0026-895X/07/7106-1598–1609$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2007 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 71:1598–1609, 2007

Vol. 71, No. 6
32961/3207406
Printed in U.S.A.

Involvement of a Dysfunctional Dopamine-D1/*N*-Methyl-D-aspartate-NR1 and Ca^{2+}/Calmodulin-Dependent Protein Kinase II Pathway in the Impairment of Latent Learning in a Model of Schizophrenia Induced by Phencyclidine[S]

Akihiro Mouri, Yukihiro Noda, Akihiro Noda, Tomonobu Nakamura, Takanobu Tokura, Yoshimitsu Yura, Atsumi Nitta, Hiroshi Furukawa, and Toshitaka Nabeshima

Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya, Japan (A.M., Y.N., A.N., T.N., T.T., Y.Y., A.N., T.N.); and Division of Clinical Science in Clinical Pharmacy Practice, Management and Research (Y.N.) and Department of Medicinal Chemistry (H.F.), Faculty of Pharmacy, Meijo University, Nagoya, Japan

Received November 23, 2006; accepted March 7, 2007

ABSTRACT

Continuous ingestion of phencyclidine (PCP) in humans produces long-lasting schizophrenic-like cognitive dysfunction. Although a malfunction of dopaminergic and/or glutamatergic neurotransmission is implicated in the etiology of schizophrenia, involvement of the dopaminergic-glutamatergic neurotransmission in the cognitive dysfunction induced by repeated PCP treatment is minor. We demonstrated that mice treated with PCP (10 mg/kg/day s.c.) for 14 days displayed an impairment of latent learning in a water-finding task and of learning-associated phosphorylation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and NR1 in the prefrontal cortex even after drug withdrawal. The infusion of a CaMKII inhibitor and NR1 antisense oligonucleotide into the prefrontal cortex produced an impairment of latent learning and decrease of learning-associated phosphorylation of CaMKII, which were observed in the PCP-treated mice. Exogenous NMDA-induced CaMKII activation was not observed in slices of the prefrontal cortex prepared from mice treated repeatedly with PCP. The potentiation of NMDA receptor function by the infusion of glycine into the prefrontal cortex ameliorated these impairments in mice treated repeatedly with PCP. The high potassium-stimulated release of dopamine from the prefrontal cortex was less extensive in the PCP-treated than saline-treated mice. The infusion of a dopamine-D1 receptor agonist into the prefrontal cortex attenuated the impairment of latent learning and decrease of learning-associated NR1 phosphorylation in the PCP-treated mice, suggesting a functional linkage between glutamatergic and dopaminergic signaling. These findings indicate that repeated PCP treatment impairs latent learning through a prefrontal cortical dysfunction of NMDA-CaMKII signaling, which is associated with dopaminergic hypofunction.

This work was supported, in part, by grants-in-aid for scientific research from the Japan Society for the Promotion of Science (14370031, 15922139, 16922036, and 17390018) and for Scientific Research on Priority Areas on "elucidation of glia-neuron network mediated information processing systems" from the Ministry of Education, Culture, Sports, Science and Technology (16047214), by funds from Integrated Molecular Medicine for Neuronal and Neoplastic Disorders [21st Century COE (Center of Excellence) program], from the Research on Regulatory Science of Pharmaceuticals and Medical Devices, from the Japan Brain Foundation, and from the Mitsubishi Pharma Research Foundation, and by an SRF (Smoking Research Foundation) Grant for Biomedical Research.

A.M. and Y.N. contributed equally to this work.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.106.032961.

[S] The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.

Schizophrenia is characterized by severe and persisting deficits in cognitive functions (Winterer and Weinberger, 2004). Several lines of evidence suggest that the *N*-methyl-D-aspartate (NMDA) receptor is involved in the pathogenesis of schizophrenic cognitive dysfunction. Postmortem studies in patients with schizophrenia have identified altered expression patterns of NMDA receptor subunits (Dracheva et al., 2001) and decreased levels of phosphorylated NR1, an essential NMDA receptor subunit (Emamian et al., 2004) in the prefrontal cortex, which is considered to be the region contributing most to the pathophysiology of schizophrenic

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; PCP, phencyclidine [1-(1-phenylcyclohexyl) piperidine hydrochloride]; KN93, 2-[*N*-(2-hydroxyethyl)]-*N*-(4-methoxybenzenesulfonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine; KN92, 2-[*N*-(4-methoxybenzenesulfonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine; NR1 subunit; *N*-methyl-D-aspartate receptor ζ subunit; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; SKF81297, (±)-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrobromide; AP, anteroposterior; ML, mediolateral; PKA, protein kinase A; ANOVA, analysis of variance.

cognitive dysfunction (Winterer and Weinberger, 2004). Some genetic analyses have disclosed that single-nucleotide or dinucleotide-repeated polymorphisms of the NMDA receptor subunit gene increase susceptibility to schizophrenia (Rice et al., 2001; Itokawa et al., 2003).

It is noteworthy that the prolonged ingestion of phencyclidine (PCP), a noncompetitive NMDA receptor antagonist, induces long-lasting neuropsychological deficits, including cognitive dysfunction for several weeks (Rainey and Crowder, 1975). In rodents, repeated PCP treatment activates the mesolimbic dopamine pathway and impairs prefrontal cortical function (Jentsch and Roth, 1999). These observations suggest that chronic PCP psychosis might be more consistent with schizophrenia than acute PCP psychosis (Javitt and Zukin, 1991; Jentsch and Roth, 1999). Experiments with animals revealed enduring cognitive dysfunction after repeated PCP treatment in a working memory task involving object retrieval with a detour and T-maze (Jentsch et al., 1997a,b) and in an associative learning task with conditioned fear (Enomoto et al., 2005). Therefore, animals treated repeatedly with PCP might be an excellent pharmacological model of schizophrenic cognitive dysfunction (Jentsch et al., 1997a,b; Enomoto et al., 2005).

Recent studies have found that schizophrenic patients have not only an explicit but also an implicit (latent) learning deficit (Exner et al., 2006). Latent learning is defined as a demonstration of learning in the absence of reinforcement. In animal experiments, the water-finding test is one of the behavioral tasks used to evaluate latent learning (Nabeshima and Ichihara, 1993; Noda et al., 2001). In the training trial of the water-finding task, a non–water-deprived animal is allowed to explore an apparatus containing a water tube, the position of which it should recognize, although there is no motivation and/or reinforcement. After the training trial, the animal is deprived of water until the test trial to promote recall of the location of the water tube in the apparatus to which it was exposed in the training trial. The trained animal recalls and finds the location of the water tube more rapidly than an animal that has not been exposed previously to this environment, indicating latency to drink from the water tube provides a measure of latent learning in mice. Latent learning in the water-finding test depends on spatial attention (Ichihara et al., 1993), because searching behavior accompanied by attention is necessary to acquire a spatial memory of the apparatus in the absence of reinforcement. This view is consistent with the clinical evidence that attention is involved in latent learning in a serial reaction time task (Jiang and Leung, 2005) used to evaluate latent learning in persons with schizophrenia (Exner et al., 2006). We have already found that acute PCP-treated mice show an impairment of latent learning in the water-finding test (Noda et al., 2001). Animals treated repeatedly with PCP might be a better model of schizophrenia than those treated acutely, because even a few days after withdrawal from PCP, mice show cognitive dysfunction related to schizophrenia (Jentsch et al., 1997a,b; Enomoto et al., 2005). However, there have been few studies of latent learning in mice treated repeatedly with PCP.

In the pathophysiology of schizophrenia, impaired functioning of the glutamatergic and dopaminergic systems in the prefrontal cortex is considered a major factor contributing to the cognitive dysfunction (Carlsson et al., 2001). The molecular mechanisms of cognitive dysfunction in mice treated repeatedly with PCP have not been investigated in detail. The present study was designed to test the hypothesis that PCP-pretreated mice develop an impairment of latent learning via a malfunction of dopaminergic-glutamatergic signaling in the water-finding test after drug withdrawal. We attempted to investigate: 1) whether latent learning is impaired after repeated administration of PCP and 2) whether such cognitive dysfunction is mediated via a malfunction of NMDA receptor signaling. Finally, we investigated the functional linkage between glutamatergic and dopaminergic signaling in the prefrontal cortices of mice treated repeatedly with PCP, because dopamine receptors modulate the increase of NMDA-mediated excitability in the prefrontal cortical neurons (Wang and O'Donnell, 2001; Tseng and O'Donnell, 2004).

Materials and Methods

Animals. Male mice of the ddY strain (Japan SLC Inc., Shizuoka, Japan), weighing approximately 30 g at the beginning of the experiments, were used. The animals were housed in plastic cages and were kept in a regulated environment (25 ± 1°C, 50 ± 5% humidity), with a 12-h light/dark cycle (lights on at 08:00 AM, off at 8:00 PM). Food (CE2; Clea Japan Inc., Tokyo, Japan) and tap water were available ad libitum. All experiments were performed in accordance with the Guidelines for Animal Experiments of the Nagoya University School of Medicine. The procedures involving animals and their care were conducted in conformity with international guidelines (Institute of Laboratory Animal Resources, 1996).

Drugs. Phencyclidine hydrochloride [1-(1-phenylcyclohexyl) piperidine hydrochloride (PCP)] was synthesized by the authors according to the method of Maddox et al. (1965) and was checked for purity. KN93 [a Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) inhibitor], KN92 (an inactive CaMKII inhibitor), and SKF81297 (a dopamine-D1 receptor agonist) were purchased from Sigma-Aldrich (St. Louis, MO). PCP, glycine, and SKF81297 were dissolved in saline solution. KN92 and KN93 were dissolved in a 0.01% dimethyl sulfoxide-containing saline solution. PCP was administered in a volume of 0.1 ml/10 g b.wt. KN93 (1 and 10 nmol/μl/mouse), KN92 (10 nmol/μl/mouse), glycine (1 μmol/μl/mouse), and SKF81297 (10 nmol/μl/mouse) were administered bilaterally into the frontal cortex [anteroposterior (AP), 1.7 mm; mediolateral (ML), ± 0.5 mm from bregma, dorsoventral (DV), 2 mm from the skull] according to the mouse brain atlas of Paxinos and Franklin (2004). The doses of KN93 used to inhibit CaMKII were based on those reported by Bevilaqua et al. (2005). These drugs were infused in a volume of 1 μl/side over 3 min under ether anesthesia. We checked that the drugs were infused into the appropriate region using a 0.1% Fast Green solution (Sigma-Aldrich) (Fig. 1A) and that there was no difference in latent learning between vehicle-infused and naive mice (data not shown).

Drug Treatment. Saline and PCP (10 mg/kg s.c.) were administered once a day for 14 days. The half-life of PCP in the brain is 30.5 min in rats treated repeatedly (Nabeshima et al., 1987). To exclude any effect of the PCP remaining in the brain on latent learning, mice were not trained in the water-finding task until 4 days after the final treatment. Mice showed no withdrawal symptoms 4 days after this abrupt withdrawal. It is noteworthy that rats undergoing long-term PCP treatment do not show withdrawal syndrome behavior (jumping, wet-dog shakes, and ptosis) 4 days after the final treatment (Nabeshima et al., 1986). KN93, KN92, glycine, and SKF81297 were administered 10 min before the training trial.

Antisense Oligonucleotide Treatment. The 18-mer phosphothionate antisense oligonucleotides were custom-synthesized at Nisshinbo Biotechnology (Tokyo, Japan) and dissolved in artificial cerebrospinal fluid (CSF; 147 mM NaCl, 3 mM $CaCl_2$, 3 mM KCl, 1.2 mM

$CaCl_2$, and 1 mM $MgCl_2$, pH 7.2). The NR1 antisense oligonucleotide (5′-CAGCAGGTGCATGGTGCT-3′) corresponds to nucleotides 4 to 21, which immediately follow the translation initiation codon. The antisense but not missense oligonucleotide has been reported to inhibit the synthesis of NR1 protein both in vivo and in vitro (Wahlestedt et al., 1993). The oligonucleotide or the corresponding vehicle was administered bilaterally into the frontal cortex as described above. The mice received four administrations of vehicle or 0.5 nmol of either the sense or antisense oligonucleotide per administration at 12-h intervals. Four hours after the last administration, mice were trained in the water-finding test.

Water-Finding Test. The apparatus consisted of an open field (30 × 50 × 15 cm high) with an alcove (10 × 10 × 10 cm high) in the middle of one of the long walls of the enclosure (Fig. 1B). The floor of the open field was divided into 15 identical squares for measuring locomotor activity (Fig. 1B). A drinking tube, identical to that used in the home cage, was inserted into the center of the alcove ceiling with its tip 6.5 cm (in the training trial) or 7.5 cm (in the test trial) above the floor to decrease the probability of its being found by chance in the test trial.

The experiments were carried out according to previous reports (Noda et al., 2001). In brief, the task consisted of two trials: a training trial (the first day) and a test trial (the second day). In the training trial, mice were placed individually into one corner of the open field of the apparatus and were allowed 3 min to explore the environment. During this time, ambulation was measured by counting the number of times the animal crossed from one square to another in the open field. The frequency of touching, sniffing, or licking of the water tube in the alcove (number of approaches) was also recorded. Animals that did not find the drinking tube during the 3-min exploratory period were omitted from the test trial. The mice were immediately returned to their home cages after the training trial and deprived of water for 24 h until the test trial. Nontrained mice were prepared for comparison with the trained mice in terms of their ability to find the water source in the same environment. In the test trial, mice were again individually placed on the test apparatus. The time taken to enter the alcove (entering latency) and the time between entering the alcove and drinking the water (finding latency) were scored (Fig. 1C). If the mice could not find the drinking tube within 5 min, the test trial was terminated.

Western Blot Analysis. Western blotting was performed as described previously (Enomoto et al., 2005). Immediately after a training trial, the mice were sacrificed by decapitation, and the brain was immediately removed. The prefrontal cortex (area surrounded with a broken line in Fig. 1A; thickness, 1.5 mm; AP, 1.5–3 mm from bregma) was rapidly dissected out on an ice-cold plate, frozen, and stored at −80°C until used.

To prepare total 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% SDS, 1% sodium deoxycholate, 1% Nonidet P-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.

Because membrane trafficking is a critical feature of the formation and plasticity of synapses, we investigated the effect of repeated PCP treatment on the expression and phosphorylation of a NMDA receptor subunit in the membrane-enriched extracts (P2 membrane proteins). The dissected brain tissue was homogenized in ice-cold 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 pellet (P2) was resuspended in ice-cold Tris buffer (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 concentration of total tissue and membrane-enriched extracts was determined using a detergent-compatible Protein Assay Kit (Bio-Rad, Richmond, CA). Samples (20 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 0.0004% bromphenol blue), separated on a polyacrylamide gel, and subsequently transferred to polyvinylidene difluoride membranes (Milli-

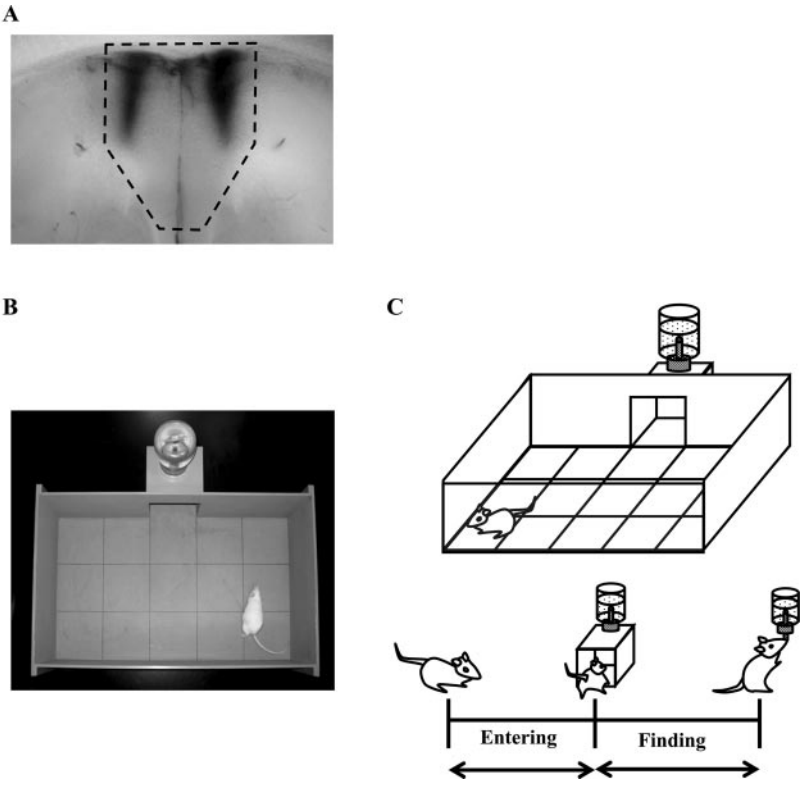

Fig. 1. Water-finding test. A, site of infusion and area dissected in the prefrontal cortex. KN93, KN92, glycine, SKF81297, and NR1 antisense oligonucleotides were injected into the prefrontal cortex of anesthetized mice and their presence was demonstrated by a dye marker, 0.1% Fast Green. The area surrounded with a broken line was dissected as the prefrontal cortex. B, the apparatus used for the water-finding task. The apparatus consisted of an open field with an alcove in the middle of one of the long walls of the enclosure. The floor of the open field was divided into 15 identical squares with black lines. A drinking tube was inserted into the center of the alcove. C, assessment of latent learning. A mouse was put in a corner of the open field and the time until it entered the alcove was measured as the entering latency, and the time between entering the alcove and drinking the water was measured as the finding latency. Latent learning was assessed by recording the entering and finding latencies in the test trial.

pore Corporation, 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; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) and exposed to X-ray film (Hyperfilm; GE Healthcare). 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 for measuring the phosphorylation ratio (protein phosphorylation/protein expression), after the protein phosphorylation 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 protein expression was detected as described above.

The primary polyclonal rabbit antibodies were anti-phospho-CaM kinase II α/β (Thr286/287) (1:1000; Upstate Biotechnology, Lake Placid, NY), anti-CaM Kinase II α (1:2000; Sigma-Aldrich), anti-phospho-NR1 (Ser897), and anti-NR1 C-terminal (1:1000; Upstate Biotechnology). The secondary antibodies, used at a dilution of 1:2000, were horseradish peroxidase-linked anti-mouse or anti-rabbit IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

Stimulation of Slices. Slices were stimulated essentially as described previously (Enomoto et al., 2005). Four days after the final PCP treatment, the mice were sacrificed by decapitation. The prefrontal cortex was dissected and sliced at a thickness of 300 μm in a McIlwain tissue chopper (Mickle Laboratory Engineering, Gomshall, Surrey, UK). After preincubation at 37°C in Ringer's buffer (10 mM HEPES-NaOH, pH 7.4, 135 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, and 10 mM glucose, gassed with 95% O_2 and 5% CO_2), each slice was stimulated with NMDA (100 μM) for 5 min. After stimulation of the NMDA receptor, the slices were homogenized as described above for Western blotting.

In Vivo Microdialysis. 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 left prefrontal cortex (15° angle away from AP +1.7; ML, +1.0 from bregma; DV, −1.5 from skull). One day after the operation, a dialysis probe (1-mm membrane length; AI-6-1; Eicom) was inserted through the guide cannula and perfused with artificial CSF (147 mM NaCl, 4 mM KCl, and 2.3 mM $CaCl_2$) at a flow rate of 1.2 μl/min. The outflow fractions were collected every 10 min. Dialysates were assayed by high-performance liquid chromatography with electrochemical detection (HTEC-300; Eicom) under the following conditions. An Eicompak PP-ODS column and a graphite electrode set at 400 mV against an Ag/AgCl reference electrode were used. The mobile phase contained 100 mM sodium phosphate buffer, pH 6.0, 500 mg/l sodium-1-decanesulfonic acid, 50 mg/l EDTA, and 1.5% (v/v) methanol. After the collection of three baseline fractions, mice were challenged with PCP (10 mg/kg s.c.). For depolarization, potassium chloride (50 mM) was locally perfused into the dialysis probe for 10 min to investigate its effect on the evoked dopamine release.

Statistical Analysis. All results were expressed as the mean ± S.E.M. for each group. The difference between groups was analyzed with a one- or two-way ANOVA, followed by the Bonferroni/Dunn multiple comparisons test. The Student's t test was used to compare two sets of data.

Results

Impairment of Latent Learning by Repeated PCP Treatment. In the training trial, there was no significant difference in exploratory behavior (number of approaches to the water tube and ambulation) among the groups (data not shown).

In the test trial, the trained mice treated repeatedly with saline ($n = 13$) showed significantly shorter latencies to enter the alcove (entering latency; $p < 0.01$; Fig. 2A) and to find the water tube and drink (finding latency; $p < 0.05$; Fig. 2A) than did the nontrained, saline-treated mice ($n = 10$), which had no exposure to the apparatus, indicating the occurrence of latent learning. The trained mice treated repeatedly with PCP ($n = 13$) (10 mg/kg s.c. once a day for 14 days; withdrawal 4 days) showed significantly shorter latencies to enter the alcove (entering latency; $p < 0.01$; Fig. 2A) than did the nontrained, PCP-treated mice ($n = 9$), indicating that the PCP-treated mice recognized the environment without any emotional deficit. In the test trial, the tip was placed 1 cm higher off the floor than in the training trial to decrease the probability of it being found by chance. The trained PCP-treated mice showed a significantly prolonged finding latency, compared with the trained saline-treated mice ($p < 0.05$; Fig. 2A), indicating that repeated PCP treatment induced an impairment of latent learning. A single PCP treatment (10 mg/kg s.c.) did not prolong finding latency 4 days after drug withdrawal ($n = 9$–10) ($p = 0.85$; Fig. 2B). It is noteworthy that after the test trial, we checked whether 24 h of water deprivation caused mice to crave water. All mice

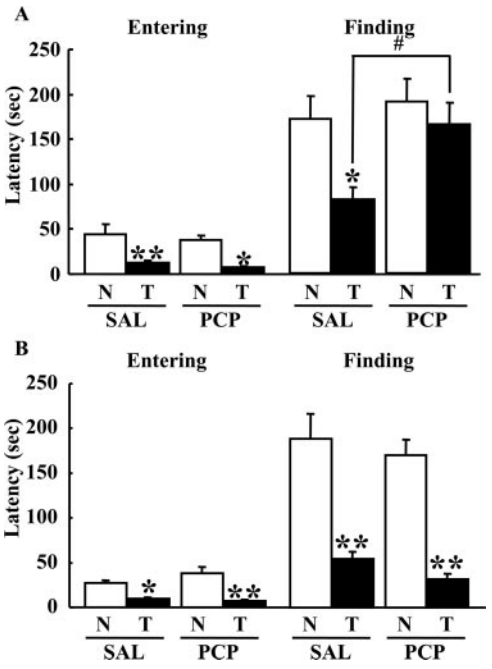

Fig. 2. Impairment of latent learning on repeated PCP treatment. A, latent learning in the mice treated repeatedly with PCP (10 mg/kg s.c. once a day for 14 days). These mice were subjected to a training trial of the water-finding task 4 days after the last PCP treatment. The entering and finding latencies were measured in the test trial 24 h after the training trial of the water-finding task. Each column represents the mean ± S.E.M. ($n = 9$–13). Results with the one-way ANOVA were: entering latency, $F_{3,41} = 8.89$; $p < 0.01$; finding latency, $F_{3,41} = 5.43$; $p < 0.01$. B, latent learning in the single PCP-treated mice. The mice receiving a single PCP treatment were subjected to a training trial of the water-finding task 4 days after PCP treatment. The entering and finding latencies were measured in the test trial 24 h after the training trial of the water-finding task. Each column represents the mean ± S.E.M. ($n = 9$–10). Results with the one-way ANOVA were: entering latency, $F_{3,35} = 10.61$, $p < 0.01$; finding latency, $F_{3,35} = 5.99$, $p < 0.01$. **, $p < 0.01$; *, $p < 0.05$ compared with corresponding nontrained mice. #, $p < 0.05$ compared with the trained, saline-treated mice. N, nontrained mice; T, trained mice; SAL, saline.

continued drinking water for more than 5 s, and there was no difference in drinking behavior among the groups.

Impairment of Learning-Associated CaMKII Activation in the Prefrontal Cortex on Repeated PCP Treatment in the Water-Finding Test. Because the NMDA/CaMKII signaling pathway plays an important role in learning and memory (Cammarota et al., 2002), we examined the learning-associated activation of CaMKII (i.e., phosphorylation of threonine 286 of the α-subunit; CaMKII phosphorylation) in the prefrontal cortex after the training trial for latent learning. Levels of phosphorylated CaMKII in the prefrontal cortex of the saline-treated mice ($n = 8$) were significantly increased immediately after the training trial, compared with those in the nontrained, saline-treated mice ($n = 8$) ($p < 0.01$; Fig. 3). However, they did not increase in the trained, PCP-treated mice ($n = 8$) above the basal level on exposure to the apparatus ($n = 7$) ($p = 0.99$, Fig. 3), and were significantly lower than those of the trained, saline-treated mice ($p < 0.01$, Fig. 3).

Impairment of Latent Learning by a CaMKII Inhibitor in the Water-Finding Test. To examine the relationship between the activation of CaMKII and latent leaning, we evaluated the effect of a CaMKII inhibitor on water-finding performance in mice. The mice microinjected with KN93, a CaMKII inhibitor, at 10 ($n = 13$) but not 1 ($n = 10$) nmol/mouse, bilaterally, into the prefrontal cortex before the training trial showed a significantly prolonged finding latency in the test trial, compared with the mice treated with vehicle ($n = 10$) (10 nmol of KN93; $p < 0.01$, 1 nmol of KN93; $p = 0.26$, Fig. 4A) or KN92 ($n = 13$; 10 nmol/mouse bilaterally), an inactive inhibitor (10 nmol of KN93; $p < 0.01$; 1 nmol of KN93, $p = 0.89$; Fig. 4A). State-dependent learning denotes the fact that information that has been learned while an animal is under the influence of a certain drug (state) can only be recalled when the animal is in the same state in which the information was learned, not in a different (i.e., undrugged) state (Zarrindast et al., 2006). The mice infused with 10 nmol of KN93 before both the training and test trials showed a significantly prolonged finding latency in the test trial compared with the mice treated with vehicle (data not shown). This prolonged finding latency was not different from that of the mice infused with KN93 only before the training trial (data not shown). It was suggested that there were no state-dependent effects of KN93 on latent learning in the water-finding task. Treatment with 10 nmol of KN93 ($n = 7$) significantly decreased the level of phosphorylated CaMKII after exposure to the apparatus, compared with the mice treated with vehicle ($n = 7$) ($p < 0.01$; Fig. 4B) or KN92 ($n = 7$) ($p < 0.01$; Fig. 4B).

Impairment by Repeated PCP Treatment of CaMKII Activation through NMDA Receptor Stimulation in Slices of the Prefrontal Cortex. To confirm that the activation of CaMKII is facilitated after stimulation of the NMDA receptor, we measured the amount of phosphorylated CaMKII in slices of the prefrontal cortex stimulated with NMDA (100 μM). Under our experimental conditions, an increase in phosphorylated CaMKII ($n = 6$) was detected 5 min after the stimulation compared with the basal level ($n = 6$) (without stimulation) in the prefrontal cortex prepared from the saline-treated mice ($p < 0.05$, Fig. 5). In the prefrontal cortex of the PCP-treated mice, however, stimulation with NMDA ($n = 6$) did not increase the level of phosphorylated CaMKII, which was significantly lower than that of the saline-treated mice ($p < 0.05$, Fig. 5).

Microinjection of Glycine into the Prefrontal Cortex Reversed the Impairment of Latent Learning and of Learning-Associated CaMKII Phosphorylation in the PCP-Treated Mice. Glycine is known as an agonist of glycine sites that stimulates NMDA receptors (Johnson and Ascher, 1987) and improves the symptoms of schizophrenia (Coyle and Tsai, 2004). Therefore, we investigated the effects of infusing glycine into the prefrontal cortex on the PCP-induced impairment of latent learning and CaMKII's activation. Glycine (1 μmol/mouse, bilaterally) treatment ($n = 9$) significantly shortened the prolonged finding latency induced by repeated PCP treatment ($n = 10$) ($p < 0.05$; Fig. 6A). In the Western blot analysis, the bilateral injection of glycine ($n = 12$) significantly increased the amount of phosphorylated CaMKII in the prefrontal cortex immediately after training in the PCP-treated mice ($n = 12$) ($p < 0.01$; Fig. 6B). The bilateral injection into the prefrontal cortex failed to affect the finding latency ($n = 7$–9) ($p = 0.33$; Supplemental Data 1A) and the learning-associated phosphorylation of CaMKII in the saline-treated mice ($n = 8$–10) ($p = 0.35$; Supplemental Data 1B).

Changes in the Phosphorylation and Expression of NMDA Receptor NR1 Subunits in the Prefrontal Cortex on Repeated PCP Treatment. To investigate whether the impairment of CaMKII's activation in the PCP-treated mice is dependent on a malfunction of NMDA receptors, the expression and activation of NR1 were examined. The NR1 expression level in the total tissue ex-

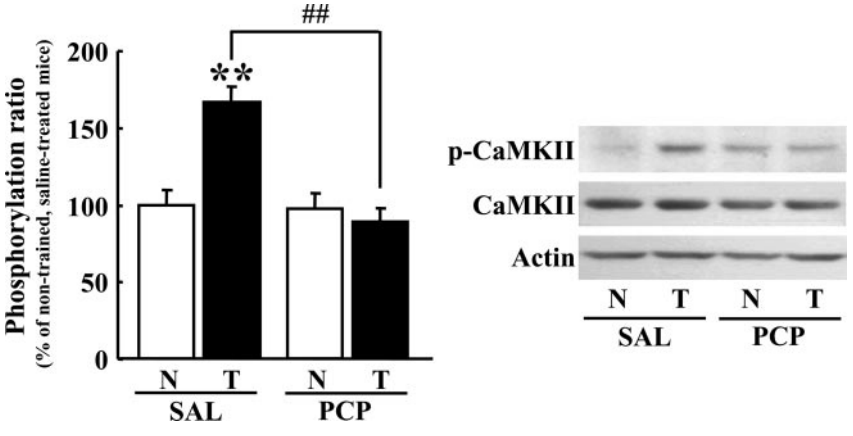

Fig. 3. Impairment of learning-associated CaMKII activation in the prefrontal cortex on repeated PCP treatment in the water-finding test. A training trial was performed 4 days after cessation of the repeated PCP treatment (10 mg/kg s.c. once a day for 14 days). Immediately after the training trial, mice were sacrificed by decapitation and CaMKII phosphorylation (Thr286; p-CaMKII) and αCaMKII expression (CaMKII) in the prefrontal cortex were detected by Western blotting. Loaded protein was normalized to actin. The phosphorylation ratio was calculated as CaMKII phosphorylation versus CaMKII expression. Each column represents the mean ± S.E.M. ($n = 7$–8). Results with the one-way ANOVA were: $F_{3,27} = 13.11$; $p < 0.01$. **, $p < 0.01$ compared with corresponding nontrained mice. ##, $p < 0.01$ compared with trained, saline-treated mice. N, nontrained mice; T, trained mice; SAL, saline.

tracts in the prefrontal cortex was significantly increased in the PCP-treated mice ($n = 6$) compared with the saline-treated mice ($n = 6$) ($p < 0.01$, Fig. 7B), whereas there was no significant difference in the NR1 expression level in the membrane-enriched extracts between the saline-treated ($n = 8$) and PCP-treated ($n = 7$) mice ($p = 0.61$; Fig. 7E). There was no significant difference in the level of phosphorylated NR1 (Ser^{897}) in the total tissue extracts between the saline-treated ($n = 6$) and PCP-treated mice ($n = 6$) ($p = 0.08$; Fig. 7A), whereas the level in the membrane-enriched extracts was significantly decreased in the PCP-treated mice ($n = 7$) compared with the saline-treated mice ($n = 8$) ($p < 0.01$, Fig. 7D). Thus, the NR1 phosphorylation ratio in total tissue and membrane-enriched extracts was significantly decreased in the PCP-treated mice compared with the saline-treated mice ($p < 0.01$, $n = 6$, Fig. 7C, $p < 0.01$, $n = 7$–8, Fig. 7F). A single PCP treatment affected neither the phosphorylation, the expression, nor the phosphorylation ratio of NR1 (data not shown).

Infusion of NR1 Antisense Oligonucleotide into the Prefrontal Cortex Impaired the Latent Learning and Learning-Associated Phosphorylation of CaMKII in the Water-Finding Test. We examined the role of the prefrontal cortical NR1 subunit in the latent learning of the water-finding task and relation to the learning-associated phosphorylation of CaMKII because the repeated PCP treatment impaired latent learning and learning-associated

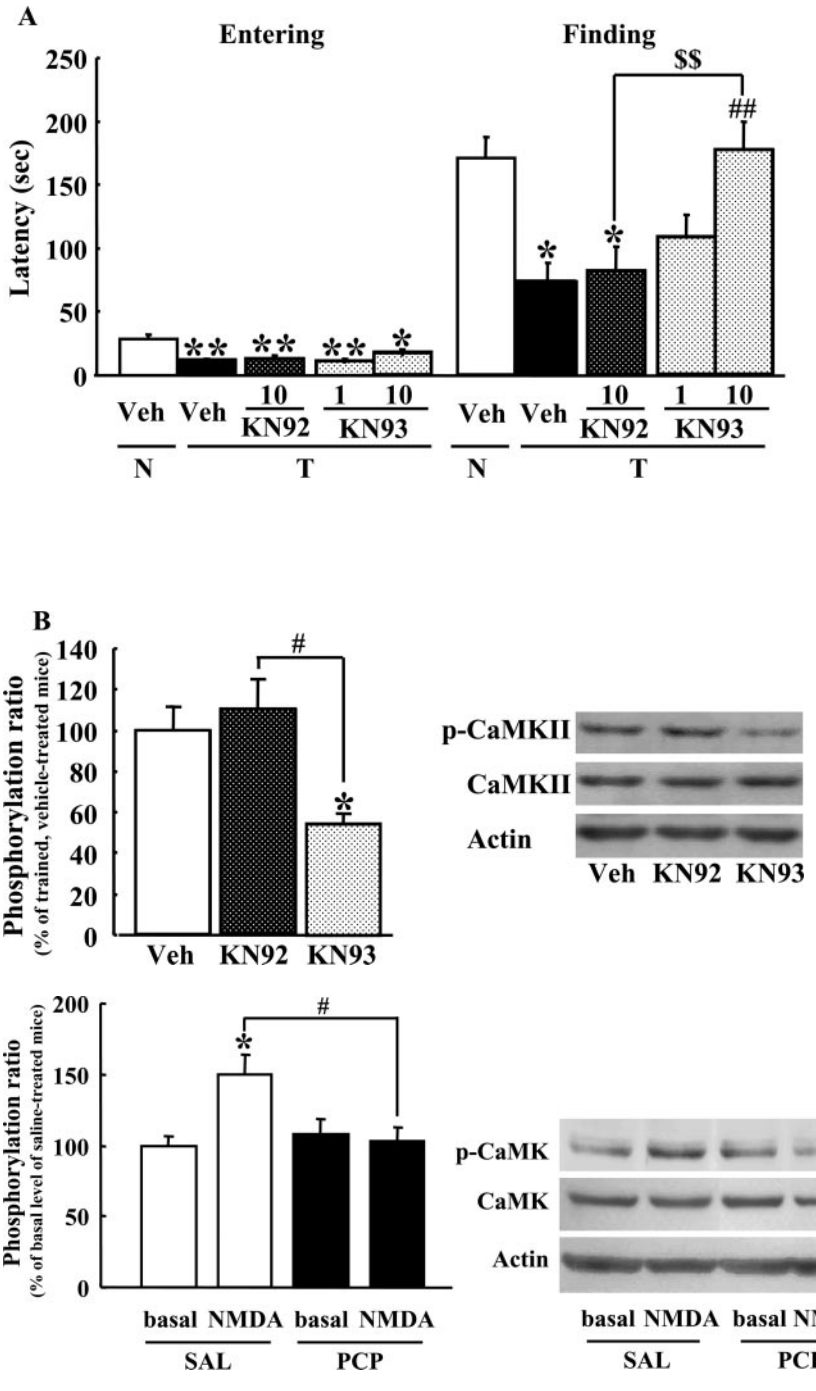

Fig. 4. Impairment of latent learning by a CaMKII inhibitor in the water-finding test. A, effect of the infusion of a CaMKII inhibitor into the prefrontal cortex on latent learning. Mice were administered a CaMKII inhibitor (KN93; 1 and 10 nmol/mouse bilaterally) 10 min before the training trial. The entering and finding latencies were measured in the test trial 24 h after the training trial of the water-finding task. Each column represents the mean ± S.E.M. ($n = 10$–13). Results with the one-way ANOVA were: entering latency, $F_{4,51} = 66.43$, $p < 0.01$; finding latency, $F_{4,51} = 7.59$, $p < 0.01$. **, $p < 0.01$; *, $p < 0.05$ compared with the nontrained, vehicle-treated mice. ##, $p < 0.01$ compared with the trained, vehicle-treated mice. $$, $p < 0.01$ compared with trained, inactive CaMKII inhibitor (KN92; 10 nmol)-treated mice. B, effect of the infusion of a CaMKII inhibitor into the prefrontal cortex on the learning-associated phosphorylation of CaMKII. Mice were administered KN93 (10 nmol) 10 min before the training trial. Immediately after the training trial, mice were sacrificed by decapitation and CaMKII phosphorylation (Thr286; p-CaMKII) and αCaMKII expression (CaMKII) in the prefrontal cortex were detected by Western blotting. Loaded protein was normalized to actin. The phosphorylation ratio was calculated as CaMKII phosphorylation versus CaMKII expression. Each column represents the mean ± S.E.M. ($n = 7$). Results with the one-way ANOVA were: $F_{2,18} = 8.10$; $p < 0.01$. *, $p < 0.05$ compared with the trained, vehicle-treated mice. #, $p < 0.05$ compared with the trained, KN92 (10 nmol)-treated mice. N, nontrained mice; T, trained mice; SAL, saline; Veh, vehicle.

Fig. 5. Impairment by repeated PCP treatment of CaMKII activation through NMDA receptor stimulation in slices of the prefrontal cortex. Slices of the prefrontal cortex were incubated in the absence or presence of 100 μM NMDA for 5 min. NMDA-stimulated phosphorylation of αCaMKII (Thr286; p-CaMKII) was detected by Western blotting. Loaded protein was normalized to actin. The phosphorylation ratio was calculated as CaMKII phosphorylation versus CaMKII expression. Each column represents the mean ± S.E.M. ($n = 6$). Results with the one-way ANOVA were: $F_{3,20} = 4.80$; $p < 0.05$. *, $p < 0.05$ compared with corresponding non-stimulated group. #, $p < 0.05$ compared with the saline-treated mice stimulated with NMDA. SAL, saline.

NMDA-CaMKII signaling through a malfunction of NR1. The performance in the water-finding test of mice that received the antisense or sense NR1 oligonucleotide in the prefrontal cortex is shown in Fig. 8A. Treatment with the antisense oligonucleotide ($n = 9$) significantly prolonged the finding latency compared with treatment with the sense oligonucleotide ($n = 9$) ($p < 0.05$; Fig. 8A). Infusion of the antisense ($n = 7$), but not sense ($n = 7$), oligonucleotide into the prefrontal cortex markedly reduced NR1 expression levels in the prefrontal cortex ($p < 0.01$, Fig. 8B) but not in other areas of the brain (hippocampus; $p = 0.96$, $n = 7$, Supplemental Data 2A, striatum; $p = 0.97$, $n = 7$, Supplemental Data 2B). It is noteworthy that the latent learning-associated phosphorylation of CaMKII was significantly decreased by treatment with the antisense NR1 oligonucleotide ($n = 7$), whereas the sense oligonucleotide ($n = 7$) had no effect ($p < 0.01$, $n = 7$; Fig. 8C).

Decrease of High Potassium- and PCP-Induced Dopamine Release from the Prefrontal Cortex in the Mice Treated Repeatedly with PCP. A hypofunctioning dopaminergic neuronal system in the prefrontal cortex is one of the causes of schizophrenia, and the dopaminergic neuronal system plays an important role in memory (Winterer and Weinberger, 2004). Therefore, changes in the amount of dopamine released in the prefrontal cortex were investigated by microdialysis in the PCP-treated mice. The amount of dopamine released in response to high potassium (50 mM) in the prefrontal cortex was significantly lower in the PCP-treated mice ($n = 6$) than in the saline-treated mice ($n = 5$) ($p < 0.05$, Fig. 9A). To clarify the cause of the reduced sensitivity to the potassium-evoked release in the PCP-treated mice, we investigated the release evoked by PCP (10 mg/kg s.c) in the prefrontal cortex. The administration of PCP significantly increased the amount of dopamine release in the mice treated repeatedly with saline ($n = 6$) but not in the mice treated repeatedly with PCP ($n = 6$) ($p < 0.01$, Fig. 9B).

Infusion of a Dopamine-D1 Receptor Agonist into the Prefrontal Cortex Rescued Impairment of Latent Learning and Learning-Associated Phosphorylation of NR1 Induced by Repeated PCP Treatment. In the next experiment, we investigated whether the infusion of a dopamine D1 receptor agonist reverses the hypofunctioning of the glutamatergic neuronal system in the prefrontal cortex and impairment of latent learning in the PCP-treated mice. Infusion of the agonist SKF81297 ($n = 8$) (10 nmol/mouse bilaterally) into the prefrontal cortex significantly shortened the prolonged finding latency in the PCP-treated mice ($n = 9$) ($p < 0.05$; Fig. 10A). This dose of SKF81297 ($n = 8$) failed to affect the finding latency of the saline-treated mice ($n = 9$) ($p = 0.96$, Supplemental Data 3A). The decrease in the learning-associated NR1 phosphorylation ratio in the PCP-treated mice ($n = 8$) was also reversed by the local infusion of SKF81297 ($n = 7$) into the prefrontal cortex ($p < 0.05$; Fig. 10B). The infusion of SKF81297 ($n = 6$) into the prefrontal cortex also elevated levels of phosphorylated NR1 in the saline-treated mice ($n = 6$) ($p < 0.01$, Supplemental Data 3B).

Discussion

In the present study, we investigated whether repeated PCP treatment causes an impairment of latent learning via a malfunction of dopaminergic-glutamatergic signaling in a

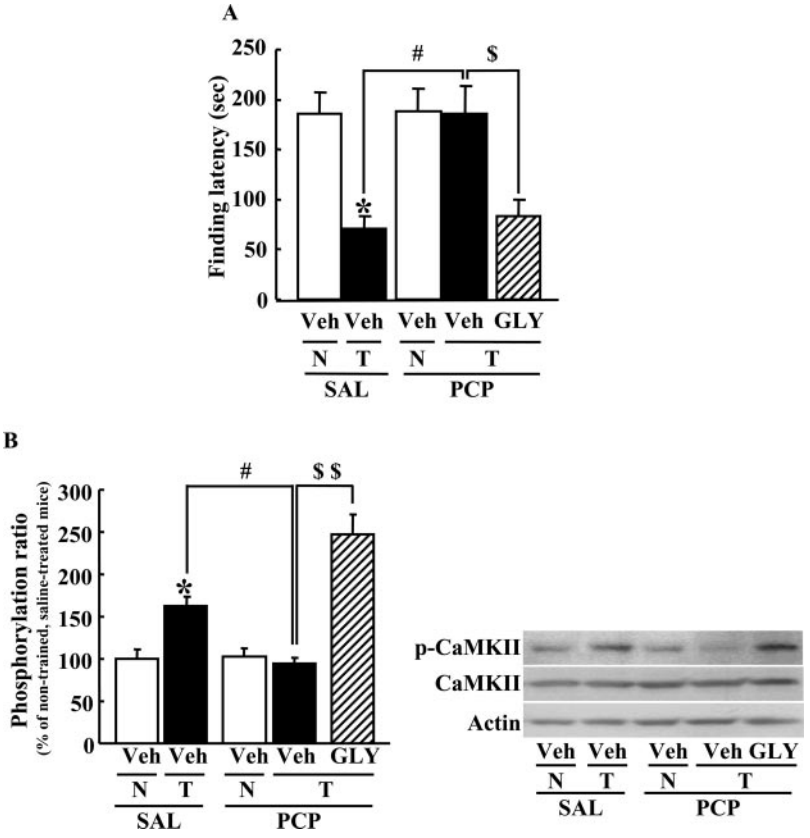

Fig. 6. Microinjection of glycine into the prefrontal cortex reversed the impairment of latent learning and of learning-associated CaMKII phosphorylation in the PCP-treated mice. A, effect of infusing glycine into the prefrontal cortex on the impairment of latent learning induced by repeated PCP treatment. The mice treated repeatedly with PCP (10 mg/kg s.c. once a day for 14 days; withdrawal 4 days) were administered glycine (1 μmol/mouse, bilaterally) 10 min before the training trial of the water-finding test. The test trial was performed 1 day after the training trial. Each column represents the mean ± S.E.M. ($n = 9$–10). Results with the one-way ANOVA were: $F_{4,44} = 8.12$; $p < 0.01$. *, $p < 0.05$ compared with the corresponding nontrained mice. #, $p < 0.05$ compared with the trained, saline-treated mice. $, $p < 0.05$ compared with the trained, PCP-treated mice. B, effect of infusing glycine into the prefrontal cortex on the impairment of learning-associated CaMKII phosphorylation in the prefrontal cortices of mice treated repeatedly with PCP. Repeated PCP-treated (10 mg/kg s.c. once a day for 14 days; withdrawal 4 days) mice were administered glycine (1 μmol/mouse, bilaterally) 10 min before the training trial of the water-finding test. Immediately after the training trial, mice were sacrificed by decapitation and CaMKII phosphorylation (Thr286; p-CaMKII) and αCaMKII expression (CaMKII) in the prefrontal cortex were detected by Western blotting. Loaded protein was normalized to actin. The phosphorylation ratio was calculated as CaMKII phosphorylation versus CaMKII expression. Each column represents the mean ± S.E.M. ($n = 11$–12). Results with the one-way ANOVA were: $F_{4,54} = 21.47$, $p < 0.01$. *, $p < 0.05$ compared with corresponding nontrained, mice. #, $p < 0.05$ compared with the trained, saline-treated mice. $$, $p < 0.01$ compared with the trained, PCP-treated mice. N, nontrained mice; T, trained mice; SAL, saline; Veh, vehicle.

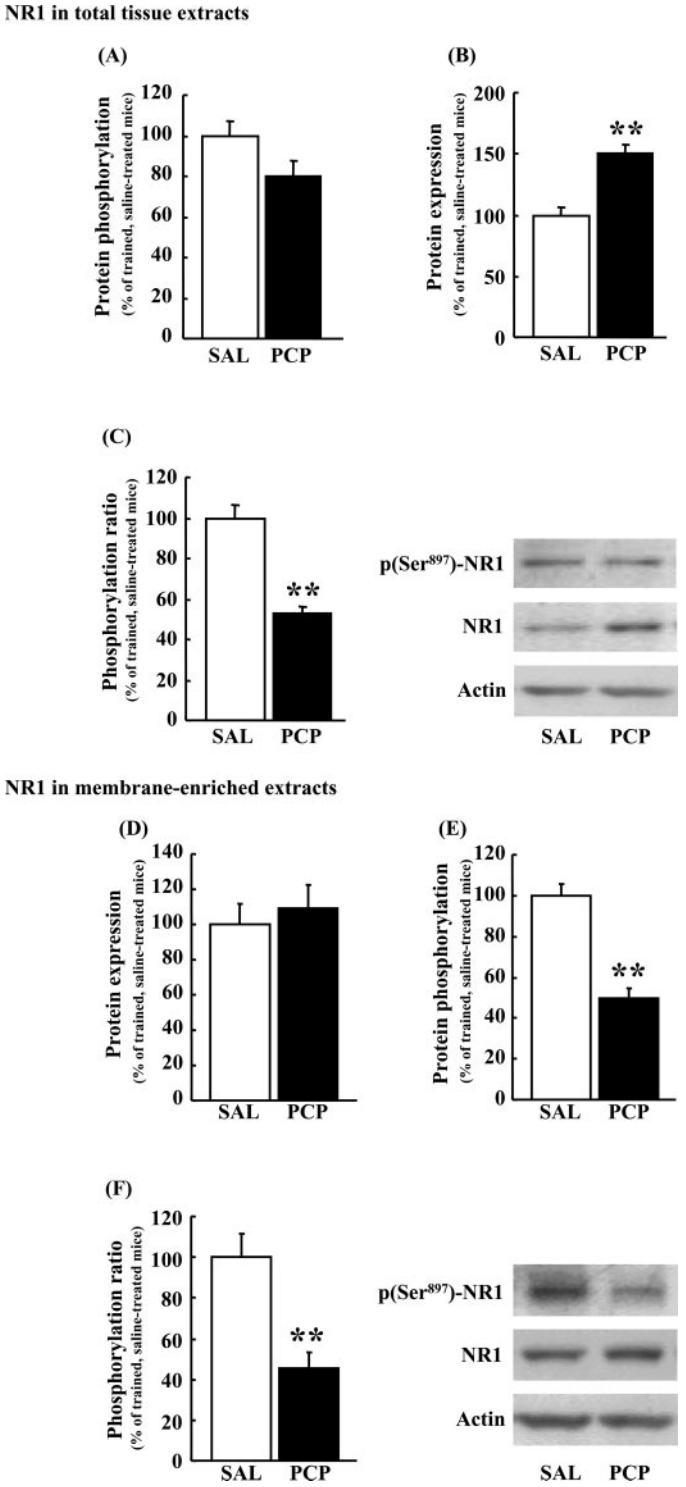

Fig. 7. Changes in the phosphorylation and expression of the NMDA receptor subunit NR1 in the prefrontal cortex on repeated PCP treatment. Immediately after the training trial, the mice treated repeatedly with PCP (10 mg/kg s.c. once a day for 14 days; withdrawal 4 days) were sacrificed by decapitation and NR1 phosphorylation (Ser^{897}; *p*-NR1) and NR1 expression (NR1) in the total tissue extracts (A–C) and membrane-enriched extracts (D–F) were detected by Western blotting. Loaded protein was normalized to actin. The phosphorylation ratio was calculated as NR1 phosphorylation versus NR1 expression. Each column represents the mean ± S.E.M. (A–C, $n = 6$; D–F, $n = 7$–8). Results are presented as the level of NR1 expression (A and D), level of NR1 phosphorylation (Ser897) (B and E), and the ratio of NR1 phosphorylation versus NR1 expression (C and F). **, $p < 0.01$ compared with the saline-treated mice (Student's *t* test). SAL, saline.

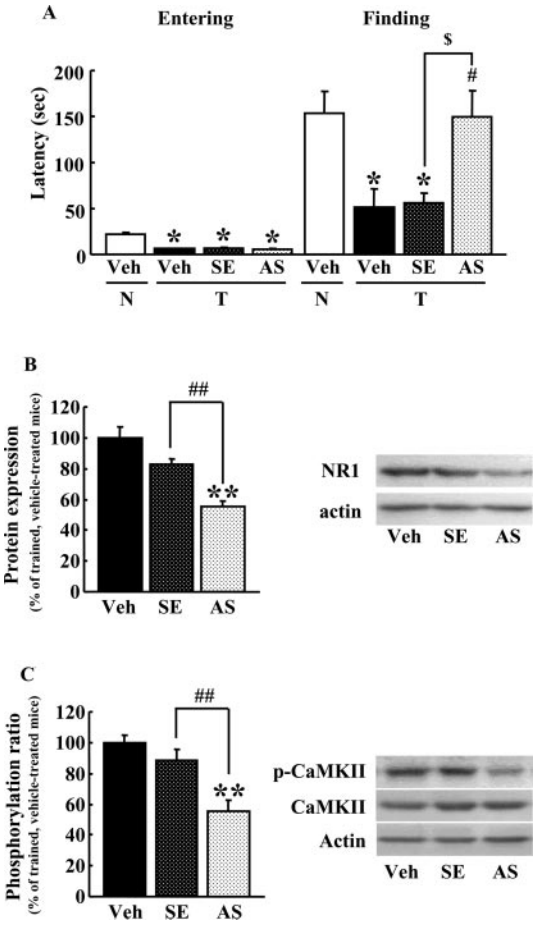

Fig. 8. NR1 antisense infusion into the prefrontal cortex impaired the latent learning and learning-associated phosphorylation of CaMKII in the water-finding test. A, effect of infusing NR1 antisense into the prefrontal cortex on latent learning. Repeated NR1 antisense (0.5 nmol/mouse, bilaterally, four times; 12-h interval)-treated mice were subjected to a training trial of the water-finding task 4 h after the last NR1 antisense treatment. The entering and finding latencies were measured in the test trial 24 h after the training trial. Each column represents the mean ± S.E.M. ($n = 9$–10). Results with the one-way ANOVA were: entering latency, $F_{3,33} = 60.14$; $p < 0.01$, finding latency, $F_{3,33} = 6.54$; $p < 0.01$. *, $p < 0.05$ compared with the nontrained, vehicle-infused mice. #, $p < 0.05$ compared with the trained, vehicle-infused mice. $, $p < 0.05$ compared with the trained, sense-infused mice. B, effect of infusing NR1 antisense into the prefrontal cortex on the expression of NR1 in the prefrontal cortex. Mice treated repeatedly with NR1 antisense (0.5 nmol/mouse, bilaterally, 4 times; 12-h interval) were subjected to a training trial of the water-finding task 4 h after the last NR1 antisense treatment. Immediately after the training trial, mice were sacrificed by decapitation. The level of NR1 expression (NR1) in the total tissue extracts was determined by Western blotting. Each column represents the mean ± S.E.M. ($n = 7$). NR1 expression was calculated as NR1 versus actin. Results with the one-way ANOVA were: $F_{2,18} = 20.17$; $p < 0.01$. **, $p < 0.01$ compared with the vehicle-infused mice. ##, $p < 0.01$ compared with the sense-infused mice. C, effect of infusing NR1 antisense into the prefrontal cortex on the learning-associated CaMKII phosphorylation. Mice treated repeatedly with NR1 antisense (0.5 nmol/mouse, bilaterally, 4 times; 12-h interval) were subjected to a training trial of the water-finding task 4 h after the last NR1 antisense treatment. Immediately after the training trial, mice were sacrificed by decapitation and CaMKII phosphorylation (Thr286; p-CaMKII) and αCaMKII expression (CaMKII) in the prefrontal cortex were detected by Western blotting. Loaded protein was normalized to actin. The phosphorylation ratio was calculated as CaMKII phosphorylation versus CaMKII expression. Each column represents the mean ± S.E.M. ($n = 7$). Results with the one-way ANOVA were: $F_{2,18} = 12.71$; $p < 0.01$. **, $p < 0.01$ compared with the vehicle-infused mice. ##, $p < 0.01$ compared with the sense-infused mice. N, nontrained mice; T, trained mice; Veh, vehicle; SE, sense; AS, antisense.

water-finding test after drug withdrawal. The mice treated repeatedly with saline took less time to find the water tube than did nontrained, saline-treated mice that were not exposed to the apparatus in a training trial. However, the PCP-treated mice had a prolonged finding latency, indicating an impairment of latent learning, in the water-finding task. Because their performance in the training trial and entering performance and drinking behavior in the test trial did not differ from that of saline-treated mice, it is unlikely that the impairment is attributable to altered anxiety processes (for example, avoidance of open fields), motor dysfunction, and/or thirst for water. The PCP-induced prolonged finding latency was attenuated by the infusion of glycine or the dopamine-D1 agonist into the prefrontal cortex only before the training trial, not before the test trial. It is suggested that repeated PCP treatment impaired latent learning (attention) of the location of the water tube in the training trial rather than a recall of memory in the test trial. The same dose of glycine or the dopamine-D1 agonist did not facilitate latent learning in the saline-treated mice, suggesting that the enhancement of the NMDA receptor and dopaminergic neuronal activities in saline-treated mice may have reached a ceiling as to acquired latent learning. The latent learning of PCP-treated mice in the water-finding task would make an excellent pharmacological model of schizophrenic cognitive dysfunction, which is related to latent learning (Exner et al., 2006) and attention (Nuechterlein and Dawson, 1984)

Accumulating evidence implicates the CaMKII pathway in cognitive functions such as learning and memory formation as well as in behavioral responses to NMDA receptor antagonists. For instance, the autophosphorylation of αCaMKII at Thr^{286} is critical for long-term potentiation and spatial memory (Giese et al., 1998) and fear memory in Pavlovian fear conditioning (Rodrigues et al., 2004). The infusion of a NMDA antagonist (2-amino-5-phosphonovalerate) into the

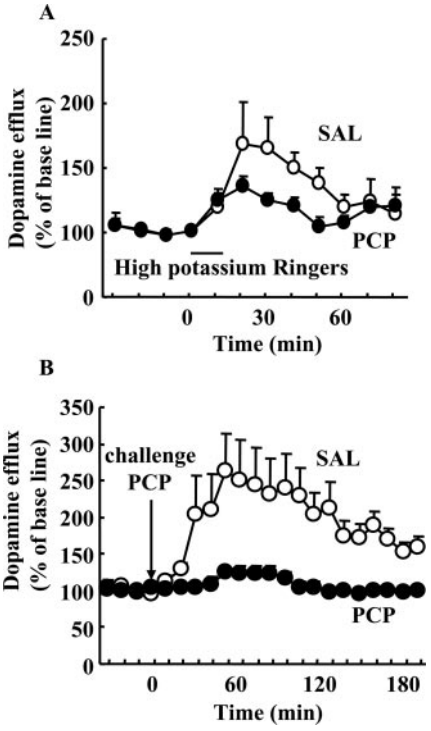

Fig. 9. Decrease of high potassium- and PCP-induced dopamine release from the prefrontal cortex in the mice treated repeatedly with PCP. A, high potassium-evoked dopamine release from the prefrontal cortex in the mice treated repeatedly with PCP. High potassium-induced dopamine release from the prefrontal cortex was measured in the mice treated repeatedly with PCP (10 mg/kg s.c. once a day for 14 days; withdrawal 4 days). Values correspond to the mean ± S.E.M. (n = 5–6). Results with the two-way ANOVA were: $F_{1,72}$ = 6.93; $p < 0.05$. B, the change of PCP-induced dopamine release from the prefrontal cortices of the mice treated repeatedly with PCP. PCP (10 mg/kg s.c.)-induced dopamine release was measured in the prefrontal cortices of mice treated repeatedly with PCP (10 mg/kg s.c. once a day for 14 days; withdrawal 4 days). Values correspond to the mean ± S.E.M. (n = 6). Results with the two-way ANOVA were: $F_{1,190}$ = 103.83; $p < 0.01$. SAL, saline. The basal levels of dopamine in the prefrontal cortex of the saline- and PCP-treated mice were 0.25 ± 0.06 and 0.27 ± 0.04 pmol/12 μl/10 min, respectively.

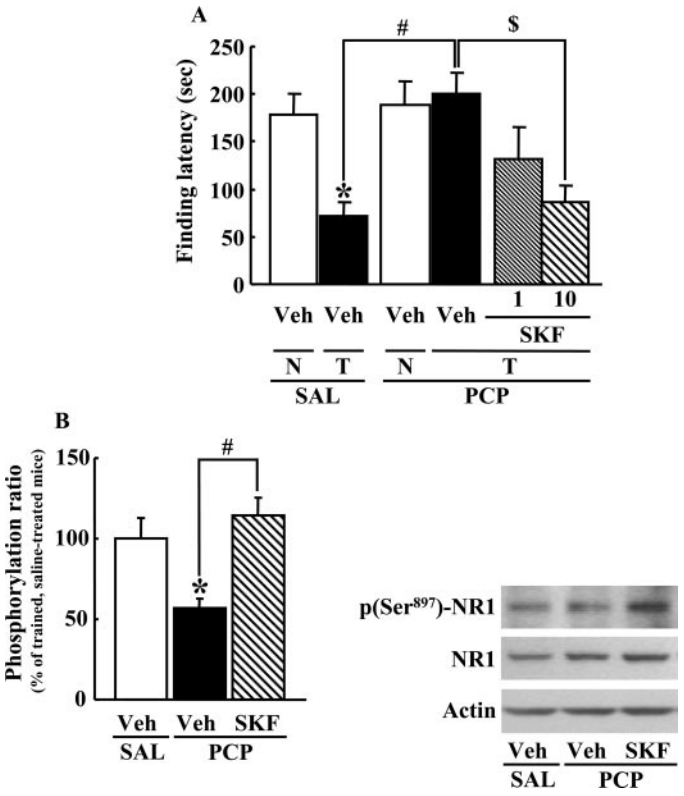

Fig. 10. Rescue, by infusion of a dopamine-D1 receptor agonist into the prefrontal cortex, of impairment of latent learning and learning-associated phosphorylation of NR1 induced by repeated PCP treatment. A, effect of the infusion of a dopamine-D1 agonist into the prefrontal cortex on the impairment of latent learning induced by repeated PCP treatment. Mice treated repeatedly with PCP (10 mg/kg s.c. once a day for 14 days; withdrawal 4 days) were administered SKF81297 (1 and 10 nmol/mouse, bilaterally) 10 min before a training trial of the water-finding test. The test trial was performed 1 day after the training trial. Each column represents the mean ± S.E.M. (n = 7–10). Results with the one-way ANOVA were: $F_{5,46}$ = 6.25; $p < 0.01$. *, $p < 0.05$ compared with the corresponding nontrained mice. #, $p < 0.05$ compared with the trained, saline-treated mice. $, $p < 0.05$ compared with the trained, PCP-treated mice. B, effect of the infusion of a dopamine-D1 agonist into the prefrontal cortex on the impairment of the NR1 phosphorylation ratio induced by repeated PCP treatment. Mice treated repeatedly with PCP (10 mg/kg s.c. once a day for 14 days; withdrawal 4 days) were administered SKF81297 (10 nmol/mouse, bilaterally) 10 min before the training trial of the water-finding test. Immediately after the training trial, mice were sacrificed by decapitation and NR1 phosphorylation (Ser897; p-NR1) and NR1 expression (NR1) in the total tissue extracts was detected by Western blotting. Loaded protein was normalized to actin. The phosphorylation ratio was calculated as NR1 phosphorylation versus NR1 expression. Each column represents the mean ± S.E.M. (n = 7–8). Results with the one-way ANOVA were: $F_{2,20}$ = 8.45; $p < 0.01$. *, $p < 0.05$ compared with the trained, saline-treated mice. #, $p < 0.05$ compared with the trained, PCP-treated mice. N, nontrained mice; T, trained mice; SAL, saline; Veh, vehicle; SKF, SKF81297.

hippocampus impaired memory consolidation in an inhibitory avoidance learning task and learning-associated phosphorylation of CaMKII (Bevilaqua et al., 2005). Although there was no difference in performance in the training trial, the present study showed that αCaMKII (Thr^{286}) was phosphorylated after the training trial in the prefrontal cortex of saline-treated mice but not PCP-treated mice and that the infusion of a CaMKII inhibitor into the prefrontal cortex of saline-treated mice impaired the latent learning. It was suggested that the phosphorylation of CaMKII in the training trial is related not to searching behavior itself but rather to attention associated with searching behavior, because the water-finding task is a latent learning task. Taken together, these results suggest that the prefrontal cortical CaMKII activation in the training trial is critical to the acquisition of latent learning, and the impairment of latent learning in PCP-treated mice is due to a failure to activate CaMKII.

A previous report has demonstrated that αCaMKII undergoes rapid phosphorylation at a threonine residue (Thr286) after the influx of Ca^{2+} mediated by the NMDA receptor, which is a ligand-gated Ca^{2+} channel (Xia and Storm, 2005). The prefrontal cortical glutamatergic transmission, particularly that meditated by NMDA receptors, participates in cognitive function (Wang, 1999). We investigated NMDA-CaMKII signaling after stimulation with exogenous NMDA in slices of the prefrontal cortex. In the prefrontal cortex prepared from saline-treated mice, levels of phosphorylated CaMKII were increased after the simulation, whereas stimulation with NMDA failed to increase the amount of phosphorylated CaMKII in the prefrontal cortex prepared from the PCP-treated mice. The prefrontal cortical infusion of glycine, which is a positive allosteric modulator for the NMDA receptor (Johnson and Ascher, 1987), alleviated the PCP-induced impairment of latent learning and learning-associated phosphorylation of CaMKII. Our findings clearly demonstrate that repeated PCP treatment disrupts the activation of CaMKII mediated via NMDA receptors and that the impairment of latent learning in the PCP-treated mice is due to dysfunctional NMDA-CaMKII signaling.

We investigated whether the dysfunctional NMDA-CaMKII signaling is accompanied by changes in the NMDA receptor subunit NR1 in the prefrontal cortex, because the level of phosphorylated NR1 (Ser897) is decreased in the frontal cortex of patients with schizophrenia (Emamian et al., 2004). In the present study, NR1 expression in total tissue extracts was enhanced in the prefrontal cortex of the PCP-treated mice, whereas NR1 (Ser897) phosphorylation in the membrane-enriched extracts and the NR1 (Ser897) phosphorylation ratio in both extracts were decreased. The phosphorylation of NR1 (Ser897) modulates the function of the NMDA receptors by facilitating expression at the cell surface of NMDA receptors from the endoplasmic reticulum (Scott et al., 2003). It is suggested that the decreased phosphorylation ratio of NR1 caused by the repeated PCP treatment results in suppressed trafficking of the NMDA receptor to the cellular surface. Furthermore, the decreased phosphorylation ratio of NR1 may be associated with the impairment of latent learning, because the infusion of the NR1-antisense oligonucleotide into the prefrontal cortex impaired latent learning and learning-associated activation of CaMKII, which were observed in the PCP-treated mice. In genetic animal experiments, mutant mice exhibited reduced NR1 functioning with schizophrenic-like behavior: NR1 knockdown mice, which express 5 to 10% of the normal level of NR1, showed an increase

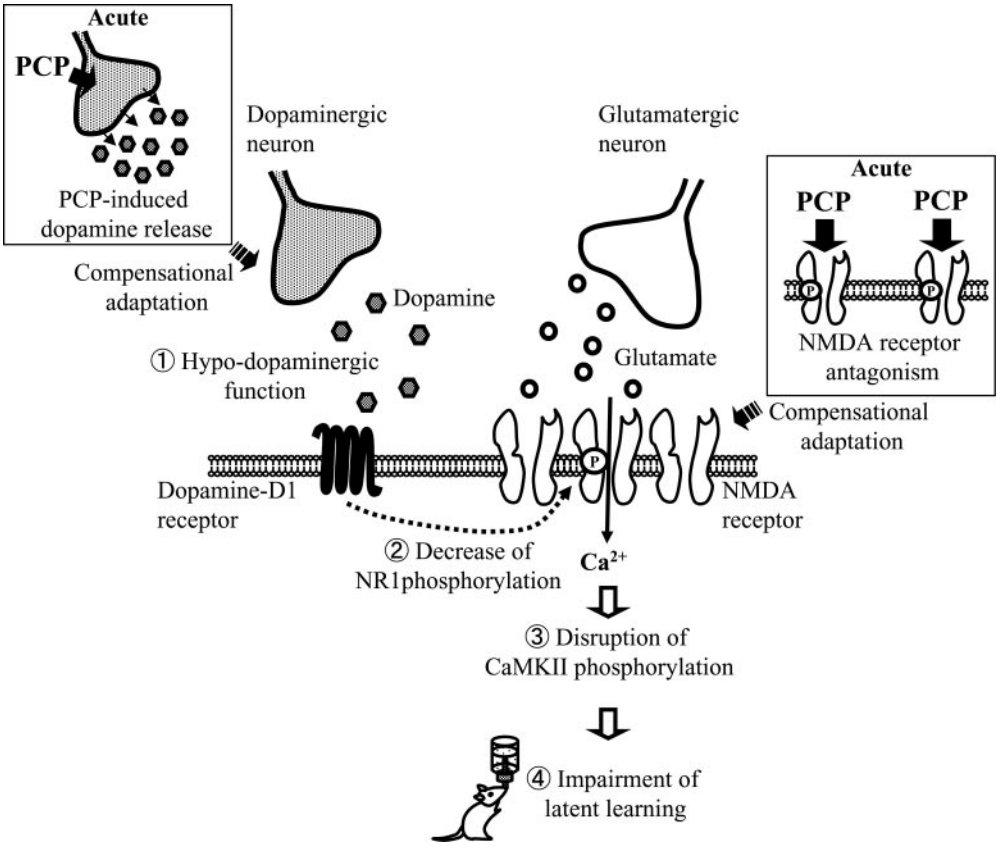

Fig. 11. Schematic representation of the molecular mechanism of latent learning impairment caused by repeated PCP treatments. A, with PCP-induced antagonism of the NMDA receptor, prefrontal cortical NR1 expression was enhanced and dopamine release was diminished. The diminished release of extracellular dopamine in the prefrontal cortex decreases dopamine-D1 signaling (①). Nevertheless, NR1 expression is enhanced, and dopamine-D1 signaling is decreased by repeated PCP treatment. Thus, NR1 (Ser897) phosphorylation is reduced (②) in the prefrontal cortex. The decreased NR1 (Ser897) phosphorylation induces a failure of learning-associated NMDA-CaMKII signaling (③), which is critical to the acquisition of latent learning (④) Thus, repeated PCP treatment impairs latent learning through a prefrontal cortical dysfunction of NMDA-CaMKII signaling, which is associated with dopaminergic hypofunction.

in locomotor activity and deficits of social and sexual interaction (Mohn et al., 1999). NR1 point-mutated mice, which have point mutations of NR1 glycine-binding site, showed hyperactivity (Ballard et al., 2002). Taken together with our reports, these results suggest that repeated PCP treatment induces impairment of latent learning by decreasing the NR1 (Ser897) phosphorylation ratio in the prefrontal cortex, and NR1 deregulation is one of the major factors in the pathogenesis of schizophrenia.

It is well established that the sensitivity of NMDA receptors is regulated by dopamine at the postsynaptic level in the prefrontal cortex. Electrophysiological experiments have indicated that NMDA-mediated excitation was enhanced by a dopamine-D1 receptor agonist in the prefrontal cortical pyramidal neurons through PKA-dependent mechanisms (Wang and O'Donnell, 2001). The dopamine-D1 receptor is coupled to G proteins, activating adenyl cyclase, increasing the level of cAMP, and phosphorylating PKA and Ser897 of NR1, which is a substrate of PKA (Tingley et al., 1997). Snyder et al. (1998) have also reported that a dopamine-D1, but not -D2, receptor agonist phosphorylated NR1 via the activation of PKA. We found that the infusion of a dopamine-D1 receptor agonist into the prefrontal cortex not only induced the phosphorylation in the NR1 (Ser^{897}) in the saline-treated mice but also attenuated the impairment of latent learning and the decrease of NR1 phosphorylation (Ser^{897}) ratio in the PCP-treated mice. These results suggest that dopaminergic function, especially dopamine-D1 receptor signaling, in the prefrontal cortex is critical for the regulation of latent learning-associated NMDA-CaMKII signaling.

Not only glutamatergic but also dopaminergic innervations of the prefrontal cortex play an important role in cognitive functions in schizophrenia (Winterer and Weinberger, 2004). In the animals treated repeatedly with PCP, dysfunctional dopaminergic transmission in the prefrontal cortex is associated with cognitive deficits (Jentsch et al., 1997a,b). In the present in vivo microdialysis experiments, the PCP-treated mice failed to release dopamine in response to high potassium stimulation or a challenge of PCP in the prefrontal cortex. Thus, it is possible that repeated PCP treatment impairs latent learning through a malfunction of NMDA-CaMKII signaling in the prefrontal cortex, which depends on the presynaptic hypofunction of dopaminergic systems.

Although short-term PCP treatment impaired latent learning (Noda et al., 2001), it failed to impair latent learning after drug withdrawal. These findings indicate that the effects of short-term PCP treatment on latent learning, neurotransmission, and/or intracerebral signaling are transient. With PCP-induced antagonism of the NMDA receptor and dopamine release, however, an enhancement of NR1 expression and a diminishment of dopamine release were observed in the prefrontal cortex of PCP-treated mice even after withdrawal (Fig. 11). These compensatory neuronal adaptations to repeated treatment might induce a malfunction of the NMDA receptor associated with hypofunctioning dopaminergic neurons in the prefrontal cortex, which is responsible for the impairment of latent learning (Fig. 11), because the infusion of the dopamine-D1 agonist into the prefrontal cortex attenuated the PCP-induced decrease in the NR1 phosphorylation ratio. Alterations in the circuitry of the prefrontal cortex may contribute to the impairments of cognitive function that are commonly observed in persons with schizophrenia (Lewis and Lieberman, 2000). These observations suggested that the neuronal changes induced by repeated PCP treatment might be more consistent with schizophrenia than the transient antagonism of the NMDA receptor induced by acute PCP treatment.

In conclusion, our results suggest that the impaired functioning of the glutamatergic and dopaminergic nervous systems in the pathogenesis of schizophrenia is mechanically linked. This repeated PCP-treated animal model will contribute to further understanding of the mechanism of cognitive dysfunction in schizophrenia.

References

Ballard TM, Pauly-Evers M, Higgins GA, Ouagazzal AM, Mutel V, Borroni E, Kemp JA, Bluethmann H, and Kew JN (2002) Severe impairment of NMDA receptor function in mice carrying targeted point mutations in the glycine binding site results in drug-resistant nonhabituating hyperactivity. *J Neurosci* **22:**6713–6723.

Bevilaqua LR, Medina JH, Izquierdo I, and Cammarota M (2005) Memory consolidation induces *N*-methyl-D-aspartic acid-receptor- and Ca^{2+}/calmodulin-dependent protein kinase II-dependent modifications in alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor properties. *Neuroscience* **136:**397–403.

Cammarota M, Bevilaqua LR, Viola H, Kerr DS, Reichmann B, Teixeira V, Bulla M, Izquierdo I, and Medina JH (2002) Participation of CaMKII in neuronal plasticity and memory formation. *Cell Mol Neurobiol* **22:**259–267.

Carlsson A, Waters N, Holm-Waters S, Tedroff J, Nilsson M, and Carlsson ML (2001) Interactions between monoamines, glutamate, and GABA in schizophrenia: new evidence. *Annu Rev Pharmacol Toxicol* **41:**237–260.

Coyle JT, and Tsai G (2004) The NMDA receptor glycine modulatory site: a therapeutic target for improving cognition and reducing negative symptoms in schizophrenia. *Psychopharmacology (Berl)* **174:**32–38.

Dracheva S, Marras SA, Elhakem SL, Kramer FR, Davis KL, and Haroutunian V (2001) *N*-methyl-D-aspartic acid receptor expression in the dorsolateral prefrontal cortex of elderly patients with schizophrenia. *Am J Psychiatry* **158:**1400–1410.

Emamian ES, Karayiorgou M, and Gogos JA (2004) Decreased phosphorylation of NMDA receptor type 1 at serine 897 in brains of patients with schizophrenia. *J Neurosci* **24:**1561–1564.

Enomoto T, Noda Y, Mouri A, Shin EJ, Wang D, Murai R, Hotta K, Furukawa H, Nitta A, Kim HC, et al. (2005) Long-lasting impairment of associative learning is correlated with a dysfunction of *N*-methyl-D-aspartate-extracellular signaling-regulated kinase signaling in mice after withdrawal from repeated administration of phencyclidine. *Mol Pharmacol* **68:**1765–1774.

Exner C, Boucsein K, Degner D, and Irle E (2006) State-dependent implicit learning deficit in schizophrenia: Evidence from 20-month follow-up. *Psychiatry Res* **142:**39–52.

Giese KP, Fedorov NB, Filipkowski RK, and Silva AJ (1998) Autophosphorylation at Thr286 of the alpha calcium-calmodulin kinase II in LTP and learning. *Science (Wash DC)* **279:**870–873.

Ichihara K, Nabeshima T, and Kameyama T (1993) Dopaminergic agonists impair latent learning in mice: possible modulation by noradrenergic function. *J Pharmacol Exp Ther* **264:**122–128.

Institute of Laboratory Animal Resources (1996) *Guide for the Care and Use of Laboratory Animals*, 7th ed. Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, Washington DC.

Itokawa M, Yamada K, Yoshitsugu K, Toyota T, Suga T, Ohba H, Watanabe A, Hattori E, Shimizu H, Kumakura T, et al. (2003) A microsatellite repeat in the promoter of the *N*-methyl-D-aspartate receptor 2A subunit (GRIN2A) gene suppresses transcriptional activity and correlates with chronic outcome in schizophrenia. *Pharmacogenetics* **13:**271–278.

Javitt DC, and Zukin SR (1991) Recent advances in the phencyclidine model of schizophrenia. *Am J Psychiatry* **148:**1301–1308.

Jentsch JD, Redmond DE Jr., Elsworth JD, Taylor JR, Youngren KD, and Roth RH (1997a) Enduring cognitive deficits and cortical dopamine dysfunction in monkeys after long-term administration of phencyclidine. *Science (Wash DC)* **277:**953–955.

Jentsch JD, and Roth RH (1999) The neuropsychopharmacology of phencyclidine: from NMDA receptor hypofunction to the dopamine hypothesis of schizophrenia. *Neuropsychopharmacology* **20:**201–225.

Jentsch JD, Tran A, Le D, Youngren KD, and Roth RH (1997b) Subchronic phencyclidine administration reduces mesoprefrontal dopamine utilization and impairs prefrontal cortical-dependent cognition in the rat. *Neuropsychopharmacology* **17:**92–99.

Jiang Y and Leung AW (2005) Implicit learning of ignored visual context. *Psychon Bull Rev* **12:**100–106.

Johnson JW and Ascher P (1987) Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature (Lond)* **325:**529–531.

Lewis DA and Lieberman JA (2000) Catching up on schizophrenia: natural history and neurobiology. *Neuron* **28:**325–334.

Maddox VH, Godefroi EF, and Parcell RF (1965) The synthesis of phencyclidine and other 1-arylcyclohexylamines. *J Med Chem* **56:**230–235.

Mohn AR, Gainetdinov RR, Caron MG, and Koller BH (1999) Mice with reduced NMDA receptor expression display behaviors related to schizophrenia. *Cell* **98:**427–436.

Nabeshima T, Fukaya H, Yamaguchi K, Ishikawa K, Furukawa H, and Kameyama T (1987) Development of tolerance and supersensitivity to phencyclidine in rats after repeated administration of phencyclidine. *Eur J Pharmacol* **135:**23–33.

Nabeshima T, and Ichihara K (1993) Measurement of dissociation of amnesic and behavioral effects of drug in mice. In Conn PM. (eds), Paradigms for the Study of Behavior, Methods in Neurosciences, vol 14. San Diego, Academic Press, pp 217–229.
Nabeshima T, Ishikawa K, Yamaguchi K, Furukawa H, and Kameyama T (1986) Methysergide-induced precipitated withdrawal syndrome in phencyclidine-dependent rats. *Neurosci Lett* **69:**275–278.
Noda A, Noda Y, Kamei H, Ichihara K, Mamiya T, Nagai T, Sugiura S, Furukawa H, and Nabeshima T (2001) Phencyclidine impairs latent learning in mice: interaction between glutamatergic systems and sigma(1) receptors. *Neuropsychopharmacology* **24:**451–460.
Nuechterlein KH and Dawson ME (1984) Information processing and attention functioning in the developmental course of schizophrenic disorders. *Schizophr Bull* **10:**160–203.
Paxinos G and Franklin KB (2004) *The Mouse Brain in Stereotaxic Coordinates*, compact 2nd ed., Elsevier Academic Press, San Diego.
Rainey JM Jr and Crowder MK (1975) Prolonged psychosis attributed to phencyclidine: report of three cases. *Am J Psychiatry* **132:**1076–1078.
Rice SR, Niu N, Berman DB, Heston LL, and Sobell JL (2001) Identification of single nucleotide polymorphisms (SNPs) and other sequence changes and estimation of nucleotide diversity in coding and flanking regions of the NMDAR1 receptor gene in schizophrenic patients. *Mol Psychiatry* **6:**274–284.
Rodrigues SM, Farb CR, Bauer EP, LeDoux JE, and Schafe GE (2004) Pavlovian fear conditioning regulates Thr286 autophosphorylation of Ca^{2+}/calmodulin-dependent protein kinase II at lateral amygdala synapses. *J Neurosci* **24:**3281–3288.
Scott DB, Blanpied TA, and Ehlers MD (2003) Coordinated PKA and PKC phosphorylation suppresses RXR-mediated ER retention and regulates the surface delivery of NMDA receptors. *Neuropharmacology* **45:**755–767.
Snyder GL, Fienberg AA, Huganir RL, and Greengard P (1998) A dopamine/D1 receptor/protein kinase A/dopamine- and cAMP-regulated phosphoprotein (M_r 32 kDa)/protein phosphatase-1 pathway regulates dephosphorylation of the NMDA receptor. *J Neurosci* **18:**10297–10303.
Tingley WG, Ehlers MD, Kameyama K, Doherty C, Ptak JB, Riley CT, and Huganir RL (1997) Characterization of protein kinase A and protein kinase C phosphorylation of the N-methyl-D-aspartate receptor NR1 subunit using phosphorylation site-specific antibodies. *J Biol Chem* **272:**5157–5166.
Tseng KY, and O'Donnell P (2004) Dopamine-glutamate interactions controlling prefrontal cortical pyramidal cell excitability involve multiple signaling mechanisms. *J Neurosci* **24:**5131–5139.
Wahlestedt C, Golanov E, Yamamoto S, Yee F, Ericson H, Yoo H, Inturrisi CE, and Reis DJ (1993) Antisense oligodeoxynucleotides to NMDA-R1 receptor channel protect cortical neurons from excitotoxicity and reduce focal ischaemic infarctions. *Nature (Lond)* **363:**260–263.
Wang J, and O'Donnell P (2001) D1 dopamine receptors potentiate NMDA-mediated excitability increase in layer V prefrontal cortical pyramidal neurons. *Cereb Cortex* **11:**452–462.
Wang XJ (1999) Synaptic basis of cortical persistent activity: the importance of NMDA receptors to working memory. *J Neurosci* **19:**9587–9603.
Winterer G, and Weinberger DR (2004) Genes, dopamine and cortical signal-to-noise ratio in schizophrenia. *Trends Neurosci* **27:**683–690.
Xia Z, and Storm DR (2005) The role of calmodulin as a signal integrator for synaptic plasticity. *Nat Rev Neurosci* **6:**267–276.
Zarrindast MR, Noorbakhshnia M, Motamedi F, Haeri-Rohani A, and Rezayof A (2006) Effect of the GABAergic system on memory formation and state-dependent learning induced by morphine in rats. *Pharmacology* **76:**93–100.

Address correspondence to: Dr. Toshitaka Nabeshima, Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466-8560, Japan. E-mail: tnabeshi@med.nagoya-u.ac.jp