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ORIGINAL ARTICLE

BEHAVIORAL AND REWARDING EFFECTS OF METHYLONE, AN ANALOG OF MDMA IN MICE

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Abstract Methylone, an analog of 3,4-methylenedioxymethamphetamine（MDMA）is a narcotic drug which is forbidden due to its abusability. However, a detailed behavioral toxicity and rewarding effect of methylone has not yet been reported to this date. The aim of this study is to evaluate the toxicity and addictive effects of methylone. In order to detect the stimulant effect to central nerous system, a mouse behavioral toxicity test and a conditioned place preference（CPP）test were conducted by administering mehtylone, MDMA and methamphetamine（MAP）. An immunohistochemical study was also performed to analyze ΔFosB, which is known to accumulate in the nucleus accumbens after chronic administration of the drugs of abuse. For determining the expression levels of Δ*fosB* mRNA in striatum, quantitative PCR analysis was also conducted by acute administration of methylone. Significant differences appeared in mice that were administered with 50 mg/kg and above of methylone. Methylone causes a similar qualitative behavioral effect as MAP, however not the same stereotyped behavior as MDMA. According to the result from CPP test, methylone of 2.5 mg/kg and above had shown a rewarding action. Chronic administration of methylone causes significant ΔFosB accumulation in the neurons of nucleus accumbens. Methylone and MDMA significantly induce Δ*fosB* mRNA in striatum. It is therefore suggested that methylone has psychoactive effects and can be considered as an addictive drug.

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Key words: methylone; MDMA; behavioral effect; rewarding effect; ΔFosB.

原　著

MDMA の類縁体である methylone の行動毒性および依存性に対する影響について

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抄録　合成麻薬 MDMA の designer drug として乱用され，麻薬指定された methylone の行動毒性や依存性について検討した報告はない．そこで，methylone の危害性を明らかにするためにマウスによる行動毒性及び場所嗜好性(CPP)試験を行った．さらに，依存性薬物投与時に前脳側坐核に蓄積するΔFosB を免疫組織学的に検討し，methylone の急性投与時における脳線条体でのΔ*fosB* mRNA 発現量を定量的 PCR で確認した．その結果，methylone による運動量及び行動の質的変化は，MDMA よりむしろ methamphethamine に類似していた．さらに methylone では，CPP 試験で依存性が確認されただけでなく，組織学的にも報酬系回路の側坐核でΔFosB のタンパクと mRNA の有意な増加が確認された．以上より，methylone は向精神作用を有する依存性薬物である可能性が示された．

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キーワード：methylone；MDMA；行動影響；報酬効果；ΔFosB.

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Introduction

Various psychoactive drugs are widely used recreationally throughout the world. Methamphetamine (MAP) is well known as a stimulant, related substance of MAP, 3,4-m ethylenedioxymethamphetamine (MDMA) has become a popular abused drug in recent years. This is because it is available in tablet form and has similar effects as MAP due to their close chemical structures. The use of non-controlled psychoactive drugs by the younger generation for excitement, euphoria and hallucination purposes have been creating many social problems. Furthermore, non-controlled psychoactive drugs or so called "gateway drugs" lead to the use of illegal drugs that yield stronger effects. Most non-controlled psychoactive drugs are designer drugs synthesized to be chemically similar to illegal drugs. Because of their similar structures, they are expected to produce similar effects on their users. In 2008, the Japanese Ministry of Health, Labour and Welfare amended the Pharmaceutical Affairs Act to control the use of some designer drugs. Although there have been accidents and misuses associated with these designer drugs, there is limited scientific research regarding their hazards.

Phenethylamine compounds such as MAP, MDMA, and their analogs are one of the most widely available drugs of abuse in the world. MDMA has a similar chemical structure to MAP (stimulant) and mescaline (hallucinogen). Even a low dose of MDMA causes psychotoxicity and dependence as well as excitement, euphoria and hallucination[1, 2]. In the Netherlands, 3,4-methylenedioxy-N-methylcathinone (methylone) first appeared on the drug market in 2004[3]. It then spread all over Europe and Japan via the Internet[4, 5]. Methylone was expected to have MDMA-like effects because of their similar chemical structures. Our group detected methylone for the first time in 2004 through the analysis of an illicit drug that was purchased from an online shop in Japan. Addiction to methylone has been reported in Japan since 2005[6, 7]. The Japanese government regulated methylone as a narcotic drug in February 2007. Since then, the metabolic pathway[8], the effects on dopamine and serotonin receptors *in vivo*[9, 10] and hepatocyte toxicity[11] on methylone have been investigated. While Dal Cason reported that methylone in rats caused similar effects to MDMA[12], Alexander Shlugin wrote on the internet that methylone does not act like MDMA. Since MDMA became an illicit drug, the usage of methylone dramatically increased because of its expected similar effects. However, there are limited scientific data on its dependence effects and toxicity. By defining the effects of methylone, the characteristics of MDMA analog substances could be estimated and thus, this information would help to assign these drugs as illicit at an earlier stage.

In this study, a mouse behavioral toxicity test and a conditioned place preference (CPP) test were conducted to evaluate the toxicity and dependence effects of methylone. An immunohistochemical study was also performed to analyze ΔFosB, which is known to accumulate in neurons of the nucleus accumbens (NAc) for a long time after chronic administration of an addictive drug[13]. In addition, we examined the expression level of Δ*fosB* mRNA in striatum with real-time PCR.

Materials and methods

Chemicals

Before such actions became illegal, methylone was easily accessible through internet, therefore methylone was purchased via an internet-order in 2005. The purity of methylone was determined to be greater than 99% using HPLC and GC-MS by the procedure of Shimizu[7]. In the present study, methamphetamine (MAP) was

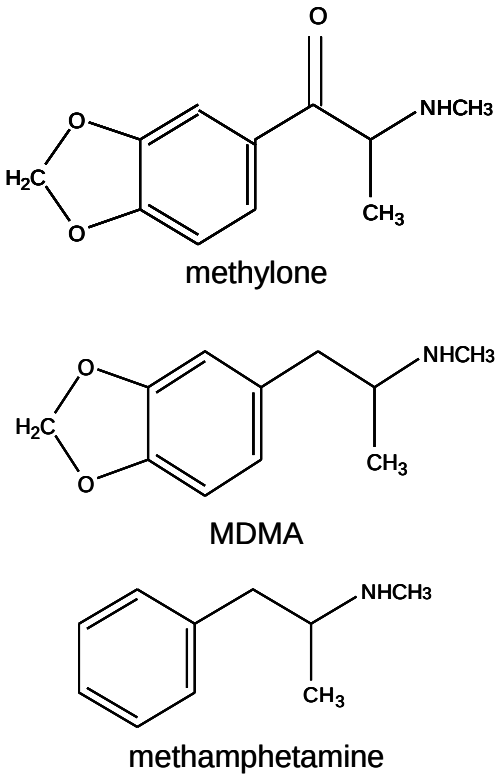

Fig. 1 Chemical structures of compounds tested.

purchased from Dainippon Sumitomo Pharma (Osaka, Japan) and MDMA was obtained by the Tokyo Metropolitan Institute of Public Health and both were used as positive control chemicals. Other chemicals used in this study were the highest grade available from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The chemical structures of methylone, MAP and MDMA are shown in Fig. 1. All these chemicals were in the monohydrochloride form.

Animals

Male ddY mice (6 to 7 weeks old) were purchased from Japan SLC Inc. (Hamamatsu, Japan) at least 7 days prior to the experiment. Three to five mice were kept in a plastic cage under controlled environmental conditions, (23 ±1°C, 50±10% humidity, 12 h light/dark cycle) and food (CE-2, Japan Clea Inc. Tokyo, Japan) and tap water were available *ad libitum*. For the study, 8- to 14-week old mice were used and all experiments were performed between 9:30 and 17:00. All animal care and use were in accordance with the guidelines for animal experimentation of Kanagawa Prefectural Institute of Public Health. There were no significant differences in mean body weight of mice between the test groups.

Locomotor activity test

Locomotor activity was measured by the SCANET SV-20 system (Melquest Ltd., Toyama, Japan). To habituate mice to the experimental environment and treatment, they were placed individually in clear plastic cages (41.5×25×18.5 cm) in SCANET and administered water forcibly using an oral sonde for 3 consecutive days.

Thirty minutes prior to the locomotor test, the overnight (16-20h) fasted mice were placed in the apparatus. The locomotor activity was measured every minute for 180 minutes after

Table 1 Functional and observational tests employed in this study.

Behavioral	Neurologic	Autonomic
Body position	Convulsion	Respiration rate
Stereotypy	Tremor	Piloerection
Parapebral closure	Gait	Lacrimation
Bizarre behavior	Tail elevation	Salivation
Pinna reflex	Body tone	Defecation
Approach response	Abdominal tone	Urination
Touch response	Limb tone	Skin color
Aggression	Grip strength	Heart rate
Vocalization	Finger splay	Body temperature
Locomotor activity	Righting reflex	
Rearing	Wire maneuver	
Transfer arousal		
Tail pinch response		
Visual placing		

oral administration of methylone dissolved in distilled water at doses of 12.5, 25, 50 or 100 mg/kg (10 mL/kg) in groups of six. The control animals were given an equal volume of water in the same way.

Functional and observational tests

The functional and observational tests were performed based on the protocol previously described by Irwin[14] with the following changes. Although these tests have been a part of neurotoxicity screening studies, the reliability and validity of these measures are influence by experimenter bias and work environment[15]. For reducing these problems, the two independent observers conducted all studies were unaware of the dose level of each mouse, and all experiments were performed between 13:00 and 17:00.

In the present study, each group included consisted of eight mice. After oral administration of methylone (at doses of 5, 12.5, 25, 50 or 100 mg/kg (10 mL/kg)) or water (control), mice were observed for 15 minutes in their home-cages. Following this, the locomotor activity was measured by the SCANET SV-20 system for 15 minutes. Mice were then transferred to an open field (45×45×30 cm) and observed for 5 minutes. The animals were observed for clinical signs and the details were recorded using the scaling system defined by our institution as follows: 0 is normal, –1 and –2 are depression and 1 and 2 are enhancement. Additional functional assessments were conducted after observations were recorded.

Rectal temperature was measured by PowerLab (Bio Research Center Co. Nagoya, Japan). The difference in body temperature of mice was determined from the difference between the rectal temperature before administration and 40 minutes after the administration. The observational and functional effects list is shown in Table 1.

To evaluate behavior following administration of methylone, MDMA and MAP, 50 mg/kg of methylone, 47 mg/kg of MDMA and 38 mg/kg MAP were administrated. These doses are 0.205mmol/kg of each drug.

Conditioned place preference (CPP) test

To clarify the rewarding effects of methylone, the CPP test was conducted by the pre-conditioning method[16, 17] with a minor modification. The test substances were dissolved in saline and administered intraperitoneally with doses of 0, 1, 2.5 or 5 mg/kg. The CPP test chamber (29×15×15 cm) was divided into two

compartments separated by a sliding partition. One compartment had black walls with a wire grid floor, whereas the other had clear walls with a white plastic floor. The test chamber was put in the SCANET SV-20LD system under using the lighting conditions of 50–70 lx on the clear compartment floor.

Briefly, to habituate the mice to the test conditions, they were placed into the test chamber without the partition. The mice stayed for 15 minutes each day during the 3-day preconditioning period, and were free to move around. On day 3, the time that the mice spent in each compartment during the 15-minute period was recorded. After 15 minutes, the non-preferred compartment was identified and the time spent in this compartment was recorded as the pre-conditioning score. The mice were treated with methylone in the non-preferred compartment. Conditioning was performed on 10 successive days starting from day 4. On day 4, the mice were intraperitoneally injected with methylone (0, 1, 2.5 or 5 mg/kg) and were enclosed into the non-preferred compartment for 30 minutes. On the following day, the mice were intraperitoneally injected with saline and placed in the preferred compartment for 30 minutes. This 2-day treatment is considered as one session and was repeated five times. The CPP test was conducted on the day after completion of the fifth conditioning session (day 14). The mice were placed in the test chamber with free access to both compartments for 15 minutes. The time that the mice stayed in the methylone-treated compartment was recorded as the post-conditioning score. The index of drug rewarding effect was calculated by subtracting the pre-conditioning score from the post-conditioning score and defined as the CPP score.

The same procedure was carried out using 0.25 mg/kg of MAP instead of methylone.

Immunohistochemistry

We performed immunohistochemistry to detect the accumulation of ΔFosB protein in NAc after chronic methylone treatment.

Mice were intraperitoneally injected with methylone at 2.5 or 5 mg/kg or saline ten times every 3 or 4 days. Three hours after the final injection, the animals were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.), and perfused transcardially with 0.1 M phosphate-buffered saline (PBS) containing heparin (5 U/mL), followed by 4% paraformaldehyde in PBS. The brains were removed quickly, postfixed overnight with 4% paraformaldehyde in PBS at 4 °C, then transferred into phosphate-buffered 30% sucrose solution until saturated. The brains were sliced into 1 mm-thick sections using a mouse brain matrix. The sections were frozen in liquid nitrogen and stored at –80° C until sectioning.

The serial sections were made with a coronal orientation and cut into 30 μm sections on a cryostat. NAc was identified according to the Mouse Brain in Stereotaxic Coordinates[18]. The free-floating sections were washed in PBS and the endogenous peroxidase activity were blocked by incubation in a 0.3% hydrogen peroxide solution for 30 minutes. After rinsing with PBS, the sections were incubated in PBS with 0.3% Triton-X and 5% normal donkey serum to minimize non-specific labeling. Tissue sections were incubated in primary antibody against pan-FosB (1:2,000, Sc-48, Santa Cruz Biotechnology, CA, USA) in PBS with 0.3% Triton-X and 0.5% bovine serum albumin for 48 h at 4° C. Sections were rinsed in PBS with 0.3% Triton-X, and then incubated in biotinylated donkey anti-rabbit antibody (1:1500, AP182B Chemicon, CA, USA) at room temperature for 2 h. Sections were washed in PBS and incubated in a streptavidin–biotin complex with horseradish peroxidase (1:100, Vector Laboratories, CA, USA) for 1 h. Sections were washed in Tris buffer and then the reaction was visualized using a Metal

Enhanced DAB Substrate Kit (Pierce, IL, USA). Sections were then washed in distilled water and mounted onto the slides. The slides were dehydrated and coverslipped with Eukit (Merck, Darmstadt, Germany).

The observers counted the serial three or four sections prior from the point of 1.10 mm anterior from the Bregma[18]. The slides were chosen randomly and the counts were conducted without any information of the animal. The FosB positive cells within the NAc (0.025 mm^2) were counted using image analysis software (WinROOF ver.5.0, Mitani Co., Tokyo, Japan). The numbers of FosB positive cells were calculated from the total amount of positive cells divided by the numbers of sections used for counting.

Real-time PCR and RT-PC

To measure the relative expression of *fosB* and Δ*fosB* mRNAs, the mice were intraperitoneally injected with drug (0.041 mmol/kg methylone or MDMA) or saline. Three hours after administration, the striatum was isolated from the mice treated with drug or saline, immersed in RNAlater (Applied Biosystems, CA, USA) and stored at -20°C until use. Total RNA was extracted using RNAiso (Takara, Tokyo, Japan) according to the manufacturer's protocol, and purified using an RNeasy Mini Kit (Qiagen, Dusseldorf, Germany). cDNA was generated from total RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

Δ*fosB* mRNA is truncated from the *fosB* gene by alternative splicing. The amount of cDNA synthesized from the target mRNA was quantified using real-time PCR. The primers used were amplified from the common area of *fosB* and Δ*fosB* (*fosB* + Δ*fosB*) mRNA or a specific area of *fosB* mRNA. *β-Actin* quantification was used as an internal control for normalization. The primers used for amplification were as follows: 5'-AGAGCCAGGCCTAGAAGACC-3' and 5'-ACGGTTCCTGCACTTAGCTG-3' for *fosB* + Δ*fosB*, 5'-ACCTGACGGCTTCTCTCTTTAC-3' and 5'-GTGAGGACAAACGAGGAAGTG-3' for *fosB* and 5'-AGAGGGAAATCGTGCGTGAC-3' and 5'-CAATAGTGACCTGGCCGT-3' for *β-actin*. Real-time PCR was performed in triplicate using a 7900HT real-time PCR system (Applied Biosystems) with a SYBR Green Master Mix (Applied Biosystems). Cycling conditions were as follows: 95°C - 10 min, 1 cycle; 95°C - 15 sec, 60°C - 60 sec, 45 cycles; melt curve from 60 - 95°C.Differences in mRNA levels from control values were calculated using the ΔΔCt method as described in the Applied Biosystems manual.

To confirm the *fosB* or Δ*fosB* mRNA, the RT-PCR was performed using Applied Biosystems 9600 PCR system with AmpliTaq Gold DNA Polymerase (Applied Biosystems). *Gapdh* was used as an internal control. The primers used for amplification were as follows: 5'-ACGGTCACCGCAATCACAAC-3' and 5'-TCCTTGTTCCTTGCGGGTTTG-3' for both long *fosB* transcripts and short spliced Δ*fosB* isoform and 5'-TGTCTTCACCA CCATGGAGAAGG-3' and 5'-GTGGATGCA GGGATGATGTTCTG-3' for *Gapdh*. Cycling conditions were as follows: 95°C - 10 min, 1 cycle; 95°C - 30 sec, 60°C - 30 sec, 72°C - 30 sec, 45 cycles; 72°C - 7 min, 1 cycle. The PCR products were applied to agarose gel electrophoresis and visualized by staining with ethidium bromide.

Statistical analysis

Animals were assigned into groups based on the results of body weight analysis with one-way ANOVA and a Bartlett test to avoid any significant differences between the groups. The rest of the data, which includes locomotor activity, body temperature, CPP score, FosB positive cells and the expression of Δ*fosB* mRNA, were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test. The observational test results were analyzed by

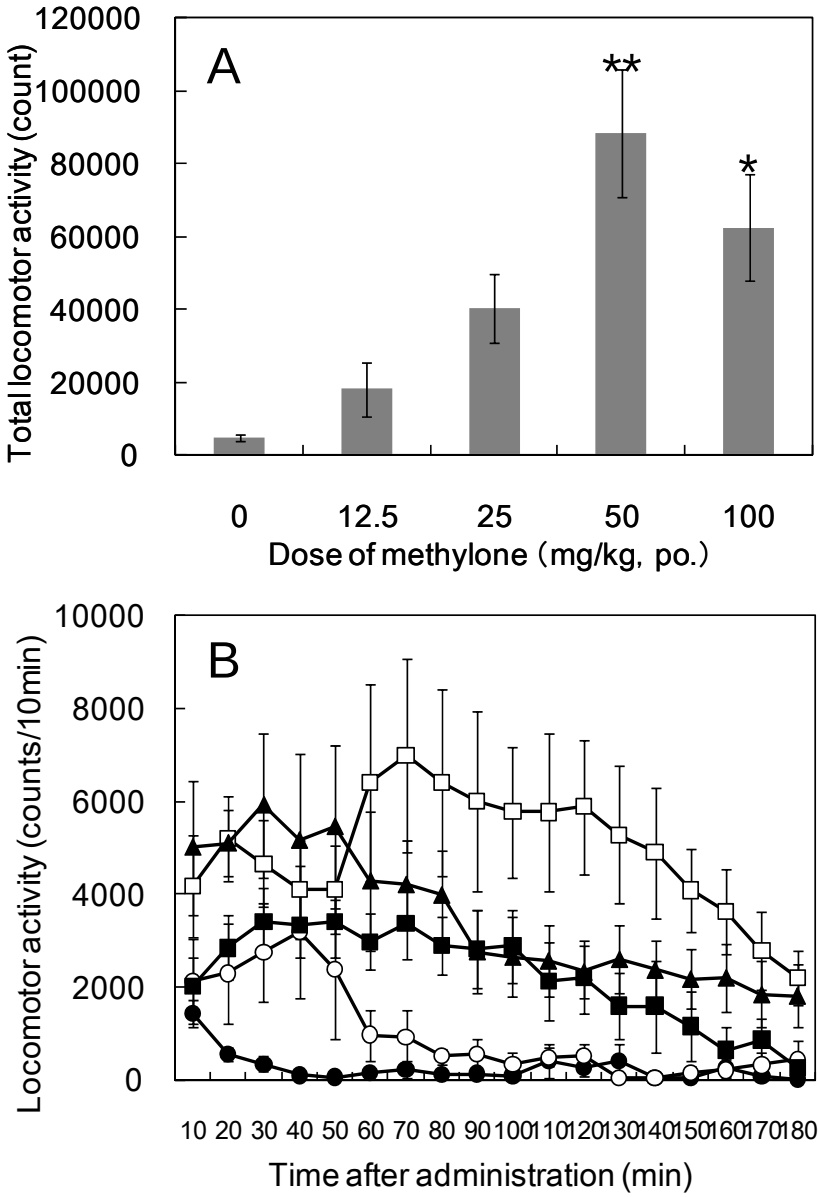

Fig. 2 Effects of methylone on the locomotor activity of mice.
(A) Total locomotor activity changes after administration of methylone 12.5, 25, 50 and 100 mg/kg, po. (B) Time course of locomotor activity 180 min. after administration of methylone. ● water, ○ 12.5 mg/kg, ■ 25 mg/kg, □ 50 mg/kg, ▲ 100 mg/kg. Data are represented as the mean ± SEM of locomotion counts of 6 mice. $^{*}P<0.05$, $^{**}P<0.01$, indicates significant difference from the water-treated group.

the Kruskal-Wallis test and followed by the Steel test. The data were presented as means ± SEM. Probability value of $P < 0.05$ was considered to be statistically significant.

Results

Locomotor activity of methylone

There were significant differences in locomotor activity between the group treated with 50 mg/kg or more methylone (F (4, 25)=8.52, $P<0.01$) (Fig. 2A). The animals administered 100 mg/

Table 2 Results of functional and observational tests of methylone in mice.

Observed behavioral and functional lists	0mg/kg	5mg/kg	12.5mg/kg	25mg/kg	50mg/kg	100mg/kg
Stereotypy	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.5 ± 0.2	1.0 ± 0.0**
Approach response	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.2	0.6 ± 0.1**
Touch response	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.2	1.0 ± 0.0**
Locomotor activity	7862 ± 369	10231 ± 1425	13004 ± 1993	19607 ± 1683**	22589 ± 2094**	19930 ± 1069*
Rearing in open field	0.0 ± 0.0	−0.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	−0.4 ± 0.2	−0.8 ± 0.2*
Transfer arousal	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.5 ± 0.2	0.6 ± 0.2	0.8 ± 0.2*
Tail pinch response	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.2	1.0 ± 0.0**
Gait	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.4 ± 0.2	0.8 ± 0.2*
Wire maneuver	1.8 ± 0.1	2.0 ± 0.1	1.8 ± 0.2	1.4 ± 0.3	0.1 ± 0.6	−1.0 ± 0.5**
Respiration rate	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.2	1.0 ± 0.0**
Piloerection	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.8 ± 0.3	0.6 ± 0.3	1.8 ± 0.2**
Salivation	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.3	1.5 ± 0.3*
Body temperature (℃)	1.1 ± 0.2	0.7 ± 0.2	0.5 ± 0.2	1.0 ± 0.4	1.5 ± 0.3	3.3 ± 0.5**

Details of observation and functional methods are described by Irwin[14]. Locomotor activity was measured for 15 min from 15 min after administration. Body temperature was calculated from the difference in rectal temperature before administration to that after open field observation at about 40 min after administration. Data are represented as the mean ± SEM of observation scores, motor activity and body temperature of 8 mice. *P<0.05, **P<0.01, indicates significant difference from the water-treated group.

kg showed decreased locomotion compared with those dosed with 50 mg/kg, probably because of the toxic effects of methylone. The locomotor activity gradually increased following administration of methylone, and the highest activity was observed around 60 minutes after the 50 mg/kg administration (Fig. 2B). In other groups, the locomotion peak appeared at about 30-40 minutes after administration (Fig. 2B).

Behavioral profile of methylone

Table 2 shows the behavior and functional changes with significant differences among the groups. Locomotor activity increased significantly for 15 minutes in the groups treated with 25 mg/kg or more methylone (F (5, 42) =14.84, P<0.01). During the observation at open field, the score of transfer arousal (X^2=17.49, df =5, P<0.01) increased dose-dependently. Mice administered methylone explored just after transfer to the open field for a short time, however they moved with pelvic elevation posture. Stereotyped behavior of animals administered with 50 mg/kg and above increased significantly. e.g. sniffing (X^2=31.59, df =5, P<0.01).

In an approach response test, when the forceps was extended toward the animal, the mice treated with higher doses of methylone showed little or no interest in it (X^2=34.25, df=5, P<0.01). Instead, they were sensitive against touch response (X^2=32.63, df=5, P<0.01) and pinch tail response (X^2=29.02, df=5, P<0.01), and escaped rapidly after the test, especially the 100 mg/kg group. When these mice were restrained in a supine position, they resisted strongly but did not show aggression.

No significant differences were found in neurological and muscle functions such as grip strength, however the number of mice that could not lift up their hind limbs on to the wire or fell easily, increased significantly in the 50 mg/kg and over dose group (X^2=24.80, df=5, P<0.01).

In observational endpoints of the automatic nervous system function, the respiratory rate (doses over 12.5 mg/kg), piloerection (over 25 mg/kg) and salivation (over 50 mg/kg), were significantly different from the control. For example, significant differences were observed in respiratory rate at methylone dosed more than

Table 3 Results of functional and observational tests of methylone, MDMA and MAP in mice

Observed behavioral and functional lists	control	Methylone	MDMA	MAP
Stereotypy	0.0 ± 0.0	0.6 ± 0.2	1.0 ± 0.0**	0.8 ± 0.2*
Approach response	0.0 ± 0.0	0.4 ± 0.2	0.6 ± 0.2	0.9 ± 0.1*
Touch response	0.0 ± 0.0	0.3 ± 0.2	1.0 ± 0.3*	1.1 ± 0.1*
Locomotor activity	7712 ± 573	22125 ± 2612**	14097 ± 1268*	10764 ± 1946
Rearing in open field	0.0 ± 0.0	−0.4 ± 0.2	−0.8 ± 0.2*	−0.4 ± 0.2
Tail pinch response	0.0 ± 0.0	0.3 ± 0.2	1.0 ± 0.0**	1.0 ± 0.0**
Wire maneuver	1.8 ± 0.1	0.4 ± 0.6	−1.2 ± 0.2*	−1.4 ± 0.5*
Piloerection	0.1 ± 0.1	0.7 ± 0.4	1.6 ± 0.4*	1.5 ± 0.3*
Salivation	0.0 ± 0.0	0.6 ± 0.3	0.0 ± 0.0	1.1 ± 0.3*
Body temperature (℃)	1.0 ± 0.2	1.7 ± 0.2	2.7 ± 0.3*	1.7 ± 0.3

Details of observation and functional methods are described by Irwin[14]. Locomotor activity was measured for 15 min beginning 15 min after administration. Body temperature was calculated from the difference in rectal temperature before administration to that after open field observation at about 40 min after administration. Data are represented as the mean ± SEM of observation scores, motor activity and body temperature of 6 mice. *P<0.05, **P<0.01, indicates significant difference from the water-treated group.

12.5 mg/kg and over (X^2=27.75, df=5, P<0.01), piloerection at 25 mg/kg and over (X^2=21.44, df=5, P<0.01), and salivation at 50 mg/kg and over (X^2=25.83, df=5, P<0.01). Significant differences in many effects were observed in the 100 mg/kg group, and it was thought that toxic effects appeared because of the fact that locomotor activity decreased in the 50 mg/kg group.

Comparison of methylone, MDMA and MAP effects on behavior

The comparison of the effects on behavior by administration of methylone, MDMA and MAP were performed at the dose of 0.205mmol/kg of each drug, which was equivalent to 50mg/kg of methylone appeared the highest locomotor activity.

Table 3 shows the behavioral and functional changes with significant differences from the treatment and control groups. Mice administered methylone showed more locomotor activity for 15 minutes (F(3,20)=12.23, P<0.01) than mice dosed with MDMA or MAP, and many of the mice administered with methylone had good stimulation and motor function. On the other hand, mice which were administered with MDMA or MAP had significantly decreased motor function, such as difficulties in lifting up hind limbs on to a wire (X^2=14.01, df=3, P<0.01). In addition, they showed an extreme sensitive response to escaping from our approach (X^2=11.54, df=3, P<0.01) and having their tail pinched (X^2=13.41, df=3, P<0.01).

Mice treated with either methylone or MAP showed stereotyped behavior, e.g. sniffing around, gait with elevated pelvic posture, momentary immobility followed by swift movement in the open field, and salivation. MDMA-treated mice showed specific movements, e.g. circling around in one direction, pointing the nose upward, sniffing around and swinging the head right-to-left, however, gait with elevated pelvic posture and frequent stopping were not observed. Of note is the fact that the mice's body temperature increased significantly 40 minutes after the MDMA administration (F(3,20)=6.07, P<0.01).

Rewarding effect of methylone

The CPP test is a screening method which is able to detect addictive substances easily as the procedure is simple and short. In this study,

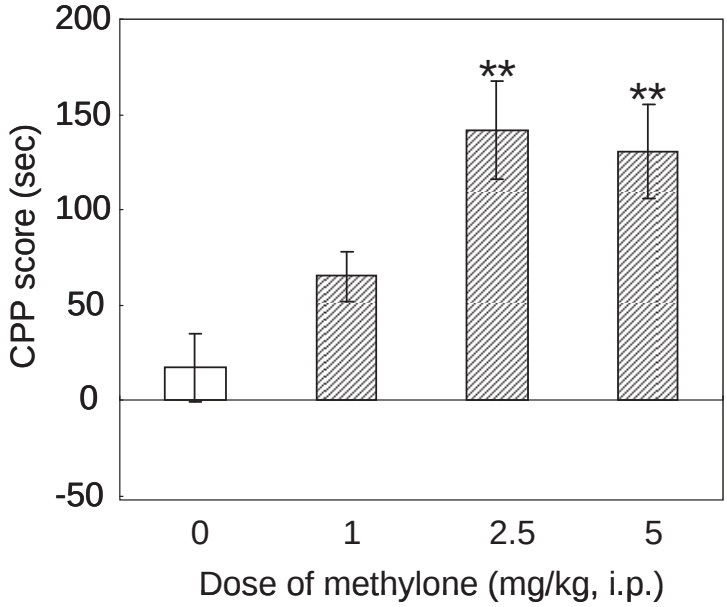

Fig. 3 Effects of methylone on the conditioned place preference test.
Mice were injected with methylone (0, 1, 2.5 or 5 mg/kg). CPP score was expressed in scores calculated as the difference between post-conditioning and preconditioning time spent in the compartment treated with methylone. Data are represented as the mean ± SEM of 14 mice.
** $P<0.01$, indicates significant difference from the saline treated group.

the CPP score of MAP treated mice (positive control) significantly increased compared with saline (negative control) treated mice (date not shown). The CPP score of methylone (2.5 mg/kg and over) treated group increased significantly ($F(3, 52)=7.82$, $P<0.01$), confirming that methylone has a rewarding effect (Fig. 3)

ΔFosB accumulation in NAc

The NAc in the forebrain is the projection area of the reward circuit. ΔFosB accumulation in NAc after chronic administration was found to last for long periods[13, 19-21]. Therefore, levels of ΔFosB accumulation were analyzed by immunohistochemistry. The anti-pan FosB antibody was employed for this study as it recognizes both FosB and ΔFosB. 5 mg/kg of methylone significantly induced FosB positive neurons in the NAc of mice brain ($F(2,15)=9.29$, $P<0.01$) (Fig. 4). The positive control of NAc of MAP treated mice showed similar accumulation of FosB (data not shown).

The expression of fosB and ΔfosB mRNAs

The relative ratio of Δ*fosB* to *fosB* mRNA increased most significantly after acute, not chronic, stimulation[22]. Therefore, we measured *fosB* and Δ*fosB* mRNA in the striatum including NAc of mice with acute administration of methylone and MDMA using quantitative PCR. The common region of *fosB* and Δ*fosB* mRNAs (*fosB* + Δ*fosB*) and the unique region in *fosB* mRNA, which includes an alternative splicing site, were amplified by using real-time PCR. As shown in Figs. 5A and 5B, the expressions of *fosB* + Δ*fosB* ($F(2, 12)=8.19$, $P<0.01$) and *fosB* ($F(2, 12)=19.59$, $P<0.01$) mRNAs were significantly up-regulated by acute administration of methylone and MDMA

Because the amplification efficiency of *fosB* + Δ*fosB* and *fosB* mRNAs were nearly equal at 111% ($R^2=0.98$) and 108% ($R^2=0.99$) respectively, the ratio of *fosB* +Δ*fosB* to *fosB* mRNA was calculated. The ratio of *fosB* +Δ*fosB* mRNA increased significantly ($F(2,12)=14.56$, $P<0.01$)

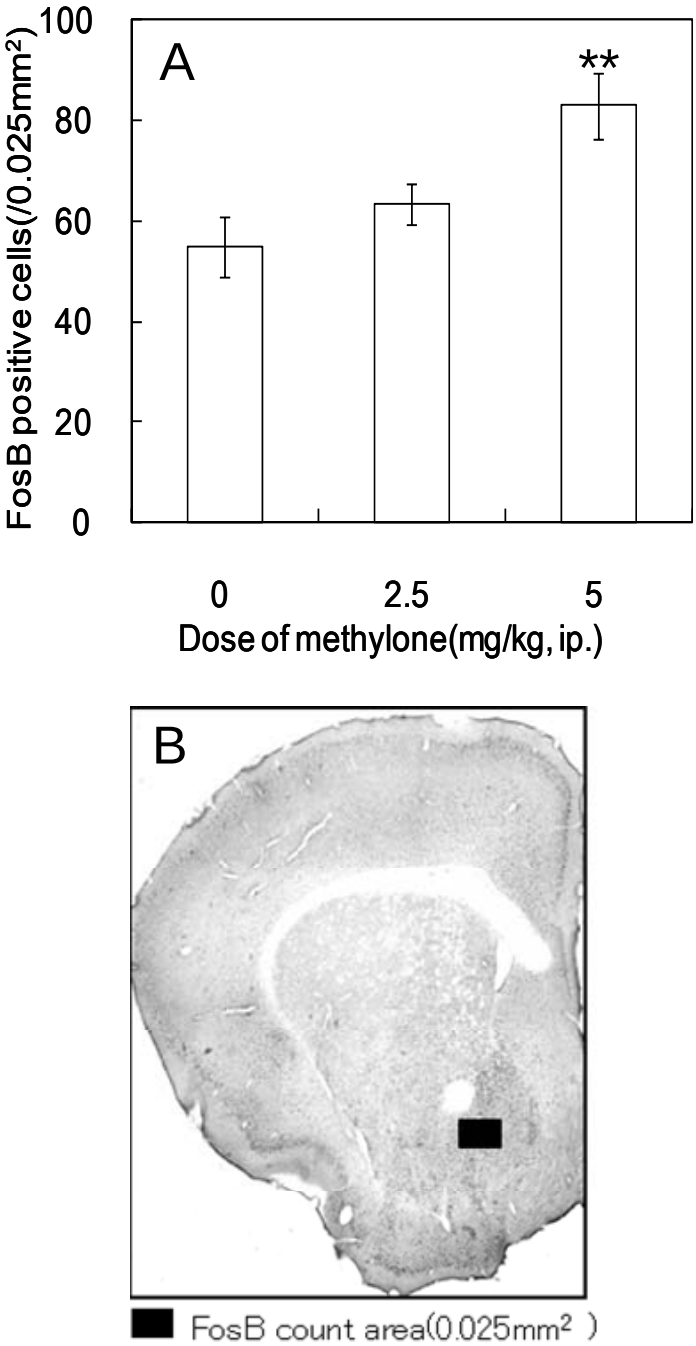

Fig. 4 Effect of methylone on the number of FosB positive cells in the nucleus accumbens of mice. (A) Data are represented as the mean ± SEM of 6 mice. $^{**}P<0.01$, indicates significant difference from the saline treated group. (B) Photographs of count area in the nucleus accumbens core.

in methylone (3.9 fold, $P<0.01$) and MDMA (3.6 fold, $P<0.01$) treated groups (Fig. 5C)

RT-PCR results (Fig. 5D) indicated that the PCR products of Δ*fosB* (749 bp) were detected in all groups, however the PCR products of *fosB* (893 bp) were detected as indistinct bands in the methylone and MDMA administered groups.

Discussion

The purpose of this study was to investigate the different effects of MDMA, MAP and

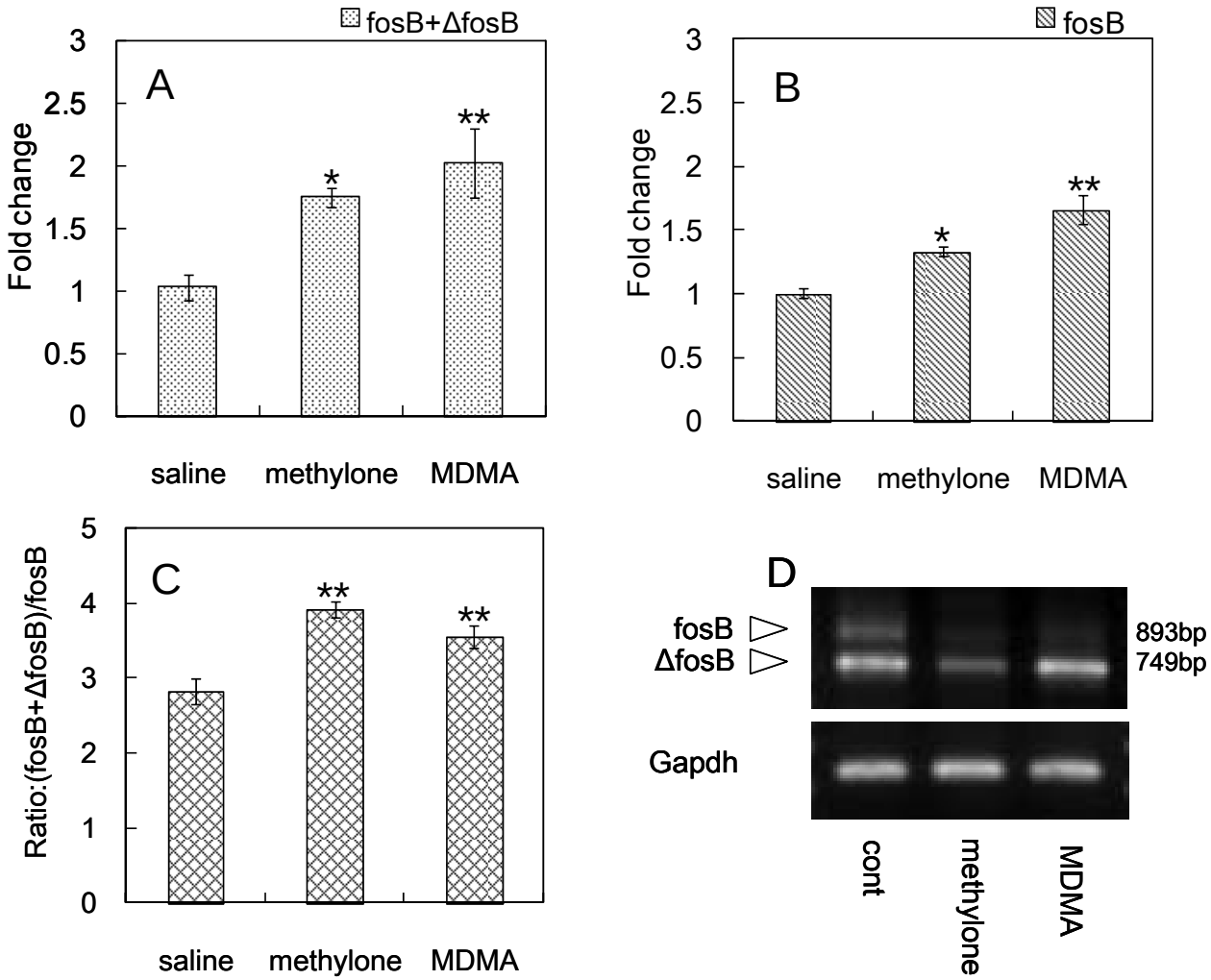

Fig. 5 Expression of *fosB* and Δ*fosB* mRNAs in the nucleus accumbens of mouse brain after acute treatment (i.p.) of methylone (10 mg/kg) or MDMA (9.4 mg/kg).
Data are represented as the mean ± SEM of 5 mice.**P*<0.05, ***P*<0.01, indicates significant difference from the saline treated group. (A) Expression level of *fosB* + Δ*fosB* mRNA using real-time PCR. (B) Expression level of *fosB* mRNA using real-time PCR. (C) The ratio of Δ*fosB* to *fosB* mRNA calculated on the basis of the results of real-time PCR. (D) RT-PCR analysis for *fosB* mRNA and Δ*fosB* mRNA.

methylone, an analog of MDMA, on behavioral toxicity in mice. In addition, mice CPP tests were conducted to detect how methylone can lead to drug addiction. This test also provides information on ΔFosB protein level in the neurons of NAc and the mRNA level of Δ*fosB*. The results indicated accumulation of ΔFosB protein and an increase in Δ*fosB* mRNA after dosing with methylone.

For humans, the usual dosage of MDMA is 100 mg, and for mice, the equivalent dosage is 2 mg/kg MDMA. After the mice were administered with 2 mg/kg, dose dependence was apparent from the results of touch response, pain response, pinna reflex and related response as well as observations of spontaneous and stereotyped behaviors[23]. Methylone dosage for humans is generally 100–200 mg, and it causes milder excitement and euphoria than 100 mg MDMA[7].

As shown in Fig. 2, animals administered methylone at from 12.5 mg/kg to 50mg/kg

showed a dose-dependent increase in locomotor activity. This implies that methylone can be classified as a stimulant similar to MDMA and MAP. Based on behavioral observations, methylone demonstrated weaker effects than MDMA or MAP while methylone created similar types of behavioral changes to MAP rather than MDMA. After methylone or MAP was administered, various degrees of the following effects were observed: sniffing, which is classified as a stereotypic behavior, elevated pelvic posture, frequently becoming immobile, swift movements, and salivation. Animals that were administered with MDMA demonstrated stereotypical behaviors such as circling in one direction, pointing the nose upward, sniffing around and swinging the head from right to left. However, the number of times that the mice reared in the open field was significantly reduced. The animals displayed normal walking posture and frequent immobility was not observed. Of note is that body temperature rose significantly 40 minutes after MDMA administration.

Unlike MAP, MDMA causes both central stimulation and hallucinogenic effects, and these effects are mainly related to the serotonergic nerve system. These effects occur because of inhibition of serotonin re-uptake and acceleration of serotonin release[24, 25]. Nagai et al.[10] reported that methylone has an inhibitory effect is only 3% of that of MDMA on the re-uptake of serotonin, while its inhibitory effects on the re-uptake of dopamine and noradrenaline are at a similar level to MDMA. They also found methylone and MDMA resulted in the release of similar levels of dopamine and norepinephrine, but the level of serotonin release of mehylone was 4% that of MDMA. High serotonin levels induce hyperthermic responses, tension, hyperreflexia, rigidity, tremors and excitement[2]. Therefore, the dissimilarity of symptoms caused by methylone and MDMA is attributed to different effects on the serotonergic nerve endings.

MAP and MDMA both cause strong physical dependence and are well known to establish rewarding effects for mice as well as humans[26, 27]. In this study, treatment with methylone also resulted in rewarding effects in mice (Fig. 3). It was reported that MDMA doses of ≥10 mg/kg in mice caused significant differences on a CPP test[27]. In this study, where methylone was administered to mice at ≥2.5 mg/kg, a significant difference was also observed for the CPP test. A simple comparison of these results is difficult, as the administrative route and mice strain used were different. In brief, methylone appears to cause dependence similar to or stronger than MDMA.

The reward circuit related to drug addiction is composed of the dopaminergic neurons, which run from the ventral tegmental area towards NAc. The NAc is the dopaminergic neurons' projection area. Accumulation of ΔFosB in the NAc is observed after chronic administration of cocaine[19], morphine[28], amphetamine[29], MAP[30], MDMA[31], Δ^9-tetrahydroxycannabinol[32] and many other drugs of abuse. ΔFosB is a member of the Fos family of proteins, encoded by Δ*fosB* mRNA, and is the truncated spliced form of FosB without the C-terminal 101 amino acids from 237th amino acid. ΔFosB suppresses Jun transcription[33], and regulates GluR2[34], Cdk5[35] and c-fos[36] transcription. In this study, immunohistochemistry was employed to prove ΔFosB intranuclear accumulation in NAc by using an antibody of FosB and ΔFosB. The result showed that the FosB positive cell number in the NAc increased significantly in animals administrated with 5 mg/kg of methylone. This finding suggests that methylone has a common effect with MAP on the reward circuit.

The animals that were administered with 2.5mg/kg of methylone showed rewarding effects on the CPP test, however, the FosB positive cell number did not show a significant increase. This finding suggests that ΔFosB accumulation may

be induced by a dosage higher than 5 mg/kg of methylone.

Δ*fosB* mRNA expression significantly increased after acute administration of amphetamine, well known as an addictive drug, but decreased after chronic administration[21]. Thus the relation between *fosB* and Δ*fosB* mRNA expression was examined by acute administration of methylone. Real-time PCR was performed using primers which amplified the common area of *fosB* and Δ*fosB* (*fosB* + Δ*fosB*) mRNA, mRNAs which were significantly increased in methylone and MDMA administered animals. The *fosB* mRNA that contains the splicing site was also increased. The ratio of *fosB* + Δ*fosB* to *fosB* mRNA were 3.9 fold in the methylone treated group and 3.6 fold in the MDMA treated group. This suggests that Δ*fosB* mRNA is up-regulated by methylone as well as MDMA.

Additionally RT-PCR was performed using the primer that amplified *fosB* (893 bp) and Δ*fosB* (749 pb). The electrophoresis shows a clearer band for Δ*fosB* (749 bp) than *fosB* (893 bp). Thus this also suggests Δ*fosB* mRNA has been increased.

In this study, the CPP test demonstrated that methylone is addictive. In addition, significant increases in the ΔFosB protein and mRNA in the NAc were observed by immunohistology. These increases are closely related to the activation of the reward circuit, which again demonstrates that methylone is an addictive drug. On the whole, methylone, with a similar chemical structure to MDMA, causes slightly weaker behavioral toxicity than MDMA. The toxicological profile of methylone appears to have greater similarity to MAP than MDMA, and causes stronger dependence than MDMA.

Drugs of the phenethylamine type are some of the most variable types of drugs. It is difficult to obtain accurate risk assessments of these designer drugs. A minor chemical structural difference may result in the drugs changing from an agonist to an antagonist. Side-chain modification may amplify the risk of addiction as is the case for morphine and heroine. Further detailed toxicological examinations are required for elucidating the effects, pathways and mechanisms of designer drugs.

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Reference

1) Ricaurte GA, McCann UD. Recognition and management of complications of new recreational drug use. Lancet 2005;365:2137-2145.

2) Cami J, Farré M, Mas M, Roset PN, Poudevida S, Mas A, San L, et al. Human pharmacology of 3,4 -methylenedioxymethamphetamine ("ecstasy"): psychomotor performance and subjective effects. J Clin Psychopharmacol 2000;20:455-466.

3) Bossong MG, Van Dijk JP, Niesink RJ. Methylone and mCPP, two new drugs of abuse? Addict Biol 2005;10:321-323.

4) Yasuda I. The identification of illegal drugs and the change of circulation products. Ann Rep Tokyo Metr Inst Pub Health 2007;58:37-45.

5) McNamara S, Stokes S, Coleman N. Head shop compound abuse amongst attendees of the Drug Treatment Centre Board. Ir Med J 2010;103:136-137.

6) Kojima T, Miyazawa M, Doi K. Restriction in the future of uncontrolled substances. Modern Media 2006;52:99-108.

7) Shimizu E, Watanabe H, Kojima T, Hagiwara H, Fujisaki M, Miyatake R, Hashimoto K. Combined intoxication with methylone and 5-MeO-MIPT. Progr Neuro-psychopharmacol Biol Psychiatry 2007;31:288-291.

8) Kamata HT, Shima N, Zaitsu K, Kamata T, Miki A, Nishikawa M, Katagi M, et al. Metabolism of the recently encountered designer drug, methylone, in humans and rats. Xenobiotica 2006;36:709-723.

9) Cozzi NV, Sievert MK, Shulgin AT, Jcob P 3rd, Ruoho AE. Inhibition of plasma membrane monoamine transporters by beta-ketoamphetamines. Eur J Pharmacol 1999;381:63-9.

10) Nagai F, Nonaka R, Satoh H, Kamimura K. The effects of non-medically used psychoactive drugs on monoamine neurotransmission in rat brain. Eur J Pharmacol 2007;559:132-137.

11) Nakagawa Y, Suzuki T, Tayama S, Ishii H, Ogata A. Cytotoxic effects of 3,4-methylenedioxy-N-alkylamphetamines, MDMA and its analogues, on isolated rat hepatocytes. Arch Toxicol 2009;83:69-80.

12) Dal Cason TA, Young R, Glennon RA. Cathinone: an investigation of several N-alkyl and methylenedioxy-substituted analogs. Pharmacol Biochem Behav 1997;58:1109-1116.

13) Nestler EJ, Barrot M, Self DW. ΔFosB: Asustained molecular switch for addiction. Proc Natl Acad Sci USA 2001;98:11042-11046.

14) Irwin S. Comprehensive observational Assessment: Ia. A systematic, quantitative procedure for assessing the behavioral and physiologic state of the mouse. Psychopharmacologia 1968;13:222-257.

15) Moser VC, Becking GC, MacPhail RC, Kuling BM. The IPCS collaborative study on neurobehavioral screening methods. Fundam Appl Toxicol 1997; 35:143-151.

16) Noda Y, Miyamoto Y, Mamiya T, Kamei H, Furukawa H, Nabeshima T. Involvement of dopaminergic system in phencyclidine-induced place preference in mice pretreated with phencyclidine repeatedly. J Pharmacol Exp Ther 1998;286:44-51.

17) Funada M. Evaluation of the rewarding effect of drug by conditioned place preference paradigm: its bases and applications. Folia Pharmacol Jpn 2005;126:10-16.

18) Paxinos G, Franklin KBJ. The Mouse Brain in Stereotaxic Coordinates. 2nd ed. San Diado; Academic press; 2001.

19) Hope BT, Nye HE, Kelz MB, Self DW, Iadarola MJ, Nakabeppu Y, Duman RS, et al. Induction of a long-lasting AP-1 complex composed of altered Fos-like proteins in brain by chronic cocaine and other chronic treatments. Neuron 1994;13:1235-1244.

20) Chen J, Nye HE, Kelz MB, Hiroi N, Nakabeppu Y, Hope BT, Nestler EJ. Regulation of delta FosB and FosB-like proteins by electroconvulsive seizure and cocaine treatments. Mol Pharmacol 1995;48:880-889.

21) Hiroi N, Brown JR, Haile CN, Ye H, Greenberg ME, Nestler EJ. FosB mutant mice: loss of chronic cocaine induction of Fos-related proteins and heightened sensitivity to cocaine's psychomotor and rewarding effects. Proc Natl Acad Sci USA 1997;94:10397-10402.

22) Alibhai IN, Green TA, Potashkin JA, Nestler EJ. Regulation of fosB and DeltafosB mRNA expression: in vivo and in vitro studies. Brain Res 2007; 27:22-33.

23) Satoh K, Fukumori N, Nonaka R, Fuwa T, Tanaka T. Development of assay systems to detect and evaluate the biological effects of (potentially) illegal drugs. Ann. Rep. Tokyo Metr Inst Pub Health 2009;60:21-35.

24) Wall SC, Gu H, Rudnick G. Biogenic amine flux mediated by cloned transporters stably expressed in cultured cell lines: amphetamine specificity for inhibition and efflux. Mol Pharmacol 1995;47:544-550.

25) Green AR, Mechan AO, Elliott JM, O'Shea E, Colado MI. The pharmacology and clinical pharmacology of 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy"). Pharmacol Rev. 2003;55:463-508.

26) Bilsky EJ, Hui YZ, Hubbell CL, Reid LD. Methylenedioxymethamphetamine's capacity to establish place preferences and modify intake of an alcoholic beverage. Pharmacol Biochem Behav 1990;37:633-638.

27) Salzmann J, Marie-Claire C, Le Guen S, Roques BP, Noble F. Importance of ERK activation in behavioral and biochemical effects induced by MDMA in mice. Br J Pharmacol 2003;140:831-838.

28) Nye HE, Nestler EJ. Induction of chronic Fos-related antigens in rat brain by chronic morphine administration. Mol Pharmacol 1996;49:636-45.

29) Murphy CA, Russig H, Pezze MA, Ferger B, Feldon J. Amphetamine withdrawal modulates FosB expression in mesolimbic dopaminergic target nuclei: effects of different schedules of administration. Neuropharmacology 2003;44:929-939.

30) McDaid J, Graham MP, Napier TC. Methamphetamine-induced sensitization differentially alters pCREB and Δ-FosB throughout the limbic circuit of the mammalian brain. Mol Pharmacol 2006;70:2064-2074.

31) Olausson P, Jentsch JD, Tronson N, Neve RL, Nestler EJ, Taylor JR. DeltaFosB in the nucleus accumbens regulates food-reinforced instrumental behavior and motivation. J Neurosci 2006;26:9196-9204.

32) Perrotti LI, Weaver RR, Robison B, Renthal W, Maze I, Yazdani S, Elmore RG, et al. Distinct patterns of deltaFosB induction in brain by drugs of abuse. Synapse 2008;62:358-369.

33) Nakabeppu Y, Nathans D. A naturally occurring truncated form of FosB that inhibits Fos/Jun transcriptional activity. Cell 1991;64:751-759.

34) Kelz MB, Chen J, Carlezon WA Jr, Whisler K, Gilden L, Beckmann AM, Steffen C, et al. Expression of the transcription factor DFosB in the brain controls sensitivity to cocaine. Nature 1999;401:272-276.

35) Bibb JA, Chen J, Taylor JR, Svenningsson P, Nishi A, Snyder GL, Yan Z, et al. Effects of chronic exposure to cocaine are regulated by the neuronal protein Cdk5. Nature 2001;410:376-380.

36) Renthal W, Carle TL, Maze I, Covington HE 3rd, Truong HT, Alibhai I, Kumar A, et al. Delta FosB mediates epigenetic desensitization of the c-fos gene after chronic amphetamine exposure. J Neurosci 2008;28:7344-7349.