Silver *et al*, 2000), although the precise mechanisms underlying the efficacy of SSRIs on these symptoms are currently unclear. We previously reported that SSRIs possess high to moderate affinity for sigma-1 receptors (Narita *et al*, 1996). Among the SSRIs, fluvoxamine was the most potent ($K_i = 36$ nM) for sigma-1 receptors, and paroxetine was weak ($K_i = 1893$ nM) for sigma-1 receptors, suggesting that these receptors may in some way play a role in the mechanisms of action of fluvoxamine (Narita *et al*, 1996; Hashimoto and Ishiwata, 2006). Multiple lines of evidence suggest that sigma-1 receptors play a role in the pathophysiology of neuropsychiatric diseases such as schizophrenia, anxiety disorders, and depression, as well as in the evolution of cognitive deficits associated with these conditions (Debonnel and de Montigny, 1996; Maurice *et al*, 2001; Su and Hayashi, 2003; Hayashi and Su, 2004; Takebayashi *et al*, 2004; Bermack and Debonnel, 2005; Hashimoto and Ishiwata, 2006).

The present study was undertaken to examine the effects of fluvoxamine and paroxetine on PCP-induced cognitive deficits in mice using the novel object recognition test. We also examined the effects of the selective sigma-1 receptor antagonist NE-100 (Okuyama and Nakazato, 1996), the selective sigma-1 receptor agonist SA4503 (Matsuno and Mita, 1998), and the endogenous sigma-1 receptor agonist dehydroepiandrosterone 3-sulfate (DHEA-S) (Urani *et al*, 2001; Takebayashi *et al*, 2004) on PCP-induced cognitive deficits in order to study the role of the sigma-1 receptor in the mechanism of action of fluvoxamine.

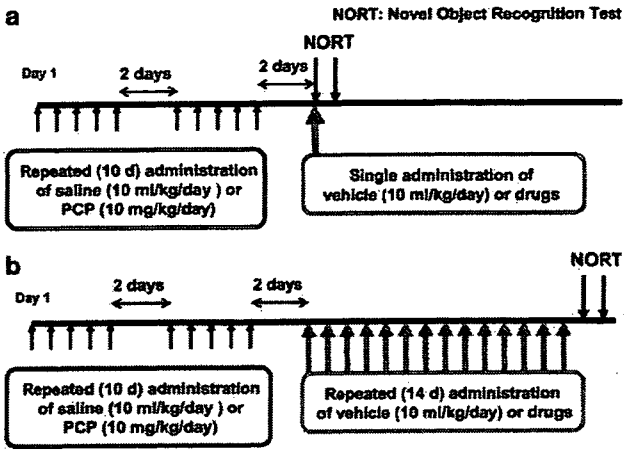

Figure 1 Treatment schedule. (a) Acute treatment. Saline (10 ml/kg) or PCP (10 mg/kg) were administered s.c. for 10 days (once daily on days 1–5 and 8–12). At 3 days (day 15) after the final administration of saline or PCP, vehicle (10 ml/kg) or drugs were administered i.p. into mice. The training session for the novel object recognition test was performed 1.5 h after administration of vehicle or drugs, and the retention test session was performed 24 h after the training session. (b) Subchronic (2-week) treatment. Saline (10 ml/kg) or PCP (10 mg/kg) were administered s.c. for 10 days (once daily on days 1–5 and 8–12). At 3 days (day 15) after the final administration of saline or PCP, vehicle (10 ml/kg) or drugs were administered i.p. into mice. This treatment was continued for 2 consecutive weeks (once daily on days 15–28). The training session for the novel object recognition test was performed 24 h after the final administration of vehicle or drugs, and the retention test session was performed 24 h after the training session.

METHODS

Animals

Male ICR mice (6 weeks old) weighing 25–30 g were purchased from SLC Japan (Hamamatsu, Shizuoka, Japan). Mice in groups of 4 or 5 were housed in clear polycarbonate cages ($22.5 \times 33.8 \times 14.0\,cm^3$) under a controlled 12/12 h light–dark cycle (light from 0700 to 1900 h), at 23 ± 1°C and 55 ± 5% humidity. The mice were given free access to water and food pellets. The experimental procedure was approved by the Animal Care and Use Committee of Chiba University Graduate School of Medicine.

Drugs

PCP hydrochloride and the selective sigma-1 receptor antagonist NE-100 were synthesized in our laboratory. Fluvoxamine maleate was obtained from Solvay Seiyaku K.K. (Tokyo, Japan); paroxetine hydrochloride and DHEA-S sodium salt from Sigma-Aldrich (St Louis, MO, USA); and the selective sigma-1 receptor agonist SA4503 from M's Science Corporation (Kobe, Japan). Other drugs were purchased from commercial sources.

Drug Administration

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

In the acute experiment, vehicle (10 ml/kg) or drugs were administered intraperitoneally (i.p.) into mice 3 days (day 15) after the final administration of saline or PCP. The training session of the novel object recognition test was performed 1.5 h after administration, and the test session was performed 24 h after the training session as described below (Figure 1a). We also used the novel object recognition test to study the effect of the withdrawal of the drug. In these tests, the training session was performed 24 h after a single administration of fluvoxamine (20 mg/kg), and the retention test session was performed 24 h after the training session.

In the subchronic (2-week) administration experiment, 3 days (day 15) after the final administration of saline or PCP, vehicle or drugs were administered i.p. into mice. This treatment was continued for 2 consecutive weeks (once daily on days 15–28). The training session of the novel object recognition test was performed 24 h after the final administration, and the retention test session was performed 24 h after the training session as described below (Figure 1b). Next, we performed the novel object recognition test in order to study the comparative effects of withdrawal of the drug and the duration of treatment. The training session was performed 1.5 h after the final administration of fluvoxamine (20 mg/kg/day for 2 weeks), and the retention test session was performed 24 h after the training session.

The doses of drugs used in this study were fluvoxamine (20 mg/kg), paroxetine (10 mg/kg), SA 4503 (1 mg/kg), NE-100 (1 mg/kg), and DHEA-S (25 mg/kg). These doses had been shown to be effective *in vivo* as sigma-1 receptor agonists or antagonists as reported previously (Sánchez and

Meier, 1997; Okuyama and Nakazato, 1996; Matsuno *et al*, 1997; Matsuno and Mita, 1998; Minabe *et al*, 1999; Zou *et al*, 2000; Urani *et al*, 2001).

Spontaneous Locomotor Activity in Mice Treated with Saline or PCP

At 3 days (day 15) or 17 days (day 29) after the final administration of saline (10 ml/kg, s.c.) or PCP (10 mg/kg, s.c.), locomotor activity was measured using an animal movement analysis system (SCANET SV-10, Melquest, Toyama, Japan) as reported previously (Fukami *et al*, 2004).

Novel Object Recognition Test

The novel object recognition test was performed as previously reported (Tang *et al*, 1999, 2001; Hashimoto *et al*, 2005a; Ozawa *et al*, 2006). The apparatus for this task consisted of a black open field box ($50.8 \times 50.8 \times 25.4\,cm^3$). Before the test, mice were habituated in the box for 3 days. During a training session, two objects (various objects differing in shape and color but similar in size) were placed in the box 35.5 cm apart (symmetrically), and each animal was allowed to explore in the box for 5 min. The animals were considered to be exploring the object when the head of the animal was facing the object within 2.54 cm of the object or when any part of the body, except for the tail, was touching the object. The time that mice spent exploring each object was recorded. After training, mice were immediately returned to their home cages, and the box and objects were cleaned with 75% ethanol to avoid any possible instinctive odorant cues. Retention tests were carried out at 1-day intervals following the respective training. During the retention test, each mouse was placed back into the same box in which one of the objects used during training was replaced by a novel one. The mice were then allowed to explore freely for 5 min, and the time spent exploring each object was recorded. Throughout the experiments, the objects were used in a counterbalanced manner in terms of their physical complexity and emotional neutrality. A preference index, that is, the ratio of the amount of time spent exploring any one of the two objects (training session) or the novel one (retention test session) over the total time spent exploring respective to both objects, was used to measure memory performance.

Statistical Analysis

Data are expressed as means ± SEM. Statistical analysis was performed using one-way analysis of variance analysis (ANOVA) and the *post hoc* Bonferroni test. *P*-values less than 0.05 were considered statistically significant.

RESULTS

First, we measured spontaneous locomotor activity in mice after repeated administration of saline (10 mg/kg/day, s.c.) or PCP (10 mg/kg/day, s.c.) (days 1–5 and 8–12). At 3 days (day 15) and 17 days (day 29) after the final administration of saline or PCP, there was no difference between the saline-treated group and the PCP-treated groups in terms of spontaneous locomotor activity (Figure 2).

In the novel object recognition test, we recently reported that the repeated administration of PCP (10 mg/kg/day for 10 days) caused significant cognitive deficits 3 days and 6 weeks after the final administration of PCP (Hashimoto *et al*, 2005a). In the training session of the present study, the exploratory preferences of the two groups were the same. However, in the retention test session, the exploratory preference (approximately 40%) of the PCP-treated group was significantly lower than that (approximately 50%) of the saline-treated group 2 weeks after the final administration of PCP (Figure 3). It seems that small deviations may be important to interpretation since the effects of PCP-induced cognitive deficits are small in this paradigm. During the training session, there were no differences between the saline- and PCP-treated groups in the total amount of time spent exploring the two objects.

In the acute experiments, the effects of a single administration of fluvoxamine (20 mg/kg), paroxetine (10 mg/kg), SA4503 (1 mg/kg), NE-100 (1 mg/kg), fluvoxamine (20 mg/kg) + NE-100 (1 mg/kg) or SA4503 (1 mg/kg) + NE-100 (1 mg/kg) were examined. The training session was performed 1.5 h after a single administration of vehicle or drug. Then, the retention test session was performed 24 h after the training session. In the training session, the

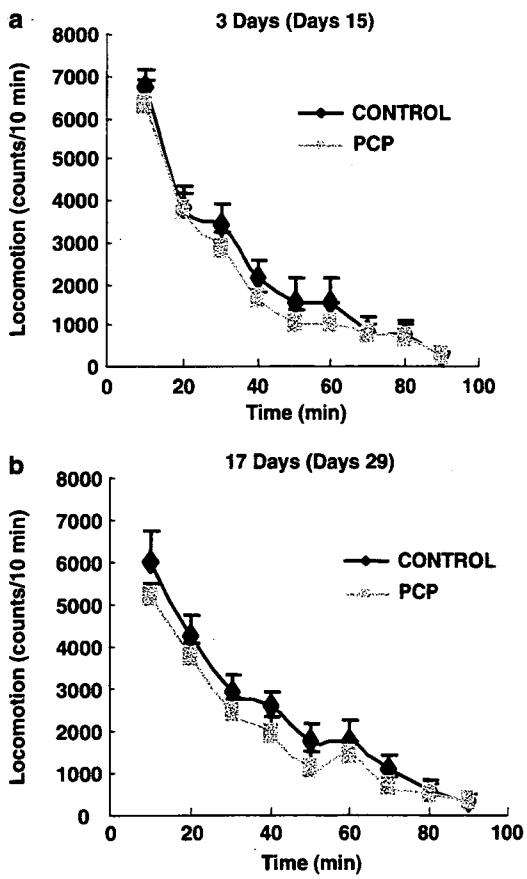

Figure 2 Spontaneous locomotion in mice treated with saline or PCP. Saline (10 ml/kg) or PCP (10 mg/kg) were administered s.c. for 10 days (once daily on days 1–5 and 8–12). Spontaneous locomotor activity in mice was measured 3 days (a: day 15) or 17 days (b: day 29) after the final administration of saline or PCP.

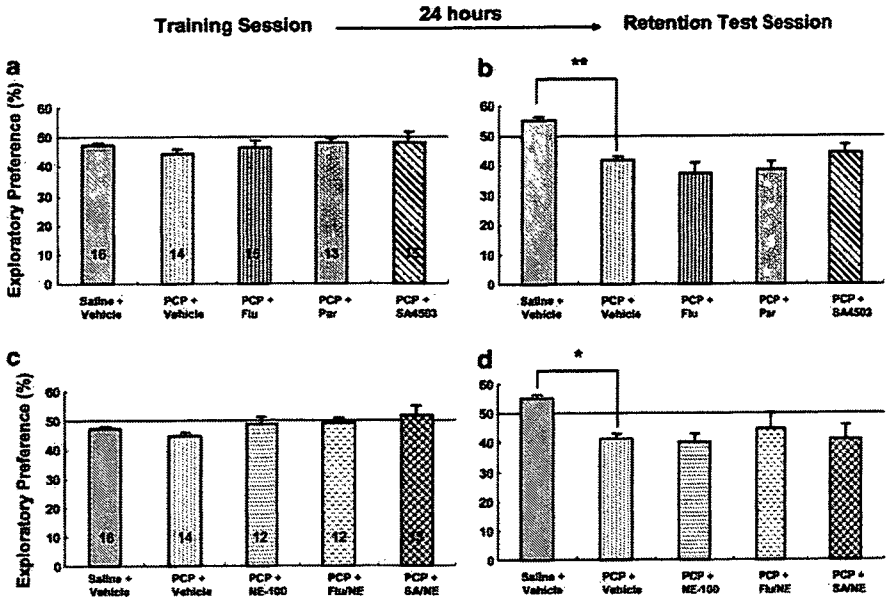

Figure 3 Effects of acute administration of drugs on PCP-induced cognitive deficits in mice. Saline (10 ml/kg) or PCP (10 mg/kg) were administered s.c. for 10 days (once daily on days 1–5 and 8–12). At 3 days (day 15) after the final administration of saline or PCP, vehicle (10 ml/kg), fluvoxamine (20 mg/kg), paroxetine (10 mg/kg), SA4503 (1 mg/kg) or a combination of one of the above with NE-100 (1 mg/kg) were administered i.p. into mice. The training session for the novel object recognition test was performed 1.5 h after administration of drugs, and the retention test session was performed 24 h after the training session. Values are means ± SEM. The numbers on the columns indicate the numbers of animals used. $*p<0.05$, $**p<0.011$ as compared with PCP + Vehicle-treated group.

exploratory preference of mice with each of the drug treatments was the same as that of the control group (Figure 3a and c). In the retention test session, none of the drugs altered the reduction of exploratory preference in mice after repeated administration of PCP (Figure 3b and d). In our study of the effect of the withdrawal of the drug, in which the training session was performed 24 h after a single administration of fluvoxamine (20 mg/kg), the single administration of fluvoxamine (20 mg/kg, 24 h after injection) did not affect PCP-induced cognitive deficits in mice (data not shown). These findings suggest that acute administration of these drugs is ineffective for PCP-induced cognitive deficits in mice.

In contrast, PCP-induced cognitive deficits were significantly improved after subsequent subchronic (2-week) administration of fluvoxamine (20 mg/kg/day). In the training session, one-way ANOVA revealed that the exploratory preferences of six groups were not significantly different ($F[5,112]=1.481$, $p=0.202$) (Figure 4a). However, one-way ANOVA did reveal significant differences in the exploratory preferences of the six groups in the retention test sessions ($F[5,112]=5.447$, $p<0.001$) (Figure 4b). The *post hoc* Bonferroni test indicated that the exploratory preference of the PCP plus vehicle-treated group was significantly ($p<0.001$) lower than that of the PCP plus fluvoxamine (20 mg/kg/day)-treated group, but not of groups treated with PCP plus fluvoxamine (20 mg/kg/day)/NE-100 (1 mg/kg/day) and PCP plus NE-100 (1 mg/kg/day) (Figure 4b). These findings suggest that sigma-1 receptors play a role in the active mechanism of fluvoxamine. Furthermore, in both the training session and the retention test session, the exploratory preferences of the group with subchronic administration of fluvoxamine (20 mg/kg/day for 2 weeks) alone were not different from that of the control group (Figure 4). In our study of the comparative effects of the withdrawal of the drug and the duration of treatment, in which the training session was performed 1.5 h after the final administration of fluvoxamine (20 mg/kg/day for 2 weeks), the fluvoxamine-treated group showed significant improvement of PCP-induced cognitive deficits (data not shown). These data suggest that the effects of fluvoxamine might depend more on the duration of treatment than on the withdrawal of the drug.

The effects of another SSRI paroxetine on PCP-induced cognitive deficits were examined. As shown in Figure 5, PCP-induced cognitive deficits were not affected by the subsequent subchronic (2-week) administration of paroxetine (10 mg/kg/day). In the retention test session, one-way ANOVA revealed that the exploratory preferences of three groups were significantly different ($F[2,54]=5.565$, $p=0.006$) (Figure 5b). The *post hoc* Bonferroni test indicated that the exploratory preference of the PCP plus vehicle-treated group was not statistically significantly different from that of the PCP plus paroxetine-treated group.

Next, we examined the effects of the selective sigma-1 receptor agonist SA4503 (1 mg/kg/day for 2 weeks) and the endogenous sigma-1 receptor agonist DHEA-S (25 mg/kg/day for 2 weeks). In the training session, one-way ANOVA revealed no difference between the exploratory preferences of the four groups (Figure 6a: $F[3,61]=0.208$, $p=0.891$; Figure 7a: $F[3,71]=0.603$, $p=0.615$). In the retention test

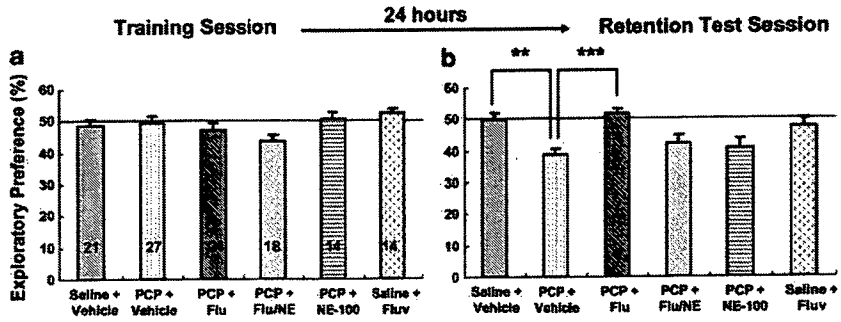

Figure 4 Effects of fluvoxamine on PCP-induced cognitive deficits in mice. Saline (10 ml/kg) or PCP (10 mg/kg) were administered s.c. for 10 days (once daily on days 1–5 and 8–12). At 3 days (day 15) after the final administration of saline or PCP, vehicle (10 ml/kg), fluvoxamine (20 mg/kg), fluvoxamine (20 mg/kg) plus NE-100 (1 mg/kg), or NE-100 (1 mg/kg) were administered i.p. into mice. The treatment was continued for 2 consecutive weeks (once daily on days 15–28). The training session for the novel object recognition test was performed 24 h (day 29) after the final administration of vehicle or drugs, and the retention test session was performed 24 h (day 30) after the training session. Values are means ± SEM. The numbers on the columns indicate the numbers of animals used. $^{**}p<0.01$, $^{***}p<0.001$ as compared with PCP-treated group.

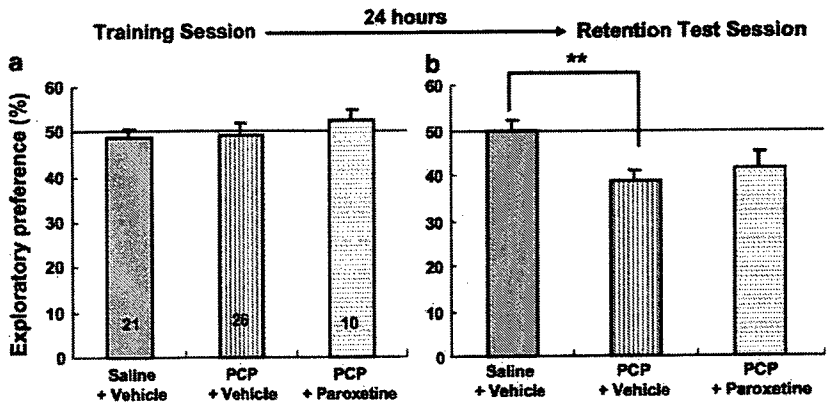

Figure 5 Effects of paroxetine on PCP-induced cognitive deficits in mice. Saline (10 ml/kg) or PCP (10 mg/kg) was administered s.c. for 10 days (once daily on days 1–5 and 8–12). At 3 days (day 15) after the final administration of saline or PCP, vehicle (10 ml/kg) or paroxetine (10 mg/kg) was administered i.p. into mice. The treatment was continued for 2 consecutive weeks (once daily on days 15–28). The training session for the novel object recognition test was performed 24 h (days 29) after the final administration of vehicle or drugs, and the retention test session was performed 24 h (days 30) after the training session. Values are means ± SEM. The numbers on the columns indicate the numbers of animals used. $^{**}p<0.01$ as compared with PCP + Vehicle-treated group.

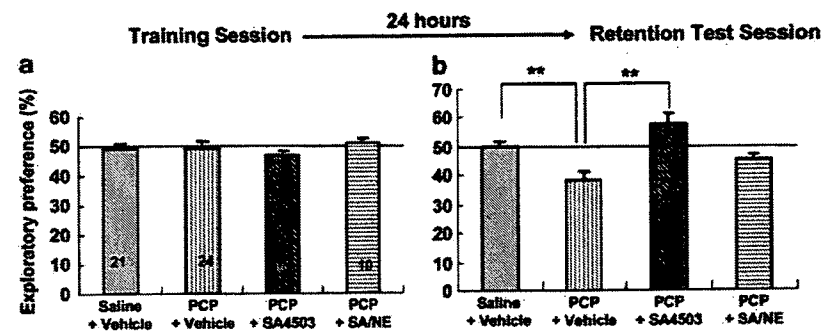

Figure 6 Effects of SA 4503 on PCP-induced cognitive deficits in mice. Saline (10 ml/kg) or PCP (10 mg/kg) was administered s.c. for 10 days (once daily on days 1–5 and 8–12). At 3 days (day 15) after the final administration of saline or PCP, vehicle (10 ml/kg), SA 4503 (1 mg/kg), or SA 4503 plus NE-100 (1 mg/kg) was administered i.p. into mice. The treatment was continued for 2 consecutive weeks (once daily on days 15–28). The training session for the novel object recognition test was performed 24 h (day 29) after the final administration of vehicle or drugs, and the retention test session was performed 24 h (day 30) after the training session. Values are means ± SEM. The numbers on the columns indicate the numbers of animals used. $^{**}p<0.01$ as compared with PCP + Vehicle-treated group.

session, one-way ANOVA revealed that the exploratory preferences of the three groups were significantly different (Figure 6b: $F[3,61]=7.622$, $p<0.001$; Figure 7b: $F[3,71]=6.034$, $p=0.001$). The *post hoc* Bonferroni test indicated that the exploratory preference of the PCP plus vehicle-treated group was significantly lower than that of

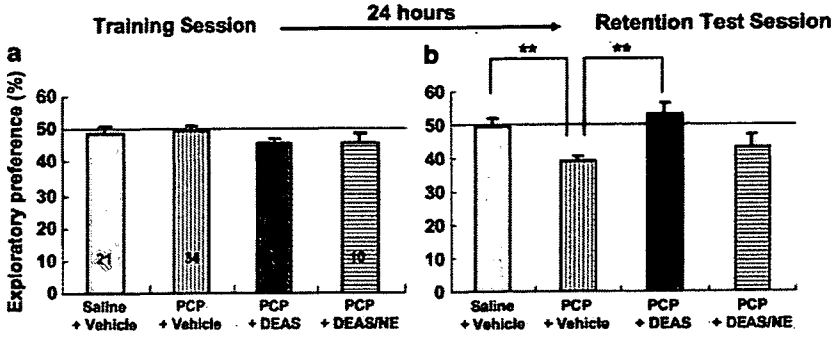

Figure 7 Effects of DHEA-S on PCP-induced cognitive deficits in mice. Saline (10 ml/kg) or PCP (10 mg/kg) was administered s.c. for 10 days (once daily on days 1–5 and 8–12). At 3 days (day 15) after the final administration of saline or PCP, vehicle (10 ml/kg), DHEA-S (25 mg/kg), or DHEA-S (25 mg/kg) plus NE-100 (1 mg/kg) were administered i.p. into mice. The treatment was continued for 2 consecutive weeks (once daily on days 15–28). The training session for the novel object recognition test was performed 24 h (day 29) after the final administration of vehicle or drugs, and the retention test session was performed 24 h (day 30) after the training session. Values are means ± SEM. The numbers on the columns indicate the numbers of animals used. $^{**}p < 0.01$ as compared with PCP + Vehicle-treated group.

the PCP plus SA4503-treated group ($p < 0.001$) or the PCP plus DHEA-S-treated group ($p = 0.009$). However, a single administration of SA4503 (1 mg/kg, 1.5 h) did not alter the exploratory preference of the PCP-treated group in either the training or the retention session (Figure 3).

DISCUSSION

We reported recently that repeated administration of PCP (10 mg/kg/day for 10 days) caused cognitive deficits in mice for more than 6 weeks after the final administration of PCP, and that the PCP-induced cognitive deficits could be improved by subsequent subchronic administration of clozapine, but not haloperidol (Hashimoto *et al*, 2005a). Therefore, reversal of PCP-induced cognitive deficits as measured by the novel object recognition test may be a potential animal model of atypical antipsychotic activity in relation to the amelioration of cognitive deficits in schizophrenia (Hashimoto *et al*, 2005a).

The major findings of the present study are that the PCP-induced cognitive deficits could be improved by subsequent subchronic administration of fluvoxamine via sigma-1 receptors. In contrast, we found that paroxetine with no affinity to sigma-1 receptors did not attenuate the PCP-induced cognitive deficits in mice. Furthermore, we found that treatment with the selective sigma-1 receptor agonist SA4503 or the endogenous sigma-1 receptor agonist DHEA-S significantly attenuated PCP-induced cognitive deficits in mice. In addition, the effects of SA4503 and DHEA-S on PCP-induced cognitive deficits were also antagonized by co-administration of the sigma-1 receptor antagonist NE-100, suggesting the role of sigma-1 receptors in the mechanisms of action of SA4503 and DHEA-S. Taken together, it is likely that sigma-1 receptor agonists are potential therapeutic drugs for the treatment of cognitive deficits in schizophrenia.

In the novel object recognition test, no differences in the total amount of time spent exploring the two objects or in exploratory preference were found between saline- and PCP-treated groups during the training session, suggesting that levels of motivation, curiosity, and interest in exploring novel objects were the same in the two groups. Repeated administration of PCP significantly decreased the exploratory preference in the retention test session, but not the training session. In the retention test session, the exploratory preference (approximately 40%) of the PCP-treated group was significantly lower (by approximately 50%) than that of the saline-treated group, suggesting that impairment of novel object exploration may not be due to memory impairment. It seems that small deviations may be important to interpretation since the effects of PCP-induced cognitive deficits are small in the novel object recognition test paradigm. Furthermore, it has been reported that the repeated administration of PCP caused social interaction deficits in animals (Mandillo *et al*, 2003; Sams-Dodd, 1998). Taken together, it is likely that our model of PCP-induced cognitive deficits using the novel object recognition test may show cognitive deficits in schizophrenia (Hashimoto *et al*, 2005a).

In this study, we found that PCP-induced cognitive deficits could be improved by subsequent subchronic (2-week), but not acute, administration of fluvoxamine. In the novel object recognition test, improvement by fluvoxamine was detected both 1.5 and 24 h after the final administration of fluvoxamine (20 mg/kg/day for 2 weeks), whereas no improvement by fluvoxamine was shown 1.5 and 24 h after a single administration of fluvoxamine. These data suggest that the effects of fluvoxamine on PCP-induced cognitive deficits in mice depend more on the duration of the treatment than the withdrawal of the drug.

Adjunctive medication to antipsychotic treatment is one approach used to improve several symptoms of schizophrenia (Silver, 2003, 2004). It has been demonstrated that fluvoxamine (relative to the other SSRIs) can improve primary negative symptoms in chronic schizophrenic patients treated with antipsychotic drugs (Silver *et al*, 2000; Silver, 2001). It is thus possible that sigma-1 receptors may be implicated in the beneficial effects of fluvoxamine, although further studies using specific sigma-1 receptor agonists would be necessary. At present, no studies have been published regarding the effects of post-treatment with fluvoxamine on PCP-induced cognitive deficits in rodents. As described above, we reported that PCP-induced cognitive deficits in mice could be improved by post-treatment with clozapine, but not haloperidol (Hashimoto *et al*,

2005a). In the present study, we found that, similar to clozapine, fluvoxamine could improve PCP-induced cognitive deficits in mice. Therefore, it would be of great interest to examine the effects of fluvoxamine on cognitive deficits in schizophrenic patients.

Possible cellular and molecular mechanisms underlying the effects of sigma-1 receptor agonists on PCP-induced cognitive deficits cannot be fully evaluated from the present study. It has been reported that sigma-1 receptors regulate Ca^{2+} release from intracellular Ca^{2+} storage sites (Hayashi *et al*, 2000; Hayashi and Su, 2001, 2004), and that injections of inhibitors of intracellular Ca^{2+} released into the brain could abolish the effects of sigma-1 receptor agonists in an animal model of depression (Urani *et al*, 2002). Therefore, it is likely that modulation of Ca^{2+} signaling by a sigma-1 receptor agonist may play a role in the active mechanism of these drugs in the cognitive deficits paradigm, although further detailed studies are necessary.

In conclusion, the present study suggests that agonistic activity of fluvoxamine at the sigma-1 receptors plays a role in the active mechanisms of fluvoxamine on PCP-induced cognitive deficits, and that sigma-1 receptor agonists could improve PCP-induced cognitive deficits in mice. Therefore, it is likely that drugs that act as sigma-1 receptor agonists, including fluvoxamine, might potentially be used for the treatment of cognitive deficits of schizophrenia.

ACKNOWLEDGEMENTS

This study was supported in part by grants from the Minister of Education, Culture, Sports, Science, and Technology of Japan (KH) and the Ministry of Health, Labor, and Welfare of Japan (KH).

REFERENCES

Bermack JE, Debonnel G (2005). The role of sigma receptors in depression. *J Pharmacol Sci* **97**: 317–336.

Coyle JT (1996). The glutamatergic dysfunction hypothesis for schizophrenia. *Harv Rev Psychiatry* **3**: 241–253.

Coyle JT, Tsai G (2004). The NMDA receptor glycine modulatory site: a therapeutic target for improving cognition and reducing negative symptoms in schizophrenia. *Psychopharmacology (Berlin)* **174**: 32–38.

Debonnel G, de Montigny C (1996). Modulation of NMDA and dopaminergic neurotransmissions by sigma ligands: possible implications for the treatment of psychiatric disorders. *Life Sci* **58**: 721–734.

Freedman R (2003). Schizophrenia. *N Engl J Med* **349**: 1738–1749.

Fukami G, Hashimoto K, Koike K, Okamura N, Shimizu E, Iyo M (2004). Effect of antioxidant *N*-acetyl-L-cysteine on behavioral changes and neurotoxicity in rats after administration of methamphetamine. *Brain Res* **1016**: 90–95.

Green MF, Nuechterlein KH, Gold JM, Barch DM, Cohen J, Essock S *et al* (2004). Approaching a consensus cognitive battery for clinical trials in schizophrenia: the NIMH-MATRICS conference to select cognitive domains and test criteria. *Biol Psychiatry* **56**: 301–307.

Hashimoto K, Fujita Y, Shimizu E, Iyo M (2005a). Phencyclidine-induced cognitive deficits in mice are improved by subsequent subchronic administration of clozapine, but not haloperidol. *Eur J Pharmacol* **519**: 114–117.

Hashimoto K, Fukushima T, Shimizu E, Komatsu N, Watanabe H, Shinoda N *et al* (2003). Decreased serum levels of D-serine in patients with schizophrenia: evidence in support of the *N*-methyl-D-aspartate receptor hypofunction hypothesis of schizophrenia. *Arch Gen Psychiatry* **60**: 572–576.

Hashimoto K, Ishiwata K (2006). Sigma receptor ligands: possible application as therapeutic drugs and as radiopharmaceuticals. *Curr Pharm Des* (in press).

Hashimoto K, Okamura N, Shimizu E, Iyo M (2004). Glutamate hypothesis of schizophrenia and approach for possible therapeutic drugs. *Curr Med Chem CNS Agents* **4**: 147–154.

Hashimoto K, Shimizu E, Iyo M (2005b). Dysfunction of glia-neuron communication in pathophysiology of schizophrenia. *Curr Psychiatry Rev* **1**: 151–163.

Hashimoto K, Tomitaka S, Bi Y, Narita N, Minabe Y, Iyo M (1997). Rolipram, a selective phosphodiesterase type-IV inhibitor, prevents induction of heat shock protein HSP-70 and hsp-70 mRNA in rat retrosplenial cortex by the NMDA receptor antagonist dizocilpine. *Eur J Neurosci* **9**: 1891–1901.

Hayashi T, Maurice T, Su TP (2000). Ca^{2+} signaling via sigma-1 receptors: novel regulatory mechanism affecting intracellular Ca^{2+} concentration. *J Pharmacol Exp Ther* **293**: 788–798.

Hayashi T, Su TP (2001). Regulating ankyrin dynamics: roles of sigma-1 receptors. *Proc Natl Acad Sci USA* **98**: 491–496.

Hayashi T, Su TP (2004). Sigma-1 receptor ligands: potential in the treatment of neuropsychiatric disorders. *CNS Drugs* **18**: 269–284.

Javitt DC (2004). Glutamate as a therapeutic target in psychiatric disorders. *Mol Psychiatry* **9**: 984–987.

Javitt DC, Balla A, Burch S, Suckow R, Xie S, Sershen H (2004). Reversal of phencyclidine-induced dopaminergic dysregulation by *N*-methyl-D-aspartate receptor/glycine-site agonists. *Neuropsychopharmacology* **29**: 300–307.

Javitt DC, Zukin SR (1991). Recent advances in the phencyclidine model of schizophrenia. *Am J Psychiatry* **148**: 1301–1308.

Jentsch JD, Roth RH (1999). The neuropsychopharmacology of phencyclidine: from NMDA receptor hypofunction to the dopamine hypothesis of schizophrenia. *Neuropsychopharmacology* **20**: 201–225.

Krystal JH, D'Souza DC, Petrakis IL, Belger A, Berman RM, Charney DS *et al* (1999). NMDA agonists and antagonists as probes of glutamatergic dysfunction and pharmacotherapies in neuropsychiatric disorders. *Harv Rev Psychiatry* **7**: 125–143.

Kurtz MM (2005). Neurocognitive impairment across the lifespan in schizophrenia: an update. *Schizophr Res* **74**: 15–26.

Mandillo S, Rinaldi A, Oliverio A, Mele A (2003). Repeated administration of phencyclidine, amphetamine and MK-801 selectively impairs spatial learning in mice: a possible model of psychotomimetic drug-induced cognitive deficits. *Behav Pharmacol* **14**: 533–544.

Matsuno K, Mita S (1998). SA 4503: a novel sigma-1 receptor agonist. *CNS Drug Rev* **4**: 1–24.

Matsuno K, Senda T, Kobayashi T, Okamoto K, Nakata K, Mita S (1997). SA4503, a novel cognitive enhancer, with sigma-1 receptor agonistic properties. *Behav Brain Res* **83**: 221–224.

Maurice T, Urani A, Phan VL, Romieu P (2001). The interaction between neuroactive steroids and the sigma-1 receptor function: behavioral consequences and therapeutic opportunities. *Brain Res Brain Res Rev* **37**: 116–132.

Minabe Y, Matsuno K, Ashby Jr CR (1999). Acute and chronic administration of the selective sigma1 receptor agonist SA4503 significantly alters the activity of midbrain dopamine neurons in rats: an *in vivo* electrophysiological study. *Synapse* **33**: 129–140.

Moghaddam B (2003). Bringing order to the glutamate chaos in schizophrenia. *Neuron* **40**: 881–884.

Morimoto T, Hashimoto K, Yasumatsu H, Tanaka H, Fujimura M, Kuriyama M *et al* (2002). Neuropharmacological profile of a novel potential atypical antipsychotic drug Y-931 (8-fluoro-12-(4-methylpiperazin-1-yl)-6H-[1]benzothieno[2,3-b][1,5] benzodiazepine maleate). *Neuropsychopharmacology* **26**: 456–467.

Narita N, Hashimoto K, Tomitaka S, Minabe Y (1996). Interactions of selective serotonin reuptake inhibitors with subtypes of sigma receptors in rat brain. *Eur J Pharmacol* **307**: 117–119.

Okamura N, Hashimoto K, Shimizu E, Kumakiri C, Komatsu N, Iyo M (2004). Adenosine A1 receptor agonists block the neuropathological changes in rat retrosplenial cortex after administration of the NMDA receptor antagonist dizocilpine. *Neuropsychopharmacology* **29**: 544–550.

Okuyama S, Nakazato A (1996). NE-100: a novel sigma receptor antagonist. *CNS Drug Rev* **2**: 226–237.

Olney JW, Farber NB (1995). Glutamate receptor dysfunction and schizophrenia. *Arch Gen Psychiatry* **52**: 998–1007.

Ozawa K, Hashimoto K, Kishimoto T, Shimizu E, Ishikura H, Iyo M (2006). Immune activation during pregnancy in mice leads to dopaminergic hyperfunction and cognitive impairment in the offspring: a neurodevelopmental animal model of schizophrenia. *Biol Psychiatry* (in press).

Sams-Dodd F (1998). Effects of continuous D-amphetamine and phencyclidine administration on social behaviour, stereotyped behaviour, and locomotor activity in rats. *Neuropsychopharmacology* **19**: 18–25.

Sánchez C, Meier E (1997). Behavioral profiles of SSRIs in animal models of depression, anxiety and aggression. Are they all alike? *Psychopharmacology (Berlin)* **129**: 197–205.

Silver H (2001). Fluvoxamine as an adjunctive agent in schizophrenia. *CNS Drug Rev* **7**: 283–304.

Silver H (2003). Selective serotonin reuptake inhibitor augmentation in the treatment of negative symptoms of schizophrenia. *Int Clin Psychopharmacol* **18**: 305–313.

Silver H (2004). Selective serotonin re-uptake inhibitor augmentation in the treatment of negative symptoms of schizophrenia. *Expert Opin Pharmacother* **5**: 2053–2058.

Silver H, Barash I, Aharon N, Kaplan A, Poyurovsky M (2000). Fluvoxamine augmentation of antipsychotics improves negative symptoms in psychotic chronic schizophrenic patients: a placebo-controlled study. *Int Clin Psychopharmacol* **15**: 257–261.

Su TP, Hayashi T (2003). Understanding the molecular mechanism of sigma-1 receptors: towards a hypothesis that sigma-1 receptors are intracellular amplifiers for signal transduction. *Curr Med Chem* **10**: 2073–2080.

Takebayashi M, Hayashi T, Su TP (2004). A perspective on the new mechanism of antidepressants: neuritogenesis through sigma-1 receptors. *Pharmacopsychiatry* **37**(Suppl 3): S208–S213.

Tang YP, Shimizu E, Dube GR, Rampon C, Kerchner GA, Zhuo M *et al* (1999). Genetic enhancement of learning and memory in mice. *Nature* **401**: 63–69.

Tang YP, Wang H, Feng R, Kyin M, Tsien JZ (2001). Differential effects of enrichment on learning and memory function in NR2B transgenic mice. *Neuropharmacology* **41**: 779–790.

Urani A, Roman FJ, Phan VL, Su TP, Maurice T (2001). The antidepressant-like effect induced by sigma-1 receptor agonists and neuroactive steroids in mice submitted to the forced swimming test. *J Pharmacol Exp Ther* **298**: 1269–1279.

Urani A, Romieu P, Portales-Casamar E, Roman FJ, Maurice T (2002). The antidepressant-like effect induced by the sigma-1 receptor agonist igmesine involves modulation of intracellular calcium mobilization. *Psychopharmacology (Berlin)* **163**: 26–35.

Zou LB, Yamada K, Sasa M, Nakata Y, Nabeshima T (2000). Effects of sigma-1 receptor agonist SA4503 and neuroactive steroids on performance in a radial arm maze task in rats. *Neuropharmacology* **39**: 1617–1627.

Neuropsychopharmacology (2007) 32, 658–664

www.neuropsychopharmacology.org

Selective Serotonin Reuptake Inhibitors, Fluoxetine and Paroxetine, Attenuate the Expression of the Established Behavioral Sensitization Induced by Methamphetamine

Yujiro Kaneko[1], Atsushi Kashiwa[1], Takashi Ito[1], Sumikazu Ishii[1], Asami Umino[1] and Toru Nishikawa*[,1]

[1]*Section of Psychiatry and Behavioral Sciences, Tokyo Medical and Dental University Graduate School, Yushima, Bunkyo-ku, Tokyo, Japan*

To obtain an insight into the development of a new pharmacotherapy that prevents the treatment-resistant relapse of psychostimulant-induced psychosis and schizophrenia, we have investigated in the mouse the effects of selective serotonin reuptake inhibitors (SSRI), fluoxetine (FLX) and paroxetine (PRX), on the established sensitization induced by methamphetamine (MAP), a model of the relapse of these psychoses, because the modifications of the brain serotonergic transmission have been reported to antagonize the sensitization phenomenon. In agreement with previous reports, repeated MAP treatment (1.0 mg/kg a day, subcutaneously (s.c.)) for 10 days induced a long-lasting enhancement of the increasing effects of a challenge dose of MAP (0.24 mg/kg, s.c.) on motor activity on day 12 or 29 of withdrawal. The daily injection of FLX (10 mg/kg, s.c.) or PRX (8 mg/kg, s.c.) from 12 to 16 days of withdrawal of repeated MAP administration markedly attenuated the ability of the MAP pretreatment to augment the motor responses to the challenge dose of the stimulant 13 days after the SSRI injection. The repeated treatment with FLX or PRX alone failed to affect the motor stimulation following the challenge of saline and MAP 13 days later. These results suggest that the intermittent and repetitive elevation of serotonergic tone may inhibit the expression of the motor sensitization induced by pretreatment with MAP. It is proposed that clinically available serotonin reuptake inhibitors could be useful for preventing the recurrence of hallucinatory-paranoid state in drug-induced psychosis and schizophrenia.

Neuropsychopharmacology (2007) **32,** 658–664. doi:10.1038/sj.npp.1301111; published online 31 May 2006

Keywords: behavioral sensitization; methamphetamine; fluoxetine; paroxetine; methamphetamine psychosis; schizophrenia

INTRODUCTION

The addiction of amphetamine, methamphetamine (MAP), cocaine, and other psychostimulants with dopamine agonist properties has been a serious worldwide health and social concern, and has been estimated to affect more than 60 million patients based on recent reports from the World Health Organization. The abuse of these drugs causes a growing intensification of craving of psychotomimetic substances, and stimulant-induced psychiatric symptoms exhibit progressive quantitative alterations from a non-psychotic to a prepsychotic and finally to a hallucinatory-paranoid state indistinguishable from that of schizophrenia (Ujike and Sato, 2004). The robust drug craving and psychotic state have been observed to easily reoccur even after long period of abstinence by reuse of a small amount of a stimulant or an unspecific stressor (Ujike and Sato, 2004). These observations indicate that the severe vulnerability to relapse of the above psychotomimetic effects may be established during stimulant abuse (Ujike and Sato, 2004). The difficult clinical problems of stimulant craving and psychosis, and their unpredictable relapses often lead to antisocial behavior and require the development of a novel treatment that can eliminate the enduring vulnerability.

One of the rational approaches to develop this type of treatment appears to explore the substances that reverse an animal model of the drug-induced craving and recurrent psychosis, psychostimulant-induced reverse tolerance, or behavioral sensitization. The behavioral sensitization is a characteristic phenomenon in that the single or repeated exposure to amphetamines and other psychostimulants results in a progressive enhancement of the psychotomimetic responses to these drugs or stress, including hyperactivity and stereotypy, in the rodents (Nishikawa *et al*, 1983; Robinson and Becker, 1986; Vanderschuren and Kalivas, 2000). The augmented behavioral responses have been shown to persist even long after drug discontinuation. Because the progressively intensifying, cross-reactive (to stimulants and stress), easily relapsing, long-lasting, and dopamine agonist-inducible nature of the behavioral sensitization of rodents seems to mimic that of stimulant-

*Correspondence: Dr T Nishikawa, Section of Psychiatry and Behavioral Sciences, Tokyo Medical and Dental University Graduate School, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8519, Japan, Tel: +81 3 5803 5237, Fax: +81 3 5803 0135, E-mail: tnis.psyc@tmd.ac.jp
Received 14 November 2005; revised 5 April 2006; accepted 19 April 2006
Online publication: 5 May 2006 at http://www.acnp.org/citations/Npp050306050677/default.pdf

induced drug craving and psychosis in humans, these animal and human abnormalities induced by stimulants have been considered to share a common pathophysiology underlying the vulnerability to their recurrences (Ujike and Sato, 2004; Vanderschuren and Kalivas, 2000). Moreover, in remitted or stable schizophrenic patients, a hallucinatory-paranoid state reappeared or was exacerbated following a small and subpsychotomimetic dose of a psychostimulant for normal volunteers (Segal and Janowsky, 1978; Snyder, 1973). These results support the idea that, like the patients with stimulant-induced psychosis, a subpopulation of schizophrenic patients may be much more sensitive to psychotomimetic effects of stimulants than normal volunteers. Taken together, the behavioral sensitization may also be a useful model for the relapse vulnerability in schizophrenic patients. Therefore, the treatment that produces a lasting inhibition of the expression of the once established behavioral sensitization can be expected to possess a prophylactic efficacy on the recurrence of psychotic states of stimulant-induced psychosis and/or schizophrenia.

Recently, the repeated systemic administration with a dopamine D1 agonist (Li *et al*, 2000), an NMDA antagonist plus dopamine D2 receptor agonist (Li *et al*, 2000), serotonin-2 (5-hydroxytryptamine-2; 5-HT_2) antagonists (Davidson *et al*, 2002a), and a 5-HT_3 antagonist (King *et al*, 1998, 2000; Davidson *et al*, 2002b), during the withdrawal period after the repetitive injection of cocaine, amphetamine, or MAP has been reported to attenuate the expression of behavioral sensitization. Although the exact mechanisms underlying these attenuating effects are still unclear, several lines of evidence indicate that the cerebral serotonergic systems could be involved in the modification of the stimulant-induced long-lasting changes in the behavioral responses. Thus, (1) the unlimited self-administration of cocaine produced a sustained decrease in the extracellular 5-HT concentration in the nucleus accumbens during the withdrawal period (Parsons *et al*, 1995), (2) the enhanced synaptic levels of serotonin by administration of a 5-HT precursor L-tryptophan or of a 5-HT selective serotonin reuptake inhibitor (SSRI) fluoxetine (FLX) reduced the reinforcing effects of cocaine (Lyness, 1983; Carroll *et al*, 1990; Richardson and Roberts, 1991; Takamatsu *et al*, 2005), and (3) repeated MAP treatment has been shown to fail to cause behavioral sensitization in the mice lacking a 5-HT transporter with an excess of extracellular 5-HT contents (Shen *et al*, 2003). These data suggest that the decreased serotonergic tone may play an important role in the maintenance of sensitization elicited by the psychostimulant drugs and, in turn, increased cerebral serotonergic transmission could suppress the expression of the established sensitization.

To test the possible suppression by 5-HT agonists, we have studied the influences of repeated administration of typical SSRIs, FLX, and paroxetine (PRX), during withdrawal of the repetitive treatment with MAP, on the ability of a challenge dose of MAP to cause an augmented motor response in mice following a drug-free period after the SSRI injections. We have chosen these clinically available SSRIs because we have considered the future clinical applications of these drugs for the purpose of the prophylaxis against the relapses of stimulant-induced craving or psychotic state and/or of schizophrenia if they could reverse the established sensitization.

MATERIALS AND METHODS

Animals

The present animal experiments were performed in strict accordance with the guidance of the Tokyo Medical and Dental University and were approved by the Animal Investigation Committee of the Institution. Male ddY mice (Clea Japan Inc., Japan) at ages ranging from postnatal days 50 to 56 weighing 32–42g were used. The animals were housed in groups of 4–5 per cage at 23.0 ± 0.5°C in a humidity-controlled room under a light-controlled (14-h/12-h light/dark cycle, lights on at 0600 hours) and had free access to food and water.

Chemicals

MAP hydrochloride was purchased from Dainippon Pharmaceutical Co., Ltd (Osaka, Japan), with official permission of the Tokyo Metropolitan Bureau of Public Health. FLX HCl and PRX maleate were purchased from TOCRIS (Avonmouth, UK). The other chemicals used were of ultrapure quality and were commercially available. Doses for the injections always refer to the free bases. Each drug was dissolved in saline (SAL) (0.9% NaCl) and subcutaneously (s.c.) injected in a volume of 0.005 ml/g body weight. The control mice were treated with SAL.

Drug Administration Schedule

Establishment and maintenance of behavioral sensitization. To confirm the establishment and maintenance of the behavioral sensitization under our experimental conditions, 16 mice were pretreated with 1.0 mg/kg of MAP (s.c.) (eight mice) or SAL (eight mice) once daily for 10 days. On days 1, 3, 7, and 10 of the pretreatment, these mice were placed into the movement measurement apparatus to count their spontaneous activities. On day 11 (12 animals) of withdrawal following pretreatment with MAP or SAL, the animals were injected with SAL and, on the next day, with a challenge dose of MAP (0.24 mg/kg, s.c.). The two experimental groups were

(1) MAP (1.0 mg/kg/day for 10 days) + MAP (0.24 mg/kg on day 12 of withdrawal) ($N = 8$) and
(2) SAL (for 10 days) + MAP (0.24 mg/kg on day 12 of withdrawal) ($N = 4$).

Treatment with SSRIs. In the experiments to study the effects of SSRIs on the MAP-induced behavioral sensitization, the mice pretreated with MAP or SAL for 10 days were repeatedly administered with FLX (10 mg/kg/day, s.c.) or PRX (8 mg/kg/day, s.c.) once daily from day 12 to 16 of pretreatment withdrawal. These animals received a challenge of MAP or SAL 13 days after the repeated treatment with FLX or PRX, respectively. Table 1 summarizes the 16 groups for the SSRI experiments.

Table 1 Schedules and Doses for Pretreatment and Challenge of MAP, FLX, PRX, and SAL and Cumulated Motor Activity after Challenge of MAP or SAL

Group (duration)	N	Pretreatment with MAP or SAL (10 days)	Withdrawal period I (11 days)	Pretreatment with SSRI or SAL (5 days)	Withdrawal period II (12 days)	Challenge	Cumulated motor activity (counts/60 min)
Fluoxetine							
1	8	SAL		SAL		SAL	1143±161
2	8	SAL		FLX		SAL	1581±300
3	8	MAP		SAL		SAL	1378±255
4	8	MAP		FLX		SAL	2682±344
5	8	SAL		SAL		MAP	2883±735
6	8	SAL		FLX		MAP	2488±800
7	8	MAP		SAL		MAP	8066±831
8	8	MAP		FLX		MAP	4689±725
Paroxetine							
1	10	SAL		SAL		SAL	1923±200
2	10	SAL		PRX		SAL	2016±657
3	10	MAP		SAL		SAL	1561±351
4	10	MAP		PRX		SAL	1822±399
5	10	SAL		SAL		MAP	3476±722
6	10	SAL		PRX		MAP	2353±620
7	10	MAP		SAL		MAP	7770±862
8	10	MAP		PRX		MAP	3843±776

The different dosing regimens for the eight groups in each experiment are summarized. Methamphetamine (MAP; 1.0 mg/kg, s.c.) or saline (SAL) was repeatedly injected during the pretreatment period. The pretreatment with FLX (fluoxetine; 10 mg/kg/day, s.c.), PRX (paroxetine; 8 mg/kg/day, s.c.), or SAL for 5 days was initiated from day 12 to 16 of withdrawal of the repeated MAP injection. The animals pretreated with MAP or SAL plus FLX, PRX, or SAL were challenged with MAP at the dose of 0.24 mg/kg or SAL (s.c.) on day 13 of withdrawal of FLX, PRX, or SAL treatment. Each cumulated motor activity is expressed as means with SEM of the data obtained from 8 to 10 determinations.

Behavioral Analyses

To evaluate the behavioral effects of MAP (1.0 or 0.24 mg/kg, s.c.), the spontaneous vertical and horizontal movements including locomotion, rearing, and head movements were quantified by automatically counting the number of heat changes in the multiple zones of the test cage by means of the heat sensor with a Supermex instrument (Muromachi-kikai Co. Ltd, Tokyo, Japan) (Masuo *et al*, 1995; Hara *et al*, 2001). The mice were placed into the acrylic test cage (24.5 × 17.5 × 12.5 cm) within a soundproof and illuminated wood box at an ambient temperature of 23.0±0.5°C. The Supermex consists of a monitor that was mounted above the test cage to detect changes in heat across multiple zones of the cage through an array of Fresnel lenses. The body heat radiated by an animal was detected by the sensor head of the monitor, which contained paired infrared light ray pyroelectric detectors. Every behavioral analysis was always performed for 120–150 min before and for 60 min after the injection of the MAP or SAL.

Statistical Analyses

Results are usually reported as means with SEM of the data. For comparison between the two groups, statistical evaluations were made using the two-tailed Student's *t*-test. Statistical differences among more than three groups were estimated by a one-way analysis of variance (ANOVA; homogeneous variance) or the Kruskal–Wallis test (heterogeneous variance) followed by the Dunnett or Scheffé *post hoc* test. The significance level was set at $p<0.05$ for all comparisons.

RESULTS

Establishment and Maintenance of Behavioral Sensitization by Repeated MAP Treatment

As shown in Figure 1a, repeated treatment of ddY mice with MAP (1 mg/kg once daily for 10 days, s.c.) resulted in a progressive and significant enhancement of the ability of MAP to increase the amounts of motor activity for 60 min on the 7th ($p<0.05$ *vs* the 1st day) and 10th ($p<0.01$) day of the drug regimen. The enhanced motor responses to MAP were also observed 12 days after discontinuation of the repeated treatment with MAP (Figure 1b). Because these observations confirmed the establishment of the MAP-induced sensitization and were consistent with those in the previous sensitization experiments (Vanderschuren and Kalivas, 2000), we routinely applied this MAP treatment schedule to the present behavioral experiments.

Effects of FLX and PRX on the Established Behavioral Sensitization after Repeated MAP Treatment

In the experiments using MAP and SSRIs (see Figures 2 and 3), the long-lasting nature of behavioral sensitization

was further verified by the results that the mice pretreated with MAP (1 mg/kg once daily for 10 days, s.c.) exhibited augmented motor responses to a challenge dose of MAP on day 29 of withdrawal (SAL + SAL + MAP *vs* MAP + SAL + MAP in Figures 2 and 3).

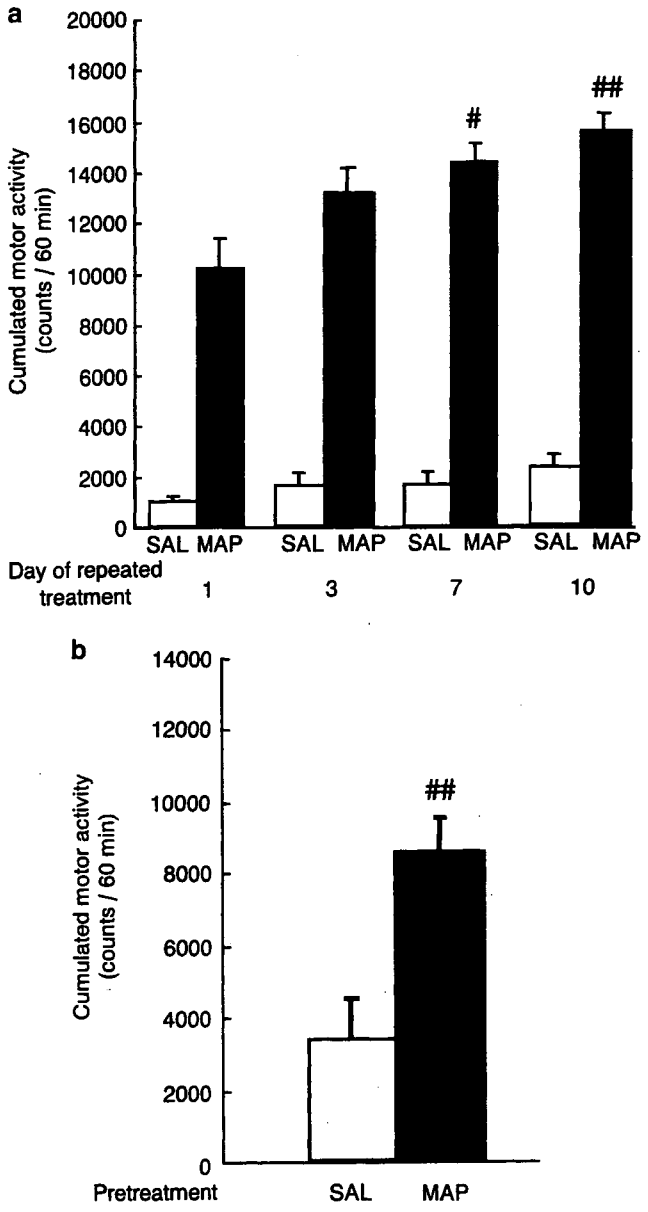

Figure 1 Changes in behavioral responses to MAP challenge during or after repeated MAP pretreatment. (a) Cumulated motor activity for 60 min following daily injection of MAP (1.0 mg/kg, s.c.) or SAL during repeated treatment for 10 days in mice. Each value is the mean with SEM of eight determinations. $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs* values obtained on the first treatment day in the respective experimental group. Statistical analysis of the present data with a homogeneous variance (Bartlett test: MAP, $\chi^2=2.3480$, df = 3, $p=0.5033$; SAL, $\chi^2=5.3896$, df = 3, $p=0.1454$) was performed using a one-way ANOVA followed by the Dunnett *post hoc* test (SAL, $F(3, 28)=1.997$, $p=0.137$ (no statistically significant difference); MAP, $F(3, 28)=5.926$, $p<0.01$ ($p=0.0029$). (b) Cumulated motor activity for 60 min following a challenge dose of MAP (0.24 mg/kg, s.c.) on day 12 of withdrawal of the repeated treatment with MAP or SAL. Each value is the mean with SEM of 4–8 determinations. $^{\#\#}p<0.01$ *vs* the respective SAL-pretreated controls. Statistical analysis of the present data with a homogeneous variance ($F=0.604$, $p=0.3670$) was performed using the two-tailed Student's *t*-test ($t=-3.35$, $p<0.01$ ($p=0.0074$)).

As shown in Figure 2, in the SAL-pretreated mice, repeated FLX administration failed to cause a significant change in the cumulated motor activity after a challenge of SAL (SAL + FLX + SAL) or MAP (SAL + FLX + MAP) on day 13 of FLX withdrawal as compared to the corresponding repetitive vehicle-treated animals (SAL + SAL + SAL and SAL + SAL + MAP). There was a trend toward, but not statistically significant, increase in the motor response to SAL challenge in the repeatedly MAP-pretreated FLX-injected mice (MAP + FLX + SAL) when compared to the repeatedly MAP-pretreated vehicle-injected mice (MAP + SAL + SAL) (Figure 2). However, in the MAP-pretreated behaviorally sensitized mice, repeated FLX injection led to significantly lower counts of spontaneous movements after a challenge dose of MAP (MAP + FLX + MAP) on day 13 of FLX withdrawal than the repeated vehicle injection (MAP + SAL + MAP) (Figure 2). No stereotyped behavior was observed after a MAP challenge in any of the experimental groups of animals. These results indicate that repeated FLX treatment may reduce the expression of the behavioral sensitization following a MAP challenge without apparent changes in the motor responses to SAL in the sensitized and the nonsensitized animals, and to MAP in nonsensitized mice.

Similarly, the repeated PRX administration inhibited the ability of a subsequent challenge of MAP to increase

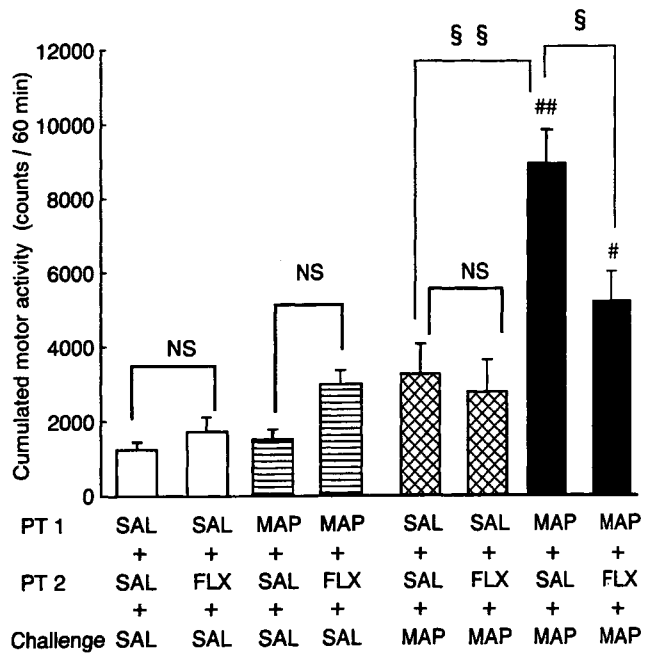

Figure 2 Effects of repeated injections of fluoxetine during withdrawal of MAP pretreatment on behavioral responses to MAP challenge. The detailed schedules of the drug administrations are shown in Table 1. The motor activity was automatically quantified and cumulated for 60 min following MAP challenge (0.24 mg/kg, s.c.) on days 29 and 13 of withdrawal of the repeated treatment with MAP (PT 1; pretreatment 1) and that with fluoxetine (PT 2; pretreatment 2), respectively. Each value is the mean with SEM of eight determinations. $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs* SAL-pretreated (for two times) and SAL-challenged animals (absolute controls). $^{\S}p<0.05$, $^{\S\S}p<0.01$ between the two groups linked with a solid line. NS: no statistically significant difference between the two groups linked with a solid line. Statistical analysis of the present data with a heterogeneous variance (Bartlett test: $\chi^2=28.5574$, df = 7, $p<0.01$ ($p=0.0002$)) was performed using the Kruskal–Wallis test ($p<0.0001$) followed by the Scheffé *post hoc* test.

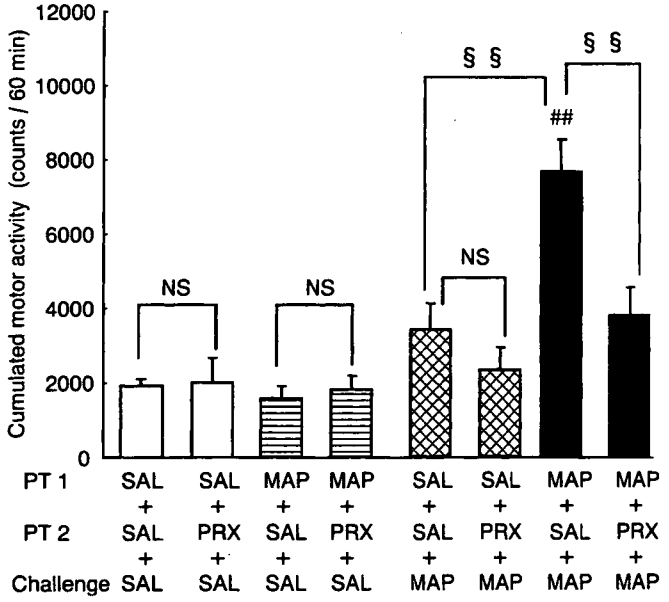

Figure 3 Effects of repeated injections of PRX during withdrawal of MAP pretreatment on behavioral responses to MAP challenge. The detailed schedules of the drug administrations are shown in Table 1. The motor activity was automatically quantified and cumulated for 60 min following MAP challenge (0.24 mg/kg, s.c.) on days 29 and 13 of withdrawal of the repeated treatment with MAP (PT 1; pretreatment 1) and that with PRX (PT 2; pretreatment 2), respectively. Each value is the mean with SEM of 10 determinations. $^{\#\#}p<0.01$ *vs* SAL-pretreated (for two times) and SAL-challenged animals (absolute controls). $^{\S\S}p<0.01$ between the two groups linked with a solid line. NS no statistically significant difference between the two groups linked with a solid line. Statistical analysis of the present data with a heterogeneous variance (Bartlett test: $\chi^2=22.2354$, $df=7$, $p<0.01$ ($p=0.0023$)) was performed using the Kruskal–Wallis test ($p<0.0001$) followed by the Scheffé *post hoc* test.

the number of movements in the MAP-pretreated mice (MAP + PRX + MAP *vs* MAP + SAL + MAP) on day 13 of PRX withdrawal (Figure 3). This PRX regimen failed to change the behavioral response to SAL challenge in the SAL- and MAP-pretreated animals (SAL + PPX + SAL and MAP + PRX + SAL) and to a challenge dose of MAP in the SAL-pretreated mice (SAL + PRX + MAP *vs* SAL + SAL + MAP) (Figure 3). The MAP challenge produced no apparent stereotyped behavior in any of the experimental groups.

DISCUSSION

In the present study, we have verified that 10 daily administrations of MAP (1 mg/kg, s.c.) produced a progressive and enduring augmentation in the increased movements elicited by a subsequent challenge of MAP, that is, behavioral sensitization. Our obtained data first demonstrate that the repeated injection of FLX or PRX to behaviorally sensitized mice by MAP pretreatment attenuates the expression of the enhanced behavioral response to a challenge dose of MAP after a 13-day drug-free interval. This attenuation suggests that FLX and PRX may be able to reverse the established behavioral sensitization following an exposure to psychostimulants.

The nonspecific phenomena including the long-term sedation or accumulation of either SSRI or its active metabolites after repeated SSRI treatment could produce the attenuating effects of the SSRIs on the challenge MAP-induced movements. FLX and its active desmethyl metabolite, nor-fluoxetine, have indeed been reported to display long halflives ranging between 1 and 4 days and between 7 and 15 days, respectively, in humans (Hiemke and Hartter, 2000; Sills *et al*, 2000). However, the above presumptions are unlikely because (1) neither the repeated FLX nor PRX treatment diminished the basal amounts of movements (SAL-induced movements) in the SAL- and MAP-pretreated mice and the ability of a MAP challenge to increase significantly the movements in the SAL-pretreated mice (Figures 2 and 3), (2) a subchronic injection of FLX for 5 days potentiated the increasing effects of an acute amphetamine application on motor activity on days 1 and 2, but not on day 5, of withdrawal (Sills *et al*, 2000), and (3) repeated treatment with FLX or PRX for 27 days enhanced the psychomotor stimulatory effects of an alcohol challenge on the 28th day of the experiments (Goeldner *et al*, 2005). Moreover, no apparent stereotypy following a challenge dose of MAP in any experimental groups seems to deny the possibility that the apparent reduction in the MAP-induced movements (Figures 2 and 3) might reflect the diminished ambulation owing to the robust sensitization with increased frequencies of the stereotyped behavior in one location.

Both repeated FLX and PRX treatment by themselves tended to reduce, although nonsignificantly, the locomotor response to acute MAP administration. These tendencies are also likely to be associated with SSRI-induced attenuation of the expression of behavioral sensitization, because the repeated SAL treatment as repeated injection stress could augment the ability of amphetamines to induce abnormal behavior. This view seems to be supported by the previous observation (Antelman *et al*, 1980) indicating that repeated mild stress (tail pressure stress) resulted in an enhanced behavioral response to *d*-amphetamine.

Based upon the fact that the common selective and potent action between FLX and PRX is 5-HT uptake inhibition, it is more likely that the reduced expression of behavioral sensitization in the animals treated with these SSRIs after the establishment of the sensitization may be connected to an enhanced serotonergic tone in the brain. Although increased synaptic 5-HT has been reported to prevent the development of the stimulant-induced behavioral sensitization and craving (see Introduction), there has so far been no study to test the effects of 5-HT agonists on the sustainment of these behavioral changes. The elevated contents of the synaptic 5-HT by SSRIs (Felton *et al*, 2003) could reverse the stimulant-induced sensitization by compensating the plausible persistent decrease in the basal extracellular release of 5-HT in the nucleus accumbens, which has been suggested to play an important role in sustaining the sensitization (Parsons *et al*, 1995).

The SSRI-induced disruption of the sensitization could be mediated by the specific 5-HT receptor subtypes that interact with the ascending dopamine neurons projecting from the ventral tegmental area to the nucleus accumbens, because these neurons have been proved to participate in the development and expression of the long-lasting locomotor sensitization (Vanderschuren and Kalivas, 2000). In terms of this interaction, it is of interest to note that 5-HT_{1B} (Yan and Yan, 2001; Yan *et al*, 2004) and 5-HT_{2A}

(Auclair *et al*, 2004; Esposito, 2006) receptors in either of the two brain areas have been well known to be involved in the control of dopamine release from the nucleus accumbens. Recent studies have further suggested the modification of the meso-accumbens dopamine neurons by the 5-HT_{1A} (Andrews *et al*, 2005), 5-HT_{2C} (Esposito, 2006), and 5-HT_3 (De Deurwaerdere *et al*, 2005) receptors. The increased vulnerability to cocaine (Rocha *et al*, 1998) and amphetamine (Bronsert *et al*, 2001) in mice lacking the 5-HT_{1B} receptor favors the possible role of 5-HT_{1B} receptor stimulation in the reversal of the stimulant-induced locomotor sensitization. However, inhibition of the expression of the established behavioral sensitization was caused by the 5-HT_3 receptor antagonist, ondansetron, and some agents with the 5-HT_{2A} receptor antagonist property including clozapine, mianserin, and ketanserin (Davidson *et al*, 2002a,b). Activation of the 5-HT_{1A} receptor was reported to prevent the development of the behavioral sensitization to L-DOPA (L-3,4-dihydroxyphenylalanine) (Tomiyama *et al*, 2005), but has not yet been tested with respect to the established sensitization phenomenon. To clarify the 5-HT receptor subtypes critical for the reversal effects of SSRIs on the expression of the MAP sensitization, further investigation is needed to try to block the reversal effects using 5-HT_{1B}, 5-HT_{2A}, 5-HT_{1A}, 5-HT_{2C}, and 5-HT_3 antagonists.

Because the long-lasting nature of the behavioral sensitization has been considered to be associated with brain plasticity, the SSRIs used in this study could modulate the plastic changes underlying behavioral sensitization through their influences on the brain growth factors (Sodhi and Sanders-Bush, 2004) and hippocampal neurogenesis, which are related to the rearrangements or remodeling of the neuron circuits (Duman *et al*, 2001). This view is supported by the findings that (1) the repetitive administration of a psychostimulant, cocaine, has been shown to decrease neurogenesis in the adult rat hippocampus (Yamaguchi *et al*, 2004), (2) the single or repeated treatment with amphetamine, MAP, or cocaine has been found to alter the levels of mRNA or proteins in the brain-derived growth factor (Meredith *et al*, 2002; Grimm *et al*, 2003; Le Foll *et al*, 2005), and (3) stress causes the suppression of neurogenesis, debranching, and shortening of the dendrites in the adult rat hippocampal dentate gyrus, which have been documented to be reversed by repeated FLX (Malberg *et al*, 2000; Malberg and Duman, 2003; Kodama *et al*, 2004).

MAP-induced behavioral sensitization has been considered to be an animal model of MAP craving or psychosis, or paranoid schizophrenia (Ellinwood *et al*, 1973; Robinson and Becker, 1986; Ujike and Sato, 2004). The patients with these disorders often suffer from relapses for many years or a lifetime even after the long discontinuance of MAP and/or the continued treatment with antipsychotic drug. The markedly reduced expression of sensitization by a temporary treatment with FLX and PRX observed here suggests that the short-term treatment with these SSRIs might attenuate the relapse of the psychotic state associated with psychostimulants and/or schizophrenia. Therefore, it would be relevant for the development of an additional pharmacotherapy for MAP psychosis and/or a group of schizophrenia to test the ability of a subchronic regimen of FLX and PRX to mitigate or prevent the recurrence of the hallucinatory-paranoid state in these psychoses. However, before start of such a clinical test, careful considerations are required of the previous data indicating that SSRI augmentation of antipsychotics in the treatment of schizophrenia improved negative symptoms of schizophrenia and had no effect on positive symptoms (Silver and Shmugliakov, 1998; Silver, 2004), although the therapeutic target of the SSRIs is not the positive symptoms by themselves but the vulnerability to their relapse. It should also be noted that some cases were omitted from the clinical trials owing to the worsening of the positive symptoms (Silver and Shmugliakov, 1998; Poyurovsky *et al*, 1999).

In conclusion, the present study indicates that a 5 days treatment with SSRIs, FLX, and PRX, during the withdrawal period of chronic MAP treatment, may, at least in part, reverse the MAP-induced behavioral sensitization. It is proposed that these SSRIs could be clinically useful as prophylactic agents against the easy reactivation of serious psychotic states in patients with MAP craving or psychosis, and/or some schizophrenic patients.

ACKNOWLEDGEMENTS

We thank Ms M Asakawa and M Kurita for their excellent assistance. This work was partly supported by a Research Grant from the Ministry of Health, Labor and Welfare (Japan), and a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture (Japan).

REFERENCES

Andrews CM, Kung HF, Lucki I (2005). The 5-HT_{1A} receptor modulates the effects of cocaine on extracellular serotonin and dopamine levels in the nucleus accumbens. *Eur J Pharmacol* **508**: 123–130.

Antelman SM, Eichler AJ, Black CA, Kocan D (1980). Interchangeability of stress and amphetamine in sensitization. *Science* **207**: 329–331.

Auclair A, Drouin C, Cotecchia S, Glowinski J, Tassin JP (2004). 5-HT_{2A} and alpha1b-adrenergic receptors entirely mediate dopamine release, locomotor response and behavioural sensitization to opiates and psychostimulants. *Eur J Neurosci* **20**: 3073–3084.

Bronsert MR, Mead AN, Hen R, Rocha BA (2001). Amphetamine-induced locomotor activation in 5-HT(1B) knockout mice: effects of injection route on acute and sensitized responses. *Behav Pharmacol* **12**: 549–555.

Carroll ME, Lac ST, Asencio M, Kragh R (1990). Intravenous cocaine self-administration in rats is reduced by dietary L-tryptophan. *Psychopharmacology (Berlin)* **100**: 293–300.

Davidson C, Lazarus C, Xiong X, Lee TH, Ellinwood EH (2002a). 5-HT_2 receptor antagonists given in the acute withdrawal from daily cocaine injections can reverse established sensitization. *Eur J Pharmacol* **453**: 255–263.

Davidson C, Lee TH, Xiong Z, Ellinwood EH (2002b). Ondansetron given in the acute withdrawal from a repeated cocaine sensitization dosing regimen reverses the expression of sensitization and inhibits self-administration. *Neuropsychopharmacology* **27**: 542–553.

De Deurwaerdere P, Moison D, Navailles S, Porras G, Spampinato U (2005). Regionally and functionally distinct serotonin3 receptors control *in vivo* dopamine outflow in the rat nucleus accumbens. *J Neurochem* **94**: 140–149.

Duman RS, Nakagawa S, Malberg J (2001). Regulation of adult neurogenesis by antidepressant treatment. *Neuropsychopharmacology* **25**: 836–844.

Ellinwood Jr EH, Sudilovsky A, Nelson LM (1973). Evolving behavior in the clinical and experimental amphetamine (model) psychosis. *Am J Psychiatry* **130**: 1088–1093.

Esposito E (2006). Serotonin–dopamine interaction as a focus of novel antidepressant drugs. *Curr Drug Targets* **7**: 177–185.

Felton TM, Kang TB, Hjorth S, Auerbach SB (2003). Effects of selective serotonin and serotonin/noradrenaline reuptake inhibitors on extracellular serotonin in rat diencephalon and frontal cortex. *Naunyn Schmiedebergs Arch Pharmacol* **367**: 297–305.

Goeldner FO, Pigatto G, Ribeiro AF, Machado HB, Boerngen-Lacerda R (2005). Influence of fluoxetine and paroxetine in behavioral sensitization induced by ethanol in mice. *Pharmacol Biochem Behav* **82**: 388–396.

Grimm JW, Lu L, Hayashi T, Hope BT, Su TP, Shaham Y (2003). Time-dependent increases in brain-derived neurotrophic factor protein levels within the mesolimbic dopamine system after withdrawal from cocaine: implications for incubation of cocaine craving. *J Neurosci* **23**: 742–747.

Hara J, Beuckmann CT, Nambu T, Willie JT, Chemelli RM, Sinton CM *et al* (2001). Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity. *Neuron* **30**: 345–354.

Hiemke C, Hartter S (2000). Pharmacokinetics of selective 5-HT reuptake inhibitors. *Pharmacol Ther* **85**: 11–28.

King GR, Xiong Z, Ellinwood Jr EH (1998). Blockade of the expression of sensitization and tolerance by ondansetron, a 5-HT_3 receptor antagonist, administered during withdrawal from intermittent and continuous cocaine. *Psychopharmacology (Berlin)* **135**: 263–269.

King GR, Xiong Z, Douglass S, Ellinwood EH (2000). Long-term blockade of the expression of cocaine sensitization by ondansetron, a 5-HT(3) receptor antagonist. *Eur J Pharmacol* **394**: 97–101.

Kodama M, Fujioka T, Duman RS (2004). Chronic olanzapine or fluoxetine administration increases cell proliferation in hippocampus and prefrontal cortex of adult rat. *Biol Psychiatry* **56**: 570–580.

Le Foll B, Diaz J, Sokoloff P (2005). A single cocaine exposure increases BDNF and D3 receptor expression: implications for drug-conditioning. *NeuroReport* **16**: 175–178.

Li Y, White FJ, Wolf ME (2000). Pharmacological reversal of behavioral and cellular indices of cocaine sensitization in the rat. *Psychopharmacology* **151**: 175–183.

Lyness WH (1983). Effect of L-tryptophan pretreatment on *d*-amphetamine self administration. *Subst Alcohol Actions Misuse* **4**: 305–312.

Malberg JE, Eisch AJ, Nestler EJ, Duman RS (2000). Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *J Neurosci* **20**: 9104–9110.

Malberg JE, Duman RS (2003). Cell proliferation in adult hippocampus is decreased by inescapable stress: reversal by fluoxetine treatment. *Neuropsychopharmacology* **28**: 1562–1571.

Masuo Y, Noguchi J, Morita S, Matsumoto Y (1995). Effects of intracerebroventricular administration of pituitary adenylate cyclase-activating polypeptide (PACAP) on the motor activity and reserpine-induced hypothermia in murines. *Brain Res* **700**: 219–226.

Meredith GE, Callen S, Scheuer DA (2002). Brain-derived neurotrophic factor expression is increased in the rat amygdala, piriform cortex and hypothalamus following repeated amphetamine administration. *Brain Res* **949**: 218–227.

Nishikawa T, Mataga N, Takashima M, Toru M (1983). Behavioral sensitization and relative hyperresponsiveness of striatal andlimbic dopaminergic neurons after repeated methamphetamine treatment. *Eur J Pharmacol* **88**: 195–203.

Parsons LH, Koob GF, Weiss F (1995). 5-HT dysfunction in the nucleus accumbens of rats during withdrawal after unlimited access to intravenous cocaine. *J Pharmacol Exp Ther* **274**: 1182–1191.

Poyurovsky M, Isakov V, Hromnikov S, Modai I, Rauchberger B, Schneidman M *et al* (1999). Fluvoxamine treatment of obsessive-compulsive symptoms in schizophrenic patients: an add-on open study. *Int Clin Psychopharmacol* **14**: 95–100.

Richardson NR, Roberts DC (1991). Fluoxetine pretreatment reduces breaking points on a progressive ratio schedule reinforced by intravenous cocaine self-administration in the rat. *Life Sci* **49**: 833–840.

Robinson TE, Becker JB (1986). Enduring changes in brain and behavior produced by chronic amphetamine administration: a review and evaluation of animal models of amphetamine psychosis. *Brain Res* **396**: 157–198.

Rocha BA, Scearce-Levie K, Lucas JJ, Hiroi N, Castanon N, Crabbe JC *et al* (1998). Increased vulnerability to cocaine in mice lacking the serotonin-1B receptor. *Nature* **393**: 175–178.

Segal DS, Janowsky DS (1978). Psychostimulant-induced behavioral effects: possible models of schizophrenia. In: Lipton MA, DiMascio A, Killam KF (eds). *Psychopharmacology: A Generation of Progress*. Raven Press: New York. pp 1113–1123.

Shen H, Hagino Y, Kobayashi H, Numachi Y, Yamamoto H, Yamamoto T *et al* (2003). Methamphetamine sensitization in dopamine (DAT) and/or 5-HT (SERT) transporter knockout mice. *The Society for Neuroscience 33rd Annual Meeting*, New Orleans, USA [2003/11/8-14].

Sills TL, Greenshaw AJ, Baker GB, Fletcher PJ (2000). Subchronic fluoxetine treatment induces a transient potentiation of amphetamine-induced hyperlocomotion: possible pharmacokinetic interaction. *Behav Pharmacol* **11**: 109–116.

Silver H, Shmugliakov N (1998). Augmentation with fluvoxamine but not maprotiline improves negative symptoms in treated schizophrenia: evidence for a specific serotonergic effect from a double-blind study. *J Clin Psychopharmacol* **18**: 208–211.

Silver H (2004). Selective 5-HT re-uptake inhibitor augmentation in the treatment of negative symptoms of schizophrenia. *Expert Opin Pharmacother* **5**: 2053–2058.

Snyder SH (1973). Amphetamine psychosis: a 'model' schizophrenia mediated by catecholamines. *Am J Psychiatry* **130**: 61–67.

Sodhi MS, Sanders-Bush E (2004). 5-HT and brain development. *Int Rev Neurobiol* **59**: 111–174.

Takamatsu Y, Yamamoto H, Hagino Y, Markou A, Ikeda K (2005). Fluoxetine as a potential pharmacotherapy for methamphetamine dependence. *Ann NY Acad Sci* (in press).

Tomiyama M, Kimura T, Maeda T, Kannari K, Matsunaga M, Baba M (2005). A serotonin 5-HT_{1A} receptor agonist prevents behavioral sensitization to L-DOPA in a rodent model of Parkinson's disease. *Neurosci Res* **52**: 185–194.

Ujike H, Sato M (2004). Clinical features of sensitization to methamphetamine observed in patients with methamphetamine dependence and psychosis. *Ann NY Acad Sci* **1025**: 279–287.

Vanderschuren LJ, Kalivas PW (2000). Alterations in dopaminergic and glutamatergic transmission in the induction and expression of behavioral sensitization: a critical review of preclinical studies. *Psychopharmacology (Berlin)* **151**: 99–120.

Yamaguchi M, Suzuki T, Seki T, Namba T, Juan R, Arai H *et al* (2004). Repetitive cocaine administration decreases neurogenesis in adult rat hippocampus. *Ann NY Acad Sci* **1025**: 351–362.

Yan QS, Yan SE (2001). Activation of 5-HT(1B/1D) receptors in the mesolimbic dopamine system increases dopamine release from the nucleus accumbens: a microdialysis study. *Eur J Pharmacol* **418**: 55–64.

Yan QS, Zheng SZ, Yan SE (2004). Involvement of 5-HT_{1B} receptors within the ventral tegmental area in regulation of mesolimbic dopaminergic neuronal activity via GABA mechanisms: a study with dual-probe microdialysis. *Brain Res* **1021**: 82–91.

Available online at www.sciencedirect.com
ScienceDirect
Neuroscience Letters 424 (2007) 116–121
Neuroscience Letters
www.elsevier.com/locate/neulet

Altered EphA5 mRNA expression in rat brain with a single methamphetamine treatment

Yohtaro Numachi [a,*], Sumiko Yoshida [b], Motoyasu Yamashita [c], Ko Fujiyama [c], Shigenobu Toda [d], Hiroo Matsuoka [c], Yasushi Kajii [e,1], Toru Nishikawa [e]

[a] *Musashi Hospital, National Center of Neurology and Psychiatry, Tokyo, Japan*
[b] *Department of Neuropsychiatry & Psychosomatic Medicine, Faculty of Medicine, Saitama Medical University, Moroyama-machi, Japan*
[c] *Department of Psychiatry, Tohoku University Graduate School of Medicine, Sendai, Japan*
[d] *Department of Neurosciences, Medical University of South Carolina, Charleston, SC, USA*
[e] *Section of Psychiatry and Behavioral Sciences, Tokyo Medical and Dental University Graduate School, Tokyo, Japan*

Received 25 May 2007; received in revised form 8 July 2007; accepted 23 July 2007

Abstract

Methamphetamine is a potent and indirect dopaminergic agonist which can cause chronic brain dysfunctions including drug abuse, drug dependence and drug-induced psychosis. Methamphetamine is known to trigger molecular mechanisms involved in associative learning and memory, and thereby alter patterns of synaptic connectivity. The persistent risk of relapse in methamphetamine abuse, dependence and psychosis may be caused by such alterations in synaptic connectivity. EphA5 receptors constitute large families of tyrosine kinase receptor and are expressed almost exclusively in the nervous system, especially in the limbic structures. Recent studies suggest EphA5 to be important in the topographic projection, development, and plasticity of limbic structures, and to be involved in dopaminergic neurotransmission. We used in situ hybridization to examine whether methamphetamine alters EphA5 mRNA expression in the brains of adult male Wister rats. EphA5 mRNA was widely distributed in the medial frontal cortex, cingulate cortex, piriform cortex, hippocampus, habenular nucleus and amygdala. Compared to baseline expression at 0 h, EphA5 mRNA was significantly decreased (by 20%) in the medial frontal cortex at 24 h, significantly increased (by 30%) in the amygdala at 9 and 24 h, significantly but transiently decreased (by 30%) in the habenular nucleus at 1 h after a single injection of methamphetamine. Methamphetamine did not change EphA5 mRNA expression in the cingulate cortex, piriform cortex or hippocampus. Our results that methamphetamine altered EphA5 mRNA expression in rat brain suggest methamphetamine could affect patterns of synaptic connectivity, which might be responsible for methamphetamine-induced chronic brain dysfunctions.

Keywords: Methamphetamine; Associative learning; Dependence; Neuronal plasticity; Synaptic connectivity; Receptor tyrosine kinase

Methamphetamine, a potent and indirect dopaminergic agonist, can cause brain dysfunctions including drug abuse, drug dependence and drug-induced psychosis [23] [28]. These disorders are chronic, since even after treatment and long periods of drug abstinence, the risk of relapsing, i.e. resuming drug use, remains high. Recent investigations have shown that methamphetamine and other abused drugs can trigger a set of molecular mechanisms normally involved in associative learning and memory: stimulation of dopamine D1 receptors, activation of the cAMP/PKA/CREB signal transduction pathway, altered gene expression, and synaptic rearrangements. Thus, the persistent tendency to relapse into drug abuse, drug dependence and drug-induced psychosis may reflect the persistence of altered patterns of synaptic connectivity, as is thought to occur with normal learning and memory formation [1].

Methamphetamine and amphetamine (which has pharmacological properties similar to those of methamphetamine) have been reported to reorganize patterns of synaptic connectivity. Amphetamine increased the length of dendrites,

* Corresponding author at: Musashi Hospital, National Center of Neurology and Psychiatry (NCNP), 4-1-1, Ogawa-Higashi, Kodaira, Tokyo 187-8551, Japan. Tel.: +81 42 341 2712x3028/81 42 346 1733; fax: +81 42 346 1705.
E-mail address: ynumachi@ncnp.go.jp (Y. Numachi).
[1] Present address: Research Laboratory I (CNS), Pharmaceuticals Research Division, Mitsubishi Pharma Corporation, Yokohama, Japan.

0304-3940/$ – see front matter © 2007 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2007.07.025

the density of dendritic spines, and the number of branched spines in the nucleus accumbens and pyramidal neurons in the medial prefrontal cortex of rats [20,21] and induced dendritic growth of dopaminergic neurons in the ventral tegmental area [19]. Methamphetamine and amphetamine also affect brain expressions of molecules associated with synaptogenesis and neuritogenesis including synaptophysin and stathmin [26], neuromodulin and synapsin I [14] and neuroglycan C [13] in rats. These reports suggest that methamphetamine can persistently alter brain synaptic connectivity.

The Eph family of receptors and ligands is the largest of all known tyrosine kinase receptor-ligand systems [10,32]. The best known function of the Eph family receptors and ligands is the guidance of axons and formation of topographic projection maps in the nervous system. In addition, roles in angiogenesis and cell migration have been described [32]. There are two major subgroups of Eph receptors; EphA and EphB. EphA5 (also known as cek7, Bsk, ehk1 and Hek7, in chickens, mice, rats and humans, respectively) was originally identified as a nervous-system-specific orphan receptor tyrosine kinase in the EphA subfamily [24,31,17,27]. EphA5 is almost exclusively expressed in the nervous system (with weak expression in the testes), especially in limbic structures [17]. EphA5 and its ligands are important in mediating axon guidance [3,15], topographic projection [2,4–6], development [7,16] and the plasticity of limbic structures [8,18,30], which play key roles in learning and memory [9]. Recent studies suggest the EphA5 receptor to be involved in dopaminergic neurotransmission. Transgenic mice with truncated forms of the EphA5 receptor showed decreased striatal dopamine and serotonin concentrations [11]. Transgenic mice over-expressing a soluble EphA5 receptor antagonist were insensitive to the locomotor stimulating effect of amphetamine [25]. These reports suggest signaling through EphA5 to have important roles in the actions of amphetamine or methamphetamine accompanied by altered dopaminergic neurotransmission. In the present study, we investigated whether methamphetamine has direct effects on EphA5 mRNA expression in rat brain.

Male Wister rats aged seven weeks, weighing 150–200 g on arrival, were obtained from Funabashi Farm (Funabashi, Japan). Two or three animals were housed in a metal cage (40 cm × 35 cm × 20 cm), with free access to food (standard rat chow F-2, Funabashi Farm) and water, in a room maintained at 24 ± 2 °C and 50 ± 10% humidity under a 12 h light-dark cycle (lights on at 08:00 h). We conducted all experiments with the approval of the Animal Ethics Committee at Tohoku University Graduate School of Medicine.

After one week of habituation to the housing conditions, the rats received an intraperitoneal injection of 4 mg/kg methamphetamine hydrochloride (Philopon, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) in saline solution during the light phase and were sacrificed by decapitation 0, 1, 3, 9 and 24 h after the injections. Following the decapitations, their brains were quickly removed and immediately frozen in isopentane and cooled at −40 °C. Each brain was sectioned on a cryostat at 14 μm, and a series of sections were mounted on poly-L-lysine coated slides. The slides were kept at −80 °C until processing for in situ hybridization.

A rat EphA5 cDNA fragment (Genbank accession X78689, n.t. 1678–2081, 404 bp) was amplified by PCR and subcloned into pGEM T Easy Vector (Promega, WI, USA). The plasmid was linearized by NcoI (New England Biolabs, MA, USA) or SpeI (New England Biolabs) for the anti-sense or sense probe, respectively. A DIG RNA labeling Kit (Roche Applied Science, IN, USA) was used for in vitro transcription with appropriate RNA polymerases (Sp6 RNA polymerase for the antisense riboprobe; T7 RNA polymerase for the sense riboprobe, Roche). The specificity of the antisense riboprobe was examined by Northern hybridization, with total RNA extracted from the entire brain of male Wister rat. Luminescent detection was used with Anti-Digoxigenin-AP and CSPD (Roche).

The stored brain sections were fixed in 4% paraformaldehyde for 1 h, followed by three washes in 2× SSC (1× SSC is 150 mM sodium chloride, 15 mM sodium citrate). The sections were then placed in a solution containing acetic anhydride (0.25%) in triethanolamine (0.1 M, pH 8) for 10 min at room temperature, rinsed in distilled water and dehydrated through

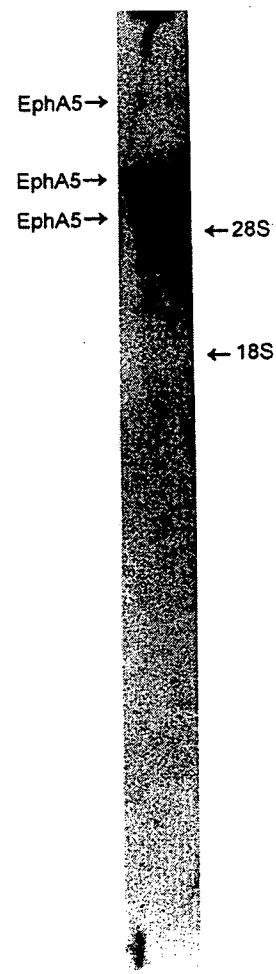

Fig. 1. Representative Northern blot of EphA5 mRNA in the brain of male Wister rat. An antisense cRNA probe against EphA5 mRNA detected transcripts of approximately 8.5, 5.9 and 5.1 kb.

graded alcohols (50, 75, 85, 95 and 100%). After air drying, the sections were hybridized with a ^{35}S-labeled cRNA probe. The probes were labeled in a reaction mixture consisting of 1 μg of linearized plasmid, 5× transcription buffer (Promega), 125 μCi [^{35}S]UTP (Amersham Bucks, UK), 125 μCi [^{35}S]CTP (Amersham), 150 μM each of ATP and GTP, 12.5 mM dithiothreitol, 20 U RNase inhibitor (Promega), and 6 U RNA polymerase (Promega). The reaction mixtures were incubated for 90 min at 37 °C. Then, the probes were separated from unincorporated nucleotides over a column (Centri-cep, QIAGEN, Hilden, Germany). The probe were diluted in hybridization buffer (containing 50% formamide, 10% dextran sulfate, 3× SSC, 50 mM sodium phosphate, 1× Denhardt's solution, 0.1 mg/ml yeast tRNA and 10 mM dithiothreitol) to yield 10^6 dpm/70 μl. The sections were cover-slipped and placed inside a humidified box overnight at 55 °C. Following hybridization, the cover-slips were removed and the sections were rinsed and washed three times in 2× SSC for 5 min each, then incubated for 1 h in RNase (200 μg/ml in Tris buffer containing 0.5 M NaCl, pH 8) at 37 °C. The sections were washed in increasingly stringent solutions of SSC, 2×, 1× and 0.5×, for 5 min each, followed by incubation for 1 h in 0.1× SSC at 62 °C. After rinsing in distilled water, the sections were dehydrated through graded alcohols, air-dried and exposed to a Kodak XAR film (Eastman Kodak, NY, USA) for 5 days. During hybridization, several sections were treated with sense riboprobe from the same plasmid insert to control for nonspecific hybridization.

Optical density measurements were quantified from X-ray film exposure of the hybridized sections. The images were captured using an MCID system (Imaging Research Inc., Ontario, Canada) and NIH image (http://rsb.info.nih.gov/nih-image/). Optical density measurements were taken for the medial frontal cortex, cingulate cortex, piriform cortex, hippocampus (CA1, CA2, CA3 and dentate gyrus), amygdala and habenular nucleus, from both sides of each coronal brain section. Eight sections per rat were used. Optical density values were corrected for background (corpus callosum), and then averaged to produce one data point for each animal. These data points were averaged per group and compared statistically. Differences in optical densities for EphA5 mRNA signals were analyzed using one-way ANOVA for time after the methamphetamine injections. For post hoc comparisons, the Tukey HSD test was used. A difference was considered to be statistically significant at $p < 0.05$.

EphA5 mRNA of approximately 8.5, 5.9 and 5.1 kb were identified by Northern blot analysis. Fig. 1 is a representative Northern image of EphA5 mRNA identified in the brain of Wister rat using the EphA5 antisense cRNA probe. Fig. 2 shows representative in situ hybridization images of EphA5 mRNA. Fig. 2a and b show EphA5 mRNA to be highly distributed in the medial frontal cortex, cingulate cortex and piriform cortex.

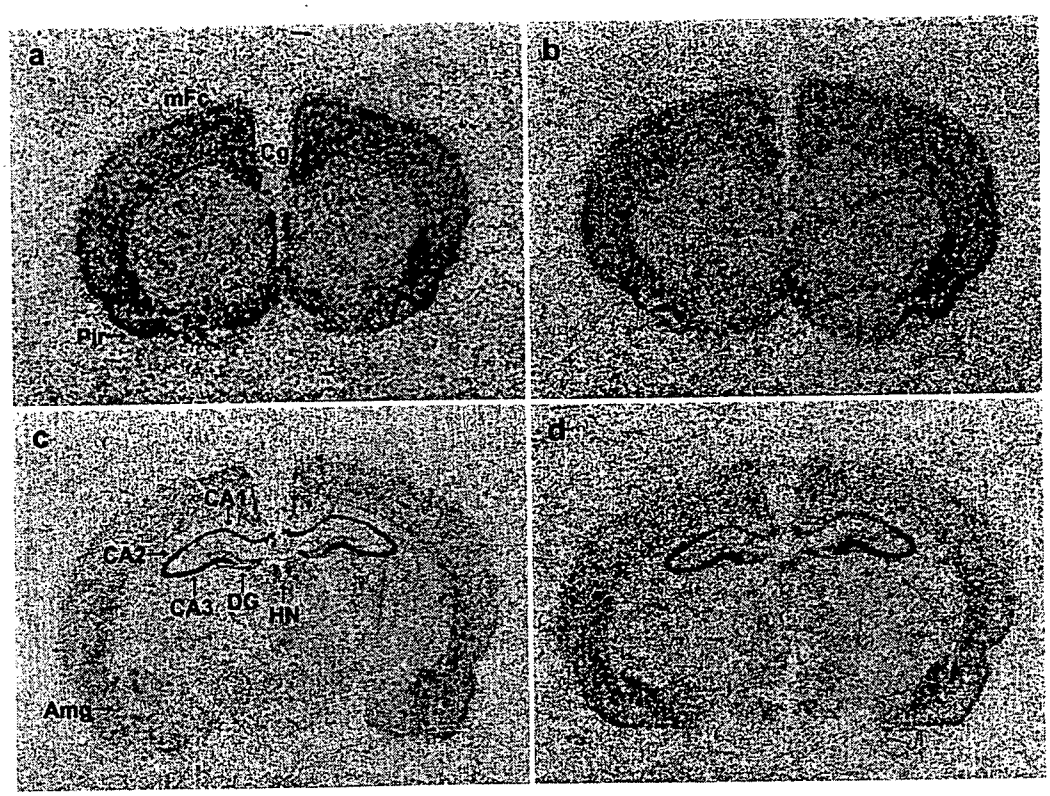

Fig. 2. Representative photomicrographs from 5-day exposure of X-ray films following in situ hybridization with an antisense cRNA probe against rat EphA5 mRNA. Fresh frozen coronal rat brain sections were hybridized with EphA5 antisense cRNA probe. Fig. 2a and c are photomicrographs before methamphetamine treatments (0 h), and Fig. 2b and d are photomicrographs 24 h after the treatments. EphA5 mRNA is abundant in the medial frontal cortex, cingulate cortex and piriform cortex (Fig. 2a), as well as in the hippocampus (CA3 > CA2 > CA1 > dentate gyrus), habenular nucleus and amygdala (Fig. 2b). mFc, medial frontal cortex; Cg, cingulate cortex; Pir, piriform cortex; CA1, 2 and 3, hippocampal subfields CA1, CA2, and CA3, respectively; DG, hippocampal subfield dentate gyrus; HN, habenular nucleus; Amg, amygdala.

Fig. 2c and d show EphA5 mRNA to be highly distributed in the hippocampus (CA3 > CA2 > CA1 > dentate gyrus), habenular nucleus and amygdala.

Fig. 3 shows alterations in optical densities for EphA5 mRNA signals after the methamphetamine injections. In the medial frontal cortex, a single injection of methamphetamine significantly decreased EphA5 mRNA expression at 24 h after treatment, i.e. by 20% as compared to 0 h ($F(4, 20) = 2.985$, $p < 0.05$ by one-way ANOVA, $p < 0.05$ by Tukey's HSD test). In the amygdala, EphA5 mRNA expression was significantly increased, by 30%, at 9 and 24 h after treatment as compared to 0 h ($F(4, 20) = 4.85$, $p < 0.01$ by one-way ANOVA, $p < 0.05$ by Tukey's HSD test). In the habenular nucleus, methamphetamine caused a significant but transient decrease of 30% in EphA5 mRNA at 1 h after treatment as compared to 0 h ($F(4, 19) = 3.133$, $p < 0.05$ by one-way ANOVA, $p < 0.05$ by Tukey's HSD test). In the cingulate cortex, piriform cortex and hippocampus, methamphetamine caused no changes in the expressions of EphA5 mRNA.

By Northern blot analysis using newly created riboprobe, we detected three mRNA transcripts for EphA5. Sizes of the transcripts were consistent with those of two previous reports [17,27]. The in situ hybridization using our riboprobe revealed strong expressions in the medial frontal cortex, cingulate cortex, piriform cortex, hippocampus (CA3 > CA2 > CA1 > dentate gyrus), habenular nucleus and amygdala. The observed distribution patterns were also similar to those reported previously [17,27]. These results suggest that our riboprobe, as used in the present study, adequately detected EphA5 mRNA.

Methamphetamine altered EphA5 mRNA expression in the brain with regional differences. In the cingulate cortex, piriform cortex and hippocampus, methamphetamine did not change the expressions of EphA5 mRNA. On the other hand, compared to baseline expression of EphA5 mRNA at 0 h, methamphetamine caused a significant decrease (20%) in the medial frontal cortex at 24 h, and a significant increase (30%) in the amygdala at 9 and 24 h after treatment. We also observed a transient but significant change in EphA5 mRNA in the habenular nucleus at 1 h after

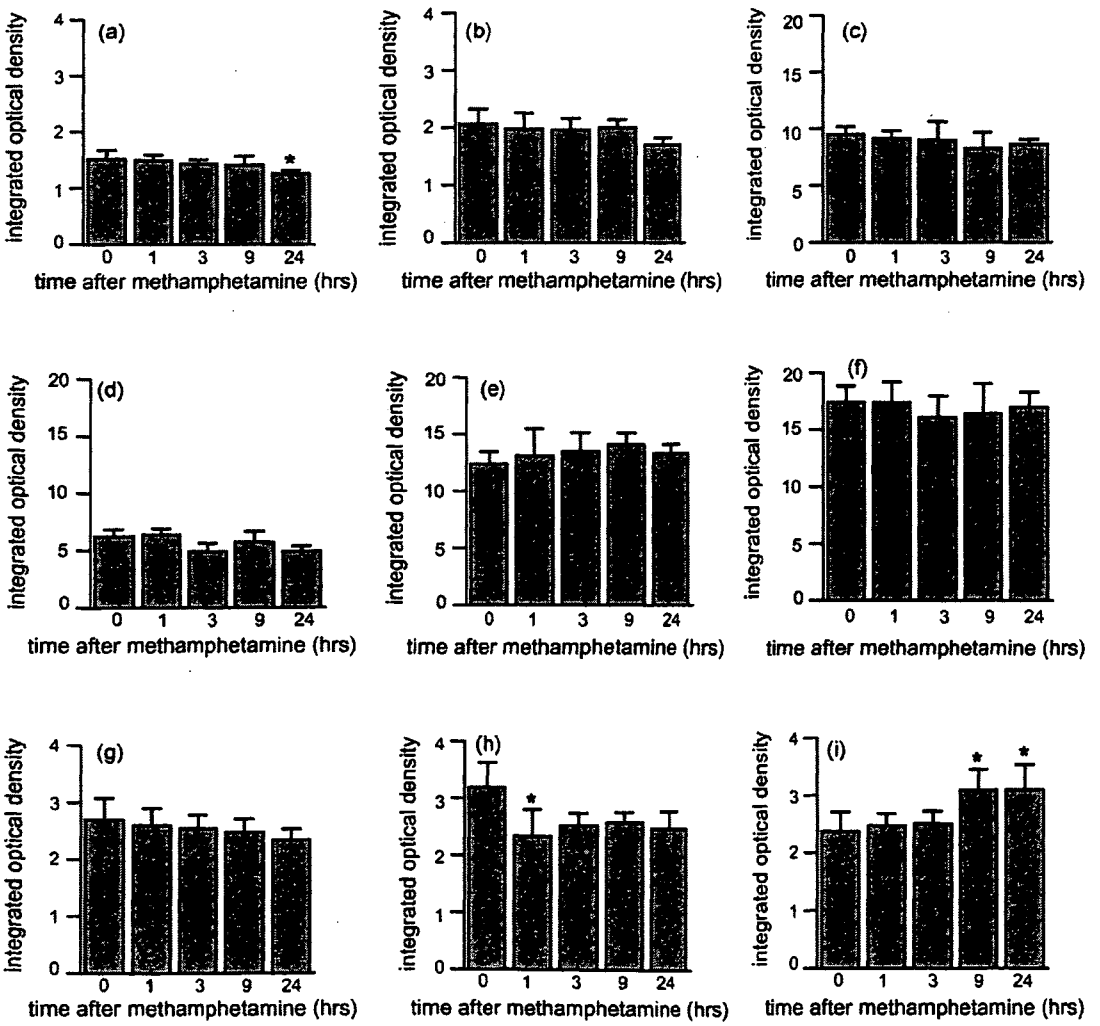

Fig. 3. Integrated optical density of radioactive signal for EphA5 mRNAs in the medial frontal cortex (a), cingulate cortex (b), piriform cortex (c), hippocampal subfields CA1 (d), CA2 (e), CA3 (f) and the dentate gyrus (g), habenular nucleus (h) and amygdala (i) in male Wister rats after acute methamphetamine treatments. Asterisks indicate a statistically significant difference. (*) $p < 0.05$; compared to 0 h (one-way ANOVA followed by Tukey's HSD test). Values are presented as means ± S.D., $n = 5$ rats per group.

methamphetamine treatment (as discussed below). The early changes in the habenular nucleus might be due to direct effects of methamphetamine (possibly via increased dopaminergic neurotransmissions). However, the later changes in the medial frontal cortex and amygdala might be attributable to indirect effects of methamphetamine.

Both of the medial frontal cortex and amygdala are parts of cortex-basal ganglia circuits which play critical roles in memory and the pathogenesis of drug abuse, drug dependence and drug-induced psychosis [1]. Previous studies also found methamphetamine to increase the expression of neuroglycan C mRNA which is associated with rearrangement of neural networks [13] and c-Fos immunoreactivity which is a marker for activated brain regions, in multiple regions of the brain including the medial frontal cortex and amygdala [29]. Methamphetamine-induced EphA5 mRNA changes in the medial frontal cortex and amygdala might be related to persistent alterations in synaptic connectivity in these brain regions. Transgenic mice with disrupted EphA receptor-ligand signaling, due to over-expression of a soluble EphA5 receptor antagonist, showed decreased densities of terminals and postsynaptic spines in the hippocampus [18]. This report is of special interest because Robinson et al. demonstrated that methamphetamine increased the lengths of dendrites, the density of dendritic spines, and in the number of branched spines in the nucleus accumbens and pyramidal neurons in the medial prefrontal cortex of rats [20,21]. Our data showing methamphetamine significantly decreased EphA5 mRNA in the medial frontal cortex might be related to methamphetamine-induced morphological changes in pyramidal neurons in the medial prefrontal cortex [20,21].

The habenular nucleus receives an abundance of afferent input from dopamine-rich forebrain areas and sends efferent output to mesencephalic dopaminergic systems, thereby possibly regulating the activity of dopamine neurons as a part of a feedback loop from the striatum to the substantia nigra [12]. Electrolytic lesions of the habenular nucleus significantly attenuated methamphetamine-induced inhibition of dopamine neurons in the substantia nigra [22]. In the present study, methamphetamine significantly but transiently decreased, by 30%, EphA5 mRNA in the habenular nucleus at 1 h after treatment. Observed changes in EphA5 mRNA in the habenular nucleus might be attributable to increased dopaminergic neurotransmission by methamphetamine, although the precise mechanisms remain unknown.

In summary, we found that methamphetamine affected expressions of EphA5 mRNA specifically in the medial frontal cortex, amygdala and habenular nucleus of the rat brain. As these brain regions are intimately involved in the long-term effects by which methamphetamine causes chronic brain dysfunctions, the observed changes in EphA5 mRNA might underlie altered patterns of synaptic connectivity in response to methamphetamine.

Acknowledgements

This study was supported in part by grant-in-aid for Scientific Research (C)(2) #14570906 (YN).

References

[1] J.D. Berke, S.E. Hyman, Addiction, dopamine, and the molecular mechanisms of memory, Neuron 25 (2000) 515–532.
[2] A. Brown, P.A. Yates, P. Burrola, D. Ortuno, A. Vaidya, T.M. Jessell, S.L. Pfaff, D.D. O'Leary, G. Lemke, Topographic mapping from the retina to the midbrain is controlled by relative but not absolute levels of EphA receptor signaling, Cell 102 (2000) 77–88.
[3] I.W. Caras, A link between axon guidance and axon fasciculation suggested by studies of the tyrosine kinase receptor EphA5/REK7 and its ligand ephrin-A5/AL-1, Cell Tissue Res. 290 (1997) 261–264.
[4] A. Dufour, J. Seibt, L. Passante, V. Depaepe, T. Ciossek, J. Frisen, K. Kullander, J.G. Flanagan, F. Polleux, P. Vanderhaeghen, Area specificity and topography of thalamocortical projections are controlled by ephrin/Eph genes, Neuron 39 (2003) 453–465.
[5] C.A. Ellsworth, A.W. Lyckman, D.A. Feldheim, J.G. Flanagan, M. Sur, Ephrin-A2 and -A5 influence patterning of normal and novel retinal projections to the thalamus: conserved mapping mechanisms in visual and auditory thalamic targets, J. Comp. Neurol. 488 (2005) 140–151.
[6] D.A. Feldheim, P. Vanderhaeghen, M.J. Hansen, J. Frisen, Q. Lu, M. Barbacid, J.G. Flanagan, Topographic guidance labels in a sensory projection to the forebrain, Neuron 21 (1998) 1303–1313.
[7] P.P. Gao, Y. Yue, J.H. Zhang, D.P. Cerretti, P. Levitt, R. Zhou, Regulation of thalamic neurite outgrowth by the Eph ligand ephrin-A5: implications in the development of thalamocortical projections, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 5329–5334.
[8] W.Q. Gao, N. Shinsky, M.P. Armanini, P. Moran, J.L. Zheng, J.L. Mendoza-Ramirez, H.S. Phillips, J.W. Winslow, I.W. Caras, Regulation of hippocampal synaptic plasticity by the tyrosine kinase receptor, REK7/EphA5, and its ligand, AL-1/Ephrin-A5, Mol. Cell Neurosci. 11 (1998) 247–259.
[9] R. Gerlai, N. Shinsky, A. Shih, P. Williams, J. Winer, M. Armanini, B. Cairns, J. Winslow, W. Gao, H.S. Phillips, Regulation of learning by EphA receptors: a protein targeting study, J. Neurosci. 19 (1999) 9538–9549.
[10] R. Gerlai, Eph receptors and neural plasticity, Nat. Rev. Neurosci. 2 (2001) 205–209.
[11] A.K. Halladay, L. Tessarollo, R. Zhou, G.C. Wagner, Neurochemical and behavioral deficits consequent to expression of a dominant negative EphA5 receptor, Brain. Res. Mol. Brain Res. 123 (2004) 104–111.
[12] G.R. Hanson, J.K. Ritter, C.J. Schmidt, J.W. Gibb, Response of mesolimbic substance P systems to methamphetamine treatment, Eur. J. Pharmacol. 128 (1986) 265–268.
[13] K. Ishikawa, A. Nitta, H. Mizoguchi, A. Mohri, R. Murai, Y. Miyamoto, Y. Noda, K. Kitaichi, K. Yamada, T. Nabeshima, Effects of single and repeated administration of methamphetamine or morphine on neuroglycan C gene expression in the rat brain, Int. J. Neuropsychopharmacol. 9 (2006) 407–415.
[14] S. Iwata, G.H. Hewlett, S.T. Ferrell, A.J. Czernik, K.F. Meiri, M.E. Gnegy, Increased in vivo phosphorylation state of neuromodulin and synapsin I in striatum from rats treated with repeated amphetamine, J. Pharmacol. Exp Ther. 278 (1996) 1428–1434.
[15] A.K. Jassen, H. Yang, G.M. Miller, E. Calder, B.K. Madras, Receptor regulation of gene expression of axon guidance molecules: implications for adaptation, Mol. Pharmacol. 70 (2006) 71–77.
[16] K. Mackarehtschian, C.K. Lau, I. Caras, S.K. McConnell, Regional differences in the developing cerebral cortex revealed by ephrin-A5 expression, Cereb. Cortex 9 (1999) 601–610.
[17] P.C. Maisonpierre, N.X. Barrezueta, G.D. Yancopoulos, Ehk-1 and Ehk-2: two novel members of the Eph receptor-like tyrosine kinase family with distinctive structures and neuronal expression, Oncogene 8 (1993) 3277–3288.
[18] A. Martinez, R. Otal, B.A. Sieber, C. Ibanez, E. Soriano, Disruption of ephrin-A/EphA binding alters synaptogenesis and neural connectivity in the hippocampus, Neuroscience 135 (2005) 451–461.
[19] D. Mueller, C.A. Chapman, J. Stewart, Amphetamine induces dendritic growth in ventral tegmental area dopaminergic neurons in vivo via basic fibroblast growth factor, Neuroscience 137 (2006) 727–735.

[20] T.E. Robinson, B. Kolb, Alterations in the morphology of dendrites and dendritic spines in the nucleus accumbens and prefrontal cortex following repeated treatment with amphetamine or cocaine, Eur. J. Neurosci. 11 (1999) 1598–1604.
[21] T.E. Robinson, B. Kolb, Structural plasticity associated with exposure to drugs of abuse, Neuropharmacology 47 (Suppl 1) (2004) 33–46.
[22] K. Sasaki, H. Suda, H. Watanabe, H. Yagi, Involvement of the entopeduncular nucleus and the habenula in methamphetamine-induced inhibition of dopamine neurons in the substantia nigra of rats, Brain Res. Bull. 25 (1990) 121–127.
[23] M. Sato, Y. Numachi, T. Hamamura, Relapse of paranoid psychotic state in methamphetamine model of schizophrenia, Schizophr. Bull. 18 (1992) 115–122.
[24] H. Shao, L. Lou, A. Pandey, M.F. Verderame, D.A. Siever, V.M. Dixit, cDNA cloning and characterization of a Cek7 receptor protein-tyrosine kinase ligand that is identical to the ligand (ELF-1) for the Mek-4 and Sek receptor protein-tyrosine kinases, J. Biol. Chem. 270 (1995) 3467–3470.
[25] B.A. Sieber, A. Kuzmin, J.M. Canals, A. Danielsson, G. Paratcha, E. Arenas, J. Alberch, S.O. Ogren, C.F. Ibanez, Disruption of EphA/ephrin-a signaling in the nigrostriatal system reduces dopaminergic innervation and dissociates behavioral responses to amphetamine and cocaine, Mol. Cell Neurosci. 26 (2004) 418–428.
[26] M. Takaki, H. Ujike, M. Kodama, Y. Takehisa, A. Yamamoto, S. Kuroda, Increased expression of synaptophysin and stathmin mRNAs after methamphetamine administration in rat brain, Neuroreport 12 (2001) 1055–1060.
[27] V. Taylor, G.C. Miescher, S. Pfarr, P. Honegger, H. Breitschopf, H. Lassmann, A.J. Steck, Expression and developmental regulation of Ehk-1, a neuronal Elk-like receptor tyrosine kinase in brain, Neuroscience 63 (1994) 163–178.
[28] H. Ujike, Stimulant-induced psychosis and schizophrenia: the role of sensitization, Curr. Psychiat. Rep. 4 (2002) 177–184.
[29] A. Umino, T. Nishikawa, K. Takahashi, Methamphetamine-induced nuclear c-Fos in rat brain regions, Neurochem. Int. 26 (1995) 85–90.
[30] Y. Yue, Z.Y. Chen, N.W. Gale, J. Blair-Flynn, T.J. Hu, X. Yue, M. Cooper, D.P. Crockett, G.D. Yancopoulos, L. Tessarollo, R. Zhou, Mistargeting hippocampal axons by expression of a truncated Eph receptor, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 10777–10782.
[31] R. Zhou, T.D. Copeland, L.F. Kromer, N.T. Schulz, Isolation and characterization of Bsk, a growth factor receptor-like tyrosine kinase associated with the limbic system, J. Neurosci. Res. 37 (1994) 129–143.
[32] R. Zhou, The Eph family receptors and ligands, Pharmacol. Ther. 77 (1998) 151–181.