

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 D₁ 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 D₁ 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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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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(A β) fragment (as an animal model of Alzheimer's disease) (Wang *et al.* 2007a) and by repeated treatment with a non-competitive *N*-methyl-D-aspartate receptor antagonist, phencyclidine (PCP) (as an animal model of schizophrenia) (Wang *et al.* 2007b). It increases the extracellular dopamine release in the hippocampus and prefrontal cortex (PFC) of A β_{25-35} -infused and PCP-treated mice, respectively. The ameliorating effects of galantamine on A β_{25-35} - and PCP-induced cognitive impairment are mediated through the augmentation of dopaminergic neurotransmission following activation of nAChRs (Wang *et al.* 2007a,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), mecamlamine 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. Mecamlamine (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.* 2007a,b) and in the report by Geerts *et al.* (2005), in which donepezil is

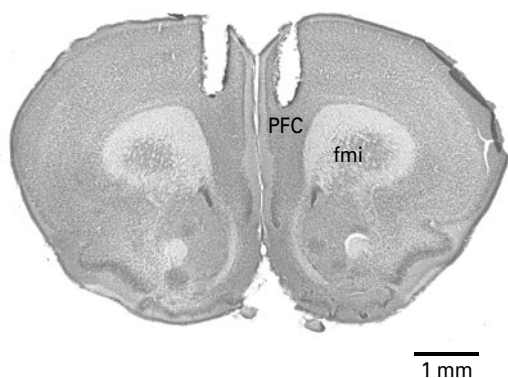


Fig. 1. Representative figure of mPFC local injection site. PFC; prefrontal cortex, fmi; forceps minor of the corpus callosum.

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.* 2007a,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: $A \text{ or } B / (B + A) \times 100 (\%)$,

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

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 CaCl₂) at a flow rate of 1 µl/min (Mouri *et al.*

2006) and 1.2 $\mu\text{l}/\text{min}$ (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 mecamlamine, 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 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin, and 10 $\mu\text{g}/\text{ml}$ 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) (Thr²⁰²/Tyr²⁰⁴) 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. 2a). Treatment with galantamine significantly improved cognitive impairment in Meth-treated mice ($p < 0.01$) (Fig. 2a). Galantamine affected neither the level of exploratory preference for the objects in the training session [$F(3, 36) = 1.188$, $p = 0.328$] (Fig. 2a) 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. 2b).

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

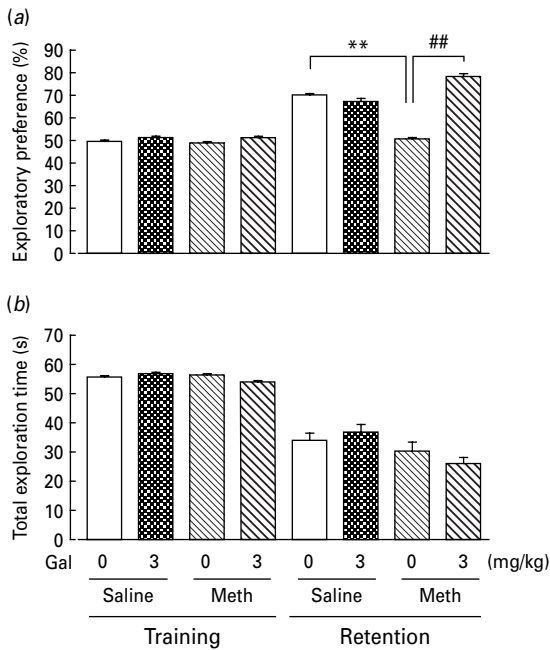


Fig. 2. Effect of galantamine on methamphetamine (Meth)-induced impairment of recognition memory in mice: (a) exploratory preference and (b) total exploration time. One day after the cessation of repeated Meth (1 mg/kg s.c.) treatment for 7 d, mice underwent the novel object recognition test. Galantamine (3 mg/kg p.o.) or saline was administered 1 h before the training session. Values indicate the mean \pm s.e. ($n=10$). One-way ANOVA, (a) training: $F(3, 36)=1.188$, $p=0.328$; retention: $F(3, 36)=63.849$, $p<0.01$; (b) training: $F(3, 36)=1.241$, $p=0.309$; retention: $F(3, 36)=2.396$, $p=0.084$. ** $p<0.01$ compared to saline + saline-treated group (Bonferroni's test). ## $p<0.01$ compared to Meth + saline-treated group (Bonferroni's test).

are due to increase of ACh levels caused by inhibition of AChE, we examined the effect of donepezil, an AChE inhibitor, on the impairment of cognition in Meth-treated mice.

Donepezil at a dose of 1 mg/kg caused about a 2-fold increase in the levels of extracellular ACh in the PFC of Meth-treated mice [$F(1, 35)=14.042$, $p<0.01$] (Fig. 3a). However, donepezil (1 mg/kg) had no effect on the level of exploratory preference for the objects in the retention sessions in Meth-treated mice (Fig. 3b). It also affected neither the level of exploratory preference for the objects in the training session [$F(2, 40)=0.159$, $p=0.854$] (Fig. 3a) nor the total exploration time in either the training [$F(2, 40)=0.296$, $p=0.746$] or retention [$F(2, 40)=0.160$, $p=0.215$] sessions in Meth-treated mice (Fig. 3c).

Involvement of nicotinic receptors, but not muscarinic receptors in the cognitive-improving effect of galantamine on Meth-treated mice

To determine whether the improving effects of galantamine on Meth-induced cognitive impairment are mediated via nAChRs, but not muscarinic AChRs (mAChRs), we examined the antagonism by using mecamylamine, a nAChR antagonist and scopolamine, a mAChR antagonist, against the cognitive-improving effects of galantamine in Meth-treated mice.

In the training session of the NOR task, there were no differences in exploratory preference for the objects in any of the groups (Fig. 4a, c). The nAChR antagonist, mecamylamine (3 mg/kg) significantly and completely prevented the improving effects of galantamine on the impairment of recognition memory in Meth-treated mice ($p<0.01$) (Fig. 4a). In saline-treated mice, mecamylamine alone at the dose used had no effect on the NOR performances (Fig. 4a). The antagonistic effect of mecamylamine on galantamine-induced improvement of exploratory preference in Meth-treated mice was not associated with changes in the total exploration time [training: $F(4, 57)=0.516$, $p=0.725$; retention: $F(4, 57)=2.403$, $p=0.060$] (Fig. 4b).

Scopolamine at a dose of 0.1 mg/kg impaired the performance of saline-treated mice in the NOR task (Fig. 4c). However, scopolamine failed to prevent the improving effects of galantamine on the impairment of recognition memory in Meth-treated mice (Fig. 4c). Treatment with any compound did not affect the total exploration time in either the training [$F(6, 77)=2.193$, $p=0.053$] or retention [$F(6, 77)=1.919$, $p=0.088$] sessions (Fig. 4d).

Effects of galantamine on the levels of extracellular dopamine in the PFC of Meth-treated mice

We examined whether galantamine at a dose of 3 mg/kg, which improved the cognitive deficit in Meth-treated mice, facilitated dopamine release in the PFC of Meth-treated mice.

There were no differences in the basal levels of extracellular dopamine in the PFC in any of the groups (Fig. 5 insert). As shown in Fig. 5, galantamine (3 mg/kg) caused a marked increase in the levels of extracellular dopamine in the PFC of Meth-treated mice (Fig. 5). The significant increase in the levels of extracellular dopamine was observed from 30 min after galantamine administration ($p<0.01$ by *post hoc* test, Fig. 5). When mecamylamine (3 mg/kg) was injected into Meth-treated mice 20 min after galantamine administration, galantamine-induced elevation of extracellular dopamine levels was significantly diminished

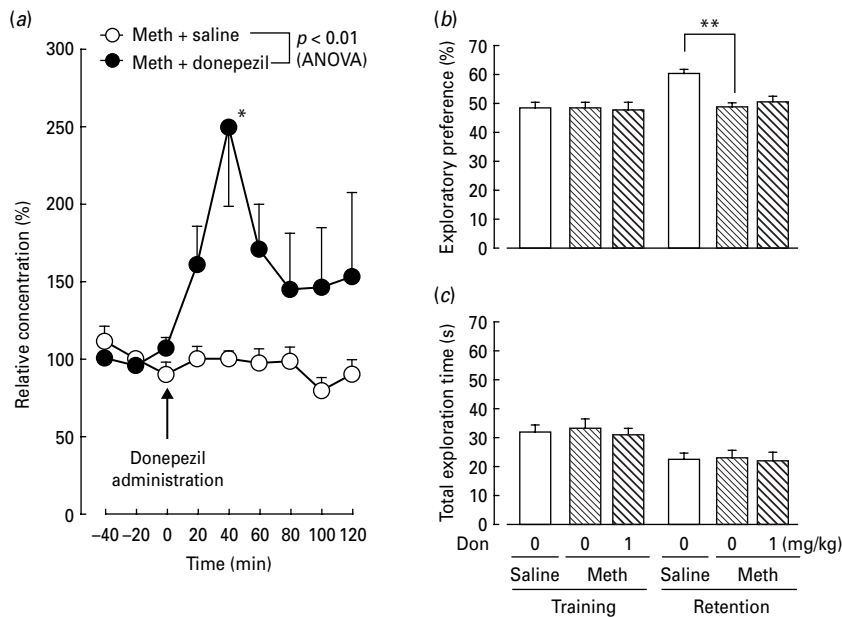


Fig. 3 Effect of donepezil on the extracellular acetylcholine (ACh) levels of the prefrontal cortex (PFC) and the impairment of recognition memory in methamphetamine (Meth)-treated mice. (a) Extracellular ACh levels of PFC in microdialysis. *In-vivo* microdialysis was performed 3 d after the final injection of Meth (1 mg/kg s.c.) treatment for 7 d. Donepezil (1 mg/kg p.o.) was administered to the Meth-treated mice (●, Meth + donepezil). In the control group, an equivalent amount of saline was given to the Meth-treated mice (○, Meth + saline). Values indicate the mean \pm S.E. ($n = 4-5$). Results with the repeated ANOVA were: time [$F(5, 35) = 1.111$, $p = 0.37$]; treatment [$F(1, 35) = 14.042$, $p < 0.01$]; time \times treatment interaction [$F(5, 35) = 0.677$, $p = 0.64$]. * $p < 0.05$ compared to Meth + saline-treated group (Bonferroni's test). The basal levels of ACh in the PFC of the Meth + saline- and Meth + donepezil-treated mice were 0.17 ± 0.05 and 0.12 ± 0.06 pmol/20 μ l per 20 min, respectively. (b) Exploratory preference in novel object recognition (NOR) test. (c) Total exploration time in NOR test. One day after the cessation of repeated Meth (1 mg/kg s.c.) treatment for 7 d, mice underwent the NOR test. Donepezil (1 mg/kg p.o.) or saline was administered 1 h before the training session. Values indicate the mean \pm S.E. ($n = 13-15$). One-way ANOVA, (b) training: $F(2, 40) = 0.159$, $p = 0.854$; retention: $F(2, 40) = 9.400$, $p < 0.01$; (c) training: $F(2, 40) = 0.296$, $p = 0.746$; retention: $F(2, 40) = 0.160$, $p = 0.215$. ** $p < 0.01$ compared to saline + saline-treated group (Bonferroni's test).

(Fig. 5). However, mecamylamine alone did not affect the extracellular dopamine levels in saline-treated mice (data not shown).

Involvement of dopaminergic systems in the cognitive-improving effect of galantamine on Meth-treated mice

Previous studies have shown that the ERK1/2 signalling pathway linked to dopamine D_1 receptors (D_1 Rs) (Valjent *et al.* 2000; Zanassi *et al.* 2001) is involved in Meth-associated contextual memory in rats (Mizoguchi *et al.* 2004) and that repeated Meth treatment induces cognitive impairment in the NOR test in mice, which is accompanied by dysfunction of the dopamine D_1 R-ERK1/2 pathway in the PFC (Kamei *et al.* 2006). To clarify whether the improving effects of galantamine on Meth-induced cognitive impairment are mediated through the activation of dopamine D_1 Rs, we investigated the antagonism by using SCH 23390, a

dopamine D_1 R antagonist, against the cognitive-improving effects of galantamine in Meth-treated mice.

SCH 23390 (0.02 mg/kg) significantly and completely prevented the improving effects of galantamine on Meth-induced cognitive impairment without affecting the exploratory preference for the objects in the training session (Fig. 6a). In saline-treated mice, SCH 23390 alone had no effect on NOR performance (Fig. 6a). SCH 23390 also had no effect on the total exploration time in either the training [$F(4, 50) = 1.520$, $p = 0.211$] or retention [$F(4, 55) = 1.943$, $p = 0.116$] sessions of Meth-treated mice (Fig. 6b).

Effect of galantamine on the defect of novelty-induced ERK1/2 phosphorylation in the PFC of Meth-treated mice

Kamei *et al.* (2006) have demonstrated novelty-induced ERK1/2 activation in the PFC when mice are

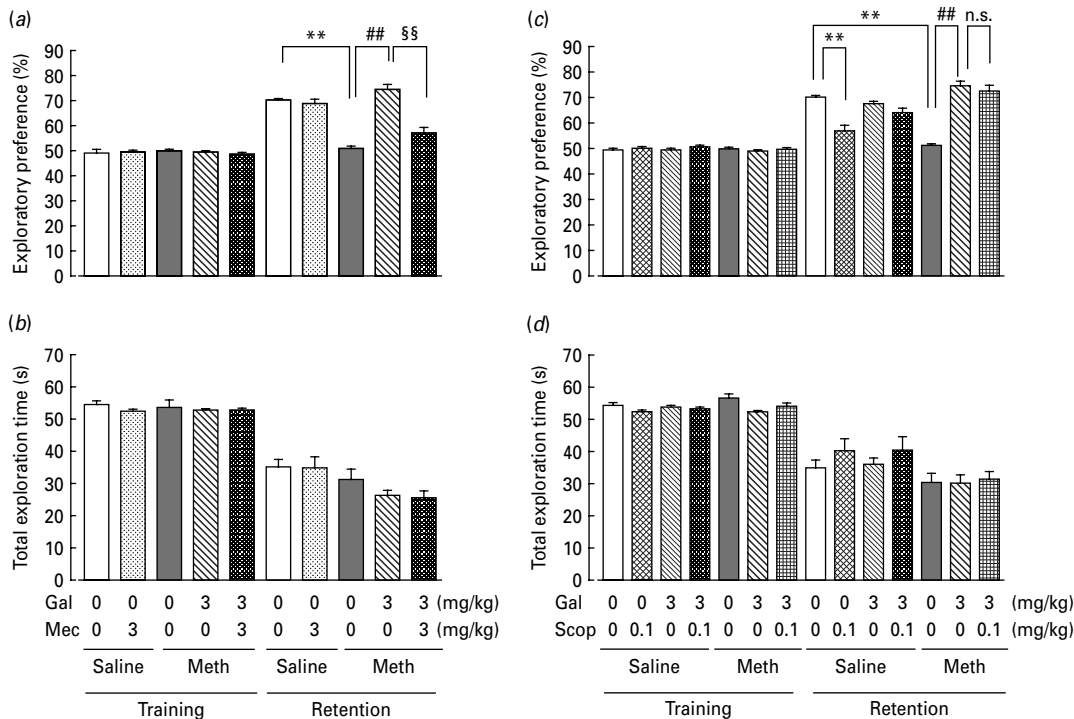


Fig. 4. Involvement of nicotinic receptors, but not muscarinic receptors in the cognitive-improving effect of galantamine on methamphetamine (Meth)-treated mice: (a, c) exploratory preference and (b, d) total exploration time. One day after the cessation of repeated Meth (1 mg/kg s.c.) treatment for 7 d, mice underwent the novel object recognition test. Galantamine (Gal; 3 mg/kg p.o.), mecamylamine (Mec; 3 mg/kg s.c.) and/or scopolamine (Scop; 0.1 mg/kg s.c.) were administered to saline- or Meth-treated mice 1 h, 40 min and/or 40 min, respectively, before the training session. Values indicate the mean \pm s.e. ($n=10-15$). One-way ANOVA, (a) training: $F(4, 57)=0.255$, $p=0.906$; retention: $F(4, 57)=28.901$, $p<0.01$; (b) training: $F(4, 57)=0.516$, $p=0.725$; retention: $F(4, 57)=2.403$, $p=0.060$; (c) training: $F(6, 77)=0.429$, $p=0.858$; retention: $F(6, 77)=20.277$, $p<0.01$; (d) training: $F(6, 77)=2.193$, $p=0.053$; retention: $F(6, 77)=1.919$, $p=0.088$. ** $p<0.01$ compared to saline + saline/saline-treated group (Bonferroni's test). ## $p<0.01$ compared to Meth + saline/saline-treated group (Bonferroni's test). §§ $p<0.01$ compared to Meth + galantamine/saline-treated group (Bonferroni's test). n.s., Not significant.

exposed to novel objects, leading to the formation of long-lasting object recognition memory. Further, memory impairment in Meth-treated mice was associated with dysfunction of ERK1/2 signalling in the PFC. In order to examine the mechanism by which galantamine ameliorates the impairment of recognition memory in Meth-treated mice, we examined the effect of galantamine on ERK1/2 phosphorylation in the PFC of Meth-treated mice when they were exposed to novel objects.

A significant increase in phosphorylation of ERK1/2 levels was observed in the PFC of saline-treated mice immediately after a 10-min exposure to novel objects (Fig. 7a, b) ($p<0.01$ vs. baseline in saline-treated mice, Student's t test), and repeated Meth treatment abolished novelty-induced ERK1/2 activation in the PFC in accord with the previous study (Kamei *et al.* 2006) ($p<0.01$) (Fig. 7a). Galantamine (3 mg/kg) significantly recovered the defect of novelty-induced activation of

ERK1/2 in the PFC of Meth-treated mice ($p<0.01$) (Fig. 7a). SCH 23390 (0.02 mg/kg) significantly blocked the improving effects of galantamine on the defect of novelty-induced ERK1/2 phosphorylation in the PFC ($p<0.01$) (Fig. 7a). SCH 23390 alone had no effect on the levels of phosphorylation and total ERK1/2 in either the baseline or exposure of saline-treated mice (Fig. 7b). The levels of total ERK1/2 did not differ in the exposed groups examined [$F(3, 16)=1.629$, $p=0.222$].

Influence of an ERK inhibitor on the cognitive-improving effect of galantamine on Meth-treated mice

We confirmed that PD98059 (2 μ g/1 μ l/bilateral) has no effect on the phosphorylation of ERK1/2 in the PFC and hippocampus of naive mice (data not shown). Then, we examined the effect of PD98059 (2 μ g/1 μ l/bilateral) administered before the training session on

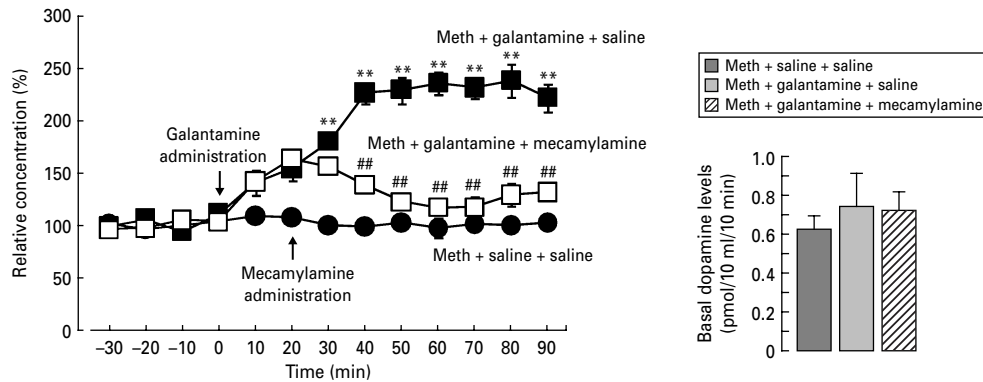


Fig. 5. Effects of galantamine on the levels of the extracellular dopamine in the PFC of methamphetamine (Meth)-treated mice. Meth (1 mg/kg, s.c.) was injected for 7 d, and 3 d after withdrawal, extracellular levels of dopamine were measured in the PFC by *in-vivo* microdialysis. Galantamine (3 mg/kg p.o.) was administered to the Meth-treated mice (■, Meth + galantamine + saline). In the control group, an equivalent amount of saline was given (●, Meth + saline + saline) to the Meth-treated mice. Mecamlamine (3 mg/kg s.c.) was injected 20 min after galantamine (□, Meth + galantamine + mecamlamine) to Meth-treated mice. The basal levels of dopamine in the PFC of the Meth + saline + saline (■), Meth + galantamine + saline (□) and Meth + galantamine + mecamlamine (▨)-treated mice were 0.62 ± 0.08 , 0.74 ± 0.18 and 0.72 ± 0.10 pmol/10 μ l per 10 min, respectively (right-hand panel). Values indicate the mean \pm S.E. ($n=3$). Results with the repeated ANOVA were time [$F(9, 54)=8.063$, $p<0.01$], treatment [$F(2, 6)=73.188$, $p<0.01$], and time \times treatment interaction [$F(18, 54)=10.802$, $p<0.01$]. ** $p<0.01$ compared to Meth + saline + saline-treated group (Bonferroni's test). ## $p<0.01$ compared to Meth + galantamine + saline-treated group (Bonferroni's test).

the cognitive-improving effect of galantamine in Meth-treated mice to determine the involvement of ERK1/2 activation in the mechanism of action of galantamine.

In the training session, bilateral microinjections of PD98059 into the PFC (1 μ g/side) of saline-treated mice did not affect the exploratory preference for the objects (Fig. 8a). In the retention session, the level of exploratory preference in PD98059-treated mice was significantly increased as for vehicle-treated mice ($p<0.01$, Fig. 8a), but it was significantly decreased compared to that in vehicle-treated mice ($p<0.05$, Fig. 8a). PD98059 had no effect on the total exploration time in either the training or retention sessions of saline-treated mice (Fig. 8b).

In Meth-treated mice, PD98059 completely blocked the ameliorating effect of galantamine on the impairment of exploratory preference for a novel object in the retention session [$F(2, 25)=27.986$, $p<0.01$] (Fig. 8c). The antagonistic effect of PD98059 on galantamine-induced improvement of exploratory preference in Meth-treated mice was not associated with changes in the total exploration time [training: $F(2, 25)=0.399$, $p=0.676$; retention: $F(2, 25)=0.015$, $p=0.985$] (Fig. 8d).

Discussion

We have reconfirmed that Meth-treated mice show impairments to their novelty discrimination ability in

the NOR test that is consistent with previous reports (Ito *et al.* 2007; Kamei *et al.* 2006). It is unlikely that the impairment in performance of Meth-treated mice in learning and memory tasks is due to changes in motivation, although various motivations are involved in the behavioural task. The fact that Meth reduced the exploratory preference for the objects in the retention session could be interpreted as neophobia. However, the possible involvement of motivation and/or neophobia can be excluded because Meth treatment had no effect on total exploration time of novel objects during the training session. Therefore, it is likely that impairment of performance in Meth-treated mice is due to learning and memory deficits.

Galantamine, a drug approved for the treatment of Alzheimer's disease, has a dual mechanism of action; it inhibits AChE and allosterically modulates nAChR as a potent APL (Eisele *et al.* 1993; Santos *et al.* 2002). We have recently reported that galantamine reverses the impairment of object recognition in $A\beta_{25-35}$ -infused mice as an animal model of Alzheimer's disease and in repeated PCP-treated mice as an animal model of schizophrenia (Wang *et al.* 2007a,b). In accord with these findings, in the present study, galantamine significantly ameliorated the cognitive impairments induced by Meth in the NOR test. Galantamine at a dose of 3 mg/kg had no effect on the total exploration time in the training session of the NOR test in Meth-treated

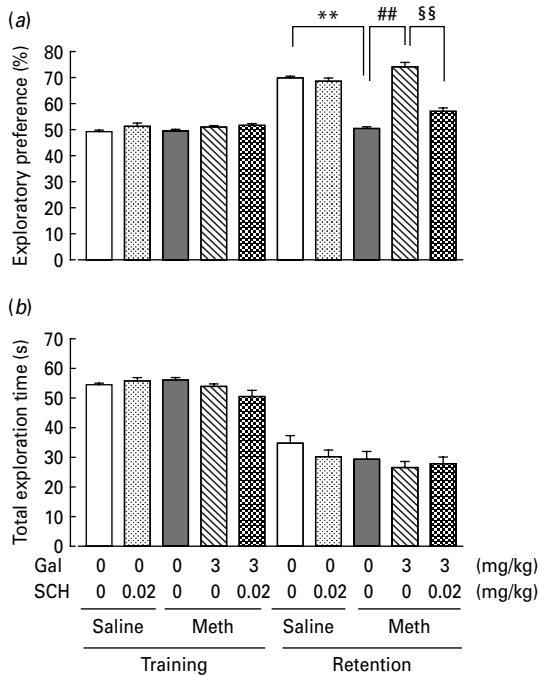


Fig. 6. Involvement of dopaminergic systems in the cognitive-improving effect of galantamine on methamphetamine (Meth)-treated mice: (a) exploratory preference and (b) total exploration time. One day after the cessation of repeated Meth (1 mg/kg s.c.) treatment for 7 d, mice underwent the novel object recognition test. Galantamine (Gal; 3 mg/kg p.o.) and SCH 23390 (SCH; 0.02 mg/kg s.c.) were administered 1 h and 30 min, respectively, before the training session. Values indicate the mean \pm s.e. ($n=10-15$). One-way ANOVA, (a) training: $F(4, 50)=1.422$, $p=0.240$; retention: $F(4, 55)=40.622$, $p<0.01$; (b) training: $F(4, 50)=1.520$, $p=0.211$; retention: $F(4, 55)=1.943$, $p=0.116$. ** $p<0.01$ compared to saline + saline/saline-treated group (Bonferroni's test). *** $p<0.01$ compared to Meth + saline/saline-treated group (Bonferroni's test). §§ $p<0.01$ compared to Meth + galantamine/saline-treated group (Bonferroni's test).

mice. Therefore, it is unlikely that the observed improvement in performance in the task brought about by galantamine is due to changes in motivation in Meth-treated mice, and it is apparently true that galantamine ameliorates learning and memory deficits caused by repeated Meth treatment in mice. The improving effects of galantamine on the performance of Meth-treated mice were prevented by treatment with mecamylamine, a nAChR antagonist, at a dose that did not significantly affect the performance of saline-treated mice. These findings support the notion that galantamine improves Meth-induced cognitive impairment via activation of nAChRs. Alternatively, the roles of mAChRs in the effects of galantamine were

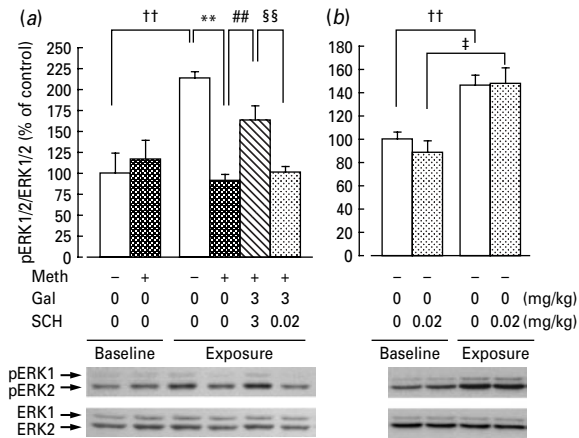


Fig. 7. Effect of galantamine on the defect of novelty-induced ERK1/2 phosphorylation in the PFC of methamphetamine (Meth)-treated mice. One hour before exposure to novel objects, galantamine (Gal; 3 mg/kg p.o.) or saline was administered to mice that had been previously treated with either saline or Meth (1 mg/kg s.c.) for 7 d. SCH 23390 (SCH; 0.02 mg/kg s.c.) was administered 30 min before exposure to novel objects. Values indicate the mean \pm S.E. ($n=4-5$). $^{\dagger\dagger} p < 0.01$ compared to saline + saline/saline-treated group that was not exposed to novel objects (baseline) (Student's t test). $^{\dagger} p < 0.05$ compared to saline + saline/SCH23390-treated group that was not exposed to novel objects (baseline) (Student's t test). One-way ANOVA: $F(3, 16) = 28.286$, $p < 0.01$. $^{**} p < 0.01$ compared to saline + saline/saline-treated group (exposure) (Bonferroni's test). $^{***} p < 0.01$ compared to Meth + saline/saline-treated group (exposure) (Bonferroni's test). $^{ss} p < 0.01$ compared to Meth + galantamine/saline-treated group (exposure) (Bonferroni's test).

also investigated in the present study. The effects of galantamine on the performance of Meth-treated mice in the NOR task were not blocked by scopolamine at the dose that impaired the performance of saline-treated mice. Although mAChR agonists improve cognitive dysfunctions in patients with Alzheimer's disease and schizophrenia (Friedman, 2004), the present result indicated that mAChRs have little influence on the effects of galantamine for this particular cognitive task. On the other hand, the activation of nAChRs may be due to an increase in the levels of ACh caused by AChE inhibition of galantamine. We investigated the effect of donepezil, which is 3–15 times more potent in AChE inhibition than that of galantamine *in vivo* (Geerts *et al.* 2005), on Meth-induced cognitive impairment. Although donepezil at 1 mg/kg caused about a 2-fold increase from basal extracellular ACh levels in the PFC of Meth-treated mice, it had no effect on behavioural performance in Meth-treated mice. From the

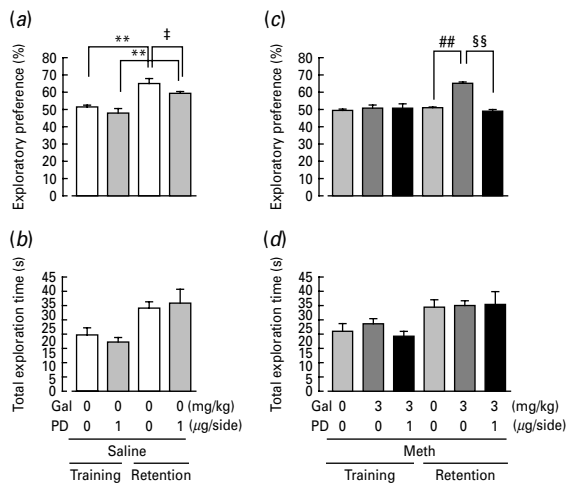


Fig. 8. Influence of an ERK inhibitor on the cognitive-improving effect of galantamine on methamphetamine (Meth)-treated mice: (a, c) exploratory preference and (b, d) total exploration time. One day after the cessation of repeated Meth (1 mg/kg s.c.) treatment for 7 d, mice underwent the novel object recognition test. Galantamine (Gal; 3 mg/kg p.o.) and PD98059 (PD; 1 μg/0.5 μl per side) were administered 1 h and 30 min, respectively, before the training session. Values indicate the mean \pm S.E. (a, b; $n=8$) (c, d; $n=9-10$). One-way ANOVA, (c) training: $F(2, 25)=0.309$, $p=0.737$; retention: $F(2, 25)=27.986$, $p<0.01$; (d) training: $F(2, 25)=0.399$, $p=0.676$; retention: $F(2, 25)=0.015$, $p=0.985$. ** $p<0.01$ compared to corresponding saline-treated training group (Student's t test). * $p<0.05$ compared to saline + saline/vehicle-treated retention group (Student's t test). ## $p<0.01$ compared to Meth + saline/vehicle-treated group (Bonferroni's test). §§ $p<0.01$ compared to Meth + galantamine/vehicle-treated group (Bonferroni's test).

present results and a report that there is only 1–12% brain AChE inhibition 1 h after s.c. injection of 3 mg/kg galantamine (Geerts *et al.* 2005), our conclusion is that galantamine induces the ameliorating effect on impairment of memory mainly by allosterically modulating the function of nAChRs, but not by AChE inhibition. However, further experiments are needed to exclude the involvement of AChE inhibition by galantamine in the ameliorating effect of it on cognitive impairment in Meth-treated mice, since the allosteric potentiating effect of nAChRs can be detected at lower doses (Geerts *et al.* 2005).

Accumulating evidence suggests that the dopaminergic system in the PFC is involved in cognitive function. For instance, disruption of dopamine transmission in the PFC by infusions of dopamine D₁R antagonists or by excitotoxic lesions impairs the performance of object retrieval-detour tasks, as well as delayed response tasks in non-human primates (Dias

et al. 1996a,b; Sawaguchi & Goldman-Rakic, 1991). A previous study with functional magnetic resonance imaging has shown that dysfunction in the PFC of Meth abusers is related to cognitive impairment (Paulus *et al.* 2002). Accordingly, cognitive impairment in Meth abusers may be associated with deficits in dopamine transmission in the PFC. Our previous findings in *in-vivo* microdialysis experiments demonstrated that galantamine increases the extracellular dopamine release in the hippocampus and PFC and that the increasing effects of galantamine on dopamine release in the hippocampus are potentiated by nicotine and antagonized by mecamylamine (Wang *et al.* 2007a). The present *in-vivo* microdialysis experiment show that galantamine significantly increased extracellular dopamine release in the PFC of Meth-treated mice. The effects of galantamine on increasing dopamine release were antagonized by mecamylamine. These results strongly suggest that galantamine ameliorates Meth-induced learning and memory deficits by activating nAChRs, and thereby stimulates release of dopamine in the PFC. Further, we found that the improving effects of galantamine were prevented by SCH 23390, a dopamine D₁R antagonist. Galantamine enhances dopaminergic neurotransmission *in vivo* via allosteric potentiation of nAChRs. These findings provide the *in-vivo* evidence that galantamine augments dopaminergic neurotransmission in the PFC through the allosteric activation of nAChRs. The present results are supported by the results published by Schilström *et al.* (2007) that effects of galantamine on dopamine cell firing are mediated by allosteric potentiation of nAChRs. Taken together, our results suggest that the PFC-dependent behaviour task was impaired due to dysfunction of dopaminergic systems induced by Meth, since the PFC is involved in object recognition behaviour (Kamei *et al.* 2006). In fact, Kamei *et al.* (2006) have already demonstrated that repeated administration of Meth in mice induces object recognition impairment, which is associated with the dopamine D₁Rs, but not dopamine D₂Rs in the PFC. However, the object recognition memory is ascribed to the perirhinal cortex and its interactions with the hippocampus (Winters *et al.* 2008). We will investigate the functional role of the perirhinal cortex in Meth-induced cognitive deficits, in the ameliorating effects of galantamine and D₁R/ERK signalling in the NOR test.

Previous studies have demonstrated that the ERK1/2 signalling pathway linked to dopamine D₁Rs (Valjent *et al.* 2000; Zanassi *et al.* 2001) is involved in the rewarding effects induced by Meth (Mizoguchi *et al.* 2004) and the behavioural sensitization and

rewarding effects induced by cocaine (Valjent *et al.* 2000). Regarding the mechanism underlying the repeated Meth-induced memory impairment, Kamei *et al.* (2006) have already demonstrated dysfunction of the ERK1/2 pathway in the PFC. Hyperphosphorylation of ERK1/2 was found in the PFC when control mice were exposed to novel objects, whereas this activation was abolished in repeated Meth-treated mice. Inhibition of ERK1/2 by the microinjection of PD98059 (4 µg/mouse/bilateral), a selective MEK inhibitor, into the PFC resulted in cognitive impairment (Kamei *et al.* 2006). Ito *et al.* (2007) have also found that another MEK1/2 inhibitor, SL327 (30 and 50 mg/kg i.p.), significantly impairs long-term recognition memory 24 h after a training session in naive mice. In this study, galantamine ameliorated the Meth-induced defect of ERK1/2 hyperphosphorylation in the PFC of mice exposed to novel objects. In addition, the ameliorating effect of galantamine on Meth-induced object recognition impairment was completely blocked by pretreatment with the ERK inhibitor PD98059 at the dose used, slightly affecting the performance of saline-treated mice. Accordingly, these results suggest that the ameliorating effect of galantamine on Meth-induced cognitive impairment is related to the activation of ERK1/2 in the PFC.

As discussed above, our findings suggest that dopamine D₁R-ERK1/2 systems are required for the effects of galantamine. Since dopamine the D₁R antagonist and ERK inhibitor impaired recognition memory based on phosphorylation of ERK in the PFC of normal mice (Kamei *et al.* 2006), dopamine D₁R-ERK1/2 systems are critical in recognition memory. If the action site of galantamine is downstream of dopamine D₁R-ERK1/2 systems, dopamine D₁R antagonists or the ERK inhibitor would fail to reverse the effect of galantamine. Accordingly, our data suggest that galantamine acts upstream of dopamine D₁R-ERK1/2 systems.

In conclusion, the ameliorating effect of galantamine on Meth-induced memory impairment is associated with indirect activation of dopamine D₁R-ERK1/2 following augmentation with dopaminergic neurotransmission in the PFC through the allosteric activation of nAChRs. Galantamine could prove to be a useful therapeutic drug for treating cognitive deficits in schizophrenia/Meth psychosis, as well as Alzheimer's disease.

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Statement of Interest

None.

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