Role of N-Methyl-D-aspartate Receptors in Antidepressant-Like Effects of σ_1 Receptor Agonist 1-(3,4-Dimethoxyphenethyl)-4-(3-phenylpropyl)piperazine Dihydrochloride (SA-4503) in Olfactory Bulbectomized Rats

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ABSTRACT

In the present study, we aimed to investigate the role of N-methyl-D-aspartate (NMDA) receptors in the antidepressant-like effects of a σ_1 receptor agonist, 1-(3,4-dimethoxyphenethyl)-4-(3-phenylpropyl)piperazine dihydrochloride (SA-4503), in the olfactory bulbectomized (OB) rat model of depression. A symptomatology-based behavioral investigation was made by reconstructing in OB rats the symptoms of depression, such as psychomotor agitation, loss of interest, and cognitive dysfunction, using a typical antidepressant, desipramine, as a positive control. Repeated treatment with SA-4503 ameliorated the behavioral deficits in OB rats resembling depression symptoms in the open-field test, sexual behavior test, and cued and contextual fear-conditioning test. SA-4503 displayed advantages over desipramine in the sexual behavior test. SA-4503 also reversed the decrease in the protein expression of NMDA receptor subunit (NR)1, but not NR2A or NR2B, in the prefrontal cortex, hippocampus, and amygdala of OB rats. The behavioral and neurochemical effects of SA-4503 were blocked by combined treatment with a specific σ_1 receptor antagonist, N,N-dipropyl-2-(4-methoxy-3-(2-phenylethoxy)phenyl)ethylamine monohydrochloride (NE-100). Furthermore, the effects of SA-4503 on the performance of OB rats in the behavioral tests were abrogated by acute treatment with an NMDA receptor antagonist, (−)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801). The present study indicated for the first time that the σ_1 receptor agonist SA-4503 may have effects on depressive symptoms such as agitation, loss of interest, and impaired cognition, which are mediated by NMDA receptors.

σ_1 Receptors are particularly concentrated in the limbic structures of the brain, which play important roles in emotion and cognition (Matsuno et al., 1996; Skuza, 2003; Skuza and Wedzony, 2004; Bermack and Debonnel, 2005; Stahl, 2005). Various antidepressants, including tricyclic compounds, selective serotonin reuptake inhibitors, and monoamine oxidase inhibitors, possess affinity and act as agonists for σ_1 receptors (Maurice et al., 2001; Su and Hayashi, 2003). Based on the above-mentioned points, it is hypothesized that σ_1 receptor agonists may act as antidepressants. SA-4503 is

ABBREVIATIONS: SA-4503, 1-(3,4-dimethoxyphenethyl)-4-(3-phenylpropyl)piperazine dihydrochloride; NMDA, N-methyl-D-aspartate; NR, N-methyl-D-aspartate receptor subunit; PFC, prefrontal cortex; Hip, hippocampus; MK-801, (−)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate; OB, olfactory bulbectomy/bulbectomized; DES/Des, desipramine; NE-100, N,N-dipropyl-2-(4-methoxy-3-(2-phenylethoxy)phenyl)ethylamine monohydrochloride; ANOVA, analysis of variance; Amg, amygdala; 5-HT, 5-hydroxytryptamine (serotonin); Sham, sham-operated; Sal, saline; (+)-SKF-10047, [2S-(2α,6α,11R*)]-1,2,3,4,5,6-hexahydro-6,11-dimethyl-3-(2-propenyl)-2,6-methano-3-benzazocin-8-ol hydrochloride.

a highly selective agonist of σ_1 receptors, with higher binding affinity than a prototypical σ_1 receptor agonist, (+)-SKF-10047 (Matsuno et al., 1996; Guitart et al., 2004). Although it has been reported that SA-4503 facilitates the release of acetylcholine or dopamine and potentiates the function of N-methyl-D-aspartate (NMDA) receptors (Bergeron and Debonnel, 1997; Urani et al., 2002) via the activation of σ_1 receptors, the mechanisms underlying the antidepressant-like effects of SA-4503 are not clear.

Although many studies on depression have focused on alterations in the levels of monoamines, recent studies have investigated postsynaptic targets. NMDA receptors play important roles in fundamental functions of neurons. However, the role of NMDA receptors in depression is still not clear. It has been reported that NMDA receptor density or the mRNA expression of NR1 decreases in the prefrontal cortex (PFC) or hippocampus (Hip) of depressive patients (Nowak et al., 1995; Law and Deakin, 2001; Nudmamud-Thanoi and Reynolds, 2004), and long-term use of a specific NMDA receptor antagonist, phencyclidine, induces symptoms of acute anxiety and depression in humans (Liden et al., 1975; De Angelis and Goldstein, 1978). The involvement of NMDA receptors in depression has also been indicated in pharmacological research: repeated treatments with NMDA receptor antagonists, e.g., phencyclidine and MK-801, not only impair performance in the forced swimming test but also prevent the behavioral and neurochemical effects of antidepressant treatments (De Montis et al., 1993; Meloni et al., 1993; Petrie et al., 2000; Javitt, 2004).

The olfactory bulbectomized (OB) rat has been proposed as a model of depression, exhibiting several essential symptomatic isomorphisms, such as psychomotor agitation, loss of interest, and impaired learning and memory (Holmes, 2003). The olfactory bulbs have extensive neural connections with the structures of the limbic system and other parts of the brain, and they influence many emotional aspects of behavioral and other brain output functions (Jesberger and Richardson, 1985). Bilateral olfactory bulbectomy in rodents produces neuroanatomical deficits analogous to the cortical/allocortical degeneration in depressive patients that is, in general, not dependent on particular structures (Holmes, 2003).

The present symptomotology-based study was conducted to investigate the antidepressant-like effects of the σ_1 receptor agonist SA-4503 and the role of NMDA receptors in the effects, using OB rats as a model of depression, since OB rats have dysfunctional glutamatergic systems (Kelly et al., 1997). In the present study, a tricyclic antidepressant, desipramine, was selected as a positive control to compare the σ_1 receptor- and non-σ_1 receptor-mediated effects, based on the fact that desipramine is a typical antidepressant and its

affinity for σ_1 receptors is the weakest of the antidepressants presently in clinical use and several hundred times weaker than that of SA-4503 (Matsuno et al., 1996; Narita et al., 1996).

Materials and Methods

Animals. Five-week-old male Sprague-Dawley rats (170–200 g), were purchased from Japan SLC (Shizuoka, Japan). All rats were housed in our Animal Experimental Center, at a room temperature of 25 ± 1°C and a relative humidity of 40 to 60%. The rooms were illuminated from 9:00 AM to 9:00 PM. All experiments were performed following the Guidelines for Animal Experiments of Nagoya University, which conformed to the international guidelines set out in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996).

Medicines and Reagents. SA-4503 was provided by M's Science Corporation (Kobe, Japan). Desipramine hydrochloride (DES), NE-100, and (+)-MK-801 were purchased from Sigma-Aldrich (St. Louis, MO). Goat polyclonal anti-NR1, NR2A, and NR2B IgG recognizing protein bands of approximately 103, 180, and 200 kDa and horseradish peroxidase-conjugated donkey anti-goat IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-NR1 antibody has an epitope mapping at the C terminus of $NR1_{011}$ (938 amino acids in rat brain; approximately 103 kDa), which is also known as $\zeta1$, NR1a, or NMDAR1-1a, and is the predominant form of the eight splices of NR1 in adult rat brain (Laurie and Seeburg, 1994; Zukin and Bennett, 1995).

Surgical Procedure. The rats were anesthetized with 60 mg/kg pentobarbital sodium, and then they were fixed on a stereotactic apparatus (Narishige, Tokyo, Japan). A midline sagittal incision was made to expose the skull overlaying the olfactory bulbs. A hole 4 mm in diameter was made through the skull 6 mm anterior to the bregma. The olfactory bulbs were cut with a microknife, and they were aspirated out using a pipette tip connected with a water suction pump, with care being taken not to damage the frontal cortex. The cavity for the olfactory bulbs was filled with hemostatic sponge. The hole in the skull was covered with a piece of gelatin gauze, and the skin was sutured. Sham-operated rats were treated in a similar way, except that the olfactory bulbs were not removed. The success of the operation was anatomically confirmed after all of the behavioral tests, and the data from the maloperated rats were excluded from the subsequent analysis.

Schedule of Drug Administration and Behavioral Tests. The schedule of drug administration and behavioral tests is shown in Fig. 1. Saline, desipramine, SA-4503, and/or NE-100 was s.c. administered daily 2 weeks after the surgery for 16 days. MK-801 was s.c. injected 30 min before testing or training trials.

Open-Field Test. The present protocol was adapted from those of Kameyama et al. (1980) and Kelly and Leonard (1994). The open field apparatus, painted gray, consisted of a square arena (60 × 60 cm) divided into 15-cm squares by black lines. The wall of the arena was 30 cm in height. A 60-watt light bulb was positioned at the center 90 cm above the base of the arena.

On the 29th day after the operation, ambulation and rearing
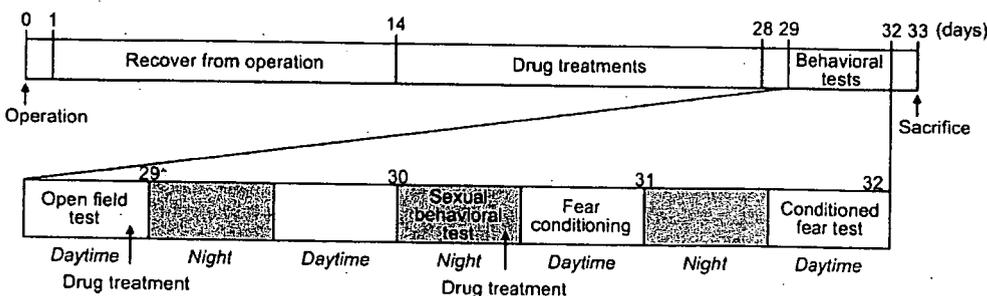

Fig. 1. Schedule of operation, drug treatment, and behavioral tests.

frequencies were recorded in the first 3 min immediately after each rat entered the arena. After each test, the apparatus was sprayed with 70% alcohol, and it was wiped thoroughly to eliminate residual odor.

Sexual Behavior Test. The present protocol was adapted from that of Breigeiron et al. (2002). The apparatus for the sexual behavior test consisted of a transparent Plexiglas box [45 (length) × 27 (width) × 39.5 (height) cm] with a black plastic base, illuminated with a red lamp. On the 30th day after the operation, the sexual behavior of individual male OB rats was observed for 30 min between 10:00 PM and 3:00 AM. A male rat was first placed in the Plexiglas box to habituate to the environment for 3 min. Then, a sexually receptive normal female rat was introduced that had been s.c. administered 0.14 mg of estradiol 72 and 48 h before the test and 0.7 mg of progesterone 4 h before the test. The following parameters of sexual behavior were recorded: starting latency of genital probing and thrusting, count of genital probing and thrusting, and the percentage of the rats that probed the female genitals or showed thrusting behavior. After each test, the apparatus was sprayed with 70% alcohol, and it was wiped thoroughly to eliminate residual odor.

Cued and Contextual Fear-Conditioning Test. The present protocol was adapted from those of Mamiya et al. (2003) and Phillips and LeDoux (1992). The apparatus consisted of a transparent Plexiglas box [45 (length) × 27 (width) × 39.5 (height) cm; the neutral box] with a black plastic base and a Perspex box [32 (length) × 26 (width) × 48 (height) cm; the conditioning box] with a steel grid floor connected to an electric shock generator (Neuroscience-Idea Co., Ltd., Osaka, Japan) and enclosed in an opaque compartment. The neutral box was illuminated with a red lamp, and the conditioning box was illuminated with a fluorescent lamp (6 watt).

For measuring basal levels of the freezing response (preconditioning phase), on the 31st day after the operation, rats were individually placed in the neutral box for 1 min and then in the conditioning box for 2 min. For conditioning (conditioning phase), a 60-s tone (75 dB) was presented as a conditioned stimulus. Just before the end of the tone, a 0.5-mA electric foot-shock lasting for 0.5 s was delivered as an unconditioned stimulus. The tone and the electric foot-shock ceased together. It should be noted that a 0.5-mA electric current lasting for 0.5 s in only one training session was not strong enough to form a stable conditioned response in all of the rats; hence, a difference in the ability to learn and memorize could be observed.

Cued and contextual tests were carried out 24 h after the conditioning. For the cued test, the freezing response was measured in the neutral box for 1 min in the presence of the tone. For the contextual test, rats were placed in the conditioning box, and the freezing response was measured for 2 min in the absence of the tone and foot shock. The freezing response was defined as follows: all four paws of the rat remaining still and the animal stooped down with fear. After each test, the apparatus was sprayed with 70% alcohol and wiped thoroughly to eliminate residual odor.

Western Blot Analysis. After all of the behavioral tests, the rats were sacrificed by decapitation. The dorsal PFC, the CA1-CA3 and dentate gyrus of the Hip, and the posteromedial and posterolateral cortical amygdaloid nuclei of the Amg were rapidly dissected out according to the atlas of the brain of the rat (Paxinos and Watson, 1998). Brain samples were frozen and stored at −80°C until used. The brain samples were homogenized in 150 μl of ice-cold lysis buffer [50 mM Tris-HCl, 150 mM NaCl, 1 mM sodium orthovanadate, 10 mM EDTA, 10 mM NaF, 0.1% SDS, 1% Igepal CA-630 (Sigma-Aldrich), 1% sodium deoxycholate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 0.5 mM DL-dithiothreitol] using an ultrasonic processor (Astrason, Farmingdale, NY). After homogenization, the lysates were kept in an ice bath for 20 min, and then they were centrifuged at 13,000 rpm for 20 min at 4°C. The protein concentration of supernatants was determined by Lowry's method (Lowry et al., 1951). Samples of equal protein concentration were made by mixing the supernatants with lysis buffer, diluting 1:1 with sample-loading buffer (100 mM Tris, 200 mM DL-dithiothreitol, 4%

SDS, 0.2% bromphenol blue, and 20% glycerol, pH 6.8), and heating at 95°C for 5 min. Different samples with a protein concentration of 30 μg/10 μl were electrophoresed by SDS-polyacrylamide gel electrophoresis (PAGE) (6–15% step-gradient resolving gel, an upper 6% gel used for the separation of NMDA receptor subunits, and a lower 15% gel for the separation of β-actin), transferred to polyvinylidene difluoride membranes, and incubated in block solution. The membranes were then incubated overnight with anti-NR1, NR2A, or NR2B antibody (1:1000) at 4°C. After wash, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h, and then they were washed thoroughly. The bands on membranes were then visualized, and the light absorbance was analyzed using an ATTO Densitograph Software Library Lane Analyzer (Atto Co., Tokyo, Japan).

Statistical Analysis. Statistical differences were evaluated with a one-way analysis of variance (ANOVA), except for the evaluation of the time-dependent change in body weight, for which a two-way ANOVA was used. The modified Tukey's test was applied after ANOVA tests for multiple comparisons. The difference in the percentage of rats that showed genital-probing and thrusting behavior among the groups in the sexual behavioral test was evaluated with the χ^2 test. The criterion for a statistically significant difference was $p < 0.05$.

Results

Antidepressant-Like Effects of SA-4503 in OB Rats

Open-Field Test. The open-field test is the behavioral test most commonly used to evaluate the antidepressant-like
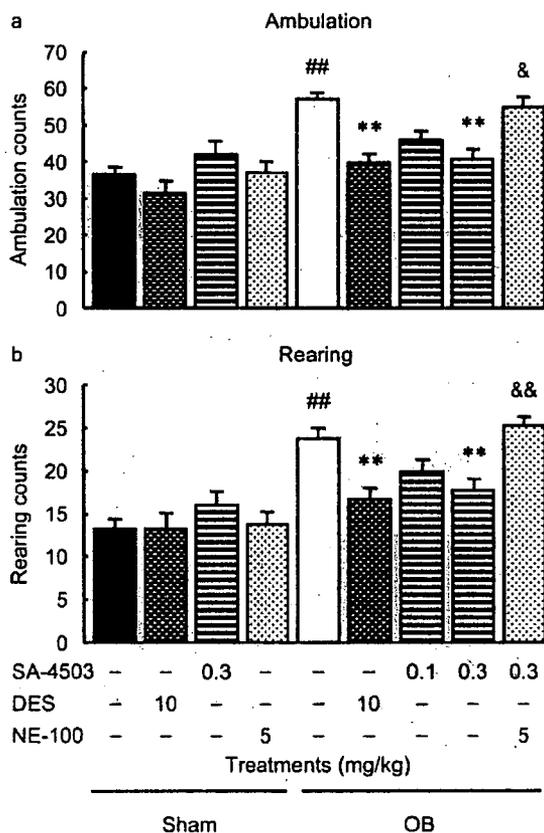

Fig. 2. Effects of repeated treatment with SA-4503 on performance of the open-field test in OB rats. a, ambulation counts in the first 3 min of the test. b, rearing counts in the first 3 min of the test. Results are expressed as means ± S.E., $n = 16$–18. ##, $p < 0.01$, versus saline-treated sham-operated rats; **, $p < 0.01$, versus saline-treated OB rats; &, $p < 0.05$ and &&, $p < 0.01$, versus SA-4503 (0.3 mg/kg)-treated OB rats. Sham, sham-operated rats.

effects of medicines using the OB rat model. As shown in Fig. 2, a and b, saline-treated OB rats exhibited significantly increased counts of ambulation [$F_{(8,141)}$ = 10.088; $p < 0.01$] and rearing [$F_{(8,141)}$ = 8.168; $p < 0.01$] in the first 3 min after being put into the open-field arena. The exploratory hyper-activity in OB rats was reversed by the repeated treatments both with 10 mg/kg desipramine and 0.3 mg/kg SA-4503. The effects of SA-4503 were abolished by the combined treatment with 5 mg/kg NE-100. Desipramine at 10 mg/kg, SA-4503 at 0.3 mg/kg, and NE-100 at 5 mg/kg did not significantly affect behavior in sham-operated rats. Interestingly, the counts of ambulation and rearing decreased in OB rats compared with sham-operated rats from 6 to 9 min after the animals were put into the open-field arena. These data are not shown because these counts are not commonly adopted as experimental indices in the open-field test using OB rats.

Sexual Behavior Test. The loss of interest shown in depressive patients is a core symptom of depression as depicted in the Diagnostic and Statistical Manual, Version IV (Seidman and Roose, 2001). As an alternative measure, we examined the effect of the σ_1 receptor agonist SA-4503 on the sexual dysfunction. As shown in Fig. 3, the starting latency of genital probing [$F_{(8,141)}$ = 29.466; $p < 0.01$] and thrusting [$F_{(8,141)}$ = 6.343; $p < 0.01$] was increased in saline-treated

OB rats (Fig. 3, a and d), whereas the number of genital-probing [$F_{(8,141)}$ = 13.300; $p < 0.01$] and thrusting events [$F_{(8,141)}$ = 4.178; $p < 0.01$] (Fig. 3, b and e) and the percentage of the rats that showed genital-probing and thrusting behavior (Fig. 3, c and f) were decreased, compared with values for sham rats. These results showed sexual deficits in OB rats.

Treatment with 0.3 mg/kg SA-4503 reduced the deficits of genital-probing and thrusting behavior in OB rats without affecting the behavior in sham-operated rats (Fig. 3). The effects of SA-4503 on sexual behavior were blocked by NE-100 (Fig. 3). Repeated treatment with desipramine at the dose of 10 mg/kg ameliorated the loss of genital-probing behavior (Fig. 3, a–c), without affecting thrusting behavior (Fig. 3, d–f).

Cued and Contextual Fear-Conditioning Test. Major depressive patients exhibit significant cognitive dysfunction, to which minor depression is not related (Murphy et al., 1998; Airaksinen et al., 2004; Stordal et al., 2004). In the preconditioning phase of the test, all the rats showed a similar freezing time either in the neutral box or in the conditioning box (data not shown). Twenty-four hours after conditioning, OB rats exhibited a significantly shortened freezing time in both the cued [$F_{(8,116)}$ = 8.783; $p < 0.01$] and contextual
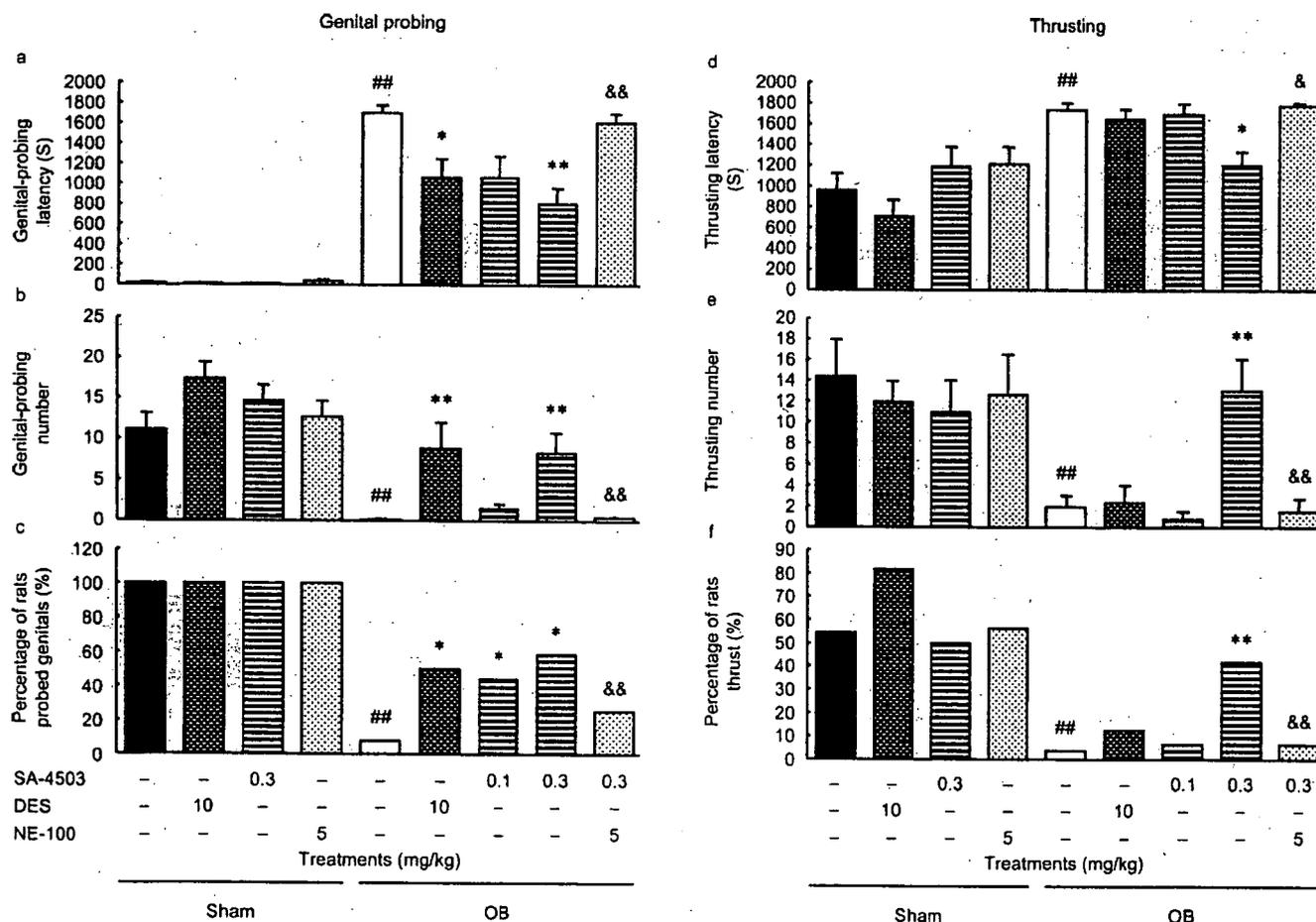

Fig. 3. Effects of repeated treatment with SA-4503 on sexual behavior in OB rats. a, genital-probing starting latency. b, genital-probing number. c, percentage of rats probing the female genital. d, thrusting latency. e, thrusting number. f, percentage of rats that showed penis-thrusting behavior. Results are expressed as means ± S.E. or percentages, n = 16–18. ##, $p < 0.01$, versus saline-treated sham-operated rats; *, $p < 0.05$ and **, $p < 0.01$, versus saline-treated OB rats; &, $p < 0.05$ and &&, $p < 0.01$, versus 0.3 mg/kg SA-4503-treated OB rats. Statistical differences of percentages among the groups were analyzed with the χ^2 test.

$[F_{(8,116)} = 10.440; \; p < 0.01]$ tests compared with sham-operated rats (Fig. 4, a and b).

In both the cued and contextual tests, the cognitive deficits in OB rats were reversed by repeated treatment with 10 mg/kg desipramine and SA-4503 (0.3 mg/kg in cued test and 0.1 and 0.3 mg/kg in contextual test; Fig. 4, a and b). The effects of SA-4503 were blocked by NE-100 at the dose of 5 mg/kg (Fig. 4, a and b). In preliminary experiments, no change in the response threshold was found in saline- and drug-treated OB rats: the minimal current intensities required to elicit flinching/running, jumping, or vocalization in saline- and drug-treated OB rats were the same as those in sham-operated control rats (data not shown).

Involvement of NMDA Receptors in the Effects of SA-4503

Because 0.1 and 0.3 mg/kg SA-4503 significantly improved the behavioral abnormalities in OB rats in a dose-dependent manner, the dose of 0.3 mg/kg was used in all subsequent experiments.

Effects of SA-4503 on Protein Expression of NRs. As shown in Fig. 5, the protein expression of NR1 decreased in the PFC $[F_{(4,50)} = 19.055; p < 0.01]$, Hip $[F_{(4,50)} = 4.274; p < 0.01]$, and Amg $[F_{(4,50)} = 19.399; p < 0.01]$ of saline-treated OB rats compared with sham-operated control rats. The loss of NR1 was ameliorated by the treatments with 10 mg/kg desipramine and 0.3 mg/kg SA-4503. The improving effects of
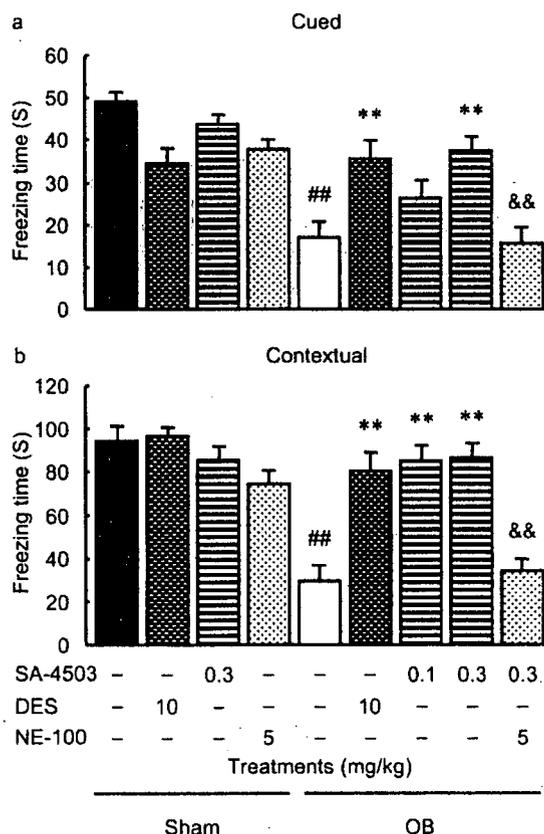
Fig. 4. Effects of repeated treatment with SA-4503 on performance of cued and contextual conditioning test in OB rats. a, freezing time in the cued test. b, freezing time in the contextual test. Results are expressed as means ± S.E., $n = 12–14$. ##, $p < 0.01$, versus saline-treated sham-operated rats; **, $p < 0.01$, versus saline-treated OB rats; &&, $p < 0.01$, versus 0.3 mg/kg SA-4503-treated OB rats.

Fig. 5. Effects of repeated treatment with SA-4503 on protein expression of NR1 in the prefrontal cortex, hippocampus, and amygdala of OB rats. a, in the PFC. b, in the Hip. c, in the Amg. Results are expressed as means ± S.E., $n = 11$. ##, $p < 0.01$, versus saline-treated sham-operated rats; **, $p < 0.01$, versus saline-treated OB rats; &&, $p <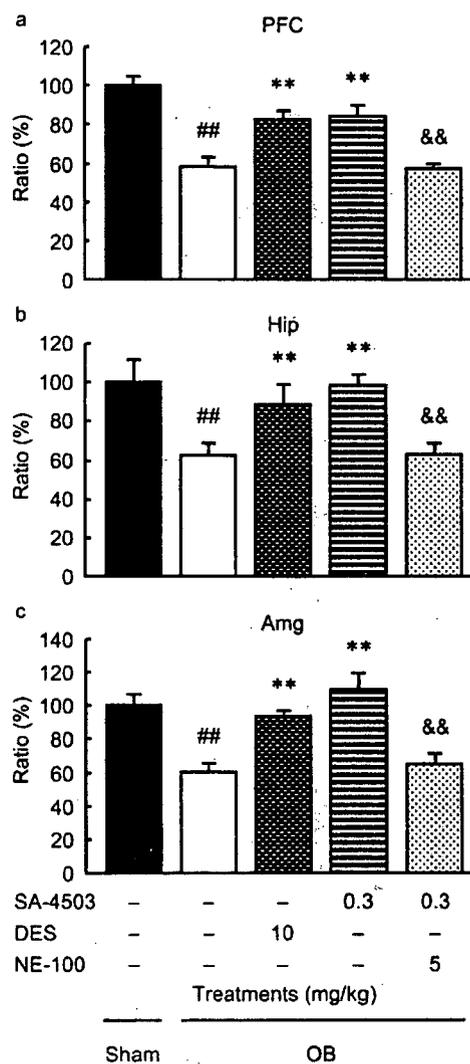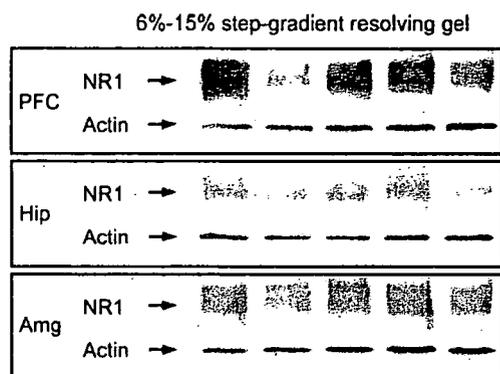 0.01$, versus 0.3 mg/kg SA-4503-treated OB rats. Proteins were separated by SDS-PAGE on 6 to 15% step-gradient resolving gels.

SA-4503 on the protein expression of NR1 in these regions in OB rats were blocked by 5 mg/kg NE-100. There was no significant difference in the protein expression of NR2A or NR2B in these regions between sham-operated and OB con-

trol rats (Fig. 6). These results are consistent with previous publications on the density and function of NMDA receptors in the brain of OB rats, and they fit well with reports that the expression of NR1 decreases in the brain of depressive patients (Kelly et al., 1997; Law and Deakin, 2001; Robichaud et al., 2001; Nudmamud-Thanoi and Reynolds, 2004).

Open-Field Test. As shown in Fig. 7, the treatment with MK-801 at 0.03 mg/kg blocked the effect of 0.3 mg/kg SA-4503 on the exploratory hyperactivity in OB rats. The treatment with MK-801 at 0.03 mg/kg tended to increase the counts of ambulation [$F_{(4,85)}$ = 11.143; p < 0.01] and rearing [$F_{(4,85)}$ = 11.230; p < 0.01] in the sham-operated rats.

Sexual Behavior Test. The effects of 0.3 mg/kg SA-4503 on the latency of genital probing [$F_{(4,75)}$ = 38.257; p < 0.01]

and thrusting [$F_{(4,75)}$ = 4.797; p < 0.01] (Fig. 8, a and d), the number of genital-probing [$F_{(4,75)}$ = 6.784; p < 0.01] and thrusting events [$F_{(4,75)}$ = 3.850; p < 0.01] (Fig. 8, b and e), and the percentage of animals that probed the female genitals and showed thrusting behavior (Fig. 8, c and f) were blocked by the treatment with MK-801 at 0.03 mg/kg, a dose that did not significantly affect sexual behavior in the sham-operated rats (Fig. 8).

Cued and Contextual Fear-Conditioning Test. The treatment with MK-801 at 0.03 mg/kg blocked the effects of 0.3 mg/kg SA-4503 in both the cued [$F_{(4,49)}$ = 15.305; p < 0.01] and contextual [$F_{(4,49)}$ = 13.493; p < 0.01] tests (Fig. 9, a and b). At the dose of 0.03 mg/kg, the treatment with MK-801 significantly impaired the performance of sham-operated rats (Fig. 9, a and b).
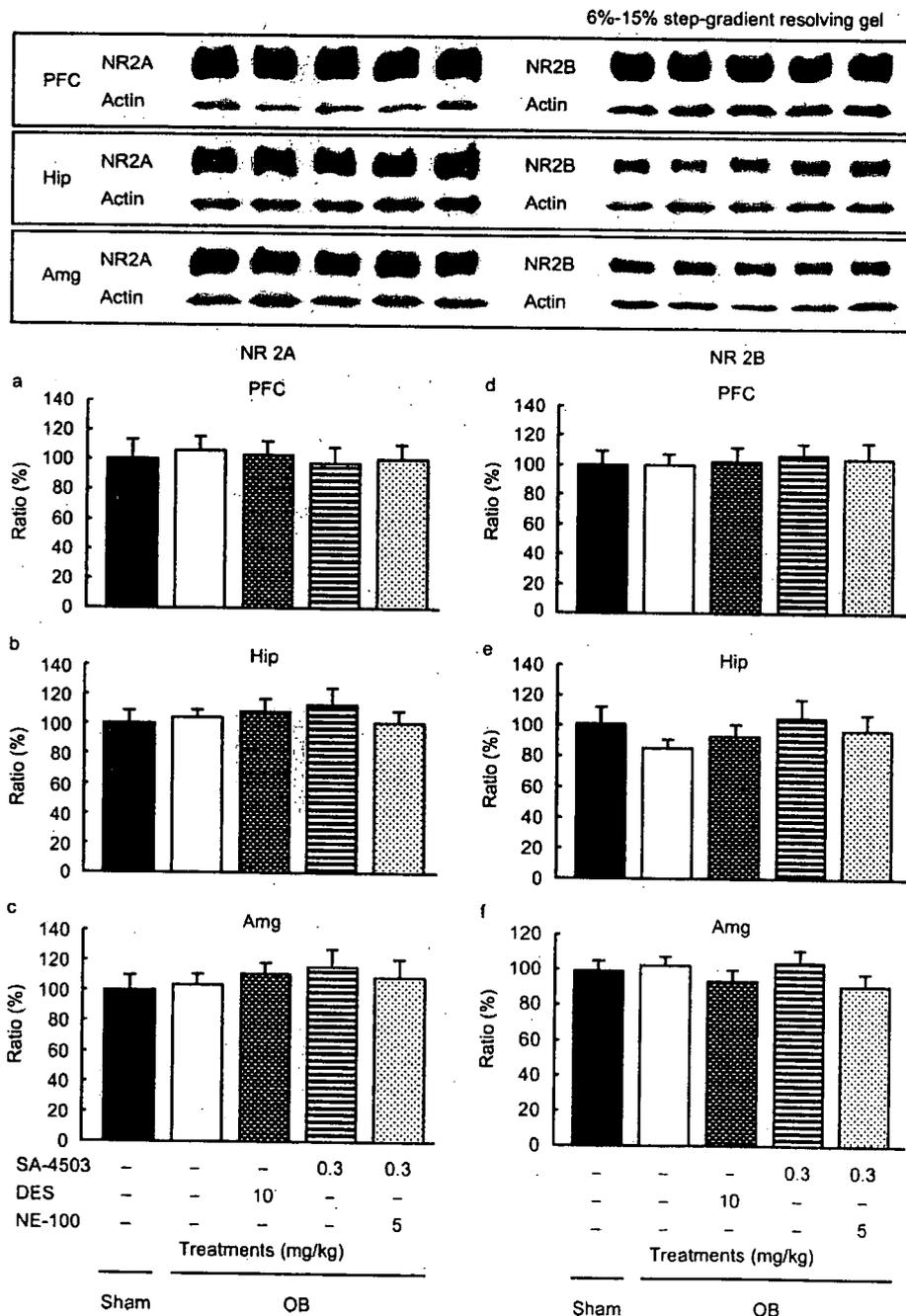
Fig. 6. Effects of repeated treatment with SA-4503 on protein expression of NR2A and NR2B in the prefrontal cortex, hippocampus, and amygdala of OB rats. a, protein expression of NR2A in the PFC. b, protein expression of NR 2A in the Hip. c, protein expression of NR2A in the Amg. d, protein expression of NR2B in the PFC. e, protein expression of NR2B in the Hip. f, protein expression of NR2B in the Amg. Results are expressed as means ± S.E., n = 10. Proteins were separated by SDS-PAGE on 6 to 15% step-gradient resolving gels.

Fig. 7. Effects of SA-4503 on performance of the open-field test in OB rats were abolished by MK-801. a, ambulation counts. b, rearing counts. Results 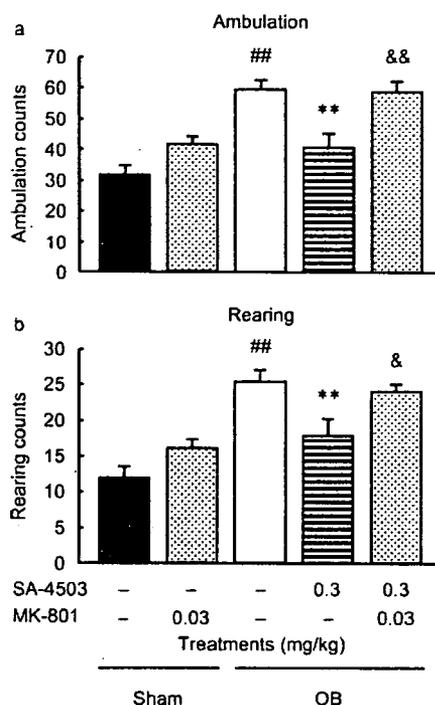are expressed as means ± S.E., $n = 18$. ##, $p < 0.01$, versus saline-treated sham-operated rats; **, $p < 0.01$, versus saline-treated OB rats; &, $p < 0.05$ and &&, $p < 0.01$, versus 0.3 mg/kg SA-4503-treated OB rats.

Changes in Body Weight of Sham-Operated and OB Rats Treated with Saline and Medicines

Olfactory bulbectomy induced the decrease of body weight in rats. Treating OB rats with SA-4503 at the dose of 0.3 mg/kg partially reversed the decrease of the body weight. In contrast to the treatment with SA-4503, repeated treatment with desipramine at the dose of 10 mg/kg decreased the body weight of OB rats [$F_{\text{group (3,2897)}} = 222.742$; $p < 0.01$; and $F_{\text{time (28,2897)}} = 485.303$; $p < 0.01$] (Fig. 10).

Discussion

The OB rat is considered to be one of the best animal models of depression in terms of construct validity (Jesberger and Richardson, 1988; Lumia et al., 1992; Kelly et al., 1997; van der Stelt et al., 2005). Chronic deprivation of olfaction, the primary sensory mode in rats, constitutes a stress of high intensity, and the behavioral deficits induced by OB are primarily the result of alterations in neuronal functions, which is supported by the phenomenon that the behavioral deficits can be reversed by antidepressant treatments although the olfactory bulbs are nonexistent (van Riezen et al., 1977; Jesberger and Richardson, 1988; Mar et al., 2000; O'Neil and Moore, 2003). The depression symptom-resembling deficits in OB rats can be normalized by chronic, not acute, antidepressant treatments (Jesberger and Richardson, 1985; Kelly et al., 1997). In previous preliminary study, treating OB rats with SA-4503 for 1 week did not significantly ameliorate the behavioral deficits, which were ameliorated after treating for 2 weeks in the present study.

Desipramine, a conventional tricyclic antidepressant that inhibits the reuptake of norepinephrine and 5-HT, was used

as a positive control. The binding affinity of desipramine ($K_i \approx 1987$ nM) for σ_1 receptors is approximately 450 times weaker than that of SA-4503 ($K_i \approx 4.4$ nM; $IC_{50} \approx 17.4$ nM) (Narita et al., 1996; Shiba et al., 2006), and it takes effects mainly by inhibiting the reuptake of norepinephrine ($IC_{50} \approx 8.3$ nM) or 5-HT ($IC_{50} \approx 17.5$ nM) at the present dose (Pi et al., 1986; Hyttel, 1993). We also treated rats with imipramine at the dose of 20 mg/kg; however, the subcutaneous or intraperitoneal treatment induced severe inflammation in the rats. Therefore, imipramine-treated rats were not fit for behavioral analyses in the emotional study. Selective serotonin reuptake inhibitors were not preferred as control agents, given that they increase the risk of suicide-related behavior, especially in adolescents (Fegert and Herpertz-Dahlmann, 2005).

The open-field test is most commonly used for screening antidepressants using OB rat model. Within the initial 3 min in a stressful environment, OB rats show hyperlocomotion, which resembles the psychomotor agitation in depression, the extreme of which is a suicidal impulse (Lumia et al., 1992; Holmes, 2003). Based on the predictive value of the open-field test, it was suggested that SA-4503 may have antidepressant-like effects, which is further supported by the results of the sexual behavioral and the fear-conditioning tests.

Patients with major depression exhibit symptoms of sexual and cognitive dysfunction, which have been proven to be unrelated to minor depression and dysthesia (Murphy et al., 1998; Seidman and Roose, 2001; Airaksinen et al., 2004; Stordal et al., 2004). Alternatively, the sexual dysfunction in OB rats resembles the loss of interest that is a core symptom of depression. The result with the sexual dysfunction in OB rats is consistent with previous publications (Mathew et al., 1980; Mathew and Weinman, 1982). Compared with SA-4503, desipramine had relatively weak effects on sexual behavior in OB rats. It improved genital-probing behavior, rather than thrusting behavior, indicating a combination of positive effects and latent side effects of desipramine and that SA-4503 may have therapeutic advantages over it.

Major depression is associated with cognitive impairments (Murphy et al., 1998; Airaksinen et al., 2004; Stordal et al., 2004). Studies have reported spared functions in depressed patients in tests tapping implicit memory (Hertel and Hardin, 1990; Danion et al., 1995), explicit memory (Bazin et al., 1994), and attention (Landro et al., 2001). In the present study, an impairment of associative learning and long-term memory involving the Hip and Amg was observed in OB rats in the fear-conditioning test, which fits well with some clinical observations demonstrating explicit memory deficits in depressive patients (Vythilingam et al., 2004; Kieseppa et al., 2005). The performance of OB rats in both the cued and contextual tests was improved by desipramine and SA-4503. These results indicated that SA-4503 may ameliorate the cognitive symptoms of depression.

NMDA receptors play fundamental roles in the mammalian nervous system. They also have neurotrophic effects, and NR1 null animals cannot survive (Augustine et al., 1987; Malenka, 1994; Mohn et al., 1999; Balazs, 2006). Dysfunctional central nervous system glutamatergic pathways may be one of pathophysiological factors in depression (Nudmamud-Thanoi and Reynolds, 2004), and magnetic resonance imaging revealed a reduced level of glutamate in the
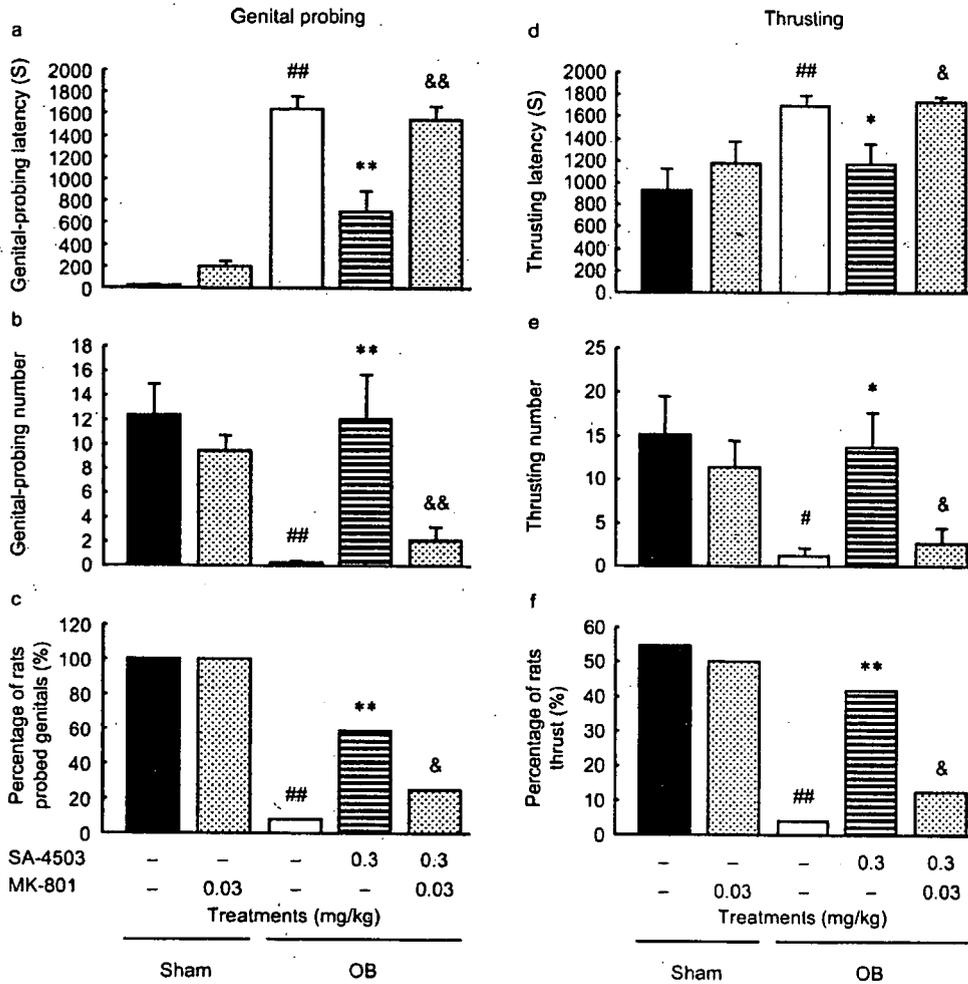
Fig. 8. Effects of SA-4503 on sexual behavior in OB rats were abolished by MK-801. a, genital-probing starting latency. b, genital-probing number. c, percentage of rats that probed the female genital. d, thrusting latency. e, thrusting number. f, percentage of rats that showed penis-thrusting behavior. Results are expressed as means $\pm$ S.E. or percentages, $n = 16$. #, $p < 0.05$ and ##, $p < 0.01$, versus saline-treated sham-operated rats; *, $p < 0.05$ and **, $p < 0.01$, versus saline-treated OB rats; &, $p < 0.05$ and &&, $p < 0.01$, versus 0.3 mg/kg SA-4503-treated OB rats. Statistical differences of percentage among the groups were analyzed with the χ^2 test.

PFC, which returned to normal following treatments with antidepressants (Bermack and Debonnel, 2005). Law and Deakin (2001) have reported that the expression of NR1 decreases in the hippocampus of depressive patients. The NMDA receptor density and the immunoreactivity of NR1 also decrease in other brain structures in depressive patients (Nudmamud-Thanoi and Reynolds, 2004). Furthermore, NMDA receptor density is decreased in the frontal cortex of suicide victims of depression, and the adaptation of the density to repeated antidepressant treatments has been ruled out (Nowak et al., 1995).

NR1 is indispensable for diverse NMDA receptors, and it is functional in a homomeric form; however, NR2 subunits require NR1 to form functional complexes (Zukin and Bennett, 1995). NR1 is distributed ubiquitously in the brain. In contrast, NR2 subunits are region-specifically distributed. NR2A is distributed widely, with relatively high levels in the cerebral cortex, the Hip, and cerebellar granule cells. NR2B is expressed selectively in the forebrain, with high levels in the cerebral cortex, hippocampal formation, septum, caudate-putamen, olfactory bulbs, and thalamus. The NR2C subunit is found predominantly in the cerebellum, whereas weak expression is detected in the olfactory bulbs and the thalamus. NR2D is expressed at much lower levels than the other subunits, and it is found in the thalamus, brainstem, and olfactory bulbs (Liu and Zhang, 2000). The deficit in the protein expression of NR1 underlies the decreased density

and function of NMDA receptors in the OB rat brain (Kelly et al., 1997; Robichaud et al., 2001). Hei et al. (2006) have reported that NR1 expression was increased by activation of NMDA receptors that coexist with and are potentiated by σ_1 receptors (Bergeron and Debonnel, 1997; Urani et al., 2002), which indicates a mechanism for the effect of SA-4503 on protein expression of NR1.

The affinities of SA-4503 for α_1, D_2, 5-HT$_{1A}$, 5-HT$_2$, H$_1$, M$_1$, and M$_2$ receptors are at least 100 times weaker than that for the σ_1 receptors (Matsuno et al., 1996). In the present study, all the effects of SA-4503 were blocked by the σ_1 receptor antagonist NE-100 at a low dose, which confirmed that the effects of SA-4503 are basically mediated by σ_1 receptors.

The behavioral effects of SA-4503 were blocked by an NMDA receptor antagonist, MK-801. This result is at least partially supported by the report that the mice expressing 5–10% NR1 exhibit sexual dysfunction (Mohn et al., 1999). The decrease in the protein expression of NR1 is just one facet of neurodegeneration in the OB rat brain, and NMDA receptors play a crucial role in the emotional effects of SA-4503, because the compensatory phenomena evident in sham-operated rats were not observed in SA-4503-treated OB rats.

Besides having a role in emotion, NMDA receptors are involved in the cognitive effects of SA-4503 in the fear-conditioning test, which is supported by the report that the Hip-regional knockout of NR1 inhibits the ability of animals
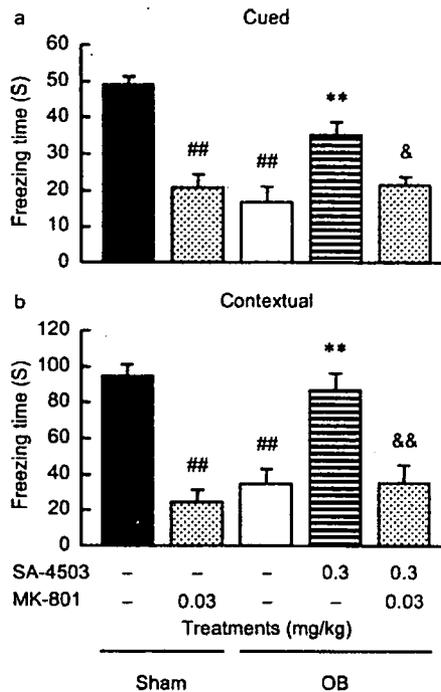
Fig. 9. Effects of repeated treatment with SA-4503 on performance of cued and contextual conditioning test in OB rats were abolished by MK-801. a, freezing time in the cued test. b, freezing time in the contextual test. Results are expressed as means ± S.E., $n = 9$–12. ##, $p < 0.01$, versus saline-treated 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.
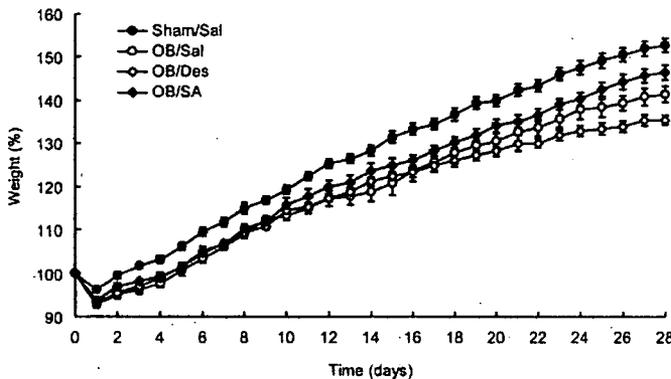

Fig. 10. Changes in body weights of sham-operated and OB rats treated with saline and medicines. Results are expressed as means ± S.E., $n = 23$–28. Sham/Sal, saline-treated sham-operated rats. OB/Sal, saline-treated OB rats. OB/Des, 10 mg/kg desipramine-treated OB rats. OB/SA, 0.3 mg/kg SA-4503-treated 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-HT$_3$ 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.

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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 Dependence

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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 place preference and sensitization by attenuating the 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-

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, ΔFosB, 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	±	±
Repeated	↑	↑
Conditioned place preference		
Wild type	↑	↑
Wild type + TNF-α	↓	↓
TNF-α (−/−)	↑↑	↑↑
TNF-α (−/−) + TNF-α	N.D.	↓
Hyperlocomotion (Single)		
Wild type	↑	↑
Wild type + TNF-α	↓	↑
TNF-α (−/−)	↑	N.D.
Locomotor sensitization (Repeated)		
Wild type	↑	↑
Wild type + TNF-α	↓	↓
TNF-α (−/−)	↑↑	N.D.
Extracellular dopamine levels		
Wild type	↑	↑
Wild type + TNF-α	↓	↓

±, No change; ↑, Significant increase; ↓, 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'-monophosphase 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 astro glial 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 loco- motor 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 treat- ment are examined by using in vivo microdialysis and the [³H]DA uptake assay, respectively. Leu-Ile inhibits the repeated METH treatment-induced increase in extra- cellular 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 hetero-zygous and TNF-α knockout mice (10, 31).

To explore the mechanisms of the inhibitory effects of Leu-Ile on MOR-induced rewarding effects and loco-motor 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, neurotropin-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 sensiti-zation, CPP, and dopamine release in the NAc (33). Infusion of BDNF into the NAc enhances the stimula-tion 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 depen-dence.

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.

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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⒮

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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.

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-hydroxy-ethyl)]-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-1H-3-benzazepine hydrobromide; AP, anteroposterior; ML, medio-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 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 13,000g 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% bromphenol blue), separated on a polyacrylamide gel, and subsequently transferred to polyvinylidene difluoride membranes (Milli-
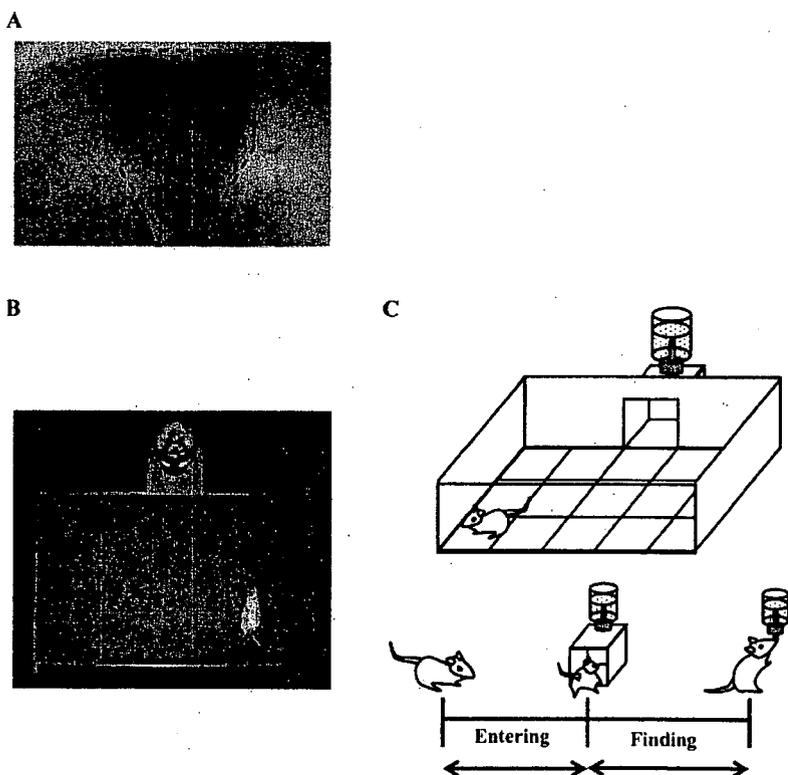
A

B

C

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
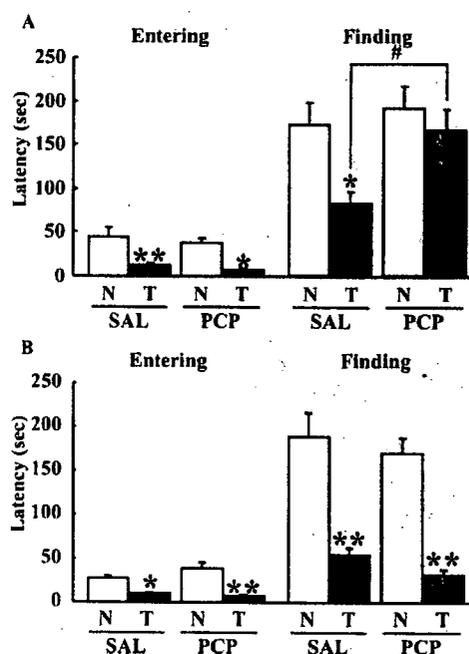

Fig. 2. Impairment of latent learning on repeated PCP treatment. A, latent learning in the mice treated repeatedly with PCP (10 mg/kg s.c. once a day for 14 days). These mice were subjected to a training trial of the water-finding task 4 days after the last PCP treatment. The entering and finding latencies 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.