Clozapine ameliorates epigenetic and behavioral abnormalities induced by phencyclidine through activation of dopamine D1 receptor

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Abstract

Accumulating evidence suggests that dysregulation of histone modification is involved in the pathogenesis and/or pathophysiology of psychiatric disorders. However, the abnormalities in histone modification in the animal model of schizophrenia and the efficacy of antipsychotics for such abnormalities remain unclear. Here, we investigated the involvement of histone modification in phencyclidine-induced behavioral abnormalities and the effects of antipsychotics on these abnormalities. After repeated phencyclidine (10 mg/kg) treatment for 14 consecutive days, mice were treated with antipsychotics (clozapine or haloperidol) or the histone deacetylase inhibitor sodium butyrate for 7 d. Repeated phencyclidine treatments induced memory impairment and social deficit in the mice. The acetylation of histone H3 at lysine 9 residues decreased in the prefrontal cortex with phencyclidine treatment, whereas the expression level of histone deacetylase 5 increased. In addition, the phosphorylation of Ca2+/calmodulin-dependent protein kinase II in the nucleus decreased in the prefrontal cortex of phencyclidine-treated mice. These behavioral and epigenetic changes in phencyclidine-treated mice were attenuated by clozapine and sodium butyrate but not by haloperidol. The dopamine D1 receptor antagonist SCH-23390 blocked the ameliorating effects of clozapine but not of sodium butyrate. Furthermore, clozapine and sodium butyrate attenuated the decrease in expression level of GABAergic system-related genes in the prefrontal cortex of phencyclidine-treated mice. These findings suggest that the antipsychotic effect of clozapine develops, at least in part, through epigenetic modification by activation of the dopamine D1 receptor in the prefrontal cortex.

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Introduction

The results of recent studies have led to the notion that epigenetic mechanisms, which exert lasting control over gene expression without altering the genetic code, could mediate stable changes in brain function (Levenson and Sweatt, 2005; Tsankova et al., 2007; Kazantsev and Thompson, 2008). In particular, chromatin remodeling by histone modification is widely recognized as a crucial mechanism with respect to numerous aspects of the brain, including neuronal differentiation, neurodegeneration, circadian rhythm, seizure, memory formation, drug addiction, and psychiatric disorders (Tsankova et al., 2006, 2007). Accumulating evidence suggests that dysregulation of histone modification is involved in the pathogenesis and/or pathophysiology of psychiatric disorders. It has been reported that in individuals with schizophrenia, the expression level of histone deacetylase 1 increases, whereas the acetylation levels of histone H3 at the lysine 9 and 14 residues decrease (Gavin et al., 2008; Sharma et al., 2008).

Several reports have demonstrated that dysregulation of histone modification is evident in the animal model of psychiatric disorders. Chronic defeat stress induces depressive behavioral abnormalities associated with the specific dimethylation of histone H3 at lysine 27 residues in the Bdnf (brain-derived neurotrophic factor) gene (Tsankova et al., 2006). Recently, we reported that an animal model combining genetic factors and environmental...
stressors may be a promising model for psychotic depression. In this mouse model, stress induced an epigenetic abnormality in the tyrosine hydroxylase gene (Niwa et al., 2013). Although antipsychotics cause histone modification (Dong et al., 2008), it remains unclear whether histone modification is required to induce the antipsychotic effect.

Dopaminergic malfunction in the prefrontal cortex plays an important role in cognitive deficits in schizophrenia (Winterer and Weinberger, 2004). In the animal models of schizophrenia, it is suggested that dysfunction of dopaminergic systems encompasses reduction of signaling via dopamine receptors (Jentsch et al., 1997a, b). Previously, we have shown that decrease of dopamine D1 receptor signaling is associated with cognitive dysfunction in animal model of schizophrenia (Mouri et al., 2007b). Dopamine D1 signaling regulates histone modification (Schroeder et al., 2008). But, it is not clear whether dysregulation of dopamine D1 receptor signaling brings epigenetic abnormalities in schizophrenia. Moreover, clinical studies have suggested that aberrations of gene expressions of other parameters are implicated in the pathogenesis and/or pathophysiology of schizophrenia (Lewis et al., 1999; Guidotti et al., 2000; Itokawa et al., 2003). Among them, aberrations of gamma-aminobutyric acid (GABA)-related gene expression were widely observed in both schizophrenia patients and animal models (Volk et al., 2000; Toyooka et al., 2002; Hashimoto et al., 2003). Previous study reported that atypical antipsychotics, especially clozapine (CLZ), attenuate the protracted hypermethylation of cortical GAD67 promoters induced by methionine (MET) administration (Dong et al., 2008). However, the involvement of epigenetic changes in the aberrations of GABA-related gene expressions in schizophrenia is still unclear.

To clarify the pathogenesis and/or pathophysiology of psychiatric disorders, validated animal models are needed. Phencyclidine (PCP), a noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist, reproduces schizophrenia-like psychosis, including positive symptoms, negative symptoms, and cognitive dysfunction in humans (Luby et al., 1959; Rainey and Crowder, 1975; Allen and Young, 1978; Cosgrove and Newell, 1991; Javitt and Zukin, 1991). In rodents, chronic PCP treatment has been shown to induce schizophrenia-like abnormal behaviors such as hyperlocomotion (Nagai et al., 2003; Takahata and Moghaddam, 2003) and impairment of social interaction (Qiao et al., 2001), motivation (Noda et al., 2000), cognitive function (Li et al., 2003; Nagai et al., 2009), and attention (Mouri et al., 2007b). Therefore, PCP-treated animals are well known to be useful pharmacological models of schizophrenia (Mouri et al., 2007a). Indeed, on the basis of a study involving such an animal model, we had proposed that an enriched environment during adolescence might prevent the suffering from schizophrenia through modification of the epigenetic machinery (Koseki et al., 2012).

The present study was designed to investigate the involvement of histone modification in the effects of antipsychotics on PCP-induced behavioral abnormalities. We found that histone H3 acetylation at lysine 9 residues decreased in the prefrontal cortex of PCP-treated mice. CLZ attenuated the PCP-induced abnormal behaviors and the histone deacetylation in a dopamine D1 receptor-dependent manner; CLZ restored the changes in the nuclear localization of histone deacetylase 5 and phosphorylated Ca2+/calmodulin-dependent protein kinase II (CaM kinase II) as well as the decrease in gamma-aminobutyric acidergic (GABAergic) nervous system-related genes expression in the prefrontal cortex of PCP-treated mice.

Materials and methods

Mice

Male Institute of Cancer Research (ICR) mice (6 wk old; n=400) were obtained from Japan SLC (Japan). The animals were housed in plastic cages and kept in a regulated environment (24±1°C, 50±5% humidity), with a 12-h light/dark cycle (lights on at 8:00 A.M.). Food and tap water were available ad libitum. All experiments were performed in accordance with the Guidelines for Animal Experiments of Meijo University, Faculty of Pharmaceutical Sciences. The procedures involving animals and their care were conducted in conformity with the international guidelines – Principles of Laboratory Animal Care (National Institutes of Health publication 85-23, revised 1985). Different batches of mice were used for behavioral and biochemical experiments.

Drugs and treatment

Piperidine hydrochloride (PCP) (1-(1-phenylcyclohexyl) PCP) was synthesized by the authors according to the method reported by Maddox et al. (1965) and was checked for purity. The PCP and sodium butyrate (SB; Wako, Japan) were dissolved in saline solution. An injectable solution of haloperidol (HAL; 5 mg/ml; Mitsubishi Tanabe Pharma, Japan) was diluted with saline. CLZ (Sigma-Aldrich, USA) was dissolved in a minimum amount of 0.1 N HCl and then diluted with saline, as previously described (Qiao et al., 2001). SCH-23390 hydrochloride (SCH; Research Biochemicals International, USA) was prepared as a stock solution of 1 mg/ml in saline and dissolved in saline before the experiments. All compounds were administered in a volume of 0.1 ml/10 g body weight. PCP (10 mg/kg, s.c.) or saline (10 ml/kg, s.c.) was injected once a day for 14 consecutive days into the 6-wk-old mice. After chronic PCP or saline treatment, HAL (1.0 mg/kg, p.o.), CLZ (10 mg/kg, p.o.), or SB (0.2, 1.0, or 2.0 g/kg, i.p.) was injected once a day for 7 consecutive days. In the present study, doses of CLZ and HAL were referred to our previous report (Qiao et al., 2001). SCH (0.1 mg/kg, s.c.) or saline was injected 30 min before the CLZ or SB injection.
Novel object recognition test

The novel object recognition test was conducted as described previously (Nagai et al., 2009), with minor modifications. The experimental apparatus consisted of a Plexiglas box (30 cm length × 30 cm width × 35 cm height), with the floor covered in sawdust. The test procedure consisted of three sessions: habituation, training, and retention. Each mouse was individually habituated to the box, with 10 min of exploration in the absence of objects each day for 3 consecutive days (habituation session). On day 4, two novel objects were symmetrically placed on the floor of the box, and each animal was allowed to explore the box for 10 min (training session). The objects differed in shape and color but were similar in size. An animal was considered to be exploring the object when its head was facing the object or it was touching or sniffing the object. After the training sessions, the mice were immediately returned to their home cages. The animals were returned to the same box with 1 of the familiar objects from the training session and 1 novel object, at 24 h after the training sessions. They were allowed to explore freely for 10 min, and the time spent exploring each object was recorded on a video tape. A preference index, a ratio of the amount of time spent exploring any 1 of the 2 objects (training session) or the novel object (retention session) over the total time spent exploring both objects, was used to measure cognitive function.

Social interaction test

The social interaction test was conducted as described previously (Qiao et al., 2001), with minor modifications. The apparatus used for the social interaction test consisted of a square open arena (25 cm length × 25 cm width × 30 cm height) made of gray non-reflecting acrylics, illuminated with lamps that could not be seen by the mice directly. The light was diffused to minimize shadows in the arena. Before the test, each mouse (including a social partner mouse) was placed alone in the apparatus for 10 min on two consecutive days (habituation). On the testing day, the test mouse was randomly assigned to another male ICR mouse of the same age, used as the unfamiliar partner. The mouse and its unfamiliar partner were placed in the test box for 10 min and the time spent in active social interaction (such as sniffing, following, mounting, and crawling under or over the partner) was measured and recorded on a video tape for 10 min.

Nuclear extraction and Western blot analysis

Western blot analysis was performed as previously described (Mouri et al., 2007b). The mice were sacrificed by decapitation, and the brain was immediately removed. The prefrontal cortex containing cingulate and prelimbic area (Bregma +2.96 to Bregma +1.34) are defined in the atlas of Franklin and Paxinos (2008). To prepare tissue extracts, the dissected brain tissue was homogenized using a pestle-type homogenizer in an ice-cold hypotonic buffer (25 mM KCl, 10 mM Tris- HCl [pH 7.4], 10 mM NaCl, 1 mM MgCl2, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM NaF, 20 μg/ml pepstatin, 20 μg/ml aprotinin, and 20 μg/ml leupeptin). After centrifugation at 1000 rpm for 10 min, the supernatant was again centrifuged at 13200 rpm for 60 min, and the supernatant was used as the cytosolic fraction. The pellet was washed in phosphate-buffered saline (PBS) and was resuspended in ice-cold lysis buffer (10 mM Tris- HCl [pH 7.4], 10% glycerol, 0.5% Triton X-100, 150 mM NaCl, 20 μg/ml pepstatin, 20 μg/ml aprotinin, and 20 μg/ml leupeptin). After centrifugation at 13200 rpm for 60 min, the pellet washed in PBS was homogenized by sonication in ice-cold radio immunoprecipitation assay (RIPA) buffer (20 mM Tris- HCl [pH 7.4], 150 mM NaCl, 50 mM NaF, 2 mM EDTA, 0.1% sodium deoxycholate [SDS], 1% sodium deoxycholate, 1% Triton X-100, 1 mM sodium orthovanadate, 20 μg/ml pepstatin, 20 μg/ml aprotinin, and 20 μg/ml leupeptin), and the solution was used as the nuclear fraction. The protein concentration was determined using a BCA Protein Assay Kit (Thermo Scientific, USA). Samples (10 μg of protein) were boiled in a sample buffer (125 mM Tris- HCl [pH 6.8], 10% 2-mercaptoethanol, 4% sodium diphosphate decahydrate, 10% sucrose, and 0.0004% Bromophenol Blue), separated on a polyacrylamide gel, and subsequently transferred to polyvinylidene difluoride membranes (Millipore Corporation, USA). The membranes were blocked with a Detector Block Kit (Kirkegaard and Perry Laboratories, USA) or 3% bovine serum albumin and probed with a primary antibody. The membranes were washed with a washing buffer (50 mM Tris- HCl [pH 7.4], 0.05% Tween 20, and 150 mM NaCl) and were subsequently incubated with a horseradish peroxidase-conjugated secondary antibody. The immune complexes were detected using ChemiDoc XRS (Bio-Rad Laboratories, Inc., USA) with a chemiluminescence system (ECL Plus Western Blotting Detection System; GE Healthcare, UK). The band intensities were analyzed by densitometry by using the ATTO Densitograph Software Library Lane Analyzer (ATTO, Japan). After detection of protein expression, the membranes were stripped with a stripping buffer (125 mM Tris- HCl [pH 6.7]) at 55 °C for 30 min, and the total protein expression was detected as described above. The primary polyclonal rabbit antibodies were anti-acetyl-histone H3 (Lys9) (1:500; Millipore Corporation), anti-acetyl-histone H3 (1:500; Millipore Corporation), anti-phospho-CaM kinase II α/β (T286/287) (1:1000; Millipore Corporation), anti-CaM kinase II α (1:2000; Sigma-Aldrich), anti-lamin A/C (1:500; Cell Signaling Technology Inc., USA), previous reports (Mouri et al., 2007b; Murai et al., 2007), the prefrontal cortex containing cingulate and prelimbic area (Bregma +2.96 to Bregma +1.34) are defined in the atlas of Franklin and Paxinos (2008). 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anti-histone deacetylase 5 (1:1000; Cell Signaling Technology Inc.), and goat anti-actin (C-11) (1:2000; Santa Cruz Biotechnology, USA) antibodies. The secondary antibodies (all from Kirkegaard and Perry Laboratories), used at a 1:2000 dilution, were horseradish peroxidase-linked anti-rabbit IgG and anti-goat IgG antibodies.

Real-time reverse transcription polymerase chain reaction

The prefrontal cortex within a month of storing at −80 °C was homogenized and total RNA was isolated using an RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer’s instructions. Isolated RNA was pure RNA because the A260/A280 ratio (purity of RNA) of extracted RNA from all samples was within the range of 1.9 to 2.1. Extracted RNA was converted into cDNA by using a SuperScript™ First-Strand System for RT-PCR Kit (Invitrogen, USA) in a final volume of 20 μl, according to the manufacturer’s instructions. A total of 1 μg of RNA per sample was used. Following first strand cDNA synthesis, the reaction volume was increased to 25 and 1 μl of this was used for each polymerase chain reaction. The levels of glutamic acid decarboxylase 1 (Gad1), GABA A receptor subunit alpha 1 (Gabra1), GABA A receptor subunit beta 2 (Gabrb2), and parvalbumin (Pvalb) mRNAs were determined by real-time reverse transcription polymerase chain reaction (RT-PCR) by using a TaqMan probe. Beta-actin (Actb) mRNA was used as the internal control. The primers used for real-time RT-PCR have been described in Table 1. In a preliminary experiment for the validation of a method based on the PCR, the amplification efficiencies, calculated from the slopes of the standard curves, of these primers using dilution series of cDNA were between 90 and 110%. Real-time RT-PCRs were performed using the Platinum TaqDNA polymerase (Invitrogen) according to the manufacturer’s instructions. The amplification consisted of an initial step at 95 °C for 5 min and then 40 cycles of denaturation for 30 s at 95 °C, annealing for 40 s at 59 °C, and an extension time of 1 min at 72 °C in an iCycler iQ Detection System (Bio-Rad Laboratories). Expression levels were calculated by the ΔΔCt method. Sample and negative controls (no template) were analyzed in duplicate.

Preparation and staining of brain slices

Histological procedures were performed as previously described, with minor modifications (Murai et al., 2007). The mice were anesthetized with urethane (1.5 g/kg i.p.) and perfused transcardially with ice-cold saline, followed by 4% paraformaldehyde in PBS. The brains were removed, postfixed in the same fixative for 12 h, and then soaked in up to 30% (w/v) sucrose in PBS. Coronal sections (20 μm thick) were cut with a Cryostar HM560 cryostat (Microm International, GmbH, Germany). For immunohistochemistry, the primary antibodies such as rabbit anti-acetyl-histone H3 (Lys9) (1:500; Millipore Corporation) and mouse anti-dopamine D1A receptor, clone SG2-D1a (1:200; Millipore Corporation), antibodies were applied to the brain slices. Fluorescently conjugated secondary antibodies (Alexa 488, 546; Invitrogen) were used. Images were acquired with a confocal microscope (LSM510; Carl Zeiss, Germany).

Statistical analysis

All results have been expressed as the mean±S.E.M. values for each group. The difference between groups was analyzed with a one-way, two-way, or repeated measured analysis of variance (ANOVA), followed by the post-hoc Tukey test. A value of p<0.05 was regarded as being statistically significant.

Results

CLZ, but not HAL, improves PCP-induced behavioral deficits

We investigated the effect of antipsychotics on the PCP-induced behavioral impairments in mice. The mice were administered PCP (10 mg/kg, s.c.) for 14 d, after which they were treated with antipsychotics for 7 d. A novel object recognition test and a social interaction test were carried out 24 h after the last treatment with antipsychotics (8 d after PCP withdrawal) (Fig. 1a). The habituation session (day 19–21) in the novel object recognition test was conducted at least 6 h later following antipsychotics and other treatment. The habituation session and test in social interaction test were conducted on day

Table 1. Primers for gamma-aminobutyric acid (GABA)-related and other genes

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>TaqMan probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gad1</td>
<td>GATTGAAAAACCAGATCAAC</td>
<td>AAAACCATCTCAAACCTCTTC</td>
<td>CCTGGAGCTGGCTATTACC</td>
</tr>
<tr>
<td>Gabra1</td>
<td>TTATACAAGAGCAGAAGTTG</td>
<td>CATACGTGTTAAACGTGAC</td>
<td>TCTGCTCAAACACTGAACGG</td>
</tr>
<tr>
<td>Gabrb2</td>
<td>CAATTCTGATTACCCTCTCT</td>
<td>AGTTGAATTCCTAATGGCAA</td>
<td>CAGCAGAGCCATGTTAATCCCA</td>
</tr>
<tr>
<td>Pvalb</td>
<td>ACTGTCGCTGAAAAACACA</td>
<td>AGAATTCTTCAACCCAAATC</td>
<td>CACATCTTGTCTCCAGGCG</td>
</tr>
<tr>
<td>Actb</td>
<td>GGGCCTATGCTCTCCCCACG</td>
<td>GTCACGCAGATTTCCCTCTC</td>
<td>CCTGGCTTGACCTGGCTTG</td>
</tr>
</tbody>
</table>

Gad1 (Glutamic acid decarboxylase 1), Gabra1 (GABA A receptor subunit alpha 1), Gabrb2 (GABA A receptor subunit beta 2), Pvalb (Parvalbumin), Actb (Actin, beta).
24, 25, and on day 26, respectively. In the training session of the novel object recognition test, PCP- and saline-treated mice spent equal amounts of time exploring either of the two objects (Fig. 1b). When the retention session was performed 24 h after the training session, the level of exploratory preference for the novel objects in the PCP-treated mice was significantly lower than that for the saline-treated mice (Fig. 1b). The 7-d treatments with CLZ (10 mg/kg, p.o.), but not HAL (1.0 mg/kg, p.o.), significantly improved the impairment of recognition memory in the PCP-treated mice (Fig. 1b). One day after the novel object recognition test, a social interaction test was carried out. The social interaction time for the unfamiliar ICR mice decreased in the PCP-treated mice (Fig. 1c). Again, the 7-d treatment with CLZ, but not HAL, significantly improved the social deficit in the PCP-treated mice.
These antipsychotics had no effect on the exploratory preference for novel object and social interaction time in the saline-treated mice (Fig. 1c). In novel object recognition test, total time spent in the exploration to objects in the training and retention session did not differ among all groups (Table 2A). These observations suggest that PCP and antipsychotics treatment has no effect on motivation, curiosity, or motor function.

Decrease in the acetylation of histone H3 at lysine 9 residues in the prefrontal cortex is associated with PCP-induced behavioral deficits

Acetylated histone H3 levels decrease in individuals with schizophrenia (Gavin et al., 2008). Hypofrontality is responsible for the emotional and cognitive deficits in PCP-treated mice (Nabeshima et al., 2006). To examine the relationship between epigenetic changes and PCP-induced behavioral impairments, we investigated the effect of repeated PCP treatments on histone H3 acetylation in the prefrontal cortex at 8 d after the withdrawal of PCP. The repeated PCP treatments significantly decreased the acetylation level of histone H3 at lysine 9 residues (Ac-H3K9) in the prefrontal cortex (Fig. 2a). Interestingly, the PCP-induced reduction in the acetylation level at H3K9 was restored by the 7-d treatments with CLZ, but not with HAL (Fig. 2a). Furthermore, the PCP-induced reduction of the acetylation level of H3K9 in the prefrontal cortex was restored by the histone deacetylase (HDAC) inhibitor SB (1.0 g/kg, i.p.) (Fig. 2b). In the expression level of H3, there was no difference among the all groups (in Fig. 2a; SAL/SAL: 100±7.36, PCP/SAL: 106.38±6.26, PCP/CLZ: 98.90±3.73, PCP/HAL: 111.00±4.55, in Fig. 2b; SAL/SAL: 100±10.58, PCP/SAL: 107.91±8.07, PCP/SB: 90.61±6.64). Repeated PCP treatments had no effect on the histone H3 acetylation at lysine 14 residues (Ac-H3K14) and the phosphorylation at serine 10 residues (P-H3S10), whereas SB significantly increased the expression levels of Ac-H3K14 and P-H3S10 in the prefrontal cortex of PCP-treated mice at 24 h after the last treatment (Table 3E). In saline-treated mice, CLZ and SB, but not HAL, increased the histone modifications (Ac-H3K9, Ac-K14, and P-H3S10) (Table 3A, D).

Next, we investigated the effect of SB on the PCP-induced behavioral impairments. The PCP-treated mice were administered SB (0.2, 1.0, or 2.0 g/kg, i.p.) for 7 d and then subjected to novel object recognition and social interaction tests (Fig. 1a). The 7-d treatments with SB significantly ameliorated the recognition memory impairment (Fig. 2c) and social deficit (Fig. 2d) in PCP-treated mice. SB did not affect the exploratory preference for novel objects and the social interaction time in salinetreated mice (Fig. 2c,d). In novel object recognition test, total time spent in the exploration to objects in the training and retention session did not differ among all groups (Table 2B). These observations suggest that SB treatment has no effect on motivation, curiosity, or motor function.

The dopamine D1 receptor is involved in the ameliorating effect of CLZ on PCP-induced epigenetic and behavioral abnormalities

We investigated the localization of Ac-H3K9 in the prefrontal cortex of PCP-treated mice at 1 h after acute CLZ...
Histone H3 acetylation was mainly observed in dopamine D1 receptor-positive cells in the prefrontal cortex of PCP/CLZ-treated mice (Fig. 3a). To explore the involvement of the dopamine D1 receptor in the CLZ-induced acetylation of histone H3, we investigated the effect of the dopamine D1 receptor antagonist SCH-23390 on the CLZ-induced histone H3 acetylation in the prefrontal cortex of the PCP-treated mice. SCH significantly blocked the CLZ-induced increase in Ac-H3K9 (Fig. 3b).

In the expression level of H3, there was no difference among the all groups (Fig. 3b; SAL/SAL: 100 ±7.75, PCP/SAL: 109.63±7.41, PCP/CLZ: 104.35±6.41, PCP/CLZ/SCH: 105.18±7.74). Although repeated PCP treatments did not affect Ac-H3K14 and P-H3S10, CLZ significantly increased P-H3S10 in the prefrontal cortex of the PCP-treated mice. SCH tended to block the CLZ-induced P-H3S10 (Table 3C). Similarly, SCH blocked the ameliorating effect of CLZ, but not of SB, on recognition memory impairment and social deficit induced by PCP (Fig. 3c,d). In novel object recognition test, total...
time spent in the exploration to objects in the training and retention session did not differ among all groups (Table 2C).

**CLZ attenuated PCP-induced changes in the intracellular localization of HDAC 5 and decrease in the phosphorylated, but not total, CaM Kinase II level in the nucleus through the dopamine D1 receptor**

A shift in intracellular HDAC distribution from the nucleus to the cytosol allows for changes in the histone acetylation level (Renthal et al., 2007). To explore this point in the modulation of histone acetylation by PCP and CLZ, we measured the HDAC 5 content in nuclear and cytosolic extracts in the prefrontal cortex of PCP- and CLZ-treated mice. Repeated PCP treatments significantly increased the HDAC 5 content in the nuclear fraction, whereas cytosolic HDAC 5 decreased in extracts from the PCP-treated mice. These changes in HDAC 5 distribution in the nuclear and cytosolic extracts were reversed by CLZ, which was blocked by SCH (Fig. 4a,b).

Phosphorylated CaM kinase II (phospho-CaM kinase II) modulates the nuclear localization of HDAC 5 (Chawla et al., 2003). We investigated the effects of PCP and CLZ treatments on the phospho-CaM kinase II content in nuclear extracts from the prefrontal cortex. Repeated PCP treatments decreased the phospho-CaM kinase II content in the nuclear extract (Fig. 4c), but this effect was reversed by CLZ (Fig. 4c). SCH partially blocked the ameliorating effect of CLZ (Fig. 4c). There was no change in the total CaM kinase II content in the nuclear extracts for all the groups (Fig. 4d).

**CLZ and SB attenuate PCP-induced down-regulation of the GABAergic system-related genes**

Since chronic administration of PCP induces GABAergic dysfunction in the prefrontal cortex (Toyooka et al., 2002), we investigated the effects of PCP and CLZ treatments on the expression of GABAergic system-related genes, such as Gad1, Gabra1, Gabrb2, and Pvalb, in the prefrontal cortex. PCP treatment significantly decreased the mRNA expression of Gad1, Gabra1, and Pvalb. CLZ attenuated the PCP-induced decrease in the mRNA expression of Pvalb but not of Gad1 or Gabra1 (Fig. 5a,b,d and Table 4B). There was no difference in the Gabrb2 mRNA level for all the groups (Fig. 5c). To examine the involvement of HDAC in the PCP-induced decrease in GABAergic system-related genes expression, we investigated the effect of SB on the PCP-induced decrease in Gad1, Gabra1, and Pvalb mRNA in the prefrontal cortex. SB (1.0 g/kg, i.p.) attenuated the PCP-induced decrease in Gabra1 and Pvalb, but not Gad1, mRNA expression (Fig. 5e,f,h and Table 4B). SB also increased the Gabrb2 mRNA expression in the prefrontal cortex (Fig. 5g). There was no significant difference between SAL/SAL and SAL/CLZ, and between SAL/SAL and SAL/SB in the four different mRNA of GABAergic genes.
Thus, CLZ and SB treatment had no effect on the expression of these genes in saline-treated mice.

Discussion

Recent studies have suggested that chromatin remodeling by histone modification may be involved in the pathogenesis and/or pathophysiology of psychiatric disorders, including schizophrenia (Tsankova et al., 2006). Long-term treatment with antipsychotics is often necessary for the improvement of schizophrenic symptoms (Lieberman et al., 2005; Lewis and Gonzalez-Burgos, 2006). Although it has been suggested that gene expression is required for the efficacy of long-term antipsychotic treatments, the involvement of epigenetic changes is still unclear. In the present study, we demonstrated the involvement of epigenetic changes in PCP-induced abnormal behaviors and the effects of antipsychotics on these abnormalities.
Repeated PCP treatments induce abnormal behaviors such as hyperlocomotion, social deficit, decrease in motivation, and cognitive dysfunctions (Javitt and Zukin, 1991; Noda et al., 2000; Qiao et al., 2001; Li et al., 2003; Nagai et al., 2003, 2009; Takahata and Moghaddam, 2003; Mouri et al., 2007b). Typical antipsychotics improve only hyperlocomotion, but atypical antipsychotics improve not only hyperlocomotion but also social deficit, decrease of motivation, and cognitive dysfunctions in this animal model (Phillips et al., 2001;
Grayson et al., 2007; Mouri et al., 2007a). Thus, the repeated PCP treatment animal model might be valuable for developing novel antipsychotics such as aripiprazole (Nagai et al., 2003, 2009; Neill et al., 2010). In this study, repeated PCP treatments (10 mg/kg/d for 14 d) induced social deficit in the social interaction test and impairment of recognition memory in the novel object recognition test; both of these effects were attenuated by the atypical antipsychotic CLZ but not by the typical antipsychotic HAL.

Gavin et al. (2008) observed dysregulation of histone modification, such as increase in histone deacetylase 1 and decrease in Ac-H3K9 and Ac-H3K14 in individuals with schizophrenia. Changes in gene expression that are associated with dysregulation of epigenetic modification lead to behavioral deficits in animal models for mental disorders (Tsankova et al., 2006; Koseki et al., 2012; Niwa et al., 2013). Antipsychotics have been shown to induce epigenetic changes in rodents (Dong et al., 2008). In the current study, we demonstrated that the PCP-induced impairment of recognition memory and social deficit was associated with suppression of Ac-H3K9 in the prefrontal cortex, which was restored by CLZ but not by HAL. In parallel, the inhibition of HDAC by SB restored the PCP-induced behavioral and epigenetic abnormalities. These results suggest that histone modifications in the prefrontal cortex play a crucial role, at least in part, in PCP-induced behavioral deficits and constitute a novel target for the antipsychotic action of CLZ. Furthermore, HDAC inhibitors might act as antipsychotics.

Our previous study demonstrated that infusion of a dopamine D1 receptor agonist into the prefrontal cortex ameliorates impairment of latent learning in PCP-treated mice (Mouri et al., 2007b). In the present experiment, localization of CLZ-induced Ac-H3K9 in the prefrontal cortex was mainly observed in dopamine D1 receptor-positive cells, which would be far more manifested by using the D1-GFP mice (Nelson et al., 2012). Our results by western blot and immunohistochemistry (Fig. 3a,b) suggested that the increase of histone H3 acetylation by
CLZ in PCP-treated mice was due to the activation of dopamine D1 receptor signaling. To confirm this suggestion, quantitative data for the double-labeling of dopamine D1 receptor and Ac-H3 cells are useful. Thus, more detailed quantitative experiments using dopamine D1 receptor, Ac-H3 and PV triple-staining are needed in the future studies. The dopamine D1 receptor antagonist blocked the ameliorative effect of CLZ on the
PCP-induced behavioral deficits and the decrease in Ac-H3K9 in the prefrontal cortex, indicating that CLZ ameliorates, at least in part, the PCP-induced abnormalities in a dopamine D1 receptor-dependent manner. SCH-23390 is widely used as a dopamine D1 receptor antagonist but may have an effect on serotonin 5-HT2 and 5-HT1C receptors (Bischoff et al., 1986, 1988; Terry and Katz, 1994). Therefore, it is necessary to analysis changes of histone acetylation level by CLZ using not only pharmacological blockade but also genetic technique such as the dopamine D1 receptor knock out/down.

Histone acetylation is modulated by histone acetyltransferases and HDACs and permits transcription. It has been reported that histone acetylation is involved in higher brain functions, including learning and memory (Levenson and Sweatt, 2005). Phosphorylated HDAC 5 is translocated from the nucleus to the extranuclear space when HDAC 5 is phosphorylated by CaM kinase II (Chawla et al., 2003; Kazantsev and Thompson, 2008). In the present study, repeated PCP treatments increased the HDAC 5 content in the nuclear fractions, whereas the level of phospho-CaM kinase II decreased. The accumulation of HDAC 5 and decrease in phospho-CaM kinase II in the nucleus were restored by CLZ in a dopamine D1 receptor-dependent manner. These findings suggest that the reduction of nuclear phospho-CaM kinase II levels after repeated PCP treatment leads to the accumulation of HDAC 5, which in turn decreases Ac-H3K9 in the prefrontal cortex. Indeed, repeated treatment with PCP has been shown to disrupt activation of CaM kinase II mediated by NMDA receptors, and the impairment of cognitive and emotional behavior in PCP-treated mice has been shown to be attributable to dysfunctional NMDA–CaM kinase II signaling (Mouri et al., 2007b; Murai et al., 2007). Since our study focused on HDAC 5, the involvement in other HDACs and kinase other than CaM kinase II and/or phosphatase in this process must be clarified in the future.

Clinical studies have suggested that prefrontal GABAergic activity contributes to symptoms in schizophrenia (Lewis et al., 1999; Guidotti et al., 2000; Volk et al., 2000; Hashimoto et al., 2003). Our previous studies have shown that single PCP-treatment-induced cognitive impairments are attenuated by increasing GABAergic interneuron precursors grafted into the prefrontal cortex of neonatal mice (Tanaka et al., 2011). Moreover, DNA microarray analysis has shown that repeated PCP treatments affect the expression of GABAergic genes such as GABA receptor α1 and GABA receptor β2 (Toyooka et al., 2002). As described above, the effects of CLZ were blocked by a dopamine D1 receptor antagonist. Furthermore, parvalbumin-containing GABAergic interneurons have the dopamine D1 receptor (Yang et al., 1999; Glausier et al., 2009). Therefore, we investigated the effects of CLZ and SB on the GABAergic neuronal system. Repeated PCP treatments decreased the mRNA expression of Gad1, Gabra1, and Pvalb in the prefrontal cortex. The reduction in Pvalb mRNA expression in PCP-treated mice was restored by both CLZ and SB. Although there is no direct evidence that Ac-H3K9 regulates Pvalb transcription, the histone modification induced by repeated PCP treatments may be associated with GABAergic activity.

In concluding, atypical antipsychotics CLZ but not typical antipsychotics HAL ameliorates behavioral abnormalities and reduction of the acetylation level of H3K9 in the prefrontal cortex associated with increase of HDAC 5 and decrease of CaMKII phosphorylation induced by PCP in a dopamine D1 receptor-dependent manner. These ameliorating effects of CLZ may be involved in Pvalb transcription via Ac-H3K9. From the present results, the epigenetic modification of and prevention of decrease in parvalbumin in the prefrontal cortex is one of the targets for the development of a therapeutic strategy.

Table 4. Effect of antipsychotics and histone deacetylase inhibitor on the expression of GABAergic genes in the prefrontal cortex

<table>
<thead>
<tr>
<th>Number of animals</th>
<th>Gad1</th>
<th>Gabra1</th>
<th>Gabrb2</th>
<th>Pvalb</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A.</strong> Saline/Saline</td>
<td>7</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Saline/Clozapine</td>
<td>8</td>
<td>0.91</td>
<td>1.01</td>
<td>0.96</td>
</tr>
<tr>
<td><strong>B.</strong> Saline/Saline</td>
<td>7</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Phencyclidine/Saline</td>
<td>6</td>
<td>0.44**</td>
<td>0.34**</td>
<td>0.44</td>
</tr>
<tr>
<td>Phencyclidine/Clozapine</td>
<td>6</td>
<td>0.46**</td>
<td>0.47*</td>
<td>0.82</td>
</tr>
<tr>
<td><strong>C.</strong> Saline/Saline</td>
<td>7</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Saline/Sodium butyrate</td>
<td>7</td>
<td>0.89</td>
<td>0.90</td>
<td>0.91</td>
</tr>
<tr>
<td><strong>D.</strong> Saline/Saline</td>
<td>7</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Phencyclidine/Saline</td>
<td>6</td>
<td>0.44**</td>
<td>0.34**</td>
<td>0.44</td>
</tr>
<tr>
<td>Phencyclidine/Sodium butyrate</td>
<td>7</td>
<td>0.57*</td>
<td>0.86##</td>
<td>1.41#</td>
</tr>
</tbody>
</table>

* p<0.05, ** p<0.01 compared with saline/saline-treated mice.
# p<0.05, ## p<0.01 compared with phencyclidine/saline-treated mice.
Acknowledgments
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Statement of Interest
None.

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