rodents, this animal model is unlikely to completely resemble the pathogenesis of schizophrenia, since at least in some cases, the pathologic abnormalities occur during development and are initiated by prenatal insults [35,39]. Therefore, it is necessary to consider the process by which the symptoms of schizophrenia develop. Based on the neurodevelopment hypothesis of psychiatric disorders, several studies have modified the classic "PCP-based animal model", with treatment using NMDA antagonists during the early development of the brain [1,12,44,49,50]. Moreover, one recent study has revealed that postnatal, but not adulthood, NMDA receptor ablation in the corticolimbic interneurons confers schizophrenia-like phenotypes in adult mice [5].

Our previous study confirmed that prenatal exposure to PCP (20 mg/kg) produced a cognitive deficit and hypersensitivity to PCP in terms of locomotor activity, which was associated with abnormal expression of the NMDA receptor [27]. Furthermore, these behavioral changes were attenuated by clozapine [27], an atypical antipsychotic that enhanced the function of glutamatergic transmission [9,30] and inhibited the up-regulation of glutamate transporters [29,48]. These findings suggest that the pre-synaptic glutamatergic system is involved in the behavioral deficits induced by prenatal PCP treatment. However, little attempt has been made to investigate the role of pre-synaptic glutamate transmission in this model. In this study, we evaluated the long-term effects of prenatal exposure to PCP on the prefrontal glutamatergic neurotransmission in mice.

2. Materials and methods

2.1. Animals

ICR female and male mice (8 weeks old) were obtained from Japan SLC (Shizuoka, Japan) and maintained on a 12/12 h, light/dark cycle (lights on from 08:00 to 20:00) with free access to food (CE2; Clea Japan Inc., Tokyo, Japan) and water. The mice were mated one pair per cage. Females were checked in the next morning and those with an embolus in their vaginas were considered pregnant [embryonic day 0 (E 0)]. The dams were randomly divided into saline- (SAL) and PCP-treated groups. All were housed individually till parturition. There were no maternal deaths and resorption or stillbirths caused by exposure to PCP in this study. At birth [postnatal day 0 (PD 0)], pups were culled to 8–10 per litter with a balance of males and females as possible. Maternal care behavior during feeding was monitored till weaning on PD 21. After weaning, pups given the same prenatal treatment were mixed by gender, and then randomly assigned into groups to do behavioral tests. Each behavioral test was involved 2–3 litters each time and repeated more than 3 times by using different mice to reduce the influence of litters. Moreover, a balanced number of males and females were used in each experiment, since no significant differences were observed between genders in our preliminary study [27].

The experiments with offspring were started at the age of 8 weeks and carried out in a sound-attenuated and air-conditioned room (23 ± 1 °C, 50 ± 5% humidity). The mice were habituated to the room for more than 40 min before behavioral experiments. All the behavioral tests were recorded with a digital versatile disc camera to analyze the results. The experiments were performed in accordance with the Guidelines for Animal Experiments of Meijo University Faculty of Pharmaceutical Sciences and the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society (2008).

2.2. Drugs

PCP hydrochloride was synthesized according to the method of [28] and checked for purity. PCP was dissolved in saline. DL-threo-β-benzyloxyaspartate (DL-TBOA, Tocris, MO) was prepared as a stock solution of 100 mM in 50% dimethyl sulfoxide (DMSO) and 100 mM NaOH, and diluted with artificial cerebrospinal fluid (aCSF) before the behavioral test, or with Ringer's solution before the microdialysis analysis [34].

2.3. Drug treatment

The dams were administered SAL or PCP (5 mg/kg, s.c.) once daily at 18:00 from E 6 to E 18, the middle and late stages of pregnancy, covering the entire period of neurodevelopment in the prenatal brain from neurulation to corticogenesis [45]. The injection by s.c. was performed gently as possible to minimize potential stress-related influences on dams. The dose of PCP (5 mg/kg) was selected according to one study [44], since it was not toxic enough to affect the sensitization of dopaminergic system or tolerance of serotonergic ataxic behaviors in adults [25].

Under light anesthesia with diethyl ether, mice received an microinjection of DL-TBOA (1 or 10 nmol/μl/site; bilaterally) into the PFC [Anteroposterior (AP): +1.7 mm from Bregma; Mediolateral (ML): ±0.5 mm from Bregma; Dorsoventral (DV): +2 mm from the skull] according to the mouse brain atlas of [15], 30 min before each behavioral test [34]. DL-TBOA was injected over a period of 30 s, and the injector was left in the place for 1 min to allow diffusion. For the analysis of microdialysis, according to the effective dose observed in the behavioral tests, DL-TBOA (1 mM) was administered through the dialysis probe at a rate of 1 μl/min for a total of 30 min.

2.4. Novel object recognition test

The novel object recognition test was performed as described previously [27]. Each mouse was individually habituated to the box (L 30 × W 30 × H 35 cm), with 10 min of exploration in the absence of objects for 3 days (habituation session). During the training session, two objects (a red painted triangular prism and a yellow painted cube) were symmetrically fixed to the floor of the box, 8 cm from the walls, and each animal was allowed to explore in the box for 10 min (day 4). An animal was considered to be exploring the object when its head was facing the object or it was touching or sniffing the object at a distance of less than 2 cm and/or touching it with the nose. The time spent exploring each object was recorded. After training, mice were immediately returned to their home cages. During the retention session, animals were placed back into the same box 24 h (day 5) after the training session, in which one of the familiar objects used during training was replaced with a novel object (a black painted golf ball). The animals were 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, the ratio of time spent exploring either of the two objects (training session) or the novel object (retention session) over the total amount of time spent exploring both the objects, was used to assess cognitive function.

2.5. Forced swimming test

The forced swimming test was done according to a previous report [34], with a minor modification. Mice were placed in a transparent glass cylinder (20 cm high, 15 cm in diameter), which contained water at 22 °C to a depth of 11.5 cm, and forced to swim for 6 min. The duration of swimming was measured by using a SCANET MV-10 AQ apparatus (Melquest Co. Ltd., Toyama, Japan). Immobility time was calculated as: immobility time (s) = 360 − swimming time.

2.6. Microdialysis analysis

Different mice from behavioral tests above were used for the following microdialysis analysis. Mice were anesthetized with pentobarbital-Na (50 mg/kg, i.p.) and fixed in a stereotaxic apparatus (David Kopf Instruments, CA). A dialysis probe (D-I-6-01; EICOM, Kyoto, Japan) was implanted into the PFC [AP: +1.7, ML: −0.05 from bregma, DV: −2 mm from the skull]. Twenty-four hours later, Ringer's solution (147 mM, NaCl, 4 mM KCl, and 2.3 mM $CaCl_2$) was perfused at a flow rate of 1 μl/min. Dialysate was collected every 10 min and the amount of glutamate was determined by an HPLC system (HTEC-500, EICOM) with electrochemical detection (ECD). For depolarization, 100 mM KCl-containing Ringer's solution (51 mM NaCl, 100 mM KCl, and 2.3 mM $CaCl_2$) was delivered through the dialysis probe for 30 min. Then, dialysate was collected for 90 min with Ringer's solution. For the rescue with DL-TBOA, after the collection of baseline fractions, 10 nmol of DL-TBOA dissolved in 10 μl of Ringer's solution was injected through the probe through the microinjection tube for 30 min.

2.7. Preparation of brain slice and staining

Histological procedures were performed as previously described with a minor modification [34]. Mice were anesthetized with chloral hydrate (200 mg/kg i.p.) and perfused transcardially with ice-cold phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in PBS and then soaked in 10–30% (w/v) sucrose in PBS. Coronal sections 20 μm thick were cut with a cryostat (CM 1850; Leica, Germany). Cresyl violet staining was performed and the areas of brains and ventricles, and the number of neurons with a visible nucleus in the layers II/III of the prelimbic area were counted using computer-based image analysis system (WinRoof, Mitani, Japan). Images were acquired with a microscope (BZ-9000, Keyence, JP).

2.8. Western blotting analysis

Western blotting was performed as described previously with a minor modification [34]. The PFC including the cingulate and prelimbic area (Bregma +2.96 to Bregma +1.34) was rapidly dissected out, frozen, and stored at −80 °C prior to assays. The brain samples were homogenized in ice-cold buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 10 mM NaF; 10 mM EDTA, 1% NP-40; 1 mM sodium orthovanadate; 10 mM sodium pyrophosphate; 0.5 mM DTT; 0.2 mM PMSF; 4 μg/ml pepstatin, 4 μg/ml aprotinin, and 4 μg/ml leupeptin). The lysate was centrifuged at 8000 × g for 10 min at 4 °C, and the suspension of precipitate was used.

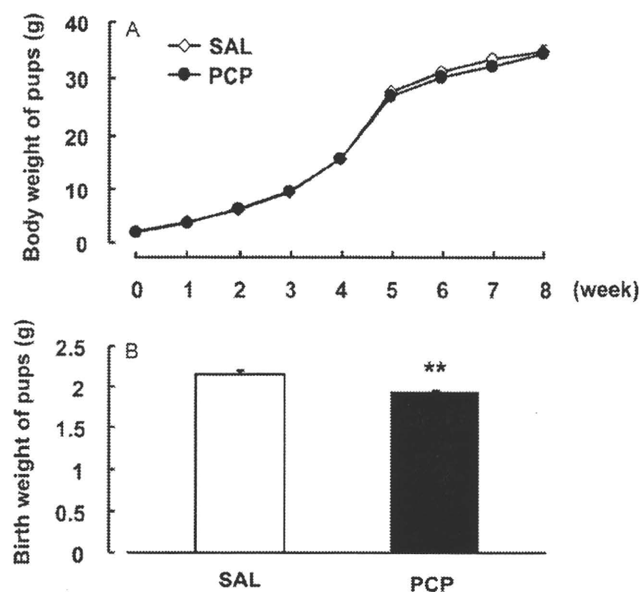

Fig. 1. Effects of prenatal PCP treatment on body weight of pups. The body weight of pups during the developing period from birth to 8 weeks old (A) (repeated one-way ANOVA with Bonferroni's test); the birth weight of pups at PD1 (B) (Student's *t*-test). $^{**}P<0.01$ compared with the prenatal SAL-treated group. Data are expressed as the mean ± S.E.M for 36–38 mice. SAL, saline; PCP, phencyclidine.

The protein concentrations were determined using a Pierce BCA Protein Assay Kit (Thermo Fishers, CA, USA). Samples were boiled at 95 °C for 5 min in sample buffer (125 mM Tris–HCl, pH 6.8, 10% 2-mercaptoethanol, 4% sodium diphosphate decahydrate, 10% sucrose and 0.0004% bromophenol blue), separated on a polyacrylamide gel, and transferred to polyvinylidene difluoride membranes (Millipore Corporation, MA, USA). The membranes were blocked with a Detector Block Kit (Kirkegaard and Perry Laboratories, MD, USA) and probed with primary anti-GLAST, anti-GLT-1 (1:1000; Upstate Biotechnology, NY, USA), and anti-GFAP (1:1000; Upstate Biotechnology) antibodies. Membranes were washed with the washing buffer (50 mM Tris–HCl, pH 7.4, 0.05% Tween 20, and 150 mM NaCl) and subsequently incubated with a secondary horseradish peroxidase-linked antibody (Kirkegaard and Perry Laboratories). Immunoreactive complexes on the membrane were detected using Western blotting detection reagents (GE Healthcare Biosciences, NJ, USA). The intensity of bands was detected by densitometry using ChemiDoc system and Quantity One Software (Bio-Rad, Hercules, CA, USA). After the GLAST, GLT-1 and GFAP proteins were detected, membranes were stripped with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris–HCl, pH 6.7) at 50 °C for 30 min, and the expression of β-actin was detected with a primary anti-actin antibody (1:1000; Santa Cruz Biotechnology, CA, USA) as described above.

2.9. Statistical analysis

All data were expressed as the mean ± S.E.M. Significant differences between two groups were determined with Student's *t*-test. That among three groups or more was determined using a one- or two-way analysis of variance (ANOVA), or repeated one- or two way ANOVA, respectively, followed by Bonferroni's test ($P<0.05$).

3. Results

3.1. Effect of prenatal PCP treatment on body weight during development

To confirm the effects of prenatal PCP administration on development, the body weight of pups was observed throughout the development. As shown in Fig. 1, there were no significant differences between SAL- or PCP-treated offspring from birth to the age of 8 weeks old, when the behavioral tests were carried out ($F_{group\,(1,576)}=1.58$, $P>0.05$; $F_{week\,(8,576)}=2584.08$, $P<0.01$; $F_{group\times week\,(8,576)}=1.24$, $P>0.05$; repeated one-way ANOVA; Fig. 1A). These results suggested that prenatal PCP treatment did not affect the growth of body weight in pups, although there is a significant decrease in the weight at birth (SAL-treated mice, 2.15 ± 0.04 g; PCP-treated mice, 1.93 ± 0.03 g; $P<0.01$, $n=36$–38; Student's *t*-test; Fig. 1B).

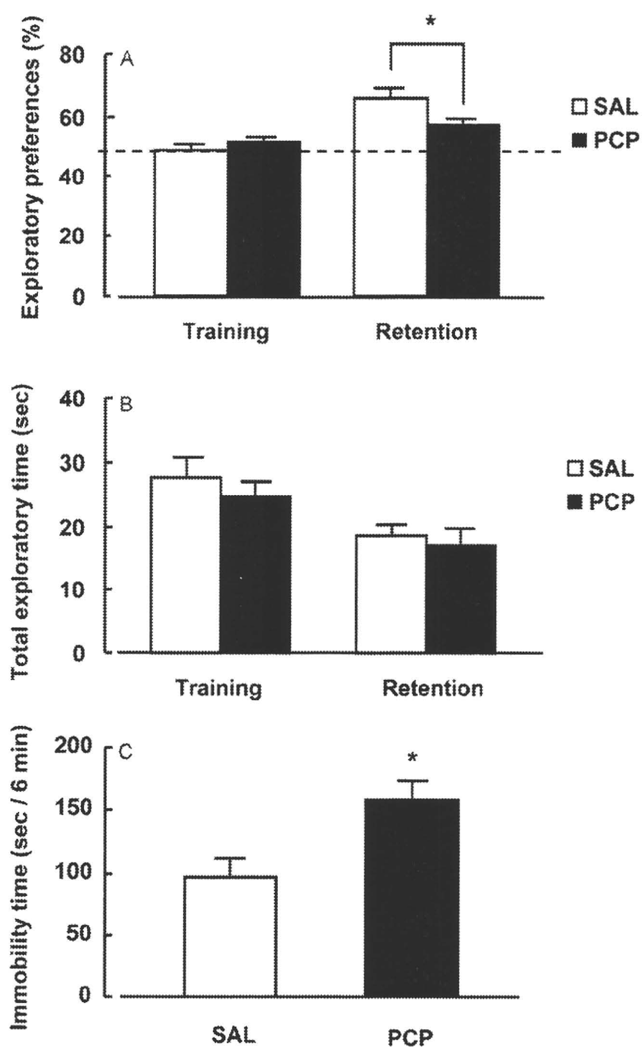

Fig. 2. Influences of prenatal PCP treatment on cognitive and emotional behavior in offspring at 8 weeks old. Exploratory preference (%) (A); and total time spent exploring the objects (s) (B) in training and retention sessions of the novel object recognition test. In the forced swimming test, immobility time (s) was assessed for 6 min (C). $^{*}P<0.05$ compared with the prenatal SAL-treated group. Data are expressed as the mean ± S.E.M. for 10–14 mice in each group (Student's *t*-test). SAL, saline; PCP, phencyclidine.

3.2. Abnormal cognitive and emotional behavior in prenatal PCP-treated mice

To investigate effects of prenatal PCP treatment on cognitive function, we performed a novel object recognition test. In the training session, the prenatal SAL- or PCP-treated mice spent almost equal amounts of time exploring either of the two objects, and there was no biased exploratory preference in each group of mice (SAL-treated mice, 49.05 ± 1.82%; PCP-treated mice, 51.76 ± 1.49%; $P>0.05$, $n=10$–14; Student's *t*-test; Fig. 2A). In addition, the total time spent on the exploration of objects in the training session did not differ between the two groups (SAL-treated mice, 27.65 ± 3.05 s; PCP-treated mice, 24.89 ± 2.38 s; $P>0.05$, $n=10$–14; Student's *t*-test; Fig. 2B). In the retention session, the PCP-treated mice showed a reduced level of exploratory preference for the novel objects compared with the SAL-treated group (SAL-treated mice, 65.95 ± 3.14%; PCP-treated mice, 57.20 ± 2.33%; $P<0.05$,

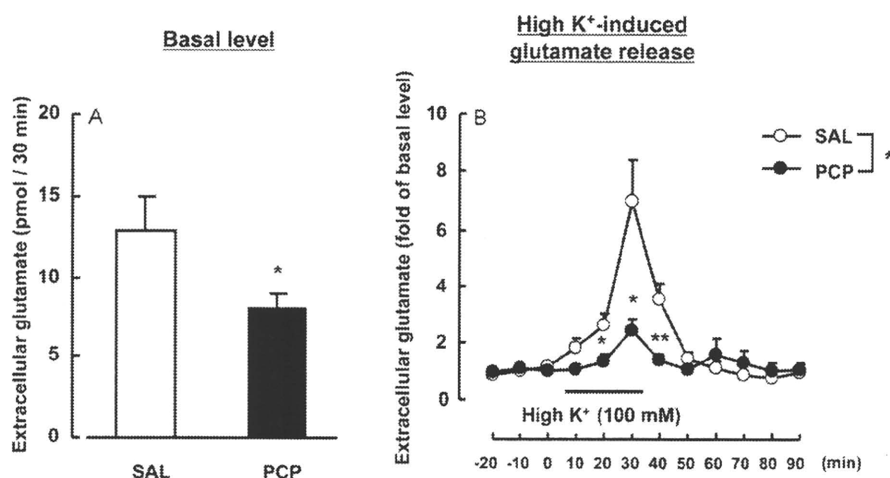

Fig. 3. Basal extracellular glutamate levels and high K^+-evoked glutamate release in the prefrontal cortex of prenatal PCP-treated mice. Basal extracellular levels of glutamate (A) and high K^+ (100 mM)-evoked glutamate release (B) in the prefrontal cortex of prenatal SAL- or PCP-treated mice were determined by microdialysis. Data are expressed as the mean ± S.E.M. for 7 mice in each group. $^*P < 0.05$, $^{**}P < 0.01$ compared with the prenatal SAL-treated group (Student's *t*-test or repeated one-way ANOVA with Bonferroni's test).

$n = 10$–14; Student's *t*-test; Fig. 2A). However, no significant difference was observed in the total exploration time (SAL-treated mice, 18.74 ± 1.72 s; PCP-treated mice, 17.08 ± 2.97 s; $P > 0.05$, $n = 10$–14; Student's *t*-test; Fig. 2B).

To investigate effects of prenatal exposure to PCP on emotional behavior, we performed the forced swimming test. The PCP-treated mice showed significantly prolonged immobility throughout the 6-min test, compared with the SAL-treated mice (SAL-treated mice, 96.8 ± 13.48 s; PCP-treated mice, 157.47 ± 15.69 s; $P < 0.05$, $n = 10$–14; Student's *t*-test; Fig. 2C), which implied that emotional deficits were induced by the prenatal exposure.

3.3. Reduced glutamate release in the prefrontal cortex of prenatal PCP-treated mice

To investigate whether pre-synaptic glutamatergic neurotransmission was adversely affected by prenatal exposure to PCP, we examined extracellular glutamate levels in the PFC using microdialysis. When the extracellular levels reached a steady state without any treatment, the basal release of glutamate was monitored for 30 min. The prenatal PCP-treated mice showed a dramatically reduced level of extracellular glutamate in the PFC, compared with the SAL-treated mice (SAL-treated mice, 12.97 ± 1.96 pmol; PCP-treated mice, 8.06 ± 0.95 pmol; $P < 0.05$, $n = 7$; Student's *t*-test; Fig. 3A). Next, we evaluated the glutamate release induced by high potassium (high K^+, 100 mM). The high K^+ treatment increased the release of extracellular glutamate in both the SAL- and PCP-treated mice. However, the increase was significantly lower in the PCP-treated mice ($F_{\text{treatment}\,(1,\,12)} = 20.63$, $P < 0.01$; $F_{\text{time}\,(4,\,12)} = 11.11$, $P < 0.01$; $F_{\text{treatment} \times \text{time}\,(4,\,12)} = 3.93$, $P < 0.01$; repeated one-way ANOVA for 10–50 min; Fig. 3B).

3.4. Overexpression of glutamate transporters in the prefrontal cortex of prenatal PCP-treated mice

To further examine whether the decrease in the extracellular glutamate level was due to changes to glutamate transporters, we investigated the protein expression of GLAST and GLT-1 in the PFC by Western blotting. The level of GLAST protein was significantly higher in the PCP-treated mice than SAL-treated mice (SAL-treated mice, 100 ± 10.24%; PCP-treated mice, 135.18 ± 9.98%; $P < 0.05$, $n = 6$–7; Student's *t*-test; Fig. 4A). However, we did not observe a significant difference in GLT-1 expression between the groups (SAL-treated mice, 100 ± 12.49%; PCP-treated mice, 121.98 ± 12.29%; $P > 0.05$, $n = 6$–7; Student's *t*-test; Fig. 4B). We further examined the level of GFAP expression, a marker of glial cell, by Western blotting. There was no significant difference in GFAP expression between the prenatal SAL- and PCP-treated mice (SAL-treated mice, 100 ± 5.19%; PCP-treated mice, 104.38 ± 6.72%; $P > 0.05$, $n = 6$–7; Student's *t*-test; Fig. 4C), suggesting that the increased expression of GLAST was not directly due to increasing numbers of glial cells in the PFC of mice.

3.5. The neuronal number in the prefrontal cortex of prenatal-PCP treated mice

To further investigate whether the reduced glutamate release was due to the decrease of number of neurons in adult mice, we examined morphological changes of neurons in the PFC by Cresyl violet staining. As shown in the results, there was only a tendency to decrease in the number of neurons in the layer II/III of the PFC at PD 56, but not significant difference regardless of prenatal PCP treatment (SAL-treated mice, 2247 ± 117; PCP-treated mice, 1925 ± 116; $P = 0.08$, $n = 6$; Student's *t*-test; Supplementary Fig. 1A and B). It suggested that the decrease in extracellular glutamate level might be not due to the changes of number of neurons in adult.

3.6. Effects of a potent glutamate transporter inhibitor on behavioral deficits and the impairment of glutamate release in prenatal PCP-treated mice

To further investigate whether the prenatal PCP-induced behavioral changes and glutamatergic dysfunction were associated with the overexpression of glutamate transporters, we examined the effects of a potent inhibitor of glutamate transporters, DL-TBOA. In the PCP-treated mice, DL-TBOA (10 nmol) attenuated the impairment of recognition memory in the novel recognition test. There was no biased exploratory preference ($F_{\text{group}\,(1,\,40)} = 0.17$, $P > 0.05$; $F_{\text{treatment}\,(2,\,40)} = 1.29$, $P > 0.05$; $F_{\text{group} \times \text{treatment}\,(2,\,40)} = 0.16$, $P > 0.05$; two-way ANOVA; Fig. 5A), and no difference in total exploration time between the two groups in the training session ($F_{\text{group}\,(1,\,40)} = 0.61$, $P > 0.05$; $F_{\text{treatment}\,(2,\,40)} = 2.71$, $P > 0.05$; $F_{\text{group} \times \text{treatment}\,(2,\,40)} = 0.57$, $P > 0.05$; two-way ANOVA; Fig. 5B). In the retention session, the impairment of recognition memory in the PCP-treated mice was significantly improved by the

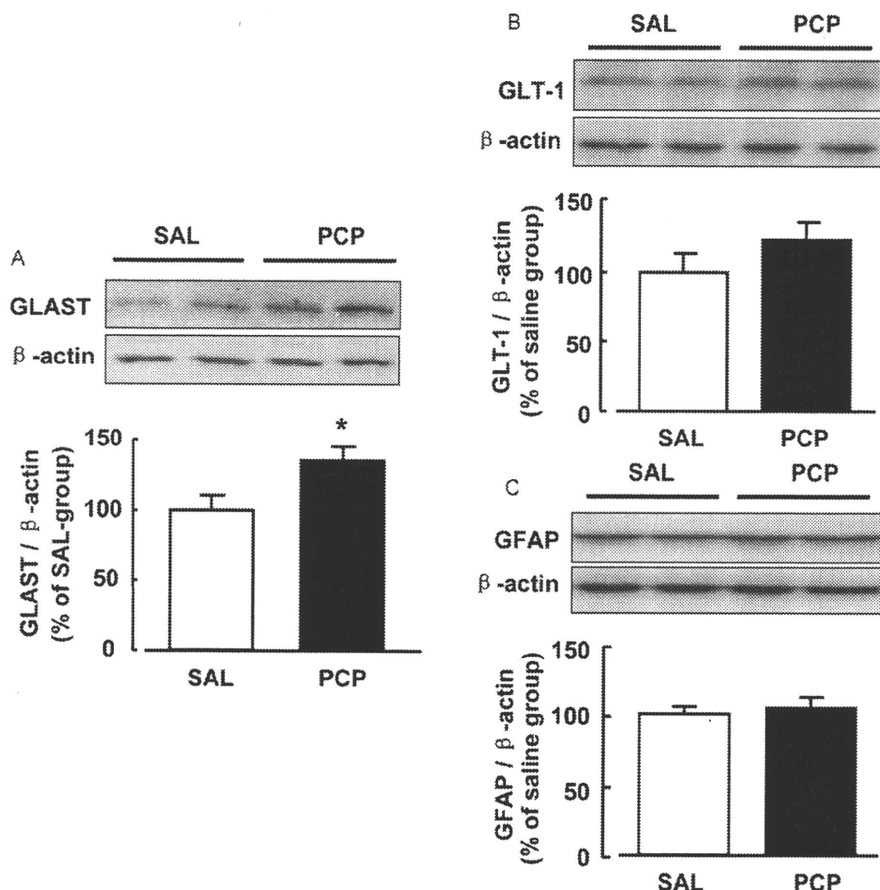

Fig. 4. Changes in the expression of glutamate transporters in the prefrontal cortex of prenatal PCP-treated mice. Representative Western blots band for the expression of GLAST, GLT-1 and GFAP. The amount of protein (30 μg/well) loaded was normalized to that of β-actin. Results are represented as the level of GLAST (A), and GLT-1 (B), as well as GFAP (C) in the prefrontal cortex. *$P < 0.05$ compared with the prenatal SAL-treated group. Data are expressed as the mean ± S.E.M. for 6–7 mice in each group (Student's t-test). SAL, saline; PCP, phencyclidine.

higher dose of DL-TBOA (10 nmol) ($F_{\text{group}\,(1,40)} = 24.66$, $P < 0.01$; $F_{\text{treatment}\,(2,40)} = 3.96$, $P < 0.05$; $F_{\text{group} \times \text{treatment}\,(2,40)} = 6.51$, two-way ANOVA; $P < 0.01$, Fig. 5C). However, there were no significant differences in total exploration time in the retention session among each group ($F_{\text{group}\,(1,40)} = 0.14$, $P > 0.05$; $F_{\text{treatment}\,(2,40)} = 0.02$, $P > 0.05$; $F_{\text{group} \times \text{treatment}\,(2,40)} = 1.27$ $P > 0.05$; two-way ANOVA; Fig. 5D). These results suggested that DL-TBOA did not affect motivation or curiosity, but ameliorated the impairment of recognition memory in the prenatal PCP-treated mice.

Next, we evaluated the effects of DL-TBOA on the prolonged immobility time in the forced swimming test in the PCP-treated mice. DL-TBOA (10 nmol) significantly reversed the prolonged immobility induced by prenatal PCP exposure in the forced swimming test ($F_{\text{group}\,(1,40)} = 18.03$, $P < 0.01$; $F_{\text{treatment}\,(2,40)} = 7.42$, $P < 0.01$; $F_{\text{group} \times \text{treatment}\,(2,40)} = 3.10$, $P = 0.06$; two-way ANOVA; Fig. 5E), but did not change immobility time in the SAL-treated mice. These results indicated DL-TBOA to be effective in correcting emotional abnormalities induced by prenatal exposure to PCP without affecting motility.

Furthermore, we continued to evaluate the effects of DL-TBOA on the reduced extracellular glutamate level and impairment of K^+-induced glutamate release induced by prenatal PCP treatment. After the basal levels of glutamate reached a steady state, DL-TBOA was given through the probe for dialysis and the basal release of glutamate was monitored for 90 min. DL-TBOA tended to normalize the level of extracellular glutamate in the prenatal PCP-treated mice ($F_{(2,18)} = 2.60$, $P = 0.10$; one-way ANOVA; Fig. 6A). Moreover, it clearly improved the high K^+ (100 mM)-induced glutamate release reduced by prenatal exposure to PCP ($F_{\text{group}\,(2,18)} = 9.09$, $P < 0.01$; $F_{\text{time}\,(4,18)} = 22.45$, $P < 0.01$; $F_{\text{group} \times \text{time}\,(8,18)} = 2.43$, $P < 0.05$; repeated two-way ANOVA for 10–50 min; Fig. 6B). These results indicated that DL-TBOA reversed the reduction in glutamatergic neurotransmission observed in the prenatal PCP-treated mice.

4. Discussion

The blockade of NMDA receptors by PCP in the developing brain has been found to impair learning and memory. For instance, prenatal exposure to PCP disrupts passive avoidance and pole-climbing avoidance responses [36], and impairs performance in the eight-arm maze and Morris water maze in adult rats [2,51]. In the present study, prenatal PCP treatment produced an impairment of memory in the novel object recognition test, consistently suggested a cognitive deficit in this model. Furthermore, the prenatal PCP-treated mice showed a prolonged immobility in the forced swimming test, which are frequently observed in PCP animal models displaying schizophrenia-like negative symptom [38]. Taken together, these results indicate that the blockade of NMDA receptors by PCP in the prenatal period triggers cognitive and emotional abnormalities in postpubertal mice.

Glutamate neurotransmission plays a critical role in synaptic activity and plasticity throughout the brain, including cognition-, emotion- and reward-related circuits [31]. In schizophrenic patients, evidence of abnormal glutamatergic transmission has been found, such as disturbances of cortical glutamate release

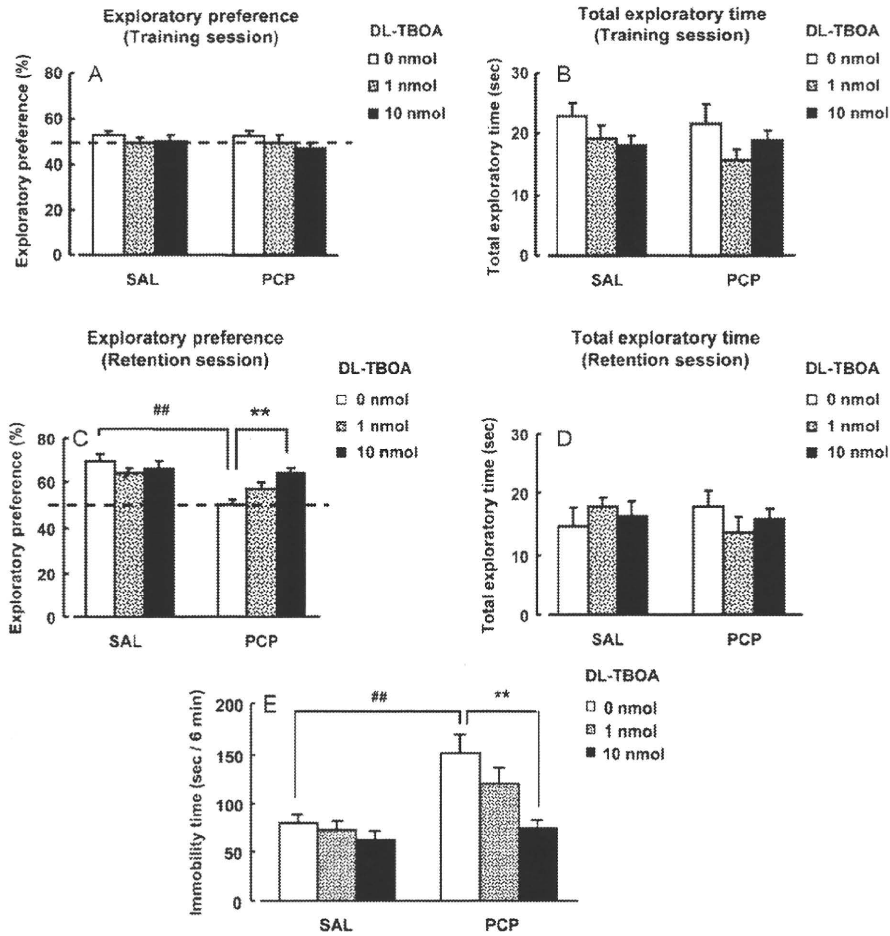

Fig. 5. Effects of DL-TBOA on the behavior in the prenatal PCP-treated mice. DL-TBOA (1 and 10 nmol) was administered by microinjection directly into the prefrontal cortex 30 min before each behavioral test. Exploratory preference (%) in the training session (A) and retention session (C). Total exploration time (s) in the training session (B) and retention session (D) of the novel object recognition test. Immobility time (s) was assessed for 6 min in the forced swimming test (E). Data are expressed as the mean ± S.E.M. for 7–8 mice in each group. ##$P < 0.01$ compared with the prenatal SAL-treated group; *$P < 0.05$, **$P < 0.01$ compared with the prenatal PCP-treated group (two-way ANOVA with Bonferroni's test). SAL, saline; PCP, phencyclidine.

[7,8,14,24], lower glutamate levels in the PFC [46], and decreased levels of glutamate in cerebrospinal fluid [19], as well as reduced glutamatergic tone in the cortex area [16]. In PCP-treated adult mice, a decrease in spontaneous extracellular glutamate release [34] and in the level of phosphorylated-NR1 [33,34], but an increase in levels of GLAST expression has been observed in the PFC [34]. In the present study, we found a decrease in both the extracellular glutamate concentration and high K^+-induced release of glutamate in the PFC of the PCP-treated mice compared with the SAL-treated mice, suggesting that prenatal exposure to PCP produced a pre-

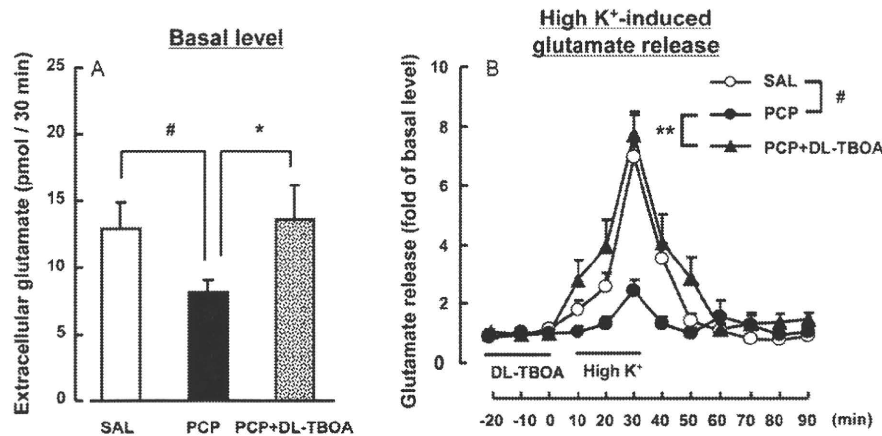

Fig. 6. Effects of DL-TBOA on the reduction of glutamate release in the prenatal PCP-treated mice. DL-TBOA (1 mM) was administered through a micodialysis tube into the prefrontal cortex of mice for 30 min (μl/min). After the administration, basal glutamate release (A) and K^+-evoked (100 mM) glutamate release (B) in the prefrontal cortex of prenatal SAL- or PCP-treated mice were determined. Data are expressed as the mean ± S.E.M. for 7 mice in each group. #$P < 0.05$ compared with the prenatal SAL-treated group; *$P < 0.05$, **$P < 0.01$ compared with the prenatal PCP-treated group (one-way ANOVA or repeated two-way ANOVA with Bonferroni's test). SAL, saline; PCP, phencyclidine.

synaptic hypofunctional glutamatergic neurotransmission. Given that glutamate neurotransmission plays a critical role in synaptic plasticity and long-term potentiation [31], it is possible that the hypofunctional glutamatergic neurotransmission was associated with these behavioral deficits induced by prenatal PCP treatment.

Glutamate transporters, GLAST and GLT-1, are considered to regulate glutamate transmission by rapidly clearing glutamate from extracellular fluid [11]. It is suggested that an increase in GLAST expression contributes to a decrease in extracellular glutamate release, because the regulation of extracellular glutamate levels by membrane transporters is important for terminating synaptic transmission in the brain [43]. Furthermore, increased numbers of binding sites and protein expression of glutamate transporters have been observed in the postmortem brain of schizophrenia patients [9,11,19,37,42]. Additionally, antipsychotics such as clozapine inhibites the increase in glutamate transporters [29,48]. In this study, we observed the overexpression of GLAST protein in the prenatal PCP-treated mice, although no significant change in GLT-1 expression. GLAST is produced during embryogenesis in rodents, while GLT-1 is expressed in the forebrain postnatally [47]. Therefore, prenatal exposure to PCP might lead to different patterns of GLAST and GLT-1 expression. Furthermore, we found that DL-TBOA, a potent glutamate transporter blocker, attenuated the cognitive and emotional deficits by normalizing the extracellular release of glutamate. These results suggested that the elevated expression of GLAST protein was, at least in part, responsible for the dysfunctional glutamate transmission associated with these behavioral changes in the prenatal PCP-treated mice.

GLAST proteins are expressed in glial cells of the adult brain and spinal cord [23,41]. However, in this study, we failed to detect a significant change in the expression of GFAP, a marker of glial cells. Thus, it is unlikely that the increase in GLAST expression is due to the activation of glial cells. It was reported that Ca^{2+} influx inhibits GLAST expression in astrocytes [26]. Since PCP inhibited the influx of Ca^{2+} by blocking the NMDA receptor, one possibility is that the disruption of Ca^{2+} influx into astrocytes is associated with the up-regulation of GLAST expression. Furthermore, any factors which enhance the gene transcription of GLAST or disrupt the protein's degradation might also contribute to the up-regulated expression. The precise mechanism remains to be elucidated.

The NMDA receptors are thought to control the differentiation and migration of immature neurons [4,20]. Many neurons undergo a stage when they are critically dependent on stimulation by glutamate through the NMDA receptors, and sustained deprivation of this input by NMDA receptor antagonists during development activates apoptosis [17]. Therefore, we could not exclude the possibility that a loss of glutamatergic neurons contributes to the glutamatergic hypofunction, although here, we did not observe any significant decrease in the total number of neurons in adult mice. Thus, any neurodevelopmental disturbances caused by prenatal exposure to PCP in development are potentially implicated in these behavioral and biochemical changes.

5. Conclusion

The present findings indicate that prenatal exposure to PCP leads to cognitive impairment and emotional dysfunction, which are accompanied by a disruption to pre-synaptic glutamate neurotransmission through the enhanced expression of glutamate transporters in the PFC. Since the abnormal glutamatergic release and the altered expression of glutamate transporters are involved in the pathophysiology of schizophrenia, this study provides further insights into how psychiatric illnesses develop.

Conflicts of interest

The authors state no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbr.2011.01.035.

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A Cannabinoid CB_1 Receptor Antagonist Ameliorates Impairment of Recognition Memory on Withdrawal from MDMA (Ecstasy)

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(+/−)-3,4-Methylenedioxymethamphetamine (MDMA, 'Ecstasy') abusers have persistent neuropsychiatric deficits including memory impairments after the cessation of abuse. On the other hand, cannabinoid CB_1 receptors have been implicated in learning/memory, and are highly expressed in the hippocampus, a region of the brain believed to have an important function in certain forms of learning and memory. In this study, we clarified the mechanism underlying the cognitive impairment that develops during MDMA withdrawal from the standpoint of the cannabinoid CB_1 receptors. Mice were administered MDMA (10 mg/kg, i.p.) once a day for 7 days. On the 7th day of withdrawal, a novel object recognition task was performed and the amount of cannabinoid CB_1 receptor protein was measured with western blotting. Recognition performance was impaired on the 7th day of withdrawal. This impairment was blocked by AM251, a cannabinoid CB_1 receptor antagonist, administered 30 min before the training trial or co-administered with MDMA. At this time, the level of cannabinoid CB_1 receptor protein increased significantly in the hippocampus but not the prefrontal cortex or striatum. This increase of CB_1 receptor protein in the hippocampus was also blocked by the co-administration of AM251. Furthermore, CB_1 receptor knockout mice showed no impairment of recognition performance on the withdrawal from MDMA. The impairment of recognition memory during withdrawal from MDMA may result from the activation of cannabinoid CB_1 receptors in the hippocampus.
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Keywords: MDMA; cannabinoid CB_1 receptors; recognition memory; novel object recognition task; withdrawal; hippocampus

INTRODUCTION

(+/−)-3,4-Methylenedioxymethamphetamine (MDMA) is widely abused throughout the world. MDMA abusers have neuropsychiatric deficits including memory impairments (McCardle *et al*, 2004; Wareing *et al*, 2007). Recent studies suggest that this neuropsychiatric deficit persists after the cessation of abuse (Ward *et al*, 2006). In addition, cocaine, amphetamine, or opiate abusers also show cognitive impairment during long-term drug abstinence (Ersche *et al*, 2006; Pace-Schott *et al*, 2008).

Cannabis usage causes deficits in attention, executive functioning, and short-term memory (O'Leary *et al*, 2002; Medina *et al*, 2007). We showed earlier that repeated treatment with Δ^9-tetrahydrocannabinol (Δ^9-THC), the main psychoactive ingredient of marijuana (*cannabis*), impaired delayed matching-to-sample performance even 24 h after the administration (Miyamoto *et al*, 1995). An other study has also found that Δ^9-THC impairs spatial memory (Lichtman and Martin, 1996). These reports suggest that the activation of the brain cannabinoid system impairs working memory. Furthermore, it has been revealed that the cannabinoid system is involved in drug dependence (Yamamoto and Takada, 2000; Yamamoto *et al*, 2004). A cannabinoid CB_1 receptor antagonist, SR141716A, attenuated the reinstatement of methamphetamine-seeking behavior (Anggadiredja *et al*, 2004; Hiranita *et al*, 2008). Moreover, cannabinoid CB_1 receptor knockout mice failed to establish cocaine, morphine, and ethanol self-administration (Cossu *et al*, 2001; Soria *et al*, 2005; Thanos *et al*, 2005). In a biochemical study, Gonzalez *et al* (2002) reported that chronic exposure to morphine increased levels of cannabinoid CB_1 receptor mRNA and CB_1 receptor binding in the brain. In addition, the hippocampal cannabinoid system seems to be activated during withdrawal from ethanol, because both endogenous cannabinoids and CB_1 receptors levels increased (Mitrirattanakul *et al*, 2007). Despite the close involvement of the cannabinoid system in

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the reward system, it is still unclear whether the system is involved in cognitive impairment on withdrawal from chronic exposure to drugs of abuse.

Here, we clarified the role of the cannabinoid system in cognitive impairment during withdrawal from MDMA using the novel object recognition task. We also investigated that the effect of MDMA on the level of cannabinoid CB_1 receptor protein correlated with a behavioral test.

MATERIALS AND METHODS

Animals

Male CD1 (wild-type) mice (Charles River, Yokohama, Japan) and cannabinoid CB_1 receptor knockout (CB_1 KO) mice on a CD1 background, provided by Dr Catherine Ledent (Institut de Recherches en Biologie Humaine et Moléculaire, Université Libre de Bruxelles), and weighing 30–35 g, were used in the present experiment. There were 117 wild-type mice and 29 CB_1 KO mice used in all experiment. We conducted each experiment with a small control group of 2–3 mice each and these control group data were combined together in the end to represent the control values. The animals were housed in plastic cages and kept in a regulated environment (23 ± 1°C), with a 12/12 h light–dark cycle (lights on at 7:00 am). Food and water were available *ad libitum*. Procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the Declaration of Helsinki and Nagasaki International University Publication, enacted in 2006.

Drugs

(+ /−)-3,4-Methylenedioxymethamphetamine HCl (MDMA; provided by Dr Tatsunori Iwamura, Matsuyama University) was dissolved in saline. AM251 (Sigma, St Louis, MO) was dissolved in a mixture of DMSO, Tween-80 and saline (1 : 1 : 18, respectively). All drugs were administered intraperitoneally (i.p.), and injected at a volume of 0.1 ml per 10 g of body weight. Saline or MDMA (10 mg/kg) was administered once or once daily for 7 days. AM251 (1.0 or 3.2 mg/kg) was co-administered with MDMA or singly administered 30 min before the training trial on the 7th day of withdrawal after the repeated administration of MDMA.

Behavioral Testing

Object recognition test. The object recognition test was carried out on the 1st or 7th day after the repeated administration of MDMA in separate groups. This test was performed in a Plexiglas open-field box (in cm 70 wide × 70 deep × 40 high) with black vertical walls and a floor. The objects to be discriminated were silver cone-shaped and bulb-shaped. Mice were habituated to the open field for 1 h (habituation trial). The next day, in the training trial, each mouse was placed in the open field and allowed to explore two identical objects for 10 min. The test trial was performed 3 h after the training trial. One familiar object and one novel object were placed in the same location as in the training trial. For the measurement on the 1st day of withdrawal, the habituation trial was conducted just before the last drug injection. The time spent exploring each object and the total amount of time spent exploring both objects were recorded. Exploration of an object was defined as placing the nose or a forepaw at or beyond marks put on the open-field at a distance of 1 cm from each object. A discrimination ratio was calculated as the difference in time spent exploring the novel and familiar object, expressed as a ratio of the total time spent exploring both objects in the test trial. Mice showing a total exploration time of <10 s during the training trial were excluded. The ambulation during the trial was measured with a digital tracking and computerized scoring system (LimeLight, Actimetrics). To determine whether the mice discriminated between novel and familiar objects, the discrimination ratios obtained under each condition were compared with those that would be expected by chance (ie, a ratio of 0.0), using one-sample t tests.

Biochemical Testing

Western blot analysis. Immediately after decapitation, the whole brain was removed from the skull, placed on ice, and the hippocampus, prefrontal cortex, and striatum were removed as described earlier (Yamaguchi *et al*, 2004). These tissues were immediately homogenized in a lysis buffer (10 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM EDTA, 250 mM Sucrose, 1 mM Dithiothreitol, 1% Triton X-100, 1% sodium cholate, and protease inhibitor cocktail). All samples were subjected to a BCA assay to adjust the amount of protein loaded before the sample buffer was added. The sample (10 μg) was applied to a 10% polyacrylamide gel (BioRad, Hercules, CA), and the proteins were transferred electrophoretically to nitrocellulose membranes (Bio Rad). The membrane was blocked with TBS-Tween 20 (0.1%) and 5% nonfat dry milk and incubated with the primary antibodies [anti-cannabinoid CB_1 (1 : 1000, Calbiochem, US and Canada) overnight at 4°C and anti-β-actin (1 : 2000, Sigma)] 1 h at room temperature. The antibodies were detected using HRP-conjugated anti-rabbit and anti-mouse IgG (GE Healthcare, Tokyo, Japan, 1 : 1000) secondary antibodies. The blots were detected using a chemiluminescence method (ECL system; GE Healthcare).

Data Analysis

Data are expressed as mean ± SE. A one-way ANOVA was used to compare means, and Bonferroni–Dunn tests were used for *post hoc* analysis. $p<0.05$ was accepted as statistically significant.

RESULTS

Novel Object Recognition Performance During MDMA Withdrawal

In the training trial, vehicle, single MDMA, and repeated (for 7 days) MDMA-treated mice on the 1st day after the last treatment spent 23.1 ± 1.8, 22.0 ± 5.6, and 21.3 ± 2.0 s exploring objects, respectively. Meanwhile, on the 7th day after treatment, the time spent exploring objects in the vehicle, single MDMA, and repeated MDMA-treated groups was 19.3 ± 2.7, 26.6 ± 5.2, and 21.4 ± 3.1 s, respectively.

Hence, the time spent exploring objects on the 1st day of withdrawal in the training trial in single MDMA- and repeated MDMA-treated groups was not significantly different from that of vehicle-treated group ($p = 0.06$ and $p = 0.55$ *vs* vehicle-treated group, respectively). In addition, on the 7th day of withdrawal, there was no significant difference in the time spent exploring objects among the three groups in the training trial ($p = 0.28$, $p = 0.94$ *vs* vehicle-treated group, respectively). In the test trial, the vehicle-treated mice spent significantly longer exploring the novel object (21.3 ± 2.3 s) than the familiar object (5.4 ± 1.0 s) ($F[1,28] = 30.8$, $p < 0.0001$ *vs* exploration time for the familiar object). On the 1st and 7th day after a single administration of MDMA, there was no significant change in the discrimination ratio ($p = 0.47$ and $p = 0.13$ *vs* control group on the 1st and 7th day, respectively). However, the discrimination ratio significantly decreased on the 1st and 7th days of withdrawal from repeated administration of MDMA (0.597 ± 0.071–0.26 ± 0.106%: $F[1,19] = 5.3$, $p < 0.05$ and 0.633 ± 0.048–0.048 ± 0.049%: $F[1,27] = 70.2$, $p < 0.001$ *vs* control group on the 1st and 7th days, respectively) (Figure 1). Discrimination ratios were significantly above chance in all groups except for mice on the 7th days of withdrawal from repeated MDMA ($p < 0.001$; control group on the 1st and 7th day, $p < 0.01$; on the 1st and 7th day of withdrawal from single MDMA, $p < 0.05$; on the 1st day of withdrawal from repeated MDMA). In this test trial, ambulation did not differ between the MDMA-treated and vehicle-treated groups ($p = 0.21$ and $p = 0.61$ on the 1st and 7th days, respectively). The decrease in the discrimination ratio on the 7th day of withdrawal was prevented by the co-administration of AM251, a cannabinoid CB_1 receptor antagonist, with MDMA in a dose-dependent manner (0.048 ± 0.049 to 0.592 ± 0.067%: $F[1,22] = 48.5$, $p < 0.001$ *vs* MDMA group) (Figure 2a). However, ambulation in AM251 co-administered group (4269 ± 269 cm) did not differ from ambulation in vehicle (4578 ± 354 cm) or MDMA (4334 ± 310 cm) groups ($p = 0.51$ and $p = 0.55$ *vs* vehicle and MDMA alone, respectively). On the other hand, a single administration of AM251 30 min before the training trial on the 7th day of withdrawal from repeated MDMA treatment stopped the reduction in the discrimination ratio in a dose-dependent manner (0.048 ± 0.049 to 0.661 ± 0.074%: $F[1,18] = 54.7$, $p < 0.001$ *vs* MDMA group) (Figure 2b). A single administration of AM251 on 7th day of withdrawal from repeated MDMA had no effect on ambulation ($p = 0.19$ and $p = 0.3$ *vs* vehicle and MDMA alone, respectively). Discrimination ratios were significantly above chance in mice co-administered and singly administered AM251 ($p < 0.001$). While, there was no significant difference in the time spent exploring objects and the discrimination ratio between vehicle-treated wild-type and CB_1 KO mice in the test trial. However, CB_1 KO mice did not exhibit a reduction in the discrimination ratio on both 1st and 7th day of withdrawal from repeated MDMA treatment (Figure 3). Discrimination ratios were significantly above chance in all groups of CB_1 KO mice ($p < 0.001$).

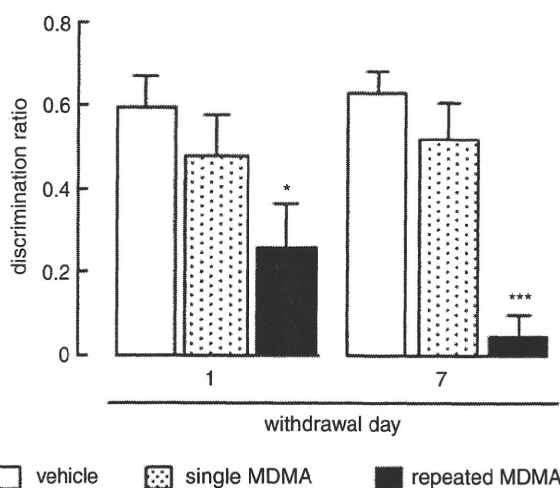

Figure 1 Novel object recognition performance in wild-type mice on the 1st or 7th day of withdrawal after single or repeated (daily for 7 day) MDMA treatment (10 mg/kg, i.p.). Each graph shows the discrimination ratio in the test trial. Data represent the mean ± SEM ($n = 5$–15). $*p < 0.05$, $***p < 0.001$ *vs* vehicle-treated mice. Vehicle includes results for mice administered saline once or repeatedly for 7 days.

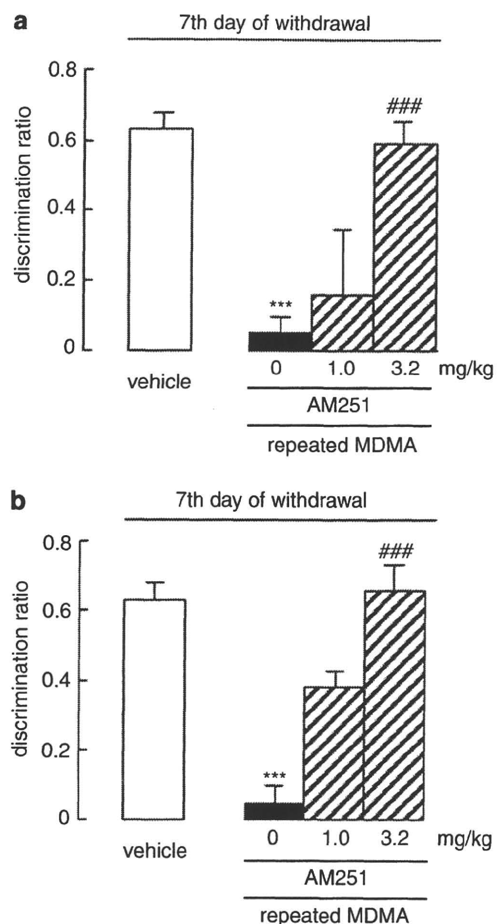

Figure 2 Effect of a cannabinoid CB_1 receptor antagonist, AM251, on cognitive impairment on the 7th day of MDMA withdrawal (10 mg/kg, i.p., daily for 7 days) in wild-type mice. (a) AM251 (1.0 or 3.2 mg/kg, i.p.) was co-administered with MDMA. Data represent the mean ± SEM ($n = 8$–15). $***p < 0.001$ *vs* vehicle-treated mice; $^{###}p < 0.001$ *vs* MDMA (10 mg/kg)-treated mice. Vehicle means results for mice administered saline. (b) AM251 (1.0 or 3.2 mg/kg, i.p.) was administered 30 min before the training trial. Data represent the mean ± SEM ($n = 5$–15). $***p < 0.001$ *vs* vehicle-treated mice; $^{###}p < 0.001$ *vs* MDMA-treated mice. Vehicle means results for mice administered saline.

Alteration of the Level of Cannabinoid CB_1 Receptor Protein During Withdrawal from Repeated MDMA Treatment

The level of cannabinoid CB_1 receptor protein did not change on the 1st day of withdrawal from repeated administration of MDMA in the hippocampus. On the 7th day of withdrawal, the level of CB_1 receptor protein in the hippocampus was significantly increased (0.48 ± 0.06–0.96 ± 0.07, $F[1,13] = 28.1$, $p < 0.001$ *vs* vehicle-treated group) (Figure 4a). This increase was prevented by co-administration of AM251 with MDMA (0.96 ± 0.07–0.60 ± 0.09, $F[1,14] = 9.6$, $p < 0.01$ *vs* MDMA-treated group) (Figure 4b). There was no significant change in the prefrontal cortex or striatum on both 1st and 7th day of withdrawal (Figure 4a).

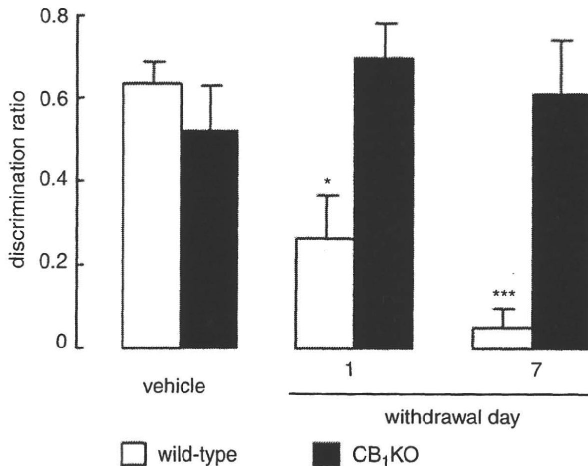

Figure 3 Comparison of novel object recognition performance in wild-type and CB_1 receptor knockout mice on the 1st and 7th day of MDMA withdrawal (10 mg/kg, i.p., daily for 7 days). Each graph shows the discrimination ratio in the test trial. Data represent the mean ± SEM ($n = 8$–15). $^*p < 0.05$, $^{***}p < 0.001$ *vs* vehicle-treated mice. Open and closed bars indicate wild-type and CB_1 receptor knockout mice, respectively. Vehicle means results for mice administered saline.

DISCUSSION

Object recognition memory in mice was impaired on withdrawal from repeated MDMA. This impairment was prevented by the co-administration of AM251, a cannabinoid CB_1 receptor antagonist, with MDMA in wild-type mice. In addition, a single treatment of AM251 on the 7th day of MDMA withdrawal ameliorated this recognition memory impairment. In CB_1 KO mice, recognition memory was not impaired on withdrawal from MDMA. These results suggest that the activation of cannabinoid CB_1 receptors is involved in the appearance of cognitive impairment on withdrawal from MDMA. In rats, it was also reported that object recognition memory is also impaired on withdrawal from MDMA similar to our present findings in mice (Morley *et al*, 2001; McGregor *et al*, 2003; Piper and Meyer, 2004). However, this is the first report to show the involvement of cannabinoid CB_1 receptors in the appearance of cognitive impairment on withdrawal from abusive drugs. Recently, Touriño *et al* (2008) indicated that CB_1 KO mice did not show the performance of MDMA self-administration. This finding suggests that the activation of CB_1 receptors is involved in the MDMA reinforcing effect.

The level of cannabinoid CB_1 receptor protein in the hippocampus was significantly increased on the 7th day of withdrawal but not on the 1st day while recognition

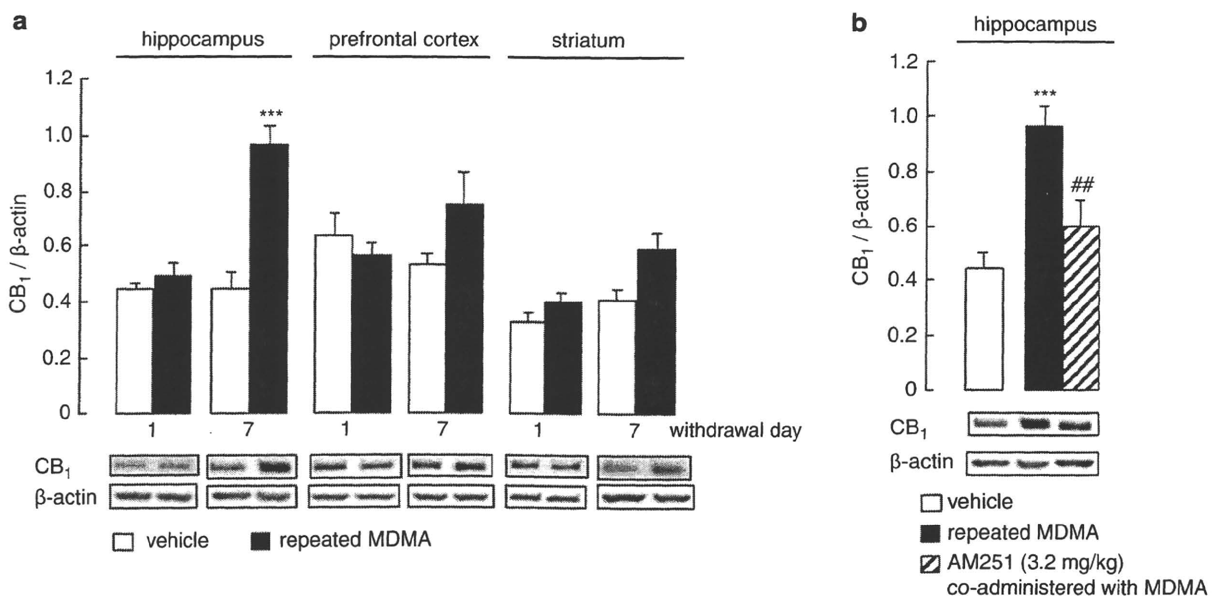

Figure 4 Effect of repeated administration of MDMA on the levels of CB_1 receptor protein in the brain in wild-type mice on the 1st and 7th day of MDMA withdrawal (10 mg/kg, i.p., daily for 7 days). (a) The levels of CB_1 receptor protein in the hippocampus, prefrontal cortex, and striatum were measured. Data represent the mean ± SEM ($n = 8$–11). (b) Effect of repeated administration of AM251 (3.2 mg/kg) with MDMA on MDMA-induced up-regulation of CB_1 receptor protein expression in the hippocampus. Open and closed bars indicate vehicle- and repeated MDMA-treated group. Data represent the mean ± SEM ($n = 5$–11). $^{***}p < 0.001$ *vs* vehicle-treated mice; $^{\#\#}p < 0.01$ *vs* MDMA-treated mice. Vehicle means results for mice administered saline.

memory was impaired on both the 1st day and 7th day of MDMA withdrawal. In this regard, cognitive impairment on the 1st day of MDMA withdrawal may be due to the increase in hippocampal endocannabinoid. Mitrirattanakul *et al* (2007) reported that the amount of endocannabinoid 2-AG in the hippocampus significantly increased in the early phase of ethanol withdrawal without any increase in CB_1 receptor expression. Our findings may be supported by this result. On the other hand, a single treatment with CB_1 receptor antagonist for MDMA withdrawal significantly ameliorated the recognition memory impairment on 7th day of MDMA withdrawal. Accordingly, CB_1 receptors may be activated with the increase in their expression at this time.

In addition, the activation of the brain cannabinoid system causes deficits in attention, executive functioning, and short-term memory (Lichtman and Martin, 1996; O'Leary *et al*, 2002; Medina *et al*, 2007). It is also demonstrated that object recognition memory was impaired by both endogenous cannabinoid Δ^9-THC and synthetic CB_1 receptor agonist WIN 55,212-2 (Schneider and Koch, 2002; Quinn *et al*, 2008). Our findings may be supported by the literatures above.

The important function of the hippocampus in cognitive functions including recognition memory is well established by earlier findings. Hippocampal damage from ibotenic acid disrupted recognition memory in the novel object recognition task and the visual paired comparison task (Clark *et al*, 2000; Broadbent *et al*, 2004). Furthermore, an intra-hippocampal WIN 55,212-2, also impaired performance of the novel object recognition task (Kosiorek *et al*, 2003; Suenaga and Ichitani, 2008).

Hampson and Deadwyler (2000) found that Δ^9-THC and WIN 55,212-2 act selectively to disrupt the encoding of events in the hippocampus during memory processing, on measuring the combined simultaneous multineuron firing rate. Recently, it was also suggested that the cannabinoids Δ^9-THC and a cannabinoid CB_1 receptor agonist CP55940 disrupted the temporal coordination of hippocampal neurons, and that this effect may correlate with memory deficits in individuals (Robbe *et al*, 2006).

Enocannabinoids are known to participate in forms of synaptic plasticity (Mackie, 2008). This phenomenon associated with endocannabinoids may help explain the mechanism by which cannabinoids impair memory. Long-term potentiation (LTP) is a form of synaptic plasticity thought to have functional roles in learning and memory processes. The importance of the hippocampal LTP in learning and memory has also been shown that hippocampal LTP is facilitated after following exposure to a novel environment but not by exposure to a familiar environment (Li *et al*, 2003). In addition, it has been shown that the cannabinoid system affects the hippocampal LTP by chronic Δ^9-THC blocking hippocampal LTP via CB_1 receptors after withdrawal (Hoffman *et al*, 2007). It has been shown that cannabinoids inhibit neurotransmitter release via presynaptic cannabinoid CB_1 receptors (Schlicker and Kathmann, 2001). Additionally, LTP disruption in the hippocampus by WIN 55,212-2 may be associated with an inhibition of hippocampal glutamatergic transmission (Misner and Sullivan, 1999). Accordingly, the appearance of cognitive impairment during MDMA withdrawal may result in dysfunction of hippocampal LTP via inhibition of glutamate release induced by an activation of CB_1 receptors. These reports strongly support our present finding that the activation of the hippocampal cannabinoid system disrupts recognition memory during MDMA withdrawal.

In conclusion, our results suggest the impairment of recognition memory during withdrawal from repeated administration of MDMA to be due to the activation of cannabinoid CB_1 receptors in the hippocampus. Moreover, these findings suggest that cannabinoid CB_1 receptor antagonists would have a therapeutic effect on cognitive dysfunction in MDMA abusers.

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DISCLOSURE

The authors declare no conflict of interest.

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A TRYPTAMINE-DERIVED CATECHOLAMINERGIC ENHANCER, (−)-1-(BENZOFURAN-2-YL)-2-PROPYLAMINOPENTANE [(−)-BPAP], ATTENUATES REINSTATEMENT OF METHAMPHETAMINE-SEEKING BEHAVIOR IN RATS

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Abstract—Relapse to drug craving is problematic in treatment for drug abuse. Evidence suggests inactivation of dopaminergic neurotransmission during drug withdrawal. Meanwhile, a tryptamine analogue, (−)-1-(benzofuran-2-yl)-2-propylaminopentane [(−)-BPAP], has been reported to enhance electrical stimulation of monoamine release. This study examined the effect of (−)-BPAP on reinstatement of methamphetamine-seeking behavior in an animal model of relapse to drug abuse. Rats were trained to i.v. self-administer methamphetamine paired with a light and tone (methamphetamine-associated cues) under a fixed-ratio 1 schedule of reinforcement for 10 days. After extinction session under saline infusions without cues, a reinstatement test under saline infusions was begun. Reinstatement induced by methamphetamine-associated cues or methamphetamine-priming injections was attenuated by repeated administration of (−)-BPAP during the extinction phase. Acute administration of (−)-BPAP on test day dose-dependently attenuated both reinstatements. Acute administration of (−)-BPAP neither reinstated methamphetamine-seeking behavior alone nor affected methamphetamine self-administration. Pretreatment with either *R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine (SCH-23390), a dopamine D_1-like receptor antagonist, or amisulpride, a dopamine D_2-like receptor antagonist, did not appreciably affected the acute effect of (−)-BPAP on both reinstatements. Co-pretreatment with the dopamine receptor antagonists failed to alter the effects of (−)-BPAP. Meanwhile, pretreatment with a dopamine D_1-like receptor agonist, (+/−)-6-chloro-7,8-dihydroxy-l-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrobromide (SKF-81297), dose-dependently attenuated reinstatement induced by the cues or methamphetamine-priming injections. In contrast to (−)-BPAP, pretreatment with SCH-23390 reversed the effects of SKF-81297. Our findings suggest activation of dopamine D_1-like receptors results in attenuation of the reinstatement of methamphetamine-seeking behavior. Additionally, our findings provide evidence to develop (−)-BPAP and dopamine D_1-like receptor agonists as an anti-relapse medication for methamphetamine abusers.
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Key words: craving, dopamine, methamphetamine, reinstatement, relapse, self-administration.

Abuse of the psychostimulant methamphetamine is a growing problem worldwide. Due to the prevalence of its abuse and lack of effective treatment in methamphetamine abuse, a clear need exists in clarifying the mechanisms underlying methamphetamine dependence. Among symptoms in drug dependence, relapse to craving is a main hurdle of treatment. In human and animal models of relapse, three different kinds of stimuli are capable of eliciting relapse: stress, cues predicting drug availability, and re-exposure to a previously self-administered drug (Shalev et al., 2002). Medications that reduce the ability of these stimuli to induce relapse may be effective in treatment of drug dependence. So far, we have reported (1) important roles of cannabinoid CB_1, nicotinic acetylcholine, and opioid receptors in reinstatement of methamphetamine-seeking behavior and (2) brain regions responsible for the reinstatement in rats (Anggadiredja et al., 2004a,b; Hiranita et al., 2004, 2006, 2008). We have reported agonists for $\alpha4\beta2$ nicotinic acetylcholine receptors and antagonists for cannabinoid CB_1 and opioid receptors as anti-relapse agents.

Evidence suggests inactivation of central dopaminergic neurotransmission during methamphetamine withdrawal. For example, clinical studies demonstrated loss of dopamine transporters in methamphetamine abusers during withdrawal (Volkow et al., 2001a,b). A post-mortem study also found reduced levels of dopamine nerve terminal markers such as dopamine, dopamine transporter, and tyrosine hydroxylase, an enzyme responsible for dopamine synthesis, in the striatum of methamphetamine abusers (Wilson et al., 1996). Recently, we demonstrated involvement of the nucleus accumbens, one of the main terminals of dopaminergic neurons, in the reinstatement of methamphetamine-seeking behavior in rats (Hiranita et al., 2006, 2008). Available reports on psychostimulants other than methamphetamine also suggest the inactivation of dopaminergic neurotransmission during drug withdrawal.

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Abbreviations: BD 1063, *N*-[2-(3, 4-dichlorophenyl) ethyl]-4-methylpiperazine; (−)-BPAP, (−)-1-(benzofuran-2-yl)-2-propylaminopentane; CHO cells, Chinese hamster ovary cells; SCH-23390, *R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; SKF-81297, (+/−)-6-chloro-7,8-dihydroxy-l-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrobromide; σ1-R, σ1 receptor.

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Thus, a decrease in basal extracellular dopamine levels in the nucleus accumbens during withdrawal from cocaine self-administration has been shown (Weiss et al., 1992). Furthermore, decreased striatal 6-fluorodopa uptake, an index of dopaminergic presynaptic activity, was associated with increased duration of cocaine withdrawal (Wu et al., 1997). In the reinstatement model, dopamine D_1-like receptor agonists have been reported to block reinstatement of cocaine-induced cocaine-seeking behavior, whereas dopamine D_2-like receptor agonists enhance this behavior in rats (Self et al., 1996, 2000). Additionally, high levels of cocaine use have been reported to be subsensitive to the ability of the dopamine D_1-like receptor agonist (+/−)-6-chloro-7,8-dihydroxy-l-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrobromide (SKF-81297) to inhibit cocaine-induced reinstatement of cocaine-seeking behavior, but supersensitive to the dopamine D_2-like receptor agonist quinpirole-induced reinstatement of cocaine seeking behavior in rats (Edwards et al., 2007). These findings suggest that inactivation of dopaminergic neurotransmission through dopamine D_1-like receptors during drug withdrawal might be pre-requisite to reinstatement of drug-seeking behavior. However, the involvement of the dopaminergic inactivation in relapse to methamphetamine-seeking behavior is not well understood.

Endogenous biogenic amines such as β-phenylethylamine and tryptamine have been found to enhance electrically stimulated release of [^{3}H] monoamines from the rat brainstem (Knoll et al., 1996). Based on the structure of these amines, (−)-1-(benzofuran-2-yl)-2-propylaminopentane [(−)-BPAP] has been synthesized and reported as a highly potent enhancer (Yoneda et al., 2001) of the electrically stimulated monoamine release (Miklya and Knoll, 2003). Dissimilar to methamphetamine (Yoneda et al., 2001) and tyramine (Shimazu et al., 2003b), however, (−)-BPAP alone does not release catecholamines. Furthermore, (−)-BPAP also has been reported to inhibit monoamine uptake (IC_{50} values: [3H] dopamine, [3H] noradrenaline, and [3H] serotonin; 42, 52, and 640 nM, respectively) (Shimazu et al., 2003b). However, none of the standard monoamine uptake inhibitors has the enhancing effect of electrically stimulated monoamine release (Miklya and Knoll, 2003). In addition, (−)-BPAP blocked tyramine-induced monoamine release from rat brain synaptosomes (Shimazu et al., 2003b). These findings suggest (−)-BPAP as an atypical monoamine uptake inhibitor. Behavioral studies demonstrated that (−)-BPAP stimulated locomotor activity in rats (Shimazu et al., 2003a) and that (−)-BPAP-induced hyperlocomotion was attenuated by pre-treatment with *R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine (SCH-23390), a dopamine D_1-like receptor antagonist (Shimazu et al., 2001). Additionally, chronic administration of (−)-BPAP ameliorated impairment of social interaction behavior following forced swimming and the ameliorating effect was blocked by pre-treatment with either SCH-23390 or sulpiride, a dopamine D_2-like receptor antagonist (Tsunekawa et al., 2008). These findings suggest that (−)-BPAP functions as the activator of dopaminergic neurotransmission. Considering reports on inhibitory action of dopamine D_1-like receptor agonists on reinstatement of cocaine-seeking behavior (Self et al., 1996, 2000), we examined whether pretreatment with (−)-BPAP would block reinstatement of methamphetamine-seeking behavior in rats in comparison with SKF-81297, a dopamine D_1-like receptor agonist.

EXPERIMENTAL PROCEDURES

Drugs

The drugs used were methamphetamine hydrochloride (Dainippon Pharmaceutical Co., LTD, Osaka, Japan), [(−)-BPAP] (gift from Fujimoto Pharmaceutical Corporation), R-(+)-SCH-23390 (a dopamine D_1-like receptor antagonist, Sigma, St. Louis, MO, USA), amisulpride (a dopamine D_2-like receptor antagonist, Sigma), and SKF-81297 (a dopamine D_1-like receptor agonist, Sigma). All of the drugs were dissolved in 0.9% saline. Methamphetamine was delivered i.v. for self-administration (0.02 mg/0.1 ml/infusion) and i.p. for priming injections (1.0 mg/kg) 30 min before test sessions. R-(+)-SCH-23390 and SKF-81297 were administered s.c. 30 and 15 min before test sessions, respectively, while (−)-BPAP and amisulpride were administered i.p. 30 min and 1 h before test sessions, respectively. Repeated administration of (−)-BPAP or saline was administered daily, i.p. 30 min after the extinction sessions for 5 days.

Subjects

At the beginning of this study, 192 male Wistar rats (250–350 g, 10 weeks old, Nippon SLC Co., Hamamatsu, Japan) were individually housed in a temperature- and humidity-controlled environment under a 12-h light/dark cycle (lights on at 7:00 AM). Food and water were available *ad libitum* in the home cage except when daily food intake was limited to 15–20 g after the catheter implantation to fix the distance between the proximal position of a catheter in the vein and the surface of the atrial auricle. Procedures for animal handling were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the Declaration of Helsinki and Faculty of Pharmaceutical Sciences, Kyushu University Publication, 1988.

Apparatus

The injector system contained a fluid swivel (Instech Lab., Inc., PA, USA) mounted on the top of each operant chamber (Neuroscience, Inc., Tokyo, Japan). One end of the swivel was connected via polyethylene tubing (Kaneka Medix Co., Osaka, Japan) encased in a protective stainless steel spring tether (Instech Laboratories, Inc., PA, USA) to the animal's catheter while the other end of the swivel was connected via polyethylene tubing to the infusion pump. The operant chambers were enclosed in ventilated, sound-attenuating cubicles and controlled by computer software (Med Associates Inc., VT, USA). The chamber's light was switched on throughout the session. Lever-pressing responses resulted in methamphetamine infusion (0.02 mg/infusion over 6 s) accompanied by light (mounted 4 cm above the lever, 200 lux) and tone (85 dB/2.9 kHz) for 26 s (methamphetamine associated-cues). The subsequent 20 s was a "time out" period during which lever presses were still recorded but not accompanied with infusions.

Surgery

Silicon catheters (Silascon; inner and outer diameter: 0.5 and 1.0 mm, respectively; Kaneka Medix Co., Osaka, Japan) were surgically implanted into the jugular vein under sodium pentobarbital (40 mg/kg i.p., Kyoritsu Seiyaku Co., Tokyo, Japan) anesthesia as

described previously (Hiranita et al., 2006). After the surgery, catheter patency was maintained by daily infusion of 0.15 ml saline solution containing heparin (30 U/ml) after each session.

Autoshaping

Autoshaping procedures to lever-press for food pellet reinforcement (45 mg; Bioserv, Holton Industries Co., Frenchtown, NJ, USA) in operant chambers under a fixed-ratio 1 schedule of reinforcement (each lever-pressing is reinforced) followed by the surgery for the self-administration training were used. Both the right and left levers were designated as active with a cue (light). The room lamp over the levers was illuminated to indicate that lever-pressing responses resulted in the immediate delivery of a food pellet and activation of the feedback tone for 0.5 s. Each session lasted for 20 min. Cessation of lever-pressing training occurred when the rat was able to deliver 30 pellets within 180 s for three consecutive sessions.

Methamphetamine self-administration, extinction, and reinstatement

Two days after surgery, rats were trained to self-administer methamphetamine under a fixed-ratio 1 schedule of reinforcement in a 2 h daily session for 10 days. Each injection was accompanied by a light and tone (methamphetamine-associated cues). During this time, inactive lever responses had no programmed consequences but were recorded. After the self-administration sessions, at least five extinction sessions (1 h) were conducted daily during which active lever responding resulted in an infusion of saline instead of methamphetamine without presentation of the methamphetamine-associated cues (and until the rats achieved the extinction criterion of less than 10 responses per session on the previously active lever). Reinstatement tests under saline infusions were carried out for 30 min from day six of extinction (or the day after rats achieved the extinction criterion) every 6 days under a fixed-ratio 1 schedule. In the cue-induced reinstatement test, immediately after the onset of the session, rats were re-exposed to the methamphetamine-associated cues and each press on the active lever resulted in presentation of the cues. In the methamphetamine-primed reinstatement test, methamphetamine (1.0 mg/kg i.p.) was injected 30 min before the test. Each response during the test session resulted in an infusion of saline but not the methamphetamine-associated cues. In the present study, subjects were mainly divided to the four following groups. The first group was used for repeated administration of (−)-BPAP (n=15) or saline (n=12) during extinction phase. The second group was used for pre-session treatment with (−)-BPAP before methamphetamine self-administration on the 10th tenth day of methamphetamine self-administration phase (n=6). The third group was used for pre-session treatment with (−)-BPAP, SKF-81297 or SCH-23390 alone after extinction phase (n=16, 18, or 18, respectively). The fourth group was used for pre-session treatment with (−)-BPAP or SKF-81297 on reinstatement test day (n=65 or 42, respectively). In the first group, subjects were further divided to sub-groups depending on treatment (eight for treatment with saline on cue presentation, six for treatment with (−)-BPAP on cue presentation, seven for treatment with saline on methamphetamine-priming injections, or six for treatment with (−)-BPAP on methamphetamine-priming injections). In the fourth group, subjects were also divided to sub-groups depending on reinstatement factors (methamphetamine-associated cues or methamphetamine-priming injections), treatment drugs, or the drug doses (see each figure legend for more detail). Each rat in the fourth group was used for two reinstatement tests first on methamphetamine-associated cues and then methamphetamine-priming injections. The sample sizes of methamphetamine-priming injections in each group were the same as or less than those of methamphetamine-associated cues because data from subjects with problems related to health or catheter issues have been removed. All of the tests were conducted with a mixed order schedule of drug doses. In order to minimize the overall number of subjects, control data were shared in the sub-groups pre-treated with (−)-BPAP or SKF-81297.

Operant task performance for food reinforcement

All subjects had sessions to lever-press for food-pellet reinforcement under a fixed-ratio 1 schedule 5 min after the self-administration or reinstatement session. Each test ended when rats had received 30 pellets or 1200 s had passed.

Data analysis

Data represent the mean±SEM of number of responses or methamphetamine infusions and were analyzed by ANOVA (a between-subjects design). The significance of effects on responding or methamphetamine infusions was assessed by ANOVA, with Dunn or Bonferroni t-test for *post-hoc* analyses as appropriate. To determine if there was a difference in effects of re-exposure to methamphetamine-associated cues or drug priming injections, a two-way (repeated administration of (−)-BPAP) and one-way (others) measures ANOVA was used. A one-way repeated measures ANOVA was used to assess the pre-session treatment effects of a single administration of (−)-BPAP on methamphetamine self-administration. A two-way repeated measures ANOVA was used to assess effects of repeated administration of (−)-BPAP on lever responses during the extinction phase. A two-way measures ANOVA was used to assess the effects of pre-session treatments of the test drugs on reinstatement of methamphetamine-seeking behavior, and food-maintained behavior (drug doses and reinstatement factors). Pearson's correlations were used to analyze correlation between total amount of methamphetamine-intake and number of responses at test sessions. Differences were considered significant at $P<0.05$.

RESULTS

On the first day of methamphetamine self-administration, the numbers of active and inactive lever responses per session were 73.8±7.3 and 70.7±38.4, respectively. During the second and third sessions, both numbers of active and inactive lever responses per session decreased from 46.5±4.0 and 17.7±10.1 to 29.1±2.1 and 4.5±0.8, respectively. During subsequent sessions, both the number of active and inactive lever responses did not alter (data not shown). Compared with lever responses, the total amount of daily methamphetamine intake was less vari-

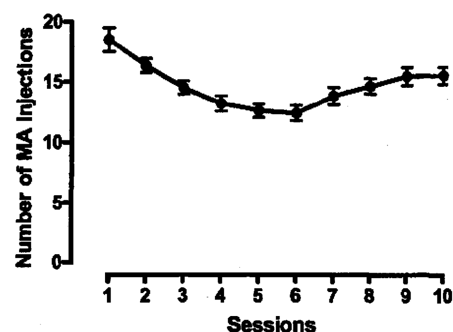

Fig. 1. Methamphetamine self-administration in rats. Rats were allowed to self-administer methamphetamine (0.02 mg/0.1 ml/injection) under a fixed ratio one schedule of reinforcement for a daily 2 h sessions for 10 days (n=180).

able (Fig. 1). On the first day of methamphetamine self-administration, the numbers of methamphetamine infusions per session were 18.5±1.0. In the subsequent sessions, the numbers of methamphetamine infusions per session decreased gradually until the sixth session (12.4±0.6). During the last four sessions, the numbers of methamphetamine infusions per session increased slightly. Total intake of methamphetamine over the course of the self-administration was 3.0±0.1 mg in rats excluding rats used for experiments on the effect of pre-session treatment with (−)-BPAP on methamphetamine self-administration. During the last three sessions of methamphetamine self-administration, both numbers of active and inactive lever responses per session were stable (23.5±1.5 and 3.1±0.1, respectively). On the first day of the extinction session, the numbers of active and inactive lever responses per session were 16.8±1.8 and 4.5±0.6, respectively. Through extinction sessions, the numbers of active lever responses per session decreased, whereas the numbers of inactive lever responses per session were relatively stable (data not shown). On the last day of extinction sessions, the numbers of active and inactive lever responses were 4.9±0.3 and 2.9±0.4, respectively in rats, excluding rats used for experiments on repeated administration of (−)-BPAP on reinstatement with methamphetamine-seeking behavior.

Effect of repeated administration of (−)-BPAP during extinction phase on reinstatement of methamphetamine-seeking behavior

During extinction sessions, the number of lever responses decreased gradually (data not shown). Two-way repeated measures ANOVA indicated significant effects of repeated administration of (−)-BPAP during extinction phase on extinction days ($F(4,100)=13.584$, $P<0.001$ and $F(4,100)=3.732$, $P=0.007$), but not drug treatment ($F(1,100)=3.265$, $P=0.083$ and $F(1,100)=0.0996$, $P=0.755$) or the interaction ($F(4,100)=0.728$, $P=0.575$ and $F(4,100)=0.0478$, $P=0.996$, active and inactive lever responses, respectively). In the saline-pretreated group as the control of (−)-BPAP administration, *post-hoc* comparison indicated significant effects on active lever responses on the first day of extinction compared with those of the third, fourth and fifth day ($t=3.269$, 3.269 and 3.897, $P=0.015$, 0.015 and 0.002, respectively). In the (−)-BPAP-pretreated group, *post-hoc* comparison indicated significant effects on active lever responses on the first day of extinction compared with those of the third, fourth and fifth day ($t=4.328$, 4.474 and 5.497, $P<0.001$, 0.001 and 0.001, respectively). In addition, *post-hoc* comparison indicated significant effects on active lever responses between saline- and (−)-BPAP groups on the first day of extinction ($t=2.036$, $P=0.044$). After extinction sessions, subsequent re-exposure to methamphetamine-associated cues and methamphetamine-priming injections increased active lever responses (Fig. 2). The increases in active lever responses in both were attenuated by repeated administration of (−)-BPAP during the extinction phase (Fig. 2). Two-way measures ANOVA indicated significant effect of (−)-BPAP treatment on the dose ($F(1,23)=122.507$, $P<0.001$), but not reinstatement factor (cues or methamphetamine-priming injections; $F(1,23)=0.133$, $P=0.179$), and significant effect on the interaction ($F(1,23)=16.203$, $P<0.001$). *Post hoc* analysis indicated significant effect of (−)-BPAP treatment on the increases in active lever responses induced by methamphetamine-associated cues ($t=5.056$, $P<0.001$) and methamphetamine-priming injections ($t=10.518$, $P<0.001$). On the other hand, methamphetamine-associated cues and methamphetamine-priming injections did not affect inactive lever responses (Fig. 2, $P\geq0.66$, both). However, two-way measures ANOVA indicated significant effect of (−)-BPAP treatment on the dose ($F(1,23)=7.562$, $P<0.011$), but not reinstatement factor ($F(1,23)=0.817$, $P=0.372$) or the interaction ($F(1,23)=0.817$, $P=0.372$). *Post hoc* analysis indicated significant effect of (−)-BPAP treatment on inactive lever responses induced by methamphetamine-associated cues ($t=2.627$, $P=0.015$) but not by methamphetamine-priming injections ($t=1.282$, $P=0.213$). In addition, the total amount of methamphetamine intake was not correlated with the increase in active lever responses induced by either methamphetamine-associated cues or methamphetamine-priming injections ($r=-0.382$ and -0.276, $P=0.350$ and 0.550, and $n=8$ and 7, respectively).

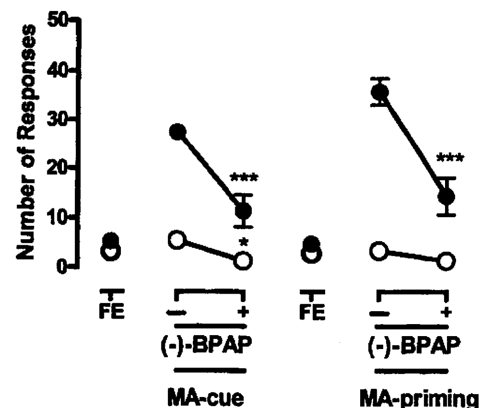

Fig. 2. Effects of repeated administration of (−)-BPAP during extinction phase (1.0 mg/kg i.p., 30 min after the extinction sessions daily for 5 days) on reinstatement of methamphetamine-seeking behavior induced by methamphetamine-associated cues or methamphetamine-priming injections. The reinstatement tests were performed 24 h after the last administration of (−)-BPAP. Closed and open circles indicate responding on active and inactive levers. * $P<0.05$, and *** $P<0.001$ versus a vehicle-treated group challenged with methamphetamine-associated cues or methamphetamine-priming injections. FE; final extinction. The order of test session was first cue presentation and then methamphetamine-priming injections. Data on FE consist of groups challenged with and without (−)-BPAP. In the test on methamphetamine-associated cues, the sample sizes of the FE session, vehicle, and (−)-BPAP pretreatment were 14, eight, and six, respectively. In the test on methamphetamine-priming injections, the sample sizes of the FE session vehicle, and (−)-BPAP pretreatment were 13, seven, and six, respectively.

Effect of acute administration of (−)-BPAP on reinstatement of methamphetamine-seeking behavior

In an experiment on controls, methamphetamine-associated cues and methamphetamine-priming injections rein-

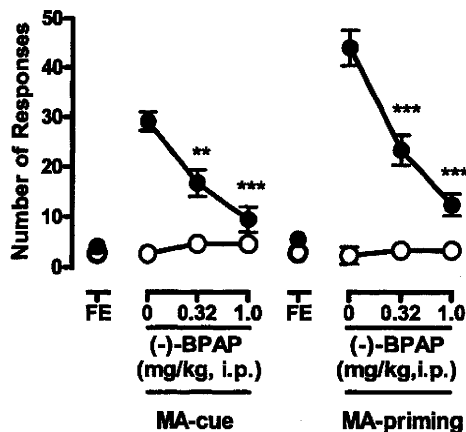

Fig. 3. Effects of acute administration of (−)-BPAP on reinstatement of methamphetamine-seeking behavior. (−)-BPAP was administered i.p. 30 min before the session. Closed and open circles indicate responding on active and inactive levers, respectively. ** $P<0.01$, and *** $P<0.001$ versus a vehicle-treated group challenged with methamphetamine-associated cues or methamphetamine-priming injections. FE; final extinction. In the test on methamphetamine-associated cues, the sample sizes of the FE session and (−)-BPAP pretreatment at the dose of 0, 0.32, and 1.0 mg/kg were 31, 10, 11, and 10, respectively. In the test on methamphetamine-priming the injection, sample sizes of the FE session and (−)-BPAP pretreatment at the dose of 0, 0.32, and 1.0 mg/kg were 23, seven, seven, and nine, respectively.

stated methamphetamine-seeking behavior (Fig. 3). One-way ANOVA indicated significant effects of methamphetamine-associated cues and methamphetamine-priming injections on active lever responses ($P<0.001$, both) but not inactive lever responses ($P=0.792$ and 0.184, respectively). *Post hoc* analysis (Dunn's method) indicated significant effects of methamphetamine-associated cues and methamphetamine-priming injections on active lever responses ($Q=5.060$, $P<0.05$, and $Q=4.208$, $P<0.05$, respectively). The total amount of methamphetamine intake was not correlated with the increase induced by either methamphetamine-associated cues or methamphetamine-priming injections ($r=0.459$ and -0.611, $P=0.182$ and 0.145, $n=10$ and 7, respectively).

Similar to the repeated administration of (−)-BPAP during the extinction phase, acute administration of (−)-BPAP (0.32 and 1.0 mg/kg i.p.) on test day also attenuated an increase in active lever responses induced by methamphetamine-associated cues and methamphetamine-priming injections (Fig. 3). Two-way measures ANOVA indicated significant effects of (−)-BPAP dose ($F(2,48)=47.455$, $P<0.001$) and reinstatement factor ($F(1,48)=14.097$, $P<0.001$), but not the interaction ($F(2,48)=2.620$, $P=0.083$) on active lever responses. The *post hoc* analysis indicated significant effect of (−)-BPAP at the dose of 0.32 and 1.0 mg/kg on active lever responses induced by methamphetamine-associated cues ($t=3.605$, $P=0.002$ and $t=5.584$, $P<0.001$) and methamphetamine-priming injections ($t=4.903$, $P<0.001$ and $t=7.969$, $P<0.001$, respectively). However, acute administration of (−)-BPAP had non-significant effects on inactive lever responses ($P\geq0.25$). Two-way measures ANOVA indicated non-significant effect of (−)-BPAP on (−)-BPAP dose, reinstatement factor, and the interaction ($F(2,48)=0.678$, $P=0.513$; $F(1,48)=0.679$, $P=0.414$; and $F(2,48)=0.074$, $P=0.929$, respectively). As indicated in Fig. 4, pre-session treatment with SCH-23390, a dopamine D_1-like receptor antagonist, (0.1, 1.0, and 10 μg/kg s.c.) did not significantly reverse the attenuating effect of pre-session treatment with (−)-BPAP on the active lever responses induced by methamphetamine-associated cues or methamphetamine-priming injections. Two-way measures ANOVA indicated non-significant effects of SCH-23390 dose, reinstatement factor, and the interaction on the (−)-BPAP-induced decrease in active lever responses ($F(3,38)=1.664$, $P=0.191$; $F(1,38)=1.845$, $P=0.182$; and $F(3,38)=2.720$, $P=0.058$, respectively). When pre-treated with (−)-BPAP, SCH-23390 did not affect inactive lever responses (Fig. 4). Two-way measures ANOVA indicated non-significant effect of the pre-session treatment with SCH-23390 on the drug dose ($F(3,38)=0.261$, $P=0.853$), reinstatement factor ($F(1,38)=2.261$, $P=0.141$), and the interaction ($F(3,38)=0.821$, $P=0.821$). Meanwhile, pretreatment with amisulpride, a dopamine D_2-like receptor antagonist, (3.2 or 10 mg/kg i.p.) failed to affect the attenuating effects of (−)-BPAP on increased active lever responses induced by methamphetamine-associated cues or methamphetamine-priming injections (Fig. 5). Two-way measures ANOVA indicated non-significant effect of amisulpride dose ($F(3,34)=2.166$, $P=0.110$) and reinstatement factor ($F(1,34)=0.576$, $P=0.453$) on the active lever responses. Pre-session treatment with amisulpride (3.2 or 10 mg/kg i.p.) failed to affect inactive lever responses (Fig. 5). Two-way measures ANOVA indicated non-significant effect of amisulpride dose ($F(3,34)=0.570$, $P=0.639$) and reinstatement factor ($F(1,34)=0.254$, $P=0.617$) on the inactive lever responses. Additionally, co-pretreatment with SCH-23390

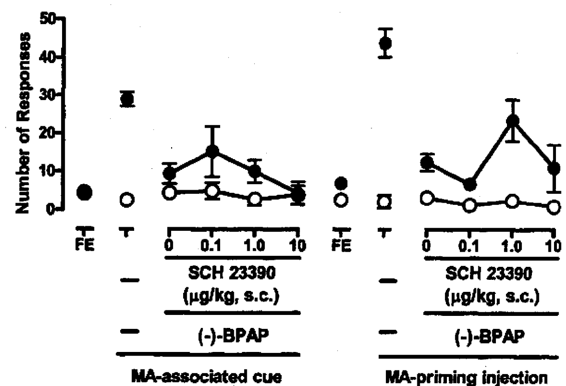

Fig. 4. Effect of acute administration of (−)-BPAP (1.0 mg/kg i.p.) with pre-session treatment with SCH-23390, a dopamine D_1-like receptor antagonist, on reinstatement of methamphetamine-seeking behavior. SCH-23390 was administered s.c. and concurrently with (−)-BPAP 30 min before the session. Closed and open circles indicate responding on active and inactive levers. FE; final extinction. In the test on methamphetamine-associated cues, the sample sizes of the FE session, vehicle alone, and (−)-BPAP co-pretreated with SCH-23390 at the dose of 0, 0.1, 1.0 and 10 mg/kg were 19, 10, five, five, five, and four, respectively. In the test on methamphetamine-priming injections, the sample sizes of the FE session, vehicle alone, and (−)-BPAP co-pretreated with SCH-23390 at the dose of 0, 0.1, 1.0 and 10 mg/kg were 10, nine, five, four, and three, respectively.

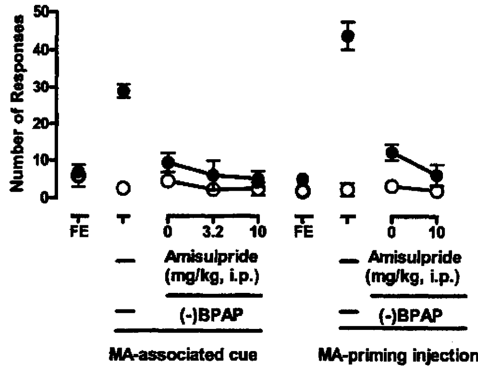

Fig. 5. Effect of acute administration of (−)-BPAP (1.0 mg/kg i.p.) with pre-session treatment of amisulpride, a dopamine D_2-like receptor antagonist, on reinstatement of methamphetamine-seeking behavior. Amisulpride and (−)-BPAP were administered i.p. 1 h and 30 min before the session, respectively. Closed and open circles indicate responding on active and inactive levers. FE; final extinction. In the test on methamphetamine-associated cues, the sample sizes of the FE session, vehicle alone, and (−)-BPAP co-pretreated with amisulpride at the dose of 0, 3.2, and 10 mg/kg were eight, 10, five, four and five, respectively. In the test on methamphetamine-priming injections, the sample sizes of the FE session, vehicle alone, and (−)-BPAP co-pretreated with amisulpride at the dose of 0, and 10 mg/kg were five, nine, five, and six, respectively.

(10 μg/kg s.c.) and amisulpride (10 mg/kg i.p.) failed to block the attenuating effect of pre-session treatment with (−)-BPAP on the increased active lever responses induced by methamphetamine-associated cues and methamphetamine-priming injections (Fig. 6). Two-way measures ANOVA indicated non-significant effect of drug treatment (F(1,24)=3.138, P=0.089), reinstatement factor (F(1,24)=0.589, P=0.450), and the interaction (F(1,24)=0.0616, P=0.806) on the active lever responses. The co-pretreatment with SCH-23390 and amisulpride also failed to alter responses on inactive levers (Fig. 6). Two-way measures ANOVA indicated non-significant effect of drug treatment (F(1,24)=0.971, P=0.334), reinstatement factor (F(1,24)=0.0277, P=0.869) and the interaction (F(1,24)=0.324, P=0.574) on the active lever responses.

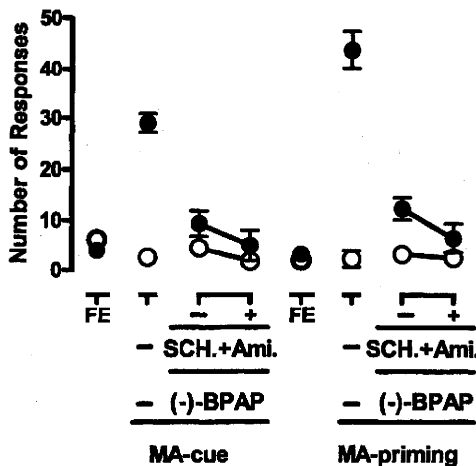

Fig. 6. Effect of pre-session treatment with (−)-BPAP (1.0 mg/kg i.p.) combined with both SCH-23390 (10 μg/kg s.c.) and amisulpride (10 mg/kg i.p.) on reinstatement of methamphetamine-seeking behavior. (−)-BPAP, and SCH-23390 were administered 30 min before the session, whereas amisulpride was administered 1 h before the session. Closed and open circles indicate responding on active and inactive levers. FE; final extinction. In the test on methamphetamine-associated cues, the sample sizes of the FE session, vehicle alone, and pre-session treatment with (−)-BPAP co-pretreated with and without combination of SCH-23390 and amisulpride were five, 10, five, and five, respectively. In the test on methamphetamine-priming injections, the sample sizes of the FE session, vehicle alone, and pre-treatment with (−)-BPAP co-pretreated with and without combination of SCH-23390 and amisulpride were four, nine, five, and four, respectively.

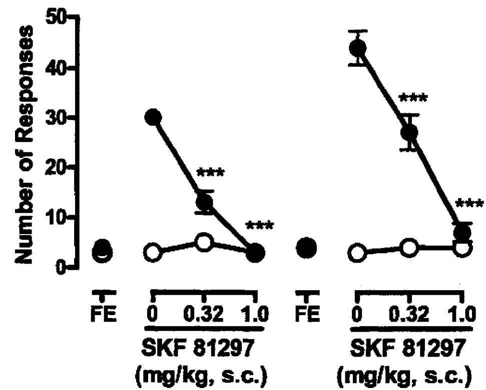

Fig. 7. Effects of pre-session treatment with SKF-81297, a dopamine D_1-like receptor agonist, on reinstatement of methamphetamine-seeking behavior. SKF-81297 was administered s.c. 15 min before the session. Closed and open circles indicate responding on active and inactive levers, respectively. *** P<0.001 versus groups challenged with methamphetamine-associated cues or methamphetamine-priming injections alone. FE; final extinction. In the test on methamphetamine-associated cues, the sample sizes of the FE session and SKF-81297 pretreatment at the dose of 0, 0.32, and 1.0 mg/kg were 24, eight, eight, and eight, respectively. In the test on methamphetamine-priming injections, the sample sizes of the FE session and SKF-81297 pretreatment at the dose of 0, 0.32, and 1.0 mg/kg were 24, eight, eight, and eight, respectively.

Effect of pre-session treatment with SKF-81297, a dopamine D_1-like receptor agonist, on reinstatement of methamphetamine-seeking behavior

In an experiment on controls, methamphetamine-associated cues and methamphetamine-priming injections reinstated methamphetamine-seeking behavior (Fig. 7). One-way ANOVA indicated significant effects of methamphetamine-associated cues on active lever responses (F(1,30)=579.836, P<0.001) and methamphetamine-priming injections on active and inactive lever responses (P<0.001 and =0.041). *Post hoc* analysis indicated significant effects of methamphetamine-associated cues on active lever responses (t=24.080, P<0.001, Bonferroni t-test) and methamphetamine-priming injections on active and inactive lever responses (Q=4.178, P<0.05, and Q=2.024, P<0.05, respectively, Dunn's Method). Total amount of methamphetamine intake was not correlated with the increase induced by either methamphetamine-associated cues or methamphetamine-priming injections (r=−0.681 and −0.401, P=0.0628 and 0.325, n=8 and 8, respectively).

Pre-session treatment with SKF-81297 (0.32–1.0 mg/kg s.c.) dose-dependently attenuated an increase in active lever responses induced by methamphetamine-associated cues and methamphetamine-priming injections (Fig. 7). Two-way measures ANOVA indicated significant effects of SKF-81297 dose ($F(2,47)=88.858$, $P<0.001$), reinstatement factor ($F(1,42)=30.898$, $P<0.001$), but not the interaction ($F(2,42)=3.163$, $P=0.053$) on increase in active lever responses induced by methamphetamine-associated cues and methamphetamine-priming injections. The *post hoc* analysis indicated significant effect of SKF-81297 at the dose of 0.32 and 1.0 mg/kg on an increase in active lever responses induced by methamphetamine-associated cues ($t=5.001$, $P<0.001$, and $t=7.911$, $P<0.001$) and methamphetamine-priming injections ($t=4.889$, $P<0.001$ and $t=10.934$, $P<0.001$, respectively). The pre-session treatment with SKF-81297 failed to affect responses on inactive levers (Fig. 7). Two-way measures ANOVA indicated non-significant effect of SKF-81297 dose ($F(2,47)=1.719$, $P=0.192$), reinstatement factor ($F(1,47)=0.0559$, $P=0.814$) and the interaction ($F(2,47)=0.753$, $P=0.477$) on the inactive lever responses. Pre-session treatment with SCH-23390 (0.1 and 1.0 μg/kg s.c.) dose-dependently blocked the attenuating effect of pre-session treatment SKF-81297 (1.0 mg/kg i.p.) on methamphetamine-associated cues as well as methamphetamine-priming injections (Fig. 8). Two-way measures ANOVA indicated significant effects of SCH-23390 dose ($F(2,30)=91.427$, $P<0.001$), reinstatement factor ($F(1,30)=8.728$, $P<0.001$), and the interaction ($F(2,30)=3.978$, $P=0.0029$) on the active lever responses induced by methamphetamine-associated cues and methamphetamine-priming injections. The *post hoc* analysis indicated significant effect of SCH-23390 at the dose of 0.1 and 1.0 μg/kg on the active lever responses induced by methamphetamine-associated cues ($t=3.049$, $P=0.014$, and $t=7.546$, $P<0.001$) and methamphetamine-priming injections ($t=5.272$, $P<0.001$, and $t=11.526$, $P<0.001$, respectively). However, SCH-23390 did not affect inactive lever responses (Fig. 8). Two-way measures ANOVA indicated significant effects of SCH-23390 dose ($F(2,30)=0.935$, $P=0.404$), reinstatement factor ($F(1,30)=1.555$, $P=0.222$), and the interaction ($F(2,30)=0.004\ 31$, $P=0.996$) on the inactive lever responses.

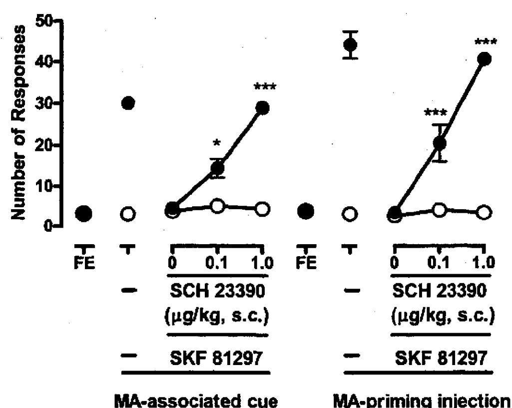

Fig. 8. Effects of pre-session treatment with SKF-81297 (1.0 mg/kg s.c.) combined with SCH-23390 (0, 0.1 and 1.0 μg/kg s.c.) on reinstatement of methamphetamine-seeking behavior. SKF-81297 and SCH-23390 were administered 15 and 30 min before the session. Closed and open circles indicate responding on active and inactive levers. * $P<0.05$ and *** $P<0.001$ versus SKF-81297-pretreated groups challenged with methamphetamine-associated cues or methamphetamine-priming injections alone. FE; final extinction. In the test on methamphetamine-associated cues, the sample sizes of the FE session, vehicle alone, and SKF-81297 co-pretreated with SCH-23390 at the dose of 0, 0.1, and 1.0 mg/kg were 26, eight, six, six, and six, respectively. In the test on methamphetamine-priming injections, the sample sizes of the FE session, vehicle alone, and (−)-BPAP co-pretreated with SKF-81297 at the dose of 0, 0.1, and 1.0 mg/kg were 26, eight, six, six, and six, respectively.

Effects of administration of (−)-BPAP, SKF-81297 or SCH-23390 alone under extinction condition in methamphetamine self-administered rats after extinction sessions

(−)-BPAP alone (1.0–3.2 mg/kg i.p.) failed to affect lever responses on active and inactive levers (Fig. 9A). At the higher dose (10 mg/kg i.p.), (−)-BPAP increased in both active and inactive lever responses (Fig. 9A). One-way ANOVA and the subsequent *post hoc* analysis with Dunn's method indicated significant effect of (−)-BPAP at the dose of 10 mg/kg on active lever responses ($Q=3.598$, $P<0.05$). In addition, one-way ANOVA indicated significant effect of (−)-BPAP at 10 mg/kg on inactive lever responses ($P=0.038$); however, the subsequent *post hoc* analysis with Dunn's method indicated non-significant effect of (−)-BPAP at 10 mg/kg on inactive lever responses ($Q=2.071$, $P\geq0.05$) compared with that at 0 mg/kg. Total amount of methamphetamine intake was correlated with responses on neither active nor inactive responses ($r=0.4598$ and 0.2169, $P=0.3589$ and 0.6797, respectively). On the other hand, neither SKF-81297 (0.032–1.0 mg/kg s.c., Fig. 9B) nor SCH-23390 (1–100 μg/kg s.c., Fig. 9C) affected responses on active and inactive levers. One-way ANOVA indicated non-significant effect of SKF-81297 and SCH-23390 on active ($F(3,32)=0.139$, $P=0.936$ and $F(3,32)=0.077$, $P=0.972$) and inactive ($F(3,32)=0.615$, $P=0.611$ and $F(3,32)=0.171$, $P=0.915$, respectively) lever responses.

Effects of pre-session treatment with (−)-BPAP on methamphetamine self-administration

On day 9 of methamphetamine self-administration, the number of methamphetamine infusions was 16.5±2.2 (Fig. 10). On day 10, pre-session treatment with (−)-BPAP (1.0 mg/kg i.p.) did not affect the number of methamphetamine infusions (Fig. 10, 16.8±1.6). Twenty four hours after the (−)-BPAP pretreatment, the number of methamphetamine infusions (Fig. 10, 16.8±2.9) was unchanged from those on the previous 2 days. Similar to the number of methamphetamine infusions, pre-session treatment with (−)-BPAP failed to affect responses on active and inactive levers during these 3 days (date not shown). One-way repeated measures ANOVA indicated non-significant effects of pre-session treatment with (−)-BPAP on total amount of methamphetamine intake ($F(5,10)=2.006$,