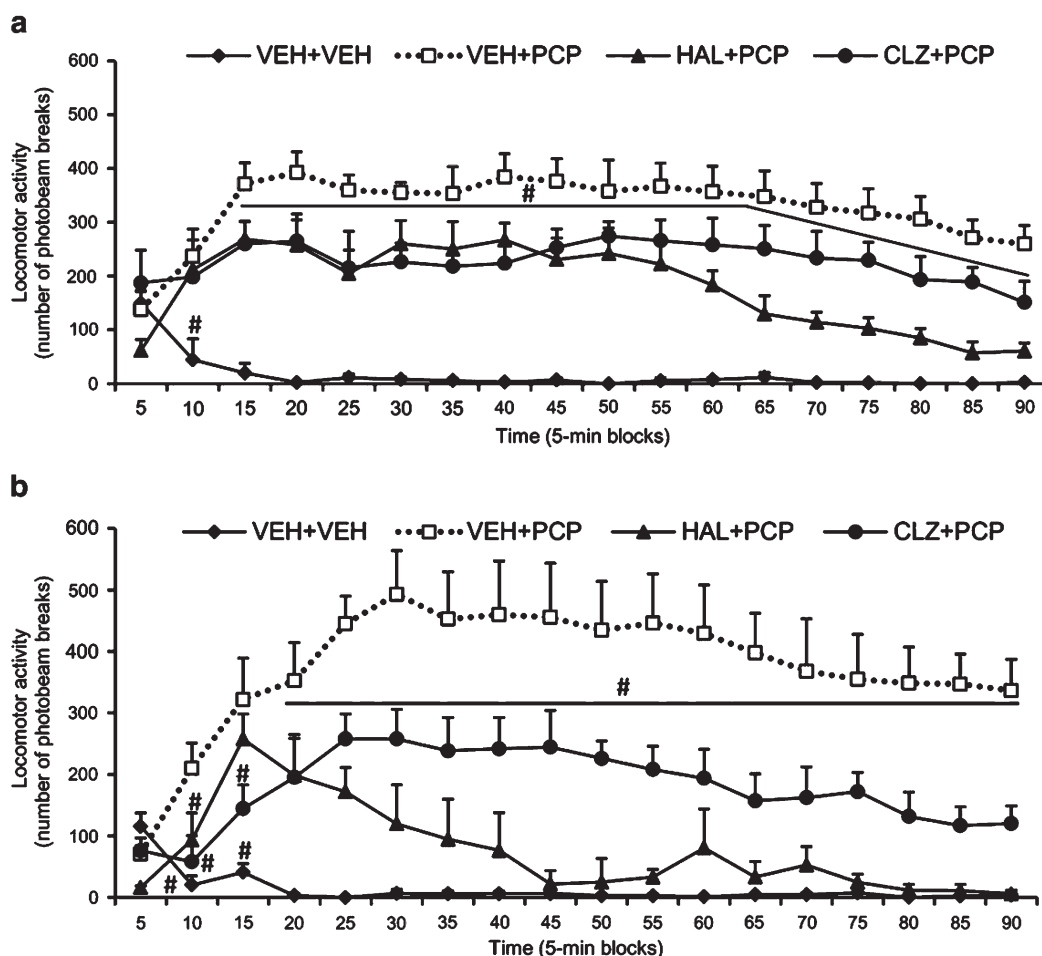


Figure 3. Time course effect of haloperidol or clozapine treatment on PCP-induced hyperlocomotion on test day 1 (a) and day 5 (b). Locomotor activity was measured over 18 5-min blocks and expressed as mean \pm SEM for each group. # $p < 0.05$ versus VEH + PCP group.

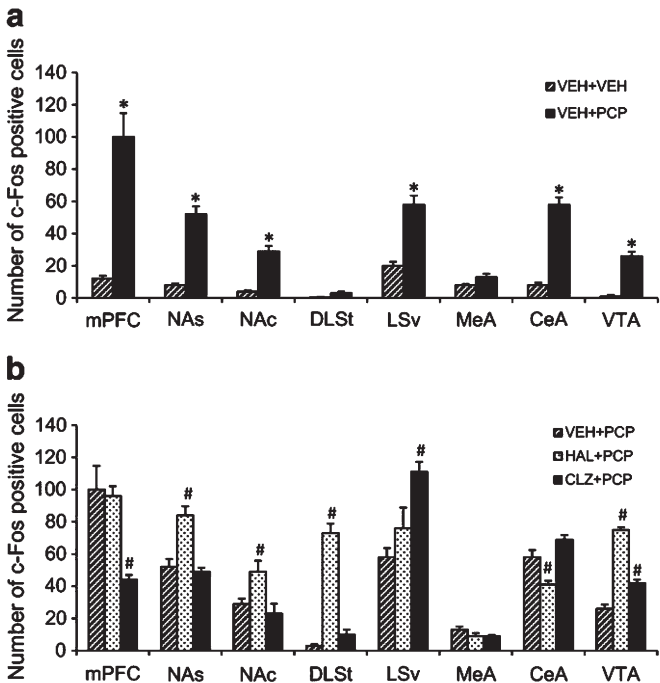


Figure 4. Effects of acute treatment of PCP alone (a), and in combination with haloperidol or clozapine (b) on c-Fos expression in rat brain. Data were expressed as mean \pm SEM. * $p < 0.05$ versus VEH + VEH control, # $p < 0.05$ versus VEH + PCP group.

but reduced it in the CeA ($p = 0.007$) (Figure 4b). HAL itself also increased c-Fos expression in the DLSt where PCP had little effect. Acute CLZ pretreatment significantly increased c-Fos expression induced by PCP in the LSv ($p = 0.002$) and VTA ($p < 0.001$), but reduced it in the mPFC ($p = 0.002$) (Figure 4b). Table 1 summarizes the distinct effects of acute HAL or CLZ pretreatment on PCP-induced c-Fos increase. Both HAL and CLZ shared a common action in the VTA in enhancing the PCP-induced c-Fos expression.

3.4. Effects of repeated HAL or CLZ treatment on PCP-induced c-Fos immunoreactivity

A one-way ANOVA revealed a significant effect in the mPFC [$F_{(3,12)} = 26.62, p < 0.001$], NAs [$F_{(3,12)} = 42.28, p < 0.001$], NAc [$F_{(3,12)} = 35.76, p < 0.001$], DLSt [$F_{(3,12)} = 62.71, p < 0.001$], LSv [$F_{(3,12)} = 51.64, p < 0.001$], CeA [$F_{(3,12)} = 94.28, p < 0.001$], VTA [$F_{(3,12)} = 85.29, p < 0.001$]. Similar to the acute effect, repeated PCP treatment significantly increased the number of c-Fos positive cells in the mPFC ($p < 0.001$), NAs ($p = 0.036$), LSv ($p < 0.001$), CeA ($p < 0.001$) and VTA ($p = 0.023$) (Figure 5a). Repeated HAL treatment resulted in an increase in PCP-induced c-Fos in the NAs, NAc and VTA (all $ps < 0.001$), while reduced it in CeA ($p < 0.001$) (Figure 5b). Repeated CLZ further increased PCP-induced c-Fos in the LSv ($p = 0.006$), CeA ($p < 0.001$) and VTA ($p = 0.001$), while reduced it in the mPFC ($p = 0.004$) (Figure 5b). Table 2 summarizes the effects of repeated HAL or CLZ treat-

Table 1. Effects of acute HAL or CLZ pretreatment on PCP-induced c-Fos expression in rat brain.

Treatment	mPFC	NAs	NAc	DLSt	LSv	MeA	CeA	VTA
VEH + PCP	Δ	Δ	Δ	\uparrow	Δ	\uparrow	Δ	Δ
HAL + PCP	—	\uparrow	\uparrow	\uparrow	—	—	\downarrow	\uparrow
CLZ + PCP	\downarrow	—	—	—	\uparrow	—	—	\uparrow

Δ , increase; \uparrow , no significant effect relative to VEH + VEH control; \uparrow , increase; \downarrow , decrease; —, no significant effect relative to VEH + PCP treatment.

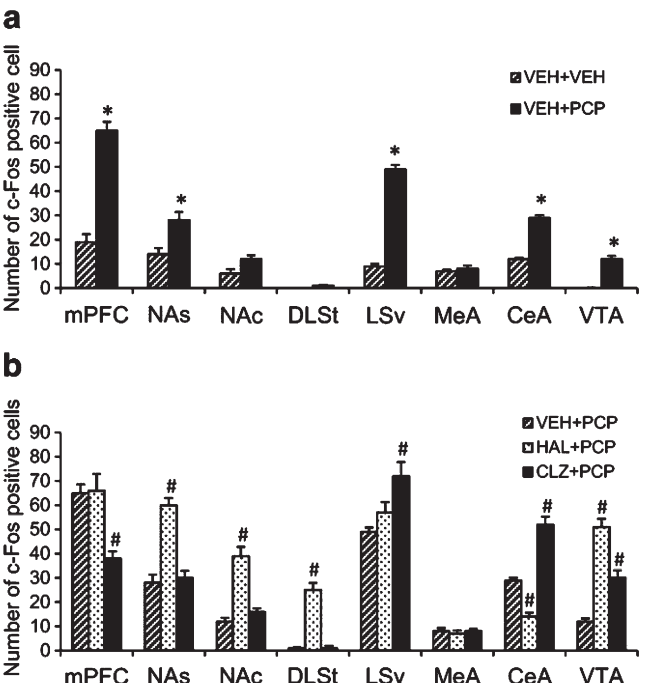


Figure 5. Effects of repeated treatment of PCP alone (a), and in combination with haloperidol or clozapine (b) on c-Fos expression in rat brain. Data were expressed as mean \pm SEM. * $p < 0.05$ versus VEH + VEH control, # $p < 0.05$ versus VEH + PCP group.

ment on PCP-induced c-Fos increase. The differences between the acute and repeated treatments of PCP, HAL and CLZ were apparent. For example, acute treatment of PCP increased Fos expression in the NAc, while repeated treatment had no effect. Repeated CLZ treatment further increased PCP-induced Fos expression in the CeA, while acute CLZ treatment had no effect (Table 1 and Table 2).

3.5. Differences in c-Fos expression between acute and repeated drug treatment

In comparison to acute effects (day 1), repeated treatment (day 5) resulted in a reduction in c-Fos immunoreactivity in most, but not all brain areas (Figure 6). The two-way ANOVA revealed a main effect of “Day” for mPFC [$F_{(1,24)} = 12.35, p = 0.002$], NAs [$F_{(1,24)} = 36.53, p < 0.001$], NAc [$F_{(1,24)} = 8.05, p = 0.009$], DLSt [$F_{(1,24)} = 70.54, p < 0.001$], LSv [$F_{(1,24)} = 20.11, p < 0.001$], CeA [$F_{(1,24)} = 91.41, p < 0.001$], and VTA [$F_{(1,24)} = 71.14, p < 0.001$]. Specifically, the number of c-Fos positive cells was significantly reduced from day 1 to day 5 in the LSv with VEH + VEH (Figure 6a, $p = 0.007$); in the mPFC ($p = 0.041$), NAs ($p = 0.006$), NAc ($p = 0.004$), CeA ($p = 0.006$) and VTA ($p = 0.003$) with VEH + PCP (Figure 6b); in the mPFC ($p = 0.015$), NAs ($p = 0.011$), DLSt ($p < 0.001$), CeA ($p < 0.001$) and VTA ($p = 0.001$) with HAL + PCP (Figure 6c); and in the mPFC ($p = 0.048$), NAs ($p = 0.003$), DLSt ($p = 0.02$), LSv ($p = 0.003$), CeA ($p = 0.007$) and VTA ($p = 0.016$) with CLZ + PCP (Figure 6d).

Table 2. Effects of repeated HAL or CLZ pretreatment on PCP-induced c-Fos expression in rat brain.

Treatment	mPFC	NAs	NAc	DLSt	LSv	MeA	CeA	VTA
VEH + PCP	Δ	Δ	\uparrow	\uparrow	Δ	\uparrow	Δ	Δ
HAL + PCP	—	\uparrow	\uparrow	\uparrow	—	—	\downarrow	\uparrow
CLZ + PCP	\downarrow	—	—	—	\uparrow	—	\uparrow	\uparrow

Δ , increase; \uparrow , no significant effect relative to VEH + VEH control; \uparrow , increase; \downarrow , decrease; —, no significant effect relative to VEH + PCP treatment.

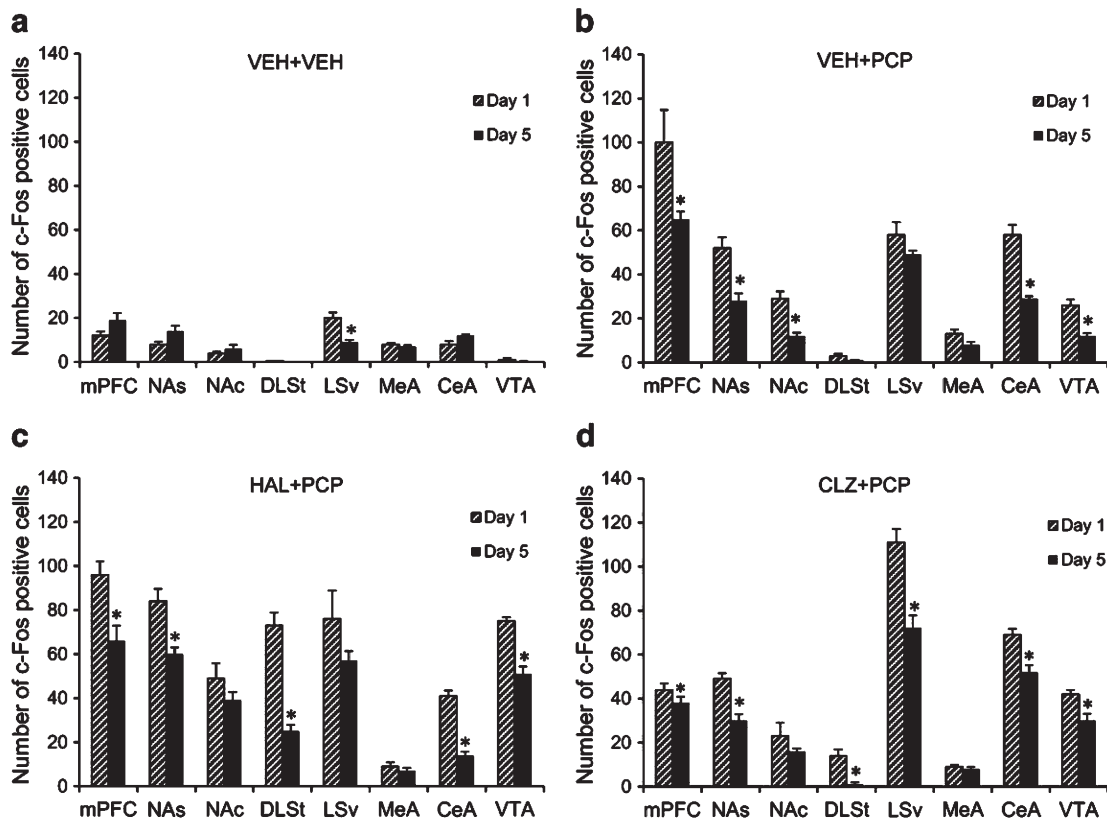


Figure 6. Effects of acute and repeated treatment of haloperidol or clozapine on PCP-induced c-Fos expression in rat brain. c-Fos expression was assessed in four distinct groups: VEH + VEH (a), VEH + PCP (b), HAL + PCP (c) and CLZ + PCP (d). Data were expressed as mean \pm SEM. * $p < 0.05$ between Day 1 and Day 5.

3.6. c-Fos identification of brain regions involved in the potentiation of inhibitory effect of repeated HAL or CLZ treatment on PCP-induced hyperlocomotion

Based on the changes of c-Fos expression, a brain region had to meet the following three criteria in order to be considered as part of the neural circuit(s) by which HAL and CLZ act to achieve their potentiated inhibitory effect on the PCP-induced hyperlocomotion. *First*, it should show altered c-Fos expression in response to both acute and repeated treatment of PCP. *Second*, it should show altered PCP-induced c-Fos expression in response to acute and repeated treatment of HAL or CLZ. *Third*, it should show a change in c-Fos expression from day 1 to day 5. Based on these criteria, three regions including NAs, CeA and VTA could be classified as part of the HAL neural circuit, and three regions including mPFC, LSv and VTA as part of the CLZ neural circuit. Table 3 summarizes the results of how these regions met these criteria.

Table 3. Brain regions considered to be part of the neural circuit through which HAL and CLZ act to achieve their potentiated inhibitory effect on PCP-induced hyperlocomotion using neuronal marker of c-Fos.

Brain regions	mPFC	NAs	LSv	CeA	VTA
Criterion 1 (PCP effect)	Δ	Δ	Δ	Δ	Δ
Criterion 2 (response to HAL)		\uparrow		\downarrow	\uparrow
Criterion 2 (response to CLZ)	\downarrow		\uparrow		\uparrow
Criterion 3 (change from acute to repeated treatment)	+	+	+	+	+

Δ , increase relative to VEH + VEH control; \uparrow , increase; \downarrow , decrease relative to VEH + PCP treatment; +, change from acute to repeated treatment.

4. Discussion

The present study differs from most other studies in the literature in which it examined the repeated effects of HAL and CLZ treatment on the neuronal activities (as indexed by the c-Fos expression) in a validated animal model of antipsychotic activity (i.e. the PCP hyperlocomotion model). Most studies in this field only focus on the *acute* effects of antipsychotic treatment in *normal* animals (Robertson and Fibiger, 1992; Robertson et al., 1994). The present study filled these knowledge gaps. Behaviorally, we replicated our previous finding that repeated antipsychotic treatment progressively potentiates the inhibition of the PCP-induced hyperlocomotion, a behavioral pattern closely resembling the characteristics of time course of antipsychotic actions in the clinic (Sun et al., 2009). Furthermore, we delineated possible neuronal mechanisms of the time course of antipsychotic action. We showed that both acute and repeated treatment of HAL significantly increased PCP-induced c-Fos expression in the NAs and VTA, while reduced it in the CeA. In contrast, CLZ treatment enhanced PCP-induced c-Fos in the LSv and VTA, but reduced it in the mPFC. More importantly, the effects of HAL and CLZ in these brain areas underwent a time-dependent reduction from day 1 to day 5, consistent with their effects at the behavioral levels. These findings indicate that these brain areas are the likely sites upon which HAL or CLZ act on to inhibit the psychomotor activation effect of PCP. Thus, we suggest that HAL may achieve its potentiated inhibitory effect on PCP-induced hyperlocomotion via acting on the NAs, CeA and VTA, while CLZ does so via the mPFC, LSv and VTA.

Phencyclidine (PCP) has been extensively used to mimic the positive and negative symptoms of schizophrenia in various animal models (Javitt and Zukin, 1991; Mouri et al., 2007; Sams-Dodd, 1998). Among many behavioral tasks, the hyperlocomotion model is commonly used as a screening tool for the

detection of antipsychotic activity. When given *acutely*, many antipsychotics inhibit the hyperlocomotor activity induced by *acute* administrations of PCP (Gleason and Shannon, 1997; Millan et al., 1999). However, this acute effect is limited in its ability to capture the intrinsic antipsychotic efficacy of a drug and mimic the time course of antipsychotic treatment in the clinic (Agid et al., 2003; Leucht et al., 2005). This is because that the inhibition of PCP induced hyperlocomotion is not exclusively the property of antipsychotics. Drugs that antagonize 5-HT_{2A} receptors but are not antipsychotics such as LY53857, ritanserin, ketanserin, fananserin, and MDL 100,907 also block PCP-induced hyperlocomotion (Gleason and Shannon, 1997; Millan et al., 1999). Therefore, the neural basis of PCP effect or antipsychotic effect identified through c-Fos immunocytochemistry based on the acute treatment model is questionable. On the other hand, repeated administration of PCP induces an array of behavioral and neurochemical changes that mimic those found in schizophrenia better than acute administration of PCP (Jentsch and Roth, 1999). More importantly, repeated antipsychotic treatment, but not anxiolytic (e.g. chlordiazepoxide) or antidepressant treatment (e.g. fluoxetine and citalopram) progressively potentiates the inhibition of the PCP-induced hyperlocomotion across sessions and prolongs this action within sessions (Redmond et al., 1999; Sun et al., 2009). Because clinical antipsychotic treatment also requires medications be taken for a prolonged period of time, and the overall antipsychotic effects show an exponential curve and reach their full therapeutic benefit in 2–3 weeks (Agid et al., 2006), we postulated that the repeated PCP-induced hyperlocomotion model based on a repeated antipsychotic treatment regimen could serve as a valid model to investigate the neurochemical and neural mechanisms of action of antipsychotic drugs. This was the rationale upon which the current c-Fos study was based on, and we believe that the brain regions identified through this approach are behaviorally and neurochemically relevant to the specific action of HAL and CLZ.

Previous work indicates that PCP treatment induces c-Fos expression in the brain in a region-specific manner. Acute PCP treatment is shown to increase c-Fos protein and mRNA expression in the mPFC, piriform, cingulate and insular cortices, nucleus accumbens (NA) and lateral septal nucleus (LS) (Gotoh et al., 2002; Habara et al., 2001; Kargieman et al., 2007; Näkki et al., 1996; Sato et al., 1997; Sugita et al., 1996). In the present study, we also found increased c-Fos expression in the mPFC, NA and LS. In addition, we found a c-Fos increase in the VTA and CeA, suggesting that the VTA and CeA, together with the mPFC, NA and LS, are parts of a neural network upon which PCP acts to achieve its behavioral effects. Indeed, PCP microinfused into the mPFC was shown to cause a hyperlocomotion, and this motor-stimulating effect was attenuated by pharmacological lesions of the mPFC (Jentsch et al., 1998). Direct infusion of PCP into the NA also increased locomotor activity (McCullough and Salamone, 1992). Systemic and site-directed injection of PCP to the NA produced an increase in extracellular dopamine levels in the NA (Carboni et al., 1989; Hernandez et al., 1988; McCullough and Salamone, 1992; Millan et al., 1999). Depletion of dopamine by microinjection of 6-hydroxydopamine (6-OHDA) into the NA or VTA inhibited the PCP-induced hyperlocomotion (French, 1986; Steinpreis and Salamone, 1993). Finally, systemic administration of HAL significantly reduced the hyperlocomotion induced by intra-accumbens injection of PCP (McCullough and Salamone, 1992). All these findings support the hypothesis that PCP affects multiple brain areas including the mPFC, NA (shell and core), CeA and VTA to achieve its psychotomimetic effect.

If this hypothesis is correct, we would also expect that pretreatment of HAL or CLZ should alter PCP-induced c-Fos expression in these brain areas. Indeed, we found that both acute and repeated pretreatment with HAL significantly increased PCP-induced c-Fos expression in the NAs and VTA, while reduced it in CeA. Similarly, CLZ pretreatment further increased PCP-induced c-Fos in the LSv and VTA, while reduced it in the mPFC (Figs. 4b, 5b). Interestingly, the c-Fos expression in

these areas underwent a time-dependent reduction from day 1 to day 5, consistent with the observed HAL and CLZ potentiated inhibition of PCP-induced hyperlocomotion from day 1 to day 5. This pattern of change in c-Fos expression over the repeated treatment period has been reported before in the studies of drugs of abuse (Hope et al., 1992; Persico et al., 1993; Rosen et al., 1994; Salminen et al., 1999), antipsychotic drugs (Atkins et al., 1999; Coppens et al., 1995; Hiroi and Graybiel, 1996; Sebens et al., 1995) and chronic stress (Melia et al., 1994; Stamp and Herbert, 1999; Umemoto et al., 1997). The exact mechanism responsible for this time-dependent c-Fos reduction is still not clear. It may be an intrinsic neuroadaptation of the brain in response to various stimuli.

The mechanism of action of PCP is complex (Jentsch and Roth, 1999). Besides blocking NMDA receptor channels, PCP can enhance serotonergic, dopaminergic and glutamatergic neurotransmission in the NA and PFC (Abekawa et al., 2007; Maurel-Remy et al., 1995; Millan et al., 1999). Because typical antipsychotics with preferential action on D₂ receptors such as HAL, and atypicals drugs with mixed D₂-like/5-HT_{2A} antagonism such as CLZ and olanzapine all inhibit the PCP-induced hyperlocomotion (Gleason and Shannon, 1997; Maurel-Remy et al., 1995; Millan et al., 1999), and other 5-HT_{2A} antagonists such as LY53857, ritanserin, ketanserin, fananserin, and MDL 100,907, but not 5-HT_{1A} or 5-HT₃ antagonists such as WAY 100,635 and zatosetron, also reduce the PCP-induced hyperlocomotion (Gleason and Shannon, 1997; Millan et al., 1999), it has been suggested that the inhibition of PCP-induced hyperlocomotion is primarily due to multiple actions of antipsychotics on dopamine D₂ and 5-HT_{2A} receptors (Gleason and Shannon, 1997; Maurel-Remy et al., 1995; Millan et al., 1999). This hypothesis is further supported by the findings that depletion of 5-HT in the NA by parachloroamphetamine abolishes PCP-induced hyperlocomotion (Millan et al., 1999); M100907, a selective 5-HT_{2A} receptor antagonist, attenuated PCP-induced c-Fos in the NA, insular cortex and piriform cortex (Habara et al., 2001); and blockade of dopamine D₂ receptors reduced the extracellular dopamine levels in the NA (McCullough and Salamone, 1992). So for HAL, we suggest that its potentiated inhibition of the PCP-induced hyperlocomotion is due to its D₂ antagonism in the NAs, CeA or VTA which leads to a reduction of PCP-induced dopamine increase. Repeated administration of CLZ causes a down-regulation of 5-HT_{2A} receptors in the PFC (Doat-Meyerhoefer et al., 2005), microinjection of PCP into the prefrontal cortex elicits hyperlocomotion (Abekawa et al., 2007), we speculate that CLZ may enhance its inhibition of the PCP-induced hyperlocomotion by down-regulating 5-HT_{2A} receptors and concomitantly decreasing PCP-induced glutamate, dopamine and 5-HT increases in the mPFC, LSv and VTA (Abekawa et al., 2007).

In conclusion, using the Fos immunoreactivity as markers of neuronal activation, we showed that typical antipsychotic HAL appears to achieve its potentiated inhibitory effect on the PCP-induced hyperlocomotion primarily via acting on the NAs, CeA and VTA, while CLZ works primarily via the mPFC, LSv and VTA. It should be pointed out that while c-Fos is an important step in illuminating the differences in neuronal actions between haloperidol and clozapine in this task, these data should be regarded as one piece of evidence toward delineating the neural basis of these drug effects. Thus, other indices such as neurotransmitter release, receptor density changes should be used to validate the current findings in future work.

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