drug treatment in Alzheimer's disease is the application of allosteric modulators of nAChRs, which up-modulates (potentiates) the channel activity of nAChRs in response to acetylcholine. Such properties are displayed by a novel class of nAChR ligands, named 'allosteric potentiating ligands' (APLs) (Maelicke and Albuquerque, 2000; Maelicke *et al*, 2000). Galantamine, an approved medication for Alzheimer's disease, has a dual mechanism of action, which inhibits acetylcholinesterase and allosterically modulates nAChR as a potent APL (Eisele *et al*, 1993; Santos *et al*, 2002). In contrast to pure agonist nicotine, the stimulation of the nAChRs by galantamine is synchronized with physiological presynaptic cholinergic activity, thereby avoiding overstimulation and desensitization, and probably leading to an optimal activation of the downstream intracellular pathways (Geerts, 2005). Galantamine augments catecholamine neurotransmission within the hippocampus by augmenting the stimulative effects of endogenous nicotinic cholinergic circuits (Sharp *et al*, 2004) Thus, galantamine has been demonstrated to have potential cognitive improving effects on Alzheimer's disease since the allosteric action of galantamine may be related, in part, to the stimulation of catecholamine neurotransmission in addition to its enhancing effects on cholinergic systems by inhibition of acetylcholinesterase.

In the hippocampus, a critical area involved in attention and memory, the significance of nicotinic–dopaminergic interactions for cognitive function has been well documented (Hefco *et al*, 2004; Levin and Simon, 1998). Some cognitive and noncognitive aspects of Alzheimer's disease arise from the dysfunction of the dopaminergic and serotonergic systems rather than the cholinergic systems (Assal and Cummings, 2002; Erkinjuntti, 2002). Acetylcholine, or its hydrolysis product choline, would activate presynaptic nAChRs, leading to a Ca^{2+}-dependent enhancement of dopamine release (Turner, 2004). From the data mentioned above, it is very important to confirm whether galantamine can improve cognitive dysfunction of Alzheimer's disease by allosterically potentiating nicotinic–dopaminergic pathway.

The present study was designed to test the hypothesis that galantamine improves cognitive dysfunction in the $A\beta_{25-35}$-injected animal model of Alzheimer's disease (Maurice *et al*, 1996), and such cognitive improving effects of galantamine are mediated via activation of nAChR-dopaminergic systems. We attempted to investigate: (1) whether cognitive improving effects of galantamine are mediated via nAChRs in the $A\beta_{25-35}$-injected mice and (2) whether galantamine augments dopamine neurotransmission within the hippocampus by activation of nAChRs.

MATERIALS AND METHODS

Subjects

Male mice of the ICR strain (Japan SLC Inc., Shizuoka, Japan), aged 5 weeks at the beginning of experiments, were used. They were housed in plastic cages, received food (CE2; Clea Japan Inc., Tokyo, Japan) and water *ad libitum*, and were maintained on a 12/12-h light/dark cycle (lights on from 0800 to 2000 hours). Behavioral experiments were carried out in a sound-attenuated and air-regulated experimental room, to which mice were habituated for at least 1 h. All experiments were performed by a blind manner 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 (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 Pharmaceutical KK (Tokyo, Japan). (−)-Nicotine di-[+]tartrate, SCH-23390 (*R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl- 2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride), *S*(−)-sulpiride, and mecamylamine were purchased from Sigma (St Louis, MO, USA). Sulpiride was initially dissolved in a minimum volume of 0.1N HCl and was then diluted with saline. $A\beta_{25-35}$ was obtained from Wako (Osaka, Japan), and was dissolved in saline at the concentration of 3 mM and stored at −20°C. The $A\beta_{25-35}$ (3 mM) were aggregated, or 'aged', by incubating in saline at 37°C for 4 days according to previous report (Maurice *et al*, 1996).

Drug Treatments

The intracerebroventricular injection of $A\beta_{25-35}$ was performed according to the protocol of Maurice *et al* (1996). Briefly, a microsyringe with a specially made 28-gauge stainless-steel needle, 3 mm in length, was used for microinjection. Mice were anesthetized lightly with ether, and inserted needle unilaterally 1 mm to the right of the midline point equidistant from each eye, at an equal distance between the eyes and the ears and perpendicular to the plane of the skull. $A\beta_{25-35}$ (9 nmol/3 μl) or saline (3 μl) was delivered gradually within 30 s. Mice exhibited normal behavior within 1 min after injection. The injection site was confirmed by injecting Indian ink in preliminary experiments. Neither insertion of the needle nor injection of the saline had a significant influence on survival, and behavioral responses or cognitive functions (Maurice *et al*, 1996).

The saline- and $A\beta_{25-35}$-injected mice were administered galantamine (p.o.), mecamylamine (s.c.), SCH-23390 (s.c.), and sulpiride (s.c.) 60, 30, 30, and 60 min, respectively, before the training session of the novel object recognition and the conditioning phase of the cued and contextual fear-conditioning tasks. The dosages used in the present study were referred to and converted from the clinical doses, our previous publications, and/or other related researches being done in the laboratory, and determined in the preliminary researches for this study. All compounds were systemically administered at a volume of 0.1 ml/10 g body weight.

Behavioral Procedures

Previous reports have shown that acute exposure of hippocampal cultures to aged $A\beta_{25-35}$ induced an apoptotic-mediated neuronal toxicity during a 6-day incubation and that acute injection of aged $A\beta_{25-35}$ also induced cognitive dysfunction in several learning and memory tests

in mice (Lockhart *et al*, 1994). In the present study, the behavioral tests started on 6 days after $A\beta_{25-35}$ injection, and were carried out sequentially according to the experimental schedule shown in Figure 1.

Novel Object Recognition Test

The task was carried out on the days 6–8 after $A\beta_{25-35}$ injection according to the protocol of Nagai *et al* (2003) and Kamei *et al* (2006) with a minor modification. The experimental apparatus consisted of a Plexiglas open-field box (40 × 40 × 29 (H) cm), the floor of which was covered with paper bedding (Japan SLC Inc., Shizuoka, Japan). The apparatus was placed in a sound-isolated room. A light bulb, fastened in the upper part of the room and cannot be seen directly by the mice, provided a constant illumination of about 40 lux at the level of the task apparatus.

The novel-object recognition task procedure consisted of three sessions: habituation, training, and retention sessions. Each mouse was individually habituated to the box, with 10 min of exploration in the absence of objects on the day 6 (habituation session). During the training session on the day 7, two objects (A and B) were placed in the back corner of the box, 10 cm 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 with two stopwatches. Exploration of an object was defined as directing the nose to the object at a distance of less than 2 cm and/or touching it with the nose. During the retention session on the day 8 (24 h after the training session), the animals were placed back into the same box, in which one (eg object A) of the familiar objects used during training 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 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 (eg A or B/(B + A) × 100 (%) in the training session, and B or C/(B + C) × 100 (%) in the retention session).

Cued and Contextual Fear-Conditioning Tests

The cued and contextual fear-conditioning tasks were carried out on the days 9–10 after $A\beta_{25-35}$ infusion according to a previous report (Enomoto *et al*, 2005) with a minor modification. For measuring basal levels of freezing response (preconditioning phase), on the day 9, mice were individually placed in a neutral cage (23 × 23 × 12 cm) for 1 min and then in the conditioning cage (25 × 31 × 11 cm) 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 0.8 mA was delivered as an unconditioned stimulus through a shock generator (Neuroscience Idea Co. Ltd, Osaka, Japan). This procedure was repeated four times with 15-s intervals. Cued and contextual tests were carried out 24 h after fear conditioning on the day 10. 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 with 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 tone and the unconditioned stimulus. The freezing response was defined as that all the paws of a mouse stayed still and stooped down with fear.

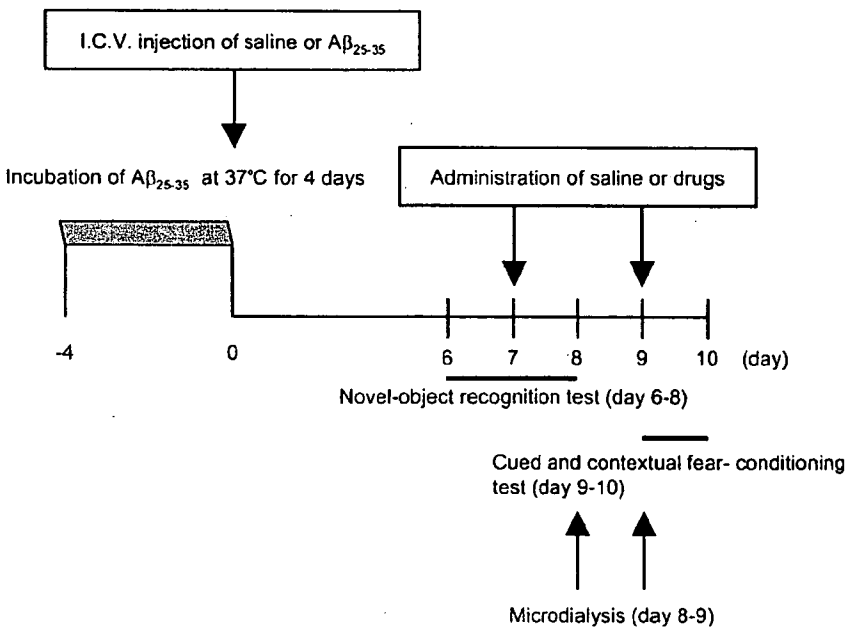

Figure 1 Behavioral experimental schedule. i.c.v., intracerebroventricular.

Determination of Extracellular Dopamine Levels in the Hippocampus

We examined the effect of galantamine on the extracellular level of dopamine in the hippocampus of saline- and $A\beta_{25-35}$-injected mice. At 7 or 8 days after $A\beta_{25-35}$ infusion, the mice were anesthetized with sodium pentobarbital (40 mg/kg) and a guide cannula (MI-AG-4; Eicom, Kyoto, Japan) was implanted into the hippocampus (coordinates: anteroposterior (AP): +3.05 mm, mediolateral (ML): +3.03 mm from bregma, dorsoventral (DV): 2.00 mm from the skull), according to the atlas of Paxinos and Franklin (2004). At 8 or 9 days after $A\beta_{25-35}$ infusion (24 h after the implantation of the guide cannula), the dialysis probe (A-I-4-02; membrane length 2 mm, Eicom) was implanted into the hippocampus and perfused with Ringer's solution (147 mM NaCl, 4 mM KCl, and 2.3 mM $CaCl_2$) at a flow rate of 1.2 µl/min. The outflow fractions were collected every 10 min. After the collection of three stable baseline fractions, mice were treated with galantamine, nicotine, and/or mecamylamine, and the dialysates were collected every 10 min for 90 min. Dopamine levels in the dialysates were assayed by HPLC equipped with Eicompak PP-ODS column and electrochemical detector (ECD-300, Eicom).

Statistical Analysis

Results are expressed as means ± SEM. Statistical difference among the experimental groups was tested using one-way analysis of variance (ANOVA) for behavioral tests or two-way ANOVA for microdialysis, and Tukey–Kramer *post hoc* test was employed for multiple comparisons. *P*-values less than 0.05 were accepted as significant.

RESULTS

Effects of Galantamine on the Impairments of Performance in Novel Object Recognition and Cued/Contextual Fear-Conditioning Tasks in the $A\beta_{25-35}$-Injected Mice

During the training session of the novel object recognition task, saline- and $A\beta_{25-35}$-injected mice treated with saline or galantamine (1 and 3 mg/kg) spent equal amount of time in exploring either of the two objects (Figure 2a), and thus there was no biased exploratory preference in the five groups without affecting total exploring time in the exploration of the objects (data not shown). When retention performance was tested 24 h after the training session, the

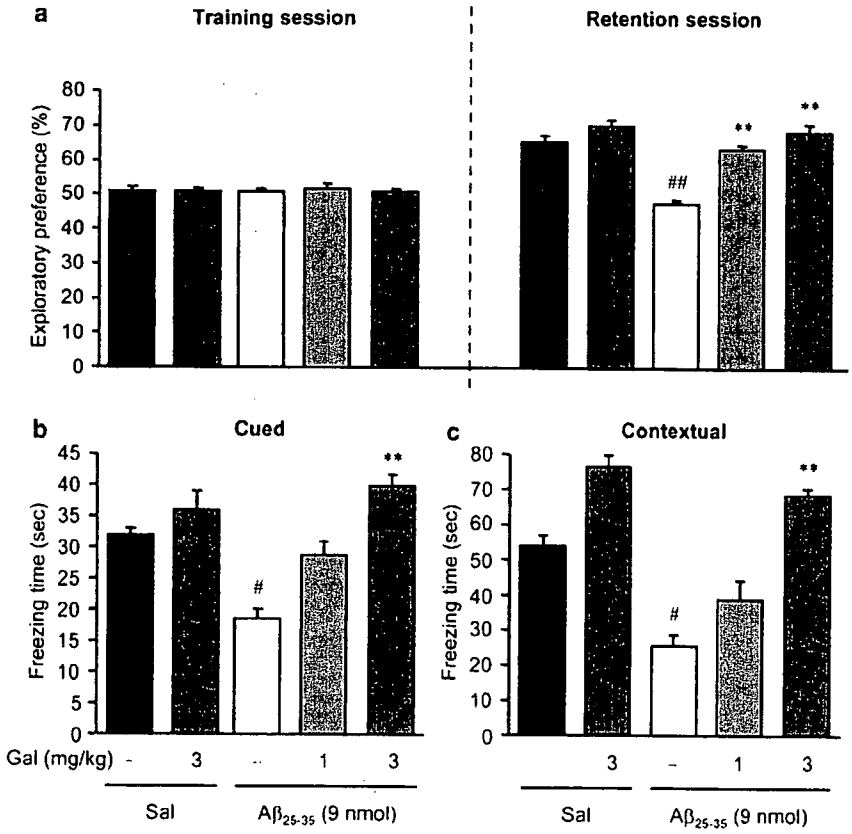

Figure 2 Effects of galantamine on behavioral deficits in $A\beta_{25-35}$-injected mice in novel object recognition, and cued and contextual fear-conditioning tasks. Galantamine (1 and 3 mg/kg p.o.) was administrated to saline- and $A\beta_{25-35}$ (9 nmol/3 µl)-injected mice 60 min before the training session of the novel object recognition task and the conditioning phase of the cued and contextual fear-conditioning tasks. (a) Novel object recognition task. In training session, $F(3,61) = 0.14$ ($p = 0.94$). In retention session, $F(3,61) = 27.44$ ($p < 0.01$). (b) Cued conditioning task, $F(3,61) = 8.68$ ($p < 0.01$). (c) Contextual conditioning task, $F(3,61) = 6.97$ ($p < 0.01$). Results were expressed as means ± SEM ($n = 13$–17), and analyzed by a one-way ANOVA, followed by Tukey–Kramer test for multiple comparisons. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs* saline-treated, saline-injected mice; $^{**}p < 0.01$ *vs* saline-treated, $A\beta_{25-35}$-injected mice.

level of exploratory preference for the novel object in the saline-treated $A\beta_{25-35}$-injected mice was significantly decreased compared to that in the saline-treated, saline-injected mice ($p<0.01$ by *post hoc* (Figure 2a)). Galantamine (1 and 3 mg/kg)-treated $A\beta_{25-35}$-injected mice spent a significantly longer time in exploring novel object than the saline-treated $A\beta_{25-35}$-injected mice ($p<0.01$ by *post hoc*) (Figure 2a), indicating that galantamine improved the recognition of novelty in mice impaired by $A\beta_{25-35}$ infusion.

In the preconditioning phase of the cued and contextual fear-conditioning task, the saline- and $A\beta_{25-35}$-injected mice treated with saline or galantamine (1 and 3 mg/kg) hardly showed the freezing response (data not shown), and there were no differences in the basal levels of freezing response among the five groups (data not shown). In the cued and contextual fear-testing trial, saline-treated, saline-injected mice exhibited marked cued and contextual freezing response, indicating that the associative learning ability in these mice is better than that in saline-treated $A\beta_{25-35}$-injected mice, which showed a significant decrease of cued and contextual freezing response 24 h after fear conditioning ($p<0.01$ by *post hoc*) (Figure 2b and c). The performance of saline-treated, $A\beta_{25-35}$-injected mice was completely reversed by the treatment with galantamine at the dose of 3 mg/kg, but not 1 mg/kg (Figure 2b and c). Galantamine (3 mg/kg) tended to increase the cognition in both asks in saline-injected mice, but not significant. No alterations of nociceptive response were found in all the groups: the minimal current required to elicit flinching/running, jumping, or vocalization was same in all the groups (data not shown). As galantamine (1 and 3 mg/kg) significantly improved the $A\beta_{25-35}$-induced cognitive impairment in dose-dependent manner, the dose of 3 mg/kg was used in subsequent experiments.

Antagonistic Effects of Mecamylamine, an nAChR Antagonist, on the Cognitive Improving Effects of Galantamine in the $A\beta_{25-35}$-Injected Mice

To determine whether the improving effects of galantamine on $A\beta_{25-35}$-induced cognitive impairments are mediated via nAChRs, we examined the antagonism by mecamylamine, an nAChRs antagonist, against the cognitive improving effects of galantamine in $A\beta_{25-35}$-injected mice.

In the training session of the novel object recognition task or the preconditioning phase of cued/contextual fear-conditioning tasks, there were no differences in the exploratory preference for the objects or the basal levels of freezing response, respectively, among all the groups. Galantamine (3 mg/kg) improved cognitive dysfunction in novel object recognition and tone cue- and context-

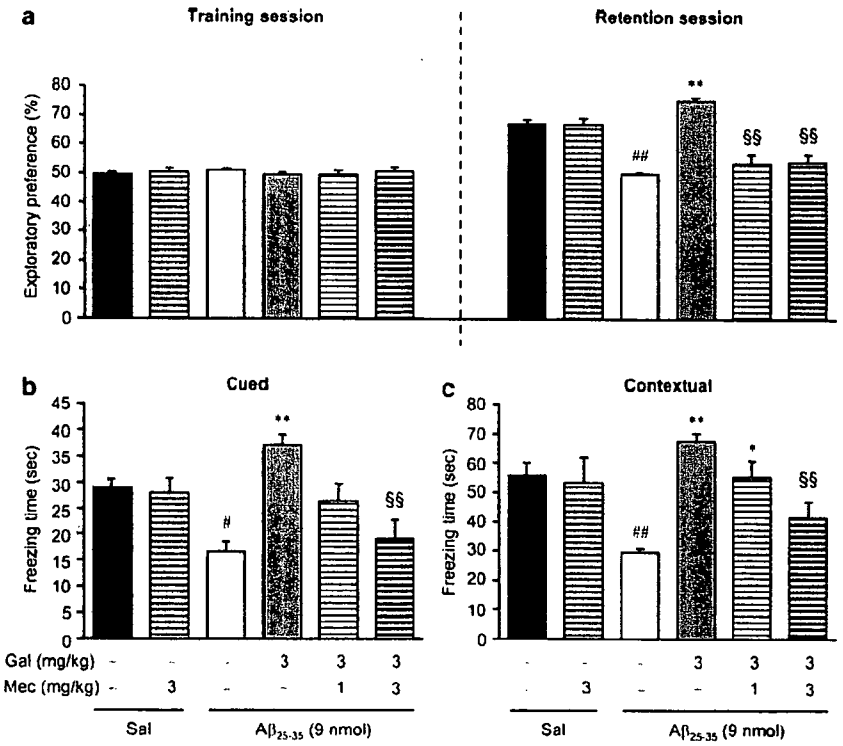

Figure 3 Antagonistic effects of mecamylamine, an nAChR antagonist, on cognitive improving effects of galantamine in $A\beta_{25-35}$-injected mice. Galantamine (3 mg/kg p.o.) and mecamylamine (Mec: 1 and 3 mg/kg) were administrated to saline- and $A\beta_{25-35}$ (9 nmol/3 μl)-injected mice 60 and 30 min, respectively, before the training session of novel object recognition task and the conditioning phase of cued and contextual fear-conditioning tasks. (a) Novel object recognition task. In training session, $F(5,63)=0.32$ ($p=0.90$). In retention session, $F(5,63)=25.83$ ($p<0.01$). (b) Cued conditioning task, $F(5,63)=7.73$ ($p<0.01$). (c) Contextual conditioning task, $F(5,63)=7.90$ ($p<0.01$). Results were expressed as means ± SEM ($n=8$–12), and analyzed by a one-way ANOVA, followed by Tukey–Kramer test for multiple comparisons. $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs* saline-treated, saline-injected mice; $^{**}p<0.01$ *vs* saline-treated, $A\beta_{25-35}$-injected mice; $^{\S\S}p<0.01$ *vs* galantamine-treated, $A\beta_{25-35}$-injected mice. Sal, saline; Gal, galantamine; Mec, mecamylamine.

conditioned fear-learning tasks in $A\beta_{25-35}$-injected mice. The nAChR antagonist mecamylamine (3 mg/kg) significantly and completely blocked the improving effects of galantamine on the impairment of recognition ($p<0.01$ by *post hoc*, Figure 3a) and cued/contextual-dependent fear learning ($p<0.01$ by *post hoc*, Figure 3b and c) in the $A\beta_{25-35}$-injected mice. In saline-injected mice, mecamylamine (3 mg/kg) by itself had no effect on the novel object recognition and cued/contextual fear-conditioning performances (Figure 3).

Effects of Scopolamine, a Muscarinic Receptor Antagonist, on the Cognitive Improving Effects of Galantamine in the $A\beta_{25-35}$-Injected Mice

To determine whether muscarinic receptors are involved in the effects of galantamine on the performance of $A\beta_{25-35}$-injected mice in the cognitive tasks, the muscarinic receptor antagonist, scopolamine (0.1 and 0.2 mg/kg), was s.c. injected to the mice 30 min after the p.o. administration of galantamine (3 mg/kg). Scopolamine at the dose of 0.2 mg/kg impaired the performance of saline-i.c.v.-injected mice in both novel object recognition and tone cue- and context-conditioned fear-learning tasks (Figure 4a–c). Galantamine (3 mg/kg) improved cognitive dysfunction in novel object recognition and tone cue- and context-conditioned fear-learning tasks in $A\beta_{25-35}$-injected mice. In the novel object recognition test, scopolamine (0.1 and 0.2 mg/kg) failed to prevent the effect of galantamine (3 mg/kg) (Figure 4a). In the cued and contextual conditioning tasks, the effects of galantamine (3 mg/kg) were not significantly prevented by scopolamine at the dosage that induces behavioral impairments in the task in saline-i.c.v.-injected mice (Figure 4b and c). In order to well understand the effects of scopolamine on the performance of mice in these tasks, we added the galantamine (3 mg/kg)-treated and galantamine (3 mg/kg)/scopolamine (0.2 mg/kg)-treated saline-i.c.v.-injected control groups. Scopolamine (0.2 mg/kg) failed to affect the performance in the novel object recognition task in galantamine-treated mice (Figure 4a). In the cued and contextual conditioning tasks, there is the tendency to inhibit the performance in galantamine (3 mg/kg)-treated mice by scopolamine (2 mg/kg), but not significant (Figure 4b and c). Although the effects of galantamine was not significantly prevented by scopolamine in the cued and contextual conditioning tasks, the performance of mice is more sensitive to scopolamine in this task than in the novel object recognition task (Figure 4a–c).

Effects of Galantamine on the Extracellular Dopamine Level in the Hippocampus of the Saline- and $A\beta_{25-35}$-Injected Mice

We examined whether galantamine at the dose of 3 mg/kg, which ameliorated the cognitive dysfunction in the $A\beta_{25-35}$-injected mice, facilitated the dopamine release in the hippocampus of saline- and $A\beta_{25-35}$-injected mice.

As shown in Figure 5a, the basal extracellular level of dopamine in the hippocampus of $A\beta_{25-35}$-injected mice

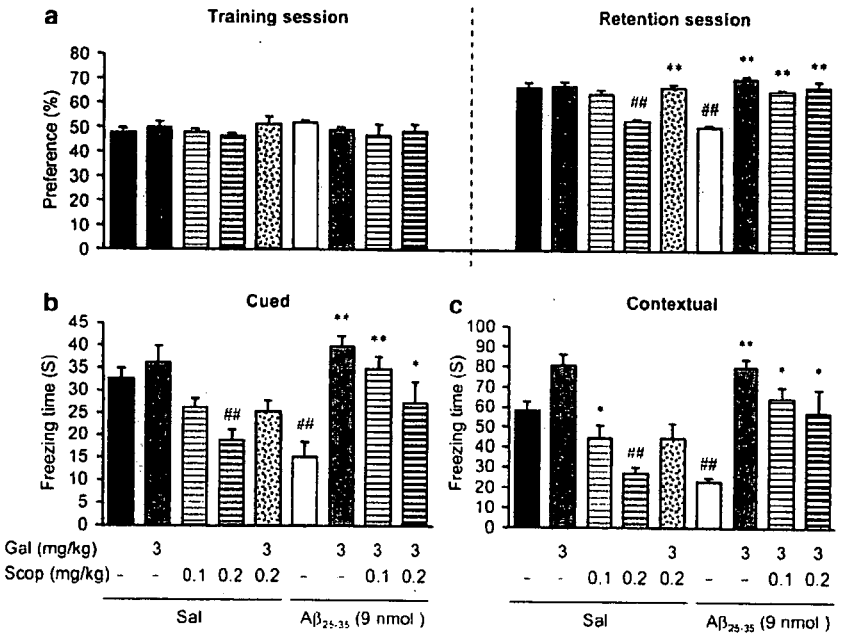

Figure 4 Effects of scopolamine, a muscarinic antagonist, on cognitive improving effects of galantamine in $A\beta_{25-35}$-injected mice. Galantamine (Gal: 3 mg/kg p.o.) and scopolamine (Scop: 0.1 and 0.2 mg/kg) were administrated to saline- and $A\beta_{25-35}$ (9 nmol/3 μl)-injected mice 60 and 30 min, respectively, before the training session of novel object recognition task and the conditioning phase of cued and contextual fear-conditioning tasks. (a) Novel object recognition task. In training session, $F(5,63)=0.32$ ($p=0.90$). In retention session, $F(8,90)=60.10$ ($p<0.01$). (b) Cued conditioning task, $F(8,90)=5.33$ ($p<0.01$). (c) Contextual conditioning task, $F(8,90)=8.04$ ($p<0.01$). Results were expressed as means ± SEM ($n=8$–12), and analyzed by a one-way ANOVA, followed by Tukey–Kramer test for multiple comparisons. $^{\#\#}p<0.01$ *vs* saline-treated saline-injected mice; $^{*}p<0.05$, $^{**}p<0.01$ *vs* saline-treated, $A\beta_{25-35}$-injected mice. Sal, saline; Gal, galantamine; Scop, scopolamine.

were significantly decreased compared to that of saline-injected mice. Galantamine (3 mg/kg) caused a marked increase in the extracellular level of dopamine in the hippocampus of saline- (Figure 5b) and $A\beta_{25-35}$-injected mice (Figure 5c). The significant increase in the extracellular level of dopamine was observed from about 20 min after galantamine administration ($p<0.01$ by *post hoc*, Figure 5b and c). When mecamylamine (3 mg/kg) was injected to saline- and $A\beta_{25-35}$-injected mice 30 min after galantamine administration, the galantamine-induced elevation of extracellular dopamine levels was significantly diminished (Figure 5b and c). However, mecamylamine by itself did not significantly affect the extracellular dopamine levels in the saline- and $A\beta_{25-35}$-injected mice (Figure 5b and c).

To confirm that dopamine release is facilitated through nAChR stimulation by galantamine, we measured extracellular dopamine level induced by nicotine in combination with galantamine in the hippocampus of $A\beta_{25-35}$-injected mice. The individual administration with nicotine at the dose of 0.4 mg/kg or galantamine at the dose of 1 mg/kg does not affect the extracellular level of dopamine in the hippocampus of $A\beta_{25-35}$-injected mice, However, the combination of nicotine (0.4 mg/kg) with galantamine (1 mg/kg) significantly increased the extracellular level of dopamine in the hippocampus of $A\beta_{25-35}$-injected mice (Figure 6). The potentiating effect of galantamine on dopamine release was antagonized by mecamylamine (3 mg/kg) administration (Figure 6). The synergistic effects of nicotine and galantamine at low doses, and the fact that

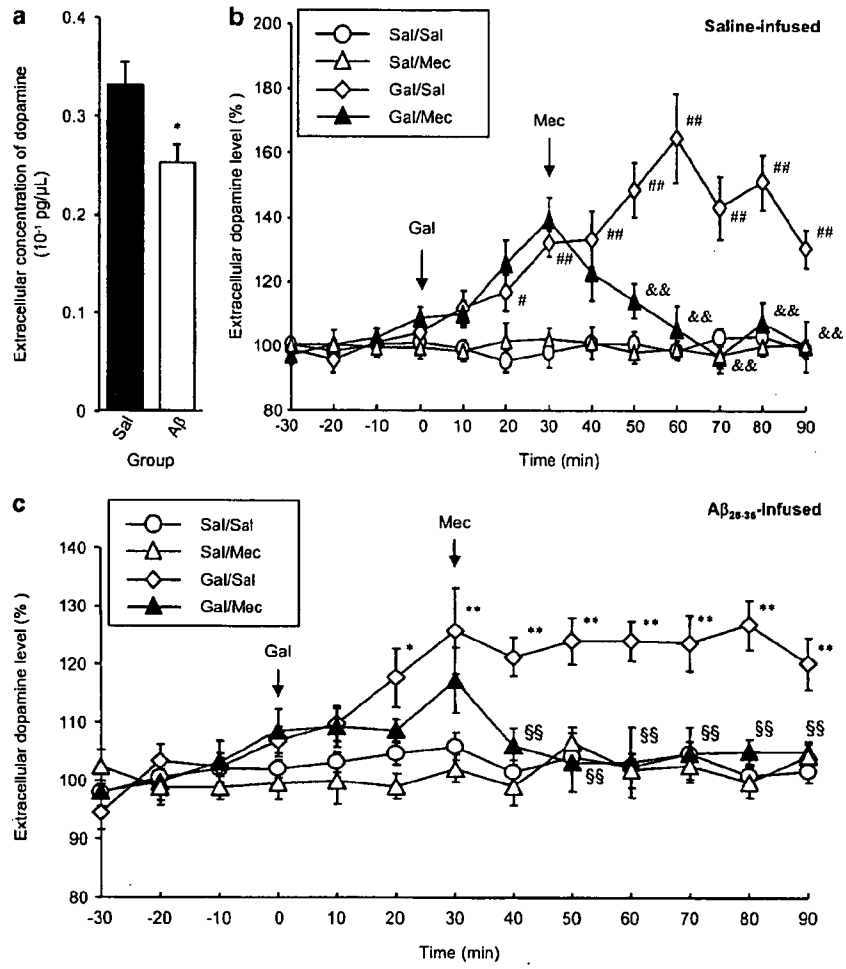

Figure 5 Effects of galantamine on extracellular dopamine level in the hippocampus of saline- and $A\beta_{25-35}$-injected mice. At 8 or 9 days after $A\beta_{25-35}$ infusion (24 h after the implantation of the guide cannula), the dialysis probe was implanted into the hippocampus. Saline- and $A\beta_{25-35}$-injected mice were treated with galantamine (Gal: 3 mg/kg p.o.) and/or mecamylamine (Mec: 3 mg/kg s.c.), and dialysates were collected every 10 min for 90 min. Dopamine levels in the dialysates were assayed by HPLC with electrochemical detection. (a) Spontaneous extracellular dopamine levels in the hippocampus of saline- and $A\beta_{25-35}$-injected mice. Results were expressed as means ± SEM, $n=8$. $^{*}p<0.05$ *vs* saline-i.c.v.-injected mice, by Student's *t*-test. (b) Effects of galantamine on extracellular dopamine level in the hippocampus of saline-injected mice. Results were expressed as means ± SEM, $n=5$–8, and analyzed by a two-way ANOVA, followed by Tukey–Kramer test for multiple comparisons, $F_{time}(12,259)=3.74$ ($p<0.01$); $F_{group}(3,259)=37.71$ ($p<0.01$). (c) Effects of galantamine on the extracellular dopamine level in the hippocampus of $A\beta_{25-35}$-injected mice. Results were expressed as means ± SEM, $n=5$–8, and analyzed by a two-way ANOVA, followed by Tukey–Kramer test for multiple comparisons, $F_{time}(12,324)=5.79$ ($p<0.01$); $F_{group}(3,324)=50.62$ ($p<0.01$). $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs* saline-treated saline-injected mice. $^{\&\&}p<0.01$ *vs* galantamine-treated saline-injected mice. $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs* saline-treated, $A\beta_{25-35}$-injected mice; $^{\S\S}p<0.01$ *vs* galantamine-treated, $A\beta_{25-35}$-injected mice. Sal, saline; Gal, galantamine; Mac, mecamylamine.

the synergy was antagonized by mecamylamine, indicated that galantamine indeed potentiates an nAChR-mediated effect.

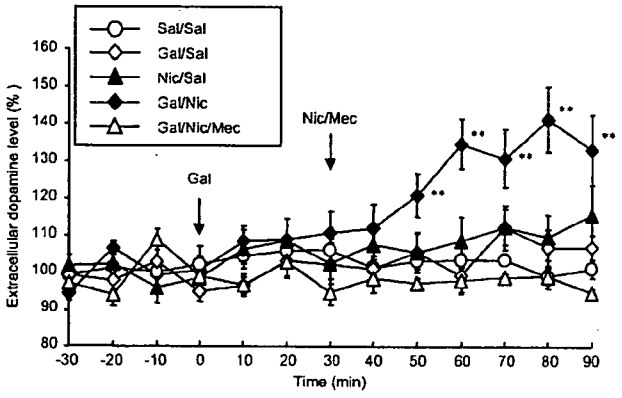

Figure 6 Effects of combined treatment with nicotine and galantamine at their non-effective doses on extracellular dopamine level in the hippocampus of $A\beta_{25-35}$-injected mice. $A\beta_{25-35}$-injected mice were administered nicotine (Nic: 0.4 mg/kg s.c.) and mecamylamine (Mec: 3 mg/kg s.c.) 30 min after galantamine (Gal: 1 mg/kg, p.o.) treatment, and dialysates were collected every 10 min for 90 min. Results were expressed as means ± SEM, $n = 5–8$, and analyzed by a two-way ANOVA, followed by Tukey-Kramer test for multiple comparisons, $F_{time}(12,376) = 4.56$ ($p < 0.01$); $F_{group}(3,376) = 22.71$ ($p < 0.01$). $^{**}p < 0.01$ *vs* saline-treated $A\beta_{25-35}$-injected mice.

Involvement of Dopaminergic Systems in the Cognitive Improving Effects of Galantamine in $A\beta_{25-35}$-Injected Mice

To clarify whether the improving effects of galantamine on $A\beta_{25-35}$-induced cognitive impairments are mediated through the activation of dopamine receptors, we investigated the antagonism of the cognitive improving effects of galantamine on $A\beta_{25-35}$-injected mice by SCH-23390, a dopamine-D1 receptor antagonist, and sulpiride, a dopamine-D2 receptor antagonist.

SCH-23390 (0.02 mg/kg) and sulpiride (12.5 mg/kg) significantly and completely antagonized the improving effects of galantamine on $A\beta_{25-35}$-induced cognitive impairment without affecting the exploratory preference for the objects in the training session of the novel object recognition task. SCH-23390 and sulpiride showed no effect on the total exploration time in either the training or retention sessions of the novel object recognition task. In addition, SCH-23390 and sulpiride by themselves had no effect on novel object recognition performance in saline-injected mice (Figure 7a).

SCH-23390 (0.02 mg/kg) and sulpiride (12.5 mg/kg) significantly blocked the ameliorating effects of galantamine on the impairments of both cued and contextual fear conditioning induced by infusion of $A\beta_{25-35}$. SCH-23390 and sulpiride by themselves had no effects on the cued and contextual freezing response in saline-injected mice (Figure 7b and c).

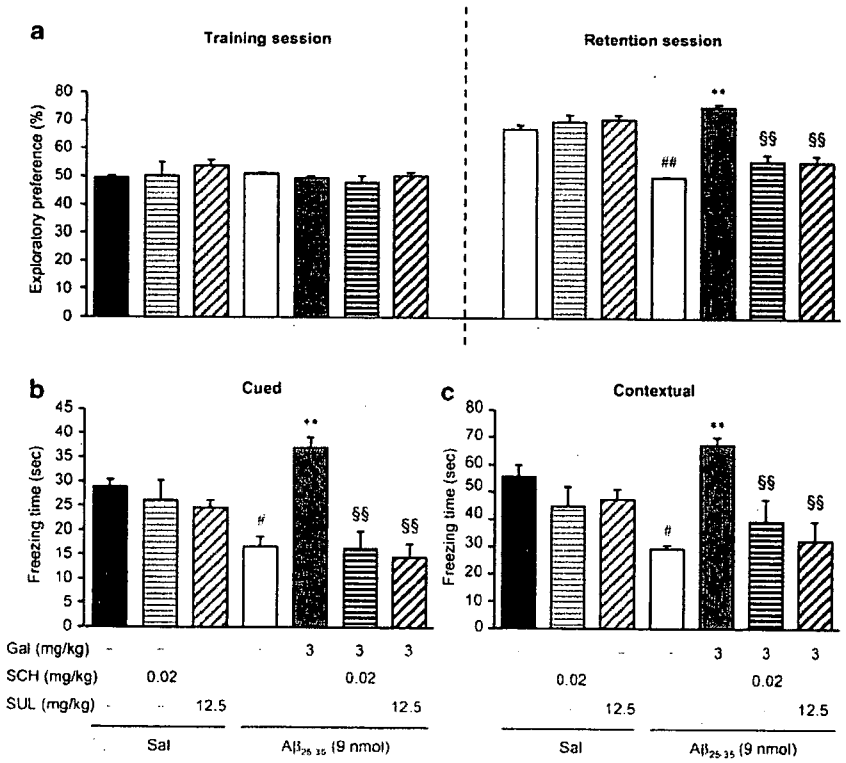

Figure 7 Involvement of dopaminergic systems in the cognitive improving effects of galantamine in $A\beta_{25-35}$-injected mice. Galantamine (p.o.), SCH-23390 (s.c.) and sulpiride (s.c.) were administrated to saline- and $A\beta_{25-35}$ (9 nmol/3 µl)-injected mice 60, 30 and 60 min respectively before the training session of novel object recognition task, and the conditioning phase of cued and contextual fear-conditioning task. (a) Novel-object recognition task. In training session, $F(6,72) = 0.73$ ($p = 0.63$). In retention session, $F(6,72) = 27.13$ ($p < 0.01$). (b) Cued conditioning task. $F(6,72) = 9.61$ ($p < 0.01$). (c) Contextual conditioning task. $F(6,72) = 6.36$ ($p < 0.01$). Results were expressed as means ± SEM, $n = 8–12$, and analyzed by a one-way ANOVA, followed by Tukey-Kramer test for multiple comparisons. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs* saline-treated saline-injected mice; $^{**}p < 0.01$ *vs* saline-treated $A\beta_{25-35}$-injected mice; $^{\S\S}p < 0.01$ *vs* galantamine-treated $A\beta_{25-35}$-injected mice. Sal: saline, Gal: galantamine, SCH: SCH-23390, SUL: sulpiride.

DISCUSSION

We found that $A\beta_{25-35}$ infusion impaired novelty-discriminating ability in the novel object recognition task and associative learning and memory in the fear-conditioning tasks. It is unlikely that the impairment of performance of the $A\beta_{25-35}$-injected mice in these tasks is due to changes in motivation or sensorimotor function, as various motivations are involved in these behavioral tasks, and different skills are required for better performance in each task. Actually, there was no difference of the total exploration time in the training session of novel object recognition task and freezing response in preconditioning phase of cued and contextual fear-conditioning tasks between the saline- and $A\beta_{25-35}$-injected mice, indicating no changes in motor function and exploratory activity. In addition, no difference in pain threshold was found between the saline- and $A\beta_{25-35}$-injected mice. Therefore, the impairment of performance in the $A\beta_{25-35}$-injected mice is due to learning and memory deficits.

Galantamine, a medication for Alzheimer's disease, has a dual mechanism of action, which inhibits acetylcholinesterase and allosterically modulates nAChR as a potent APL (Eisele *et al*, 1993; Santos *et al*, 2002). Previous paper has reported that galantamine reverses nAChR antagonist-induced deficits in delay classical conditioning of the eye blink reflex in young rabbits (Woodruff-Pak *et al*, 2003) and impairment of spatial accuracy of APP23 transgenic mouse during probe trial of Morris water maze (Van Dam and De Deyn, 2006). In the present study, galantamine significantly ameliorated the cognitive impairments induced by $A\beta_{25-35}$ infusion in the novel object recognition and fear-conditioning tasks. Galantamine at 3 mg/kg had no effect on the total exploration time in the training session of novel object recognition task and freezing response in preconditioning phase of cued and contextual fear-conditioning tasks, and sensitivity to electric footshock in the fear-conditioning phase of cued and contextual fear-conditioning tasks in the $A\beta_{25-35}$-injected mice. Therefore, it is unlikely that the observed improvement of performance by galantamine in both tasks is due to changes in sensorimotor function and/or motivation in the $A\beta_{25-35}$-injected mice, and it is apparently valid that galantamine ameliorates learning and memory deficits caused by the infusion of $A\beta_{25-35}$ into the cerebral ventricle in mice. The improving effects of galantamine on the performance of $A\beta_{25-35}$-injected mice were prevented by the treatment with mecamylamine, an nAChR antagonist, at the dose that did not significantly affect the performance of saline-i.c.v.-injected mice. These findings support the notion that galantamine improves $A\beta_{25-35}$-induced cognitive impairment via activation of nAChRs. The roles of muscarinic receptors in the effects of galantamine were also investigated in the present study. The effects of galantamine on the performance of $A\beta_{25-35}$-injected mice in the novel object recognition task were not prevented by scopolamine at the dose which impaired the performance of saline-i.c.v.-injected mice. This indicated that muscarinic receptors are not very important for effects of galantamine on this cognitive task. Our conclusion is supported by the reports that there is only 1–12% brain AChE inhibition 1 h after s.c. injection of 3 mg/kg galantamine (Geerts *et al*, 2005), and that galantamine is an nAChR-allosteric modulator (Eisele *et al*, 1993; Santos *et al*, 2002). In other words, although the brain concentration of acetylcholine is only weakly increased by galantamine at the dose of 3 mg/kg, it shows effects mainly by allosterically modulating the function of nAChR. Our results also indicated that the potentiation of the nAChR function can compensate the hypofunction of muscarinic receptors in the present cognitive tasks, especially in the novel object recognition task. As scopolamine at a dose of 0.2 mg/kg showed some effect, but not significant, on the performance of galantamine-treated mice in the cued and contextual conditioning tasks, it was indicated that muscarinic receptors may differentially regulate the observed effects of galantamine, depending on specific behavioral tasks. Although mecamylamine blocks the effects of galantamine more strongly than scopolamine, it is hard to entirely exclude the role of muscarinic receptors in the effects of galantamine, as nAChR antagonists not only block the function of nAChRs but also eliminate the desensitization of nAChR-induced increase of the function of muscarinic receptors (Wan *et al*, 2003).

The mechanism of learning and memory impairments in the $A\beta_{25-35}$-injected mice is still not clear. Harkany *et al* (1998) have shown that bilateral injection of $A\beta[Phe(SO_3H)^{24}]_{25-35}$ peptide, a metabolically stable analog of $A\beta_{25-35}$, into the rat nucleus basalis magnocellularis causes a reduction of cortical acetylcholinesterase-positive projections. In PC12 cells, $A\beta$ has been found to suppress the expression of nAChRs, such as the decrease of nAChR-binding sites, subunit proteins, and mRNA levels (Guan *et al*, 2001, 2003). In the present study, *in vivo* microdialysis experiment revealed that the basal extracellular level of dopamine in the hippocampus of $A\beta_{25-35}$-injected mice was decreased compared to that of saline-injected mice. Furthermore, we have previously demonstrated by *in vivo* microdialysis that continuous infusion of $A\beta_{1-40}$ markedly decreased high potassium- and nicotine-induced release of acetylcholine and dopamine in the hippocampus, cerebral cortex, and striatum, respectively (Itoh *et al*, 1996). These findings confirmed that the deposition of $A\beta$ in the brain is in some way related to the impairment of cognition and cholinergic–dopaminergic degeneration and suggest that dysfunctions of cholinergic and dopaminergic systems are responsible, at least in part for the $A\beta$-induced learning and memory deficits.

It has been reported that galantamine (10 μM, 4 days) dose not significantly affect the mRNA level and protein expression of nAChR subunits (Kume *et al*, 2005). Galantamine increases cholinergic function mainly in two ways: (a) increasing the concentration of acetylcholine through a competitive reversible inhibition of acetylcholine hydrolysis by acetylcholinesterase, which will increase the extracellular acetylcholine concentration and (b) allosteric modulation of nAChRs (Woodruff-Pak and Santos, 2000). The potential cognitive improving effects of galantamine on Alzheimer's disease may be related, in part, to the stimulation of dopamine neurotransmission in addition to its enhancing effects on cholinergic systems by inhibition of acetylcholinesterase. The *in vivo* microdialysis experiment showed that galantamine significantly increased the dopamine release in the hippocampus of saline- and $A\beta_{25-35}$-injected mice. The effect of nicotine on dopamine release

was strengthened by galantamine, at their noneffective doses, and antagonized by mecamylamine. Accordingly, it is plausible that galantamine ameliorates the $A\beta_{25-35}$-induced learning and memory deficits by activating nAChR, and thereby stimulates release of dopamine in the brain. Further, we found that the improving effects of galantamine were prevented by SCH-23390, a dopamine-D1 receptor antagonist, and sulpiride, a dopamine-D2 receptor antagonist. Taken together, our results suggest that these hippocampus-dependent performance in these tasks were impaired by $A\beta_{25-35}$ infusion as the result of failure of nAChR and dopamine responses, as the hippocampus is involved in the object recognition behavior (Rampon *et al*, 2000; Hammond *et al*, 2004) and the contextual fear conditioning (Daumas *et al*, 2004). These findings provide the first *in vivo* evidence that galantamine augments dopaminergic neurotransmission within the hippocampus through the allosteric activation of nAChR.

The deficit in the nAChR-dopaminergic systems is one of the facets of general degeneration in neurons induced by $A\beta_{25-35}$ treatment. The cognitive improving effects of galantamine at the present dose depend on the function of nAChR-dopaminergic systems, therefore the effects are prone to be blocked by the antagonism of the nAChR-dopaminergic systems. However, in the normal animals, neurons and their functions are almost intact: the functions and the homeostasis in neurons slightly impaired by the antagonists of the nAChR-dopaminergic systems at relatively low doses, as they can somewhat be restored by compensating mechanisms that are not very clear until now.

Because the improving-effects of galantamine on the cognitive dysfunction induced by $A\beta_{25-35}$ i.c.v. injection may be mediated through the activation of, at least in part, dopaminergic systems, we postulate that galantamine may activate dopaminergic neurotransmission in Alzheimer's disease by augmenting the activation of nAChR. This is supported by the fact that galantamine potentiated the hippocampal dopamine release in the $A\beta_{25-35}$-injected model of Alzheimer's disease, and the effects of galantamine on cognition and dopamine release was antagonized by an AChR antagonist, mecamylamine. Because the dopaminergic dysfunction has been implicated in the progress of Alzheimer's disease, dopaminergic agents may be beneficial in the treatment, and enhancement of dopamine release may be part of the mechanisms underlying the therapeutic benefit of galantamine. The results also suggest that in addition to Alzheimer's disease, galantamine may be valuable in the treatment of other diseases involving the dysfunction of dopaminergic systems, such as Parkinson's disease and neuropsychiatric dysfunctions including anxiety, depression, apathy, and psychosis.

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REINFORCING EFFECTS OF MORPHINE ARE REDUCED IN TISSUE PLASMINOGEN ACTIVATOR-KNOCKOUT MICE

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Abstract—Tissue plasminogen activator (tPA) plays a key role in neuroplasticity. We have recently demonstrated that the tPA-plasmin system is involved in the rewarding effects of drugs of abuse by regulating the release of dopamine in the nucleus accumbens. In the present study, we investigated whether tPA is involved in the reinforcing properties of morphine in a paradigm of drug self-administration. Eight-week-old tPA knockout and wild-type control mice were subjected to a single 24-h session of morphine self-administration under a fixed ratio (FR) 2 or a progressive ratio (PR) schedule of reinforcement after eight daily 30-min sessions of nose-poke training. tPA knockout mice responded significantly more often for morphine self-administration in a dose-dependent manner as compared with wild-type control mice. Under the PR schedule of morphine reinforcement, however, tPA knockout mice showed a lower breaking point than wild-type control mice. There was no significant difference in food-reinforced operant behavior, breaking points to food pellets, and saline self-administration between the two genotypes. The increased responding in tPA knockout mice under the FR2 schedule was significantly attenuated by the dopamine D1 receptor antagonist SCH23390 (0.3 mg/kg), whereas SCH23390, at a dose range of 0.03–2.0 mg/kg, demonstrated biphasic effects on morphine self-administration in wild-type control mice. Our findings suggest that the reinforcing effects of morphine are reduced in tPA knockout mice. Modulation of the tPA system in the brain may be a potential target against drugs of abuse.

Key words: tPA knockout mice, drug self-administration, morphine, reinforcing properties, dopamine D1 receptor antagonist.

Tissue plasminogen activator (tPA) has been detected in the CNS (Teesalu et al., 2004). It has well been characterized that tPA, as an extracellular protease, converts zymogen plasminogen to the active protease plasmin and plays an important role not only in neuronal migration (Moonen et al., 1982; Seeds et al., 1999) and synaptic outgrowth (Krystosek and Seeds, 1981) during the development of the brain, but also in the synaptic plasticity and neurotoxicity of the mature brain, including visual cortical plasticity (Mataga et al., 2002), long-term potentiation (Frey et al., 1996; Huang et al., 1996; Baranes et al., 1998), stress-induced anxiety-like behavior (Pawlak et al., 2003), cerebellar motor learning (Seeds et al., 2003), excitotoxic cell death (Chen and Strickland, 1997; Nicole et al., 2001), and drug dependence (Ito et al., in press; Nagai et al., 2004, 2005a,b, 2006; Pawlak et al., 2005; Yamada et al., 2005). Thus, tPA has been suggested to be a neuromodulator in the CNS (Yamada et al., 2005).

In the nervous system, tPA was found to be packaged in and released directly from catecholamine storage vesicles in response to stimuli using PC12 cells in culture (Gualandris et al., 1996; Parmer et al., 1997; Lochner et al., 2006). Recently, it has been documented that the rapid and activity-dependent induction of tPA expression is controlled by an mRNA translation mechanism mediated by the metabotropic glutamate receptor (Shin et al., 2004). Different downstream cascades are involved in the distinct effects of tPA in the brain, including neuronal damage mediated by the NMDA receptor (Nicole et al., 2001) or protease-activated receptor 1 (PAR-1) (Junge et al., 2003), neurovascular toxicity controlled by activated protein C (APC) (Liu et al., 2004), neural remodeling related to annexin II (Siao and Tsirka, 2002), and neural plasticity via low density lipoprotein receptor-related protein (LRP) (Zhuo et al., 2000; Wang et al., 2003).

We have recently demonstrated that the tPA-plasmin system is involved in the rewarding effects of morphine (MOR) (Ito et al., in press; Nagai et al., 2004, 2005b), methamphetamine (Nagai et al., 2005a) and nicotine (Nagai et al., 2006). Accordingly, tPA knockout mice show a reduction in MOR-, methamphetamine- and nicotine-induced conditioned place preference and locomotor sensitization. Furthermore, exogenous recombinant human tPA or plasmin restored the defect of MOR-induced dopamine release in the nucleus accumbens and hyperactivity in tPA knockout mice (Nagai et al., 2004). Exogenous recombinant tPA and plasmin potentiate, whereas plasminogen activator inhibitor-1 (PAI-1) inhibits, the MOR-induced release of dopamine in the nucleus accumbens and dopamine-dependent hyperlocomotion in wild-type control mice (Nagai et al., 2005b). PAR-1 is involved in the regulation of MOR-induced dopamine release by the tPA–plasmin system (Ito et al., in press). Taken together, it is suggested that tPA plays an important role in the rewarding effects of MOR by regulating dopaminergic transmission (Nagai et al., 2004; Yamada et al., 2005). In the present study, we investigated whether tPA is involved in the reinforcing

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Abbreviations: ANOVA, analysis of variance; FR, fixed ratio; MOR, morphine; PAR-1, protease-activated receptor 1; PR, progressive ratio; tPA, tissue plasminogen activator.

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properties of MOR using a paradigm of drug self-administration. Our findings suggest that the reinforcing effects of MOR are reduced in tPA knockout mice. Modulation of the tPA system in the brain may be a potential target against drugs of abuse.

EXPERIMENTAL PROCEDURES

Subjects and drug treatment

tPA knockout (n=120) and wild-type control mice (n=138) (male and 8 weeks old; Jackson Laboratory, Bar Harbor, ME, USA) weighing 20–25 g were used. All mice were kept in a regulated environment (23±0.5 °C; 50±0.5% humidity) with a reversed 12-h light/dark cycle (lights on at 9:00 A.M.). Water and food were available *ad libitum* in home cages, but unavailable in chambers for 24-h or 12-h test sessions. To minimize the number of mice used and their suffering, all experiments were performed in accordance with Guidelines for Animal Experiments of the Nagoya University School of Medicine, the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

MOR hydrochloride (Dainippon Pharmaceutical, Tokyo, Japan) was dissolved in sterile saline. Under standard experimental conditions, MOR was self-administered in a volume of 2.1 μl per infusion over 5 s. The unit dose for MOR self-administration was based on a previous report (Elmer et al., 2002) and our pilot studies. R-(+)-SCH23390 hydrochloride, a dopamine D1 receptor antagonist, was purchased from Sigma (Sigma-Aldrich Co., St. Louis, MO, USA) and dissolved in sterile saline immediately before injection. During a single 24-h session of self-administration, the mouse was disconnected from the operant system and administered i.v. with SCH23390 or vehicle via the catheter at two time points: immediately after the 5th and 12th h. The first time point was selected to make sure that before SCH23390 treatment, separate groups of mice demonstrated similar nose-pokeresponses after eight daily 30-min sessions of nose-poke training, whereas the second time point was selected to potentiate the pharmacologic effects of SCH23390 on MOR self-administration since the effects of the first infusion of SCH2390 on MOR self-administration were partial during a single 24-h session of MOR self-administration in our pilot studies.

Apparatus for operant self-administration and nose-poke training prior to MOR (or saline) self-administration

Food-reinforced operant behavior, nose-poke training, and MOR (or saline) self-administration were conducted in standard mouse operant conditioning chambers (ENV-307A, Med Associates, Georgia, VT, USA) located within a ventilated sound attenuation cubicle as described previously (Yan et al., 2006).

Except for the groups of tPA knockout and wild-type mice in the food-reinforced operant behavior, all naïve mice were initially subjected to eight daily 30-min sessions of nose-poke training prior to a single 24-h session of MOR (or saline) self-administration under a fixed ratio (FR) 2 schedule or three daily 12-h sessions of MOR/food-reinforced operant responding under a progressive ratio (PR) schedule. The nose-poke training sessions were conducted in the standard operant chamber mentioned above after food deprivation for 12 h. During the nose-poke training sessions, responses for a food pellet under the FR schedules were from FR1 (days 1–5), to FR2 (days 6–7), and then FR3 (day 8), in which active and inactive holes for nose-poke were assigned randomly throughout a session. After acquiring stable nose-poke responses for food pellets (the active responses account for more than 75% of the total number of nose-poke responses), the mice were returned to home cages for 48 h before implantation of the catheter.

Catheter implantation

After remaining in their home cages for 48 h following the nose-poke training, the mice were anesthetized with pentobarbital sodium (50 mg/kg, i.p.). Indwelling catheters were constructed of micro-silicone tubing (inner diameter, 0.50 mm; outer diameter, 0.7 mm; IMG, Imamura Co., Ltd., Tokyo, Japan) and polyethylene tubing (inner diameter, 0.50 mm; outer diameter, 0.8 mm). Incisions were made on the skin of the head and ventral neck, and the right jugular vein was externalized. The end of the catheter was inserted into the jugular vein via a small incision and was secured to the vein and surrounding tissue with silk sutures. The exit port of the catheter passed s.c. to the top of the skull where it was attached to a modified 24-gauge cannula, which was secured to the mouse's skull with quick self-curing acrylic resin (Shofu Inc., Kyoto, Japan). To extend catheter patency, the catheters were flushed immediately after surgery, and in the morning and evening of the following days, with 0.03 ml of an antibiotic solution of cefmetazole sodium (20.0 mg/ml; Sankyo Co., Ltd., Tokyo, Japan) dissolved in heparinized saline (70 units/ml; Leo Pharmaceutical Products, Ltd., Japan). The catheter patency was confirmed by taking blood back from the catheter after the tests for operant behavior.

Experiment 1: food-reinforced operant behavior in tPA knockout and wild-type control mice

To investigate operant behavior for natural reinforcement in tPA knockout and wild-type control mice, naïve animals (n=7 for each genotype) were deprived of food for 12 h and then subjected to 15 daily 30-min sessions of food-reinforced operant responding under an FR1 schedule of reinforcement, in which active and inactive nose-poke holes were assigned randomly throughout a session. The house light was illuminated throughout the session. Nose-poke responses in the active hole resulted in a pellet being delivered from the dispenser and inactivation of the cue- and hole-lamps. After each delivery, there was a 5-s timeout period. Nose-poke responses in the inactive hole and in the active hole during the timeout period had no programmed consequences but were recorded by the software MED-PC for Windows (Med Associates). Food pellets (dustless precision pellets, 20 mg) were purchased from Holton Industries Co., Frenchtown, NJ, USA.

Experiment 2: dose-response curve for MOR self-administration under an FR2 schedule of reinforcement in tPA knockout and wild-type control mice

After eight daily 30-min sessions of nose-poke training and recovering from surgery to implant a catheter into the jugular vein for at least 2 days, each genotype was assigned randomly into four groups (n=7–8 for each group) according to a between-subjects design. tPA knockout and wild-type control mice were then subjected to MOR (or saline) self-administration in a single 24-h session under an FR2 schedule of reinforcement with a 5-s timeout period after each infusion (infusion volume, 2.1 μl), in which active and inactive nose-poke holes were fixed for each mouse throughout the 24-h session. During a single 24-h session, catheters were connected to liquid swivels via a joint FEP tube (inner diameter 0.25 mm, outer diameter 0.55 mm; Eicom Co., Ltd., Kyoto, Japan), which was encased in steel spring leashes (Instech, Plymouth Meeting, PA, USA). The swivels were suspended above the chamber and were connected to infusion pumps. The house light was illuminated throughout the session. Nose-poke responses in the active hole resulted in a 5-s activation of the infusion pump and inactivation of the cue- and hole-lamps. Nose-

poke responses in the inactive hole and in the active hole during the timeout period had no programmed consequences but were recorded by the software MED-PC for Windows (Med Associates).

Experiment 3: MOR and food-reinforced operant behavior under the PR schedule in tPA knockout and wild-type control mice

After eight daily 30-min sessions of nose-poke training and recovering from surgery to implant a catheter into the jugular vein for at least 2 days, according to a within-subjects design, separate groups of tPA knockout and wild-type control mice (n=7–8 for each genotype) were then subjected to three daily 12-h sessions of MOR self-administration under the PR schedule, in which nose-poke responses required to earn an infusion escalated according to the following series (Roberts and Bennett, 1993): 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 492, 603, 737, etc. Each mouse was subjected to MOR self-administration as follows: 0.3 mg/kg/infusion (the first test: MOR 0.3 (the 1st test)), then 1.0 mg/kg/infusion (MOR 1.0), and 0.3 mg/kg/infusion once again (MOR 0.3 (the 2nd test)). The breaking point refers to the final ratio to earn the last infusion of MOR during the 12-h session.

To give a control for performance of tPA knockout mice under the PR schedule of MOR reinforcement, separate groups of tPA knockout and wild-type control mice (n=8 for each genotype) were subjected to three daily 12-h sessions of food-reinforced operant responding under the PR schedule as described above.

Experiment 4: effects of SCH23390 on MOR self-administration under the FR2 schedule in tPA knockout and wild-type control mice

After eight daily 30-min sessions of nose-poke training and recovering from surgery to implant a catheter into the jugular vein for at least 2 days, separate groups of tPA knockout and wild-type control mice were assigned randomly into 12 groups (n=6–7 for each group in each genotype) according to a between-subjects design. Both genotypes were then subjected to MOR (0.3 mg/kg/infusion) self-administration in a single 24-h session under the FR2 schedule. During a single 24-h session of self-administration, SCH23390 or vehicle was administered i.v. via the catheter at two time points: immediately after the 5th and 12th h by disconnecting a mouse from the operant system.

Experiment 5: effects of SCH23390 on saline self-administration under the FR2 schedule in tPA knockout and wild-type control mice

After eight daily 30-min sessions of nose-poke training and recovering from surgery to implant a catheter into jugular vein for at least 2 days, separate groups of tPA knockout and wild-type control mice were assigned randomly into four (tPA knockout) or six (wild-type control) groups (n=6–8 for each group) according to a between-subjects design. Both genotypes were then subjected to saline self-administration in a single 24-h session under the FR2 schedule. During a single 24-h session of self-administration, SCH23390 or vehicle was administered i.v. via the catheter at two time points: immediately after the 5th and 12th h by disconnecting the mouse from the operant system.

Statistical analysis

All data were expressed as mean±S.E.M. For food pellet, saline, and MOR self-administration, the differences between the two genotypes of mice were determined with a repeated measures two-way analysis of variance (ANOVA), and the differences between the active and the inactive nose-pokes in the same group were determined with Student's t-test. Statistical differences in the number or rate of active nose-poke responses at different doses of MOR self-administration in the two genotypes of mice, and the effects of SCH23390 on the active nose-poke responses for MOR self-administration in the two genotypes of mice were determined by one-way ANOVA, followed post hoc by the Bonferroni/Dunn tests. In all cases, the level of significance was set at $P<0.05$.

RESULTS

No significant difference in food-reinforced operant behavior was observed between tPA knockout and wild-type control mice

As shown in Fig. 1A, during the test for food-reinforced operant behavior, both genotypes of animals (n=7 for each genotype) failed to discriminate active from inactive nose-poke responses at the early phase. After 1 week, both genotypes could discriminate active from inactive nose-poke responses and then gradually showed stable responses for

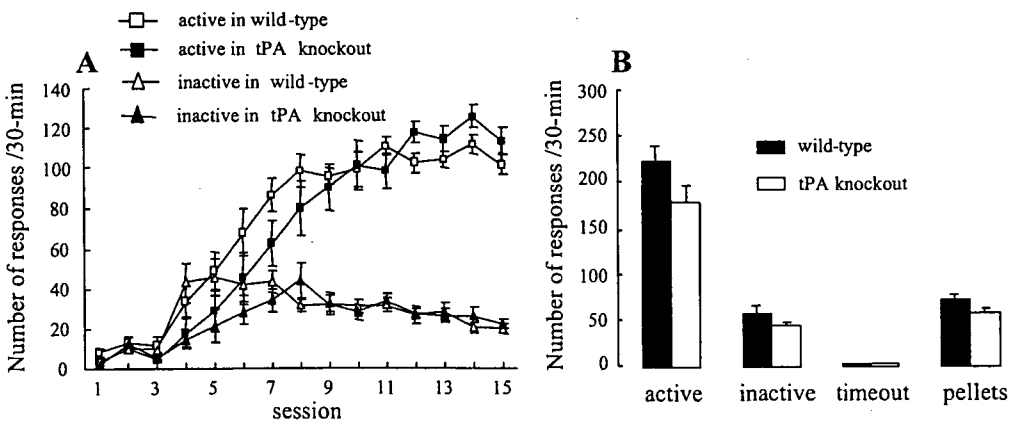

Fig. 1. No significant difference in natural reinforcement was observed between tPA knockout and wild-type control mice. A indicates active and inactive nose-poke responses under an FR1 schedule during 15 daily 30-min sessions of food-reinforced operant behavior between the two genotypes of animals (n=7 for each genotype). B indicates the total number of nose-poke responses under an FR3 schedule during the last session of nose-poke training prior to a single 24-h session of MOR self-administration between tPA knockout and wild-type control mice (n=7 for each genotype). Data are presented as mean±S.E.M.

food pellets. Importantly, there was no significant difference in active and inactive nose-poke responses between tPA knockout and wild-type control mice throughout the test.

Before a single 24-h session of MOR self-administration at a dose of 0.3 mg/kg/infusion, both tPA knockout and wild-type control mice (n=7–8 for each genotype) experienced the same nose-poke training for 8 days. As shown in Fig. 1B, during the last session of nose-poke training under an FR3 schedule, there was no significant difference in active, inactive, and timeout nose-poke responses as well as food pellets earned between the two genotypes, although tPA knockout mice tended to show a lower rate of nose-poke responses. Taken together, these observations suggest that there was no significant difference in nose-poke responses for natural reinforcement between tPA knockout and wild-type control mice.

tPA knockout mice showed an increase in nose-poke responding for MOR self-administration under the FR2 schedule in a bell-shaped manner

According to a between-subjects design, during saline self-administration, both genotypes of animals failed to discriminate active from inactive hole. Between tPA knockout and wild-type control mice, there was no difference in active or inactive nose-poke responses for saline self-administration (Fig. 2A). As shown in Fig. 2A, however, both genotypes of animals demonstrated significantly more active than inactive nose-poke responses for MOR self-administration at the dose of 0.1 mg/kg/infusion (Student's t-test, $P<0.05$ for tPA knockout and $P<0.01$ for wild-type mice), although there was no difference in active or inactive nose-poke responses between tPA knockout and wild-type control mice (two-way ANOVA, a main effect of genotype, $F_{(1,27)}=3.49$, $P=0.074$). At higher doses of MOR (0.3 and 1.0 mg/kg/infusion), tPA knockout mice showed more active nose-poke responses than wild-type control mice (one-way ANOVA, $P<0.05$), whereas there was no significant difference in inactive (without MOR reinforcement) nose-poke responses between the two genotypes. The active nose-poke responses for MOR self-administration in tPA knockout mice were reduced at the dose of 1.0 mg/kg/infusion as compared with those at the dose of 0.3 mg/kg/infusion (one-way ANOVA, $P<0.05$). As shown in Fig. 2B, at the dose of 0.1 mg/kg/infusion, there was no difference in total MOR intake between tPA knockout and wild-type control mice during a single 24-h session of self-administration (8.90±1.77 mg/kg in tPA knockout versus 7.61±1.48 mg/kg in wild-type mice). In contrast, at higher doses (0.3 and 1.0 mg/kg/infusion), tPA knockout mice self-administered a much greater amount of MOR than wild-type control mice during a single 24-h session of self-administration (one-way ANOVA, $P<0.05$ at the dose of 0.3 mg/kg/infusion (57.56±11.12 versus 27.47±6.94 mg/kg); and $P<0.01$ at the dose of 1.0 mg/kg/infusion (77.71±10.16 versus 40.75±5.68 mg/kg)).

As shown in Fig. 3, either tPA knockout mice or wild-type control mice failed to discriminate active from inactive nose-poke responses for saline self-administration throughout a single 24-h session. In contrast, both genotypes of animals could discriminate active from inactive nose-poke responses for MOR self-administration throughout a single 24-h session at the dose of 0.3 mg/kg/infusion (Fig. 3B for wild-type control mice, two-way ANOVA with repeated measures, a main effect of nose-poke hole, $F_{(1,335)}=3.72$, $P<0.01$; and Fig. 3D for tPA knockout mice, two-way ANOVA with repeated measures, a main effect of nose-poke hole, $F_{(1,335)}=14.38$, $P<0.001$). Importantly, the increased nose-poke responses for MOR reinforcement in tPA

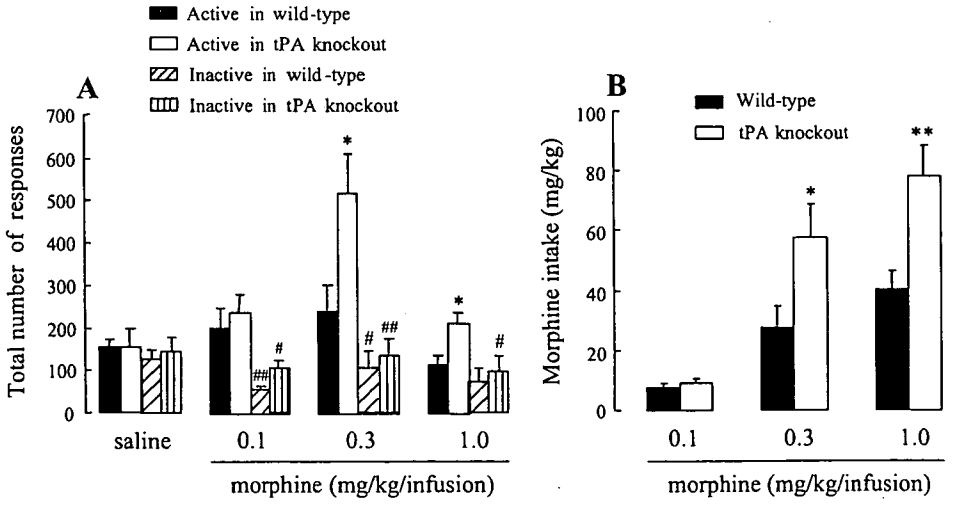

Fig. 2. tPA knockout mice showed an increase in active nose-poke responses for MOR self-administration in a bell-shaped manner. After eight daily 30-min sessions of nose-poke training, tPA knockout and wild-type control mice were subjected to saline or MOR self-administration in a single 24-h session under an FR2 schedule, according to a between-subjects design (n=6–8 for each genotype at one dose point). A indicates total active and inactive nose-poke responses for saline or MOR self-administration during the tests for dose-response curve between the two genotypes. B indicates total MOR intake during the tests for dose-response curve between the two genotypes. Data are presented as mean±S.E.M. * $P<0.05$ and ** $P<0.01$ versus corresponding wild-type control mice at the same dose point (one-way ANOVA). # $P<0.05$ and ## $P<0.01$ versus corresponding active nose-poke responses in wild-type and tPA knockout mice.

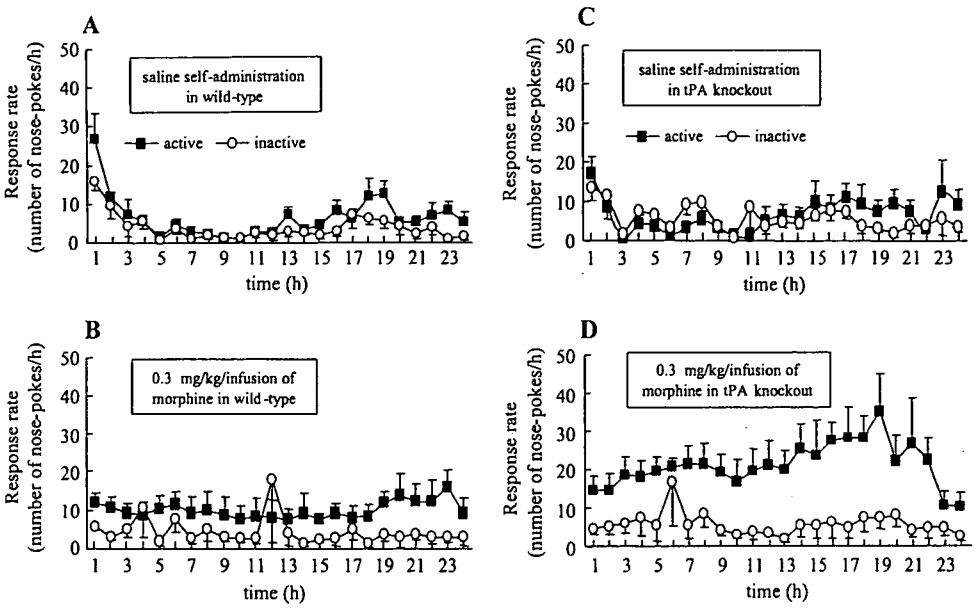

Fig. 3. Time course of changes in nose-poke responses for saline or MOR self-administration in a single 24-h session between the two genotypes. A and C indicate the response rate (nose-pokes per hour) for saline self-administration in wild-type and tPA knockout mice (n=7 for each genotype), respectively. B and D indicate the response rate (nose-pokes per hour) for MOR (0.3 mg/kg/infusion) self-administration in wild-type and tPA knockout mice (n=7 for each genotype), respectively. Data are presented as mean±S.E.M.

knockout mice appeared to be maintained throughout the single 24-h session (Fig. 3D). Taken together, the dose-response curve for MOR self-administration in a single 24-h session shifted upward in tPA knockout mice.

tPA knockout mice showed a decrease in breaking points for MOR self-administration, but not for food reinforcement, under the PR schedule

According to a within-subjects design, separate groups of animals (n=7–8 for each genotype) were subjected to three daily 12-h sessions of MOR self-administration under the PR schedule after daily 30-min sessions of nose-poke training for 8 days. As shown in Fig. 4A, during the first two sessions (at a dose of 0.3 or 1.0 mg/kg/infusion), there was no significant difference in breaking points for MOR self-administration under the PR schedule between the two genotypes of animals. When both genotypes of animals were subjected to the third 12-h session of MOR self-administration at the dose of 0.3 mg/kg/infusion, wild-type control mice showed a greater breaking point than tPA knockout mice to take MOR under the PR schedule (two-way ANOVA with repeated measures, a main effect of genotype, $F_{(1,39)}$=4.23, P<0.01). Accordingly, total MOR intake was greater in wild-type control mice than in tPA knockout mice during the third

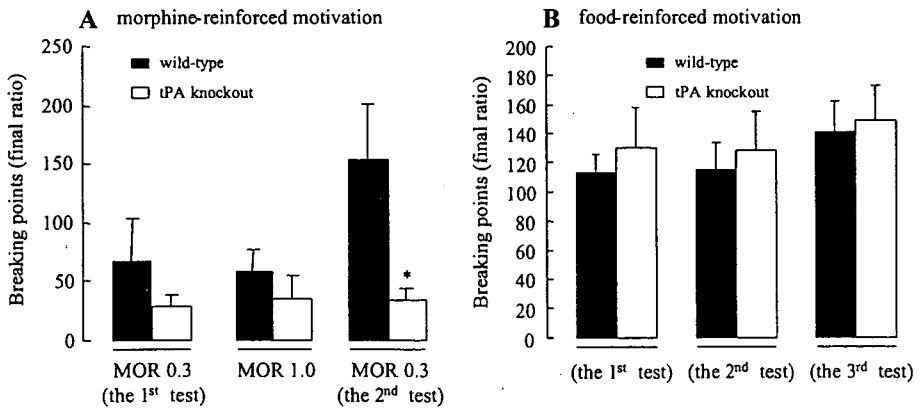

Fig. 4. Reduced breaking points for MOR self-administration, but not for food reinforcement, under the PR schedule in tPA knockout mice. After eight daily 30-min sessions of nose-poke training, separate groups of tPA knockout and wild-type control mice were subjected to MOR self-administration in a single 12-h session under the PR schedule, according to a within-subjects design (n=7 for wild-type and n=8 for tPA knockout mice). The mice were subjected to MOR self-administration at a dose of 0.3 mg/kg/infusion (1st test), then 1.0 mg/kg/infusion, and 0.3 mg/kg/infusion once again (2nd test). A indicates the breaking points for MOR reinforcement. B indicates the breaking points for food reinforcement. Data are presented as mean±S.E.M. * P<0.05 versus corresponding wild-type mice.

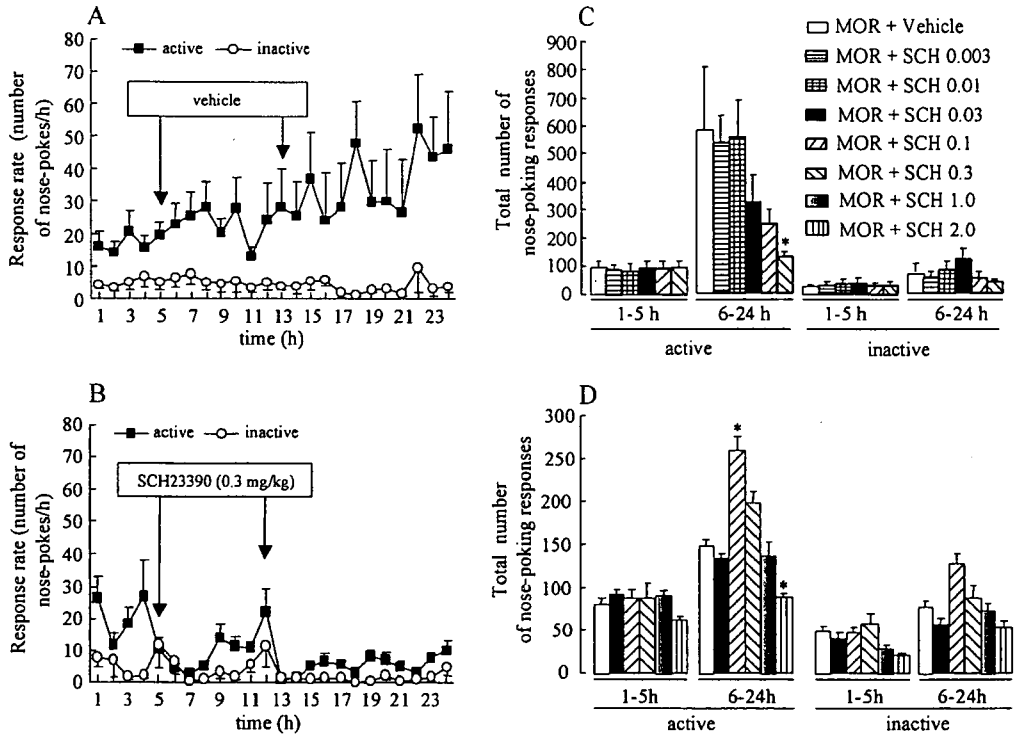

Fig. 5. Effects of the dopamine D1 receptor antagonist SCH23390 on nose-poke responses for MOR self-administration in tPA knockout and wild-type mice. After eight daily 30-min sessions of nose-poke training, separate groups of tPA knockout and wild-type control mice were subjected to MOR (0.3 mg/kg/infusion) self-administration in a single 24-h session under an FR2 schedule, according to a between-subjects design. *Arrows* in A and B indicate the time points for the i.v. injection of vehicle or SCH23390 during a single 24-h session. A indicates the time course of changes in the effects of vehicle on nose-poke responses for MOR (0.3 mg/kg/infusion) self-administration in tPA knockout mice. B indicates the time course of changes in the effects of SCH23390 (0.3 mg/kg) on nose-poke responses for MOR (0.3 mg/kg/infusion) self-administration in tPA knockout mice. C indicates the effects of SCH23390 on nose-poke responses for MOR self-administration in tPA knockout mice (n=6–7 for each dose point). D indicates the effects of SCH23390 on nose-poke responses for MOR self-administration in wild-type control mice (n=6 for each dose point). Data are presented as mean±S.E.M. * P<0.05 versus the group injected with vehicle injection.

session of MOR self-administration at the dose of 0.3 mg/kg/infusion (Student's t-test, P<0.01, data not shown). These observations suggest that tPA knockout mice had less motivation to take MOR than wild-type mice under the PR schedule of MOR reinforcement.

According to a within-subjects design, new groups of tPA knockout and wild-type control mice (n=8 for each genotype) were subjected to three daily 12-h sessions of food-reinforced operant behavior under the PR schedule using similar procedures. As shown in Fig. 4B, tPA knockout mice showed similar breaking points for food reinforcement to wild-type control mice throughout the three daily 12-h sessions. Taken together, these observations suggest that tPA knockout mice specifically showed lower breaking points for MOR self-administration under the PR schedule than wild-type control mice.

Dopamine D1 receptor antagonist SCH23390 inhibited the increased responding for MOR self-administration in tPA knockout mice, but demonstrated biphasic effects on MOR self-administration in wild-type control mice

After eight daily 30-min sessions of nose-poke training, according to a within-subjects design, separate groups of animals were subjected to a single 24-h session of MOR (0.3 mg/kg/infusion) self-administration. As shown in Fig. 5A and 5B, a dopamine D1 receptor antagonist SCH23390 was i.v. administrated via the catheter at two time points, immediately after the 5th and 12th hours. Prior to the administration of SCH2390 or vehicle (i.v.), tPA knockout mice (n=6–7 for each genotype) could discriminate active from inactive nose-poke responses (Fig. 5C, Student's t-test, P<0.05) and showed similar levels of nose-poke responses for MOR self-administration (1–5 h) (Fig. 5C, one-way ANOVA, P=0.994). However, the active nose-poke responses for MOR self-administration (6–24 h) in tPA knockout mice were attenuated by the dopamine D1 receptor antagonist SCH23390 at the dose of 0.3 mg/kg (Fig. 5B and 5C, one-way ANOVA, P<0.05), although much lower doses of SCH23390 (0.003 and 0.01 mg/kg) failed to affect active nose-poke responses for MOR self-administration in tPA knockout mice. Across the range of doses examined, SCH23390 treatment had little effect on inactive nose-poke responses. As shown in Fig. 5D, before the treatment with SCH23390 (1–5 h), wild-type control mice (n=6 for each group) showed no significant differences in active nose-poke responses for MOR self-administration (1–5 h). However, a lower dose of

SCH23390 (0.1 mg/kg) significantly increased active nose-poke responses for MOR reinforcement in wild-type mice (6–24 h, one-way ANOVA, $P<0.05$), whereas a higher dose of SCH23390 (2.0 mg/kg) significantly inhibited the active nose-poke responses in wild-type control mice (6–24 h, one-way ANOVA, $P<0.05$).

No significant effects of the dopamine D1 receptor antagonist SCH23390 on nose-poke responses for saline self-administration in the two genotypes of animals

After eight daily 30-min sessions of nose-poke training, according to a within-subjects design, separate groups of animals ($n=7–8$ for each group in tPA knockout mice and $n=6–7$ for each group in wild-type control mice) were subjected to a single 24-h session of saline self-administration. The dopamine D1 receptor antagonist SCH23390 was i.v. administrated via the catheter at two time points, immediately after the 5th and 12th hours. As shown in Fig. 6, SCH23390 (at doses of 0.03–2.0 mg/kg) had no significant effect on saline self-administration in either tPA knockout mice or wild-type control mice, suggesting that pharmacological effects of SCH23390 on active nose-poke responses for MOR self-administration in tPA knockout and wild-type mice are specifically attributable to changes in dopamine transmission, rather than general disruption of motor activity.

DISCUSSION

By using a novel single 24-h session of self-administration, we have demonstrated that tPA knockout mice showed an increase in active nose-poke responses for MOR self-administration under an FR2 schedule, but exhibited a decrease in breaking points (motivation) for MOR reinforcement under a PR schedule. In contrast, both genotypes of animals showed similar performance for food reinforcement under an FR or a PR schedule. The increased responding for MOR self-administration in tPA knockout mice was blocked by dopamine D1 receptor antagonist SCH23390 at the dose of 0.3 mg/kg, whereas the active nose-poke responses for MOR self-administration in wild-type control mice are attenuated by much higher dose of SCH23390 (2.0 mg/kg) but enhanced by a lower dose of SCH23390 (0.1 mg/kg). Our findings suggest that the reinforcing effects of MOR are reduced in tPA knockout mice.

In the present study, the dose-response curve for MOR self-administration under an FR2 schedule in tPA knockout mice shifted upward. This observation is consistent with a report that tPA knockout mice show an increase in active nose-poke responses for cocaine self-administration under a consecutive FR2 schedule of reinforcement (Ripley et al., 1999). In a paradigm of drug self-administration under an FR schedule of reinforcement, animals attempt to maintain a constant level of drug effect by increasing drug intake when drug impact is reduced and by decreasing intake when drug impact is increased. We have previously demonstrated, by using an *in vivo* dialysis technique, that the MOR-induced release of dopamine in the nucleus accumbens is reduced in tPA knockout mice as compared with wild-type control mice (Nagai et al., 2004). Thus, it is reasonable to postulate that in the present study, tPA knockout mice find a given dose of MOR (especially in the 0.3 mg/kg/infusion range) less reinforcing and therefore self-administer more of it, whereas at the lowest dose (0.1 mg/kg/infusion), neither wild-type nor tPA knockout mice found it particularly reinforcing, suggesting a subthreshold dose. In a paradigm of drug self-administration under a PR schedule, the "breaking point," expressed as the final ratio (the number of active nose-poke responses needed to earn the last infusion of MOR), may reflect the intensity of motivation for drug infusion or reinforcing effi-

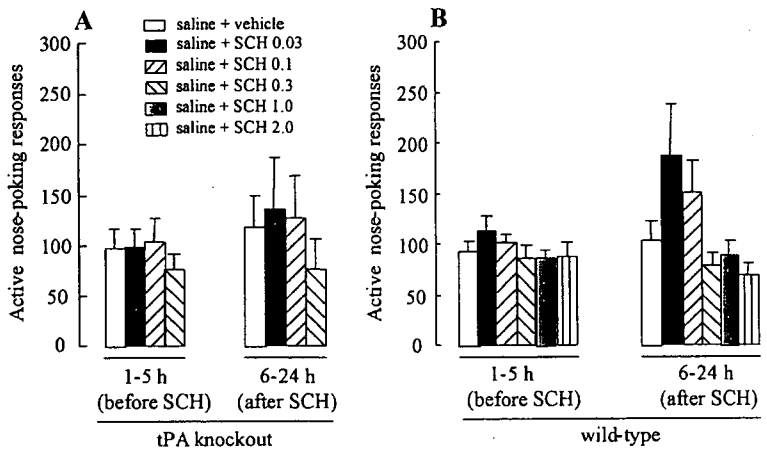

Fig. 6. Effects of the dopamine D1 receptor antagonist SCH23390 on nose-poke responses for saline self-administration in tPA knockout and wild-type mice. After eight daily 30-min sessions of nose-poke training, separate groups of tPA knockout and wild-type control mice were subjected to saline self-administration in a single 24-h session under an FR2 schedule, according to a between-subjects design. A indicates the effects of SCH23390 on nose-poke responses for saline self-administration in tPA knockout mice ($n=7–8$ for each dose point). B indicates the effects of SCH23390 on nose-poke responses for saline self-administration in wild-type control mice ($n=6–7$ for each dose point). SCH23390 was i.v. administered via a catheter at two time points as described above during a single 24-h session of saline self-administration. Data are presented as mean±S.E.M.

ciency of addictive drugs in animals (Roberts and Bennett, 1993). In our study, tPA knockout mice showed lower breaking points for MOR self-administration under the PR schedule than wild-type control mice (Fig. 4A). This observation further supports our hypothesis that reinforcing effects of MOR in tPA knockout mice are reduced as compared with wild-type control mice. It seem to be unlikely that the lower breaking points for MOR reinforcement in tPA knockout mice are derived from prior nose-poke training, since there was no difference in breaking points for natural reinforcement (food pellets) between the two genotypes of animals after the similar schedule of nose-poke training (Fig. 4B). These findings are consistent with our previous one that rewarding effects of MOR, which was assessed by a conditioned place preference test, are reduced in tPA knockout mice compared with wild-type control mice (Nagai et al., 2004).

It is well established that the dopamine transmission in the mesolimbic system in the brain plays a key role in the reinforcing properties of addictive substances such as MOR (e.g. Di Chiara and Imperato, 1988; Elmer et al., 2002; Self, 2004). It has been reported that, by the partial blockade of dopaminergic transmission, pretreatment with dopamine D1 receptor antagonists (SCH23390 and SCH39166) and a dopamine D2 receptor antagonist (eticlopride) leads to an increase in lever-presses for cocaine self-administration in rats (Caine and Koob, 1994; Barrett et al., 2004). In the present study, the low dose of SCH23390 (0.1 mg/kg) reduced the reinforcing effect of MOR to a level that supports an increase in nose-poke responses in order to regain the desired drug effects in wild-type control animals. As the dose of SCH23390 is increased (2.0 mg/kg) in wild-type control mice, dopamine D1 receptors are sufficiently blocked such that MOR is no longer reinforcing and the nose-poke responses are reduced. In tPA knockout mice, the increase in active nose-poke responses for MOR self-administration was attenuated by a lower dose of SCH23390 (0.3 mg/kg), suggesting that the combination of the dopamine D1 receptor antagonist and the mutation-induced reduction in the reinforcing effects of MOR (due presumably to a reduction in dopamine release in the nucleus accumbens, Nagai et al., 2004) resulted in a decrease in the reinforcing impact of MOR to a level that did not support nose-poke responses (hence SCH23390 dose-dependently decreased nose-poke responses). During the single 24-h session of MOR self-administration, the total number of active nose-poke responses in tPA knockout mice reached 600 or so (Fig. 5A and 5C). We therefore postulate that ceiling effects of MOR on active nose-poke responses in tPA knockout mice might mask an increase in active nose-poke responses induced by the lower doses of SCH23390. It is unlikely that the suppressive effect of SCH23390 on the increased responses for MOR self-administration in tPA knockout mice is due to a disruption of general operant behavior or nonspecific motor effects since, as compared with vehicle treatment, SCH23390 treatment had no inhibitory effects on saline self-administration in either genotype (Fig. 6). It has been reported that doses of up to and greater than 0.025 mg/kg of SCH23390 reduce cocaine- and amphetamine-induced hyperlocomotion in mice (O'Neill and Shaw 1999; O'Neill et al., 2003). Consistent with these findings, an impairment of general operant behavior by higher doses of SCH23390 (greater than 0.1 mg/kg, i.v.) was also observed immediately after the infusion of SCH23390 in our study. However, such a transient inhibition of operant performance is difficult to explain by the inhibitory action of SCH23390 on the increased responding for MOR self-administration in tPA knockout mice. One may argue that the doses of SCH23390 in our study are too high as compared with previous reports. For instance, in one report, the doses of SCH23390 to inhibit active responding for cocaine self-administration (0.01–0.1 mg/kg by pre-treatment; 1–3 h session; Corrigall and Coen, 1991; Caine and Koob, 1994) were lower than those used in the present study (0.3 mg/kg in tPA knockout mice and 2.0 mg/kg in wild-type controls during a single 24-h session; by post-treatment). The differences may be explained by the timing of administration (pre- versus post-administration of the antagonist) as well as the experimental conditions (a session of a few hours versus 24 h).

There is a body of evidence to suggest that tPA is involved in some forms of learning and memory. tPA knockout mice show deficits in hippocampal-dependent and cerebellar motor learning (Madani et al., 1999; Seeds et al., 2003). During the test for food-reinforced operant behavior (Fig. 1A) and the period of nose-poke training prior to a single 24-h session of MOR self-administration (Fig. 1B), no significant difference in active nose-poke responses for natural reinforcement was observed between tPA knockout and wild-type control mice. This observation further suggests that the increased active nose-poke responses for MOR self-administration in tPA knockout mice are related to the alteration of MOR-induced reinforcing effects, not to general deficits in learning and memory. In the present study, the single 24-h sessions of MOR self-administration were performed after eight daily 30-min sessions of nose-poke training. At the beginning of the single 24-h sessions, active nose-poke responses for MOR reinforcement might be facilitated by the habitual operant behavior or food extinction effects. Such habitual nose-poke responses or food extinction effects may mask the difference in the ability to acquire MOR self-administration behavior between the two genotypes. However, it is unlikely that, throughout the single 24-h session, the increased nose-poke responses for MOR self-administration in tPA knockout mice contributed to the extinction effects derived from the nose-poke training, since with the same procedure, no such pattern of nose-poke responses was observed for saline self-administration in the two genotypes (Fig. 2A). In both genotypes, the active nose-poke responses for saline self-administration were greater only at the beginning of the single 24-h session, but rapidly declined and then remained lower level (Fig. 3A and 3C). In contrast, active nose-poke responses for MOR self-administration were maintained at higher levels throughout the single 24-h session in both genotypes (Fig. 3B and D).

A previous report has demonstrated that a single administration of cocaine altered synaptic plasticity in the ventral tegmental area for several days (Ungless et al., 2001). Furthermore, rats given a single 2-h session of cocaine self-administration exhibit drug-seeking behavior for up to 1 year after the intake of cocaine (Ciccocioppo et al., 2004). Together with the single-session cocaine self-administration reported by Olsen and Winder (2006), the single 24-h session of self-administration used in the present study may provide an alternative experimental design to investigate genetic factors involved in the reinforcing properties of addictive substances by using genetically modified strains of mice.

CONCLUSION

In conclusion, our findings suggest that tPA is critically involved in the reinforcing properties of MOR in mice. The reinforcing effects of MOR are reduced in tPA knockout mice. The modulation of the tPA system in the brain may be a potential target against drug abuse.

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