object recognition test, Morris water maze test, and Cued and contextual fear conditioning tests.

Spontaneous Alternation in a Y-Maze Test

The maze was made of black-painted wood; each arm was 40 cm long, 12 cm high, 3 cm wide at the bottom, and 10 cm wide at the top. The arms converged at an equilateral triangular central area that was 4 cm at its longest axis. Each mouse was placed at the center of the apparatus and allowed to move freely through the maze during an 8-min session. The series of arm entries was recorded visually. Alternation was defined as successive entry into the three arms, on overlapping triplet sets. The alternation behavior (%) was calculated as the ratio of actual alternations to possible alternations (defined as the number of arm entries minus two), multiplied by 100.

Novel-Object Recognition Test

The test procedure consisted of three sessions: habituation, training, and retention. Each mouse was individually habituated to the Plexiglas box (30 × 30 × 35 cm^3 high), with 10 min of exploration in the absence of objects for 3 days (habituation session). During the training session, two objects were placed in the back corner of the box. The objects were a golf ball, wooden cylinders, and square pyramids, which were different in shape and color but similar in size. A mouse was then placed midway at the front of the box and the total time spent exploring the two objects was recorded for 10 min. An animal was considered to be exploring the object when its head was facing the object or it was touching or sniffing the object. During the retention session, the animals were placed back into the same box 24 h after the training session, in which one of the familiar objects used during training was replaced with a novel object. The animals were then allowed to explore freely for 10 min and the time spent exploring each object was recorded. Throughout the experiments, the objects were used in a counterbalanced manner in terms of their physical complexity and emotional neutrality. A preference index, a ratio of the amount of time spent exploring any one of the two objects (training session) or the novel object (retention session) over the total time spent exploring both objects was used to measure cognitive function.

Morris Water Maze Test

The Morris water maze test was conducted in a circular pool 1.2 m in diameter and filled with water at a temperature of 22 ± 1°C. A hidden platform (7 cm in diameter) was used. The mice were given two trials (one block), 60 s each trial, for 10 consecutive days, during which the platform was left in the same position. The time taken to reach to the escape platform (escape latency) was determined in each trial by using the Etho Vision system (Brainscience Idea, Osaka, Japan). Three hours after the last training trial, the mice were given a probe test without the platform and were allowed 60 s to search the pool.

Cued and Contextual Fear Conditioning Tests

For measuring basal levels of freezing response (preconditioning phase), mice were individually placed in a neutral cage (17 × 27 × 12.5 cm^3 high) for 1 min and then in the conditioning cage (25 × 31 × 11 cm^3 high) for 2 min. For training (conditioning phase), mice were placed in the conditioning cage, and then a 15-s tone (80 dB) was delivered as a conditioned stimulus. During the last 5 s of the tone stimulus, a foot shock of 0.6 mA was delivered as an unconditioned stimulus through a shock generator (Brainscience Idea). This procedure was repeated four times with 15-s intervals. Cued and contextual tests were carried out 1 day after fear conditioning. For the cued test, the freezing response was measured in the neutral cage for 1 min in the presence of a continuous-tone stimulus identical to the conditioned stimulus. For the contextual test, mice were placed in the conditioning cage, and the freezing response was measured for 2 min in the absence of the conditioned stimulus.

Western Blot Analysis

Western blotting was performed as previously described (Mouri et al., 2007b). The mice were sacrificed by decapitation, and the brain was immediately removed. The hippocampus was rapidly dissected out on an ice-cold plate, frozen, and stored at −80°C until used. To prepare tissue extracts, the dissected brain tissue was homogenized by sonication in an ice-cold lysis buffer [20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 50 mM NaF, 2 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, 1% NP-40, 1 mM sodium orthovanadate, 20 μg/ml pepstatin, 20 μg/ml aprotinin, and 20 μg/ml leupeptin]. The homogenate was centrifuged at 13,000*g* for 20 min and the supernatant was used. The protein concentration was determined using a DC Protein Assay Kit (Bio-Rad, Richmond, CA). Samples (10–100 μg of protein) were boiled in sample buffer (125 mM Tris–HCl, pH 6.8, 10% 2-mercaptoethanol, 4% sodium diphosphate decahydrate, 10% sucrose, and 0.004% bromophenol blue), separated on a polyacrylamide gel, and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). The membranes were blocked with a Detector Block Kit (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and probed with a primary antibody. Membranes were washed with the washing buffer [50 mM Tris–HCl (pH 7.4), 0.05% Tween 20, and 150 mM NaCl] and subsequently incubated with a horseradish peroxidase-conjugated secondary antibody. The immune complexes were detected based on chemiluminescence (ECL kit, Amersham Biosciences, Piscataway, NJ) and exposed to X-ray film (Hyperfilm, Amersham Biosciences). The band intensities on the film were analyzed by densitometry using the ATTO Densitograph Software Library Lane Analyzer (ATTO, Tokyo, Japan). To confirm equal loading of each protein, membranes were stripped with stripping buffer [100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris–HCl (pH 6.7)] at 50°C

for 30 min, and GAPDH protein expression was detected as described above.

The primary antibodies were a rabbit anti-CyP D [1:1,000; synthesized by the authors using a peptide (aa 43–57) of Cyp D], a mouse anti-MAP2 (1:1,000; Chemicon, Temecula, CA), a rabbit antigrowth associated protein (GAP)-43 (1:1,000; Chemicon), a mouse antiglial fibrillary acidic protein (GFAP) (1:1,000; Chemicon), a rabbit antisynaptophysin (1:1,000; Dako, Glostrup, Denmark), a guinea pig antiglutamate transporter GLAST and a guinea pig anti-GLT-1 (1:1,000; Chemicon), a mouse antiglutaminase (GLS) (1:500; Abnova, Taipei, Taiwan), a mouse anti-NR1 CT (1:1,000; Upstate Biotechnology, Lake Placid, NY), a mouse anti-NMDAR2A and a mouse anti-NMDAR2B (1:1,000; BD Pharmingen, San Diego, CA), a rat anti-ChAT (Calbiochem, San Diego, CA), and a goat anti-ACHE (E-19) (1:500; Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibodies, used at a dilution of 1:2,000, were horseradish peroxidase-linked antimouse, antirabbit, antirat, or antiguinea pig IgG (Kirkegaard and Perry Laboratories).

Preparation of Brain Slice and Staining

Histological procedures were performed as previously described with a minor modification (Murai et al., 2007). Mice were anesthetized with chloral hydrate (150 mg/kg i.p.) and perfused transcardially with ice-cold phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in PBS. The brains were removed, postfixed in the same fixative for 2 h, and then soaked in 20% (w/v) sucrose in PBS. Coronal sections 15 μm thick were cut with a Cryostar HM560 cryostat (Microm International, GmbH, Walldorf, Germany). For immunohistochemistry, the primary antibodies that were applied in the brain slices included a rabbit anti-Cyp D (1:500; synthesized by the authors), a mouse antineuron-specific nuclear antigen (NeuN) (1:500; Chemicon) and mouse anti-GFAP (1:500; Chemicon) antibody. Fluorescently conjugated secondary antibodies (Alexa 488, 546, Invitrogen, Carlsbad, CA) were used for detecting chromagen. For Nissl staining, sections were cut at 40-μm intervals and staining was done according to standard procedures (Murai et al., 2007). Images were acquired with a confocal microscope (LSM510; Carl Zeiss, Jena, Germany) and a light microscope (Axiocam HRc; Carl Zeiss).

In Vivo Microdialysis

In vivo microdialysis was performed as previously described (Mouri et al., 2007b; Murai et al., 2007). Mice were anesthetized with sodium pentobarbital (40 mg/kg i.p.) before the stereotaxic implantation of a guide cannula (AG-6, Eicom, Kyoto, Japan) into the ventral hippocampus (−2.8 mm anteroposterior, ±3.0 mm mediolateral from the bregma, −2.0 mm dorsoventral from the skull). One day after the operation, a dialysis probe (AI-4–2; 2-mm membrane length, Eicom) was inserted through the guide cannula and perfused with CSF (147 mM NaCl, 4 mM KCl, and 2.3 mM $CaCl_2$) at a flow rate of 1 μl/min. The dialysate was colleted every 20 min. Dialysates were assayed by HPLC with electrochemical detection (HTEC-500, Eicom) under the following conditions. Three samples were taken to establish baseline levels of extracellular neurotransmitter. For depolarization stimulation, 50 mM KCl-containing Ringer solution was delivered through the dialysis probe for 20 min to induce the K^+-evoked release of glutamate and acetylcholine (ACh). Then dialysate was collected for 20 min with ringer solution.

Statistic Analysis

All results were expressed as the mean ± SEM for each group. The difference between groups was analyzed with a one-way, two-way, or repeated ANOVA, followed by the Bonferroni/Dunn multiple range-test. The Student *t*-test was used to compare two sets of data.

RESULTS

General Characteristics of Cyp $D^{-/-}$ Mice

The genotype for the Cyp D locus was assessed by PCR (Fig. 1A). Cyp $D^{-/-}$ mice were confirmed to lack Cyp D protein by Western blotting (Fig. 1B). Cyp $D^{-/-}$ mice were healthy and showed no changes in physical characteristics (body weight, or appearance of fur and whiskers) at 3 months (Fig. 1C). Although Luvisetto et al. (2008) have recently reported that Cyp $D^{-/-}$ mice show adult onset obesity, our mice did not show gross changes in physical characteristics, including body weight, with age (data not shown).

Histological Appearance of the Hippocampus in Cyp $D^{-/-}$ Mice

Strong immunoreactivity for Cyp D was observed in the granule cell layer and pyramidal cell layer in the hippocampus (Fig. 1D). In higher resolution image of CA1 regions, Cyp D immunoreactivity was localized in NeuN positive neuronal cells (Fig. 1E). With enhanced sensitivity of microscope, Cyp D immunoreactivity was also observed in astrocytes, though sparsely (Fig. 1E). No Cyp D protein was pressed in the brains of Cyp $D^{-/-}$ mice as confirmed by immunofluorescent staining (Fig. 1D). Nissl staining and immunostaining for the astrocyte marker GFAP showed neither gross structural abnormalities (Fig. 1D) nor any morphological abnormality of neuronal cells and astrocytes (Fig. 1F). The expression levels of the dendritic marker MAP2, neuronal growth cone marker GAP-43, presynaptic marker synaptophysin, and GFAP remained unchanged in hippocampal homogenates from Cyp $D^{-/-}$ mice as compared

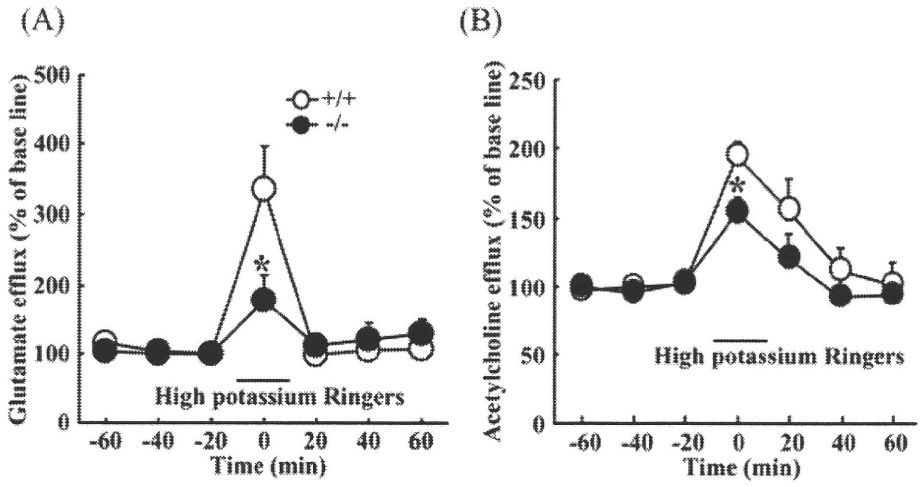

FIGURE 2. Deficiency of Cyclophilin D inhibits the release of glutamate and acetylcholine in the hippocampus. High potassium-evoked release of glutamate (A) and acetylcholine (B) from the hippocampus in the Cyp $D^{-/-}$ and wild type mice; High potassium-induced release of glutamate from the hippocampus was measured in the Cyp $D^{-/-}$ and wild-type mice. Values correspond to mean ± SEM (wild-type mice, $n = 6$; Cyp $D^{-/-}$ mice, $n = 7$). Results with repeated ANOVA were as follows: time, $F(3,33) = 11.36$, $P < 0.01$; Cyp D deficiency, $F(1,33) = 2.28$, $P = 0.16$; interaction time with Cyp D deficiency, $F(3,33) = 4.48$, $P < 0.01$. High potassium-induced release of acetylcholine from the hippocampus was measured in the Cyp $D^{-/-}$ mice. Values correspond to mean ± SEM (wild-type mice, $n = 10$; Cyp $D^{-/-}$ mice; $n = 11$). Results with repeated ANOVA were as follows: time, $F(3,57) = 11.54$, $P < 0.01$; Cyp D deficiency, $F(1,57) = 2.16$, $P = 0.16$; interaction of time with Cyp D deficiency, $F(3,57) = 0.45$, $P = 0.71$, $^*P < 0.05$ versus wild-type mice. +/+, wild-type mice; −/−, Cyp $D^{-/-}$ mice.

with those from wild-type mice (Fig. 1G; $n = 10$ per group; Student t-test).

Deficiency of Cyclophilin D Inhibits the Release of Glutamate and ACh in the Hippocampus

The release of neurotransmitters in the hippocampus plays an important role in learning and memory (Stefani and Gold, 2001; Mereu et al., 2003). Therefore, changes in the amounts of glutamate and ACh released in the hippocampus were investigated by microdialysis in the Cyp $D^{-/-}$ mice. The basal levels of glutamate in the hippocampus of the wild-type and Cyp $D^{-/-}$ mice were 0.55 ± 0.13 and 0.46 ± 0.21 pmol/10 µl/10 min, respectively (mean ± SEM; $n =$ 6–7 per group). The amount of glutamate released in response to high potassium (50 mM) in the hippocampus was significantly lower in the Cyp $D^{-/-}$ mice than in the wild-type mice (Fig. 2A; repeated ANOVA, post hoc Bonferroni/Dunn multiple range-test, $P < 0.05$). The basal levels of ACh in the hippocampus of the wild-type and Cyp $D^{-/-}$ mice were 0.15 ± 0.04 and 0.17 ± 0.03 nmol/10 µl/10 min, respectively (mean ± SEM; $n =$ 10–11 per group). The amount of ACh released in response to high potassium (50 mM) in the hippocampus was significantly lower in the Cyp $D^{-/-}$ mice than in the wild-type mice (Fig. 2B; $P < 0.05$). These results indicate that a deficiency of Cyp D results in inhibition of the potassium-induced release of glutamate and ACh in the hippocampus.

No Influence of the Cyclophilin D Deficiency on Glutamatergic and Cholinergic Nervous System-Related Protein Expression in the Hippocampus

As demonstrated above, Cyp $D^{-/-}$ mice showed a hypoglutamatergic and hypocholinergic response in the hippocampus in the presence of high potassium. It was possible that the deficiency in Cyp D affected the expression of these neuronal system-related proteins such as receptors, synthetases, degradation enzymes, and transporters. To test this possibility, the expression of NMDA receptor subunits, glutaminase (GLS), glutamate transporters, choline acetyltransferase (ChAT), and acetyltransferase (AChE), was analyzed by Western blotting. There was no significant difference in NR1, NR2A, NR2B, GLS, GLAST, GLT-1, ChAT, and AChE protein levels in the hippocampus between wild-type and Cyp $D^{-/-}$ mice (Fig. 3; $n = 10$ per group; Student t-test). These data show that the hypoglutamatergic and hypocholinergic responses observed in Cyp $D^{-/-}$ mice were not due to changes in expression levels of proteins involved in these neurotransmitters response.

Impairment by Cyclophilin D Deficiency of Learning and Memory

Spontaneous alternation in the Y-maze test

We evaluated short-term memory using a Y-maze test. There was no significant difference in the number of arm entries between the two groups (Fig. 4A; $n =$ 16–17 per group; Student

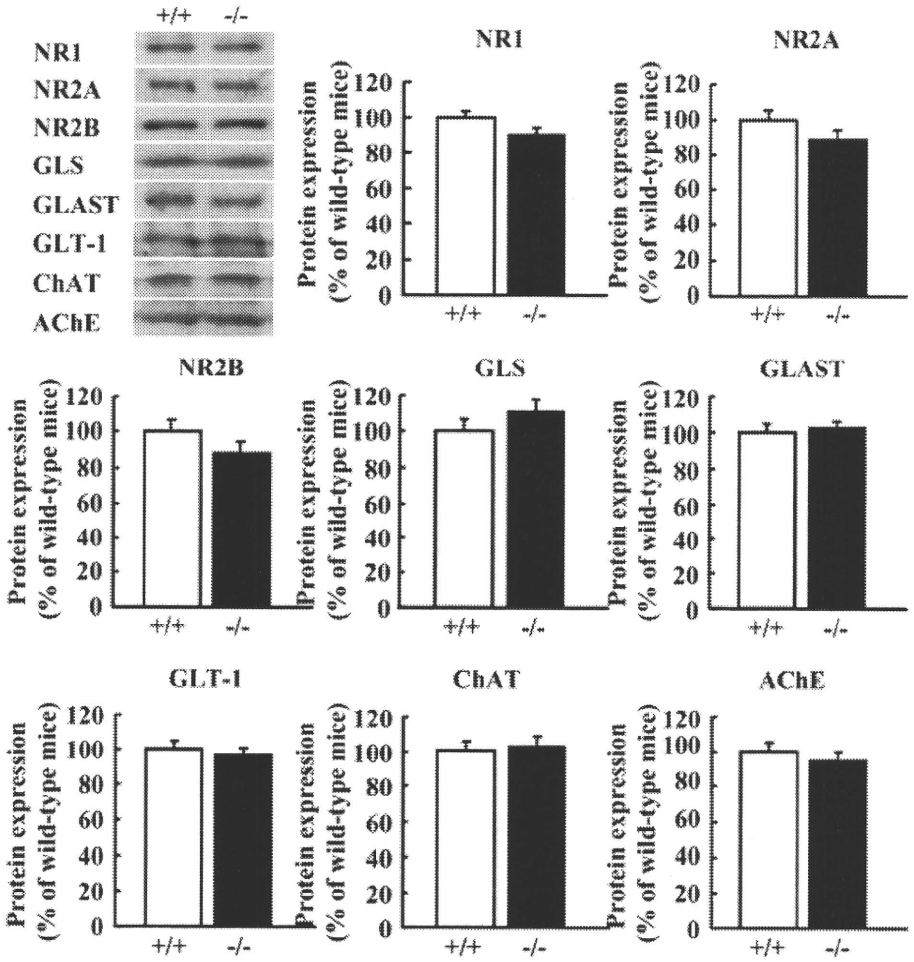

FIGURE 3. No influence of Cyclophilin D deficiency on glutamatergic nervous system-related protein expression in the hippocampus. Western blot analysis of homogenates from the hippocampus of the Cyp $D^{-/-}$ and wild-type mice. No difference was observed between the two genotypes (wild-type mice, $n = 10$; Cyp $D^{-/-}$ mice, $n = 10$; Student t-test). NR1, NMDA receptor 1 subunit; NR2A, NMDA receptor 2A subunit; NR2B, NMDA receptor 2B subunit; GLS, glutaminase; GLAST, glutamate-aspartate transporter; GLT-1, glial glutamate transporter-1; ChAT, choline acetyltrasferase; AChE, acetylcholinesterase; +/+, wild-type mice; −/−, Cyp $D^{-/-}$ mice.

t-test), suggesting that all mice have the same levels of motivation, curiosity, and motor function. However, Cyp $D^{-/-}$ mice showed significantly reduced spontaneous alternation behavior in the Y-maze compared with wild-type mice (Fig. 4B; $P < 0.05$), indicating an impairment of short-term memory.

Object recognition in the novel-object recognition test

We evaluated the visual recognition memory of Cyp $D^{-/-}$ mice using the novel-object recognition test. During the training session, there were no significant differences in exploratory preference between the two objects (Fig. 5A; n = 16–17 per group; repeated ANOVA, post hoc Bonferroni/Dunn multiple range-test) and the total time spent exploring both objects between the two groups (Fig. 5B), suggesting that all mice have

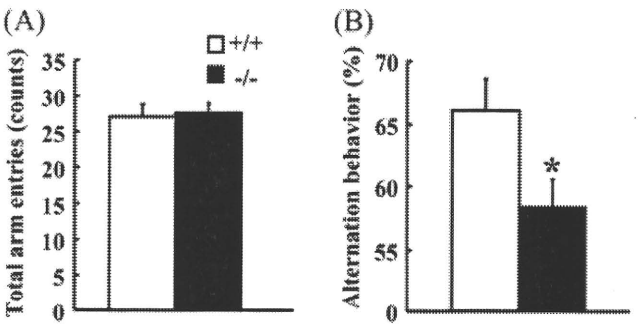

FIGURE 4. Impairment of short-term memory by Cyclophylin D deficiency in the Y-maze test. (A) Total arm entries; (B) Alternation behavior. Percent alternation during an 8-min session in the Y-maze test was measured. Values indicate mean ± SEM (wild-type mice, $n = 17$; Cyp $D^{-/-}$ mice; $n = 16$). *$P < 0.05$ versus wild-type mice (Student t-test). +/+, wild-type mice; −/−, Cyp $D^{-/-}$ mice.

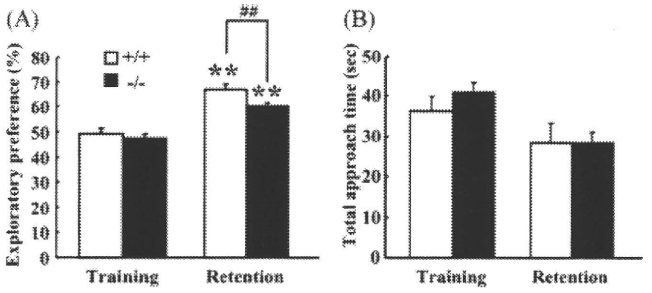

FIGURE 5. Impairment of object recognition memory by Cyclophylin D deficiency in the novel-object recognition test. (A) Exploratory preference; (B) Total approach time. The retention session was carried out 24 h after the training. Exploratory preference during a 10-min session in the novel-object recognition test was measured. Values indicate mean ± SEM (wild-type mice, n = 17; Cyp $D^{-/-}$ mice; n = 16). Results with the repeated ANOVA were as follows; exploratory preference: training/retention, $F(1,31)$ = 122.53, $P < 0.01$; Cyp D deficiency, $F(1,31)$ = 9.52, $P < 0.01$; interaction of training/retention with Cyp D deficiency, $F(1,31)$ = 2.22, $P = 0.14$; total approach time: training/retention, $F(1,31)$ = 28.32, $P < 0.01$; Cyp D deficiency, $F(1,31)$ = 0.62, $P = 0.44$; interaction of training/retention with Cyp D deficiency, $F(1,31)$ = 1.10, $P = 0.30$, $^{**}P < 0.01$ versus training. $^{\#\#}P < 0.01$ versus trained, wild-type mice. +/+, wild-type mice; −/−, Cyp $D^{-/-}$ mice.

the same levels of motivation, curiosity, and interest in exploring novel objects.

For the retention session, both groups of mice took longer time to explore the novel object than the familiar object (Fig. 5A, $P < 0.01$). However, the level of exploratory preference for the novel objects was significantly decreased in Cyp $D^{-/-}$ mice compared to wild-type mice (Fig. 5A, $P < 0.01$), indicating an impairment of visual recognition memory.

Reference memory in the Morris water maze test

We evaluated reference memory using the Morris water maze test. Both groups of mice managed to learn the position of the hidden platform (Fig. 6A). However, Cyp $D^{-/-}$ mice took significantly longer time and distance to reach the platform than wild-type mice (Fig. 6A; n = 16–17 per group; repeated ANOVA, post hoc Bonferroni/Dunn multiple range-test; $P < 0.01$), indicating an impairment of reference memory. When the probe test was carried out following the tenth block of training, wild-type mice searched preferentially in the trained quadrant (Fig. 6B; repeated ANOVA, post hoc Bonferroni/Dunn multiple range-test; $P < 0.01$), but Cyp $D^{-/-}$ mice did not. The decreased ability did not reflect a loss of swimming ability and motivation, because swimming speed and distance in the probe test were similar to those in wild-type mice (swimming speed; wild-type mice: 19.14 ± 0.78 cm/s, Cyp $D^{-/-}$ mice: 16.12 ± 1.33 cm/s, swimming distance; wild-type mice: 1,141 ± 47 cm, Cyp $D^{-/-}$ mice: 962 ± 80 cm).

Associative learning in the cued and contextual fear conditioning tests

We evaluated associative learning in the conditioned fear learning test. In the preconditioning phase, the mice of both groups hardly showed any freezing response, and there were no differences in basal levels of the freezing response between the groups (Figs. 7A,B; n = 16–17 per group; repeated ANOVA, post hoc Bonferroni/Dunn multiple range-test). In

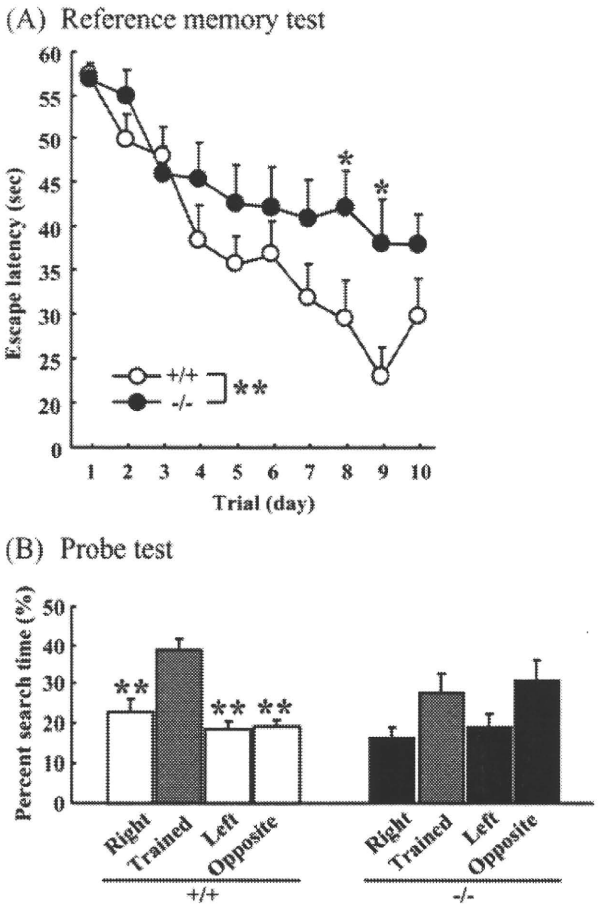

FIGURE 6. Impairment of reference memory by Cyclophylin D deficiency in the Morris water maze test. (A) Reference memory test. Escape latency during a 60-s session in the water maze test was measured. Values indicate mean ± SEM (wild-type mice, n = 17; Cyp $D^{-/-}$ mice; n = 16). Results with the repeated ANOVA were as follows: trial, $F(9,279)$ = 11.96, $P < 0.01$; animal group, $F(1,31)$ = 7.27, $P < 0.05$; interaction of trial with animal group, $F(9,279)$ = 1.13, $P = 0.34$. $^{**}P < 0.01$, $^{*}P < 0.05$ versus wild-type mice. (B) Probe test. The probe test was performed after training on day 10 in the Morris water maze test. Percent search time during a 60-s session in the water maze test was measured. Values indicate mean ± SEM (wild-type mice, n = 17; Cyp $D^{-/-}$ mice; n = 16). Results with the repeated ANOVA were as follows: quadrant, $F(3,93)$ = 5.57, $P < 0.01$; Cyp D deficiency, $F(1,31)$ = 0.01, $P = 0.91$; interaction of quadrant with Cyp D deficiency, $F(3,93)$ = 2.95, $P < 0.05$. $^{**}P < 0.01$ versus trained quadrant. +/+, wild-type mice; −/−, Cyp $D^{-/-}$ mice.

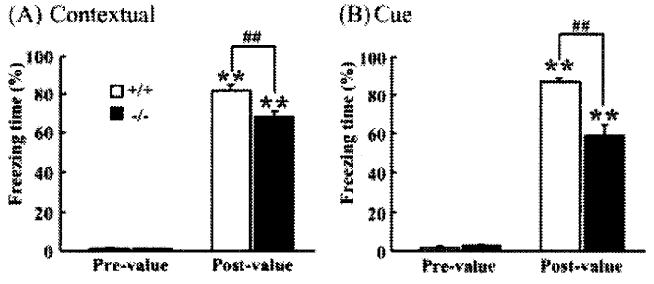

FIGURE 7. Impairment of associative learning by Cyclophylin D deficiency in the conditioned fear learning test. The test session was carried out 24 h after the conditioning. Context-dependent (A) and Cue-dependent (B) freezing times were measured. Values indicate mean ± SEM (wild-type mice, n = 17; Cyp D$^{-/-}$ mice; n = 16). Results with the repeated ANOVA were as follows; context-dependent test: conditioning, $F(1,31)$ = 1,127.89, $P < 0.01$; Cyp D deficiency, $F(1,31)$ = 9.12, $P < 0.01$; interaction of conditioning with Cyp D deficiency, $F(1,31)$ = 9.12, $P < 0.01$; cue-dependent test: conditioning, $F(1,31)$ = 703.45, $P < 0.01$; Cyp D deficiency, $F(1,31)$ = 17.14, $P < 0.01$; interaction of conditioning with Cyp D deficiency, $F(1,31)$ = 29.96, $P < 0.01$, $^{**}P < 0.01$ versus pre-conditioning. $^{\#\#}P < 0.01$ versus conditioned, wild-type mice. +/+, wild-type mice; −/−, Cyp D$^{-/-}$ mice.

the contextual learning test, both groups showed a marked contextual freezing response 24 h after fear conditioning (Fig. 7A; $P < 0.01$). However, Cyp D$^{-/-}$ mice exhibited less freezing response in the contextual tests (Fig. 7A; $P < 0.01$), indicating an impairment of associative learning. In the cued learning test, Cyp D$^{-/-}$ mice exhibited less freezing (Fig. 7B; $P < 0.01$), indicating an impairment of associative learning. Furthermore, no aberrant nociceptive responses to electric footshocks were observed in the Cyp D$^{-/-}$ mice: the footshock thresholds in the Cyp D$^{-/-}$ mice (flinching, 0.28 ± 0.02 mA; vocalizing, 0.62 ± 0.05 mA; jumping, 0.78 ± 0.08 mA) were the same as those in wild-type mice (flinching, 0.22 ± 0.03 mA; vocalizing, 0.77 ± 0.05 mA; jumping, 0.71 ± 0.11 mA).

Recapitulation of Cyclophilin D Deficiency by Infusion of Cyclosporine A Into the Hippocampus

To examine the role of hippocampal Cyp D in learning and memory, we microinjected CsA, an inhibitor of Cyp D, into the hippocampus, and evaluated its effect on performance in the novel-object recognition test and conditioned fear learning test. In the novel-object recognition test, the mice were microinjected with CsA (100 pmol/mouse/unilateral) 10 min before the training trial. During the training session, there were no significant differences in exploratory preference between the two objects and total exploratory time between the two groups (Figs. 8A,B; n = 8–9 per group; repeated ANOVA, post hoc Bonferroni/Dunn multiple range-test). However, the level of exploratory preference for the novel objects in mice treated with CsA was significantly decreased compared to that in mice treated with vehicle (Fig. 8A, $P < 0.05$), indicating a role for hippocampal Cyp D in this form of learning and memory. In the conditioned fear learning test, CsA was microinjected into the hippocampus 10 min before the conditioning trial. The mice treated with CsA exhibited less freezing response 24 h after fear conditioning in the contextual tests, which is known to be hippocampus-dependent (Fig. 8C; n = 16–17 per group; Student t-test; $P < 0.01$). But there was no difference in the cued freezing response 24 h after fear conditioning among the groups in the cued learning test, which is known to be hippocampus-independent (Fig. 8D). These results indicate that hippocampal Cyp D plays a role in the hippocampal-dependent form of learning and memory.

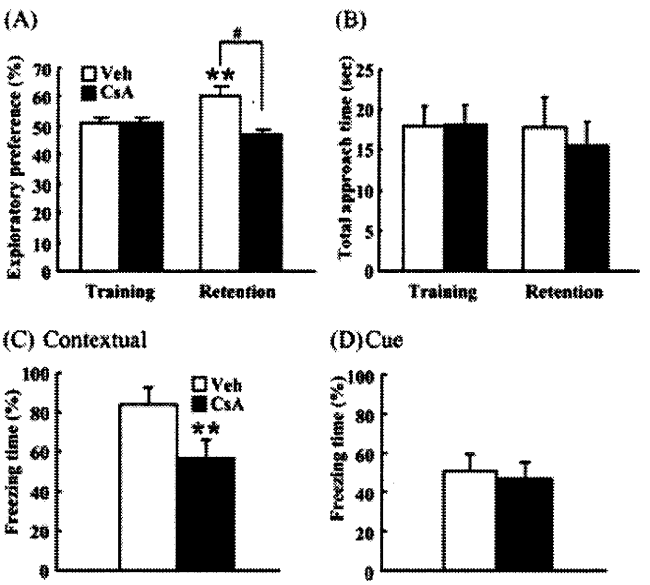

FIGURE 8. Recapitulation of Cyclophilin D deficiency by infusion of CsA into the hippocampus. Novel object recognition test: (A) Exploratory preference; (B) Total approach time. The retention session was carried out 24 h after the training. CsA (100 pmol/mouse/unilateral) was infused into the hippocampus 10 min before the training trial. Exploratory preference during a 10-min session in the novel-object recognition test was measured. Values indicate mean ± SEM (vehicle-treated mice, n = 9; CsA-treated mice, n = 8). Results with the repeated ANOVA were as follows; exploratory preference: training/retention, $F(1,15)$ = 2.67, P = 0.12; treatment, $F(1,15)$ = 7.17, $P < 0.05$; interaction training/retention with treatment, $F(1,15)$ = 12.83, $P < 0.05$; total approach time: training/retention, $F(1,15)$ = 0.12, P = 0.64; treatment, $F(1,15)$ = 0.06, P = 0.81; interaction training/retention with treatment, $F(1,15)$ = 0.89, P = 0.36. $^{**}P < 0.01$ versus training. $^{\#}P < 0.05$ versus trained, vehicle-treated mice. Conditioned fear learning test: The test session was carried out 24 h after the conditioning. Context-dependent (C) and cue-dependent (D) freezing times were measured. Values indicate mean ± SEM (vehicle-treated mice, n = 10; Cyclosporine A-treated mice, n = 10). $^{**}P < 0.01$ versus vehicle-treated mice (Student t-test). Veh, vehicle-treated mice; CsA, Cyclosporine A-treated mice.

DISCUSSION

In this study, we analyzed mice with a deficiency of Cyp D to define its role in cognitive functions. Our behavioral data showed that Cyp $D^{-/-}$ mice have subtle but significant impairments of short-term memory in the Y-maze test, visual recognition memory in the novel-object recognition test, reference memory in the water maze test, and associative learning in the conditioned fear learning test. It is unlikely that the impaired performance of Cyp $D^{-/-}$ mice in learning and memory tests is due to changes in motivation or sensorimotor function, since the motivation for each of these behavioral tests is different, and different skills are required for a good performance in each test. Actually, there were no differences in total arm entries in the Y-maze test, total time spent exploring objects in the novel object test, swimming speed in the Morris water maze test, and freezing response in the preconditioning phase and nociceptive response between the wild-type and Cyp $D^{-/-}$ mice.

Cyp D immunoreactivity is abundant in neuronal layers but sparse in astrocytes in the adult mouse brain. Naga et al. (2007) also showed that Cyp D is present at high levels in neurons and low levels in astrocytes in adult rat brain using immunohistochemistry and in primary rat neuron and astrocyte cultures using Western blotting. Although it is possible that Cyp D deficiency-induced developmental abnormalities in the neuronal and astrocytic architecture lead to cognitive dysfunctions, we did not observe histopathological abnormalities on Nissl staining and GFAP immunostaining or the irregular expression of neuronal (MAP2a-c, GAP-43, and synaptophysin) and glial (GFAP) marker proteins on Western blotting. Hippocampus as well as perirhinal and prefrontal cortex is crucial for recognition memory in the novel-object recognition test (Rampon et al., 2000; Winters and Bussey, 2005; Nagai et al., 2007). Reference memory in the Morris water maze test (Morris et al., 1982) and associative learning in the contextual, but not cued conditioned fear learning test (Phillips and LeDoux, 1992), are dependent on the hippocampus. Hippocampal infusion of CsA, an inhibitor of Cyp D (Halestrap and Davidson, 1990), replicated the hippocampus-dependent behavioral cognitive dysfunctions (impairments of recognition memory in the novel-object recognition test and of associative learning in the contextual test) observed in Cyp $D^{-/-}$ mice, except for the impairment of associative learning in the cued conditioned fear learning test, which is known to depend on the amygdala (Phillips and LeDoux, 1992). Taken together, these results strongly indicate that the role of Cyp D in cognitive functions is functional rather than developmental in nature.

Mitochondrial calcium buffering is an important regulator of synaptic function (Tang and Zucker, 1997; Billups and Forsythe, 2002). Recent studies have suggested that Cyp D-regulated MPT plays an important role in mitochondrial synaptic Ca^{2+} buffering, hippocampal synaptic plasticity, and learning and memory (Weeber et al., 2002; Levy et al., 2003; Naga et al., 2007). Mitochondria from synaptosomes, isolated from rat cerebral cortex, have less Ca^{2+} buffering ability than the nonsynaptic pool of mitochondria (Brown et al., 2006). This difference reflects the higher levels of Cyp D in synaptic than nonsynaptic mitochondria (Naga et al., 2007). The application of CsA, a deficiency of Cyp D, and a deficiency of VDAC all increase Ca^{2+} uptake capacity in isolated mitochondria (Levy et al., 2003; Naga et al., 2007). Interestingly, the application of CsA and a deficiency of VDAC impair paired-pulse facilitation and LTP (Weeber et al., 2002; Levy et al., 2003). The phenomena of paired pulse facilitation are generally accepted as a model of the presynaptic component of synaptic plasticity (Gottschalk et al., 1998). These results suggest that the cognitive dysfunction and impaired neurotransmission observed in Cyp $D^{-/-}$ mice is ascribable to deficiency of Cyp D-dependent MPT.

Excitatory transmitters such as ACh and glutamate change neural information processing by regulating the release of synaptic transmitters and modifying long-term synaptic plasticity (Giocomo and Hasselmo, 2007). In addition, the release of glutamate and ACh in hippocampus is related to cognitive performance in behavioral tests (Stefani and Gold, 2001; Mereu et al., 2003). In the present study, Cyp $D^{-/-}$ mice had lower extracellular glutamate and ACh levels in response to high potassium in the hippocampus than did the wild-type mice. Previously, a decrease in spontaneous extracellular glutamate release and increase in levels of the glutamate transporter GLAST were observed in schizophrenic animal models, which show impairments of memory (Mouri et al., 2007b; Murai et al., 2007). In present study, there was no difference in protein expression of neurotransmitter synthesis, metabolism and uptake in the glutamatergic and cholinergic neuronal system in hippocampus between wild-type and Cyp $D^{-/-}$ mice. Thus, it is unlikely that deficiency of Cyp D decrease glutamate and ACh response by modulation of these protein expressions of GLS, GLAST or GLT-1 between wild-type and Cyp $D^{-/-}$ mice. Although we have no detailed data about neurotransmitter levels in response to other potassium concentration, activities of neurotransmitters enzymes, and transporters in Cyp $D^{-/-}$ and hippocampal CsA-infused mice, our results along with other recent findings suggest that Cyp D and MPT play important roles in synaptic transmission.

Luvisetto et al. (2008) have reported that the Cyp $D^{-/-}$ mice generated by Basso et al. (2005) show adult onset obesity, increased anxiety/emotionality in the open field test and elevated plus maze test, and a facilitation of learning in the active and passive avoidance test at 10 months. As far as the obesity is concerned, we did not observe a significant difference in the body weight of Cyp $D^{-/-}$ mice up to 40 months, as compared with control littermates. We do not know the reason for this difference. Although our results are consistent with some of the results described by Luvisetto et al. (2008), notably that Cyp $D^{-/-}$ mice exhibited increased anxiety/emotionality in the elevated plus maze test (unpublished data), we did not observe any facilitation of learning and memory in our Cyp $D^{-/-}$ mice. It is conceivable that the avoidance behavior of Cyp $D^{-/-}$ mice is due to greater anxiety rather than greater learning ability. Du et al. (2008) have reported that the Cyp $D^{-/-}$ mice generated by Bains et al. (2005) show normal synaptic

plasticity and spatial memory in radial water maze test. These differences between Du's and our data might be due to different behavioral test, because the CypD$^{-/-}$ mice shows normal but slight increase of error in the behavior test at 6 months and decrease of long-term potentiation at 12–13 months (Du et al., 2008). More extensive investigation will be necessary to clarify these differences.

In summary, mice lacking Cyp D display cognitive dysfunction probably caused by the hypofunction of neurotransmission without developmental abnormalities. In pathological process, blockade of Cyp D could be a potent therapeutic strategy for degenerative disorders such as Alzheimer's disease, ischemia, and multiple sclerosis. It is possible that blockade of Cyp D impairs rather than facilitates cognitive function in normal condition. Our findings could contribute understanding not only the physiological roles of Cyp D in cognition but also appropriate use of Cyp D blocker for degenerative disorders.

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ARTICLE

CINP

Prenatal exposure to phencyclidine produces abnormal behaviour and NMDA receptor expression in postpubertal mice

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Abstract

Several studies have shown the disruptive effects of non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonists on neurobehavioural development. Based on the neurodevelopment hypothesis of schizophrenia, there is growing interest in animal models treated with NMDA antagonists at developing stages to investigate the pathogenesis of psychological disturbances in humans. Previous studies have reported that perinatal treatment with phencyclidine (PCP) impairs the development of neuronal systems and induces schizophrenia-like behaviour. However, the adverse effects of prenatal exposure to PCP on behaviour and the function of NMDA receptors are not well understood. This study investigated the long-term effects of prenatal exposure to PCP in mice. The prenatal PCP-treated mice showed hypersensitivity to a low dose of PCP in locomotor activity and impairment of recognition memory in the novel object recognition test at age 7 wk. Meanwhile, the prenatal exposure reduced the phosphorylation of NR1, although it increased the expression of NR1 itself. Furthermore, these behavioural changes were attenuated by atypical antipsychotic treatment. Taken together, prenatal exposure to PCP produced long-lasting behavioural deficits, accompanied by the abnormal expression and dysfunction of NMDA receptors in postpubertal mice. It is worth investigating the influences of disrupted NMDA receptors during the prenatal period on behaviour in later life.

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Key words: Antipsychotic, behaviour, neurodevelopment, NMDA receptor, PCP, prenatal.

Introduction

Neurodevelopmental abnormalities are considered part of the pathogenesis of psychological disturbances. Exposure to environmental insults during pregnancy increases the probability of neuropsychiatric disorders in later life (Brown & Susser, 2002; Green *et al.* 1994). According to the neurodevelopmental hypothesis of schizophrenia, disruption of the prenatal brain predisposes the neural systems to long-lasting structural and functional abnormalities, leading to the emergence of psychopathological behaviour in adulthood (Ashdown *et al.* 2006).

The *N*-methyl-D-aspartate (NMDA) receptor, a kind of ligand-gated ion channel, is a heteromeric assembly comprising a core NR1 subunit and several modulatory subunits. At the cell surface, including synapses, NMDA receptors are anchored and clustered forming larger complexes (Husi & Grant, 2001). Stimulation of NMDA receptors during development

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is critical for the survival, differentiation and migration of immature neurons (Behar *et al.* 1999; Komuro & Rakic, 1993), and controls structure and plasticity (Scheetz & Constantine-Paton, 1994), as well as establishing normal neural networks in the developing brain (Deutsch *et al.* 1998). It has been found that pharmacological inhibition of NMDA receptors during development disturbs neural functions in the brain (Bellinger *et al.* 2002). Post-mortem studies have identified the abnormal expression (Akbarian *et al.* 1996; Dracheva *et al.* 2001) and phosphorylation (Emamian *et al.* 2004) of NMDA receptors in the prefrontal cortex (PFC) of schizophrenia patients.

In clinical tests, abuse of phencyclidine (PCP), a non-competitive NMDA receptor antagonist, causes a schizophrenic psychosis in normal volunteers and exacerbates symptoms in schizophrenia patients (Javitt & Zukin, 1991). In adult rodents, PCP produces abnormal behaviour and biochemical alterations resembling schizophrenia including positive symptoms, negative symptoms, and cognitive deficits (Mouri *et al.* 2007*a*, *c*; Noda *et al.* 1995). However, several lines of evidence suggest that abnormal architectural arrangements of nerve cells, or cortical layers (Bogerts, 1993), an absence of normal cerebral structural asymmetry (Crow *et al.* 1989), and gliosis (Jones *et al.* 1994) are involved in the pathology of schizophrenia. This suggests schizophrenia to be a developmental disorder rather than a progressive degenerative disease (Bogerts, 1993).

Therefore, although many schizophrenia-like symptoms are observed in adult rodents repeatedly treated with PCP, it is unlikely that these abnormalities completely resemble the pathogenesis of schizophrenia, since at least in some cases, they occur in the developing period initiated by prenatal insults (Murray *et al.* 1992; Pilowski *et al.* 1993). Therefore, based on the neurodevelopmental hypothesis, several studies have modified this classic 'PCP animal model', through treatment with NMDA antagonists early in the development of the brain. For instance, perinatal PCP treatment in rats enhanced hyperlocomotion elicited by PCP and impaired the acquisition of a delayed spatial alternation task in adolescent offspring, associated with the disruption of neurodevelopment (Deutsch *et al.* 1998; Wang *et al.* 2001). Prenatal exposure to (+)-MK-801 has been reported to reduce the density of parvalbumin-immunoreactive interneurons and enhance PCP-induced hyperlocomotion in postpubertal rats (Abekawa *et al.* 2007). However, it is unclear whether prenatal exposure to PCP leads to behavioural and NMDA receptor dysfunction in mice.

In this study, we investigated the influences of prenatal exposure to PCP during the middle and late stages of pregnancy [embryonic days 6–18 (E6–E18)], covering the entire neurodevelopment period in the prenatal brain from neurulation to corticogenesis (Theiler, 1989). PCP-induced hyperlocomotion, recognition memory, and the expression and phosphorylation of NR1 protein were investigated from age 7 wk. In addition, the effects of antipsychotics on these behavioural abnormalities were further evaluated.

Materials and methods

Animals

Pregnant ICR dams (E5) obtained from SLC Japan (Shizuoka, Japan) were maintained on a 12-h light/dark cycle (lights on 08:00 hours) with free access to food (CE2; Clea Japan Inc., Japan) and water. The dams were randomly divided into saline-treated and PCP-treated groups. All were housed individually until parturition. There was no increase in maternal deaths and resorption or stillbirths on exposure to PCP in this study. At birth [postnatal day 0 (PD 0)], pups were culled to eight per litter with a balance of males and females wherever possible. Pups were weighed weekly until weaning and maternal care behaviour during feeding was monitored. After weaning at PD 21, pups given the same prenatal treatment were mixed by gender and then randomly assigned to each group for behavioural testing at the age of 7–8 wk. All groups of mice had litters of 2–3 and the test was repeated more than three times to reduce the influence of litters. Moreover, a balanced number of males and females were used in each experiment, since there were no significant differences between genders in this study.

The experiments with offspring commenced at the age of 7 wk and were performed in a sound-attenuated, air-conditioned room (23 ± 1 °C, 50 ± 5% humidity). The mice were habituated to the room for 40 min before the behavioural experiments. All the behavioural tests were recorded with a digital camera to re-analyse the results. The experiments were performed in accordance with the Guidelines for Animal Experiments of Meijo University Faculty of Pharmaceutical Sciences and the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society (2008).

Drugs

PCP hydrochloride was synthesized according to the method of Maddox *et al.* (1965) and checked for purity.

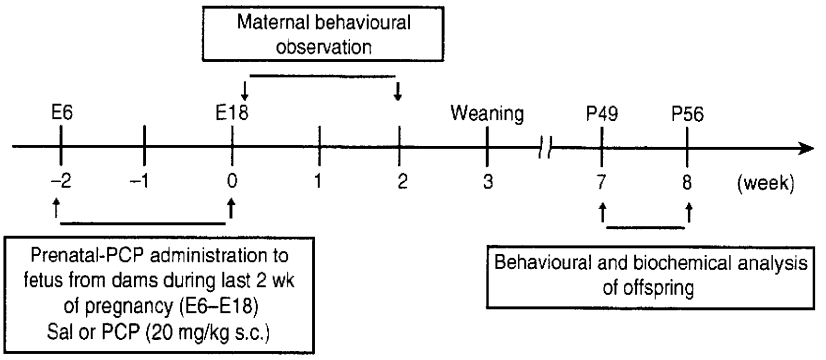

Fig. 1. Experimental protocol of this study.

The PCP was dissolved in saline prior to use. Clozapine (Sigma, USA) was dissolved in a minimum amount of 0.1 N HCl and then diluted with saline (adjusted to pH 6–7 with 0.1 N NaOH), as previously described (Qiao *et al.* 2001). An injectable solution of haloperidol (5 mg/ml; Tanabe Seiyaku, Japan) was diluted with saline. All compounds were administered in a volume of 0.1 ml/10 g body weight.

Drug treatment

The dams were administered saline or PCP (20 mg/kg s.c.) once daily at 18:00 hours on E6–E18. The injection was made as gentle as possible to minimize potential stress-related influences on dams. In the fetal brain, the density of NMDA receptors is relatively low (Monyer *et al.* 1994; Watanabe *et al.* 1992), and the affinity for PCP, as well as the distribution of PCP, remains unclear. According to dose-dependent responses in our preliminary study (2.5–20 mg/kg), the dose of 20 mg/kg was selected in the present study, since it produced more obvious and similar behavioural and biochemical changes in relation to schizophrenia (L. Lu *et al.*, unpublished data).

Based on previous studies (Mouri *et al.* 2007*a*), a low dose of PCP (3 mg/kg) or saline was used to challenge mice 30 min after habituation in PCP-induced locomotion; clozapine (1 and 3 mg/kg) or haloperidol (0.1 and 0.3 mg/kg) were injected 30 min before each behavioural test, and PCP (3 mg/kg) was injected into all mice to evaluate the effects of antipsychotics on it.

Different batches of mice were used for different experiments to avoid disruption. The experiments were performed according to the protocol shown in Fig. 1.

Measurement of locomotor activity

Locomotor activity was measured at the age of 7 wk. Mice were placed individually in a transparent acrylic cage with a black frosted Plexiglas floor (45 × 26 × 40 cm) for 120 min, and locomotor activity was measured in 5-min intervals using digital counters with infrared sensors (Scanet SV-10; Melquest Ltd, Japan) as previously reported (Lu *et al.* 2009). Locomotor activity was defined as the total number of beam cuts due to horizontal movement measured by the photo sensors.

Novel object recognition test (NORT)

As previously described (Mouri *et al.* 2007*b*), the test procedure consisted of three sessions: habituation, training, and retention. Each mouse was individually habituated to the box (L 30 × W 30 × H 35 cm), with 10 min of exploration in the absence of objects for 3 d (habituation session). During the training session, two objects (a red painted triangular prism and a yellow painted quadratic prism) were symmetrically fixed to the floor of the box, 8 cm from the walls, and each animal was allowed to explore the box for 10 min (day 4). An animal was considered to be exploring the object when its head was facing the object or it was touching or sniffing the object at a distance of <2 cm and/or touching it with its nose. The time spent exploring each object was recorded. After training, mice were immediately returned to their home cages. During the retention session, animals were returned to the same box 24 h (day 5) after the training session, in which one of the familiar objects used during training was replaced with a novel object (a black painted golf ball). The animals were allowed to explore freely for 5 min and the time spent exploring each object was recorded. Throughout the experiments, the objects were used in a counterbalanced manner in terms of their physical complexity and emotional neutrality. A preference index, the ratio of time spent exploring either of the two objects (training session) or the novel object (retention session) over the total amount of time

spent exploring both objects, was used to assess cognitive function.

Western blot analysis

Western blotting was performed as previously described (Mouri *et al.* 2007*c*). Dissected brain tissue obtained 24 h after the NORT test, was homogenized in ice-cold Tris buffer A [10 mM Tris–HCl (pH 7.4), 5 mM EDTA, 320 mM sucrose, 1 mM EGTA, 0.1 mM sodium orthovanadate, 1 mM NaF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin] and centrifuged at 700 *g* for 10 min. The supernatant was centrifuged again at 37 000 *g* for 40 min, and the membrance-enriched extracts were re-suspended in Tris buffer B [10 mM Tris–HCl (pH 7.4), 0.1 mM sodium orthovanadate, 1 mM NaF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin], and the suspension was used.

The protein concentrations were determined using a Pierce BCA Protein Assay kit (Thermo, USA). Samples were boiled at 95 °C for 5 min in the 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 transferred to polyvinylidene difluoride membranes (Millipore Corporation, USA). The membranes were blocked with a Detector Block kit (Kirkegaard & Perry Laboratories, USA) and probed with a primary antiphospho-NR1 (Ser^{897}) antibody (1:1000; Upstate Biotechnology, USA). Membranes were washed with the washing buffer [50 mM Tris–HCl (pH 7.4), 0.05% Tween-20, and 150 mM NaCl] and subsequently incubated with a secondary horseradish peroxidase-linked antibody (Kirkegaard & Perry Laboratories). The immune complexes were detected with an ECL kit (GE Healthcare, UK) and exposed to X-ray film (Hyperfilm, GE Healthcare). The intensity of bands was analysed by Atto Densitogram Software Library Lane Analyzer (Atto, Japan). After the phosphorylated-NR1 was detected, membranes were stripped with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris–HCl, pH 6.7) at 50 °C for 30 min, and NR1 expression was detected with a primary anti-NR1 antibody (1:1000; Santa Cruz Biotechnology, USA).

Preparation of brain slices and staining

Histological procedures were performed as described with a minor modification (Murai *et al.* 2007). Mice were anaesthetized with pentobarbital sodium (50 mg/kg i.p.) and perfused transcardially with ice-cold phosphate-buffered saline (PBS), followed by 4% paraformaldehyde and then soaked in 10–30% (w/v) sucrose. Coronal sections (20-μm thick) were cut with a cryostat (CM 1850; Leica, Germany). According to a previous method (Shen *et al.* 2008), Cresyl Violet staining was performed and the sizes of ventricles and brains were quantified with a computer-based image analysis system (WinRoof, Mitani, Japan). Apoptosis was detected with an *in-situ* cell-death detection kit, POD (Roche, Germany), and TUNEL-positive cells in layers II/III of the prelimbic area were counted using image analysis software. Images were acquired with a microscope (BZ-9000; Keyence, Japan).

Statistical analysis

All data were expressed as the mean ± S.E.M. The statistical significance of differences between two groups was determined by Student's *t* test. The significance of differences among more than three groups was determined using a two-way analysis of variance (ANOVA) or ANOVA with repeated measures, followed by Bonferroni's test. Pearson's correlation analysis was used to identify the relationship; $p<0.05$ was regarded as statistically significant.

Results

Effect of prenatal-PCP treatment on PCP-induced hyperlocomotion

To investigate the effects of prenatal exposure to PCP on drug-induced sensitization, PCP-induced hyperlocomotion was examined at age 7 wk. In the habituation period, no significant differences were observed among groups. After the 30-min habituation, prenatal saline- or PCP-treated mice were administered a low dose of PCP (3 mg/kg) or saline. The time-course of change in prenatal saline-treated mice revealed that the PCP challenge rapidly and significantly increased locomotion compared to the administration of saline. PCP-induced hyperlocomotion was significantly potentiated in the prenatal PCP-treated mice compared to the prenatal saline-treated mice over 5-min intervals after habituation (prenatal treatment: $F_{1,37}=9.54$, $p<0.01$; PCP challenge: $F_{1,37}=64.85$, $p<0.01$; prenatal treatment × PCP challenge: $F_{1,37}=5.73$, $p<0.05$; time: $F_{17,629}=45.82$, $p<0.01$; time × prenatal treatment: $F_{17,629}=2.33$, $p<0.01$; time × PCP challenge: $F_{17,629}=20.20$, $p<0.01$; time × prenatal treatment × PCP challenge: $F_{17,629}=1.13$, $p>0.05$, repeated two-way ANOVA; Fig. 2*a*), and the entire 90 min (30–120 min) ($F_{\text{group}(1,37)}=9.20$, $p<0.01$; $F_{\text{treatment}(1,37)}=65.19$, $p<0.01$; $F_{\text{group}\times\text{treatment}(1,37)}=5.73$, $p<0.05$, two-way ANOVA; Fig. 2*b*).

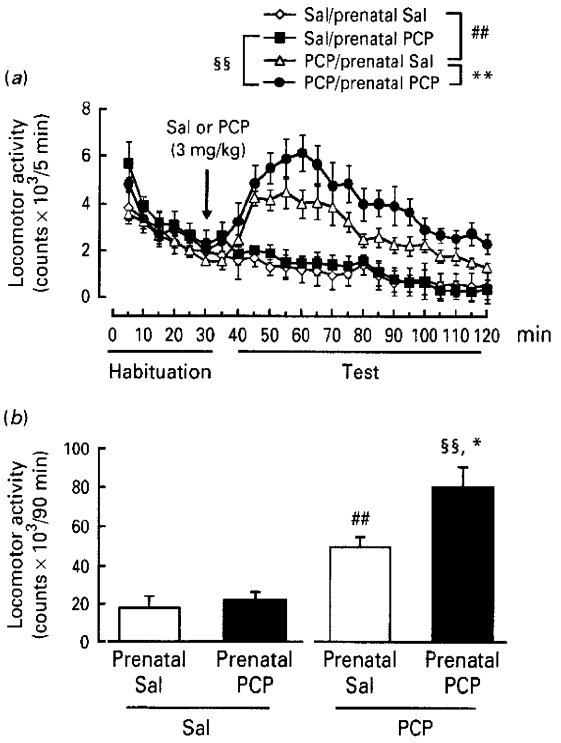

Fig. 2. Effect of prenatal phencyclidine (PCP) treatment on PCP-induced hyperlocomotion. PCP (3 mg/kg) or saline (Sal) was administered after 30 min of habituation. The locomotor activity of mice was assessed over 5-min intervals during the last 90 min after habituation (prenatal treatment: $F_{1,37}=9.54$, $p<0.01$; PCP challenge: $F_{1,37}=64.85$, $p<0.01$; prenatal treatment × PCP challenge: $F_{1,37}=5.73$, $p<0.05$; time: $F_{17,629}=45.82$, $p<0.01$; time × prenatal treatment: $F_{17,629}=2.33$, $p<0.01$; time × PCP challenge: $F_{17,629}=20.20$, $p<0.01$; time × prenatal treatment × PCP challenge: $F_{17,629}=1.13$, $p>0.05$, repeated two-way ANOVA) (*a*) and the entire 90 min (30–120 min) ($F_{\text{group}(1,37)}=9.20$, $p<0.01$; $F_{\text{treatment}(1,37)}=65.19$, $p<0.01$; $F_{\text{group × treatment}(1,37)}=5.73$, $p<0.05$, two-way ANOVA) (*b*). $^{\#\#}p<0.01$ compared to Sal/prenatal Sal group. $^{*}p<0.05$, $^{**}p<0.01$ compared to PCP/prenatal Sal group. $^{\S\S}p<0.01$ compared to Sal/prenatal PCP group. Data are expressed as the mean ± S.E.M. for 10–11 mice (Bonferroni's test).

Effect of prenatal-PCP treatment on cognitive function in the NORT

To investigate the effects of prenatal PCP treatment on cognitive function, recognition memory was evaluated in the NORT. In the training session, the prenatal saline- or PCP-treated mice spent equal amounts of time exploring either of the two objects, and there was no biased exploratory preference in each group (prenatal saline-treated mice, 50.4±1.7%; prenatal PCP-treated mice, 53.6±2.6%; $p>0.05$, Fig. 3*a*). In addition, the total time spent in exploration of objects in the training session did not differ between these two groups (prenatal saline-treated mice, 28.3±2.7 s; prenatal PCP-treated mice, 32.0±3.7 s; $p>0.05$, Fig. 3*b*). However, when retention performance was tested, the prenatal PCP-treated mice showed a reduced level of exploratory preference for the novel objects compared to the prenatal saline-treated group (prenatal saline-treated mice, 70.6±2.1%; prenatal PCP-treated mice, 54.5±2.9%; $p<0.01$, Fig. 3*c*). There was no significant difference in total exploration time in the retention session (prenatal saline-treated mice, 17.4±2.3 s; prenatal PCP-treated mice, 16.6±2.3 s; $p>0.05$, Fig. 3*d*).

The sizes of lateral ventricles and brain in prenatal PCP-treated mice

We examined whether prenatal exposure to PCP induced any architectural abnormalities of lateral ventricles and brain at age 7 wk. However, there were no obvious differences between the prenatal saline- and PCP-treated mice in the ratio of brain to body weight (prenatal saline-treated mice, 6.12±0.15%; prenatal PCP-treated mice, 6.02±0.17%; $p>0.05$, Supplementary Fig. S2*e*, available online), the size of lateral ventricles (prenatal treatment: $F_{1,6}=1.93$, $p>0.05$; bregma: $F_{3,18}=493.88$, $p<0.01$; prenatal treatment × bregma: $F_{3,18}=0.85$, $p>0.05$, repeated one-way ANOVA; Suppl. Fig. S2*f*) and of whole brain (prenatal treatment: $F_{1,6}=0.25$, $p>0.05$; bregma: $F_{3,18}=4.44$, $p<0.05$; prenatal treatment × bregma: $F_{3,18}=0.14$, $p>0.05$, repeated one-way ANOVA; Suppl. Fig. S2*g*), as well as the ratio of lateral ventricles to brain size (prenatal treatment: $F_{1,6}=3.93$, $p>0.05$; bregma: $F_{3,18}=564.37$, $p<0.01$; prenatal treatment × bregma: $F_{3,18}=1.98$, $p>0.05$, repeated one-way ANOVA; Suppl. Fig. S2*h*). These suggested the architecture of lateral ventricles was not affected by the prenatal treatment.

Changes in the expression and phosphorylation of the NR1 subunit of NMDA receptors of prenatal PCP-treated mice

We postulated that the abnormal behaviour was accompanied by a malfunction of NMDA receptors, since PCP as a non-competitive NMDA antagonist might inhibit NMDA receptors during development. The level of NR1 protein was significantly increased in the prenatal PCP-treated mice compared to that in the prenatal saline-treated mice (PFC: 100.0±7.6% *vs.* 152.0±12.9%; $p<0.01$, Fig. 4*a*; hippocampus: 100.0±10.8% *vs.* 140.9±12.2%; $p<0.05$, Fig. 5*e*; striatum: 100.0±10.9% *vs.* 138.3±10.8%; $p<0.05$, Fig. 4*i*). In contrast, the level of NR1 phosphorylated at Ser^{897} was

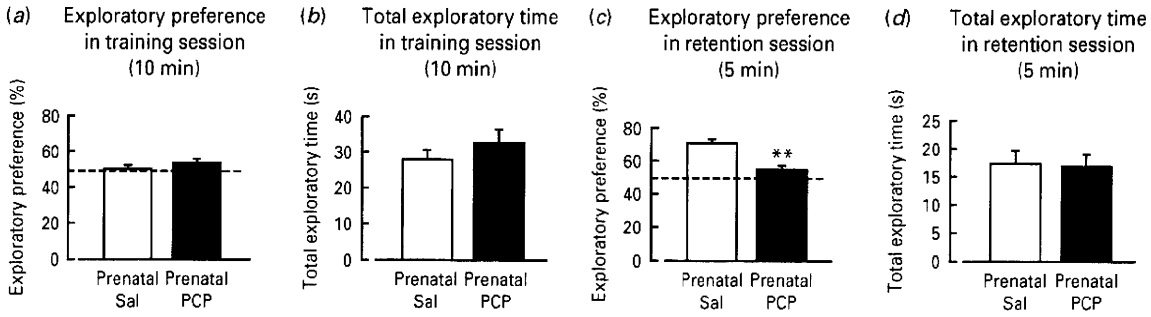

Fig. 3. Effect of prenatal phencyclidine (PCP) treatment on cognitive function in the novel object recognition test. Exploratory preference in (*a*) the training session and (*c*) the retention session. Total time spent exploring the objects in (*b*) the training session and (*d*) the retention session. ** $p<0.01$ compared to the prenatal saline (Sal) group. Data are expressed as the mean ± S.E.M. for 11–12 mice (Student's *t* test).

decreased in prenatal PCP-treated mice (PFC: 100.0 ± 5.9% *vs.* 75.4 ± 7.1%; $p<0.05$, Fig. 4*b*; hippocampus: 100.0 ± 10.5% *vs.* 67.8 ± 9.5%; $p<0.05$; Fig. 4*f*; striatum: 100.0 ± 7.3% *vs.* 87.1 ± 10.1%; $p>0.05$, Fig. 4*j*). Furthermore, the proportion of phosphorylated-NR1 was also significantly reduced in prenatal PCP-treated mice (PFC: 100.0 ± 8.5% *vs.* 50.1 ± 6.7%; $p<0.01$, Fig. 4*c*; hippocampus: 100.0 ± 13.4% *vs.* 48.8 ± 10.5%; $p<0.05$, Fig. 4*g*; striatum: 100.0 ± 14.3% *vs.* 55.0 ± 5.8%; $p<0.05$, Fig. 4*k*). Moreover, between the cognitive deficit in the NORT and the decreased level of phosphorylated NR1, there was a significant correlation in the PFC ($r=0.587$, $p=0.045$, Pearson's correlation; Fig. 4*d*), and a positive and almost significant correlation in the hippocampus ($r=0.569$, $p=0.054$, Pearson's correlation; Fig. 4*h*), but no correlation in the striatum ($r=0.325$, $p>0.05$, Pearson's correlation; Fig. 4*l*).

However, a lower dose of prenatal PCP exposure (5 mg/kg) did not affect the expression of phosphorylated NR1 in the PFC of postpubertal mice (100 ± 4.23% *vs.* 91.41 ± 6.69%; $p>0.05$, Suppl. Fig. S3). Additionally, the behavioural test itself did not affect the expression or phosphorylation of NR1 ($p>0.05$, Suppl. Fig. S4).

The neurotoxicity of prenatal-PCP treatment in the developing brain

To evaluate the neurotoxic effects of prenatal PCP treatment during neurodevelopment, the TUNEL-positive cells in the PFC were counted at PD 0, PD 7 and PD 49. As shown by the results, apoptosis was significantly increased at PD 0 (253.4 ± 19.9 *vs.* 338.8 ± 28.2; $p<0.05$, Suppl. Fig. S1*a*, *d*), but was not observed at either PD 7 (31.5 ± 3.9 *vs.* 37.2 ± 3.5; $p>0.05$, Suppl. Fig. S1*b*, *e*), or PD 49 (35.7 ± 5.1 *vs.* 39.1 ± 4.0; $p>0.05$, Suppl. Fig. S1*c*, *f*).

Effect of antipsychotics on the behavioural abnormalities in prenatal PCP-treated mice

We evaluated whether the prenatal PCP-induced behavioural changes were sensitive to both the atypical antipsychotic clozapine (Clz) and the typical antipsychotic haloperidol (Hal). The results showed that clozapine selectively attenuated the PCP-induced hypersensitivity over the 5-min intervals after habituation (30–120 min) in the prenatal PCP-treated mice (prenatal treatment: $F_{1,62}=15.41$, $p<0.01$; Clz: $F_{2,62}=29.07$, $p<0.01$; prenatal treatment × Clz: $F_{2,62}=5.23$, $p<0.01$; time: $F_{\text{time}(17,1054)}=70.46$, $p<0.01$; time × prenatal treatment: $F_{17,1054}=1.96$, $p<0.05$; time × Clz: $F_{34,1054}=3.03$, $p<0.01$; time × prenatal treatment × Clz: $F_{34,1054}=1.05$, $p>0.05$, repeated two-way ANOVA; Fig. 5*a*). However, haloperidol reduced the hyperlocomotion of mice in both the prenatal saline- and PCP-treated groups (prenatal treatment: $F_{1,63}=6.88$, $p<0.05$; Hal: $F_{2,63}=17.35$, $p<0.01$; prenatal treatment × Hal: $F_{2,63}=1.30$, $p>0.05$; time: $F_{\text{time}\,17,1071}=29.46$, $p<0.01$; time × prenatal treatment: $F_{17,1071}=1.66$, $p<0.05$; time × Hal: $F_{34,1071}=3.15$, $p<0.01$; time × prenatal treatment × Hal: $F_{34,1071}=0.92$, $p>0.05$, repeated two-way ANOVA; Fig. 5*c*). Furthermore, in terms of the entire 120-min period, the higher dose of clozapine (3 mg/kg) and haloperidol (0.1 and 0.3 mg/kg) reduced the locomotion in both the first 30 min and the last 90 min (Clz: 0–30 min: $F_{\text{group}(1,62)}=3.23$, $p>0.05$; $F_{\text{treatment}(2,62)}=5.98$, $p<0.01$; $F_{\text{group}\times\text{treatment}(2,62)}=0.12$, $p>0.05$, two-way ANOVA; 30–120 min: $F_{\text{group}(1,62)}=14.84$, $p<0.01$; $F_{\text{treatment}(2,62)}=29.07$, $p<0.01$; $F_{\text{group}\times\text{treatment}(2,62)}=5.23$, $p<0.01$, two-way ANOVA; Fig. 5*b*; Hal: 0–30 min: $F_{\text{group}(1,63)}=1.23$, $p>0.05$; $F_{\text{treatment}(2,63)}=14.90$, $p<0.01$; $F_{\text{group}\times\text{treatment}(2,63)}=0.56$, $p>0.05$, two-way ANOVA; 30–120 min: $F_{\text{group}(1,63)}=7.43$, $p<0.01$; $F_{\text{treatment}(2,63)}=17.28$, $p<0.01$; $F_{\text{group}\times\text{treatment}(2,63)}=1.30$, $p>0.05$, two-way

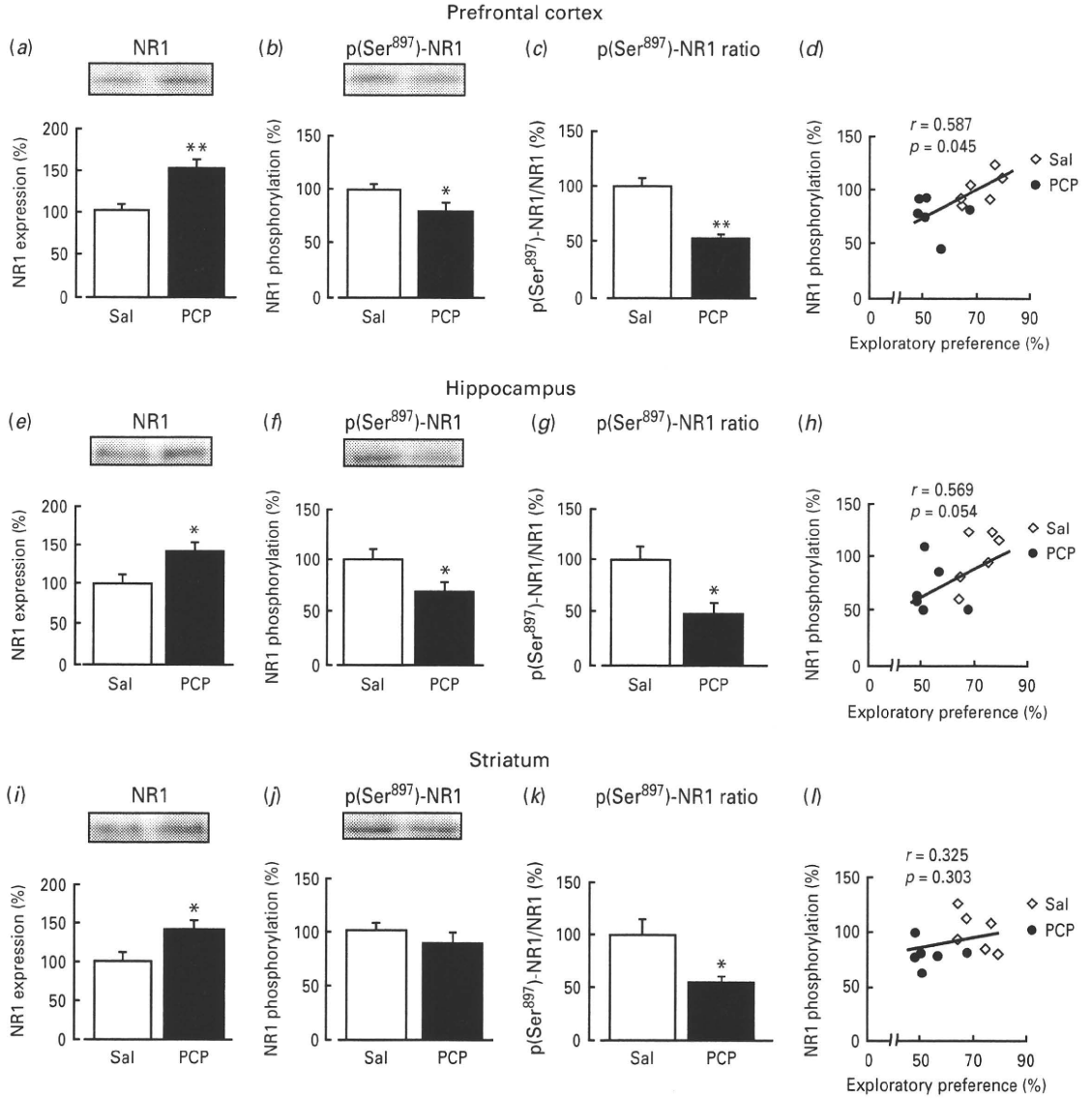

Fig. 4. Changes in the expression and phosphorylation of the NR1 subunit of the NMDA receptor of prenatal phencyclidine (PCP)-treated mice. Expression of NR1 and phosphorylated NR1 [p(Ser^{897})-NR1] was detected by Western blotting. Loaded protein was normalized to β-actin. The phosphorylation ratio was calculated as NR1 phosphorylation *vs.* NR1 expression. Results are represented as the level of NR1 expression in (*a*) the PFC, (*e*) hippocampus and (*i*) striatum; the level of NR1 phosphorylation (Ser^{897}) in (*b*) the PFC, (*f*) hippocampus and (*j*) striatum; and the ratio of NR1 phosphorylation *vs.* NR1 expression in (*c*) the PFC, (*g*) hippocampus and (*k*) striatum. The correlation of phosphorylated NR1 (Ser^{897}) with exploratory preference in the retention session of the novel object recognition test in (*d*) the PFC, (*h*) hippocampus, and (*l*) striatum. * $p<0.05$, ** $p<0.01$ compared to the prenatal saline (Sal) group. Data are expressed as the mean $\pm$ S.E.M. for six mice in each group (Student's *t* test).

ANOVA; Fig. 5*d*). However, the lower dose of clozapine (1 mg/kg) did not affect the locomotion of prenatal saline-treated mice during the 120 min (0–30 min, 30–120 min; $p>0.05$, respectively).

Next, we evaluated the effects of antipsychotics on the impairment of recognition memory. There was no bias in exploratory preference (Clz: $F_{\text{group}(1,53)}=0.42$, $p>0.05$; $F_{\text{treatment}(2,53)}=0.32$, $p>0.05$; $F_{\text{group}\times\text{treatment}(2,53)}=0.23$, $p>0.05$, two-way ANOVA; Fig. 6*a*; Hal: $F_{\text{group}(1,50)}=0.05$, $p>0.05$; $F_{\text{treatment}(2,50)}=0.23$, $p>0.05$; $F_{\text{group}\times\text{treatment}(2,50)}=1.27$, $p>0.05$, two-way ANOVA; Fig. 6*e*), or total exploration time after clozapine (1 mg/kg) and haloperidol (0.1 mg/kg) treatment in the training session, although the higher

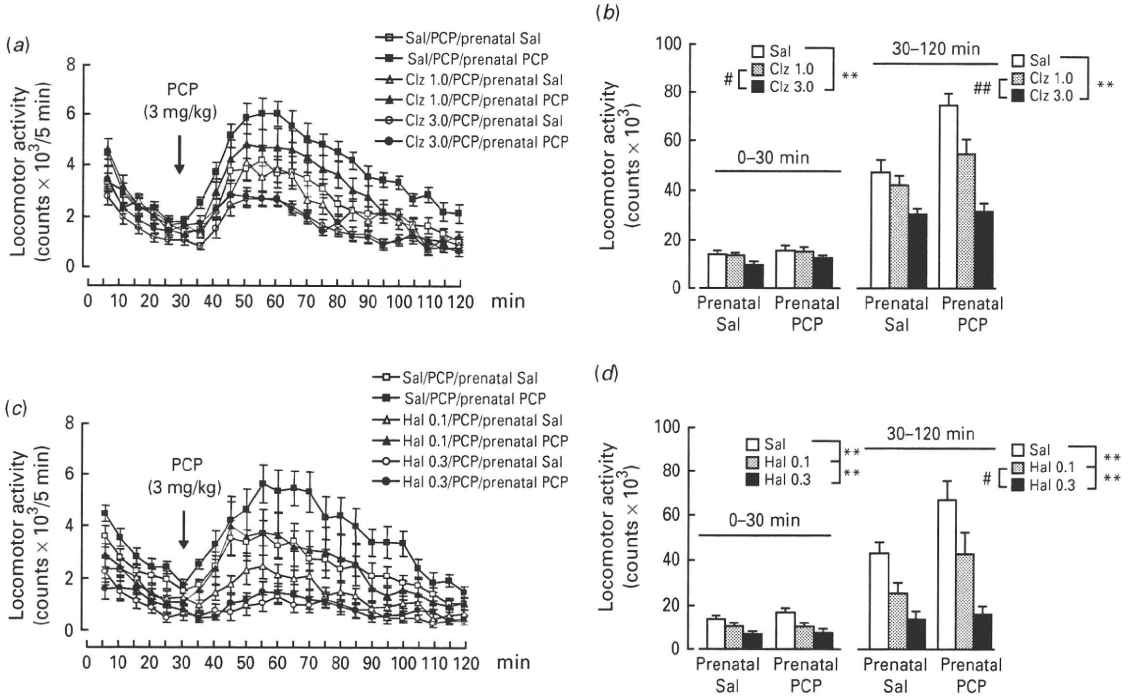

Fig. 5. Effects of antipsychotics on phencyclidine (PCP)-induced hyperlocomotion in prenatal PCP-treated mice. Clozapine (Clz; 1 or 3 mg/kg) and haloperidol (Hal; 0.1 or 0.3 mg/kg) were administered to mice 30 min before the test. After 30 min habituation, mice were challenged with PCP (3 mg/kg). The effects of clozapine or haloperidol on the PCP-induced hyperlocomotion were assessed over 5-min intervals during the last 90 min after habituation (prenatal treatment: $F_{1,62}=15.41$, $p<0.01$; Clz: $F_{2,62}=29.07$, $p<0.01$; prenatal treatment × Clz: $F_{2,62}=5.23$, $p<0.01$; time: $F_{\text{time}(17,1054)}=70.46$, $p<0.01$; time × prenatal treatment: $F_{17,1054}=1.96$, $p<0.05$; time × Clz: $F_{34,1054}=3.03$, $p<0.01$; time × prenatal treatment × Clz: $F_{34,1054}=1.05$, $p>0.05$, repeated two-way ANOVA) (*a*) and haloperidol treatment (prenatal treatment: $F_{1,63}=6.88$, $p<0.05$; Hal: $F_{2,63}=17.35$, $p<0.01$; prenatal treatment × Hal: $F_{2,63}=1.30$, $p>0.05$; time: $F_{\text{time}(17,1071)}=29.46$, $p<0.01$; time × prenatal treatment: $F_{17,1071}=1.66$, $p<0.05$; time × Hal: $F_{34,1071}=3.15$, $p<0.01$; time × prenatal treatment × Hal: $F_{34,1071}=0.92$, $p>0.05$, repeated two-way ANOVA) (*c*); and the entire 120 min (0–30 min, 30–120 min) by clozapine treatment (Clz: 0–30 min: $F_{\text{group}(1,62)}=3.23$, $p>0.05$; $F_{\text{treatment}(2,62)}=5.98$, $p<0.01$; $F_{\text{group}\times\text{treatment}(2,62)}=0.12$, $p>0.05$, two-way ANOVA; 30–120 min: $F_{\text{group}(1,62)}=14.84$, $p<0.01$; $F_{\text{treatment}(2,62)}=29.07$, $p<0.01$; $F_{\text{group}\times\text{treatment}(2,62)}=5.23$, $p<0.01$, two-way ANOVA) (*b*); and haloperidol treatment (Hal: 0–30 min: $F_{\text{group}(1,63)}=1.23$, $p>0.05$; $F_{\text{treatment}(2,63)}=14.90$, $p<0.01$; $F_{\text{group}\times\text{treatment}(2,63)}=0.56$, $p>0.05$, two-way ANOVA; 30–120 min: $F_{\text{group}(1,63)}=7.42$, $p>0.05$; $F_{\text{treatment}(2,63)}=17.28$, $p<0.01$; $F_{\text{group}\times\text{treatment}(2,63)}=1.30$, $p>0.05$, two-way ANOVA) (*d*). ** $p<0.01$ compared to saline (Sal) treatment. # $p<0.05$, ## $p<0.01$ compared to the lower dose of clozapine (1 mg/kg) or haloperidol (0.1 mg/kg) treatments. Data are expressed as the mean ± S.E.M. for 8–14 mice (Bonferroni's test).

dose had a slight effect (Clz: $F_{\text{group}(1,53)}=0.02$, $p>0.05$; $F_{\text{treatment}(2,53)}=4.27$, $p<0.05$; $F_{\text{group}\times\text{treatment}(2,53)}=0.59$, $p>0.05$, two-way ANOVA; Fig. 6*b*; Hal: $F_{\text{group}(1,50)}=3.24$, $p>0.05$; $F_{\text{treatment}(2,50)}=25.84$, $p<0.01$; $F_{\text{group}\times\text{treatment}(2,50)}=0.35$, $p>0.05$, two-way ANOVA; Fig. 6*f*). Interestingly, the impairment of recognition memory in prenatal PCP-treated mice was significantly improved by clozapine (Clz: $F_{\text{group}(1,53)}=16.11$, $p<0.01$; $F_{\text{treatment}(2,53)}=3.42$, $p<0.05$; $F_{\text{group}\times\text{treatment}(2,53)}=4.04$, $p<0.05$, two-way ANOVA; Fig. 6*c*), but not by haloperidol (Hal: $F_{\text{group}(1,50)}=56.22$, $p<0.01$; $F_{\text{treatment}(2,50)}=0.09$, $p>0.05$; $F_{\text{group}\times\text{treatment}(2,50)}=0.16$, $p>0.05$, two-way ANOVA; Fig. 6*g*). However, there were no differences in total exploration time in the retention sessions (Clz: $F_{\text{group}(1,53)}=1.72$, $p>0.05$; $F_{\text{treatment}(2,53)}=0.25$, $p>0.05$; $F_{\text{group}\times\text{treatment}(2,53)}=0.16$, $p>0.05$, two-way ANOVA; Fig. 6*d*; Hal: $F_{\text{group}(1,50)}=1.09$, $p>0.05$; $F_{\text{treatment}(2,50)}=0.20$, $p>0.05$; $F_{\text{group}\times\text{treatment}(2,50)}=0.10$, $p>0.05$, two-way ANOVA; Fig. 6*h*).

Discussion

Hypersensitivity to NMDA receptor antagonists has been demonstrated in adult rodents after repeated administration of PCP (Nabeshima *et al.* 1987; Nagai *et al.* 2003), and observed in schizophrenia patients. Perinatal exposure to PCP and prenatal exposure to

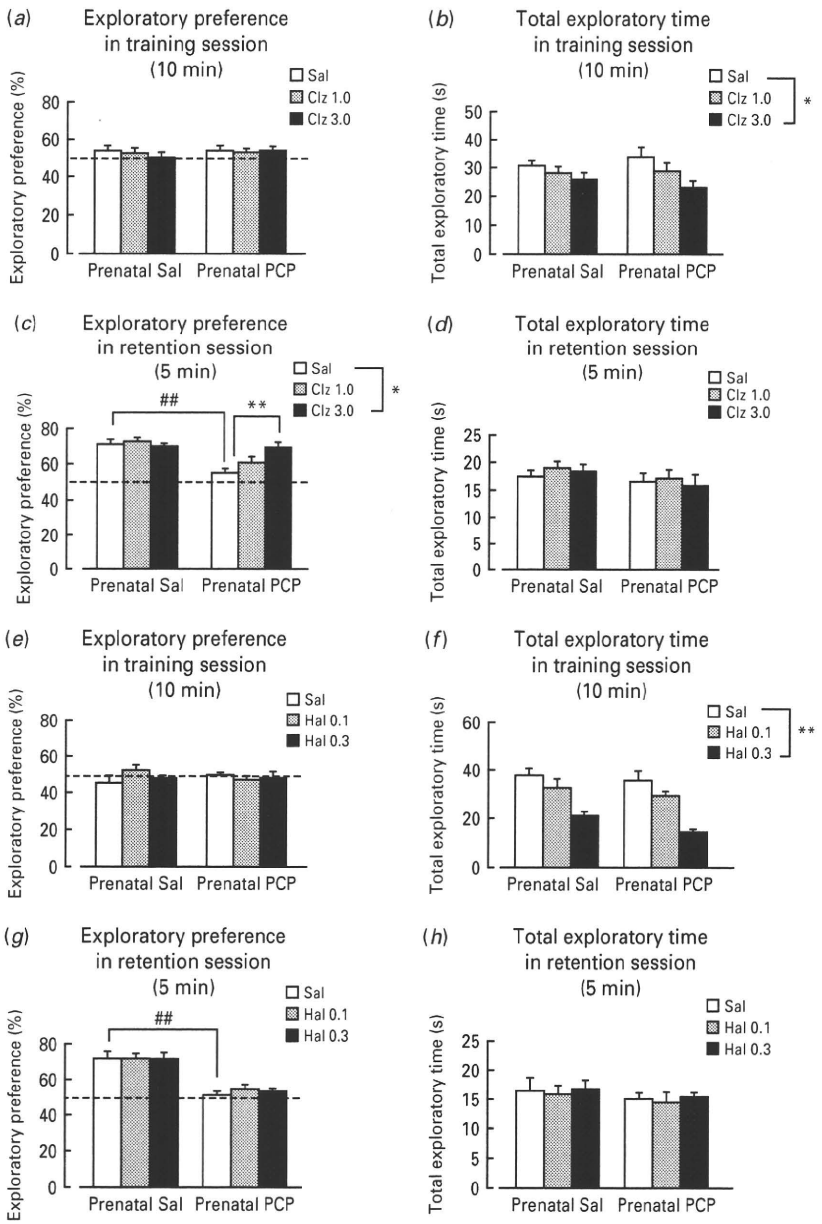

Fig. 6. Effects of antipsychotics on cognitive dysfunction in prenatal PCP-treated mice. Clozapine (Clz; 1 or 3 mg/kg) and haloperidol (Hal; 0.1 or 0.3 mg/kg) were administered 30 min before the training session. For clozapine treatment: (*a*) exploratory preference in the training session ($F_{\text{group}(1,53)}=0.42$, $p>0.05$; $F_{\text{treatment}(2,53)}=0.32$, $p>0.05$; $F_{\text{group}\times\text{treatment}(2,53)}=0.23$, $p>0.05$, two-way ANOVA), and (*c*) retention session ($F_{\text{group}(1,53)}=16.11$, $p<0.01$; $F_{\text{treatment}(2,53)}=3.42$, $p<0.05$; $F_{\text{group}\times\text{treatment}(2,53)}=4.04$, $p<0.05$, two-way ANOVA). Total exploration time in (*b*) the training session ($F_{\text{group}(1,53)}=0.02$, $p>0.05$; $F_{\text{treatment}(2,53)}=4.27$, $p<0.05$; $F_{\text{group}\times\text{treatment}(2,53)}=0.59$, $p>0.05$, two-way ANOVA) and (*d*) retention session ($F_{\text{group}(1,53)}=1.72$, $p>0.05$; $F_{\text{treatment}(2,53)}=0.25$, $p>0.05$; $F_{\text{group}\times\text{treatment}(2,53)}=0.16$, $p>0.05$, two-way ANOVA). For haloperidol treatment: (*e*) exploratory preference in the training session ($F_{\text{group}(1,50)}=0.05$, $p>0.05$; $F_{\text{treatment}(2,50)}=0.23$, $p>0.05$; $F_{\text{group}\times\text{treatment}(2,50)}=1.27$, $p>0.05$, two-way ANOVA) and (*g*) retention session ($F_{\text{group}(1,50)}=56.22$, $p<0.01$; $F_{\text{treatment}(2,\ 50)}=0.09$, $p>0.05$; $F_{\text{group}\times\text{treatment}(2,50)}=0.16$, $p>0.05$, two-way ANOVA). Total exploration time in (*f*) the training session ($F_{\text{group}(1,50)}=3.24$, $p>0.05$; $F_{\text{treatment}(2,50)}=25.84$, $p<0.01$; $F_{\text{group}\times\text{treatment}(2,50)}=0.35$, $p>0.05$, two-way ANOVA) and (*h*) retention session ($F_{\text{group}(1,50)}=1.09$, $p>0.05$; $F_{\text{treatment}(2,50)}=0.20$, $p>0.05$; $F_{\text{group}\times\text{treatment}(2,50)}=0.10$, $p>0.05$, two-way ANOVA). * $p<0.05$, ** $p<0.01$ compared to saline (Sal) treatment. ## $p<0.01$ compared to the prenatal Sal-treated group. Data are expressed as the mean ± S.E.M for 8–12 mice (Bonferroni's test).

(+)-MK-801, enhanced PCP-induced hyperlocomotion in rats (Abekawa *et al.* 2007; Wang *et al.* 2001). In the present study, mice with prenatal exposure to PCP showed hypersensitivity to PCP at age 7 wk and this hypersensitivity was reversed by antipsychotics. PCP easily crosses the placenta (Kaufman *et al.* 1983; Nicholas *et al.* 1982). Fico & Vanderwende (1988) found that PCP was rapidly transported into the fetal brain and disappeared in 8 h after maternal exposure during pregnancy. These findings suggest that prenatal PCP exposure results in a behavioural hypersensitivity similar to the neonatal and adulthood exposure.

A blockade of NMDA receptors by antagonists during development impairs cognitive function. For instance, prenatal exposure to PCP disrupts the passive avoidance response and pole-climbing avoidance response (Nabeshima *et al.* 1988), and impairs performance in the eight-arm maze and Morris water maze in adult rats (Yanai *et al.* 1992). In the present study, prenatal PCP exposure caused an impairment of recognition memory. Since NMDA receptors play a critical role in memory formation (Rao & Finkbeiner, 2007) and the hypofunction of NMDA receptors to be involved in the cognitive deficits in PCP-treated adult mice (Enomoto *et al.* 2005; Mouri *et al.* 2007*c*), we postulated that prenatal exposure to PCP results in a disturbance of NMDA receptors, associated with cognitive dysfunction.

To test this hypothesis, we evaluated the expression and function of NMDA receptors. Phosphorylated NR1 modulates the activity and function of NMDA receptors (Scott *et al.* 2003), and its expression is down-regulated in the post-mortem brains of schizophrenia patients (Emamian *et al.* 2004). In the present study, prenatal PCP-treated mice showed an increase in NR1 expression but a reduction in the level and proportion of NR1 phosphorylated at Ser^{897}. The up-regulation of NR1 expression is consistent with the inhibition of NMDA receptors in the developing brain causes an up-regulation of NMDA receptors (Anastasio & Johnson, 2008; Haberny *et al.* 2002; Slikker *et al.* 2007; Wang *et al.* 2001). It is likely that the up-regulated expression of NR1 is due to a compensatory attempt to re-establish the delicate balance of the neurotransmitter network. However, a decreased level of phosphorylated NR1 suggests the function of NMDA receptors is impaired. Moreover, there was a clearly shown positive correlation between decreased NR1 phosphorylation and memory deficits in the PFC. In addition, D-serine, a NMDA receptor agonist, is reported to reverse the spatial memory deficits in perinatal PCP-treated rats (Andersen & Pouzet, 2004). These results suggest that the impairment of recognition memory is associated with the disturbance of NMDA receptors.

In clinical tests, atypical antipsychotics are used to control both the positive and negative symptoms of schizophrenia, especially cognitive dysfunction. It has been found that atypical antipsychotics attenuate cognitive dysfunction in PCP-treated adult mice (Amitai *et al.* 2007; Nagai *et al.* 2009), and perinatal PCP-treated rats (Anastasio & Johnson, 2008; Wang *et al.* 2001). In the present study, clozapine, but not haloperidol, selectively attenuated the PCP-induced hyperlocomotion and improved the cognitive dysfunction in prenatal PCP-treated mice. Clozapine promotes the function of NMDA receptors by increasing NMDA receptor-mediated excitatory postsynaptic potentials (EPSCs) (Chen & Yang, 2002), regulating protein kinase A (PKA)-cAMP signal transduction (Leveque *et al.* 2000), and specifically phosphorylating Ser^{897} of the NR1 subunit (Raman *et al.* 1996), as well as enhancing NMDA-mediated glutamatergic release (Millan, 2005). Furthermore, clozapine facilitated long-term potentiation in the PFC (Gemperle *et al.* 2003). Therefore, a reversed hypofunction of NMDA receptors might be responsible for the beneficial effect on schizophrenia-related cognitive deficits caused by prenatal PCP exposure.

Many neurons undergo a stage when they are critically dependent on stimulation by glutamate through the NMDA receptors, and sustained deprivation of this input during development activates apoptosis (Ikonomidou *et al.* 1999). Apoptosis is dependent on the stage of development, which occurs only in late fetal and early neonatal life (Ikonomidou *et al.* 1999). In our study, we found that enhanced apoptosis occurred at PD 0, but disappeared at PD 7 and PD 49, and there were no obvious architectural abnormalities of ventricles and brain in adults. These results suggest that neurotoxicity is involved in these behavioural changes, although it is relatively temporary and not sufficiently severe to alter the ventricular architecture. Therefore, it is possible that such neurotoxicity induces developmental changes that give rise to neuronal loss, or results in cytoarchitectural abnormalities implicated in abnormal behaviour in later life. Moreover, other factors implicated in neurodevelopment are also probably involved, since the inhibition of NMDA receptors by antagonists during development disrupts neuronal migration (Komuro & Rakic, 1993), inhibits neuronal proliferation (Behar *et al.* 1999), and reduces neuronal numbers and volume (Komuro & Rakic, 1993). In addition, abnormalities of some neurodevelopmental markers, such as brain-derived

neurotropic factor (BDNF) and reelin, which plays a critical role in neurodevelopment and is implicated in schizophrenia (Angelucci *et al.* 2005; Impagnatiello *et al.* 1998), are also quite likely to be involved in these changes. However, the exact effects of them need to be investigated further.

In conclusion, our findings suggest that prenatal exposure to PCP produces long-term behavioural changes accompanied by abnormal expression and impaired function of NR1. Since the altered expression of NMDA receptors in the developing brain is considered part of the pathogenesis of schizophrenia, the present study might provide further insight into the influences of neurodevelopmental abnormalities during the prenatal period on behaviour in later life, via the disruption of NMDA receptors.

Note

Supplementary material accompanies this paper on the Journal's website (http://journals.cambridge.org/pnp).

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Statement of Interest

None.

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