

Fig. 9. Effects of repeated conditioning treatment with SA-4503 on performance of cued and contextual tests in OB rats were abolished by MK-801. a, Freezing time in the cued test. b, Freezing time in the contextual test. Results are expressed as means \pm S.E., $n = 9-12$. ##, $p < 0.01$, versus saline-treated sham-operated rats; **, $p < 0.01$, versus saline-treated OB rats; &, $p < 0.05$ and &&, $p < 0.01$, versus 0.3 mg/kg SA-4503-treated OB rats.

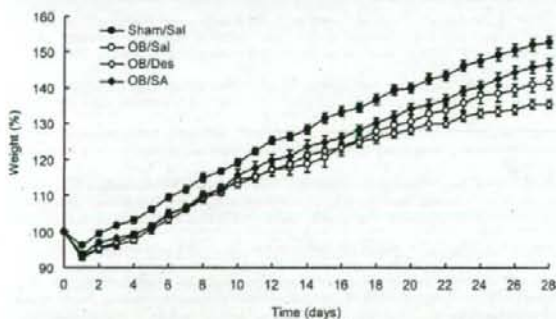


Fig. 10. Changes in body weights of sham-operated and OB rats treated with saline and medicines. Results are expressed as means \pm S.E., $n = 23-28$. Sham/Sal, saline-treated sham-operated rats. OB/Sal, saline-treated OB rats. OB/Des, 10 mg/kg desipramine-treated OB rats. OB/SA, 0.3 mg/kg SA-4503-treated OB rats.

to learn a new set of tasks within a specific context (Greene, 2005). Because MK-801 at the low dose of 0.03 mg/kg not only blocked the effects of SA-4503 but also impaired the performance of sham-operated rats in the fear-conditioning test, NMDA receptors play little of a compensable role in the performance of this test, unlike in the other behavioral tests. The MK-801-induced behavioral change seems less extensive in the SA-4503-treated group than that in the sham-operated group, indicating that NMDA receptors may be partially involved in the effect of SA-4503 in the test.

Because the dose of MK-801 that we used in the present study (0.03 mg/kg) is very low, it shows a relatively specific antagonizing effect at NMDA receptors. By contrast, NMDA

receptor antagonists ketamine and memantine have been reported to have antidepressant-like effects (Berman et al., 2000; Skuza and Rogoz, 2006). Although ketamine (i.v.) and its active metabolite norketamine with 20 to 30% activity have half-lives of approximately 1 and 6 h, respectively, antidepressant-like effect of ketamine reaches a significant level several hours after the intravenous infusion, and it gradually increases for several days (Berman et al., 2000; Zarate et al., 2006). Furthermore, ketamine also has an affinity for the μ opiate receptors, and it is an agonist for the σ receptors (Berman et al., 2000). Besides being a NMDA receptor antagonist, memantine also acts as uncompetitive antagonists at the 5-HT₂ and the nicotinic acetylcholine receptors, with potencies similar to or more than that for the NMDA receptors (Buisson and Bertrand, 1998; Rammes et al., 2001; Aracava et al., 2005). Therefore, the antidepressant-like effects of ketamine and memantine may not be mediated by NMDA receptors. The following phenomena best support the conception of the present study that NMDA receptors play fundamental roles in brain function, and although the receptors are used extensively in daily life, people usually do not become depressed after extensive thinking or learning.

Acknowledgments

We thank Hiroyuki Mizoguchi, Akihiro Mori, and Rina Murai in our laboratory for providing assistance.

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Current Perspective

The Roles of Glial Cell Line-Derived Neurotrophic Factor, Tumor Necrosis Factor- α , and an Inducer of These Factors in Drug DependenceMinae Niwa^{1,2}, Atsumi Nitta¹, Kiyofumi Yamada^{1,3}, and Toshitaka Nabeshima^{1,2,*}¹Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya 466-8560, Japan²Department of Chemical Pharmacology, Meijo University Graduate School of Pharmaceutical Sciences, Nagoya 468-8503, Japan³Laboratory of Neuropsychopharmacology, Division of Life Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kanazawa 920-1192, Japan

Received January 26, 2007; Accepted April 17, 2007

Abstract. There are few efficacious medications for drug dependence at present. Recent evidence has suggested that various cytokines are involved in the effects of abused drugs, suggesting that these factors play a role in drug dependence. In this article, the roles of glial cell line-derived neurotrophic factor (GDNF) and tumor necrosis factor- α (TNF- α) in drug dependence are discussed. GDNF inhibits the cocaine-induced upregulation of tyrosine hydroxylase activity in the ventral tegmental area and blocks behavioral responses to cocaine. TNF- α attenuates rewarding effects and locomotor sensitization induced by methamphetamine (METH) and morphine (MOR). Moreover, we mentioned the potential of Leu-Ile, which induces the expression of GDNF and TNF- α , as a novel therapeutic agent for drug dependence. Leu-Ile inhibits not only the development but also the maintenance of METH- or MOR-induced place preference and locomotor sensitization in mice. The inhibitory effect of Leu-Ile on METH- or MOR-induced place preference is not observed in GDNF heterozygous and TNF- α knockout mice. Leu-Ile inhibits METH- or MOR-induced increase in extracellular dopamine levels in the nucleus accumbens via the induction of GDNF and TNF- α expression. These findings suggest that Leu-Ile could be a novel therapeutic agent for drug dependence.

Keywords: glial cell line-derived neurotrophic factor, tumor necrosis factor- α , methamphetamine, morphine, Leu-Ile

Introduction

Drugs of abuse are able to elicit compulsive drug-seeking behaviors upon repeated administration, which ultimately leads to the phenomenon of addiction (1). In terms of lost lives and productivity, drug addiction remains one of the most serious threats to a nation's public health (2). There are currently few efficacious medications for drug dependence. Recently, it has been reported that BP897, a dopamine (DA) D₃-receptor

partial agonist, affects cocaine-associated stimulus-induced drug-seeking behavior in rats (3). The opioid κ -receptor agonist TRK-820 inhibits not only the rewarding effects of morphine (MOR) and cocaine, but also a mecamylamine-precipitated nicotine-withdrawal aversive effect (4). These medications should be effective even when they are administered after the development of drugs of abuse.

Cytokines, which are known to influence synaptic transmission and neuronal morphology (5), may be involved in alterations of the morphology of dendrites and their spines in the prefrontal cortex, nucleus accumbens (NAc), and hippocampus after repeated injection of psychostimulants (6, 7). Glial cell line-

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Published online in J-STAGE: May 31, 2007

doi: 10.1254/jphs.CP0070017

derived neurotrophic factor (GDNF) inhibits the cocaine-induced upregulation of tyrosine hydroxylase (TH) activity in the ventral tegmental area (VTA) and blocks behavioral responses to cocaine (8). Furthermore, we have previously demonstrated that tumor necrosis factor- α (TNF- α), an inflammatory cytokine, attenuates rewarding effects and locomotor sensitization induced by methamphetamine (METH) and MOR (9, 10). Taken together, GDNF and TNF- α would be candidates for therapeutic agents against drug dependence. However, there are serious obstacles to their therapeutic application: it is difficult to deliver GDNF from the periphery to the brain, since it is a macromolecule that cannot penetrate the blood-brain barrier (11), and is easily broken down by proteases in the blood stream. In addition, TNF- α damages the peripheral tissues, because it triggers the expression of other cytokines (12). Therefore, GDNF and TNF- α cannot be used directly as therapeutic tools for drug dependence. We hypothesized that a low-molecular-weight compound, which can easily penetrate the blood-brain barrier and induces production of GDNF and TNF- α in the brain, could be a novel therapeutic agent for drug dependence.

Roles of GDNF in drug dependence

GDNF produced by a glial cell line is a factor that plays important roles in the dopaminergic neurons (13). GDNF promotes the survival and function of DA neurons *in vivo*, in both the intact rat brain and after nigrostriatal lesioning (14, 15). It has been also shown that GDNF is secreted in the target (striatum) and transported retrogradely to the DA cell bodies in the mesencephalon (16). These findings suggest that GDNF could be effective against dopaminergic degeneration, and is expected to be useful for dopaminergic neurological disorders.

Transplantation of simian virus-40 glial cells, which produces and secretes GDNF, or delivery of GDNF-conjugated nanoparticles into dorsal and ventral striatum impairs the acquisition of cocaine self-administration in rats (17, 18). The upregulation of the GDNF pathway in the midbrain, is the molecular mechanism by which the putative anti-addiction drug ibogaine mediates its desirable action of reducing ethanol consumption (19). Infusion of GDNF into the VTA blocks certain biochemical adaptations (induction of TH, NR1 subunit of *N*-methyl D-aspartate receptors, Δ FosB, and protein kinase A catalytic subunit) to chronic cocaine or MOR treatment as well as cocaine-induced place preference (8). Conditioned place preference (CPP) paradigm, in which animals learn the association of an environment paired with drug exposure, is usually used as a measure

of the rewarding properties of abused drugs (8–10). Conversely, responses to cocaine are enhanced in rats by intra-VTA infusion of anti-GDNF antibody and in GDNF heterozygous knockout mice (8). Chronic exposure to cocaine or MOR causes a dramatic decrease in GDNF signaling as evidenced by a 65%–70% reduction in the levels of tyrosine-phosphorylated Ret in the VTA (8). Taken together, these findings support the scheme that the drug-induced decrease in GDNF signaling in the VTA removes a homeostatic feedback (or counteracting) mechanism on this neural pathway and thereby contributes to sensitized biochemical and behavioral responses to drugs of abuse.

Roles of TNF- α in drug dependence

TNF, a potent proinflammatory cytokine, plays a critical role in the immune response that leads to effective clearance of a variety of pathogens or to immunopathology and autoimmune disease (20). TNF- α also affects the central nervous system (CNS) directly or indirectly through the stimulation of vagal afferents (21). Thus, this cytokine is emerging as a modulator of CNS function. Regarding the behavioral effects of TNF- α , transgenic mice expressing high levels of TNF- α in the brain show several changes in exploratory activity and emotional behavior in association with reduced TH immunoreactivity in the striatum without neuronal cell death (22). In contrast, mice with targeted deletions of the TNF- α gene (TNF- α knockout mice) show anxiogenic-like behavior accompanied by an increase in serotonin metabolism (23).

METH or cocaine induces the expression of TNF- α mRNA in human brain endothelial cells (24, 25). METH induces the expression of TNF- α mRNA and protein in the NAc, striatum, hippocampus, and amygdala of rats (9). Expression of the TNF- α in the brain of mice is induced 3 h after METH injection and remains elevated for up to 6 h of METH exposure (26). Repeated METH or MOR treatment increases TNF- α mRNA and protein levels in neurons of the NAc through the activation of DA receptors (9, 10). Recently, we have demonstrated that TNF- α plays a neuroprotective role in METH-induced dependence and neurotoxicity (9) (Table 1). Moreover, we have found that TNF- α inhibits MOR-induced rewarding effects and sensitization to the locomotor-stimulating effects, which is argued to reflect one neuroadaptive process associated with dependence (10) (Table 1). Conversely, responses to METH or MOR are enhanced in TNF- α knockout mice. Furthermore, TNF- α attenuates the repeated METH or MOR treatment-induced increase in extracellular DA levels in the NAc (9, 10). TNF- α itself potentiates synaptosomal and

Table 1. Summary of the effects of TNF- α on methamphetamine- or morphine-induced changes in biomarkers and behavior

	Methamphetamine	Morphine
TNF- α mRNA		
Single	\pm	\pm
Repeated	\uparrow	\uparrow
Conditioned place preference		
Wild type	\uparrow	\uparrow
Wild type + TNF- α	\downarrow	\downarrow
TNF- α (-/-)	$\uparrow\uparrow$	$\uparrow\uparrow$
TNF- α (-/-) + TNF- α	N.D.	\downarrow
Hyperlocomotion (Single)		
Wild type	\uparrow	\uparrow
Wild type + TNF- α	\downarrow	\uparrow
TNF- α (-/-)	\uparrow	N.D.
Locomotor sensitization (Repeated)		
Wild type	\uparrow	\uparrow
Wild type + TNF- α	\downarrow	\downarrow
TNF- α (-/-)	$\uparrow\uparrow$	N.D.
Extracellular dopamine levels		
Wild type	\uparrow	\uparrow
Wild type + TNF- α	\downarrow	\downarrow

\pm , No change; \uparrow , Significant increase; \downarrow , Significant decrease; N.D., Not determined.

vesicular DA uptake and diminishes the METH-induced decrease in synaptosomal and vesicular DA uptake (9). These findings suggest that TNF- α plays a neuroprotective role in METH- or MOR-induced dependence by activating plasmalemmal DA transporter as well as vesicular monoamine transporter-2 and by inhibiting METH- or MOR-induced DA release (9, 10) (Table 1).

Effects of Leu-Ile, an inducer for GDNF and TNF- α , on METH-induced dependence

GDNF and TNF- α inhibit drug-induced dependence and would be candidates for therapeutic agents against drug dependence. Furthermore, a previous study has demonstrated that inflammatory stimuli such as TNF- α and lipopolysaccharide induces the synthesis of GDNF cultured astrocytes from mouse brain (27). However, as described at the beginning of the article, GDNF and TNF- α as anti-addictive factors cannot be used directly as therapeutic tools for drug dependence. Therefore, we hypothesized that a low molecular weight compound that can induce production of GDNF and TNF- α in the brain would be useful as a novel therapeutic agent for drug dependence. We investigated whether certain

hydrophobic dipeptides, Leu-Ile, Leu-Pro, and Pro-Ile, that partially resemble the immunophilin-binding site on FK506 have neuroprotective effects both in vivo and in vitro attributable to the induction of GDNF (28). Nitta et al. have demonstrated that only Leu-Ile induces GDNF, but not Leu-Pro and Pro-Ile (28). Leu-Ile upregulates GDNF expression by activating heat shock protein 90 / Akt / cyclic adenosine 3',5'-monophosphate response element binding protein signaling (29). Leu-Ile induces TNF- α mRNA in the NAc (10), although the mechanism underlying the upregulation of TNF- α induced by Leu-Ile remains to be elucidated. Taken together, Leu-Ile is expected to induce the production of not only GDNF but also TNF- α and to inhibit drug dependence.

The levels of GDNF mRNA, GDNF, and TNF- α protein are significantly increased 24 h after addition of Leu-Ile compared with those in the control group in primary cultured hippocampal neurons (30). In vivo, Leu-Ile treatment not only in combination with METH, but also after withdrawal from repeated treatment with METH increases GDNF and TNF- α levels in the NAc of mice (30). GDNF is expressed in the neuronal and astroglial cells of the NAc after the repeated co-administration of Leu-Ile and METH, whereas TNF- α is expressed in the neurons in mouse brain (30).

Leu-Ile treatment, in combination with METH and after withdrawal from repeated treatment with METH, inhibits place preference and locomotor sensitization to METH. GDNF and TNF- α are involved in the inhibitory effects of Leu-Ile on the rewarding effects of METH because no effects of Leu-Ile are observed in the GDNF heterozygous and TNF- α knockout mice (30).

To explore the mechanisms of the inhibitory effects of Leu-Ile on METH-induced rewarding effects and locomotor sensitization, the effects of Leu-Ile on the increase in extracellular DA levels and the decrease in DA uptake into synaptosomes induced by repeated METH treatment are examined by using in vivo microdialysis and the [3 H]DA uptake assay, respectively. Leu-Ile inhibits the repeated METH treatment-induced increase in extracellular DA levels in the NAc and negates the METH-induced inhibition of DA uptake in the midbrain. These findings suggest that Leu-Ile inhibits METH-induced rewarding effects and locomotor sensitization by regulating extracellular DA levels via the induction of GDNF and TNF- α expressions (30) (Table 2).

Effects of Leu-Ile, an inducer for GDNF and TNF- α , on MOR-induced dependence

Leu-Ile potentiates the MOR-induced increase in GDNF levels in the NAc of mice (31). Moreover,

Table 2. Summary of the effects of Leu-Ile on methamphetamine- or morphine-induced changes in biomarkers and behavior

	Methamphetamine	Morphine
GDNF levels		
Vehicle	↑	↑
Leu-Ile	↑↑	↑↑
TNF- α levels		
Vehicle	↑	↑
Leu-Ile	↑↑	↑↑
Conditioned place preference		
Vehicle	↑	↑
Leu-Ile	↓	↓
Hyperlocomotion (Single)		
Vehicle	↑	↑
Leu-Ile	↑	↑
Locomotor sensitization (Repeated)		
Vehicle	↑	↑
Leu-Ile	↓	↓
Extracellular dopamine levels		
Vehicle	↑	↑
Leu-Ile	↓	↓

↑, Significant increase; ↓, Significant decrease.

Leu-Ile treatment, in combination with MOR and after withdrawal from repeated treatment with MOR, also increases TNF- α levels in the neuronal cells of the NAc (10).

The effects of Leu-Ile on the MOR-induced rewarding effects and locomotor sensitization are examined in the CPP paradigm and locomotor activity test, respectively. Leu-Ile treatment, in combination with MOR and after withdrawal from repeated treatment with MOR, inhibits place preference and locomotor sensitization to MOR. GDNF and TNF- α are involved in the inhibitory effects of Leu-Ile on the rewarding effects of MOR, because no effects of Leu-Ile are observed in the GDNF heterozygous and TNF- α knockout mice (10, 31).

To explore the mechanisms of the inhibitory effects of Leu-Ile on MOR-induced rewarding effects and locomotor sensitization, the effects of Leu-Ile on the increase in extracellular DA levels induced by repeated MOR treatment are examined by using *in vivo* microdialysis. Leu-Ile inhibits the repeated MOR treatment-induced increase in extracellular DA levels in the NAc (10). These findings suggest that Leu-Ile inhibits MOR-induced rewarding effects and locomotor sensitization by regulating extracellular DA levels via the induction of GDNF and TNF- α expression (10, 31) (Table 2).

Conclusions

GDNF and TNF- α play important roles in the rewarding effects of both METH and MOR (8–10) (Table 2). Since the deletion of GDNF or TNF- α genes results in an enhancement of the rewarding effects, these cytokines may reduce the rewards of METH and MOR (8–10) (Table 1). As reviewed in this article, some cytokines like GDNF and TNF- α act as anti-addictive factors, whereas others such as tissue plasminogen activator (tPA), metalloproteinase (MMP)-2, MMP-9, basic fibroblast growth factor, neurotrophin-3, and brain-derived neurotrophic factor (BDNF) act as pro-addictive factors (32–34). The tPA-plasmin system, for instance, potentiates the rewarding and locomotor-stimulating effects of METH, MOR, and nicotine by regulating release of DA (34–36). MMP-2 or -9-deficient mice show reduced responses to METH in locomotor sensitization, CPP, and dopamine release in the NAc (33). Infusion of BDNF into the NAc enhances the stimulation of locomotor activity by cocaine in rats, whereas the development of sensitization and CPP is delayed in heterozygous BDNF knockout mice compared with their wild-type littermates (37, 38). The balance of levels between anti-addictive and pro-addictive factors induced by drugs of abuse seems to be important to the development of drug dependence (32). We propose that the dynamic changes and balance of levels in anti-addictive and pro-addictive factors in the brain are some of the determinants of the susceptibility to drug dependence.

As described at the beginning of the article, GDNF and TNF- α as anti-addictive factors cannot be used directly as therapeutic tools for drug dependence. In this article, we demonstrated that Leu-Ile, a low molecular weight compound that induces production of GDNF and TNF- α in the brain, inhibits both METH- and MOR-induced rewarding effects and locomotor sensitization by regulating extracellular DA levels via the induction of GDNF and TNF- α expression (10, 30, 31) (Table 2). Therefore, Leu-Ile could be a novel therapeutic agent for METH- and MOR-induced dependence.

Acknowledgments

This study was supported in part by a Grant-in-Aid for Scientific Research and Special Coordination Funds for Promoting Science and Technology, Target-Oriented Brain Science Research Program; by a Grant-in-aid for Scientific Research (B) and Young Scientists (A); by the 21st Century Center of Excellence Program "Integrated Molecular Medicine for Neuronal and Neoplastic Disorders" from the Ministry of Education, Culture,

Sports, Science, and Technology of Japan; by a Grant-in-Aid for Health Science Research on Regulatory Science of Pharmaceuticals and Medical Devices, and Comprehensive Research on Aging and Health from the Ministry of Health, Labor, and Welfare of Japan; by a Smoking Research Foundation Grant for Biomedical Research; by the Mochida Memorial Foundation for Medical and Pharmaceutical Research; by a grant from the Brain Research Center from the 21st Century Frontier Research Program funded by the Ministry of Science and Technology, Republic of Korea; and by Japan Canada Joint Health Research Program.

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Involvement of a Dysfunctional Dopamine-D1/*N*-Methyl-D-aspartate-NR1 and Ca²⁺/Calmodulin-Dependent Protein Kinase II Pathway in the Impairment of Latent Learning in a Model of Schizophrenia Induced by Phencyclidine^S

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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 Ca²⁺/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 SRP (Smoking Research Foundation) Grant for Biomedical Research.

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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, Ca²⁺/calmodulin-dependent protein kinase II; SKF81297, (\pm)-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrobromide; AP, anteroposterior; ML, medial-lateral; 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 molec-

ular 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 Ca²⁺/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 Bevilacqua et al. (2005). These drugs were infused in a volume of 1 μl/site 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 Nishinbo Biotechnology (Tokyo, Japan) and dissolved in artificial cerebrospinal fluid (CSF; 147 mM NaCl, 3 mM CaCl₂, 3 mM KCl, 1.2 mM

CaCl₂, and 1 mM MgCl₂, pH 7.2). The NR1 antisense oligonucleotide (5'-CAGCAGGTGCATGGTCT-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 (Wahlstedt 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 13,000g 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 700g for 10 min. The supernatant was centrifuged again at 37,000g 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% bromophenol blue), separated on a polyacrylamide gel, and subsequently transferred to polyvinylidene difluoride membranes (Milli-

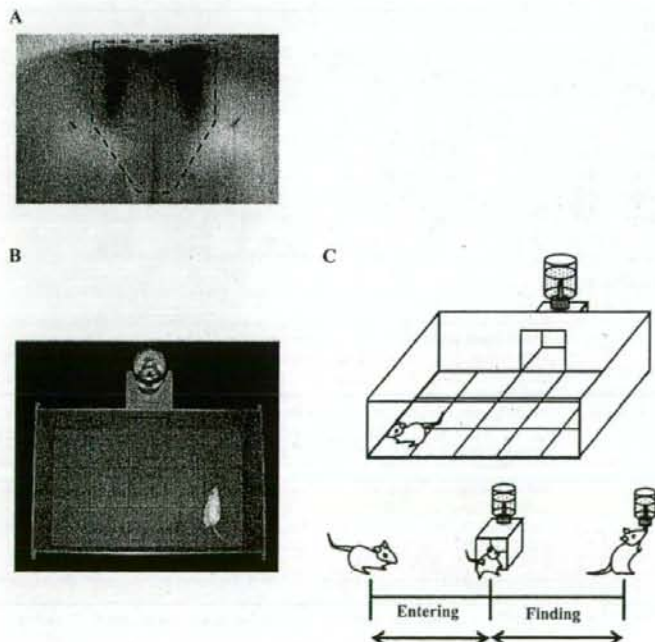


Fig. 1. Water-finding test. **A**, site of infusion and area dissected in the prefrontal cortex. KN93, KN92, glycine, SKP81297, and NR1 antisense oligonucleotides were injected into the prefrontal cortex of anesthetized mice and their presence was demonstrated by a dye marker, 0.1% Fast Green. The area surrounded with a broken line in Fig. 1A 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 μ m 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 CaCl₂, and 10 mM glucose, gassed with 95% O₂ and 5% CO₂), each slice was stimulated with NMDA (100 μ M) 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 CaCl₂) at a flow rate of 1.2 μ l/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 \pm 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

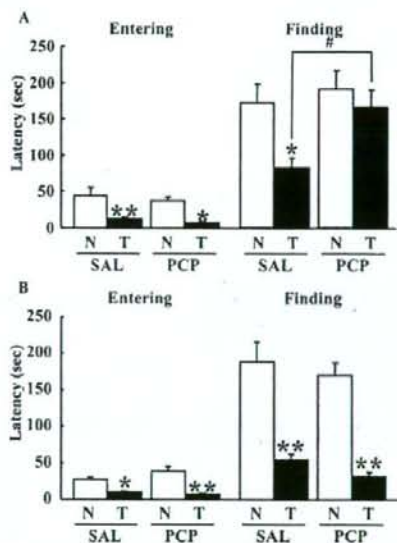


Fig. 2. Impairment of latent learning on repeated PCP treatment. **A**, latent learning in the mice treated repeatedly with PCP (10 mg/kg s.c. once a day for 14 days). These mice were subjected to a training trial of the water-finding task 4 days after the last PCP treatment. The entering and finding latencies were measured in the test trial 24 h after the training trial of the water-finding task. Each column represents the mean \pm 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 \pm 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 learning, 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 μ M). 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 μ mol/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-

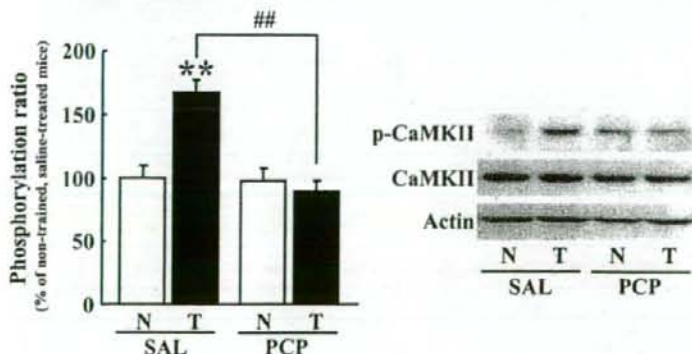


Fig. 3. Impairment of learning-associated CaMKII activation in the prefrontal cortex on repeated PCP treatment in the water-finding test. A training trial was performed 4 days after cessation of the repeated PCP treatment (10 mg/kg s.c. once a day for 14 days). Immediately after the training trial, mice were sacrificed by decapitation and CaMKII phosphorylation (Thr286; p-CaMKII) and α -CaMKII 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 \pm 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 (Ser⁸⁹⁷) 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-en-

riched 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

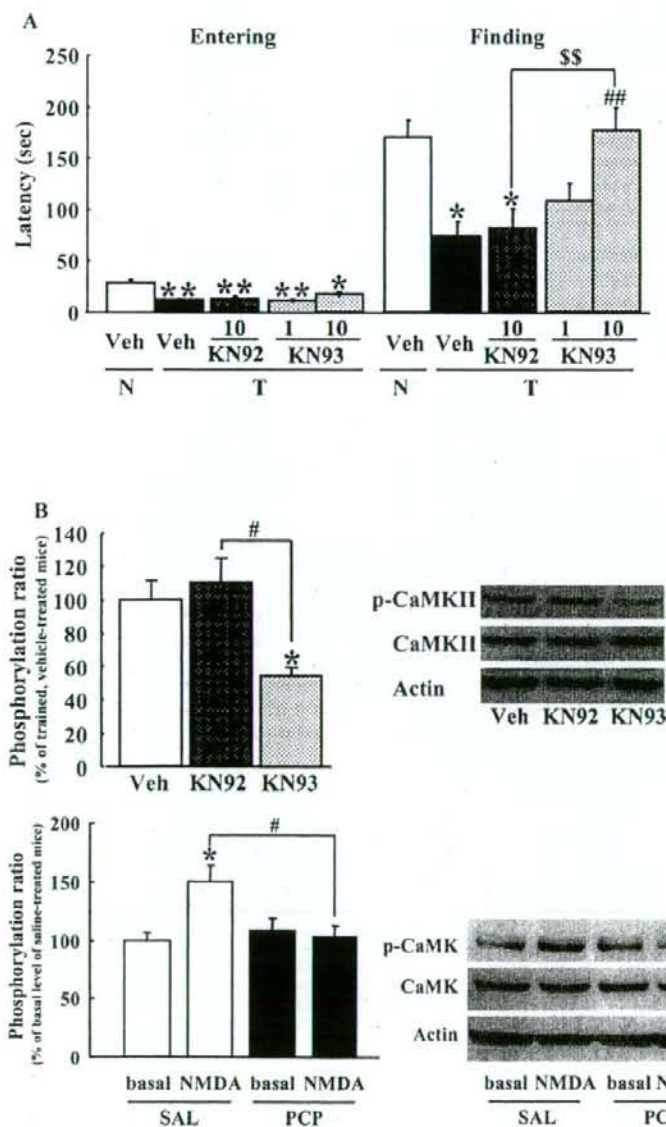


Fig. 4. Impairment of latent learning by a CaMKII inhibitor in the water-finding test. **A**, effect of the infusion of a CaMKII inhibitor into the prefrontal cortex on latent learning. Mice were administered a CaMKII inhibitor (KN93; 1 and 10 nmol/mouse bilaterally) 10 min before the training trial. 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 \pm S.E.M. ($n = 10-13$). Results with the one-way ANOVA were: entering latency, $F_{2,51} = 66.43$, $p < 0.01$; 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 α -CaMKII 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 \pm S.E.M. ($n = 7$). Results with the one-way ANOVA were: $F_{2,15} = 8.10$; $p < 0.01$. *, $p < 0.05$ 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 μ M NMDA for 5 min. NMDA-stimulated phosphorylation of α -CaMKII (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 \pm 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

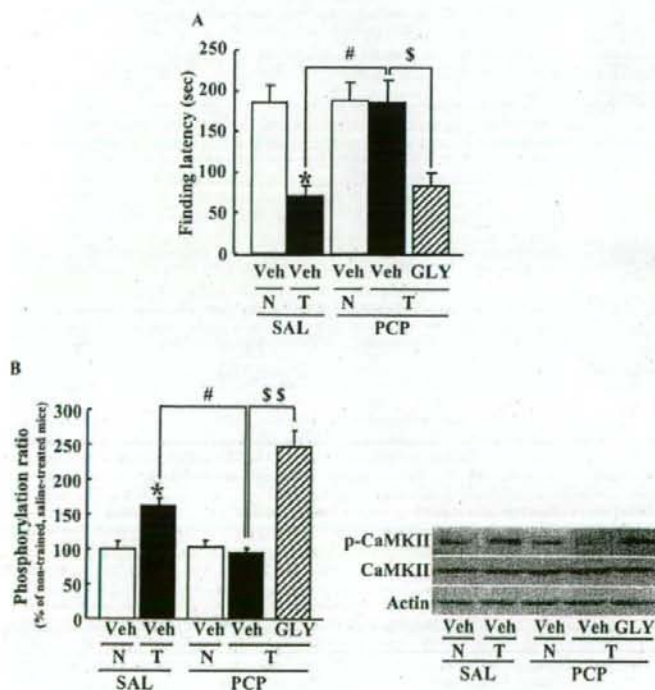
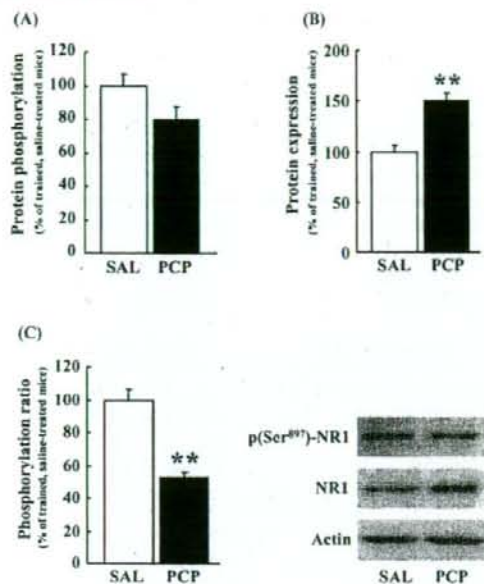


Fig. 6. Microinjection of glycine into the prefrontal cortex reversed the impairment of latent learning and of learning-associated CaMKII phosphorylation in the PCP-treated mice. **A**, effect of infusing glycine into the prefrontal cortex on the impairment of latent learning induced by repeated PCP 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 μ mol/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 \pm 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 μ mol/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 CaMKII 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 \pm 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.

NR1 in total tissue extracts



NR1 in membrane-enriched extracts

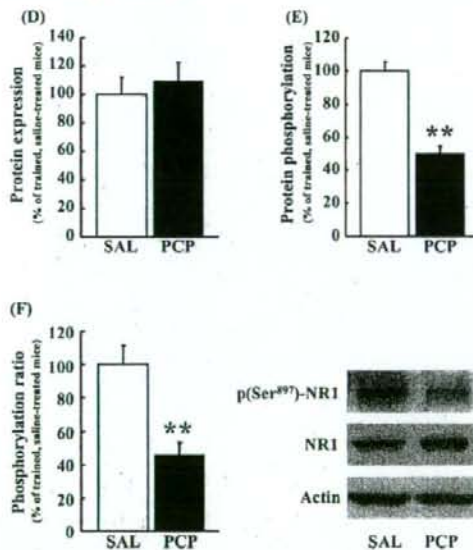


Fig. 7. Changes in the phosphorylation and expression of the NMDA receptor subunit NR1 in the prefrontal cortex on repeated PCP treatment. Immediately after the training trial, the mice treated repeatedly with PCP (10 mg/kg s.c. once a day for 14 days; withdrawal 4 days) were sacrificed by decapitation and NR1 phosphorylation (Ser⁸⁹⁷; 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 \pm 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.

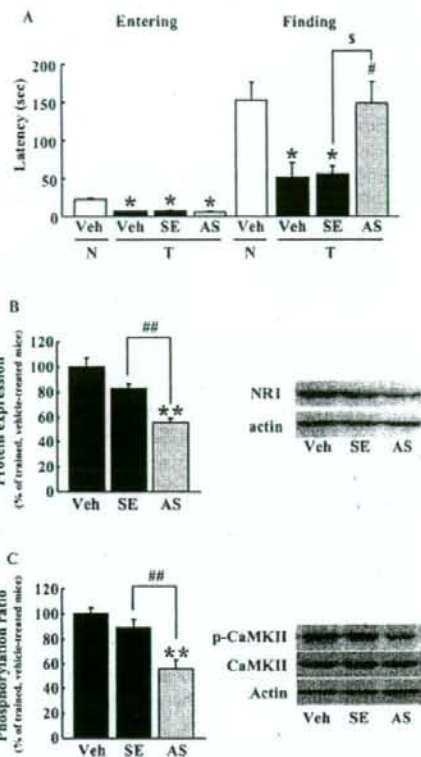


Fig. 8. NR1 antisense infusion into the prefrontal cortex impaired the latent learning and learning-associated phosphorylation of CaMKII in the water-finding test. **A**, effect of infusing NR1 antisense into the prefrontal cortex on latent learning. Repeated NR1 antisense (0.5 nmol/mouse, bilaterally, 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 \pm 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, 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 \pm 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 α -CaMKII 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 \pm 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 α CaMKII at Thr²⁸⁶ 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

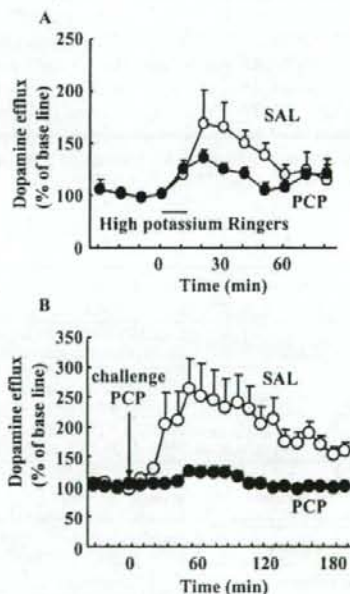


Fig. 9. Decrease of high potassium- and PCP-induced dopamine release from the prefrontal cortex in the mice treated repeatedly with PCP. **A**, high potassium-evoked dopamine release from the prefrontal cortex in the mice treated repeatedly with PCP. High potassium-induced dopamine release from the prefrontal 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 \pm 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 \pm S.E.M. ($n = 6$). Results with the two-way ANOVA were: $F_{1,199} = 103.83$; $p < 0.01$. 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 μ l/10 min, respectively.

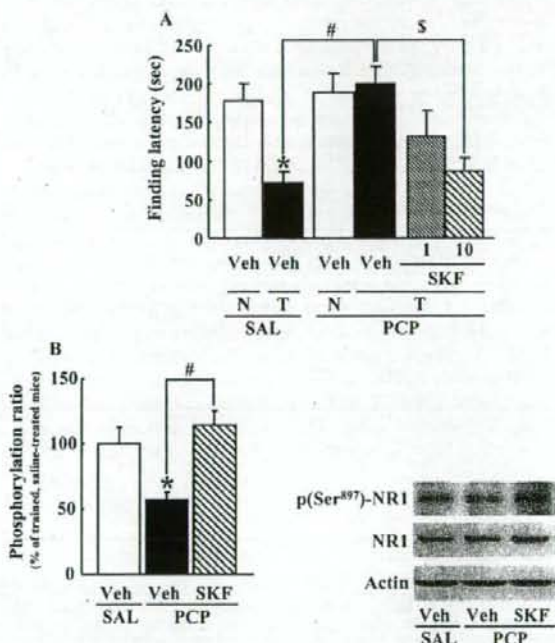


Fig. 10. Rescue, by infusion of a dopamine-D1 receptor agonist into the prefrontal cortex, of impairment of latent learning and learning-associated phosphorylation of NR1 induced by repeated PCP treatment. **A**, effect of the infusion of a dopamine-D1 agonist into the prefrontal cortex on the impairment 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 \pm S.E.M. ($n = 7-10$). Results with the one-way ANOVA were: $F_{6,46} = 6.25$; $p < 0.01$. *, $p < 0.05$ 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 \pm 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 α CaMKII (Thr²⁸⁶) 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 α CaMKII 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 mediated 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 stimulation, 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

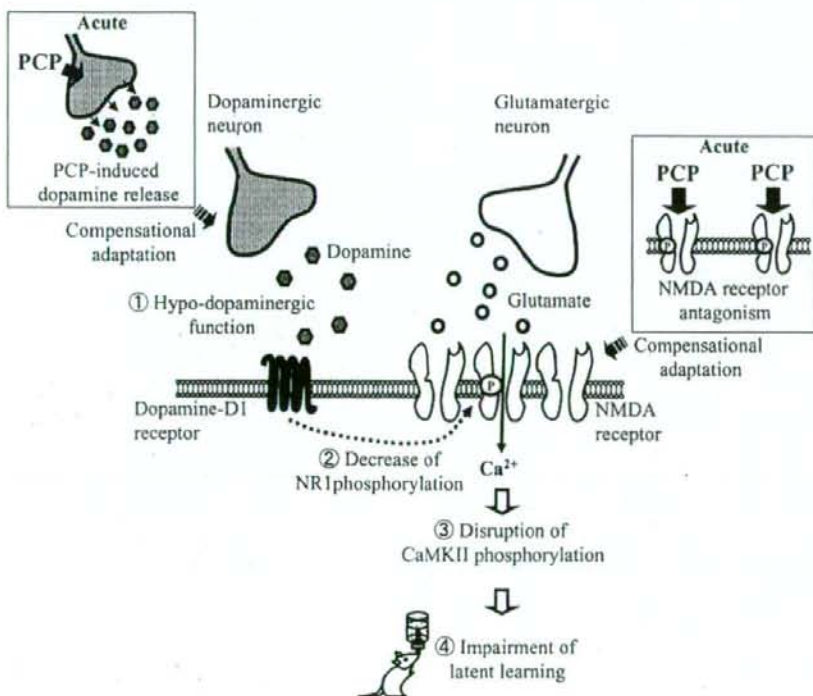


Fig. 11. Schematic representation of the molecular mechanism of latent learning impairment caused by repeated PCP treatments. A, with PCP-induced antagonism of the NMDA receptor, prefrontal cortical NR1 expression was enhanced and dopamine release was diminished. The diminished release of extracellular 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 adenylyl 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 (Ser⁸⁹⁷) in the saline-treated mice but also attenuated the impairment of latent learning and the decrease of NR1 phosphorylation (Ser⁸⁹⁷) 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 schizophre-

nia (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.

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