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$GABA_B$ receptor agonist baclofen improves methamphetamine-induced cognitive deficit in mice

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ABSTRACT

In this study, we investigated the effects of $GABA_A$ and $GABA_B$ receptor agonists on the methamphetamine-induced impairment of recognition memory in mice. Repeated treatment with methamphetamine at a dose of 1 mg/kg for 7 days induced an impairment of recognition memory. Baclofen, a $GABA_B$ receptor agonist, ameliorated the repeated methamphetamine-induced cognitive impairment, although gaboxadol, a $GABA_A$ receptor agonist, had no significant effect. $GABA_B$ receptors may constitute a putative new target in treating cognitive deficits in patients suffering from schizophrenia, as well as methamphetamine psychosis.

1. Introduction

Methamphetamine is a highly addictive drug of abuse, and addiction to methamphetamine has increased to epidemic proportions worldwide (Cretzmeyer et al., 2003; Rawson et al., 2002). Chronic use of methamphetamine causes psychiatric symptoms, such as hallucination and delusions, and long-term cognitive deficits (Simon et al., 2000; Kalechstein et al., 2003; Nordahl et al., 2003; Srisurapanont et al., 2003), which are indistinguishable from paranoid schizophrenia (Yui et al., 2002; Srisurapanont et al., 2003). In a previous study, we demonstrated that repeated methamphetamine treatment caused an enduring impairment of recognition memory in a novel object recognition test in mice, and that methamphetamine-induced cognitive impairment was reversed by an atypical antipsychotic, clozapine, but not haloperidol (Kamei et al., 2006). Furthermore, the same treatment in rats resulted in a significant impairment of spatial working memory, which was ameliorated by clozapine but not haloperidol (Nagai et al., 2007). Thus, methamphetamine-induced memory impairment in rodents may be a useful model for cognitive deficits in methamphetamine abusers and schizophrenic patients.

The GABA receptor system is known to play a significant role in modulating the dopamine system (Tepper and Lee, 2007). Several studies have demonstrated that GABA receptor agonists can inhibit the effects of drugs of abuse. For example, baclofen has been shown to attenuate amphetamine-induced increase in dopamine levels in the nucleus accumbens (Brebner et al., 2005), and $GABA_A$ receptors on ventral tegmental area dopamine neurons play a significant role in attenuating the effects of drugs of abuse in a similar manner to that of $GABA_B$ receptors (Westerink et al., 1996). Although many studies have examined the effects of GABA receptor agonists on hyperdopaminergic conditions induced by psychostimulant drugs, few studies have investigated the effects of GABA receptors on cognitive deficits induced by drugs of abuse.

Recent studies suggest that alterations of GABA systems are related to the pathophysiology of schizophrenia (Lewis, 2000; Benes and Berretta, 2001). Moreover, it is suggested that impairment in GABA-mediated inhibition in the prefrontal cortex may provide a mechanism of disturbance in cognitive processes, such as working memory, in individuals with schizophrenia (Lewis, 2000; Benes and Berretta, 2001). Cognitive dysfunction is considered a core feature of schizophrenia (Elvevåg and Goldberg, 2000), and the degree of cognitive deficit may be the best predictor of long-term functional outcome for individuals with schizophrenia (Green, 1996). Despite the clinical

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importance of cognitive dysfunction in schizophrenia, there are no appropriate drug therapies.

In this study, to develop novel pharmacotherapy for cognitive deficits in schizophrenia patients and methamphetamine abusers, we examined the effects of $GABA_A$ and $GABA_B$ receptor agonists on methamphetamine-induced impairment of recognition memory in mice.

2. Materials and methods

Male ICR mice (7–8 weeks old) were obtained from Japan SLC Inc. (Shizuoka, Japan). The animals were housed in plastic cages and kept in a regulated environment (23±1 °C, 50±5% humidity) with a 12 h light–dark cycle (lights on at 9:00 am). Food and tap water were available ad libitum. All animal care and use was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee of Kanazawa University.

Methamphetamine hydrochloride (Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan), *R*(+)-baclofen hydrochloride (Sigma-Aldrich Co., St Louis, MO) and gaboxadol hydrochloride (Sigma-Aldrich) were dissolved in saline. All drugs were administered in a volume of 0.1 ml/10 g body weight. Mice were given methamphetamine (1 mg/kg, s.c.) daily once for 7 days. One day after the last treatment of methamphetamine, novel object recognition test commenced as described below.

Novel object recognition test was carried out as described previously (Kamei et al., 2006; Mizoguchi et al., 2008). The experimental apparatus consisted of a Plexiglas box (30×30×35 cm high), with a sawdust-covered floor. The apparatus was located in a sound-attenuated room and was illuminated with a 20 W bulb.

The novel object recognition test procedure consisted of three sessions: habituation, training, and retention. Each mouse was individually habituated to the box, with 10 min of exploration in the absence of objects for 3 consecutive days (habituation session, days 1–3). During the training session, two novel objects were symmetrically fixed to the floor of the box, 8 cm from the walls, and each animal was allowed to explore the box for 10 min (day 4). The objects were a golf ball, a wooden column and a wall socket, which were different in shape and color but similar in size. The animals were considered to be exploring the object when the head of the animal was facing the object or the animal was touching or sniffing the object. The time spent exploring each object was recorded. After training, mice were immediately returned to their home cages. During the retention sessions, the animals were placed back into the same box 24 h (day 5) after the training session, in which one of the familiar objects used during training was replaced by a novel object. The animals 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 counterbalanced manner in terms of their physical complexity and emotional neutrality. A preference index in the retention session, the ratio of the amount of time spent exploring the novel object over the total time spent exploring both objects, was used to measure cognitive function. In the training session, the preference index was calculated as a ratio of the time spent exploring the object that was replaced by the novel object in the retention session, over the total exploring time.

Baclofen (1 and 2 mg/kg, s.c.) and gaboxadol (1 and 3 mg/kg, s.c.) were administered once 15 min before the training session in novel object recognition test (day 4). No drugs were given during the habituation (day 1–3) and the retention sessions (day 5) in the novel object recognition test.

To investigate effect of baclofen on motor function, locomotor activity was measured. Mice were given saline or methamphetamine at a dose of 1 mg/kg for 7 days. Mice were placed in home cage for 15 min following injection of saline or baclofen (2 mg/kg, s.c.) after the 3 day-withdrawal of repeated methamphetamine treatment, and then locomotor activity was measured for 10 min in a standard transparent rectangular rodent cage (25×30×18 high cm) using an infrared sensor (NS-AS01; Brainscience, Osaka, Japan) placed over the cage (Kamei et al., 2006; Mizoguchi et al., 2008).

All data were expressed as the mean±S.E.M.. Statistical analysis was carried out by one-way or two-way ANOVA, followed by Student–Newman–Keuls test for multigroup comparisons. *P* values less than 0.05 were taken to indicate significant differences.

3. Results

Repeated methamphetamine treatment (1 mg/kg, s.c.) for 7 days resulted in a significant reduction of the preference index in the retention session but not training session as compared with saline-treated control (Figs. 1A and 2A) although it had no effect on total exploratory time (Figs. 1B and 2B). The $GABA_A$ receptor agonist, gaboxadol, at doses of 1 mg/kg, failed to ameliorate the methamphetamine-induced reduction of exploratory preference to the novel object in the retention session of novel object recognition test (Fig. 1A). Although there was no difference between gaboxadol 1 mg/kg and 3 mg/kg in methamphetamine-treated animal ($P=0.10$), there was a tendency of recovery in gaboxadol-treated group at 3 mg/kg ($P=0.06$). Thus, we

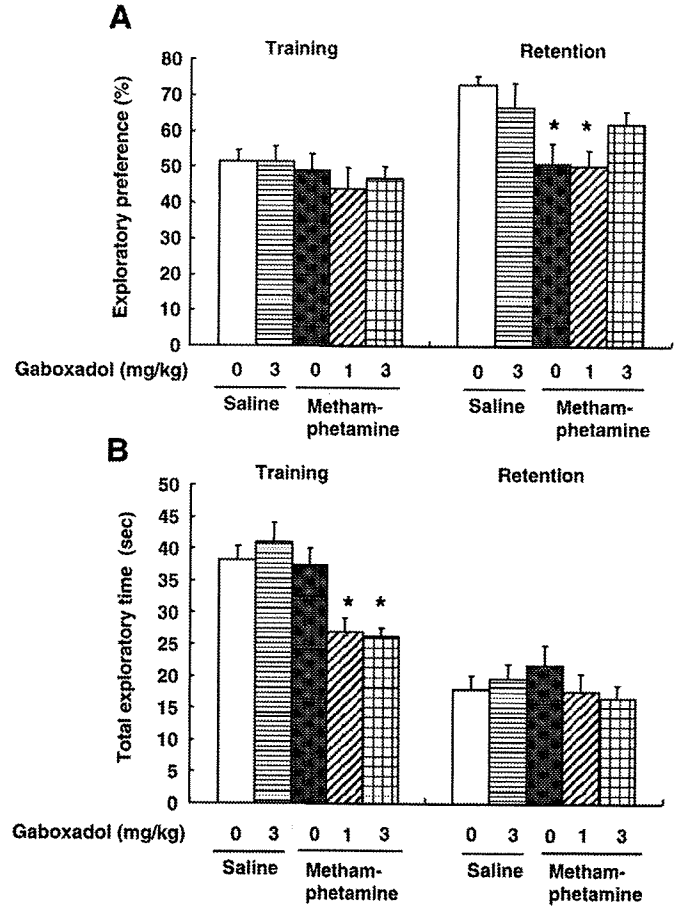

Fig. 1. Effect of gaboxadol on methamphetamine-induced impairment of recognition memory in mice. After the cessation of repeated methamphetamine (1 mg/kg, s.c.) treatment for 7 days, mice were subjected to the novel-object recognition test. Gaboxadol (1 and 3 mg/kg, s.c.), or saline was administered 15 min before the training session. (A) Exploratory preference. (B) Total exploration time. Values indicate the mean±S.E.M. (saline/saline, $n=13$; saline/Gaboxadol 3 mg/kg, $n=8$; methamphetamine/saline, $n=12$; methamphetamine/Gaboxadol 1 mg/kg, $n=7$; methamphetamine/Gaboxadol 3 mg/kg, $n=14$). ANOVA: (A, training) $F(4,49)=0.488$, $P=0.7448$; (A, retention) $F(4,49)=4.6$, $P<0.01$; (B, training) $F(4,49)=7.876$, $P<0.01$; (B, retention) $F(4,49)=0.637$, $P=0.6389$. *$P<0.05$ compared with the saline/saline-treated group (Student–Newman–Keuls test).

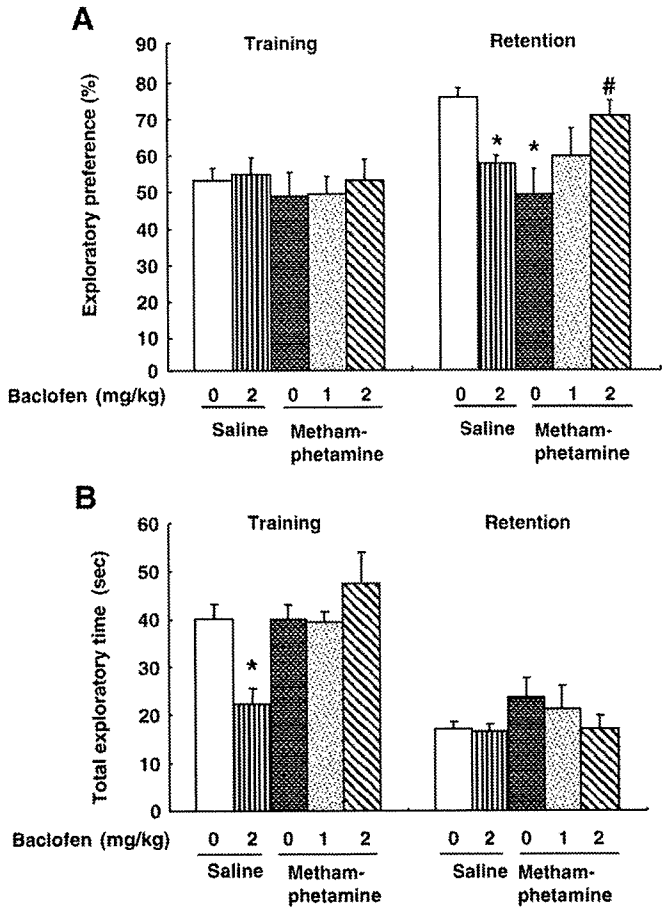

Fig. 2. Effect of baclofen on methamphetamine-induced impairment of recognition memory in mice. After the cessation of repeated methamphetamine (1 mg/kg) treatment for 7 days, mice were subjected to the novel-object recognition test. Baclofen (1 and 2 mg/kg, s.c.) or saline was administered 15 min before the training session. (A) Exploratory preference. (B) Total exploration time. Values indicate the mean ± S.E.M. (saline/saline, $n=12$; saline/Baclofen 2 mg/kg, $n=13$; methamphetamine/saline, $n=8$; methamphetamine/Baclofen 1 mg/kg, $n=8$; methamphetamine/Baclofen 2 mg/kg, $n=10$). ANOVA: (A, training) $F(4,46)=0.242$, $P=0.9133$; (A, retention) $F(4,46)=5.56$, $P<0.01$; (B, training) $F(4,46)=7.752$, $P<0.01$; (B, retention) $F(4,46)=1.2$, $P=0.3238$. *$P<0.05$ compared with the saline/saline-treated group. #$P<0.05$ compared with the methamphetamine/saline-treated group (Student–Newman–Keuls test).

also examined the effect of gaboxadol at 10 mg/kg. However, because high-dose gaboxadol at 10 mg/kg markedly reduced the exploratory activity of mice in the training session, they were not subjected to novel object recognition test (data not shown). Gaboxadol at 3 mg/kg had no effect on the exploratory preference (Fig. 1A) and total exploratory time (Fig. 1B) in both training and retention sessions in saline-treated control mice.

Next, we examined the effect of baclofen on methamphetamine-induced cognitive impairment. The $GABA_B$ receptor agonist dose-dependently improved the reduction of exploratory preference to the novel object in methamphetamine-treated mice (Fig. 2A). Baclofen at 2 mg/kg significantly ameliorated methamphetamine-induced cognitive impairment (Fig. 2A). Baclofen had no effect on the level of exploratory preference for the novel object in the training session or the total exploration time in both the training and retention sessions in methamphetamine-treated mice. Treatment with baclofen at 2 mg/kg in saline-treated control group resulted in a significant decrease in total exploratory time to novel objects in the training session (Fig. 2B), leading to a significant impairment of novel object recognition in the retention session (Fig. 2A). This is probably due to an insufficient exploratory behaviors in the training session, which could result in a poor discrimination of a novel object.

4. Discussion

We have previously demonstrated that repeated methamphetamine treatment in mice induces enduring recognition memory impairment, which is associated with dysfunction of the dopamine D_1 receptor-ERK1/2 pathway in the prefrontal cortex. Clozapine, but not haloperidol, completely restored the cognitive impairment induced by methamphetamine treatment when repeatedly administered for 7 days after withdrawal from methamphetamine, although acute treatment with these antipsychotics had no effect (Kamei et al., 2006). The data are consistent with clinical evidence that clozapine is superior to typical neuroleptics in improving cognitive deficits in schizophrenic patients (Lee et al., 1999). Thus, we propose that methamphetamine-induced cognitive impairment in mice may be a useful model for cognitive deficits in methamphetamine abusers and schizophrenic patients. In this study, we found that acute treatment with baclofen improved methamphetamine-induced cognitive deficit without affecting motor function, whereas repeated treatment was necessary for the effect of clozapine. These results suggest that $GABA_B$ receptor agonists may be more useful for the treatment of cognitive deficit in schizophrenia patients and methamphetamine abusers than clozapine and other antipsychotic drugs. In contrast, gaboxadol, a $GABA_A$ receptor agonist, had no effect on methamphetamine-induced cognitive deficits. However, gaboxadol is known to preferentially activate the $GABA_A$ receptor subtype containing the delta subunit, which mediated tonic inhibition. Therefore, gaboxadol may not be an ideal agonist for a global activation of $GABA_A$ receptors. Further studies are required to test this assumption.

Additionally, we think that the ameliorating effect of baclofen is not related to the effect on motor function. In fact, we examined the effect of baclofen at a dose of 2 mg/kg on locomotor activity of mice that had been treated with saline or methamphetamine (1 mg/kg) for 7 days. Baclofen had no effect on behavioral locomotion of repeated methamphetamine-treated group (saline/saline group ($n=7$), 399 ± 39.9 counts/10 min; saline/baclofen group ($n=7$), 343.7 ± 51.4 counts/10 min; methamphetamine/saline group ($n=7$), 429.1 ± 21.4 counts/10 min; methamphetamine/baclofen group ($n=7$), 346.3 ± 41.6 counts/10 min; $F(3,24)=1.08$, $P=0.37$). In Figs. 1 and 2, we showed the total exploratory time, which means locomotor activity in training and retention phase, respectively. Baclofen had no effect on the level of exploratory preference for the novel object in the training session or the total exploration time in both the training and retention sessions in methamphetamine-treated mice. These results suggest that baclofen has no effect on motor function in methamphetamine-treated mice. There was an apparent difference in sensitivity to baclofen between saline-treated control and methamphetamine-treated group: Baclofen at 2 mg/kg significantly reduced the total exploratory time in the training session in control mice, while the drug had no effect in the methamphetamine-treated mice. Regarding to this phenomenon, it is reported that repeated cocaine treatment decreases baclofen-stimulated [^{35}S]GTPγS binding to G protein in the nucleus accumbens, indicating desensitization of $GABA_B$ receptors (Xi et al., 2003). Thus, it is possibly that repeated methamphetamine treatment causes desensitization of $GABA_B$ receptor as does cocaine treatment.

There are some studies suggesting that $GABA_B$ receptors play an important role in regulating dopamine neurons, while the role of $GABA_A$ receptors has been unclear. For example, previous studies showed that baclofen reduced the reinforcing effects of many substances of abuse, such as cocaine, nicotine, heroin, and alcohol (Cousins et al., 2002), possibly through $GABA_B$-mediated modulation of mesolimbic dopamine transmission (Bartholini, 1985). In fact, baclofen is known to stabilize the firing pattern of dopamine neurons (Erhardt et al., 2002). It was demonstrated that chronic coadministration of baclofen and amphetamine blocked the development of sensitization to the locomotor stimulation effect of amphetamine

(Bartoletti et al., 2005), and acute treatment with baclofen inhibited the expression of amphetamine-induced locomotor sensitization (Bartoletti et al., 2004). Moreover, a recent study showed that acute treatment with baclofen ameliorated ethanol-induced memory deficit in mice (Escher and Mittleman, 2004). Moreover, we have recently demonstrated that baclofen, but not gaboxadol, ameliorates methamphetamine- and MK-801-induced impairment of prepulse inhibition of the acoustic startle reflex in mice (Arai et al., 2008). These results support our findings that baclofen ameliorates repeated methamphetamine treatment-induced cognitive deficits. Taken together, the ameliorating effect of baclofen on cognitive impairment in methamphetamine-treated mice may be attributable to its effects on $GABA_B$ receptors in midbrain dopamine neurons.

In conclusion, we demonstrated that baclofen acutely ameliorated the cognitive deficit in repeated methamphetamine-treated mice, an animal model for cognitive deficits in methamphetamine abuse and schizophrenia. Our results suggest that baclofen may be superior to clozapine and other antipsychotic drugs that mainly affect dopamine D_2 and 5-HT_2 receptors. $GABA_B$ receptors may constitute a putative new target for treating cognitive deficits in patients suffering from schizophrenia, as well as methamphetamine psychosis. Further studies are necessary to clarify the molecular mechanisms of the action of baclofen.

Conflict of interest

There are no conflicts of interest in this study.

Acknowledgments

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The Role of Cyclophilin D in Learning and Memory

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ABSTRACT: **Cyclophilin D (Cyp D) is implicated in cell death pathway and blockade of Cyp D could be a potent therapeutic strategy for degenerative disorders such as Alzheimer's disease, ischemia, and multiple sclerosis, but physiological role of Cyp D remains elusive. Here, we investigated the ability of learning and memory in several behavioral tasks in mice that lacked Cyp D (Cyp $D^{-/-}$) and the relationship between ability of learning and memory and hippocampal architecture or neuronal transmission in Cyp $D^{-/-}$ mice. Cyp $D^{-/-}$ mice showed impairments of short-term memory in the Y-maze, object recognition memory in the novel-object recognition test, reference memory in the water maze test, and associative learning in the conditioned fear learning test. Hippocampal infusion of Cyclosporine A, which binds to Cyp D, replicated the defect in hippocampus-dependent cognition observed in Cyp $D^{-/-}$ mice. The Cyp $D^{-/-}$ mice did not show histopathological abnormalities upon Nissl staining and GFAP immunostaining or irregular expression of neuronal and glial marker proteins on Western blotting. However, release of glutamate and acetylcholine was decreased from the hippocampus in response to high-potassium treatment in the Cyp $D^{-/-}$ mice than in the wild-type mice. These results suggest a physiological role for Cyp D in learning and memory via the regulation of neurotransmission.** © 2009 Wiley-Liss, Inc.

KEY WORDS: **Cyclophilin D (Cyp D); Cyclosporine A (CsA); mitochondrial membrane permeability transition (MPT); neurotransmission; learning and memory; hippocampus**

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INTRODUCTION

Mitochondria are important in the control of both cell survival and cell death, and the mitochondrial dysfunction is implicated in neurodegenerative disorders as well as in acute brain disease (Schinzel et al., 2005; Norenberg and Rao, 2007; Du et al., 2008; Forte et al., 2008). Dysregulation of mitochondrial membrane permeability transition (MPT) leads to apoptosis or necrosis (Norenberg and Rao, 2007). MPT is a regulated Ca^{2+}-dependent increase in the permeability of the mitochondrial membrane, which results in a loss of membrane potential, mitochondrial swelling, and rupture of the outer membrane (Zoratti and Szabò, 1996; Halestrap et al., 2002). MPT is proposed to occur after the opening of a channel termed the permeability transition pore and putatively composed of the voltage-dependent anion channel (VDAC) in the mitochondrial outer membrane, the adenine nucleotide translocase (ANT) in the inner membrane, and Cyclophilin D (Cyp D) in the matrix (Crompton et al., 1998; Woodfield et al., 1998; Kokoszka et al., 2004). Although the involvement of VDAC and ANT in MPT is still controversial, experiments with Cyp D gene (*ppif*)-deficient mice indicate that Cyp D is involved in MPT, at least a cyclosporine-inhibitable form of MPT (Bairns et al., 2005; Basso et al., 2005; Nakagawa et al., 2005; Shinzel et al., 2005). Cyp D is a peptidylprolyl *cis-trans*-isomerase thought to facilitate conformational change of putative targets such as ANT to trigger MPT (Leung and Halestrap, 2008). Cyp D, encoded by peptidylprolyl *cis-trans*-isomerase (*ppif*), Cyp D-deficient cells are primarily protected from necrotic, caspase-independent cell death but not from caspase-dependent apoptosis (Baines et al., 2005; Basso et al., 2005; Nakagawa et al., 2005). Cyp D deficiency provides substantial protection from damage caused by ischemia/reperfusion to both heart and brain (Baines et al., 2005; Nakagawa et al., 2005; Schinzel et al., 2005). Recent studies reported that Cyp D deficiency protects from experimental autoimmune encephalomyelitis-induced axonal injury and motor dysfunctions as a model of multiple sclerosis (Forte et al., 2008) and amyloid-β-induced neuronal apoptosis in cultured neuron and impairments of cognitive function and plasticity in amyloid precursor protein transgenic mice as a model of Alzheimer's disease (Du et al., 2008). In

pharmacological approach, Cyclosporine A (CsA), which binds to Cyp D to inhibit MPT (Halestrap and Davidson, 1990), and its analogs (*N*-methyl-val[4]-cyclosprin and FR901459) prevent neuronal degeneration in ischemia models (Matsumoto et al., 1999; Muramatsu et al., 2007). These results suggest that Cyp D and MPT is therapeutic target for these degenerative disorders.

Although, as aforementioned, a pathological role of Cyp D and MPT has been uncovered, their physiological role remains elusive. Mitochondria assist in maintaining Ca^{2+} homeostasis by sequestering and releasing Ca^{2+} (Bernardi, 1999; Nicholls and Budd, 2000). Synaptic mitochondria are synthesized in the cell body of neurons and transported to axons and dendrites (Morris and Hollenbeck, 1993; Kang et al., 2008). Mitochondria are present at high concentrations in presynaptic terminals (Shepherd and Harris, 1998; Rowland et al., 2000; Brown, 2006). Neurotransmitter release is driven by an elevation of the Ca^{2+} concentration within the presynaptic terminal (Dodge and Rahamimoff, 1967; Long et al., 2008). Thus, some researchers have reported that mitochondria play a pivotal role in the release of neurotransmitters and short-term plasticity (Tang and Zucker, 1997; Billups and Forsythe, 2002; Lee et al., 2007; Kang et al., 2008). Under physiological conditions, Cyp D-dependent MPT has been suggested to be involved in Ca^{2+} buffering and thus to play an important role in learning and memory, and synaptic plasticity (Weeber et al., 2002; Levy et al., 2003). CsA impairs long-term potentiation (LTP) and prepulse facilitation (Levy et al., 2003), and mice lacking VDAC isoforms show deficits in spatial and associative learning and synaptic plasticity (Weeber et al., 2002). Conversely, mice lacking Cyp D shows enhanced response in avoidance tests (Luvisetto et al., 2008) and normal synaptic plasticity and spatial memory in radial water maze test (Du et al., 2008). Further behavioral experiments are needed for exploring the roles of CypD in learning and memory.

In the present study, we investigated the performance of several learning and memory tasks in mice lacking Cyp D and mice infused with CsA into the hippocampus, and found cognitive dysfunction.

MATERIALS AND METHODS

Mice

Male C57BL/6J mice (7 weeks old) were obtained from Japan SLC (Shizuoka, Japan). Mice lacking Cyp D were described by Nakagawa et al. (2005). The homozygous mutant male mice (−/−; 3 months of age) and the littermate wild-type male mice (+/+; 3 months of age) were obtained by crossing F10 heterozygous Cyp D mutant mice (±) having a 99.99% pure C57BL/6J genetic background. The genotypes of mice were determined by PCR (Fig. 1A). The wild-type allele (553 bp) was detected using 5′-GCAGATCAAGCTCCC GACTG-3′ as a forward primer and 5′-ACTTGGGAAGCC GAGGTG-3′ as a reverse primer. To detect the mutant allele (206 bp), a neomycin-specific reverse primer (5′-GCAGCG CATCGCCTTCTATC-3′) was used in combination with the wild-type forward primer as described by Nakagawa et al. (2005). The animals were housed in plastic cages and kept in a regulated environment (24 ± 1°C, 50 ± 5% humidity), with a 12-h light/dark cycle (lights on at 9:00 AM). Food and tap water were available ad libitum. All experiments were performed in accordance with the Guidelines for Animal Experiments of Meijo University. The procedures involving animals and their care were conducted in conformity with the international guidelines, Principles of Laboratory Animal Care (National Institutes of Health publication 85–23, revised 1985).

Surgery

Under anesthesia (pentobarbital 40 mg/kg, i.p.), C57BL/6J mice were placed in a stereotaxic apparatus and bilaterally implanted with a guide cannula (12 mm, 0.4 mm in inner diameter, 0.5 mm in outer diameter; Eicom) in the hippocampus (−2.2 mm anteroposterior, ±2.0 mm mediolateral from the bregma, −1.5 mm dorsoventral from the skull) according to the atlas of Paxinos and Franklin (2004). A dummy cannula (0.3 mm in diameter; Eicom) was left in place throughout the experiment. Five days after the operation, mice were subjected to the novel object recognition test or conditioned fear learning test.

Drug Treatment

CsA obtained from Novartis Pharmaceuticals (Basel, Switzerland) was dissolved in ethyl alcohol/polyethoxylated castor oil (35/65). For microinjection into the hippocampus, a 28-gauge injection cannula (Eicom) cut to extend 1.0 mm beyond the guide cannula was inserted through the guide cannula. Among the known targets of CsA, Cyp D has one of the lowest Ki values in vitro (Galat, 1993). However, one specific concern with the use of CsA to block Cyp D is the possibility of inhibition of the protein phosphatase, calcineurin. Levy et al. (2003) have reported that high doses (250 μM) of CsA inhibit calcineurin in the hippocampal slice by measuring the phosphorylation state of a calcineurin substrate, synapsin I. Considering these reports and diffusion of CsA in hippocampus, CsA was diluted with artificial cerebrospinal fluid (CSF: 147 mM NaCl, 4 mM KCl, and 2.3 mM $CaCl_2$) at a concentration of 100 μM and injected bilaterally (100 pmol/1.0 μl/side) over a 5-min period 10 min before the training session in the novel object recognition test or the conditioning session in the conditioned fear learning test.

Behavioral Analysis

A battery of behavioral experiments was carried out according to previous reports (Mouri et al., 2007a). The behavioral tests were carried out sequentially with the Y-maze test, Novel-

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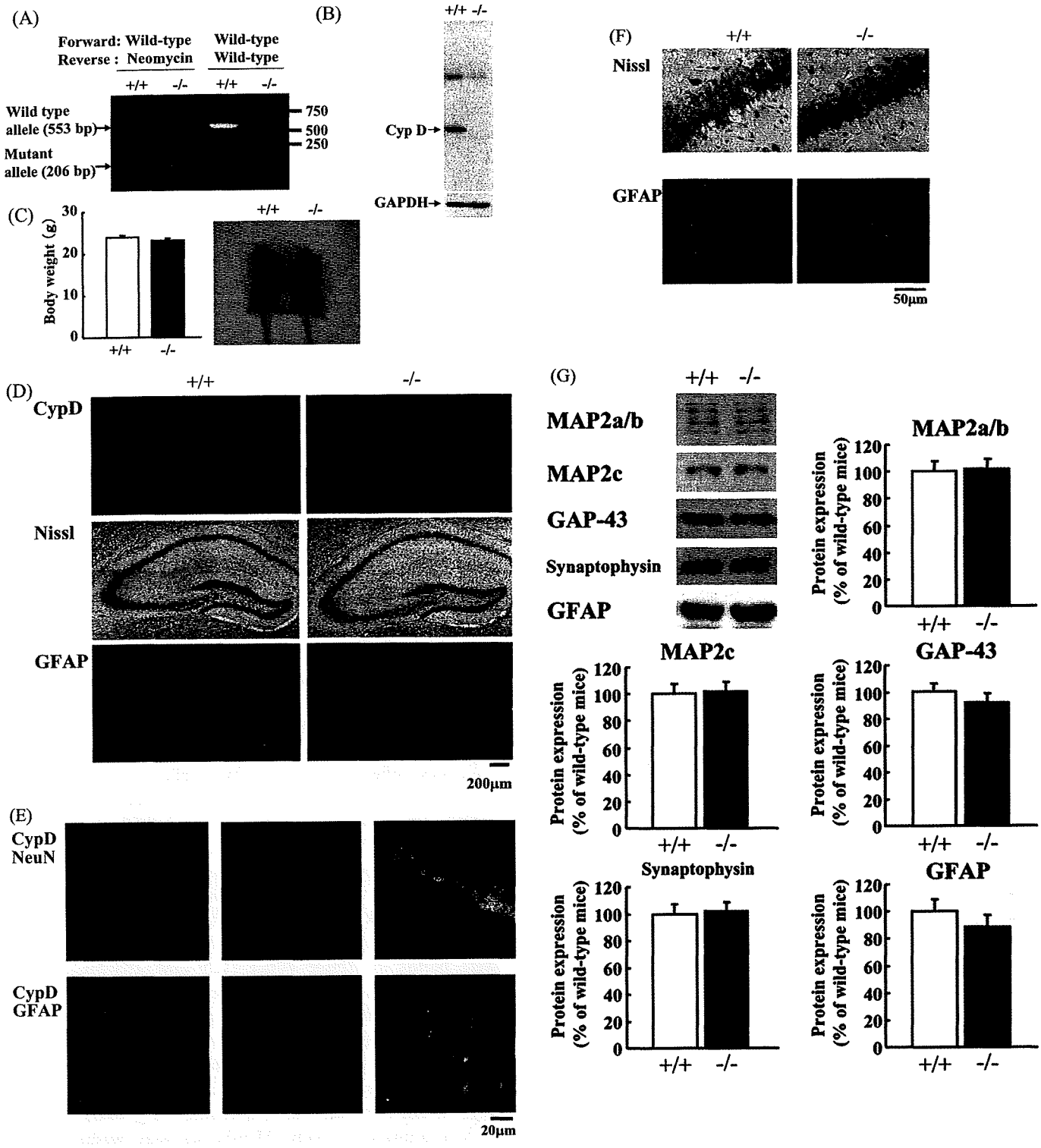

FIGURE 1. Histological characterization of Cyclophilin D and Cyclophilin D-deficient mice. (A) Determination of genotype: Genotyping was performed by PCR and gel electrophoresis. The wild-type allele (553 bp) and mutant allele (206 bp) were identified by PCR using wild-type forward and reverse primers and the wild-type forward primer and neomycin-specific reverse primer, respectively. (B) Protein expression of Cyp D in Cyp $D^{-/-}$ mice: Protein extracts from the whole brain of Cyp $D^{-/-}$ and wild type mice were examined by Western blotting. (C) Representative examples of wild-type and Cyp $D^{-/-}$ mice at 3 month: Body weights of wild-type and Cyp $D^{-/-}$ mice were 23.9 ± 0.44 and 23.0 ± 0.65 g, respectively. (D) Cyp D-, GFAP-immunostaining, and Nissl staining in the Cyp $D^{-/-}$ and wild-type mice: Slices of hippocampus obtained from Cyp $D^{-/-}$ mice showed no immunoreactivity to anti-Cyp D antibody and no abnormal structure or glial distribution. (E) Localization of Cyp D in the hippocampus: Confocal immunofluorescent images obtained from coronal sections of wild-type mice. Cyp D immunoreactivity was evident in neuronal cell layers in CA1 and localized to cells positive for NeuN, a marker of neuronal cells. Enhanced sensitivity revealed Cyp D immunoreactivity in cells positive for GFAP, a marker of astrocytes. (F) A higher magnitude image of the hippocampal CA1 region. There was no morphological change to neuronal cells and astrocytes in Cyp $D^{-/-}$ mice. (G) Western blotting of homogenates from the hippocampus for proteins described. No difference was observed between the two genotypes (wild-type mice, $n = 10$; Cyp $D^{-/-}$ mice, $n = 10$; Student t-test). +/+, wild-type mice; −/−, Cyp $D^{-/-}$ mice.

object recognition test, Morris water maze test, and Cued and contextual fear conditioning tests.

Spontaneous Alternation in a Y-Maze Test

The maze was made of black-painted wood; each arm was 40 cm long, 12 cm high, 3 cm wide at the bottom, and 10 cm wide at the top. The arms converged at an equilateral triangular central area that was 4 cm at its longest axis. Each mouse was placed at the center of the apparatus and allowed to move freely through the maze during an 8-min session. The series of arm entries was recorded visually. Alternation was defined as successive entry into the three arms, on overlapping triplet sets. The alternation behavior (%) was calculated as the ratio of actual alternations to possible alternations (defined as the number of arm entries minus two), multiplied by 100.

Novel-Object Recognition Test

The test procedure consisted of three sessions: habituation, training, and retention. Each mouse was individually habituated to the Plexiglas box (30 × 30 × 35 cm^3 high), with 10 min of exploration in the absence of objects for 3 days (habituation session). During the training session, two objects were placed in the back corner of the box. The objects were a golf ball, wooden cylinders, and square pyramids, which were different in shape and color but similar in size. A mouse was then placed midway at the front of the box and the total time spent exploring the two objects was recorded for 10 min. An animal was considered to be exploring the object when its head was facing the object or it was touching or sniffing the object. During the retention session, the animals were placed back into the same box 24 h after the training session, in which one of the familiar objects used during training was replaced with a novel object. The animals were then allowed to explore freely for 10 min and the time spent exploring each object was recorded. Throughout the experiments, the objects were used in a counterbalanced manner in terms of their physical complexity and emotional neutrality. A preference index, a ratio of the amount of time spent exploring any one of the two objects (training session) or the novel object (retention session) over the total time spent exploring both objects was used to measure cognitive function.

Morris Water Maze Test

The Morris water maze test was conducted in a circular pool 1.2 m in diameter and filled with water at a temperature of 22 ± 1°C. A hidden platform (7 cm in diameter) was used. The mice were given two trials (one block), 60 s each trial, for 10 consecutive days, during which the platform was left in the same position. The time taken to reach to the escape platform (escape latency) was determined in each trial by using the Etho Vision system (Brainscience Idea, Osaka, Japan). Three hours after the last training trial, the mice were given a probe test without the platform and were allowed 60 s to search the pool.

Cued and Contextual Fear Conditioning Tests

For measuring basal levels of freezing response (preconditioning phase), mice were individually placed in a neutral cage (17 × 27 × 12.5 cm^3 high) for 1 min and then in the conditioning cage (25 × 31 × 11 cm^3 high) for 2 min. For training (conditioning phase), mice were placed in the conditioning cage, and then a 15-s tone (80 dB) was delivered as a conditioned stimulus. During the last 5 s of the tone stimulus, a foot shock of 0.6 mA was delivered as an unconditioned stimulus through a shock generator (Brainscience Idea). This procedure was repeated four times with 15-s intervals. Cued and contextual tests were carried out 1 day after fear conditioning. For the cued test, the freezing response was measured in the neutral cage for 1 min in the presence of a continuous-tone stimulus identical to the conditioned stimulus. For the contextual test, mice were placed in the conditioning cage, and the freezing response was measured for 2 min in the absence of the conditioned stimulus.

Western Blot Analysis

Western blotting was performed as previously described (Mouri et al., 2007b). The mice were sacrificed by decapitation, and the brain was immediately removed. The hippocampus was rapidly dissected out on an ice-cold plate, frozen, and stored at −80°C until used. To prepare 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% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, 1% NP-40, 1 mM sodium orthovanadate, 20 μg/ml pepstatin, 20 μg/ml aprotinin, and 20 μg/ml leupeptin]. The homogenate was centrifuged at 13,000*g* for 20 min and the supernatant was used. The protein concentration was determined using a DC Protein Assay Kit (Bio-Rad, Richmond, CA). Samples (10–100 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 0.004% bromophenol blue), separated on a polyacrylamide gel, and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, 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, Amersham Biosciences, Piscataway, NJ) and exposed to X-ray film (Hyperfilm, Amersham Biosciences). 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, membranes were stripped with stripping buffer [100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris–HCl (pH 6.7)] at 50°C

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for 30 min, and GAPDH protein expression was detected as described above.

The primary antibodies were a rabbit anti-CyP D [1:1,000; synthesized by the authors using a peptide (aa 43–57) of Cyp D], a mouse anti-MAP2 (1:1,000; Chemicon, Temecula, CA), a rabbit antigrowth associated protein (GAP)-43 (1:1,000; Chemicon), a mouse antiglial fibrillary acidic protein (GFAP) (1:1,000; Chemicon), a rabbit antisynaptophysin (1:1,000; Dako, Glostrup, Denmark), a guinea pig antiglutamate transporter GLAST and a guinea pig anti-GLT-1 (1:1,000; Chemicon), a mouse antiglutaminase (GLS) (1:500; Abnova, Taipei, Taiwan), a mouse anti-NR1 CT (1:1,000; Upstate Biotechnology, Lake Placid, NY), a mouse anti-NMDAR2A and a mouse anti-NMDAR2B (1:1,000; BD Pharmingen, San Diego, CA), a rat anti-ChAT (Calbiochem, San Diego, CA), and a goat anti-ACHE (E-19) (1:500; Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibodies, used at a dilution of 1:2,000, were horseradish peroxidase-linked antimouse, antirabbit, antirat, or antiguinea pig IgG (Kirkegaard and Perry Laboratories).

Preparation of Brain Slice and Staining

Histological procedures were performed as previously described with a minor modification (Murai et al., 2007). Mice were anesthetized with chloral hydrate (150 mg/kg i.p.) and perfused transcardially with ice-cold phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in PBS. The brains were removed, postfixed in the same fixative for 2 h, and then soaked in 20% (w/v) sucrose in PBS. Coronal sections 15 μm thick were cut with a Cryostar HM560 cryostat (Microm International, GmbH, Walldorf, Germany). For immunohistochemistry, the primary antibodies that were applied in the brain slices included a rabbit anti-Cyp D (1:500; synthesized by the authors), a mouse antineuron-specific nuclear antigen (NeuN) (1:500; Chemicon) and mouse anti-GFAP (1:500; Chemicon) antibody. Fluorescently conjugated secondary antibodies (Alexa 488, 546, Invitrogen, Carlsbad, CA) were used for detecting chromagen. For Nissl staining, sections were cut at 40-μm intervals and staining was done according to standard procedures (Murai et al., 2007). Images were acquired with a confocal microscope (LSM510; Carl Zeiss, Jena, Germany) and a light microscope (Axiocam HRc; Carl Zeiss).

In Vivo Microdialysis

In vivo microdialysis was performed as previously described (Mouri et al., 2007b; Murai et al., 2007). 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 ventral hippocampus (−2.8 mm anteroposterior, ±3.0 mm mediolateral from the bregma, −2.0 mm dorsoventral from the skull). One day after the operation, a dialysis probe (AI-4–2; 2-mm membrane length, Eicom) was inserted through the guide cannula and perfused with CSF (147 mM NaCl, 4 mM KCl, and 2.3 mM $CaCl_2$) at a flow rate of 1 μl/min. The dialysate was colleted every 20 min. Dialysates were assayed by HPLC with electrochemical detection (HTEC-500, Eicom) under the following conditions. Three samples were taken to establish baseline levels of extracellular neurotransmitter. For depolarization stimulation, 50 mM KCl-containing Ringer solution was delivered through the dialysis probe for 20 min to induce the K^+-evoked release of glutamate and acetylcholine (ACh). Then dialysate was collected for 20 min with ringer solution.

Statistic Analysis

All results were expressed as the mean ± SEM for each group. The difference between groups was analyzed with a one-way, two-way, or repeated ANOVA, followed by the Bonferroni/Dunn multiple range-test. The Student *t*-test was used to compare two sets of data.

RESULTS

General Characteristics of Cyp $D^{-/-}$ Mice

The genotype for the Cyp D locus was assessed by PCR (Fig. 1A). Cyp $D^{-/-}$ mice were confirmed to lack Cyp D protein by Western blotting (Fig. 1B). Cyp $D^{-/-}$ mice were healthy and showed no changes in physical characteristics (body weight, or appearance of fur and whiskers) at 3 months (Fig. 1C). Although Luvisetto et al. (2008) have recently reported that Cyp $D^{-/-}$ mice show adult onset obesity, our mice did not show gross changes in physical characteristics, including body weight, with age (data not shown).

Histological Appearance of the Hippocampus in Cyp $D^{-/-}$ Mice

Strong immunoreactivity for Cyp D was observed in the granule cell layer and pyramidal cell layer in the hippocampus (Fig. 1D). In higher resolution image of CA1 regions, Cyp D immunoreactivity was localized in NeuN positive neuronal cells (Fig. 1E). With enhanced sensitivity of microscope, Cyp D immunoreactivity was also observed in astrocytes, though sparsely (Fig. 1E). No Cyp D protein was pressed in the brains of Cyp $D^{-/-}$ mice as confirmed by immunofluorescent staining (Fig. 1D). Nissl staining and immunostaining for the astrocyte marker GFAP showed neither gross structural abnormalities (Fig. 1D) nor any morphological abnormality of neuronal cells and astrocytes (Fig. 1F). The expression levels of the dendritic marker MAP2, neuronal growth cone marker GAP-43, presynaptic marker synaptophysin, and GFAP remained unchanged in hippocampal homogenates from Cyp $D^{-/-}$ mice as compared

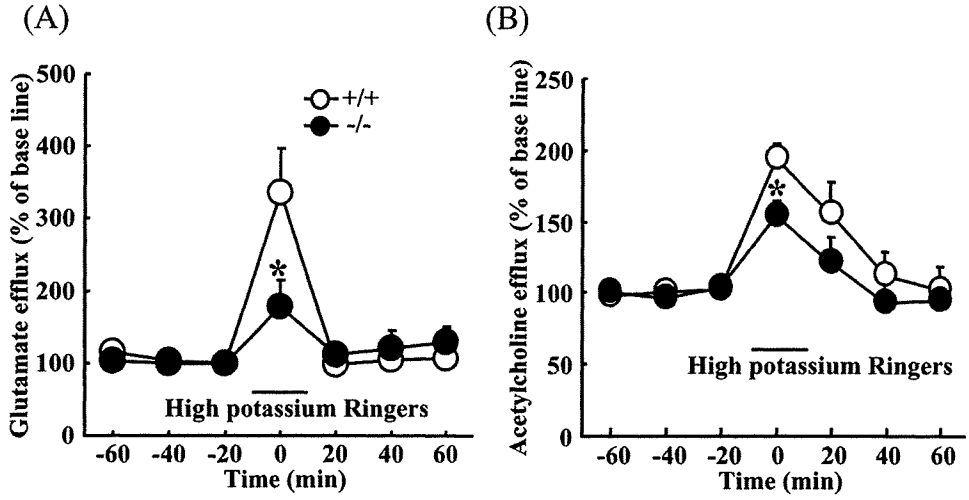

FIGURE 2. Deficiency of Cyclophilin D inhibits the release of glutamate and acetylcholine in the hippocampus. High potassium-evoked release of glutamate (A) and acetylcholine (B) from the hippocampus in the Cyp $D^{-/-}$ and wild type mice; High potassium-induced release of glutamate from the hippocampus was measured in the Cyp $D^{-/-}$ and wild-type mice. Values correspond to mean ± SEM (wild-type mice, $n = 6$; Cyp $D^{-/-}$ mice, $n = 7$). Results with repeated ANOVA were as follows: time, $F(3,33) = 11.36$, $P < 0.01$; Cyp D deficiency, $F(1,33) = 2.28$, $P = 0.16$; interaction time with Cyp D deficiency, $F(3,33) = 4.48$, $P < 0.01$. High potassium-induced release of acetylcholine from the hippocampus was measured in the Cyp $D^{-/-}$ mice. Values correspond to mean ± SEM (wild-type mice, $n = 10$; Cyp $D^{-/-}$ mice; $n = 11$). Results with repeated ANOVA were as follows: time, $F(3,57) = 11.54$, $P < 0.01$; Cyp D deficiency, $F(1,57) = 2.16$, $P = 0.16$; interaction of time with Cyp D deficiency, $F(3,57) = 0.45$, $P = 0.71$, $^*P < 0.05$ versus wild-type mice. +/+, wild-type mice; −/−, Cyp $D^{-/-}$ mice.

with those from wild-type mice (Fig. 1G; $n = 10$ per group; Student t-test).

Deficiency of Cyclophilin D Inhibits the Release of Glutamate and ACh in the Hippocampus

The release of neurotransmitters in the hippocampus plays an important role in learning and memory (Stefani and Gold, 2001; Mereu et al., 2003). Therefore, changes in the amounts of glutamate and ACh released in the hippocampus were investigated by microdialysis in the Cyp $D^{-/-}$ mice. The basal levels of glutamate in the hippocampus of the wild-type and Cyp $D^{-/-}$ mice were 0.55 ± 0.13 and 0.46 ± 0.21 pmol/10 µl/10 min, respectively (mean ± SEM; n = 6–7 per group). The amount of glutamate released in response to high potassium (50 mM) in the hippocampus was significantly lower in the Cyp $D^{-/-}$ mice than in the wild-type mice (Fig. 2A; repeated ANOVA, post hoc Bonferroni/Dunn multiple range-test, $P < 0.05$). The basal levels of ACh in the hippocampus of the wild-type and Cyp $D^{-/-}$ mice were 0.15 ± 0.04 and 0.17 ± 0.03 nmol/10 µl/10 min, respectively (mean ± SEM; n = 10–11 per group). The amount of ACh released in response to high potassium (50 mM) in the hippocampus was significantly lower in the Cyp $D^{-/-}$ mice than in the wild-type mice (Fig. 2B; $P < 0.05$). These results indicate that a deficiency of Cyp D results in inhibition of the potassium-induced release of glutamate and ACh in the hippocampus.

No Influence of the Cyclophilin D Deficiency on Glutamatergic and Cholinergic Nervous System-Related Protein Expression in the Hippocampus

As demonstrated above, Cyp $D^{-/-}$ mice showed a hypoglutamatergic and hypocholinergic response in the hippocampus in the presence of high potassium. It was possible that the deficiency in Cyp D affected the expression of these neuronal system-related proteins such as receptors, synthetases, degradation enzymes, and transporters. To test this possibility, the expression of NMDA receptor subunits, glutaminase (GLS), glutamate transporters, choline acetyltransferase (ChAT), and acetyltransferase (AChE), was analyzed by Western blotting. There was no significant difference in NR1, NR2A, NR2B, GLS, GLAST, GLT-1, ChAT, and AChE protein levels in the hippocampus between wild-type and Cyp $D^{-/-}$ mice (Fig. 3; $n = 10$ per group; Student t-test). These data show that the hypoglutamatergic and hypocholinergic responses observed in Cyp $D^{-/-}$ mice were not due to changes in expression levels of proteins involved in these neurotransmitters response.

Impairment by Cyclophilin D Deficiency of Learning and Memory

Spontaneous alternation in the Y-maze test

We evaluated short-term memory using a Y-maze test. There was no significant difference in the number of arm entries between the two groups (Fig. 4A; n = 16–17 per group; Student

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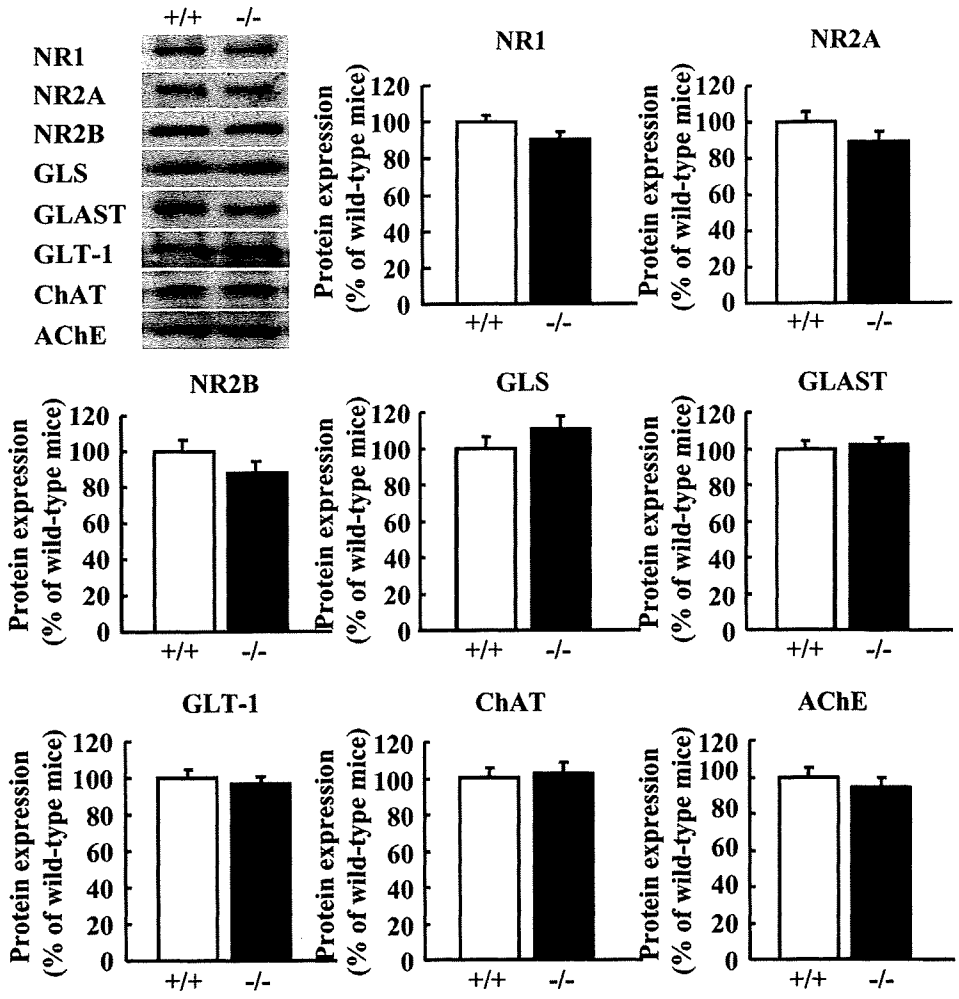

FIGURE 3. No influence of Cyclophilin D deficiency on glutamatergic nervous system-related protein expression in the hippocampus. Western blot analysis of homogenates from the hippocampus of the Cyp $D^{-/-}$ and wild-type mice. No difference was observed between the two genotypes (wild-type mice, $n = 10$; Cyp $D^{-/-}$ mice, $n = 10$; Student t-test). NR1, NMDA receptor 1 subunit; NR2A, NMDA receptor 2A subunit; NR2B, NMDA receptor 2B subunit; GLS, glutaminase; GLAST, glutamate-aspartate transporter; GLT-1, glial glutamate transporter-1; ChAT, choline acetyltrasferase; AChE, acetylcholinesterase; +/+, wild-type mice; −/−, Cyp $D^{-/-}$ mice.

t-test), suggesting that all mice have the same levels of motivation, curiosity, and motor function. However, Cyp $D^{-/-}$ mice showed significantly reduced spontaneous alternation behavior in the Y-maze compared with wild-type mice (Fig. 4B; $P < 0.05$), indicating an impairment of short-term memory.

Object recognition in the novel-object recognition test

We evaluated the visual recognition memory of Cyp $D^{-/-}$ mice using the novel-object recognition test. During the training session, there were no significant differences in exploratory preference between the two objects (Fig. 5A; $n = 16$–17 per group; repeated ANOVA, post hoc Bonferroni/Dunn multiple range-test) and the total time spent exploring both objects between the two groups (Fig. 5B), suggesting that all mice have

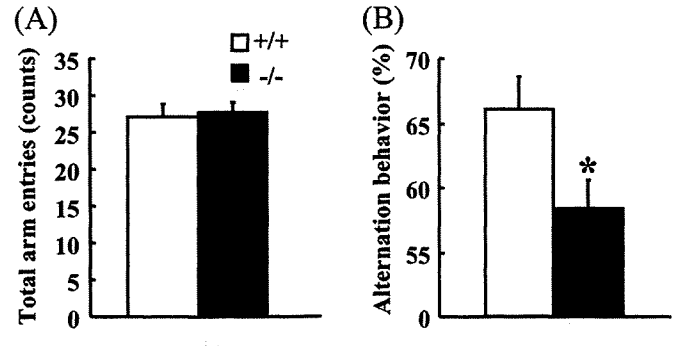

FIGURE 4. Impairment of short-term memory by Cyclophylin D deficiency in the Y-maze test. (A) Total arm entries; (B) Alternation behavior. Percent alternation during an 8-min session in the Y-maze test was measured. Values indicate mean ± SEM (wild-type mice, $n = 17$; Cyp $D^{-/-}$ mice; $n = 16$). *$P < 0.05$ versus wild-type mice (Student t-test). +/+, wild-type mice; −/−, Cyp $D^{-/-}$ mice.

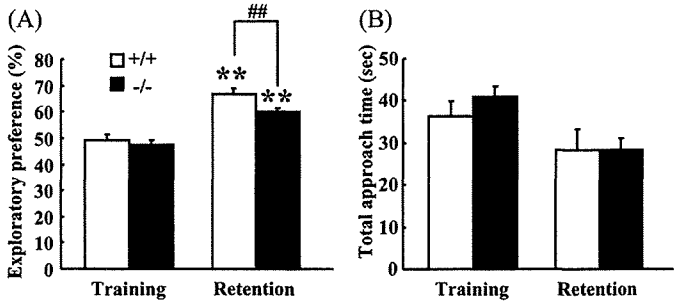

FIGURE 5. Impairment of object recognition memory by Cyclophylin D deficiency in the novel-object recognition test. (A) Exploratory preference; (B) Total approach time. The retention session was carried out 24 h after the training. Exploratory preference during a 10-min session in the novel-object recognition test was measured. Values indicate mean ± SEM (wild-type mice, n = 17; Cyp D$^{-/-}$ mice; n = 16). Results with the repeated ANOVA were as follows; exploratory preference: training/retention, $F(1,31)$ = 122.53, $P < 0.01$; Cyp D deficiency, $F(1,31)$ = 9.52, $P < 0.01$; interaction of training/retention with Cyp D deficiency, $F(1,31)$ = 2.22, $P = 0.14$; total approach time: training/retention, $F(1,31)$ = 28.32, $P < 0.01$; Cyp D deficiency, $F(1,31)$ = 0.62, $P = 0.44$; interaction of training/retention with Cyp D deficiency, $F(1,31)$ = 1.10, $P = 0.30$, $^{**}P < 0.01$ versus training. $^{\#\#}P < 0.01$ versus trained, wild-type mice. +/+, wild-type mice; −/−, Cyp D$^{-/-}$ mice.

the same levels of motivation, curiosity, and interest in exploring novel objects.

For the retention session, both groups of mice took longer time to explore the novel object than the familiar object (Fig. 5A, $P < 0.01$). However, the level of exploratory preference for the novel objects was significantly decreased in Cyp D$^{-/-}$ mice compared to wild-type mice (Fig. 5A, $P < 0.01$), indicating an impairment of visual recognition memory.

Reference memory in the Morris water maze test

We evaluated reference memory using the Morris water maze test. Both groups of mice managed to learn the position of the hidden platform (Fig. 6A). However, Cyp D$^{-/-}$ mice took significantly longer time and distance to reach the platform than wild-type mice (Fig. 6A; n = 16–17 per group; repeated ANOVA, post hoc Bonferroni/Dunn multiple range-test; $P < 0.01$), indicating an impairment of reference memory. When the probe test was carried out following the tenth block of training, wild-type mice searched preferentially in the trained quadrant (Fig. 6B; repeated ANOVA, post hoc Bonferroni/Dunn multiple range-test; $P < 0.01$), but Cyp D$^{-/-}$ mice did not. The decreased ability did not reflect a loss of swimming ability and motivation, because swimming speed and distance in the probe test were similar to those in wild-type mice (swimming speed; wild-type mice: 19.14 ± 0.78 cm/s, Cyp D$^{-/-}$ mice: 16.12 ± 1.33 cm/s, swimming distance; wild-type mice: 1,141 ± 47 cm, Cyp D$^{-/-}$ mice: 962 ± 80 cm).

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Associative learning in the cued and contextual fear conditioning tests

We evaluated associative learning in the conditioned fear learning test. In the preconditioning phase, the mice of both groups hardly showed any freezing response, and there were no differences in basal levels of the freezing response between the groups (Figs. 7A,B; n = 16–17 per group; repeated ANOVA, post hoc Bonferroni/Dunn multiple range-test). In

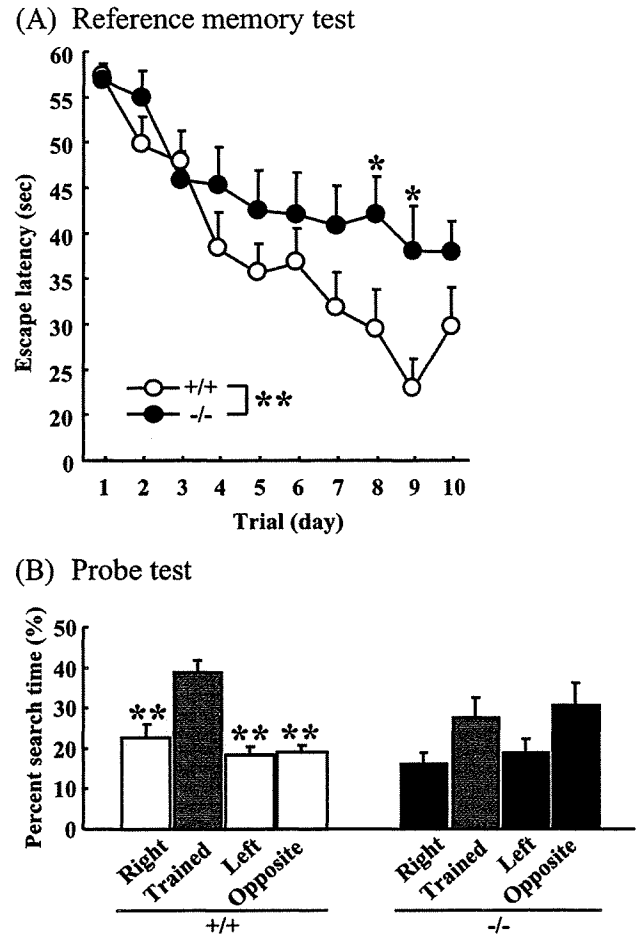

FIGURE 6. Impairment of reference memory by Cyclophylin D deficiency in the Morris water maze test. (A) Reference memory test. Escape latency during a 60-s session in the water maze test was measured. Values indicate mean ± SEM (wild-type mice, n = 17; Cyp D$^{-/-}$ mice; n = 16). Results with the repeated ANOVA were as follows: trial, $F(9,279)$ = 11.96, $P < 0.01$; animal group, $F(1,31)$ = 7.27, $P < 0.05$; interaction of trial with animal group, $F(9,279)$ = 1.13, $P = 0.34$. $^{**}P < 0.01$, $^{*}P < 0.05$ versus wild-type mice. (B) Probe test. The probe test was performed after training on day 10 in the Morris water maze test. Percent search time during a 60-s session in the water maze test was measured. Values indicate mean ± SEM (wild-type mice, n = 17; Cyp D$^{-/-}$ mice; n = 16). Results with the repeated ANOVA were as follows: quadrant, $F(3,93)$ = 5.57, $P < 0.01$; Cyp D deficiency, $F(1,31)$ = 0.01, $P = 0.91$; interaction of quadrant with Cyp D deficiency, $F(3,93)$ = 2.95, $P < 0.05$. $^{**}P < 0.01$ versus trained quadrant. +/+, wild-type mice; −/−, Cyp D$^{-/-}$ mice.

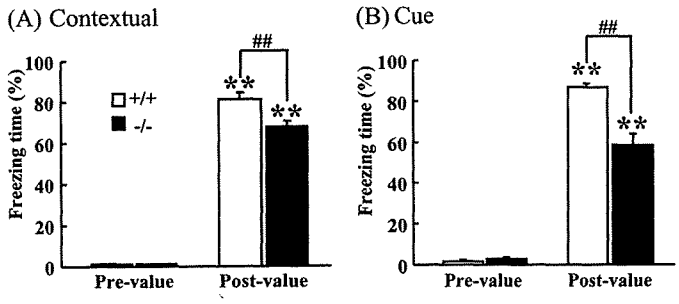

FIGURE 7. **Impairment of associative learning by Cyclophylin D deficiency in the conditioned fear learning test. The test session was carried out 24 h after the conditioning. Context-dependent (A) and Cue-dependent (B) freezing times were measured. Values indicate mean ± SEM (wild-type mice, n = 17; Cyp D$^{-/-}$ mice; n = 16). Results with the repeated ANOVA were as follows; context-dependent test: conditioning, $F(1,31)$ = 1,127.89, $P < 0.01$; Cyp D deficiency, $F(1,31)$ = 9.12, $P < 0.01$; interaction of conditioning with Cyp D deficiency, $F(1,31)$ = 9.12, $P < 0.01$; cue-dependent test: conditioning, $F(1,31)$ = 703.45, $P < 0.01$; Cyp D deficiency, $F(1,31)$ = 17.14, $P < 0.01$; interaction of conditioning with Cyp D deficiency, $F(1,31)$ = 29.96, $P < 0.01$, $^{**}P < 0.01$ versus pre-conditioning. $^{\#\#}P < 0.01$ versus conditioned, wild-type mice. +/+, wild-type mice; −/−, Cyp D$^{-/-}$ mice.**

the contextual learning test, both groups showed a marked contextual freezing response 24 h after fear conditioning (Fig. 7A; $P < 0.01$). However, Cyp D$^{-/-}$ mice exhibited less freezing response in the contextual tests (Fig. 7A; $P < 0.01$), indicating an impairment of associative learning. In the cued learning test, Cyp D$^{-/-}$ mice exhibited less freezing (Fig. 7B; $P < 0.01$), indicating an impairment of associative learning. Furthermore, no aberrant nociceptive responses to electric footshocks were observed in the Cyp D$^{-/-}$ mice: the footshock thresholds in the Cyp D$^{-/-}$ mice (flinching, 0.28 ± 0.02 mA; vocalizing, 0.62 ± 0.05 mA; jumping, 0.78 ± 0.08 mA) were the same as those in wild-type mice (flinching, 0.22 ± 0.03 mA; vocalizing, 0.77 ± 0.05 mA; jumping, 0.71 ± 0.11 mA).

Recapitulation of Cyclophilin D Deficiency by Infusion of Cyclosporine A Into the Hippocampus

To examine the role of hippocampal Cyp D in learning and memory, we microinjected CsA, an inhibitor of Cyp D, into the hippocampus, and evaluated its effect on performance in the novel-object recognition test and conditioned fear learning test. In the novel-object recognition test, the mice were microinjected with CsA (100 pmol/mouse/unilateral) 10 min before the training trial. During the training session, there were no significant differences in exploratory preference between the two objects and total exploratory time between the two groups (Figs. 8A,B; n = 8–9 per group; repeated ANOVA, post hoc Bonferroni/Dunn multiple range-test). However, the level of exploratory preference for the novel objects in mice treated with CsA was significantly decreased compared to that in mice treated with vehicle (Fig. 8A, $P < 0.05$), indicating a role for hippocampal Cyp D in this form of learning and memory. In the conditioned fear learning test, CsA was microinjected into the hippocampus 10 min before the conditioning trial. The mice treated with CsA exhibited less freezing response 24 h after fear conditioning in the contextual tests, which is known to be hippocampus-dependent (Fig. 8C; n = 16–17 per group; Student t-test; $P < 0.01$). But there was no difference in the cued freezing response 24 h after fear conditioning among the groups in the cued learning test, which is known to be hippocampus-independent (Fig. 8D). These results indicate that hippocampal Cyp D plays a role in the hippocampal-dependent form of learning and memory.

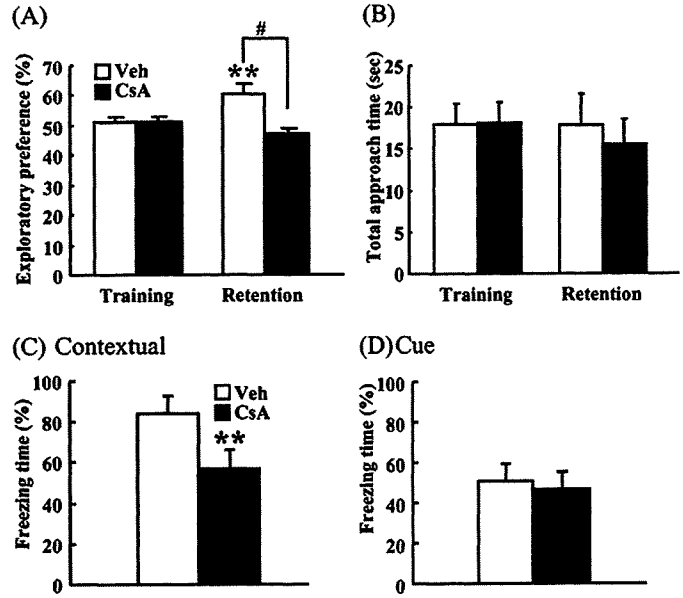

FIGURE 8. **Recapitulation of Cyclophilin D deficiency by infusion of CsA into the hippocampus. Novel object recognition test: (A) Exploratory preference; (B) Total approach time. The retention session was carried out 24 h after the training. CsA (100 pmol/mouse/unilateral) was infused into the hippocampus 10 min before the training trial. Exploratory preference during a 10-min session in the novel-object recognition test was measured. Values indicate mean ± SEM (vehicle-treated mice, n = 9; CsA-treated mice, n = 8). Results with the repeated ANOVA were as follows; exploratory preference: training/retention, $F(1,15)$ = 2.67, P = 0.12; treatment, $F(1,15)$ = 7.17, $P < 0.05$; interaction training/retention with treatment, $F(1,15)$ = 12.83, $P < 0.05$; total approach time: training/retention, $F(1,15)$ = 0.12, P = 0.64; treatment, $F(1,15)$ = 0.06, P = 0.81; interaction training/retention with treatment, $F(1,15)$ = 0.89, P = 0.36. $^{**}P < 0.01$ versus training. $^{\#}P < 0.05$ versus trained, vehicle-treated mice. Conditioned fear learning test: The test session was carried out 24 h after the conditioning. Context-dependent (C) and cue-dependent (D) freezing times were measured. Values indicate mean ± SEM (vehicle-treated mice, n = 10; Cyclosporine A-treated mice, n = 10). $^{**}P < 0.01$ versus vehicle-treated mice (Student t-test). Veh, vehicle-treated mice; CsA, Cyclosporine A-treated mice.**

DISCUSSION

In this study, we analyzed mice with a deficiency of Cyp D to define its role in cognitive functions. Our behavioral data showed that Cyp $D^{-/-}$ mice have subtle but significant impairments of short-term memory in the Y-maze test, visual recognition memory in the novel-object recognition test, reference memory in the water maze test, and associative learning in the conditioned fear learning test. It is unlikely that the impaired performance of Cyp $D^{-/-}$ mice in learning and memory tests is due to changes in motivation or sensorimotor function, since the motivation for each of these behavioral tests is different, and different skills are required for a good performance in each test. Actually, there were no differences in total arm entries in the Y-maze test, total time spent exploring objects in the novel object test, swimming speed in the Morris water maze test, and freezing response in the preconditioning phase and nociceptive response between the wild-type and Cyp $D^{-/-}$ mice.

Cyp D immunoreactivity is abundant in neuronal layers but sparse in astrocytes in the adult mouse brain. Naga et al. (2007) also showed that Cyp D is present at high levels in neurons and low levels in astrocytes in adult rat brain using immunohistochemistry and in primary rat neuron and astrocyte cultures using Western blotting. Although it is possible that Cyp D deficiency-induced developmental abnormalities in the neuronal and astrocytic architecture lead to cognitive dysfunctions, we did not observe histopathological abnormalities on Nissl staining and GFAP immunostaining or the irregular expression of neuronal (MAP2a-c, GAP-43, and synaptophysin) and glial (GFAP) marker proteins on Western blotting. Hippocampus as well as perirhinal and prefrontal cortex is crucial for recognition memory in the novel-object recognition test (Rampon et al., 2000; Winters and Bussey, 2005; Nagai et al., 2007). Reference memory in the Morris water maze test (Morris et al., 1982) and associative learning in the contextual, but not cued conditioned fear learning test (Phillips and LeDoux, 1992), are dependent on the hippocampus. Hippocampal infusion of CsA, an inhibitor of Cyp D (Halestrap and Davidson, 1990), replicated the hippocampus-dependent behavioral cognitive dysfunctions (impairments of recognition memory in the novel-object recognition test and of associative learning in the contextual test) observed in Cyp $D^{-/-}$ mice, except for the impairment of associative learning in the cued conditioned fear learning test, which is known to depend on the amygdala (Phillips and LeDoux, 1992). Taken together, these results strongly indicate that the role of Cyp D in cognitive functions is functional rather than developmental in nature.

Mitochondrial calcium buffering is an important regulator of synaptic function (Tang and Zucker, 1997; Billups and Forsythe, 2002). Recent studies have suggested that Cyp D-regulated MPT plays an important role in mitochondrial synaptic Ca^{2+} buffering, hippocampal synaptic plasticity, and learning and memory (Weeber et al., 2002; Levy et al., 2003; Naga et al., 2007). Mitochondria from synaptosomes, isolated from rat cerebral cortex, have less Ca^{2+} buffering ability than the nonsynaptic pool of mitochondria (Brown et al., 2006). This difference reflects the higher levels of Cyp D in synaptic than nonsynaptic mitochondria (Naga et al., 2007). The application of CsA, a deficiency of Cyp D, and a deficiency of VDAC all increase Ca^{2+} uptake capacity in isolated mitochondria (Levy et al., 2003; Naga et al., 2007). Interestingly, the application of CsA and a deficiency of VDAC impair paired-pulse facilitation and LTP (Weeber et al., 2002; Levy et al., 2003). The phenomena of paired pulse facilitation are generally accepted as a model of the presynaptic component of synaptic plasticity (Gottschalk et al., 1998). These results suggest that the cognitive dysfunction and impaired neurotransmission observed in Cyp $D^{-/-}$ mice is ascribable to deficiency of Cyp D-dependent MPT.

Excitatory transmitters such as ACh and glutamate change neural information processing by regulating the release of synaptic transmitters and modifying long-term synaptic plasticity (Giocomo and Hasselmo, 2007). In addition, the release of glutamate and ACh in hippocampus is related to cognitive performance in behavioral tests (Stefani and Gold, 2001; Mereu et al., 2003). In the present study, Cyp $D^{-/-}$ mice had lower extracellular glutamate and ACh levels in response to high potassium in the hippocampus than did the wild-type mice. Previously, a decrease in spontaneous extracellular glutamate release and increase in levels of the glutamate transporter GLAST were observed in schizophrenic animal models, which show impairments of memory (Mouri et al., 2007b; Murai et al., 2007). In present study, there was no difference in protein expression of neurotransmitter synthesis, metabolism and uptake in the glutamatergic and cholinergic neuronal system in hippocampus between wild-type and Cyp $D^{-/-}$ mice. Thus, it is unlikely that deficiency of Cyp D decrease glutamate and ACh response by modulation of these protein expressions of GLS, GLAST or GLT-1 between wild-type and Cyp $D^{-/-}$ mice. Although we have no detailed data about neurotransmitter levels in response to other potassium concentration, activities of neurotransmitters enzymes, and transporters in Cyp $D^{-/-}$ and hippocampal CsA-infused mice, our results along with other recent findings suggest that Cyp D and MPT play important roles in synaptic transmission.

Luvisetto et al. (2008) have reported that the Cyp $D^{-/-}$ mice generated by Basso et al. (2005) show adult onset obesity, increased anxiety/emotionality in the open field test and elevated plus maze test, and a facilitation of learning in the active and passive avoidance test at 10 months. As far as the obesity is concerned, we did not observe a significant difference in the body weight of Cyp $D^{-/-}$ mice up to 40 months, as compared with control littermates. We do not know the reason for this difference. Although our results are consistent with some of the results described by Luvisetto et al. (2008), notably that Cyp $D^{-/-}$ mice exhibited increased anxiety/emotionality in the elevated plus maze test (unpublished data), we did not observe any facilitation of learning and memory in our Cyp $D^{-/-}$ mice. It is conceivable that the avoidance behavior of Cyp $D^{-/-}$ mice is due to greater anxiety rather than greater learning ability. Du et al. (2008) have reported that the Cyp $D^{-/-}$ mice generated by Bains et al. (2005) show normal synaptic

plasticity and spatial memory in radial water maze test. These differences between Du's and our data might be due to different behavioral test, because the CypD$^{-/-}$ mice shows normal but slight increase of error in the behavior test at 6 months and decrease of long-term potentiation at 12–13 months (Du et al., 2008). More extensive investigation will be necessary to clarify these differences.

In summary, mice lacking Cyp D display cognitive dysfunction probably caused by the hypofunction of neurotransmission without developmental abnormalities. In pathological process, blockade of Cyp D could be a potent therapeutic strategy for degenerative disorders such as Alzheimer's disease, ischemia, and multiple sclerosis. It is possible that blockade of Cyp D impairs rather than facilitates cognitive function in normal condition. Our findings could contribute understanding not only the physiological roles of Cyp D in cognition but also appropriate use of Cyp D blocker for degenerative disorders.

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ARTICLE

CINP

Galantamine ameliorates the impairment of recognition memory in mice repeatedly treated with methamphetamine: involvement of allosteric potentiation of nicotinic acetylcholine receptors and dopaminergic-ERK1/2 systems

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Abstract

Galantamine, a drug used to treat Alzheimer's disease, inhibits acetylcholinesterase (AChE) and allosterically modulates nicotinic acetylcholine receptors (nAChRs) resulting in stimulation of catecholamine neurotransmission. In this study, we investigated whether galantamine exerts cognitive-improving effects through the allosteric modulation of nAChRs in an animal model of methamphetamine (Meth) psychosis. The mice treated with Meth (1 mg/kg.d) for 7 d showed memory impairment in a novel object recognition test. Galantamine (3 mg/kg) ameliorated the memory impairment, and it increased the extracellular dopamine release in the prefrontal cortex (PFC) of Meth-treated mice. Donepezil, an AChE inhibitor (1 mg/kg) increased the extracellular ACh release in the PFC, whereas it had no effect on the memory impairment in Meth-treated mice. The nAChR antagonist, mecamylamine, and dopamine D_1 receptor antagonist, SCH 23390, blocked the ameliorating effect of galantamine on Meth-induced memory impairment, whereas the muscarinic AChR antagonist, scopolamine, had no effect. The effects of galantamine on extracellular dopamine release were also antagonized by mecamylamine. Galantamine attenuated the defect of the novelty-induced activation of extracellular signal-regulated kinase 1/2 (ERK1/2). The ameliorating effect of galantamine on recognition memory in Meth-treated mice was negated by microinjection of an ERK inhibitor, PD98059, into the PFC. These results suggest that the ameliorating effect of galantamine on Meth-induced memory impairment is associated with indirect activation of dopamine D_1 receptor-ERK1/2 following augmentation with dopaminergic neurotransmission in the PFC through the allosteric activation of nAChRs. Galantamine could be a useful therapeutic agent for treating cognitive deficits in schizophrenia/Meth psychosis, as well as Alzheimer's disease.

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Key words: Allosteric potentiation of nicotinic acetylcholine receptors, cognitive impairment, dopamine, extracellular signal-regulated kinase 1/2, galantamine, methamphetamine.

Introduction

Galantamine, a potent allosteric potentiating ligand (APL) and a drug approved for treatment of Alzheimer's disease, has a dual mechanism of action; it inhibits AChE and allosterically modulates nicotinic acetylcholine receptors (nAChRs) (Eisele *et al.* 1993; Santos *et al.* 2002). We have found that galantamine has ameliorating effects on the impairment of performance in the novel object recognition (NOR) and/or conditioned fear learning tasks caused by a single intracerebroventricular infusion of amyloid-β peptide

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(Aβ) fragment (as an animal model of Alzheimer's disease) (Wang *et al.* 2007*a*) and by repeated treatment with a non-competitive *N*-methyl-D-asparatate receptor antagonist, phencyclidine (PCP) (as an animal model of schizophrenia) (Wang *et al.* 2007*b*). It increases the extracellular dopamine release in the hippocampus and prefrontal cortex (PFC) of Aβ_{25-35}-infused and PCP-treated mice, respectively. The ameliorating effects of galantamine on Aβ_{25-35}- and PCP-induced cognitive impairment are mediated through the augmentation of dopaminergic neurotransmission following activation of nAChRs (Wang *et al.* 2007*a*,*b*). These studies provide the *in-vivo* evidence that galantamine augments dopaminergic neurotransmission in the hippocampus/PFC through the allosteric activation of nAChRs. Thus, galantamine shows potential as a novel therapeutic agent for cognitive impairments associated with schizophrenia, as well as Alzheimer's disease, although the molecular mechanism of action remains to be determined in detail.

Methamphetamine (Meth) is a highly addictive drug of abuse, and addiction to Meth has increased to epidemic proportions worldwide (Cretzmeyer *et al.* 2003; Rawson *et al.* 2002). Chronic Meth users show psychotic signs such as hallucinations and delusions, which are indistinguishable from paranoid schizophrenia (Sato *et al.* 1983; Srisurapanont *et al.* 2003; Yui *et al.* 2002). Recent studies have suggested that chronic use of Meth causes long-term cognitive deficits (Kalechstein *et al.* 2003; Nordahl *et al.* 2003; Simon *et al.* 2000). We have found that repeated Meth treatment in mice impairs long-term recognition memory after withdrawal, which is associated with the dysfunction of the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway in the PFC, and that Meth-induced cognitive impairment is reversed by an atypical antipsychotic, clozapine, but not haloperidol (Kamei *et al.* 2006). Meth-induced cognitive impairment in mice may be a useful animal model for cognitive deficits in Meth abusers and/or schizophrenia patients.

The present study was designed to test the hypothesis that galantamine improves cognitive deficit in the Meth-treated animal model of Meth psychosis and/or schizophrenia (Kamei *et al.* 2006), and that such cognitive-improving effects are mediated via activation of nAChR-dopaminergic-ERK1/2 pathways. We attempted to investigate: (1) whether cognitive-improving effects of galantamine are mediated via nAChRs in Meth-treated mice and (2) whether galantamine augments dopamine neurotransmission in the PFC by activation of nAChRs.

Methods

Animals

Male mice of the ICR strain (Japan SLC Inc., Japan), aged 6 wk at the beginning of experiments, were used. They were housed in plastic cages, received food (CE2; Clea Japan Inc., Japan) and water *ad libitum*, and were maintained on a 12-h light/dark cycle (lights on 08:00 hours). Behavioural experiments were performed in a sound-attenuated and air-regulated experimental room, to which mice were habituated for at least 1 h. All experiments were conducted blind to treatment and in accordance with the Guidelines for Animal Experiments of Nagoya University Graduate School of Medicine. The procedures involving animals and their care conformed to the international guidelines set out in the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

Drugs

Galantamine hydrobromide (4a,5,9,10,11,12-hexahydro-3-methoxy-11-methyl-6H-benzofuro [3a,3,2-ef] benzazepin-6-ol hydrobromide) was supplied by Janssen Pharmaceutica (Tokyo, Japan). Galantamine, donepezil hydrochloride (Toronto Research Chemicals Inc., Canada), methamphetamine hydrochloride (Dainippon Sumitomo Pharma Co. Ltd, Japan), mecamylamine hydrochloride (Sigma-Aldrich, USA), (−)scopolamine hydrobromide (Sigma-Aldrich) and R(+)-SCH 23390 hydrochloride [R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride] (Sigma-Aldrich) were dissolved in saline. PD98059 (Sigma-Aldrich) was dissolved in 60% dimethylsulfoxide (DMSO) saline.

Drug treatment

The mice were administered Meth (1 mg/kg.d s.c.) or saline once a day for 7 consecutive days (Kamei *et al.* 2006). The NOR test and microdialysis experiment were started 1 d and 3 d, respectively after the withdrawal of Meth treatment. The saline- or Meth-treated mice were administered galantamine (3 mg/kg p.o.) or donepezil (1 mg/kg p.o.) 1 h before the training session of the NOR test, or immediately after baseline collections in the microdialysis experiment. Mecamylamine (3 mg/kg s.c.), scopolamine (0.1 mg/kg s.c.) and SCH 23390 (0.02 mg/kg s.c.) were injected 20, 20 and 30 min, respectively, after treatment with galantamine. The doses of galantamine and donepezil used in the present study were as determined in previous experiments (Wang *et al.* 2007*a*,*b*) and in the report by Geerts *et al.* (2005), in which donepezil is

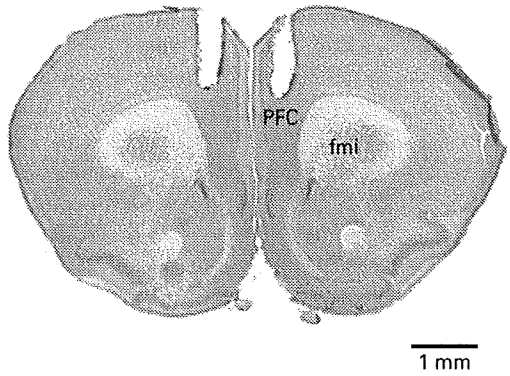

Fig. 1. Representative figure of mPFC local injection site. PFC; prefrontal cortex, fmi; forceps minor of the corpus callosum.

3–15 times more potent than galantamine in inhibiting brain AChE *in vivo*. The doses of antagonists were selected based on our previous publications (Kamei *et al.* 2006; Wang *et al.* 2007*a*, *b*). All compounds except for PD98059 were systemically administered at a volume of 0.1 ml/10 g body weight. Control mice received the same volume of saline.

For local microinjection into the PFC, mice were anaesthetized with diethyl ether and fixed on the stereotactic apparatus (Narishige, Japan) 30 min before the training session. An L-shaped injection cannula (27 gauge) with a bevel tip at its short end was grasped with forceps and implanted into the PFC (+0.3 mm mediolateral from the midpoint on the line linking the two rear canthi, −2.5 mm in depth). PD98059 at a dose of 2 μg/1 μl/bilateral or vehicle (60% DMSO/2 μl/bilateral) was infused into the PFC for 45 s using a Hamilton microsyringe connected to the cannula via a Teflon tube, and the connection was maintained for another 45 s after the injection. After the behavioural experiments, the mice were decapitated, and the brains were removed. The brains were transversely cut along the direction of the vertical insertion of the cannula to confirm the injection site, which was obvious due to its dark red colour, and easily recognized as shown in Fig. 1. Misinjected mice were excluded from subsequent data analysis.

NOR test

The task was carried out on days 1–3 after the final injection of Meth in accordance with the method of Kamei *et al.* (2006) with a minor modification. The experimental apparatus consisted of a Plexiglas open-field box (40 × 40 × 29 high cm), the floor of which was covered with paper bedding. The apparatus was placed in a sound-isolated room. A light bulb, located in the upper part of the room and which could not be seen directly by the mice, provided constant illumination of about 40 lx at the level of the task apparatus.

The NOR task procedure consisted of three sessions: habituation, training, retention. Each mouse was individually habituated to the box, with 10 min exploration in the absence of objects on day 1 (habituation session). During the training session on day 2, two objects (A and B) were placed in the back corner of the box, 10 cm away from the side wall. A mouse was then placed in the middle front of the box and the total time spent in exploring the two objects was recorded for 10 min by the experimenter using two stopwatches. Exploration of an object was defined as directing the nose to the object at a distance of <2 cm and/or touching it with the nose. During the retention session on day 3, the animals were returned to the same box 24 h after the training session, in which one of the familiar objects (e.g. object A) used during the training session was replaced by a novel object C. The animals were then allowed to explore freely for 10 min and the time spent exploring each object was recorded. Throughout the experiments, the objects were used in a balanced manner in terms of their physical complexity and emotional neutrality. A preference index, the ratio of the amount of time spent exploring any one of the two objects (training session) or the novel object (retention session) over the total time spent exploring both objects, was used to measure cognitive function, e.g.

training session: A or $B/(B+A) \times 100$ (%),

retention session: B or $C/(B+C) \times 100$ (%).

Determination of extracellular acetylcholine (ACh) and dopamine levels in the PFC

In-vivo microdialysis was performed 3 d after the final injection of Meth. One day before microdialysis, mice were anaesthetized with sodium pentobarbital (50 mg/kg i.p.) and a guide cannula (MI-AG-6; Eicom Corp., Japan) was implanted into the mPFC (+1.9 mm anteroposterior, +1.0 mm mediolateral from bregma, −1.5 mm dorsoventral from the skull, +15° angle from vertical) according to the atlas of Franklin & Paxinos (1997). One day after the operation, the dialysis probe of ACh (A-I-4-02; 2 mm membrane length; Eicom Corp.) and dopamine (A-I-6-01; 1 mm membrane length; Eicom Corp.) was inserted through the guide cannula, and perfused with artificial cerebrospinal fluid (aCSF; 147 mM NaCl, 4 mM KCl, 2.3 mM $CaCl_2$) at a flow rate of 1 μl/min (Mouri *et al.*

2006) and 1.2 μl/min (Shintani *et al.* 1993), respectively. The outflow fractions of ACh and dopamine were collected every 20 min and 10 min, respectively. When the difference of each fraction was <20%, we considered this a stable baseline. Following the collection of three stable baseline fractions of ACh and dopamine, mice were treated with donepezil, galantamine and/or mecamylamine, and then dialysates of ACh and dopamine were collected every 20 min for 120 min and every 10 min for 90 min, respectively. ACh and dopamine levels in the dialysates were analysed using an HPLC system equipped with an electrochemical detector (Mouri *et al.* 2007, 2006).

Western blotting

We examined activation of ERK1/2 in the brain of mice that were exposed to the novel objects during the training session. Phosphorylation of ERK1/2 was examined by Western blotting as described previously (Kamei *et al.* 2006; Mizoguchi *et al.* 2004). Immediately after a training session, the mice were sacrificed by decapitation, and the brain was immediately removed. The PFC was rapidly dissected out on an ice-cold plate, frozen, and stored at −80 °C until required. Tissue samples from the PFC were homogenized by sonication at 4 °C in a lysis buffer composed of 20 mM Tris–HCl, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM sodium orthovanadate, 0.1% SDS, 1% sodium deoxycholate, 0.5 mM dithiothreitol, 10 mM sodium pyrophosphate decahydrate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin (pH 7.4). The homogenate was centrifuged at 13 000 *g* for 20 min and the supernatant was used. The protein concentration of tissue extracts was determined using a DC Protein Assay kit (Bio-Rad, USA). Samples (20 μg protein) were boiled in a sample buffer [0.125 M Tris–HCl (pH 6.8), 2% SDS, 5% glycerol, 0.002% Bromphenol Blue, and 5% 2-mercaptoethanol], applied onto a 10% polyacrylamide gel, subsequently transferred to a polyvinylidene difluoride membrane (Millipore Corporation, USA) or a nitrocellulose membrane (GE Healthcare Biosciences, USA), and blocked with a Detector Block kit (Kirkegaard and Perry Laboratories, USA). Membranes were incubated with anti-phospho-ERK1/2 [phospho-p44/42 mitogen-activated protein kinase (MAPK) (Thr^{202}/Tyr^{204}) Antibody no. 9101] (1:1000 dilution; Cell Signaling Technology Inc., USA) and washed with Tris-buffered saline (TBS)-Tween 20 [10 mM Tris–HCl (pH 7.5), 100 mM NaCl, and 1% Tween 20] three times for 10 min each. After incubation with a 1:2000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (secondary antibody) for 1 h, membranes were washed with TBS-Tween 20 three times for 10 min each. The immune complex was detected using ECL Western blotting detection reagents (GE Healthcare Biosciences). The same membranes were stripped with a stripping buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM disodium hydrogen phosphate, 12-water, 1.5 mM potassium dihydrogen phosphate, and 0.2% 2-mercaptoethanol) at 55 °C for 30 min, incubated with anti-ERK1/2 (1:1000 dilution, p44/42 MAPK Antibody no. 9102, Cell Signaling Technology Inc.), and treated as described above.

Statistical analysis

Statistical significance was determined using a one-way analysis of variance (ANOVA) or a two-way ANOVA with repeated measures, followed by Bonferroni's test for multigroup comparisons. Statistical differences between two sets of groups were determined with the Student's *t* test. *p* values <0.05 were taken to indicate statistically significant differences.

Results

Effect of galantamine on Meth-induced impairment of recognition memory in mice

We examined whether Meth-induced cognitive impairment was reversed by galantamine. One day after the cessation of repeated Meth (1 mg/kg.d s.c.) treatment for 7 d, mice were subjected to the NOR test. Galantamine (3 mg/kg p.o.) was acutely administered 1 h before the training session.

As shown in Fig. 2, repeated Meth treatment significantly reduced the exploratory preference for a novel object in the retention session ($p<0.01$) (Fig. 2*a*). Treatment with galantamine significantly improved cognitive impairment in Meth-treated mice ($p<0.01$) (Fig. 2*a*). Galantamine affected neither the level of exploratory preference for the objects in the training session [$F(3,36)=1.188$, $p=0.328$] (Fig. 2*a*) nor the total exploration time in either the training [$F(3,36)=1.241$, $p=0.309$] or retention [$F(3,36)=2.396$, $p=0.084$] sessions in Meth-treated mice (Fig. 2*b*).

Effect of donepezil on the extracellular ACh levels of the PFC and the impairment of recognition memory in Meth-treated mice

To determine whether the improving effects of galantamine on Meth-induced cognitive impairment