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Research Report

Phencyclidine-induced cognitive deficits in mice are ameliorated by subsequent subchronic administration of donepezil: Role of sigma-1 receptors

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ABSTRACT

This study was undertaken to examine the effects of two acetylcholinesterase inhibitors (donepezil and physostigmine) on cognitive deficits in mice after repeated administration of the NMDA receptor antagonist phencyclidine (PCP). In the novel object recognition test, PCP (10 mg/kg/day for 10 days)-induced cognitive deficits were significantly improved by subsequent subchronic (14 days) administration of donepezil (1.0 mg/kg/day), but not donepezil (0.1 mg/kg/day). Furthermore, the effect of donepezil (1.0 mg/kg/day) on PCP-induced cognitive deficits was significantly antagonized by co-administration of the selective sigma-1 receptor antagonist NE-100 (1.0 mg/kg/day), suggesting the role of sigma-1 receptors in the active mechanisms of donepezil. In contrast, PCP-induced cognitive deficits were not improved by subsequent subchronic (14 days) administration of physostigmine (0.25 mg/kg/day). Moreover, repeated administration of PCP significantly caused the reduction of sigma-1 receptors in the hippocampus. The present study suggests that agonistic activity of donepezil at sigma-1 receptors plays a role in the active mechanisms of donepezil on PCP-induced cognitive deficits in mice. Therefore, it is likely that donepezil would be potential therapeutic drugs for the treatment of the cognitive deficits in schizophrenia.

1. Introduction

Cognitive deficits in patients with schizophrenia are core features of the illness, and predict vocational and social disabilities for patients (Freedman, 2003; Coyle and Tsai, 2004; Green et al., 2004; Kurtz, 2005). Multiple lines of evidence suggest that a dysfunction in glutamatergic neurotransmission via the N-methyl-D-aspartate (NMDA) receptors might be involved in the pathophysiology of schizophrenia (Javitt and Zukin, 1991; Olney and Farber, 1995; Coyle, 1996; Krystal et al., 1999; Hashimoto et al., 2003, 2004, 2005b; Mandillo et al., 2003). NMDA receptor antagonists such as phencyclidine (PCP) are known to induce schizophrenia-like symptoms including cognitive deficits and negative symptoms in healthy subjects

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(Javitt and Zukin, 1991); consequently, PCP has been used widely in animal models of schizophrenia (Javitt et al., 2004; Jentsch and Roth, 1999; Hashimoto et al., 2005a, 2007, 2008). We recently found that PCP-induced cognitive deficits in the novel object recognition test (NORT) could be significantly improved by subsequent subchronic (14 days) administration of clozapine, but not haloperidol. Our observations suggested that reversal of PCP-induced cognitive deficits as measured by NORT may be a potential animal model of atypical antipsychotic activity in relation to the amelioration of cognitive deficits in schizophrenia (Hashimoto et al., 2005a).

Accumulating evidence suggests that sigma-1 receptors play a role in the pathophysiology of psychiatric diseases such as schizophrenia, anxiety disorders, and depression as well as in the evolution of cognitive deficits associated with these conditions (Maurice et al., 2001; Su and Hayashi, 2003; Hayashi and Su, 2004; Guitart et al., 2004; Bermack and Debonnel, 2005; Hashimoto and Ishiwata, 2006; Monnet and Maurice, 2006). Recently, we reported that, in NORT, PCP-induced cognitive deficits could be significantly ameliorated by subsequent subchronic (14 days) administration of sigma-1 receptor agonists (fluvoxamine, SA4503, and dehydroepiandrosterone (DHEA)-sulfate), and that the effects of these sigma-1 receptor agonists were significantly antagonized by co-administration of the selective sigma-1 receptor antagonist NE-100 (Hashimoto et al., 2007). These findings suggest that sigma-1 receptor agonists would be potential therapeutic drugs for cognitive deficits in schizophrenia (Hashimoto, in press; Hashimoto et al., 2007).

Donepezil is the most widely prescribed drug for Alzheimer's disease (Blennow et al., 2006; Seltzer, 2007). The main mechanism of action through which it influences cognition and function is presumed to be the inhibition of acetylcholinesterase (AChE) in the brain; however, it has been reported that donepezil binds to sigma receptors in the brain (Kato et al., 1999). Recent studies suggest that sigma-1 receptors have been implicated in the anti-depressive, anti-amnesic and neuroprotective effects of donepezil against mouse-forced swimming test (Maurice et al., 2006), and CO gas-induced (Meunier et al., 2006a) and amyloid β_{25-35}-induced neurotoxicity (Meunier et al., 2006b). Recently, we reported that donepezil, but not physostigmine, significantly potentiated the nerve growth factor (NGF)-induced neurite outgrowth in PC12 cells, and that potentiation of NGF-induced neurite outgrowth by donepezil was significantly blocked by co-administration of the selective sigma-1 receptor antagonist NE-100 (Ishima et al., 2008). Taken together, it is likely that the pharmacological actions of donepezil as both an AChE inhibitor and sigma-1 receptor agonist might contribute to the efficacy of this drug in patients with Alzheimer's disease.

The present study was undertaken to study whether or not sigma-1 receptors play a role in the mechanisms of action of donepezil on cognitive deficits in schizophrenia. First, we examined the binding affinity of donepezil and other AChE inhibitor physostigmine at sigma-1 receptors in the mouse brain. Second, we examined the effects of donepezil and physostigmine on PCP-induced cognitive deficits in mice using NORT. We also examined the effects of the selective sigma-1 receptor antagonist NE-100 (Okuyama and Nakazato, 1996) on PCP-induced cognitive deficits in order to study the role of the sigma-1 receptor in the mechanism of action of donepezil. Third, using the immunohistochemistry, we examined whether or not repeated PCP administration alters the density of sigma-1 receptors in the regions (CA1–3 and dentate gyrus) of mouse hippocampus which might be involved in the PCP-induced cognitive deficits.

2. Results

2.1. *Effects of donepezil and physostigmine on [^{3}H](+)-pentazocine binding to mouse brain*

It has been reported that donepezil (IC_{50} = 14.6 ± 0.5 nM) has a high affinity for [^{3}H]DTG binding to sigma receptors (sigma-1 and sigma-2 receptors) whereas physostigmine (IC_{50} > 10,000 nM) showed no effect (Kato et al., 1999). In this study, we examined the effects of these two AChE inhibitors on [^{3}H](+)-pentazocine binding to sigma-1 receptors since [^{3}H]DTG is not selective for sigma-1 receptors. We found that donepezil had a high affinity (IC_{50} = 29.1 ± 0.2 nM (n = 3)) for [^{3}H](+)-pentazocine binding to sigma-1 receptors in the mouse brain (Fig. 1). Physostigmine had no affinity for [^{3}H](+)-pentazocine binding to sigma-1 receptors in mouse brain (Fig. 1).

2.2. *Effects of donepezil on PCP-induced cognitive deficits in mice*

In the training session, the exploratory preferences of the all groups were the same. In the retention test session, the exploratory preference of the PCP-treated group was significantly lower than that of the saline-treated group (Fig. 2). PCP-induced cognitive deficits in mice were significantly improved

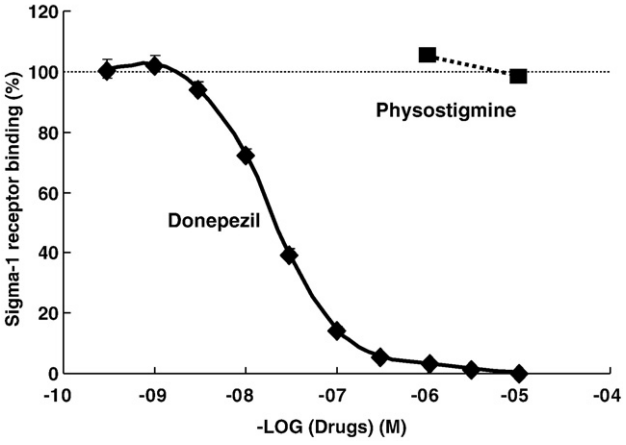

Fig. 1 – Effects of donepezil and physostigmine on [^{3}H](+)-pentazocine binding to mouse brain membranes. Inhibition of [^{3}H](+)-pentazocine binding to mouse brain membranes by donepezil and physostigmine was performed as described in Experimental procedures. The IC_{50} value of donepezil was 29.12 ± 0.24 nM. Physostigmine was very weak. The data is the mean ± S.E.M. of three experiments done in duplicate.

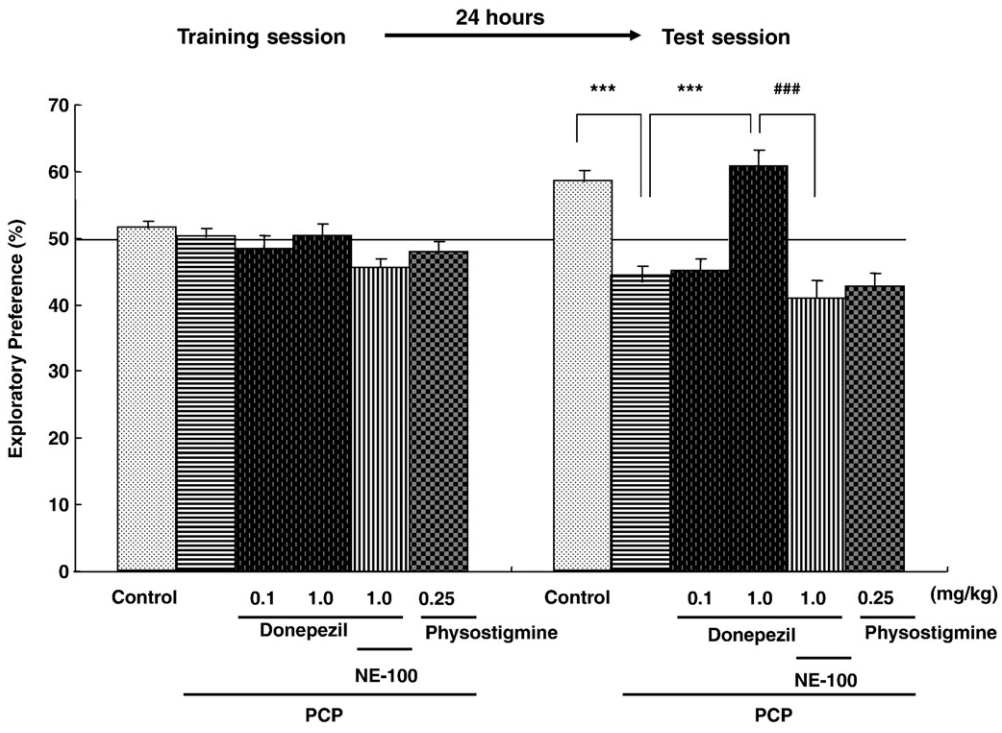

Fig. 2 – Effects of donepezil on PCP-induced cognitive deficits in mice. Saline (10 ml/kg) or PCP (10 mg/kg) was administered s.c. for 10 days (once daily on days 1–5, 8–12). Three days (day 15) after the final administration of saline or PCP, vehicle (10 ml/kg), donepezil (0.1 or 1.0 mg/kg), donepezil (1.0 mg/kg) plus NE-100 (1.0 mg/kg), or physostigmine (0.25 mg/kg) was administered i.p. into mice. The treatment was continued for 2 consecutive weeks (once daily on days 15–28). The training session for the novel object recognition test (NORT) was performed 24 h (day 29) after the final administration of vehicle or drugs, and the retention test session was performed 24 h (day 30) after the training session. Values are means ± S.E.M. (n = 10–20). $^{***}p<0.001$ as compared with PCP-treated group. $^{###}p<0.001$ as compared with PCP + donepezil-treated group.

after subsequent subchronic (14 days) administration of donepezil (1.0 mg/kg/day), but not the low dose (0.1 mg/kg/day). In the training session, one-way ANOVA revealed that the exploratory preferences of six groups were not significantly different (F [5,100] = 1.96, p = 0.091) (Fig. 2). However, one-way ANOVA did reveal significant differences in the exploratory preferences of the six groups in the retention test sessions (F [5,100] = 16.05, $p<0.001$) (Fig. 2). The *post hoc* Bonferroni test indicated that the exploratory preference of the PCP plus vehicle-treated group was significantly ($p<0.001$) lower than that of the PCP plus donepezil (1.0 mg/kg/day)-treated group, but not of groups treated with PCP plus donepezil (1.0 mg/kg/day)/NE-100 (1.0 mg/kg/day) (Fig. 2). In contrast, PCP-induced cognitive deficits in mice were not improved after subsequent subchronic (14 days) administration of physostigmine (0.25 mg/kg/day). These findings suggest that sigma-1 receptors play a role in the active mechanism of donepezil for PCP-induced cognitive deficits.

Next, we examined the effects of subchronic (14 days) administration of donepezil (1.0 mg/kg/day), physostigmine (0.25 mg/kg/day), or NE-100 (1.0 mg/kg/day) on the exploratory preference in the control mice. As shown in Fig. 3, in both training session and retention session, subchronic administration of these drugs alone did not alter the exploratory preference in the control mice.

2.3. *Effects of repeated PCP administration on sigma-1 receptors in the mouse brain*

Immunohistochemistry revealed that the immunoreactivity of sigma-1 receptors in the region of CA1 (F [1,21] = 0.159, p = 0.006), CA3 (F [1,21] = 0.113, $p<0.001$) and dentate gyrus (F [1,21] = 0.011, p = 0.005) of mice treated with PCP (10 mg/kg/day for 10 days) was significantly lower than that of control mice (Fig. 4).

3. Discussion

The major findings of the present study are that PCP-induced cognitive deficits could be improved by subsequent subchronic administration of the AChE inhibitor donepezil via sigma-1 receptors. In contrast, we found that the AChE inhibitor physostigmine with no affinity to sigma-1 receptors did not attenuate the PCP-induced cognitive deficits in mice, and that subchronic administration of donepezil or NE-100 did not alter cognition in control mice. Thus, it is unlikely that sigma-1 receptor agonist or antagonist could affect cognition in control mice, consistent with a previous report that sigma-1 receptor agonist fluvoxamine did not affect cognition of control mice (Hashimoto et al., 2007). Taken together, it is likely that sigma-

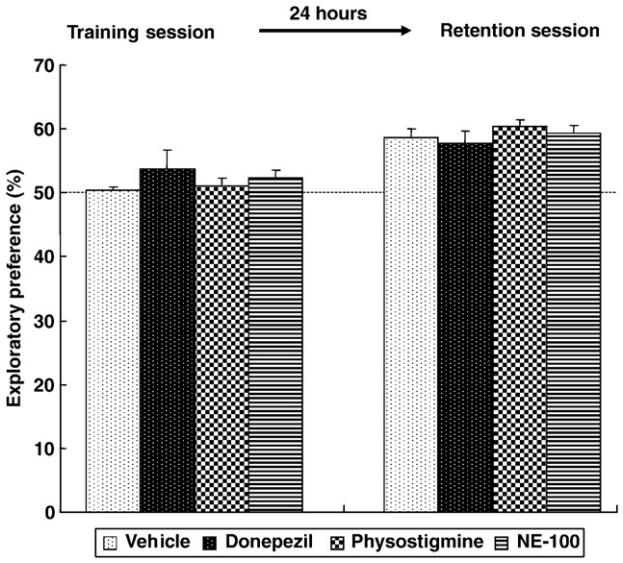

Fig. 3 – Effects of subchronic administration of donepezil, physostigmine, and NE-100 in the control mice. Vehicle (10 ml/kg), donepezil (1.0 mg/kg), physostigmine (0.25 mg/kg), or NE-100 (1.0 mg/kg) was administered i.p. into mice for 2 consecutive weeks. The training session for the NORT was performed 24 h after the final administration of vehicle or drugs, and the retention test session was performed 24 h after the training session. Values are means ± S.E.M. ($n = 10$).

1 receptors play a role in the mechanisms of action of donepezil on PCP-induced cognitive deficits in mice. Recently, we reported that sigma-1 receptor agonists (fluvoxamine, SA4503 and DHEA-sulphate) could attenuate PCP-induced cognitive deficits in mice (Hashimoto et al., 2007). Therefore, it is possible that donepezil would be a potential therapeutic drug for the treatment of cognitive deficits in schizophrenia.

Adjunctive medication to antipsychotic treatment is one approach used to improve several symptoms of schizophrenia. Although some studies have evaluated donepezil as an adjunct to antipsychotic medications in schizophrenic patients, the results have proved inconclusive. Four studies revealed that donepezil shows some cognitive improvement in schizophrenic patients (Buchanan et al., 2003; MacEwan et al., 2001; Risch et al., 2001; Chung et al., 2009); but six double-blind, placebo-controlled trials provided no beneficial effects (Friedman et al., 2002, Friedman, 2004; Tugal et al., 2004; Freudenreich et al., 2005; Fagerlund et al., 2007; Keefe et al., 2008; Akhondzadeh et al., 2008). Interestingly, the donepezil-treated group had significantly greater improvement in the negative symptoms of schizophrenic patients (Risch et al., 2007; Akhondzadeh et al., 2008), suggesting donepezil as a potential adjunctive treatment strategy for negative symptoms in schizophrenia. Recently, we reported a case of improvement of cognitive deficits in schizophrenic patient by sigma-1 receptor agonist fluvoxamine (Iyo et al., 2008). Taken together, the beneficial effects of donepezil for cognitive deficits in schizophrenic patients are currently inconclusive. Further detailed placebo-controlled studies using a large sample are necessary.

In the present study, we found that repeated PCP administration led to a reduction in sigma-1 receptors in the mouse hippocampus. This is the first investigation to demonstrate that the administration of PCP significantly reduced the density of sigma-1 receptors in the CA1, CA3,

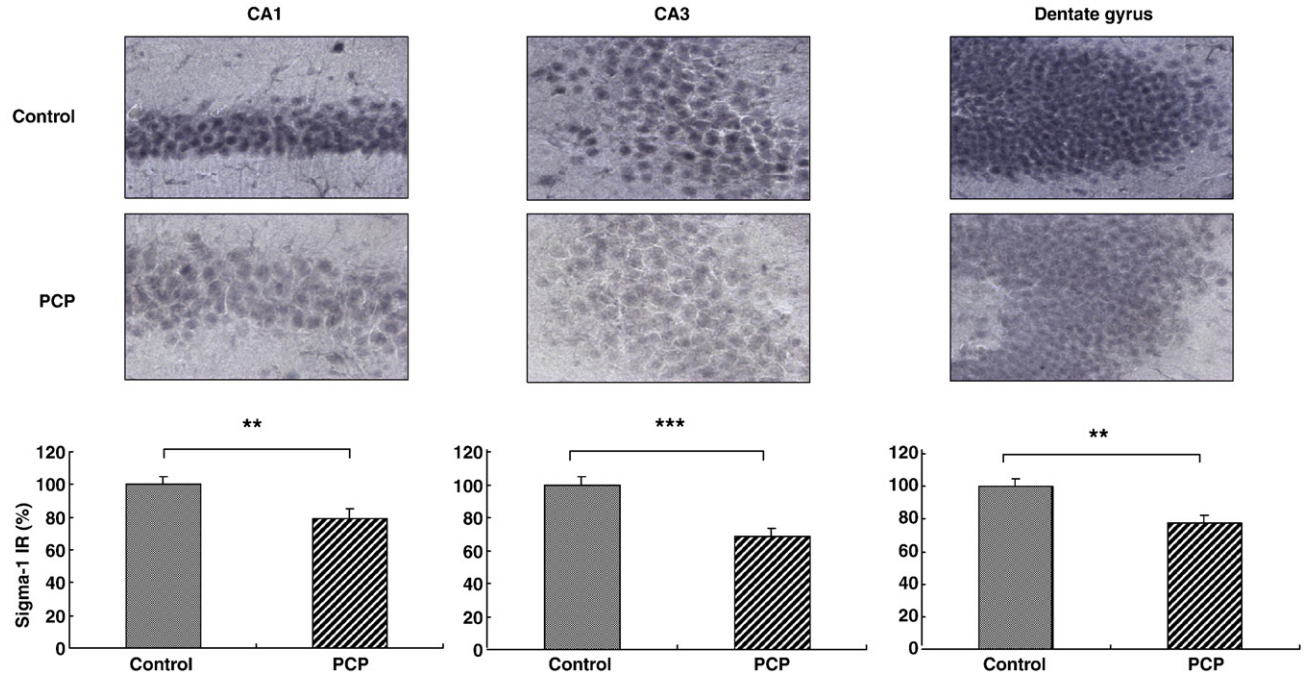

Fig. 4 – Effects of repeated PCP administration on sigma-1 receptors in the mouse hippocampus. Saline (10 ml/kg) or PCP (10 mg/kg) was administered s.c. for 10 days (once daily on days 1–5, 8–12). Three days (day 15) after the final administration of saline or PCP, the mice were perfused. Immunohistochemistry was performed as described in Experimental procedures. Values are means ± S.E.M. ($n = 11$–12). $^{**}p < 0.01$, $^{***}p < 0.001$ as compared with the saline-treated group (control).

and dentate gyrus regions of hippocampus. Recently, Western blot analysis showed that the density of sigma-1 receptors in the frontal cortex and hippocampus was significantly decreased by repeated PCP treatment (Ishima et al., 2009), consistent with the present results. There is evidence that sigma-1 receptors regulate the function of NMDA receptors in the brain (Hayashi and Su, 2004; Bermack and Debonnel, 2005; Hashimoto and Ishiwata, 2006; Monnet and Maurice, 2006). Therefore, it is likely that chronic treatment with the NMDA receptor antagonist PCP might cause the down-regulation of sigma-1 receptors in the brain although the precise mechanisms underlying how repeated PCP administration might modulate sigma-1 receptors in the brains are currently unknown. Further study of the cross-talk between sigma-1 receptors and NMDA receptors in the brain is still necessary.

Postmortem human brain studies on brains from schizophrenic patients have indicated that the density of sigma receptors was lower in the temporal cortex (Weissman et al., 1991), but higher in the superior parietal cortex (Shibuya et al., 1992) than in the control tissue, although chronic neuroleptic treatment may contribute to these differences (Simpson et al., 1991; Hashimoto and London, 1994). Furthermore, it is also reported that [^{3}H]nemonapride binding to sigma receptors in the caudate of postmortem brain of schizophrenic patients was significantly decreased as compared with control group (Helmeste et al., 1996). Currently, a specific radioligand [^{11}C] SA4503 for imaging sigma-1 receptors in the intact human brain using positron emission tomography (PET) is available (Ishiwata et al., 2006; Ishikawa et al., 2007). It is, therefore, of great interest to study whether or not sigma-1 receptors are altered in the intact brain of schizophrenic patients. The [^{11}C] SA4503-PET study in the patients with schizophrenia is now in progress.

Endoplasmic reticulum (ER) stress, such as ER Ca^{2+} depletion, has been reported to be relevant to the pathogenesis of neuropsychiatric diseases such as Alzheimer's disease (Nakagawa et al., 2000; Mattson and Chan, 2001). Communication between the ER and mitochondrion is important for bioenergetics and cellular survival. Very recently, Hayashi and Su (2007) demonstrated that the ER protein sigma-1 receptor is a Ca^{2+}-sensitive and ligand-operated receptor chaperone at mitochondrion-associated ER membrane. Normally, sigma-1 receptors form a complex at mitochondrion-associated ER membrane with another chaperone, BiP. Upon ER Ca^{2+} depletion or via agonist stimulation, sigma-1 receptors dissociate from BiP, leading to a prolonged Ca^{2+} signaling into mitochondria via IP3 receptors These findings suggest that sigma-1 receptors play important roles in the ER-mitochondrial interorganelle Ca^{2+} signaling and in cell survival (Hayashi and Su, 2007). Furthermore, it has been reported that protein levels of subunits (NR1 and NR2) of NMDA receptors in ER fraction of developing rat cortex were increased after subchronic PCP administration, suggesting that up-regulation may be because of increased synthesis of these subunits (Anastasio and Johnson, 2008). Taken together, it seems that cross-talk between sigma-1 receptors and NMDA receptors in ER may play a role in the cognitive deficits after the repeated PCP administration although a further detailed study is necessary.

In conclusion, the present study suggests that repeated PCP administration might cause the reduction of sigma-1 receptors in the mouse brain, and that agonistic activity of donepezil at the sigma-1 receptors plays a role in the active mechanisms of donepezil on PCP-induced cognitive deficits. Very recently, we have demonstrated a high occupancy of sigma-1 receptors in the human brain after a single oral administration of donepezil (5 or 10 mg) (Ishikawa et al., submitted). Therefore, it is possible that donepezil could potentially be used for the treatment of cognitive deficits of schizophrenia.

4. Experimental procedures

4.1. Animals

Male ICR mice (6 weeks old) weighing 25–30 g were purchased from SLC Japan (Hamamatsu, Shizuoka, Japan). Mice in groups of 4 or 5 were housed in clear polycarbonate cages (22.5×33.8×14.0 cm) under a controlled 12/12-h light–dark cycle (light from 7:00 AM to 7:00 PM), at 23±1 °C and 55±5% humidity. The mice were given free access to water and food pellets. The experimental procedure was approved by the Animal Care and Use Committee of Chiba University Graduate School of Medicine.

4.2. Drugs

PCP hydrochloride and the selective sigma-1 receptor antagonist NE-100 were synthesized in our laboratory. Donepezil hydrochloride was provided from Eisai Co., Ltd (Tokyo, Japan). Physostigmine hydrochloride and [^{3}H](+)-pentazocine (1.07 GBq/mmol) were purchased from Sigma-Aldrich (St Louis, MO, USA) and PerkinElmer, Inc. (Boston, MA, USA), respectively. Other drugs were purchased from commercial sources.

4.3. Drug administration

Saline (10 ml/kg) or PCP (10 mg/kg expressed as a hydrochloride salt) was administered subcutaneously (s.c.) for 10 days (once daily on days 1–5, 8–12).

In the subchronic (14 days) administration experiment, three days (day 15) after the final administration of saline or PCP, vehicle or drugs were administered i.p. into mice. This treatment was continued for 14 consecutive days (once daily on days 15–28). The training session of NORT was performed 24 h after the final administration, and the retention test session was performed 24 h after the training session. The number of animals (Fig. 2) is control group (n=20), PCP+vehicle (n=19), PCP+donepezil (0.1 mg/kg) (n=19), PCP+donepezil (1.0 mg/kg) (n=17), PCP+donepezil (1.0 mg/kg)/NE-100 (1.0 mg/kg) (n=18), and PCP+physostigmine (0.25 mg/kg) (n=10). The number of animals (Fig. 3) is control group (n=10), donepezil (1.0 mg/kg) (n=10), physostigmine (0.25 mg/kg) (n=10), and NE-100 (1.0 mg/kg) (n=10).

The doses of drugs used in this study were donepezil (0.1 and 1.0 mg/kg), NE-100 (1.0 mg/kg), and physostigmine (0.25 mg/kg). These doses had been shown to be effective *in*

vivo as reported previously (Csernansky et al., 2005; Maurice et al., 2006; Meunier et al., 2006a,b; Okuyama and Nakazato, 1996; Hashimoto et al., 2007; Senda et al., 1997). The dose (0.1 mg/kg) chosen for donepezil was selected because this dose was found to induce no effects via sigma-1 receptor mechanisms *in vivo* (Csernansky et al., 2005).

4.4. *Novel object recognition test (NORT)*

NORT was performed as previously reported (Hashimoto et al., 2005a, 2007, 2008). The apparatus for this task consisted of a black open field box (50.8×50.8×25.4 cm). Before the test, mice were habituated in the box for 3 days. During a training session, two objects (various objects differing in shape and color but similar in size) were placed in the box 35.5 cm apart (symmetrically), and each animal was allowed to explore in the box for 5 min. The animals were considered to be exploring the object when the head of the animal was facing the object within 2.54 cm of the object or when any part of the body, except for the tail, was touching the object. The time that mice spent exploring each object was recorded. After training, mice were immediately returned to their home cages, and the box and objects were cleaned with 75% ethanol to avoid any possible instinctive odorant cues. Retention tests were carried out at one-day intervals following the respective training. During the retention test each mouse was placed back into the same box in which one of the objects used during training was replaced by a novel one. The mice were then allowed to explore freely for 5 min, and the time spent exploring each object was recorded. Throughout the experiments, the objects were used in a counter-balanced manner in terms of their physical complexity and emotional neutrality. A preference index, i.e., the ratio of the amount of time spent exploring any one of the two objects (training session) or the novel one (retention test session) over the total time spent exploring respective to both objects, was used to measure memory performance.

4.5. *[^{3}H](+)-Pentazocine binding to sigma-1 receptors*

Binding assays for sigma-1 receptors were performed according to the method published previously (Hashimoto and London, 1995; Narita et al., 1996). Male ICR mice (25–35 g) were killed by decapitation, and the brains were rapidly removed. The brains were homogenized in 20 volumes of ice-cold 50 mM Tris–HCl (pH 8.0 at 25 °C) with a Polytron homogenizer at setting 5 for 30 s. The homogenate was centrifuged at 48,000 *g* for 10 min (4 °C). The resulting pellet was resuspended in the buffer and recentrifuged. This procedure was repeated once more. The final pellet was stored at −80 °C until use. At the day of binding assay, the pellet was suspended in 20 volumes of 50 mM Tris–HCl (pH 8.0 at 25 °C). For assays of sigma-1 receptors, aliquots of crude membranes (approximately 350–400 μg protein) were incubated with [^{3}H](+)-pentazocine (5 nM), drugs (donepezil: 0.3–10,000 nM, physostigmine; 1000 and 10,000 nM), and 50 mM Tris–HCl (pH 8.0 at 25 °C) in a final volume of 0.5 ml for 2 h at 25 °C. After addition of 4 ml of ice-cold buffer, the membranes were rapidly filtered, using a Brandell 24-channel cell harvester (Biochemical Research Laboratories, Gaithersburg, MD, USA), through Whatman GF/B filters pretreated with 0.5% polyethyleneimine for at least 2 h. The filters were washed three times with 4 ml of ice-cold buffer. The radioactivity trapped by the filters was determined by a liquid scintillation counter. Non-specific binding was estimated in the presence of 10 μM NE-100. Specific binding was more than 90% of total binding.

4.6. *Immunohistochemistry*

Three days after the final administration of saline (10 ml/kg/day for 10 days; days 1–5 and days 8–12) or PCP (10 ml/kg/day for 10 days; days 1–5 and days 8–12) (i.e., on the day 15), the mice were killed with sodium pentobarbital and perfused transcardially with 50 ml of isotonic saline, followed by 50 ml of ice-cold 4% paraformaldehyde in 0.1 mol/l phosphate buffer (PH 7.4). The brains were removed from skulls and postfixed overnight at 4 °C in the same fixative. For the immunohistochemical analysis, 50 μm-thick serial coronal sections of brain were cut in ice-cold 0.01 mol/l phosphate buffer saline (PBS; pH 7.5) with a vibrating blade microtome (VT1000S, Leica Microsystems AG, Wetzlar, Germany). Free-floating sections were treated with 0.3% hydrogen peroxide (H_2O_2) in 0.1 mol/l phosphate buffer saline (PBS) for 30 min and blocked in PBS containing 0.1% Triton X-100 (PBST), 0.1% BSA and normal rabbit serum for 30 min at room temperature. The samples were then incubated for 24 h at 4 °C with goat anti-sigma-1 receptor antibody (1:50, sc-16203, Santa Cruz Biotechnology, Inc., USA). The sections were washed twice PBS and processed according to the avidin–biotin–peroxidase method (Vectastain Elite ABC, Vector Laboratories, Burlingame, CA, USA). The sections were then reacted for 5 min in a solution of diaminobenzidine (0.25 mg/ml) containing 0.01% H_2O_2. Alternate sections, incubated in the absence of primary antibody as an immunohistochemical control, showed no immunostaining. The sections were mounted on gelatinized slides, dehydrated, cleared, and coverslipped under Entellan New (Merck K GaA, Damnstadt, Germany).

Quantitative analysis of the hippocampus was performed by image analysis. The optical density was analyzed using a light microscope equipped with a CCD camera (Carl Zeiss, HAL100, Germany) and Scion Image. Images were calibrated to a known optical density gray scale (Kodak step tablet #2). Background optical densities were measured in unlabeled fields from the corpus callosum and subtracted from the raw optical densities of the examined tissue. Eight slices of each region from each mouse were prepared. Average counts from three sections per animal were used.

4.7. *Statistical analysis*

Data are expressed as means±S.E.M. Receptor binding data were performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Statistical analysis was performed by using the Student *t* test or one-way analysis of variance analysis (ANOVA) and the *post hoc* Bonferroni test. *p* values less than 0.05 were considered statistically significant.

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