Measurement of locomotor activity

Each mouse was placed in a standard transparent rectangular rodent cage (25 × 30 × 18 high cm). Locomotor activity was then measured for 1h, using an infrared sensor (NS-AS01; Neuroscience, Tokyo, Japan) placed over the cage (Ito et al. 2007a).

Novel object recognition test

The novel object recognition test was performed according to previously reported methods (Nagai et al. 2003; Tang et al. 1999). The experimental apparatus consisted of a Plexiglas open-field box (30 × 30 × 35 high cm), the floor of which was covered in sawdust. The apparatus was located in a sound-attenuated room and illuminated with a 20-W bulb.

The procedure for the novel object recognition test consisted of three different sessions: habituation, training, and retention. Each mouse was individually habituated to the box, with 10min of exploration in the absence of objects each day for three consecutive days (habituation session, days1–3). In the training session, two different novel objects were symmetrically fixed to the floor of the box, 8cm from the walls, and each animal was allowed to explore the box for 10min (day4). The objects were a golf ball, wooden cylinders, and square pyramids, which were different in shape and color but similar in size. An animal was considered to be exploring the object when its head was facing the object or it was touching or sniffing the object. The time spent exploring each object was recorded. After training, mice were immediately returned to their home cages. In the retention sessions, the animals were placed back into the same box 24h (day5) after the training session, but with one of the familiar objects used during training replaced by a novel object. The animals were then allowed to explore freely for 5min, and the time spent exploring each object was recorded. Throughout the experiments, the objects were balanced 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.

Drug treatment

For effect of single treatment on locomotor activity, 0.1% CMC, aripiprazole (0.1–1.0mg/kg) or haloperidol (0.3–1.0mg/kg) was orally (p.o.) administered 1h before the experiment. The number of animals included in each drug treatment was as follows: CMC (n = 12), 0.1mg/kg aripiprazole (n = 12), 0.3mg/kg aripiprazole (n = 12), 1.0mg/kg aripiprazole (n = 11) for Fig. 1b,c; CMC (n = 10), 0.3mg/kg haloperidol (n = 10), 1.0mg/kg haloperidol (n = 10) for Fig. 1d,e.

For effect of repeated treatment on locomotor activity, 0.1% CMC, aripiprazole (0.01–0.1mg/kg), or haloperidol (0.3–1.0mg/kg) was p.o. administered for 7days; the experiment was performed 24h after last treatment. Locomotor activity was recorded for 1h. The number of animals included in each drug treatment was as follows: CMC (n = 10), 0.01mg/kg aripiprazole (n = 10), 0.03mg/kg aripiprazole (n = 10), 0.1mg/kg aripiprazole (n = 10) for Fig. 2b,c; CMC (n = 10), 0.3mg/kg haloperidol (n = 10), 1.0mg/kg haloperidol (n = 10) for Fig. 2d,e.

We have previously demonstrated that repeated treatment with PCP (10mg/kg/day s.c. for 14days) induces several schizophrenia-like behavioral and neurochemical abnormalities in mice (Murai et al. 2007; Noda et al. 1995, 1997, 2000; Qiao et al. 2001; Mouri et al. 2007b; Enomoto et al. 2005). Therefore, in typical experimental conditions, mice were given the same regimen of PCP (10mg/kg, s.c., for 14days, days1–14). Five days after the last treatment with PCP, the novel object recognition test was performed, including habituation (i.e., days19–21), training (i.e., day22), and retention (i.e., day23) sessions.

To study the single effects of antipsychotics, aripiprazole (0.01–1.0mg/kg) or haloperidol (0.3–1.0mg/kg) was administered p.o. (i.e., day22) to mice that had been previously treated with PCP for 14days (day1–14). One hour after treatment with antipsychotics, the training session using the novel object recognition test was conducted. The number of animals included in each drug treatment was as follows: saline + CMC (n = 16), PCP + CMC (n = 14), PCP + 0.01mg/kg aripiprazole (n = 10), PCP + 0.03mg/kg aripiprazole (n = 9), PCP + 0.1mg/kg aripiprazole (n = 8), PCP + 0.3mg/kg aripiprazole (n = 10), PCP + 1.0mg/kg aripiprazole (n = 11) for Fig. 3b,d; saline + CMC (n = 14), PCP + CMC (n = 13), PCP + 0.3mg/kg haloperidol (n = 7), PCP + 1.0mg/kg haloperidol (n = 9) for Fig. 3c,e.

To study the subchronic effects of antipsychotics, aripiprazole (0.01–0.1mg/kg) or haloperidol (0.3–1.0mg/kg) was administered p.o. once a day for seven consecutive days (i.e., days15–21) to mice that had been previously treated with PCP for 14days (days1–14). During habituation session of the novel object recognition test (i.e., days19–21), mice were administered antipsychotics after the habituation session. One day after the last treatment with antipsychotics (i.e., day22), the training session of the novel object recognition test was conducted. The number of animals included in each drug treatment was as follows: saline + CMC (n = 11), PCP + CMC (n = 10), PCP + 0.01mg/kg aripiprazole (n = 11), PCP + 0.03mg/kg aripiprazole (n = 8), PCP + 0.1mg/kg aripiprazole (n = 8) for Fig. 4b,d; saline + CMC (n = 15), PCP + CMC (n = 14), PCP + 0.3mg/kg haloperidol (n = 8), PCP + 1.0mg/kg haloperidol (n = 9) for Fig. 4c,e.

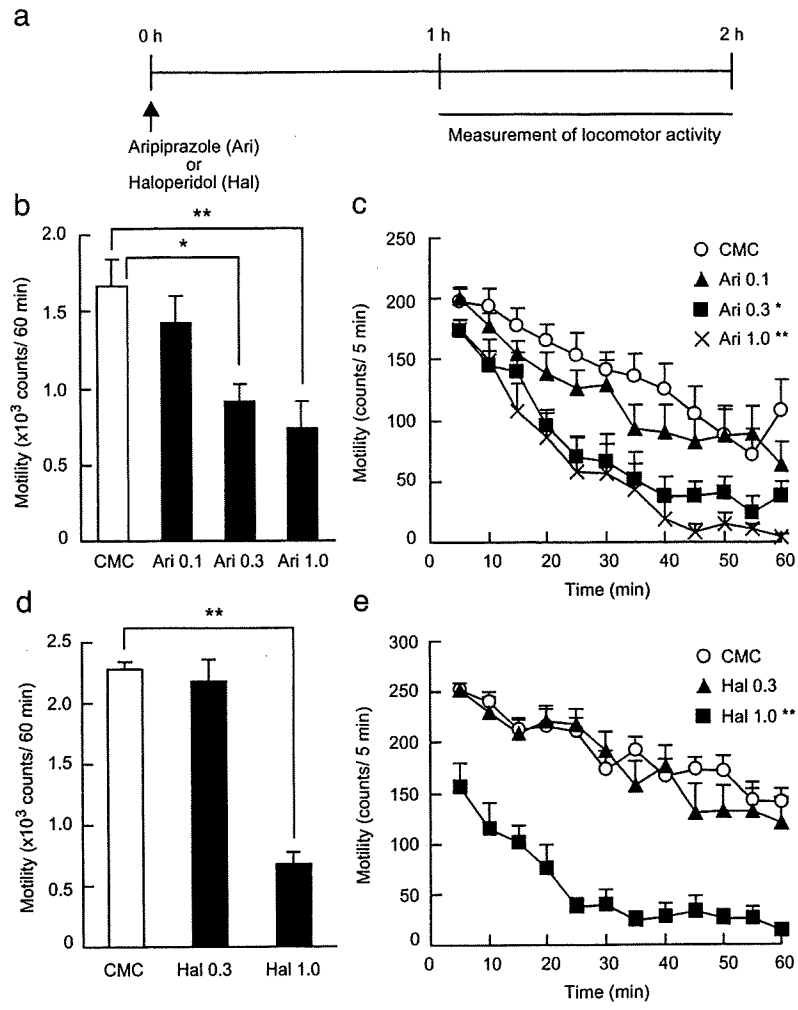


Fig. 1 Effects of single administration of aripiprazole and haloperidol on locomotor activity. **a** Experimental schedule for the measurement of locomotor activity. Mice were administered aripiprazole (*Ari*, 0.1–1.0 mg/kg, p.o.), haloperidol (*Hal*, 0.3–1.0 mg/kg, p.o.) or vehicle (0.1% *CMC*) 1 h before the measurement of locomotor activity. **b** and **c** Effect of single administration of aripiprazole on locomotor activity. **d** and **e** Effect of single administration of haloperidol on locomotor activity. **b** and **d** Total locomotor activity for 1 h. **c** and **e**: Time course of changes in locomotor activity. Values indicate the mean±SE (n=11–12). Analysis of variance: group, $F(3,43)=7.323$, $p<0.01$ for (**c**); time, $F(11,473)=58.971$, $p<0.01$ for (**c**); group × time, $F(33,473)=1.168$, $p=0.24$ for (**c**); group, $F(2,27)=46.806$, $p<0.01$ for (**e**); time, $F(11,297)=24.709$, $p<0.01$ for (**e**); group × time, $F(22,297)=1.370$, $p=0.13$ for (**e**). *$p<0.05$ and **$p<0.01$ compared with CMC group

To examine the role of dopamine D_1, D_2, and serotonin 5-HT_{1A} receptors in ameliorating the effect of aripiprazole on PCP-induced cognitive impairment, SCH23390 (0.03mg/kg), a dopamine D_1 receptor antagonist, raclopride (0.3mg/kg), a dopamine D_2 receptor antagonist, or WAY100635 (0.6mg/kg), a serotonin 5-HT_{1A} receptor antagonist, was administered intraperitoneally (i.p.) 30min after treatment with aripiprazole (0.1mg/kg, p.o.) for 7days because brain concentration of aripiprazole is the maximum at 2–3h after the oral administration, and declined $t_{1/2}$ of 1.8–2.0h in rats (Shimokawa et al. 2005). One day after the last treatment with aripiprazole and dopamine or serotonin receptor antagonists, the novel object recognition test was performed. The number of animals included in each drug treatment was as follows: saline + CMC + DW (n = 11), PCP + CMC + DW (n = 11), PCP + CMC + SCH23390 (n = 11), PCP + aripiprazole + DW (n = 11), PCP + aripiprazole + SCH23390 (n = 11) for Fig. 5b,d; saline + CMC + DW (n = 9), PCP + CMC + DW (n = 9), PCP + CMC + raclopride (n = 10), PCP + aripiprazole + DW (n = 9), PCP + aripiprazole + raclopride (n = 10) for Fig. 5c,e; saline + CMC + DW (n = 10), PCP + CMC + DW (n = 10), PCP + CMC + WAY100635 (n = 9), PCP + aripiprazole + DW (n = 10), PCP + aripiprazole + WAY100635 (n = 10) for Fig. 6.

Statistical analysis

All data were expressed as the mean±SEM. Statistical significance was determined using analysis of variance (ANOVA) with repeated measures (Figs. 1c,e, and 2c,e) or one-way (Figs. 1b,d, 2b,d, and 3–6), followed by the Bonferroni/Dunn test when F ratios were significant ($p<0.05$).

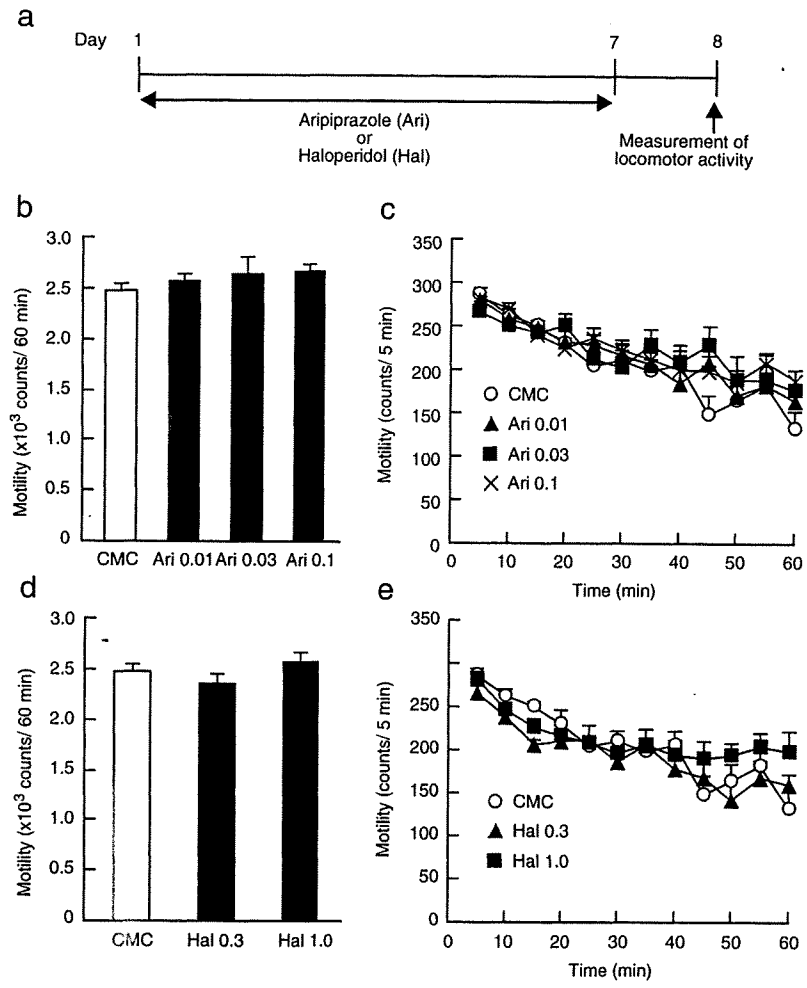


Fig. 2 Effects of repeated administration of aripiprazole and haloperidol on locomotor activity. **a** Experimental schedule for the measurement of locomotor activity. Mice were administered aripiprazole (*Ari*, 0.01–0.1 mg/kg, p.o.), haloperidol (*Hal*, 0.3–1.0 mg/kg, p.o.) or vehicle (0.1% *CMC*) for 7 days. Locomotor activity was measured 24 h after the last treatment. **b** and **c** Effect of repeated administration of aripiprazole on locomotor activity. **d** and **e** Effect of repeated administration of haloperidol on locomotor activity. **b** and **d** Total locomotor activity for 1 h. **c** and **e** Time course of changes in locomotor activity. Values indicate the mean±SE (n=10). Analysis of variance: group, $F(3,35)=0.743$, $p=0.53$ for (c); time, $F(11,385)=24.376$, $p<0.01$ for (c); group × time, $F(33,375)=1.099$, $p=0.33$ for (c); group, $F(2,27)=1.290$, $p=0.29$ for (e); time, $F(11,297)=18.444$, $p<0.01$ for (e); group × time, $F(22,297)=1.318$, $p=0.16$ for (e)

Results

Effects of administration of aripiprazole and haloperidol on locomotor activity

To explore the dose of aripiprazole and haloperidol which did not cause sedation in mice, we measured locomotor activity after oral administration of aripiprazole. Figure 1b and c shows the effect of a single administration of aripiprazole on locomotor activity in mice. Treatment with aripiprazole decreased total locomotor activity in a dose-dependent manner [$F(3,43)=7.323$, $p<0.01$, Fig. 1b]. The time course of changes in locomotor activity revealed that aripiprazole at doses of 0.3 and 1.0 mg/kg caused marked locomotor suppression 1 h after treatment [effect of group: $F(3,43)=7.323$, $p<0.01$; effect of time: $F(11,473)=58.971$, $p<0.01$; effect of interaction between group and time: $F(33,473)=1.168$, $p=0.24$ by two-way ANOVA with repeated measures, Fig. 1c]. Single treatment with haloperidol also decreased total locomotor activity in a dose-dependent manner [$F(2,27)=46.806$, $p<0.01$, Fig. 1d]. The time course of changes in locomotor activity revealed that haloperidol at the dose of 1.0 mg/kg caused marked locomotor suppression 1 h after treatment [effect of group: $F(2,27)=46.806$, $p<0.01$; effect of time: $F(11,297)=24.709$, $p<0.01$; effect of interaction between group and time: group × time, $F(22,297)=1.370$, $p=0.13$ by two-way ANOVA with repeated measures, Fig. 1e].

Effects of repeated administration of aripiprazole and haloperidol on locomotor activity were also examined. Mice were administered 0.1% CMC, aripiprazole (0.01–0.1 mg/kg, p.o.) or haloperidol (0.3–1.0 mg/kg, p.o.) was administered for 7 days. Locomotor activity was recorded 24 h after the last treatment. In contrast to the single treatment, repeated treatment with aripiprazole (0.01–0.1 mg/kg) and haloperidol (0.3–1.0 mg/kg) had no effect on the locomotor activity (Fig. 2).

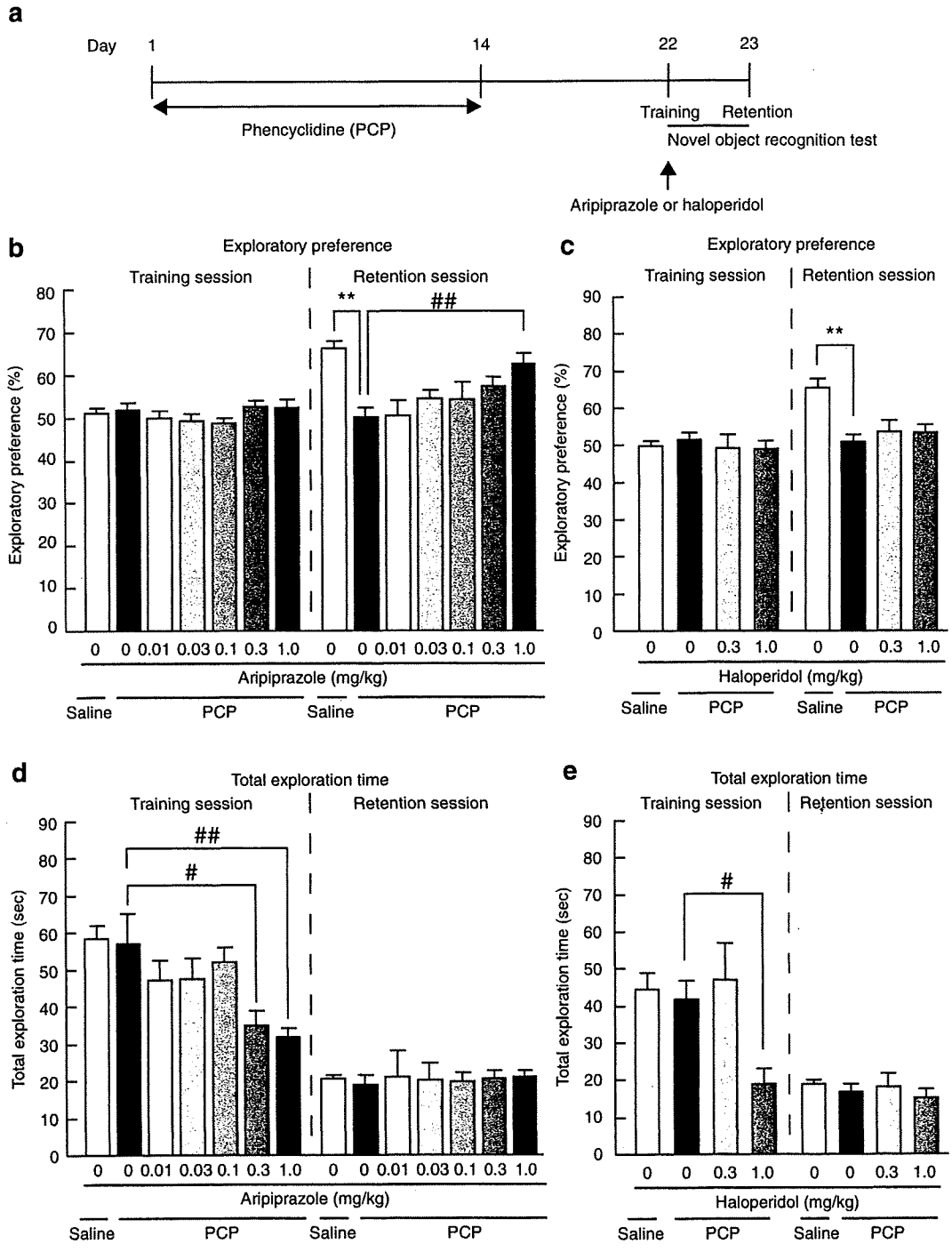


Fig. 3 Effects of single administrations of aripiprazole and haloperidol on PCP-induced cognitive impairment in novel object recognition. **a** Experimental schedule for the novel object recognition test. Eight days after withdrawal from repeated PCP (10 mg/kg, s.c., for 14 days) treatment, mice were subjected to the novel-object recognition test. Aripiprazole (0.01–1.0 mg/kg, p.o.), haloperidol (0.3–1.0 mg/kg, p.o.), or vehicle (0.1% CMC) was administered 1 h before the training session. **b** and **d** Effect of aripiprazole on PCP-induced cognitive impairment. **c** and **e** Effect of haloperidol on PCP-induced cognitive impairment. **b** and **c** Exploratory preference. **d** and **e** Total exploration time. Values indicate the mean±SE (n=8–16). Analysis of variance: $F(6,77)=0.911$, $p=0.49$ for (**b**) training; $F(6,77)=7.304$, $p<0.01$ for (**b**) retention; $F(3,39)=0.303$, $p=0.82$ for (**c**) training; $F(3,39)=8.69$, $p<0.01$ for (**c**) retention; $F(6,77)=5.009$, $p<0.01$ for (**d**) training; $F(6,77)=0.057$, $p=0.99$ for (**d**) retention; $F(3,39)=4.665$, $p<0.01$ for (**e**) training; $F(3,39)=0.600$, $p=0.62$ for (**e**) retention. $^{**}p<0.01$ compared with saline + vehicle group. $\#p<0.05$ and $\#\#p<0.01$ compared with PCP + vehicle group

Effect of PCP on performance in novel object recognition

Eight days after the last treatment with PCP (10 mg/kg, for 14 days), the novel object recognition test was performed. During habituation, no behavioral change was observed between PCP- and saline-treated mice. In the training session, PCP-treated and saline-treated mice spent equal amounts of time exploring either of the two objects (Fig. 3b,c), and thus there was no biased exploratory preference in either group of animals. In addition, total time spent in the exploration of objects in the training session did not differ between PCP- and saline-treated mice (Fig. 3d,e). These observations suggest that repeated PCP treatment has no effect on motivation, curiosity, or motor function.

When retention session was performed 24 h after the training session, the level of exploratory preference for the novel objects in the PCP-treated mice was significantly decreased compared to that in the saline-treated mice ($p<0.01$, Fig. 3b,c). The total exploration time did not differ between the two groups in the retention session (Fig. 3d,e). These results suggest that repeated PCP treatment for 14 days induces recognition memory impairment 8 days after the withdrawal of PCP.

Effects of single and repeated administrations of aripiprazole and haloperidol on PCP-induced cognitive impairment in novel object recognition

We examined whether PCP-induced cognitive impairment was reversed by aripiprazole, an atypical antipsychotic, or haloperidol, a typical antipsychotic, treatment. After the cessation of repeated PCP treatment (10 mg/kg s.c., for 14 days), mice were subjected to the novel object recognition test. Aripiprazole (0.01–1.0 mg/kg, p.o.) or haloperidol (0.3–1.0 mg/kg, p.o.) was acutely administered 1 h before the training session. A one-way ANOVA revealed that single treatment with aripiprazole dose-dependently improved cognitive impairment in PCP-treated mice [$F(5,56)=3.474$, $p<0.01$, Fig. 3b]. In contrast, single treatment with haloperidol had no effect on the cognitive impairment induced by repeated PCP treatment (Fig. 3c). Neither aripiprazole nor haloperidol affected the level of exploratory preference for the novel object in the training session (Fig. 3b,c). However, single treatment with aripiprazole and haloperidol decreased total exploration time in the training session of PCP-treated mice [one-way ANOVA: $F(6,77)=5.009$, $p<0.01$, Fig. 3d; $F(3,39)=4.665$, $p<0.01$, Fig. 3e]. Single treatment with aripiprazole (0.3 and 1.0 mg/kg) or haloperidol (1.0 mg/kg) significantly decreased the total exploration time in the training session of PCP-treated mice ($p<0.05$ and $p<0.01$, respectively, Fig. 3d; $p<0.05$, Fig. 3e). In the saline-treated mice, neither aripiprazole nor haloperidol affected the level of exploratory preference for the novel object in the training session (data not shown). However, single treatment with aripiprazole and haloperidol decreased total exploration time in the training session of saline-treated mice [one-way ANOVA: $F(5,66)=6.532$, $p<0.01$ for aripiprazole; $F(2,30)=22.346$, $p<0.01$ for haloperidol].

Next, we examined the effect of repeated treatment with antipsychotics on PCP-induced cognitive impairment. Aripiprazole (0.01–0.1 mg/kg) or haloperidol (0.3 and 1.0 mg/kg) was repeatedly administered p.o. for 7 days to mice that had been previously treated with PCP for 14 days. As shown in Fig. 4, repeated treatment with aripiprazole dose-dependently improved cognitive impairment in PCP-treated mice [$F(4,43)=9.166$, $p<0.01$], and a significant change was observed with doses of 0.03 and 0.1 mg/kg ($p<0.05$ and $p<0.01$, respectively, Fig. 4b). In contrast, repeated treatment with haloperidol failed to improve PCP-induced cognitive impairment (Fig. 4c). Repeated treatment with aripiprazole and haloperidol affected neither the level of exploratory preference for the novel object in the training session nor the total exploration time in either the training or retention sessions for PCP-treated mice (Fig. 4). In the saline-treated mice, repeated treatment with aripiprazole or haloperidol alone showed no effect on performance in the novel object recognition test (data not shown).

Effects of dopamine D_1 and D_2 receptor antagonists on ameliorative effect of aripiprazole against PCP-induced cognitive impairment

We have previously demonstrated that repeated PCP treatment in mice induces the dysfunction of dopamine neurotransmission in the prefrontal cortex which is necessary for the recognition memory (Mouri et al. 2007b; Nagai et al. 2007). Therefore, we investigated whether activation of dopamine receptors was involved in the ameliorating effect of aripiprazole on memory impairment in PCP-treated mice. SCH23390 (0.05 mg/kg i.p.), a dopamine D_1 receptor antagonist, or raclopride (0.3 mg/kg i.p.), a dopamine D_2 receptor antagonist, was co-administered with aripiprazole for 7 days, and the training session of the novel object recognition test was performed 1 day after the last treatment.

SCH23390 significantly blocked the ameliorating effect of aripiprazole on the impairment of exploratory preference for a novel object in PCP-treated mice ($p<0.01$, Fig. 5b), although it had no effect on PCP-induced impairment of memory retention (Fig. 5b). Treatment with SCH23390 did not affect the total exploration time in either the training or retention sessions (Fig. 5d). In contrast, treatment with raclopride had no effect on exploratory preference or total exploration time in the training and retention sessions (Fig. 5c,e).

Effect of serotonin 5-HT_{1A} receptor antagonist on ameliorative effect of aripiprazole against PCP-induced cognitive impairment

It has been reported that aripiprazole also has partial agonistic activity for serotonin 5-HT_{1A} receptors in parallel to its actions at dopamine D_2 receptors (Jordan et al. 2002); therefore, we examined whether 5-HT_{1A} receptors were involved in the ameliorative effect of aripiprazole on memory impairment in PCP-treated mice. The 5-HT_{1A} receptor antagonist WAY100635 (0.6 mg/kg, i.p.) was co-administered with aripiprazole for 7 days, and the training session of the novel object recognition test was performed 1 day after the last treatment.

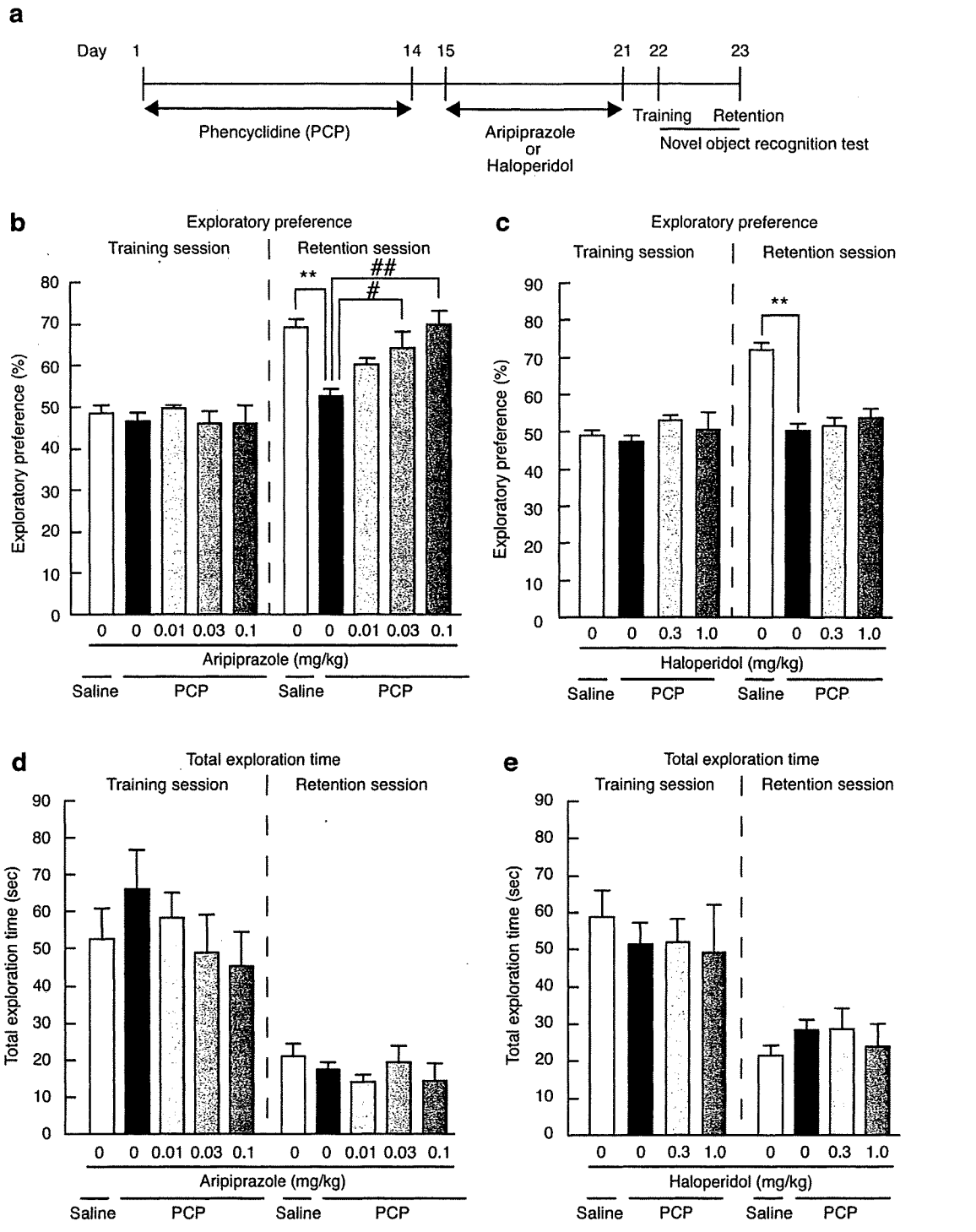
In the training session, treatment with WAY100635 alone did not affect the exploratory preference for objects in PCP-treated mice (Fig. 6b). In the retention session, WAY100635 blocked the ameliorating effect of aripiprazole on the impairment of exploratory preference for a novel object in PCP-treated mice ($p<0.01$, Fig. 6b), although it had no effect on PCP-induced impairment of memory retention (Fig. 6b). The antagonistic effect of WAY100635 on aripiprazole-induced improvement of exploratory preference in PCP-treated mice was not associated with changes in total exploration time (Fig. 6c).

Discussion

Cognitive deficits, including memory impairment, are regarded as a core feature in schizophrenia (Tamminga 2006). Recognition memory is a fundamental facet of the ability to remember and an integral component of the class of memory lost in amnesia (Aggleton and Brown 1999). The ability to discriminate familiar from novel stimuli is supported by this form of memory. To assess the effect of a novel atypical antipsychotic, aripiprazole, on cognitive dysfunction, we used a novel object recognition task, which is similar to visual recognition tests widely used in subhuman primates (Ennaceur and Delacour 1988). In the present study, an object 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 object, was used to measure cognitive function. However, it is possible that the ratio misleads the results when based on a low amount a little second on average for the whole group of mice. Therefore, we also run a paired comparisons test for each group comparing the time spent on a novel with that spent on a familiar object. The saline-treated mice spent an equal time exploring the two objects (object A and object B) in the training session (Supplemental Fig. 1a), but a significantly greater time exploring the novel object (object C) versus the familiar object (object A) in the retention session (Supplemental Fig. 1b), showing that they were able to discriminate the novel object during the retention session. PCP + vehicle-treated mice also spent an equal time exploring the two objects in training session (Supplemental Fig. 1a). However, PCP + vehicle-treated mice spent equivalent times exploring the novel and the familiar objects in retention session (Supplemental Fig. 1b), whereas PCP + single aripiprazole-treated mice spent greater time exploring the novel (Supplemental Fig. 1b). These observations agree with the results using an object preference index ratio. Taken together, it is unlikely that the ratio misleads the results in this study.

In the present study, repeated PCP treatment for 14 days induced recognition memory impairment 8 days after the withdrawal of PCP, and PCP-induced cognitive impairment was ameliorated by aripiprazole, but not haloperidol. The results are consistent with a previous report that PCP-induced cognitive deficits were improved by atypical antipsychotics, such as clozapine and perospiron, but not a typical antipsychotic, haloperidol, in a novel object recognition test (Hagiwara et al. 2008; Hashimoto et al. 2005). It is possible that the doses of haloperidol (0.3–1.0 mg/kg) used in the present study is probably too high in terms of occupancy compared to that produced by aripiprazole (0.01–1.0 mg/kg) and the relative affinities for D_2 receptors (Hirose and Kikuchi 2005). Haloperidol at the dose of 0.3 mg/kg was used as the maximal dose which did not cause locomotor suppression in this study, but it had no effect on PCP-induced memory impairment. In addition, it has been demonstrated that more low doses of haloperidol (0.05–0.1 mg/kg) do not improve PCP-induced memory impairment in the novel object recognition test (Grayson et al. 2007; Hashimoto et al. 2005). Taken together, these findings suggest that aripiprazole, but not haloperidol, may be useful for the treatment of cognitive dysfunction in schizophrenia.

Fig. 4 Effects of repeated administrations of aripiprazole and haloperidol on PCP-induced cognitive impairment in novel object recognition. **a** Experimental schedule for the novel object recognition test. Aripiprazole (0.01–0.1 mg/kg, p.o.), haloperidol (0.3–1.0 mg/kg, p.o.), or vehicle (0.1% CMC) was administered for 7 days to mice that had previously been treated with saline or PCP (10 mg/kg, s.c.) for 14 days. The novel-object recognition test was performed 24 h after the last treatment with aripiprazole or saline. **b** and **d** Effect of aripiprazole on PCP-induced cognitive impairment. **c** and **e** Effect of haloperidol on PCP-induced cognitive impairment. **b** and **c** Exploratory preference. **d** and **e** Total exploration time. Values indicate the mean±SE (n=8–15). Analysis of variance: $F(4,43)=0.851$, $p=0.50$ for (**b**) training; $F(4,43)=9.166$, $p<0.01$ for (**b**) retention; $F(3,42)=1.049$, $p=0.38$ for (**c**) training; $F(3,42)=25.898$, $p<0.01$ for (**c**) retention; $F(4,43)=1.157$, $p=0.34$ for (**d**) training; $F(4,43)=1.029$, $p=0.40$ for (**d**) retention; $F(3,42)=0.305$, $p=0.82$ for (**e**) training; $F(3,42)=0.915$, $p=0.44$ for (**e**) retention. $^{**}p<0.01$ compared with saline + vehicle group. $^{\#}p<0.05$ and $^{\#\#}p<0.01$ compared with PCP + vehicle group



It has been reported that aripiprazole acts as a dopamine D_2 receptor antagonist in the state of excessive dopamine neurotransmission and as a dopamine D_2 receptor agonist in the state of low dopaminergic neurotransmission (Burris et al. 2002; Kikuchi et al. 1995; Inoue et al. 1996). Single treatment with aripiprazole (1.0 mg/kg) ameliorated PCP-induced impairment of recognition memory, although the treatment significantly decreased total exploration time in

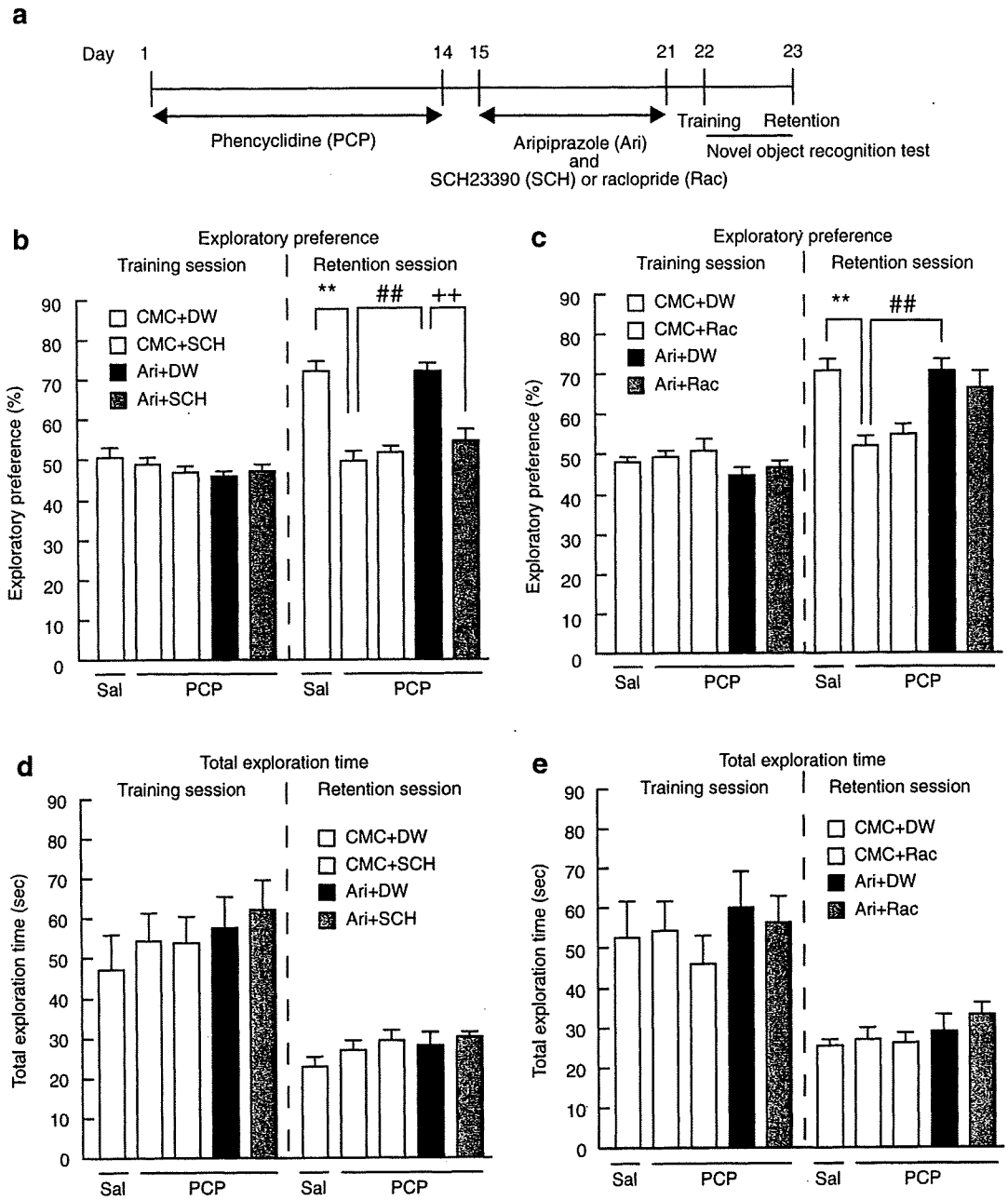


Fig. 5 Effect of dopamine D_1 and D_2 receptor antagonists on ameliorative effect of aripiprazole against PCP-induced cognitive impairment. **a** Experimental schedule for the novel object recognition test using dopamine D_1 and D_2 receptor antagonists. Aripiprazole (Ari, 0.1 mg/kg, p.o.) or vehicle (0.1% CMC) was administered for 7 days to mice that had previously been treated with saline (Sal) or PCP (10 mg/kg, s.c.) for 14 days. SCH23390 (SCH, 0.05 mg/kg, i.p.), Raclopride (Rac, 0.3 mg/kg, i.p.) or distilled water (DW) was administered 30 min after aripiprazole treatment for 7 days. The novel-object recognition test was performed 24 h after the last treatment with Ari. **b** and **d** Effect of SCH on ameliorative effect of Ari against PCP-induced cognitive impairment. **c** and **e** Effect of Rac on ameliorative effect of Ari against PCP-induced cognitive impairment. **b** and **c** Exploratory preference. **d** and **e** Total exploration time. Values indicate the mean±SE (n=9–11). Analysis of variance: $F(4,50)=0.951$, $p=0.44$ for (**b**) training; $F(4,50)=20.732$, $p<0.01$ for (**b**) retention; $F(4,42)=1.212$, $p=0.32$ for (**c**) training; $F(4,42)=8.520$, $p<0.01$ for (**c**) retention; $F(4,50)=0.527$, $p=0.72$ for (**d**) training; $F(4,50)=1.261$, $p=0.30$ for (**d**) retention. $F(4,42)=0.426$, $p=0.79$ for (**e**) training; $F(4,42)=1.210$, $p=0.32$ for (**e**) retention. $^{**}p<0.01$ compared with Sal + CMC + DW group. $^{\#\#}p<0.01$ compared with PCP + CMC + DW group. $^{++}p<0.01$ compared with PCP + Ari + DW group

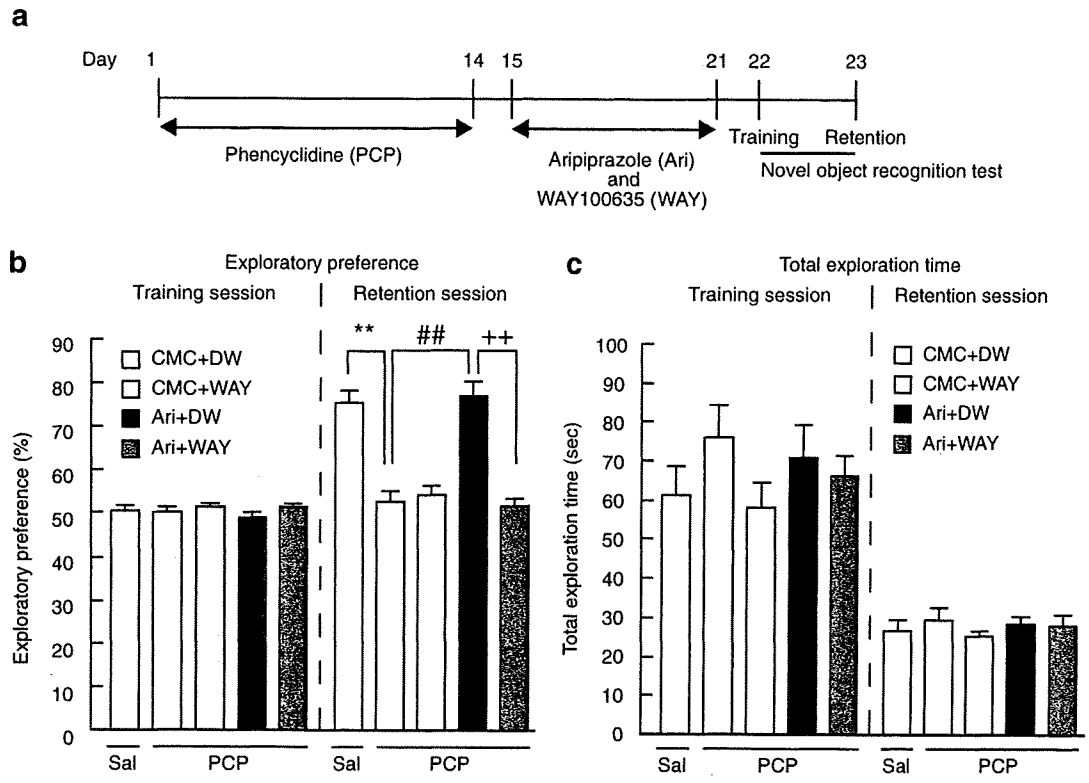


Fig. 6 Effect of serotonin 5-HT_{1A} receptor antagonist on ameliorative effect of aripiprazole against PCP-induced cognitive impairment. **a** Experimental schedule for the novel object recognition test using a serotonin 5-HT_{1A} receptor antagonist. Aripiprazole (Ari, 0.1 mg/kg, p.o.) or vehicle (0.1% CMC) was administered for 7 days to mice that had previously been treated with saline (Sal) or PCP (10 mg/kg, s.c.) for 14 days. WAY100635 (WAY, 0.6 mg/kg, i.p.) or distilled water (DW) was administered 30 min after Ari treatment for 7 days. The novel-object recognition test was performed 24 h after the last treatment with Ari. **b** Exploratory preference. **c** Total exploration time. Values indicate the mean±SE (n=9–10). Analysis of variance: $F(4,44)=0.921$, $p=0.46$ for (**b**) training; $F(4,44)=25.562$, $p<0.01$ for (**b**) retention; $F(4,44)=0.915$, $p=0.46$ for (**c**) training; $F(4,44)=0.327$, $p=0.86$ for (**c**) retention. $^{**}p<0.01$ compared with Sal + CMC + DW group. $^{\#\#}p<0.01$ compared with PCP + CMC + DW group. $^{++}p<0.01$ compared with PCP + Ari + DW group

the training session. Therefore, aripiprazole at 0.1 mg/kg was used in the repeated treatment as the maximal dose which did not cause locomotor suppression in mice.

Accumulating evidence suggests that the dopaminergic system in the prefrontal cortex is involved in cognitive function. For instance, disruption of dopamine transmission in the prefrontal cortex by infusions of dopamine D_1 receptor antagonists or by excitotoxic lesions impairs the performance of object retrieval detour tasks, as well as delayed response tasks in nonhuman primates (Sawaguchi and Goldman-Rakic 1991; Dias et al. 1996a, b). A recent study with functional magnetic resonance imaging showed that dysfunction in the prefrontal cortex of schizophrenic patients is related to cognitive impairment (Tan et al. 2007). Accordingly, cognitive impairment in schizophrenia may be associated with deficits in dopamine transmission in the prefrontal cortex. In the present study, the ameliorative effect of aripiprazole on PCP-induced cognitive impairment was prevented by a dopamine D_1 receptor antagonist, but not a dopamine D_2 receptor antagonist. Our previous study has demonstrated that stimulation with dopamine D_1 receptors is necessary for long-term retention of recognition memory in the prefrontal cortex (Kamei et al. 2006; Nagai et al. 2007). Taken together, these findings indicated that dopamine D_1 receptor in the prefrontal cortex may play a critical role in the ameliorative effect of aripiprazole on PCP-induced cognitive impairment.

Although aripiprazole has high affinity for dopamine D_2 receptors, a dopamine D_2 receptor antagonist had no effect on the ameliorative effect of aripiprazole on PCP-induced cognitive impairment. One possible reason for this discrepancy is that, the ability of dopamine D_1 receptor stimulation to improve cognition is due to a particular cellular localization in cortical networks: It has been demonstrated that dopamine D_1 receptors preferentially localize to non-pyramidal neurons, while dopamine D_2 receptors localize to both nonpyramidal and pyramidal cells in the prefrontal cortex of rats (Vincent et al. 1995). The other possible reason is that the ameliorative effect of aripiprazole on PCP-induced cognitive impairment may be involved in

receptors other than the dopamine D_2 receptor, since aripiprazole interacts with not only dopamine D_2 receptor, but also a large number of biogenic amine receptors (Shapiro et al. 2003). However, we cannot exclude the possibility that a part of ameliorative effect of aripiprazole on PCP-induced cognitive impairment is through dopamine D_2 receptor. Further studies are needed by using other dopamine D_2 antagonists or dopamine D_2 receptor knockout mice.

Accumulating evidence has suggested that serotonin 5-HT_{1A} receptors are an important target for cognitive dysfunction in schizophrenia (Bantick et al. 2001; Meltzer 1999). The density of 5-HT_{1A} receptor binding is altered in the hippocampus and cerebral cortex of the postmortem brain of schizophrenic patients (Burnet et al. 1996; Gurevich and Joyce 1997; Joyce et al. 1993; Lopez-Figueroa et al. 2004). Adjunctive treatment with tandospirone, a selective 5-HT_{1A} receptor agonist, is associated with improvements in some types of memory function as well as the cognitive performance of schizophrenic patients (Sumiyoshi et al. 2001a, b). Preclinical studies on the action of aripiprazole at 5-HT_{1A} receptors have shown partial agonist activity in vitro and in vivo (Jordan et al. 2002; Shapiro et al. 2003; Stark et al. 2007). In the present study, 5-HT_{1A} receptor antagonist blocked the ameliorating effect of aripiprazole on cognitive impairment in PCP-treated mice. Therefore, these results supported that atypical antipsychotic drugs, such as aripiprazole, clozapine, ziprasidone, and quetiapine, which have 5-HT_{1A} receptor agonist activity, are useful for cognitive impairment in schizophrenia (Jordan et al. 2002; Newman-Tancredi et al. 2001; Rollema et al. 2000; Sprouse et al. 1999)

The mechanisms by which aripiprazole ameliorates PCP-induced cognitive dysfunction through serotonin 5-HT_{1A} and dopamine D_1 receptors remain to be determined; however, it is known that the activation of 5-HT_{1A} receptors in the prefrontal cortex enhances the activity of dopaminergic neurons in the ventral tegmental area and mesocortical dopamine release (Díaz-Mataix et al. 2005). Aripiprazole increases the release of dopamine in the prefrontal cortex of rats and mice through the activation of 5-HT_{1A} receptors (Bortolozzi et al. 2007; Li et al. 2004; Zocchi et al. 2005). Recently, we have also observed that microinjection of 5-HT_{1A} receptor antagonist into the prefrontal cortex blocked the ameliorating effect of aripiprazole on cognitive impairment in PCP-treated mice (unpublished data). Accordingly, it is likely that stimulation of 5-HT_{1A} receptors in the prefrontal cortex induces dopamine D_1 receptor activation through the mesocortical dopaminergic pathway, which is involved in the ameliorating effect of aripiprazole on PCP-induced cognitive dysfunction.

Since aripiprazole has the 5-HT_{2A} receptor antagonistic activity displayed by atypical antipsychotics, such as clozapine, olanzapine, and risperidone (McQuade et al. 2002), involvement of 5-HT_{2A} receptors in the ameliorating effect of aripiprazole remains to be determined. However, it has been reported that 5-HT_{2A} receptor blockade increases dopamine release in the prefrontal cortex by atypical antipsychotics, and the increase of dopamine release is partly or totally antagonized by 5-HT_{1A} antagonist and by a defect of the 5-HT_{1A} receptor gene (Ichikawa et al. 2001; Díaz-Mataix et al. 2005). Therefore, it is likely that atypical antipsychotics through 5-HT_{2A} blockade, regardless of intrinsic 5-HT_{1A} affinity, may promote the ability of 5-HT_{1A} receptor stimulation to increase dopamine release.

In conclusion, we demonstrated that repeated PCP treatment impaired the recognition memory of novel objects. Single treatment with aripiprazole (1.0 mg/kg) ameliorated PCP-induced impairment of recognition memory, although it significantly decreased the total exploration time in the training session. Repeated treatment with aripiprazole at doses of 0.03 and 0.1 mg/kg for 7 days showed a significant ameliorating effect on PCP-induced impairment of recognition memory without affecting the total exploration time in training and retention sessions. In contrast, both single and repeated treatment with haloperidol (0.3 and 1.0 mg/kg) failed to reverse PCP-induced cognitive impairment. The ameliorating effect of aripiprazole on recognition memory in PCP-treated mice was blocked by dopamine D_1 and serotonin 5-HT_{1A} receptor antagonists; however, dopamine D_2 receptor antagonist had no effect on the ameliorating effect of aripiprazole. These results suggest that the ameliorative effect of aripiprazole on PCP-induced cognitive impairment is associated with dopamine D_1 and serotonin 5-HT_{1A} receptors.

Acknowledgements This work was supported, in part, as Research on Regulatory Science of Pharmaceuticals and Medical Devices, and Research on Risk of Chemical Substances, Health and Labour Sciences Research Grants from the Ministry of Health, Labor and Welfare, Japan, Academic Frontier Project for Private Universities (2007–2011) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and from Uehara Memorial Foundation, International Research Project supported by The Meijo Asian Research Center (MARC). We are grateful to Otsuka Pharmaceutical Co. for providing the aripiprazole.

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International Journal of Neuropsychopharmacology, Page 1 of 12. Copyright © CINP 2010
doi:10.1017/S1461145710000222

ARTICLE

CINP

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

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Abstract

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

Received 5 June 2009; Reviewed 15 July 2009; Revised 19 January 2010; Accepted 3 February 2010

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

Introduction

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

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

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

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

Methods

Animals

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

Drugs

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

Drug treatment

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

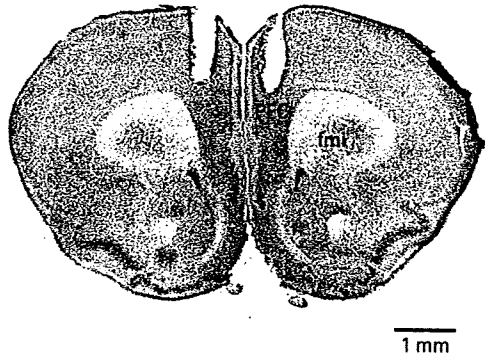

Fig. 1. Representative figure of mPFC local injection site. PFC; prefrontal cortex, fmi; forceps minor of the corpus callosum.

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

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

NOR test

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

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

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

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

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

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

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

Western blotting

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

Statistical analysis

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

Results

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

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

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

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

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

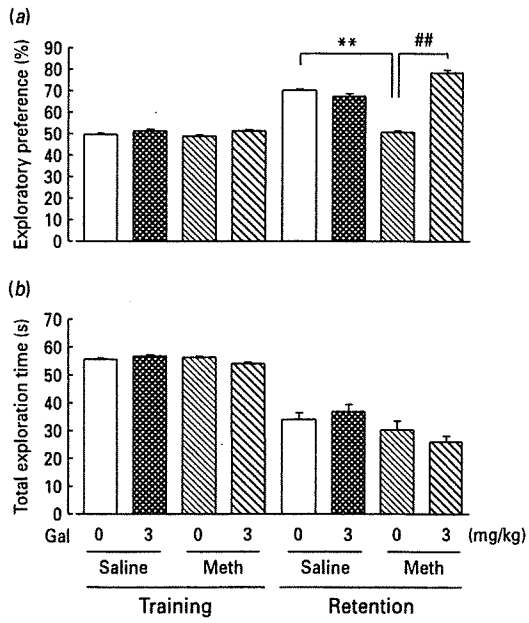

Fig. 2. Effect of galantamine on methamphetamine (Meth)-induced impairment of recognition memory in mice: (*a*) exploratory preference and (*b*) total exploration time. One day after the cessation of repeated Meth (1 mg/kg s.c.) treatment for 7 d, mice underwent the novel object recognition test. Galantamine (3 mg/kg p.o.) or saline was administered 1 h before the training session. Values indicate the mean ± 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. 3*a*). 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. 3*b*). 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. 3*a*) 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. 3*c*).

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. 4*a*,*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. 4*a*). In saline-treated mice, mecamylamine alone at the dose used had no effect on the NOR performances (Fig. 4*a*). 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. 4*b*).

Scopolamine at a dose of 0.1 mg/kg impaired the performance of saline-treated mice in the NOR task (Fig. 4*c*). However, scopolamine failed to prevent the improving effects of galantamine on the impairment of recognition memory in Meth-treated mice (Fig. 4*c*). 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. 4*d*).

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

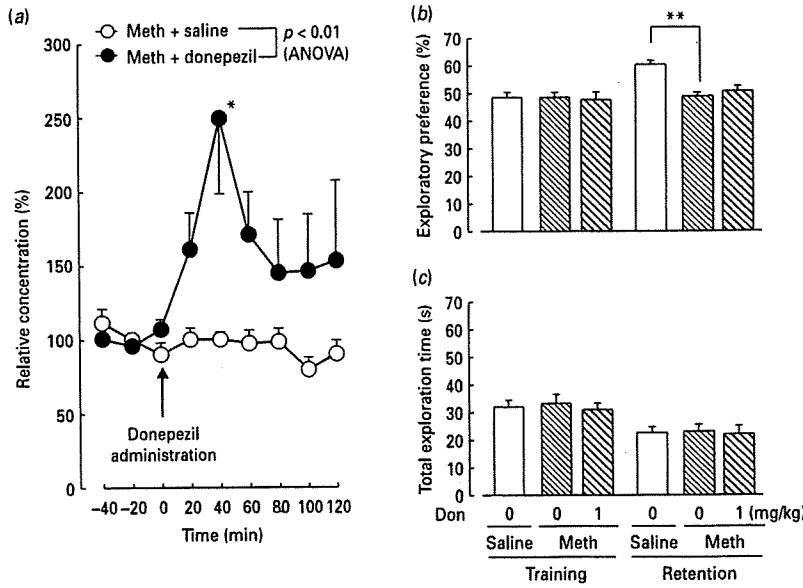

Fig. 3 Effect of donepezil on the extracellular acetylcholine (ACh) levels of the prefrontal cortex (PFC) and the impairment of recognition memory in methamphetamine (Meth)-treated mice. (*a*) Extracellular ACh levels of PFC in microdialysis. *In-vivo* microdialysis was performed 3 d after the final 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 ± 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 × 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 μl 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 ± 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_1Rs) (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_1R-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_1Rs, we investigated the antagonism by using SCH 23390, a dopamine D_1R 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. 6*a*). In saline-treated mice, SCH 23390 alone had no effect on NOR performance (Fig. 6*a*). 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. 6*b*).

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

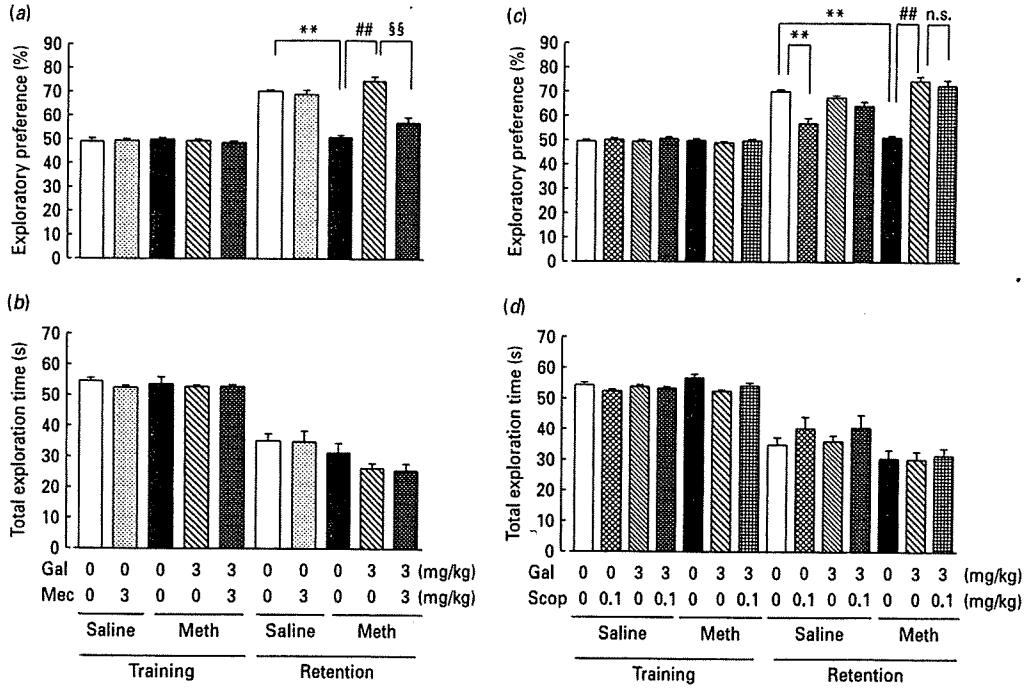

Fig. 4. Involvement of nicotinic receptors, but not muscarinic receptors in the cognitive-improving effect of galantamine on methamphetamine (Meth)-treated mice: (*a*, *c*) exploratory preference and (*b*, *d*) total exploration time. One day after the cessation of repeated Meth (1 mg/kg s.c.) treatment 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 ± 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. 7*a*, *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. 7*a*). 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. 7*a*). 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. 7*a*). 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. 7*b*). 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 μg/1 μl/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 μg/1 μl/bilateral) administered before the training session on

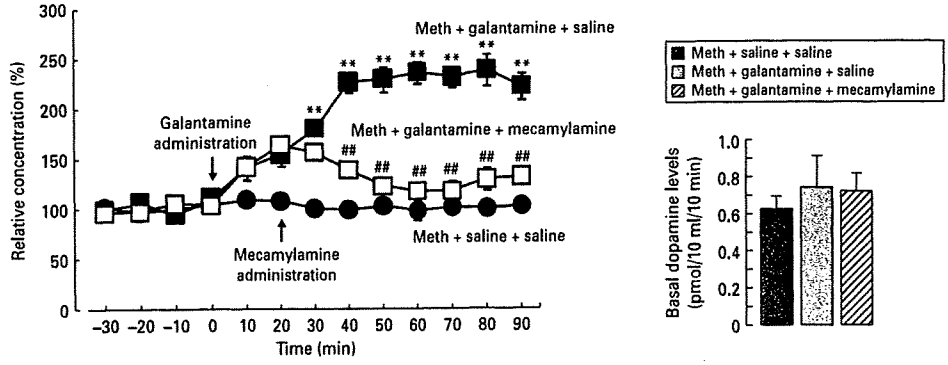

Fig. 5. Effects of galantamine on the levels of the extracellular dopamine in the PFC of methamphetamine (Meth)-treated mice. Meth (1 mg/kg, s.c.) was injected for 7 d, and 3 d after withdrawal, extracellular levels of dopamine were measured in the PFC by *in-vivo* microdialysis. Galantamine (3 mg/kg 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. Mecamylamine (3 mg/kg s.c.) was injected 20 min after galantamine (□, Meth + galantamine + mecamylamine) to Meth-treated mice. The basal levels of dopamine in the PFC of the Meth + saline + saline (■)-, Meth + galantamine + saline (▣)- and Meth + galantamine + mecamylamine (▨)-treated mice were 0.62 ± 0.08, 0.74 ± 0.18 and 0.72 ± 0.10 pmol/10 μl per 10 min, respectively (right-hand panel). Values indicate the mean ± 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 × 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 μg/side) of saline-treated mice did not affect the exploratory preference for the objects (Fig. 8*a*). 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. 8*a*), but it was significantly decreased compared to that in vehicle-treated mice ($p < 0.05$, Fig. 8*a*). PD98059 had no effect on the total exploration time in either the training or retention sessions of saline-treated mice (Fig. 8*b*).

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. 8*c*). 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. 8*d*).

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.* 2007*a*, *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