Role of NMDA receptors in antidepressant-like effects of sigma₁ receptor agonist SA-4503 in olfactory bulbectomized rats

Dayong Wang, Yukihiro Noda, Hiroko Tsunekawa, Yuan Zhou, Masayuki Miyazaki, Koji Senzaki, Atsumi Nitta, Toshitaka Nabeshima

Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya, Japan. (D. W., Y. N., H. T., Y. Z., M. M., K. S., A. N., T. N.)

Division of Clinical Science in Clinical Pharmacy Practice, Management and Research, Faculty of Pharmacy, Meijo University, Nagoya, Japan. (Y. N.)

Division of Scientific Affairs, Japanese Society of Pharmacopoeia, Tokyo, Japan. (D. W.)

Japanese Drug Organization of Appropriate Use and Research, Nagoya, Japan. (T. N.)

Department of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, Meijo University, Nagoya, Japan. (T. N.)

Research Institute, Fujimoto Pharmaceutical Corporation, Osaka, Japan. (H. T.)
**Running title:** Roles of NMDA and sigma₁ receptors in antidepressant treatment

**Address correspondence to:**

Toshitaka Nabeshima

Professor and Chairman, Department of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, Meijo University, Nagoya 468-0069, Japan.

Phone/Fax: +81-52-839-2735, Email: tnabeshi@ccmfs.meijo-u.ac.jp

**Document statistics:**

Number of text pages including figure legends: 36
Number of tables: 0
Number of figures: 10
Number of references: 69
Number of words in the abstract: 218
Number of words in the introduction: 530
Number of words in the discussion: 1493

**Non-standard abbreviations:**

OB, olfactory bulbectomy (-ized); NMDA, N-methyl-D-aspartate; NR, NMDA receptor subunit; PFC, prefrontal cortex; Hip, hippocampus; Amg, amygdala; PCP, phencyclidine; DES, desipramine; HRP, horseradish peroxidase; α₁, alpha₁ adrenergic; D, dopamine; 5-HT, serotonin; H, histamine; M: muscarinic; SSRI, selective serotonin reuptake inhibitor; Sal, 0.9% saline.
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 sigma₁ receptor agonist, 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 1 (NR1), but not NR2A or NR2B, in the prefrontal cortex (PFC), hippocampus (Hip), and amygdala (Amg) of OB rats. The behavioral and neurochemical effects of SA-4503 were blocked by combined treatment with a specific sigma₁ receptor antagonist, NE-100. Further, the effects of SA-4503 on the performance of OB rats in the behavioral tests were abrogated by acute treatment with a NMDA receptor antagonist, MK-801. The present study indicated for the first time that the sigma₁ 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.
INTRODUCTION

Sigma$_1$ receptors are particularly concentrated in the limbic structures of the brain which play important roles in emotion and cognition (Bermack and Debonnel 2005; Matsuno et al. 1996; Skuza 2003; Skuza and Wedzony 2004; Stahl 2005). Various antidepressants, including tricyclic compounds, selective serotonin reuptake inhibitors (SSRI), and monoamine oxidase inhibitors, possess affinity and act as agonists for sigma$_1$ receptors (Maurice et al. 2001; Su and Hayashi 2003). Based on the above, it is hypothesized that sigma$_1$ receptor agonists may act as antidepressants. SA-4503 is a highly selective agonist of sigma$_1$ receptors, with higher binding affinity than a prototypical sigma$_1$ receptor agonist, (+)-SKF-10047 (Guitart et al. 2004; Matsuno et al. 1996). Although it has been reported that SA-4503 facilitates the release of acetylcholine or dopamine and potentiates the function of NMDA receptors (Bergeron and Debonnel 1997; Urani et al. 2002) via the activation of sigma$_1$ 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. N-methyl-D-aspartate (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 (Law and Deakin 2001; Nowak et al. 1995; Nudmamud-Thanoi and Reynolds 2004), and
long term use of a specific NMDA receptor antagonist, phencyclidine (PCP), induces symptoms of acute anxiety and depression in humans (De Angelis and Goldstein 1978; Liden et al. 1975). The involvement of NMDA receptors in depression has also been indicated in pharmacological research: repeated treatments with NMDA receptor antagonists, e.g. PCP 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; Javitt 2004; Meloni et al. 1993; Petrie et al. 2000).

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 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 general, not dependent on particular structures (Holmes 2003).

The present symptomotology-based study was conducted to investigate the antidepressant-like effects of the sigma$_1$ 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 \( \text{sigma}_1 \) receptor- and non-\( \text{sigma}_1 \) receptor-mediated effects, based on the fact that desipramine is a typical antidepressant and its affinity for \( \text{sigma}_1 \) receptors is the weakest of the antidepressants presently in clinical use and several hundred times weaker than that of SA-4503 (Matsuno K 1996; Narita et al. 1996).
METHODS

Animals

Five-week-old male Sprague-Dawley rats, 170-200g, 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 40-60%. 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” (ILAR-NRC publication, revised in 1996).

Medicines and reagents

SA-4503 \{1-(3,4-dimethoxyphenethyl)-4-(3-phenylpropyl)piperazine dihydrochloride\} was provided by M’s Science Corporation (Kobe, Japan). Desipramine hydrochloride \{DES, 10,11-Dihydro-5-[3-(methylamino)propyl]-5H-dibenz[b,f]azepine hydrochloride\}, NE-100 \{N,N-dipropyl-2-(4-methoxy-3-(2-phenylethoxy)phenyl)ethylamine monohydrochloride\}, and (+)-MK-801 \{(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate\} were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Goat polyclonal anti-NR1, NR2A, and NR2B IgG recognizing protein bands of about 103, 180, and 200 kD, and horseradish peroxidase (HRP)-conjugated donkey anti-goat IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-NR1 antibody has an epitope mapping at the C-terminus of NR1\textsubscript{101} (938 amino acids in rat brain, about 103 kDa) which is also known as ζ1, NR1a, or NMDAR1-1a and is the predominant form of the 8 splices of NR1 in adult rat brain.
Surgical procedure

The rats were anesthetized with pentobarbital sodium (60 mg/kg), and 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 micro-knife, and aspirated out using a pipette tip connected with a water suction pump, care being taken not to damage the frontal cortex. The cavity for the olfactory bulbs was filled with haemostatic 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 Figure 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
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 high. A 60-W 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 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 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 (L) × 27 (W) × 39.5 (H) 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 22:00 and 3:00. 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 which had been s.c. administered 0.14 mg of estradiol 72 and 48 hours before the test and 0.7 mg of progesterone 4 hours 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 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 (L) × 27 (W) × 39.5 (H) cm, the neutral box] with a black plastic base and a Perspex box [32 (L) × 26 (W) × 48 (H) 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 W).

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-sec 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 sec 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 sec 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 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 rat’s brain (Paxinos and Watson 1998), 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% sodium dodecyl sulfate (SDS), 1% Igepal CA-630, 1% sodium deoxycholate, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin and 0.5 mM DL-dithiothreitol (DTT)) using an Astrason® ultrasonic processor (Farmingdale, NY). After homogenization, the lysates were kept in an ice bath for 20 min, then 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 mmol/L Tris, 200 mmol/L DTT, 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-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 PVDF
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 HRP-conjugated secondary antibodies at room temperature for 1 hour, and 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 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 Chi-square 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 effects of medicines using the OB rat model. As shown in Figure 2a and 2b, 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 hyperactivity in OB rats was reversed by the repeated treatments both with desipramine (10 mg/kg) and SA-4503 (0.3 mg/kg). The effects of SA-4503 were abolished by the combined treatment with NE-100 (5 mg/kg). Desipramine (10 mg/kg), SA-4503 (0.3 mg/kg) and NE-100 (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, which data are not shown since 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 DSM-IV (Seidman and Roose 2001). As an alternative measure, we examined the effect of the sigma$_1$ receptor agonist SA-4503 on the sexual dysfunction. As shown in Figure 3, the starting latency of genital probing ($F_{(8,141)} = 29.466, p < 0.01$) and thrusting ($F_{(8,141)} = 6.343, p < 0.01$) were increased in saline-treated OB rats (Figure 3a, d), whereas the number of genital probing ($F_{(8,141)} = 13.300, p < 0.01$) and thrusting events ($F_{(8,141)} = 4.178, p < 0.01$) were decreased in OB rats compared with sham-operated rats.
0.01) (Figure 3b, e) and the percentage of the rats that showed genital-probing and thrusting behavior (Figure 3c, f) were decreased, compared to values for sham rats. These results showed sexual deficits in OB rats.

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

Cued and contextual fear-conditioning test Major depressive patients exhibit significant cognitive dysfunction, to which minor depression is not related (Airaksinen et al. 2004; Murphy et al. 1998; 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 \(F(8,116) = 10.440, p < 0.01\) tests compared with sham-operated rats (Figure 4a, b).

In both the cued and contextual tests, the cognitive deficits in OB rats were reversed by repeated treatment with desipramine (10 mg/kg) and SA-4503 (0.3 mg/kg in cued test, 0.1 and 0.3 mg/kg in contextual test) (Figure 4a, b). The effects of SA-4503 were blocked by NE-100 at the dose of 5 mg/kg (Figure 4a, 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

Since SA-4503 (0.1 and 0.3 mg/kg) 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 Figure 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 desipramine (10 mg/kg) and SA-4503 (0.3 mg/kg). The improving effects of SA-4503 on the protein expression of NR1 in these regions in OB rats were blocked by NE-100 (5 mg/kg). There was no significant difference in the protein expression of NR2A or NR2B in these regions between sham-operated and OB control rats (Figure 6). These results are consistent with previous publications on the density and function of NMDA receptors in the brain of OB rats, and fit well with reports that the expression of NR1 decreases in the brain of depressive patients (Kelly et al. 1997; Robichaud et al. 2001; Law and Deakin 2001; Nudmamud-Thanoi and Reynolds 2004).

Open field test

As shown in Figure 7, the treatment with MK-801 at 0.03 mg/kg blocked the effect of SA-4503 (0.3 mg/kg) 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 SA-4503 (0.3 mg/kg) on the latency of genital probing ($F_{(4,75)} = 38.257, p < 0.01$) and thrusting ($F_{(4,75)} = 4.797, p < 0.01$) (Figure 8a,d), the number of genital probing ($F_{(4,75)} = 6.784, p < 0.01$) and thrusting events ($F_{(4,75)} = 3.850, p < 0.01$) (Figure 8b,e), and the percentage of animals that probed the female genitals and showed thrusting behavior (Figure 8c,f) were blocked by the treatment with MK-801 at 0.03 mg/kg, a dose which did not significantly affect sexual behavior in the sham-operated rats (Figure 8).

**Cued and contextual fear-conditioning test** The treatment with MK-801 at 0.03 mg/kg blocked the effects of SA-4503 (0.3 mg/kg) in both the cued ($F_{(4,49)} = 15.305, p < 0.01$) and contextual ($F_{(4,49)} = 13.493, p < 0.01$) tests (Figure 9a,b). At the dose of 0.03 mg/kg, the treatment with MK-801 significantly impaired the performance of sham-operated rats (Figure 9a,b).

**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 ($F_{\text{group}} (3,2897) = 222.742, p < 0.01$; $F_{\text{time}} (28,2897) = 485.303, p < 0.01$) (Figure 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; Kelly et al. 1997; Lumina et al. 1992; 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 resulted from 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 (Mar et al. 2000; O’Neil and Moore 2003; Richardson 1988; van Riezen et al. 1977). 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 $\sigma_1$ receptors is about 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 (Hyttel 1993; Pi et al. 1986). 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 (SSRI) were not preferred as control agents, given that they increase the risk of suicide-related behavior especially in adolescents (Fergert and Herpertz-Dahlmann 2005).

The open field test is most commonly employed for screening antidepressants using OB rat model. Within 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 (Lumina 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 proved to be unrelated to minor depression and dysthesia (Airaksinen et al. 2004; Murphy et al. 1998; Seidman and Roose 2001; Stordal et al. 2004). Alternatively, the sexual dysfunction in OB rats resembles the loss of interest which is a core symptom of depression. The result with the sexual dysfunction in OB rats is consistent with previous publications (Mathew and Weinman, 1982; Mathew et al. 1980). Compared with SA-4503, desipramine had relatively weak effects on sexual 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 (Stordal et al. 2004; Murphy et al. 1998; Airaksinen 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 can not survive (Augustine et al. 1987; Balazs 2006; Malenka 1994; Mohn et al. 1999). Dysfunctional CNS 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 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). Further, 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 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-putaman, olfactory bulbs, and thalamus. The NR2C subunit is found predominately in the cerebellum, while weak expression is detected in the olfactory bulbs and the thalamus. NR2D is expressed at much lower levels than the other subunits, and 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 2001). Hei et al. (2006) have reported that NR1 expression was increased by activation of NMDA receptors that co-exist with and are potentiated by sigma1 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 α1, D2, 5-HT1A, 5-HT2, H1, M1 and M2 receptors are at least 100 times weaker than that for the sigma1 receptors (Matsuno et al. 1996). In the present study, all the effects of SA-4503 were blocked by the sigma1 receptor antagonist NE-100 at a low dose, which
confirmed that the effects of SA-4503 are basically mediated by \( \sigma_1 \) receptors.

The behavioral effects of SA-4503 were blocked by a 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, since 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 animals’ ability to learn a new set of tasks within a specific context (Greene 2005). Since 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.

Since the dose of MK-801 (0.03 mg/kg) that we used in the present study 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 20-30% activity have half-lives of about 1 and 6 h respectively, antidepressant-like effect of ketamine reaches a significant level several hours after the intravenous infusion and gradually increases for several days (Berman et al. 2000; Zarate et al. 2006). Further, ketamine also has an affinity for the µ opiate receptors, and is an agonist for the sigma receptors (Berman et al. 2000). Besides being a NMDA receptor antagonist, memantine also acts as uncompetitive antagonists at the 5-HT₃ and the nicotinic acetylcholine receptors, with potencies similar to or more than that for the NMDA receptors (Aracava et al. 2005; Buisson and Bertrand 1998; Rammes et al. 2001). Therefore, the antidepressant-like effects of MK-801 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.
Acknowledgements

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


Holmes PV (2003): Rodent models of depression: reexamining validity without anthropomorphic


*Nord J Psychiatry* 47(suppl 30):5-12


Laurie DJ and Seeburg PH (1994): Regional and developmental heterogeneity in splicing the rat brain NMDAR1 mRNA. *J Neurosci* 14:3180-3194.


Shiba K, Ogawa K, Ishiwata K, Yajima K, Mori H (2006): Synthesis and binding affinities of...
methylvesamicol analogs for the acetylcholine transporter and sigma receptor. *Bioorg Med Chem* 14:2620-2626.


Su TP and Hayashi T (2003): Understanding the molecular mechanism of sigma-1 receptor: towards a hypothesis that sigma-1 receptors are intracellular amplifiers for signal transduction. *Curr Med Chem* 10:2063-2080.


Footnotes

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) (17390018), by a Grant-in-aid 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 program), 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, by the Japan Brain Foundation, by the Mitsubishi Pharma Research Foundation, by an SRF Grant for Biomedical Research, and by the Brain Research Center of the 21st Century Frontier Research Program of the Ministry of Science and Technology, Republic of Korea.
Legends for Figures

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

Figure 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 ± SE, n = 16-18. *** p < 0.01, vs saline-treated sham-operated rats; ** p < 0.01, vs saline-treated OB rats; & p < 0.05 and && p < 0.01, vs SA-4503 (0.3 mg/kg)-treated OB rats. Sham: sham-operated rats. OB: olfactory bulbectomized. DES: desipramine.

Figure 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 ± SE or percentages, n = 16-18. *** p < 0.01, vs saline-treated sham-operated rats; * p < 0.05 and ** p < 0.01, vs saline-treated OB rats; & p < 0.05 and && p < 0.01, vs SA-4503 (0.3 mg/kg)-treated OB rats. Statistical differences of percentages among the groups were analyzed with the Chi-square test. Sham: sham-operated rats. OB: olfactory bulbectomized. DES: desipramine.

Figure 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 ± SE, n = 12-14. #p < 0.01, vs saline-treated sham-operated rats; **p < 0.01, vs saline-treated OB rats; &&p < 0.01, vs SA-4503 (0.3 mg/kg)-treated OB rats. Sham: sham-operated rats. OB: olfactory bulbectomized. DES: desipramine.

Figure 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 prefrontal cortex (PFC); (b) in the hippocampus (Hip); (c) in the amygdala (Amg). Results are expressed as means ± SE, n = 11. #p < 0.01, vs saline-treated sham-operated rats; **p < 0.01, vs saline-treated OB rats; &&p < 0.01, vs SA-4503 (0.3 mg/kg)-treated OB rats. Proteins were separated by SDS-PAGE on 6%-15% step-gradient resolving gels. NR1: NMDA receptor subunit 1. Sham: sham-operated rats. OB: olfactory bulbectomized. DES: desipramine.

Figure 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 NR 2A in the prefrontal cortex (PFC); (b) Protein expression of NR 2A in the hippocampus (Hip); (c) Protein expression of NR 2A in the amygdala (Amg). (d) Protein expression of NR 2B in the prefrontal cortex (PFC); (e) Protein expression of NR 2B in the hippocampus (Hip); (f) Protein expression of NR 2B in the amygdala (Amg). Results are expressed as means ± SE, n = 10. Proteins were separated by SDS-PAGE on 6%-15% step-gradient resolving gels. NR2A: NMDA receptor subunit 2A. NR2B: NMDA receptor subunit 2B. Sham: sham-operated rats. OB:
olfactory bulbectomized. DES: desipramine.

Figure 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 ± SE, n = 18. 

\[ # p < 0.01, \text{vs saline-treated sham-operated rats}; **p < 0.01, \text{vs saline-treated OB rats}; \& p < 0.05 \text{ and } \&\& p < 0.01, \text{vs SA-4503 (0.3 mg/kg)-treated OB rats. Sham: sham-operated rats. OB: olfactory bulbectomized. DES: desipramine.} \]

Figure 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 ± SE or percentages, n = 16. 

\[ # p < 0.05 \text{ and } # p < 0.01, \text{vs saline-treated sham-operated rats}; * p < 0.05 \text{ and } ** p < 0.01, \text{vs saline-treated OB rats}; \& p < 0.05 \text{ and } \&\& p < 0.01, \text{vs SA-4503 (0.3 mg/kg)-treated OB rats. Statistical differences of percentage among the groups were analyzed with the Chi-square test. Sham: sham-operated rats. OB: olfactory bulbectomized. DES: desipramine.} \]

Figure 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 ± SE, n = 9-12. 

\[ # p < 0.01, \text{vs saline-treated sham-operated rats}; ** p < 0.01, \text{vs saline-treated OB rats}; \& p < 0.05 \text{ and } \&\& p < 0.01, \text{vs SA-4503 (0.3 mg/kg)-treated OB rats.} \]
vs SA-4503 (0.3 mg/kg)-treated OB rats. Sham: sham-operated rats. OB: olfactory bulbectomized.

DES: desipramine.

Figure 1
Figure 2

(a) Ambulation counts

(b) Rearing counts

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sham</th>
<th>OB 0.1</th>
<th>OB 0.3</th>
<th>OB 0.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA-4503</td>
<td>30</td>
<td>35</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>DES</td>
<td>15</td>
<td>20</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>NE-100</td>
<td>10</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7

(a) Ambulation counts

(b) Rearing counts

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>OB</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA-4503</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>MK-801</td>
<td>0.03</td>
<td>0.03</td>
</tr>
</tbody>
</table>
Figure 9

Freezing time (S)

SA-4503
MK-801

Treatments (mg/kg)

Sham          OB

Contextual
Figure 10