

Withdrawal from Fixed-Dose Injection of Methamphetamine Decreases Cerebral Levels of 3-Methoxy-4-hydroxyphenylglycol and Induces the Expression of Anxiety-Related Behavior in Mice

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Abstract A variety of drug treatment regimens have been proposed to model the dysphoric state observed during methamphetamine (METH) withdrawal in rats, but little has been established in experiments using mice. In male ICR mice, a fixed-dose injection regimen of METH (1.0 or 2.5 mg/kg, i.p., twice daily for 10 consecutive days) induced a significant decrease in the time spent in open arms in an elevated plus maze after 5 days of drug abstinence. Under an escalating-dose injection regimen (0.2–2.0 mg/kg, i.p., 3 times daily for 4 days, total: 15 mg/kg/animal) or continuous subcutaneous administration with osmotic mini-pumps (15 or 76 mg/kg of METH for 2 weeks), no significant behavioral change was observed after 5 days of drug abstinence, compared with control animals. Reduced gains in body weight were observed during repeated treatment with METH in the fixed-dose injection and mini-pump treatment regimens, but not the escalating-dose injection regimen. HPLC analysis revealed significant decreases in the level

of cerebral 3-methoxy-4-hydroxyphenylglycol, a norepinephrine metabolite, and norepinephrine turnover, which may be attributed to the expression of anxiety-related behavior in the elevated plus maze. These observations suggest that the mice treated with a fixed-dose of METH may model the anxiety-related behavior observed in the dysphoric state induced by METH withdrawal in humans.

Keywords Amphetamine withdrawal · Anxiety-related behavior · Norepinephrine · Methamphetamine · Animal model

Introduction

Amphetamine-type drugs (AMPH) cause mood enhancement, which is often followed by the opposite state of mood when the drugs are withdrawn. The mood state induced by AMPH withdrawal, dysphoria, has been recognized to involve depressive syndromes including anhedonia, depression, anxiety, agitation, and social inhibition [1, 2]. The putative molecular basis of the expression of withdrawal syndromes such as depression includes changes in monoaminergic neurotransmission in the brain (especially that of the serotonin (5-hydroxytryptamine, 5-HT) and norepinephrine (NE) systems), based on preclinical and clinical observations using antidepressants that inhibit the activity of 5-HT and/or NE transporters [3–5]. Depression-related physiological symptoms including locomotor inactivity and fatigue are marked in the first week of methamphetamine (METH) abstinence among METH-dependent individuals [6]. It was reported in 2000 that 49.4% of self-reported METH-dependent arrestees in a United States sample had suicidal thoughts [7]. Therefore, medication to treat the dysphoria expressed during drug

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withdrawal is important for AMPH abusers to prevent adverse outcomes, including impulsive self-injurious behavior or acts that are committed with unconscious or uncontrolled suicidal intention. Currently, no effective treatment for AMPH abuse exists [8, 9].

To achieve success in AMPH withdrawal pharmacotherapy, it is important to investigate the regulation of monoaminergic neurotransmission, which is correlated with the expression of the negative symptoms of AMPH withdrawal. It has proven very difficult to model all the negative symptoms of humans AMPH withdrawal using one animal model [10–12]. Of the dysphoric symptoms seen during AMPH withdrawal, anxiety-related behavior has been investigated least because of a lack of valid and reliable animal models (for review see ref. [13]). In addition, it is well recognized that the design of the drug treatment program (dose, route, number of injections, duration between injections, and time of behavioral assessment relative to drug exposure) is important in determining the subsequent neurochemical [13] and behavioral effects in rodents [14–17]. For instance, mice administered with a fixed-dose of AMPH (5 mg/kg, i.p. twice daily for 10 consecutive days) followed by 1-day drug withdrawal showed a significant anhedonic response and depression-like behavior in the intracranial self-stimulation test and the forced swim test, respectively, but no anxiety-related behavior in the shuttle escape learning test, fear-related behavior in the acoustic startle response test, or decreased locomotor activity [10]. Depression-like behavior was also observed in mice subjected to the tail suspension test after being administered with AMPH released from implanted mini-pumps at a rate of 5 or 10 mg/kg/day for 1 week followed by 1-day AMPH withdrawal [18].

To investigate the association between the neurochemical and behavioral aspects of anxiety in AMPH withdrawal, it is necessary to induce significant changes in the behavioral parameters relevant to the symptoms of AMPH withdrawal in animal models. In the present study, we set the postdrug period at 5 days because the disrupted acoustic startle response (i.e. fear-related behavior, which may be associated with anxiety) is observed on days 4–55 of drug withdrawal in rats [19, 20], which is longer than the period used in the studies that did not detect any fear-related or anxiety-related responses in mice [10]. Mice were used in this study because rats have been preferentially used in AMPH withdrawal experiments in the literature, but little evidence regarding the expression of anxiety during AMPH withdrawal has been produced using mice. In addition, three different treatment regimens (fixed-dose injections, escalating-dose injections, and continuous infusion from implanted mini-pumps) were tested to examine whether anxiety-related behavior was induced. HPLC analyses were also performed in order to examine the involvement of

monoaminergic neurotransmission in the expression of anxiety during AMPH withdrawal. In this article, AMPH and METH (both *d*-isomers) are treated as drugs with equivalent behavioral properties when the same dose of drug is administered to rodents, since the stimulating effects of AMPH and METH on caudate dopamine (DA) release are similar and cause similar behavioral responses including stereotypical licking and biting [21, 22].

Experimental Procedures

Subjects

Male ICR mice ($n = 210$, 11–13 weeks old; Japan SLC, Shizuoka, Japan) were housed in groups of eight (cage size, $37 \times 22 \times 15$ cm) in a temperature ($22 \pm 2^\circ\text{C}$) and humidity ($50 \pm 10\%$) controlled environment under a 12 h light/dark cycle (lights on at 0700 hours) with food and water available ad libitum except during testing. Animal handling and care were conducted according to the *Guide for the Care and Use of Laboratory Animals* (7th edition, Institute of Laboratory Animal Resources-National Research Council, National Academy Press 1996), and all experiments were reviewed and approved by our Institutional Animal Research Committee. The mice were used only once (body weight on day 1: 38–53 g) after at least one-week habituation in the facility.

Reagents

METH hydrochloride was purchased from Dainippon Sumitomo Pharma Co., Ltd (Osaka, Japan). All standard reagents for HPLC were from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals used were of the highest purity commercially available.

METH Administration and Drug-Free Periods

METH hydrochloride was dissolved in sterile saline and administered intraperitoneally (i.p.; injection volume: 0.1 ml/10 g) under the fixed-dose and escalating-dose injection regimens (Table 1). The doses of METH refer to the weight of salt. Following the habituation period, groups of animals were exposed to drug administration. For fixed-dose injection, the animals received two injections daily (0900 and 2100 hours) of saline or doses of METH (1.0 or 2.5 mg/kg) for 10 consecutive days (20 injections in total; $n = 30$ per group). For the escalating-dose injections, the animals received three injections (time: 0900, 1500, and 2100 hours) each day of saline or doses of METH. The injection schedule increased from 0.2 to 2.0 mg/kg during the first 10 injections and was then sustained at 2.0 mg/kg

Table 1 Schedule of methamphetamine (METH) administration

| Treatment regimen | Administration | Drug-free period | Test | Total METH administered (mg/kg animal) |
|--|------------------------------------|------------------|--------|---|
| Fixed-dose injection regimen (30) | Days 1–10 | Days 11–15 | Day 16 | 20 (1.0 mg/kg/inj) or 50 (2.5 mg/kg/inj) |
| | Inj × 2/day (09:00/21:00) | No inj | Test | |
| Escalating-dose injection regimen (20) | Days 1–4 | Days 5–9 | Day 10 | 15 (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.0, and 2.0 mg/kg/inj) |
| | Inj × 3/day (09:00/15:00/21:00) | No inj | Test | |
| Osmotic mini-pump (20) | Days 1–14 | Days 15–19 | Day 20 | 15 (1.1 mg/kg/day) or 76 (5.4 mg/kg/day) |
| | s.c. pump | No inj | Test | |

Parentheses indicate the number of subjects used per group. Sterile saline was used as a vehicle

Inj injection, *s.c. pump* METH-containing osmotic mini-pump implanted subcutaneously, *test* elevated plus maze and tail suspension tests

for the following 2 injections (i.e. 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.0, and 2.0 mg/kg) for 4 consecutive days (12 injections in total; $n = 20$ per group). Control animals for both the fixed-dose and escalating-dose groups were injected with the identical volume of saline at the same time as the mice in the METH groups. After the final injection, the animals received no injections and stayed in their home cages for 5 days (drug-free period, or *drug withdrawal*).

For osmotic mini-pump delivery of METH, the mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and an osmotic mini-pump (Alzet model 1002, Durect Co., Cupertino, CA, USA) was inserted subcutaneously (into the back of the animal parallel to the spine) with the flow-moderator directed posteriorly (Table 1). The pumps were filled with either saline or METH solution ($n = 20$ per group). The concentration of METH was adjusted according to the weight of the animal and the pumping rate to deliver a dose of 1.1 or 5.4 mg/kg/day. The wound was stapled with stainless steel clips, and an antibacterial preparation was applied to the incision area. On day 15, the pumps were surgically removed under pentobarbital anesthesia. The wounds were reclipped and treated with the antibacterial preparation. One group of mice ($n = 20$) underwent a sham operation that did not involve the insertion of the mini-pumps. After pump removal, the mice were allowed to recover in their home cages for 5 days with no treatment.

Elevated Plus Maze

The elevated plus maze test was carried out as described previously [23]. The apparatus was made of four acrylic arms (two enclosed arms of $30 \times 5 \times 15$ cm that formed a cross shape with the two open arms of $30 \times 5 \times 0.5$ cm, which were linked by a common central platform (neutral zone), 5×5 cm). The maze was raised 50 cm above the floor and illuminated by a dim light above the apparatus

(10–12 lux). The mice were placed on the central platform facing an open arm and allowed to explore the apparatus for 5 min. The placing of all four paws on an arm qualified as an entry, and the cumulative time spent in each arm type or neutral zone after entry was measured. The maze was wiped clean between trials with 10% ethanol. The animals were only exposed to the maze once. All experimental testing sessions were conducted between 1200 and 1700 hours.

Tail Suspension Test

After spending more than 1 h after the elevated plus maze test in their home cages, the mice were immediately suspended by the tail from a horizontal ring (distance from the floor = 25 cm) in a white acrylic box ($25 \times 25 \times 30$ cm) using adhesive tape affixed 2 cm from the tip of the tail. The testing room was brightly lit (130 lux outside; 65 lux inside the box). A 6-min test session was employed, and the data were recorded with a Supermex[®] sensor (infrared pyroelectric sensors developed by Muromachi Kikai Co.; ref. [24]) positioned 6 cm in front of the body of the animal using the CompACT FSS software for Windows version 2.40 (Muromachi Kikai Co.) installed on a PC computer (OS: Windows XP[®]). The behavioral parameter measured was the duration of immobility, which was defined as the time when the animal was not engaged in escape-like behavior. The animals were only exposed to the test once. All experimental testing sessions were conducted between 1300 and 1830 hours.

Brain Dissection and HPLC

Ten mice per group were randomly selected immediately after the tail suspension test and sacrificed by cervical dislocation, and their brains were removed. In our preliminary experiments, the tissue levels of monoamines were not significantly different between mice exposed to

and not exposed to behavioral tests after a period of METH abstinence (data not shown). The striatum, nucleus accumbens, and cerebral cortices were isolated, weighed, and frozen in liquid nitrogen until they were assayed by HPLC. The brain regions were selected since withdrawal after repeated injections of amphetamines such as METH and *d*-amphetamine significantly affect the levels of monoamine levels in these areas [13].

HPLC analysis was performed according to a previously reported method [24, 25]. Each frozen brain sample was homogenized with a Teflon/glass homogenizer in 10 volumes (w/v) of ice-cold 0.1 N perchloric acid with 30 μ M Na₂EDTA containing 3,4-dihydroxybenzylamine hydrobromide and isoproterenol as internal standards for catechols and for indoles, respectively. The homogenates were centrifuged at 10,000 \times *g* for 10 min at 4°C, and the supernatants were filtered through a 0.20- μ m membrane filter (Millipore Co., Bedford, MA, USA). The filtrates (10 μ l) were injected directly into an HPLC system (system controller: model SCL-10A; auto-injector: model SIL-10A; pump: model LC-10AD; Shimadzu Co., Kyoto, Japan) equipped with a reversed-phase ODS-column (MCM column 150; 4.6 \times 150 mm; MC Medical, Inc., Osaka, Japan) and an electrochemical detector (Coulchem Model 5100A, ESA, Inc., Chelmsford, MA, USA). The column temperature was maintained at 24°C, and the detector potentials were set at +0.40, +0.15, and -0.35 V on the conditioning cell, and Detectors 1 and 2, respectively. The mobile phase was a 1000:35.2:85.8 (v/v) mixture of a buffer (50 mM Na₂HPO₄, 50 mM citric acid, 4.4 mM 1-heptanesulfonic acid, and 0.1 mM Na₂EDTA,

pH 3.0), acetonitrile and methanol, and the flow rate was set at 0.9 ml/min.

Statistics

Values are presented as the mean \pm the standard error of the means (SEM). Statistical analysis was performed using mixed factor analysis of variance (ANOVA) with or without repeated measures followed by the Bonferroni/Dunn or Student's *t* test (Statview 5.0 for Apple Macintosh, SAS Institute, Inc., Cary, NC, USA). Statistical significance was set at $P < 0.05$.

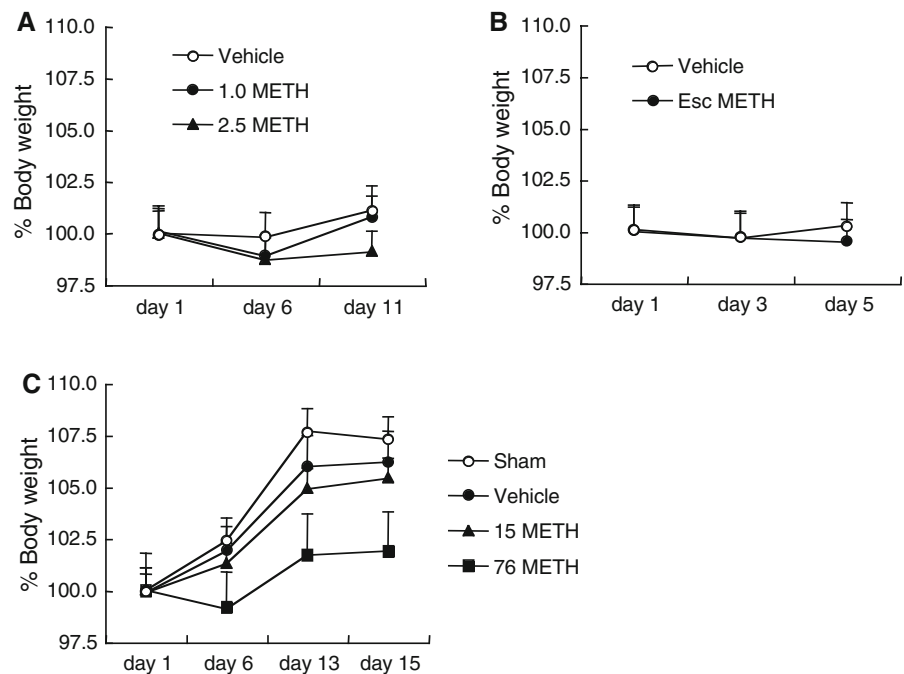
Results

Body Weight

The mice that received METH via fixed-dose or continuous s.c. infusion had a tendency to have a lower body weight than their control group throughout the experiment, but the differences were not statistically significant.

Regarding the fixed-dose injection regimen (Fig. 1a), two-way repeated-measures ANOVA (Treatment \times Time) showed a significant main effect of Time ($F(2,261) = 10.605$, $P < 0.0001$) but not Treatment ($F(2,261) = 0.766$, $P = 0.4680$). The ANOVA with repeated-measures also indicated significant Treatment \times Time interactions ($F(4,261) = 3.177$, $P < 0.05$). Regarding the escalating-dose injection regimen (Fig. 1b), two-way repeated-measures ANOVA (Treatment \times Time) showed no significant

Fig. 1 Changes in the body weights (100% = animal weight on day 1) of mice under various drug treatment regimens. **a** Fixed-dose injection regimen ($n = 30$ per group); **b** escalating-dose injection regimen ($n = 20$ per group); **c** osmotic mini-pump delivery of METH ($n = 20$ per group). Values are shown as the means \pm SEM. * $P < 0.05$, significant difference between the groups indicated (repeated-measures two-way ANOVA followed by Bonferroni/Dunn test)



main effects of Time or Treatment ($F(2,114) = 0.531$, $P = 0.5903$ and $F(1,114) = 0.027$, $P = 0.8708$), and ANOVA with repeated-measures indicated no significant Treatment \times Time interactions ($F(2,114) = 1.186$, $P = 0.3111$). Regarding treatment with the osmotic mini-pump (Fig. 1c), two-way repeated-measures ANOVA (Treatment \times Time) showed a significant main effect of Time ($F(3,304) = 5.379$, $P < 0.0001$) but not Treatment ($F(3,304) = 1.496$, $P = 0.2224$). ANOVA with repeated-measures also indicated significant Treatment \times Time interactions ($F(9,304) = 5.379$, $P < 0.0001$).

Tissue Levels of Monoamines and Their Metabolites

To investigate the relationship between METH treatment and alterations in the monoaminergic neuronal systems, the tissue contents of monoamines and their metabolites in the striatum + nucleus accumbens (NAc) and the cerebral cortex were measured by HPLC.

Fixed-Dose Injection Regimen

Table 2 shows the tissue contents of monoamines and their metabolites in mice treated with METH under the fixed-dose injection regimen. In the striatum + NAc, the DA, 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine (3-MT), and homovanillic acid (HVA) contents were increased significantly in the METH (1.0 or 2.5 mg/kg doses)-treated mice compared with the vehicle-treated mice. The 5-HT content was significantly increased in the 1.0 mg/kg METH-treated mice, but not in the 2.5 mg/kg METH-treated mice. The 3-methoxy-4-hydroxyphenylglycol (MHPG) content was significantly decreased in the 2.5 mg/kg dose of

METH-treated mice. The NE and 5-HIAA contents did not differ among the treatment groups. In the cerebral cortex, the MHPG content was significantly decreased in the METH (1.0 and 2.5 mg/kg doses)-treated mice in a dose-dependent manner. The contents of the remaining monoamines and their metabolites in the cerebral cortex did not differ among the treatment groups.

One-way ANOVA (Treatment) applied to the striatum + NAc showed significant main effects ($F(2,27) = 4.769$, $P < 0.05$; $F(2,27) = 8.878$, $P < 0.0001$; $F(2,27) = 15.298$, $P < 0.0001$; $F(2,27) = 30.063$, $P < 0.0001$; $F(2,27) = 21.072$, $P < 0.0001$; and $F(2,27) = 4.407$, $P < 0.05$) on MHPG, DA, DOPAC, 3-MT, HVA, and 5-HT, respectively. However, ANOVA showed no significant main effects of Treatment on NE or 5-HIAA ($F(2,27) = 0.932$, $P = 0.4061$; and $F(2,27) = 2.160$, $P = 0.1349$, respectively). One-way ANOVA (Treatment) applied to the cerebral cortex showed a significant main effect on MHPG ($F(2,27) = 35.817$, $P < 0.0001$), while ANOVA indicated no significant main effects on the remaining monoamines or their metabolites ($F(2,27) = 0.469$, $P = 0.6304$; $F(2,27) = 0.450$, $P = 0.6424$; $F(2,27) = 0.282$, $P = 0.7563$; $F(2,27) = 0.125$, $P = 0.8828$; $F(2,27) = 0.143$, $P = 0.8671$; $F(2,27) = 2.891$, $P = 0.0728$; and $F(2,27) = 0.737$, $P = 0.4879$ for NE, DA, DOPAC, 3-MT, HVA, 5-HT, or 5-HIAA, respectively).

Escalating-Dose Injection Regimen

Table 3 shows the tissue contents of monoamines and their metabolites in mice treated with METH under the escalating-dose injection regimen. In the striatum + NAc, the contents of NE, DA, DOPAC, HVA, 5-HT, and 5-HIAA

Table 2 Tissue contents of monoamines and their metabolites in the striatum + nucleus accumbens (Str/NAc) and cerebral cortex (Cx) of mice treated with methamphetamine (METH) under a fixed-dose injection regimen

| Treatment | NE | MHPG | DA | DOPAC | 3-MT | HVA | 5-HT | 5-HIAA |
|----------------|-----------------|-------------------------------|------------------|------------------|------------------|------------------|------------------|-----------------|
| Vehicle | | | | | | | | |
| Str/NAc | 1.63 \pm 0.07 | 4.64 \pm 0.29 | 11.1 \pm 0.49 | 0.85 \pm 0.03 | 0.26 \pm 0.04 | 0.27 \pm 0.03 | 0.90 \pm 0.04 | 0.46 \pm 0.01 |
| Cx | 1.35 \pm 0.06 | 2.40 \pm 0.08 | 3.36 \pm 0.42 | 0.36 \pm 0.02 | 0.13 \pm 0.02 | 0.13 \pm 0.02 | 0.65 \pm 0.02 | 0.23 \pm 0.01 |
| 1 mg/kg METH | | | | | | | | |
| Str/NAc | 1.81 \pm 0.12 | 3.57 \pm 0.16 | 13.7 \pm 0.58* | 1.05 \pm 0.05* | 0.60 \pm 0.03* | 0.50 \pm 0.03* | 1.07 \pm 0.04* | 0.52 \pm 0.02 |
| Cx | 1.37 \pm 0.06 | 1.95 \pm 0.10* | 2.95 \pm 0.41 | 0.34 \pm 0.03 | 0.14 \pm 0.02 | 0.12 \pm 0.02 | 0.60 \pm 0.02 | 0.24 \pm 0.01 |
| 2.5 mg/kg METH | | | | | | | | |
| Str/NAc | 1.78 \pm 0.11 | 2.93 \pm 0.15* | 14.3 \pm 0.74* | 1.19 \pm 0.05* | 0.65 \pm 0.05* | 0.49 \pm 0.03* | 1.02 \pm 0.04 | 0.49 \pm 0.02 |
| Cx | 1.43 \pm 0.08 | 1.39 \pm 0.07* [†] | 2.90 \pm 0.42 | 0.35 \pm 0.03 | 0.13 \pm 0.01 | 0.14 \pm 0.02 | 0.60 \pm 0.02 | 0.23 \pm 0.01 |

The brain regions of each mouse group were dissected just after the end of tail suspension test on day 16. Values are expressed as nanograms per milligram of wet tissues (mean \pm SEM; $n = 30$ for each group)

DA dopamine, DOPAC 3,4-dihydroxyphenylacetic acid, 5-HIAA 5-hydroxyindolacetic acid, 5-HT 5-hydroxytryptamine (serotonin), HVA homovanillic acid, MHPG 3-methoxy-4-hydroxyphenylglycol, 3-MT 3-methoxytyramine, NE norepinephrine

* $P < 0.05$, compared with Vehicle (Bonferroni/Dunn test after one-way ANOVA)

[†] $P < 0.05$, compared with 1 mg/kg METH (Bonferroni/Dunn test after one-way ANOVA)

Table 3 Tissue contents of monoamines and their metabolites in the striatum + nucleus accumbens (Str/NAc) and cerebral cortex (Cx) of mice treated with methamphetamine (METH) under an escalating-dose injection regimen

| Treatment | NE | MHPG | DA | DOPAC | 3-MT | HVA | 5-HT | 5-HIAA |
|-----------|--------------|--------------|--------------|--------------|-------------|--------------|--------------|--------------|
| Vehicle | | | | | | | | |
| Str/NAc | 0.86 ± 0.05 | 0.78 ± 0.11 | 5.73 ± 0.22 | 0.57 ± 0.02 | 0.50 ± 0.06 | 0.29 ± 0.01 | 0.83 ± 0.04 | 0.32 ± 0.01 |
| Cx | 0.67 ± 0.05 | 1.11 ± 0.08 | 1.02 ± 0.10 | 0.17 ± 0.02 | 0.11 ± 0.01 | 0.15 ± 0.01 | 0.50 ± 0.02 | 0.16 ± 0.00 |
| Esc METH | | | | | | | | |
| Str/NAc | 1.25 ± 0.05* | 0.42 ± 0.02* | 6.92 ± 0.19* | 0.74 ± 0.03* | 0.59 ± 0.02 | 0.41 ± 0.02* | 1.07 ± 0.04* | 0.43 ± 0.02* |
| Cx | 0.64 ± 0.02 | 1.00 ± 0.10 | 1.28 ± 0.24 | 0.20 ± 0.02 | 0.14 ± 0.01 | 0.22 ± 0.02* | 0.61 ± 0.02* | 0.23 ± 0.03 |

The brain regions of each mouse group were dissected just after the end of tail suspension test on day 10. Values are expressed as nanograms per milligram of wet tissues (mean ± SEM; $n = 20$ for each group)

Esc escalating-dose, DA dopamine, DOAPC 3,4-dihydroxyphenylacetic acid, 5-HIAA 5-hydroxyindolacetic acid, 5-HT 5-hydroxytryptamine (serotonin), HVA homovanillic acid, MHPG 3-methoxy-4-hydroxyphenylglycol, 3-MT 3-methoxytyramine, NE norepinephrine

* $P < 0.05$, compared with Vehicle (Student's t test after one-way ANOVA)

were significantly increased in the escalating doses of METH (0.2–2.0 mg/kg doses)-treated mice; whereas, the MHPG content was significantly decreased. The 3-MT content did not differ among the treatment groups. In the cerebral cortex, the contents of HVA and 5-HT were significantly increased in the METH-treated mice; whereas, the contents of the remaining monoamines and their metabolites did not differ among the treatment groups.

One-way ANOVA (Treatment) applied to the striatum + NAc showed significant main effects ($dF = 18$; $t = -3.144$, $P < 0.01$; $t = 4.042$, $P < 0.001$; $t = 4.358$, $P < 0.001$; $t = 5.257$, $P < 0.0001$; $t = 4.830$, $P < 0.0001$; and $t = 4.715$, $P < 0.001$) on MHPG, DA, DOPAC, HVA, 5-HT, and 5-HIAA, respectively, but showed no significant main effect of Treatment on 3-MT ($dF = 18$; $t = 1.352$, $P = 0.1932$). One-way ANOVA (Treatment) applied to the

cerebral cortex showed significant main effects of Treatment on HVA and 5-HT ($dF = 18$; $t = 3.047$, $P < 0.01$ and $t = 4.478$, $P < 0.001$, respectively), while ANOVA indicated no significant main effects of the remaining monoamines or their metabolites ($dF = 18$; $t = -0.559$, $P = 0.5829$; $t = -0.866$, $P = 0.3979$; $t = 1.097$, $P = 0.2870$; $t = 1.020$, $P = 0.3214$; $t = 1.663$, $P = 0.1135$; and $t = 2.099$, $P = 0.0502$ for NE, MHPG, DA, DOPAC, 3-MT, and 5-HIAA, respectively).

Implanted Osmotic Mini-Pumps

Table 4 shows the tissue contents of monoamines and their metabolites in the mice treated with METH released from s.c. implanted osmotic mini-pumps. No statistical differences were detected in the content of monoamines or their

Table 4 Tissue contents of monoamines and their metabolites in the striatum + nucleus accumbens (Str/NAc) and cerebral cortex (Cx) of mice treated with methamphetamine (METH) released from subcutaneously implanted osmotic mini-pumps

| Treatment | NE | MHPG | DA | DOPAC | 3-MT | HVA | 5-HT | 5-HIAA |
|---------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Vehicle | | | | | | | | |
| Str/NAc | 0.86 ± 0.05 | 0.48 ± 0.04 | 6.41 ± 0.29 | 0.63 ± 0.05 | 0.50 ± 0.05 | 0.36 ± 0.02 | 0.93 ± 0.06 | 0.35 ± 0.02 |
| Cx | 0.75 ± 0.03 | 0.85 ± 0.06 | 0.77 ± 0.10 | 0.22 ± 0.02 | 0.09 ± 0.01 | 0.12 ± 0.01 | 0.62 ± 0.04 | 0.26 ± 0.04 |
| Sham | | | | | | | | |
| Str/NAc | 0.83 ± 0.07 | 0.62 ± 0.08 | 6.22 ± 0.38 | 0.59 ± 0.05 | 0.50 ± 0.02 | 0.36 ± 0.02 | 0.86 ± 0.05 | 0.34 ± 0.02 |
| Cx | 0.82 ± 0.03 | 0.93 ± 0.04 | 1.22 ± 0.12 | 0.26 ± 0.03 | 0.10 ± 0.02 | 0.19 ± 0.04 | 0.66 ± 0.06 | 0.35 ± 0.05 |
| 15 mg/kg METH | | | | | | | | |
| Str/NAc | 0.83 ± 0.04 | 0.48 ± 0.04 | 6.03 ± 0.36 | 0.60 ± 0.05 | 0.49 ± 0.04 | 0.36 ± 0.02 | 0.83 ± 0.02 | 0.33 ± 0.01 |
| Cx | 0.84 ± 0.03 | 1.07 ± 0.13 | 1.26 ± 0.24 | 0.25 ± 0.02 | 0.12 ± 0.02 | 0.14 ± 0.01 | 0.68 ± 0.05 | 0.29 ± 0.03 |
| 76 mg/kg METH | | | | | | | | |
| Str/NAc | 0.76 ± 0.04 | 0.46 ± 0.04 | 6.25 ± 0.24 | 0.61 ± 0.05 | 0.50 ± 0.03 | 0.39 ± 0.02 | 0.83 ± 0.04 | 0.35 ± 0.02 |
| Cx | 0.82 ± 0.05 | 1.05 ± 0.03 | 0.89 ± 0.12 | 0.20 ± 0.02 | 0.12 ± 0.02 | 0.16 ± 0.03 | 0.73 ± 0.05 | 0.25 ± 0.03 |

The brain regions of each mouse group were dissected just after the end of tail suspension test on day 16. Values are expressed as nanograms per milligram of wet tissues (mean ± SEM; $n = 20$ for each group). No statistically significant differences were found

DA dopamine, DOAPC 3,4-dihydroxyphenylacetic acid, 5-HIAA 5-hydroxyindolacetic acid, 5-HT 5-hydroxytryptamine (serotonin), HVA homovanillic acid, MHPG 3-methoxy-4-hydroxyphenylglycol, 3-MT 3-methoxytyramine, NE norepinephrine

metabolites measured in the striatum + NAc or the cerebral cortex among the experimental groups.

One-way ANOVA (Treatment) applied to the striatum + NAc showed no significant main effects ($F(3,36) = 0.679, P = 0.5706; F(3,36) = 1.913, P = 0.1449; F(3,36) = 0.273, P = 0.844; F(3,36) = 0.102, P = 0.9585; F(3,36) = 0.043, P = 0.9880; F(3,36) = 0.539, P = 0.6585; F(3,36) = 1.155, P = 0.3404; \text{and } F(3,36) = 0.261, P = 0.8532$) on NE, MHPG, DA, DOPAC, 3-MT, HVA, 5-HT, or 5-HIAA, respectively. One-way ANOVA applied to the cerebral cortex also showed no significant main effects of Treatment ($F(3,36) = 0.945, P = 0.4291; F(3,36) = 1.864, P = 0.1532; F(3,36) = 2.768, P = 0.0557; F(3,36) = 1.299, P = 0.2897; F(3,36) = 0.643, P = 0.5926; F(3,36) = 1.288, P = 0.2932; F(3,36) = 0.857, P = 0.4723; \text{and } F(3,36) = 1.288, P = 0.2932$) on

NE, MHPG, DA, DOPAC, 3-MT, HVA, 5-HT, or 5-HIAA, respectively.

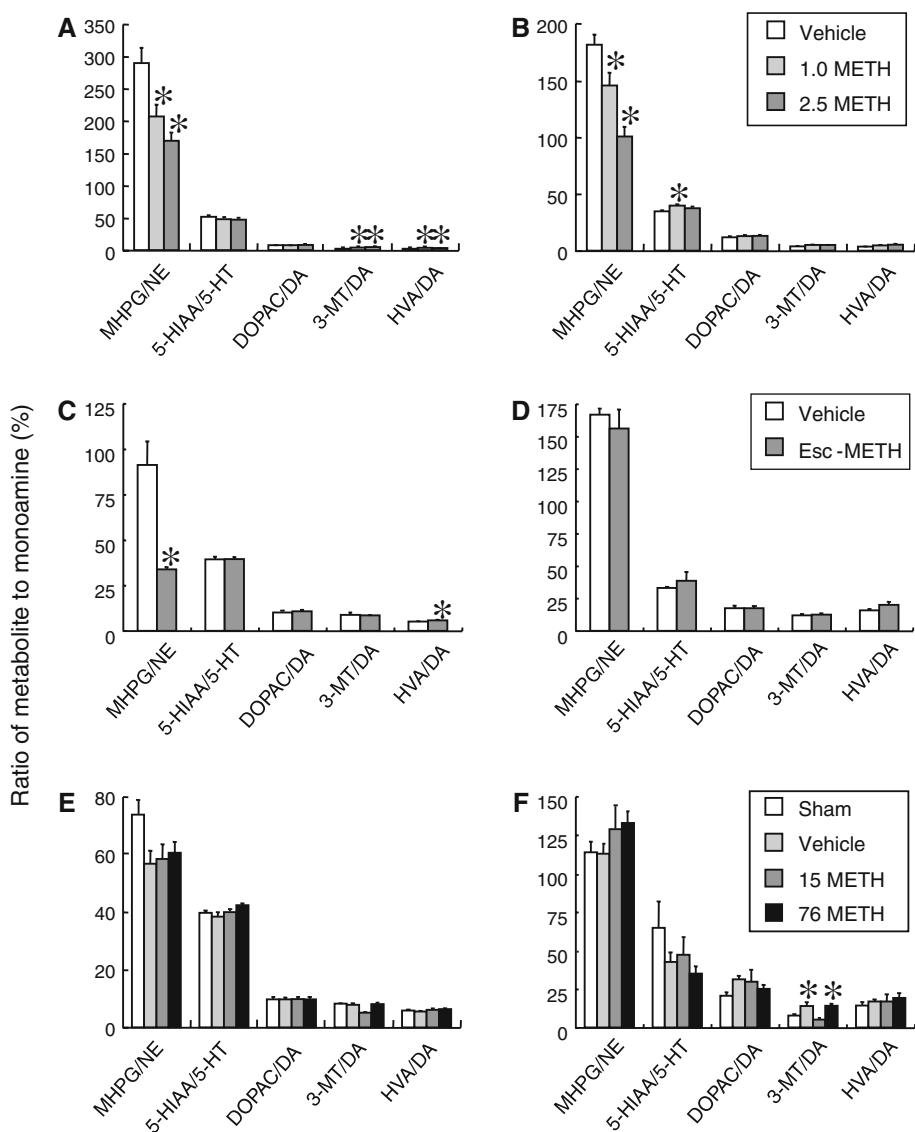
Monoamine Turnover

Monoamine metabolism was evaluated by calculating the ratio of the tissue levels of monoamine metabolites to those of their parent amines shown in Tables 2, 3, and 4.

Fixed-Dose Injection Regimen

Figure 2a, b show monoamine turnover, as calculated from the data shown in Table 2. Both in the striatum + NAc (Fig. 2a) and in the cerebral cortex (Fig. 2b), a dose-dependent decrease in the ratio of MHPG to NE was observed. Significant increases in the ratio of 3-MT to DA

Fig. 2 Apparent monoamine turnover in the striatum + NAc (a, c, e) and the cerebral cortex (b, d, f) of mice treated with METH under fixed-dose injection regimen (a, b), with METH under an escalating-dose injection regimen (c, d), and with METH released from s.c. implanted osmotic mini-pumps (e, f), respectively. Each column represents the means \pm SEM ($n = 10$ per group). DA dopamine, DOPAC 3,4-dihydroxyphenylacetic acid, 5-HIAA 5-hydroxyindolacetic acid, 5-HT 5-hydroxytryptamine (serotonin), HVA homovanillic acid, MHPG 3-methoxy-4-hydroxyphenylglycol, METH methamphetamine, 3-MT 3-methoxytyramine, NE norepinephrine. * $P < 0.05$, compared with vehicle (Bonferroni/Dunn test; a and b); compared with vehicle (Student's t test; c and d); and compared with sham (Bonferroni/Dunn test; e and f)



and HVA to DA were also observed in the striatal regions of the mice. The ratio of 5-HIAA to 5-HT in the cerebral cortex appeared to be affected in a somewhat biphasic manner; an increase in the ratio was observed at 1.0 mg/kg of dose, but no change from the vehicle level was seen after treatment with 2.5 mg/kg.

One-way ANOVA (Treatment) applied to the striatum + NAc showed significant main effects ($F(2,27) = 10.851$, $P < 0.001$; $F(2,27) = 19.205$, $P < 0.0001$; and $F(2,27) = 11.851$, $P < 0.001$) on MHPG/NE, 3-MT/DA, and HVA/DA, respectively, but no significant main effects of Treatment on 5-HIAA/5-HT or DOPAC/DA ($F(2,27) = 1.808$, $P = 0.1834$ and $F(2,27) = 2.404$, $P = 0.1095$, respectively). One-way ANOVA (Treatment) applied to the cerebral cortex showed significant main effects on MHPG/NE and 5-HIAA/5-HT ($F(2,27) = 17.422$, $P < 0.0001$ and $F(2,27) = 7.330$, $P < 0.001$, respectively), while ANOVA indicated no significant main effects of Treatment on DOPAC/DA, 3-MT/DA, or HVA/DA ($F(2,27) = 0.430$, $P = 0.6551$; $F(2,27) = 2.745$, $P = 0.0822$; and $F(2,27) = 2.307$, $P = 0.1188$, respectively).

Escalating-Dose Injection Regimen

Figure 2c, d show apparent monoamine turnover, as calculated from the data shown in Table 3. In the striatum + NAc (Fig. 2c), a marked decrease in the ratio of MHPG to NE was observed; whereas, the ratio of HVA to DA was significantly increased. In the cerebral cortex (Fig. 2d), no significant changes in the ratios were observed.

One-way ANOVA (Treatment) applied to the striatum + NAc showed significant main effects ($dF = 18$; $t = -4.353$, $P < 0.01$ and $t = 2.625$, $P < 0.05$, respectively). However, ANOVA showed no significant main effects of Treatment on 5-HIAA/5-HT, DOPAC/DA, or 3-MT ($dF = 18$; $t = 0.086$, $P = 0.9327$; $t = 1.191$, $P = 0.2493$; and $t = -0.308$, $P = 0.7614$, respectively). One-way ANOVA (Treatment) applied to the cerebral cortex showed no significant main effects for any of the monoamine turnovers calculated ($dF = 18$; $t = -0.686$, $P = 0.5016$; $t = 0.790$, $P = 0.4398$; $t = -0.030$, $P = 0.9765$; $t = 0.388$, $P = 0.7025$; and $t = 1.746$, $P = 0.0979$ for MHPG/NE, 5-HIAA/5-HT, DOPAC/DA, 3-MT/DA, and HVA/DA, respectively).

Implanted Osmotic Mini-Pumps

Figure 2e, f show monoamine turnover, as calculated from the data shown in Table 4. In the striatum + NAc, no significant changes in the ratios were observed. Significant increases in the ratios of 3-MT/DA in the cerebral cortex were observed at 0 (vehicle) and 76 mg/kg/2 week of

METH, but not 15 mg/kg/2 weeks of METH. The other ratios remained unchanged in both the striatum + NAc (Fig. 2e) and the cerebral cortex (Fig. 2f).

One-way ANOVA (Treatment) applied to the striatum + NAc showed no significant main effects on any of the monoamine turnover ratios calculated ($F(3,36) = 2.707$, $P = 0.0596$; $F(3,36) = 2.412$, $P = 0.0822$; $F(3,36) = 0.007$, $P = 0.9992$; $F(3,36) = 0.180$, $P = 0.9093$; and $F(3,36) = 1.296$, $P = 0.2906$ for MHPG/NE, 5-HIAA/5-HT, DOPAC/DA, 3-MT/DA, and HVA/DA, respectively). One-way ANOVA (Treatment) applied to the cerebral cortex showed a significant main effect on 3-MT ($F(3,36) = 3.634$, $P < 0.05$), while ANOVA indicated no significant main effects of Treatment on the remaining monoamine turnover ratios ($F(3,36) = 1.094$, $P = 0.3642$; $F(3,36) = 1.374$, $P = 0.2662$; $F(3,36) = 1.188$, $P = 0.3282$; and $F(3,36) = 0.413$, $P = 0.7448$ for MHPG/NE, 5-HIAA/5-HT, DOPAC/DA, and HVA/DA, respectively).

Elevated Plus Maze Test

Significant decreases in the time spent in open arms and the ratio of the time spent in the open arms to the total time spent (%time) were observed in the mice treated with fixed-doses of METH (1.0 and 2.5 mg/kg; Fig. 3a), while the time spent in closed arms and neutral zones was not affected by the drug treatment (Fig. 3b). In contrast, the times spent in open and closed arms and in the neutral zones were unaffected by treatment with escalating-doses (Fig. 3c, d) or continuous s.c. infusion of METH (Fig. 3e, f).

One-way ANOVA (Treatment) showed significant main effects on time spent in open arms and the percentage of time spent in open arms in the mice treated with fixed-doses of METH ($F(2,87) = 7.763$, $P < 0.001$ and $F(2,87) = 3.511$, $P < 0.05$, respectively), while ANOVA showed no significant main effects on the time spent in closed arms or neutral zones ($F(2,87) = 0.421$, $P = 0.6578$ and $F(2,87) = 3.046$, $P = 0.0527$, respectively) (Fig. 3a, b). ANOVA showed no significant main effects on any of the parameters tested ($F(1,38) = 0.072$, $P = 0.7900$; $F(1,38) = 0.311$, $P = 0.5804$; $F(1,38) = 0.135$, $P = 0.7154$; and $F(1,38) = 0.002$, $P = 0.9654$ for time spent in open arms, percentage of time spent in open arms, time spent in closed arms, and time spent in neutral zone, respectively) in mice treated with METH under the escalating-dose injection regimen (Fig. 3c, d). ANOVA showed no significant main effects on any of the parameters tested ($F(3,76) = 1.959$, $P = 0.1272$; $F(3,76) = 1.618$, $P = 0.1921$; $F(3,76) = 0.594$, $P = 0.6211$; and $F(3,76) = 2.038$, $P = 0.1157$ for time spent in open arms, percentage of time spent in open arms, time spent in closed arms, and time spent in neutral zone, respectively) in the mice treated with METH released from s.c. implanted osmotic mini-pumps (Fig. 3e, f).

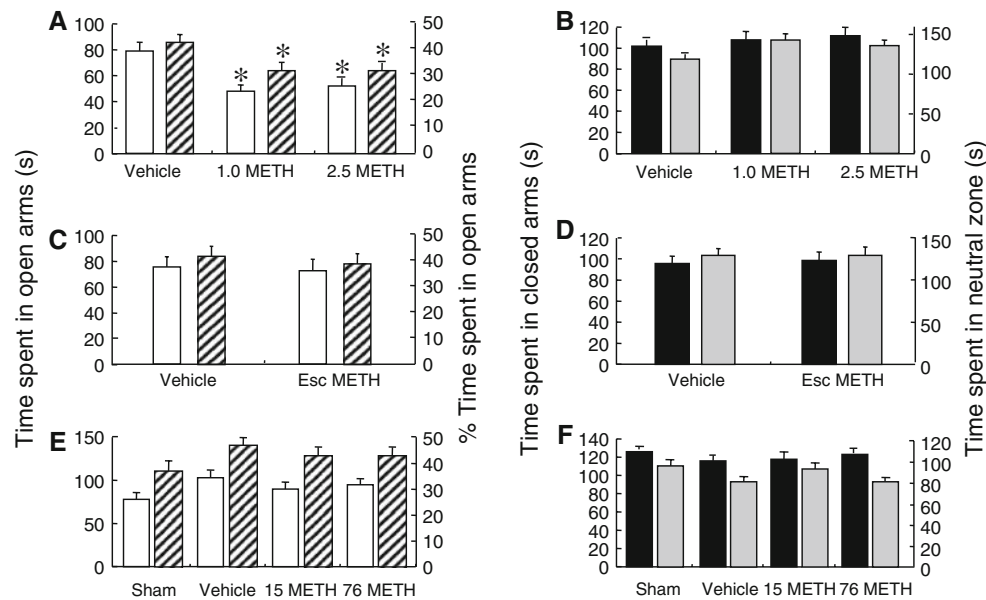


Fig. 3 Elevated plus maze in mice treated with METH under fixed-dose injection regimen (**a**, **b**), with METH under an escalating-dose injection regimen (**c**, **d**), and with METH released from s.c. implanted osmotic mini-pumps (**e**, **f**), respectively. Mice were treated with METH under an assigned injection regimen followed by 5-day drug withdrawal and were then subjected once to a maze for 5 min. The results represent the time spent in *open arms* (open columns shown in **a**, **c**, **e**), *closed arms* (closed columns shown in **b**, **d**, **f**), and *neutral*

zone (shaded columns shown in **b**, **d**, **f**), and the percentage time spent in *open arms* (hatched columns shown in **a**, **c**, **e**). Values are shown as the means \pm SEM ($n = 30, 20,$ and 20 per group of mice with METH under fixed-dose injection regimen (**a**, **b**), with METH under an escalating-dose injection regimen (**c**, **d**), and with METH released from s.c. implanted osmotic mini-pumps (**e**, **f**), respectively). * $P < 0.05$, compared with vehicle (Bonferroni/Dunn test; **a**)

Tail Suspension Test

The immobility time tended to decrease in a dose-dependent manner in mice that received fixed-doses of METH (1.0 and 2.5 mg/kg) followed by 5-day-withdrawal ($F(2,87) = 4.525$, $P < 0.05$, evaluated by One-way ANOVA (Treatment)) (Fig. 4a), but was not affected in the mice that received escalating-doses ($F(1,38) = 1.262$, $P = 0.2683$) (Fig. 4b) or continuous s.c. infusion of METH followed by 5-day-withdrawal ($F(3,76) = 0.393$, $P = 0.7585$) (Fig. 4c).

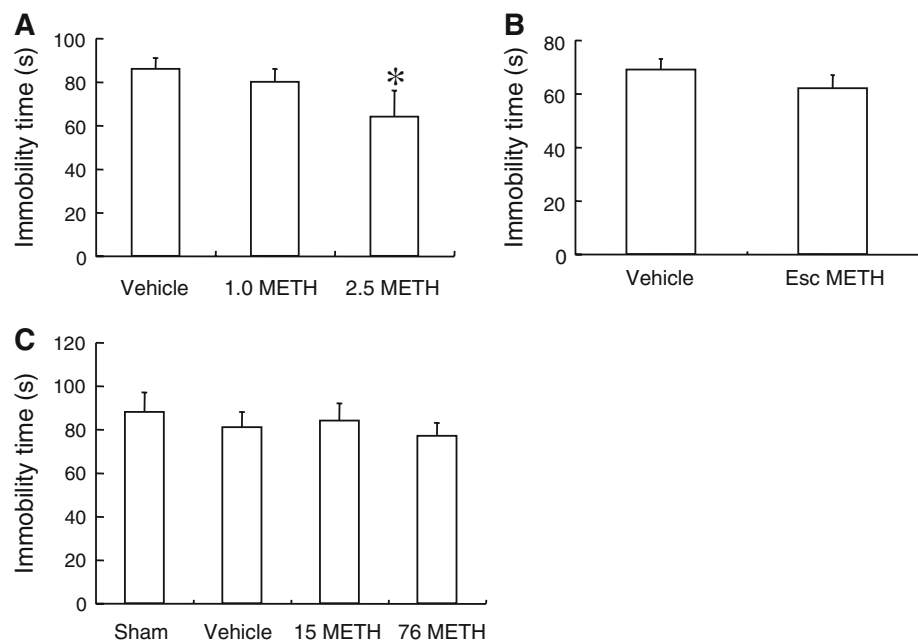
Discussion

Treatment of mice with a fixed-dose of METH for 10 consecutive days (1.0 or 2.5 mg/kg, two injections daily) followed by 5-day withdrawal significantly decreased the cerebral MHPG levels (Table 2) and NE turnover (Fig. 2b) in a dose-dependent manner. It is possible that the excitability of norepinephrine neurons may have been decreased in the postdrug period because of the decreased tissue levels of MHPG. This group of mice showed a significant decrease in the time spent in open arms (and the ratio of the time spent in open arms to the total time of the test) in the elevated plus maze (Fig. 3a), suggesting the expression of anxiety-related behavior. As expected on the

basis of human observations [26, 27], it is likely that decreased norepinephrine tone induces a dysphoric state during AMPH withdrawal including anxiety. Therefore, mice treated with a fixed-dose of METH are a good animal model of anxiety-related behavior in AMPH withdrawal in humans.

In spite of their importance, which has been reported previously [28] and is discussed below, the roles of NE neurotransmission have been less investigated with regard to the expression of anxiety during AMPH withdrawal. The mechanism(s) through which the tissue levels of MHPG and MHPG/NE are dose-dependently decreased after a fixed-dose injection regimen followed by 5-day withdrawal must be clarified. Regarding this, there are three possibilities to be considered: (1) damage to norepinephrine neurons, (2) reduced enzyme activity of brain monoamine metabolism, and (3) reduced NE biosynthesis. (1) In the present study, it is unlikely that the fixed-dose of METH induced damage or the death of neurons containing NE, since the tissue levels of NE were not affected by METH treatment (Table 2). In contrast to this observation, the depletion of brain NE was observed when higher fixed-doses than the doses used in this study were administered to mice (AMPH 10 mg/kg, twice daily for 5 days; ref. [29]). Since the total amount of the drug administered to each mouse by Short and Shuster [29] (i.e. 100 mg/kg animal) was twice as high as that used in the

Fig. 4 Tail suspension test. Animals under 5-day-withdrawal after administration of METH under a fixed-dose injection regimen (a), escalating-dose injection regimen (b), and via osmotic mini-pumps (c) were exposed to the test for 6 min. Values are shown as the means \pm SEM ($n = 30, 20,$ and 20 per group in a, b, and c, respectively). * $P < 0.05$, compared with vehicle (Bonferroni/Dunn test)



present study, it is natural to presume that the metabolism of NE or the viability of norepinephrinergic neurons might have been affected differently from those in our experiments. (2) In the brain, monoamine oxidase (MAO) followed by aldehyde reductase and catechol-*O*-methyltransferase (COMT) metabolizes NE to form MHPG. It is unlikely that reduced MAO and/or COMT activity were primarily responsible for the decreased MHPG levels since treatment with a fixed-dose of METH did not decrease the levels of DOPAC, 3-MT, HVA, or 5-HIAA, which are all formed by MAO and/or COMT functions (Table 2). (3) As mentioned below, the levels of urinary MHPG correlate positively with brain NE biosynthesis in humans [30, 31]. In line with these observations, it is interesting to speculate that the tissue levels of MHPG and the MHPG/NE ratio in the cerebral cortex may correlate positively with brain NE biosynthesis. Brain NE biosynthesis may be reduced by decreased NE neuronal excitability. In the present study, decreased NE neuronal excitability may have occurred possible, since the tissue levels of NE metabolite (i.e., MHPG) decreased; whereas, the tissue levels of the precursor molecule for NE (i.e., DA) were unchanged (Table 2), resulting in reduced brain NE biosynthesis and hence reduced NE release. Decreased cerebral NE neuronal excitability may play a role in the expression of anxiety-related behavior. It was noted that the tissue levels of NE, MHPG, and DA in the vehicle treatment group (i.e. basal values) were different between the fixed-dose injection group and the two other treatment groups (Table 2 vs. Tables 3, 4). The different treatment regimens may account for these observations. In particular, the effects of needle injection time should be considered.

As shown in Fig. 2a, apparent DA turnover in the striatum + NAc was also affected after METH withdrawal period of the fixed-dose treatment regimen. This alteration in dopaminergic pathway can also be observed in mice behaviorally sensitized to METH (1.0 mg/kg, i.p., once daily for five consecutive days; [32]). Mice showing behavioral sensitization to METH do not express symptoms of dysphoric state during METH withdrawal. Therefore, it is unlikely that alteration in apparent DA turnover in the striatum + NAc might result in the anxiety-related behavior.

In the present study, no depression-like behavior was observed in the tail suspension test in the mice under three different METH treatment regimens followed by 5-day drug withdrawal (Fig. 4). Rather, augmented efforts to escape from the tail suspension (i.e., decreased immobility time) were induced by the fixed-dose of METH (Fig. 4a), but not by the escalating-dose or continuous s.c. infusion (Fig. 4b, c). In contrast to the present results, Cryan et al. [18] reported that depression-like behavior (i.e., a significant increase in immobility time) was observed in mice after METH administration (5 and 10 mg/kg/day for 7 days, released from mini-pumps) followed by 1-day withdrawal. Their observations and ours (Fig. 4) clearly demonstrate that the period of withdrawal (1 vs. 5 days) and/or the infusion period (7 vs. 14 days) are crucial factor(s) determining the expression of depression-like behavior in mice because the total amounts of AMPH were similar (35 and 70 vs. 15 and 76 mg/kg animal). In addition, mice administered with a fixed-dose of AMPH (5 mg/kg, i.p., twice daily for 10 consecutive days; total amount of AMPH = 100 mg/kg/animal) followed by 1-day withdrawal were found to

show depression-like behavior in the forced swim test [10]. These observations suggest the importance of the withdrawal period in the expression of depression-like behavior. We noted that there were no differences in the tissue levels of monoamines and their metabolites between 1-day and 5-day withdrawal in the literature [13]. The apparent escape reaction to tail suspension (Fig. 4a) appeared to be similar to the defensive response recognized in an animal model of panic anxiety [33]. Generally, anxiety is induced by hyperexcitability in the amygdalar and hippocampal neural circuits under certain circumstances, and benzodiazepines act as anxiolytic agents by enhancing presynaptic inhibition mediated by γ -aminobutylic acid (GABA) in neural circuits especially in the medial nucleus of the amygdala [34]. It is interesting to speculate that the decrease in the immobility time in the tail suspension test (Fig. 4a) may have resulted from the enhancement of escape response induced by neural excitation during AMPH withdrawal. This remains to be clarified with regard to the relationship between NE and GABA neurotransmission, since several stress-related negative symptoms including anxiety during the drug withdrawal period are thought to be elicited by changes in NE neurotransmission in the extended amygdala [35].

The involvement of the NE system in general anxiety disorders has been verified by the successful treatment of a variety of anxiety disorders with antidepressants such as venlafaxine, milnacipran, and duloxetine (antidepressants that inhibit both 5-HT and NE reuptake) [5]. Similarly, the expression of anxiety during AMPH withdrawal [1, 2] may be attributed to changes in NE neurotransmission in the cerebral cortex during the postdrug period. This possibility is supported by evidence that the urinary excretion of MHPG decreased after AMPH withdrawal in humans [26, 27] because urinary MHPG excretion serves as an index of brain NE synthesis and metabolism [30, 31]. The expression of anxiety-related behavior in parallel with decreases in the cerebral level of MHPG and NE turnover is an important finding in mice after the administration of a fixed dose of METH, since there is little evidence available regarding the association between neurochemistry and the behavioral aspects of anxiety during AMPH withdrawal in mice [13]. This animal model will help us understand the regulation of NE neuronal excitability in anxiety disorders caused by AMPH withdrawal in humans.

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