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Abuse potential of 2-(4-iodo-2, 5-dimethoxyphenyl)−N-(2-methoxybenzyl) ethanamine (25I−NBOMe); *in vivo* and *ex vivo* approaches

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ABSTRACT

25I−NBOMe ("25-I", "N-Bomb"), one of new psychoactive substances (NPSs), is being abused for recreational purpose. However, the liability for abuse or dependence has not been systematically studied yet. The objective of the present study was to evaluate rewarding and reinforcing effects of 25I−NBOMe using conditioned place preference (CPP) and self-administration (SA) paradigms. In addition, ultrasonic vocalizations (USVs) were measured to investigate relationships between USVs and emotional state regarding dependence on psychoactive substances. To understand molecular mechanism involved in its action, dopamine (DA) level changes were analyzed using synaptosomes extracted from the striatal region of the brain. Expression level changes of SGK1 (serum/glucocorticoid regulated kinase 1) and PER2 (period circadian protein homolog 2), two putative biomarkers for drug dependence, were also analyzed. Results showed that 25I−NBOMe increased both CPP (0.3 mg/kg) and SA (0.03 mg/kg/infusion) and produced higher frequencies in USVs analysis. It also increased DA levels in the striatal region and changed expression levels of SGK1 and PER2. Results of the present study suggest that 25I−NBOMe might produce rewarding and reinforcing effects, indicating its dependence liability. In addition, frequencies of USV might be associated with emotional state of mice induced by psychoactive substances regarding substance dependence. This is the first systemic preclinical report on the dependence liability of 25I−NBOMe and the first attempt to introduce a possible relationship between USVs and emotional state of mice regarding substance dependency. Further studies are needed to clarify the mechanism involved in 25I−NBOMe dependency and determine the usefulness of USV measurement as a method for evaluating dependence liability.

1. Introduction

As one of substituted phenethylamines, 2-(4-Iodo-2,5-dimethoxyphenyl)−N-(2-methoxybenzyl)ethanamine (25I−NBOMe) is structurally related to 2,5-Dimehoxy-4-iodophenethylamine (commonly known as 2C−I). New psychoactive substances belonging to −NBOMe series including 25I−NBOMe are relatively new. This class of hallucinogens is used for recreational purposes (Adam, 2017; Hanks and González-Maeso, 2013). Street names and media nicknames for this class of compounds include "N-Bomb", "Solaris", "Smiles", "Wizard", and so forth. They are often sold on blotter papers that are small pieces of paper infused with substances (Forrester, 2014; Suzuki et al., 2015). 25I−NBOMe is extremely potent as an agonist for serotonin 2A (5-HT_{2A}) receptor. It produces similar psychological and somatic effects to lysergic acid diethylamide (LSD). Its activity has shorter duration than LSD. However, it lasts longer than two other known hallucinogens, psilocin and 2C−B. According to previous review articles, adverse effects in users include high levels of agitation, tachycardia, hypertension, and convulsions (Forrester, 2014; Suzuki et al., 2015; Wood et al., 2015). However, such ill effects reported in these articles were mostly based on anecdotal events. Science-based information on effects of 25I−NBOMe is limited, especially regarding its action in the central nervous system (CNS) and dependence potential. These are essential factors for scheduling the substance by law.

Brain-stimulation reward paradigm plays a major role in initiating the idea of brain DA, a central substrate of brain reward. Conditioned place preference test and self-administration test have been the representative animal behavioral models for investigating rewarding

Abbreviations: CPP, conditioned place preference; SA, self-administration; CNS, central nervous system; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; ECD, electrochemical detector; USV, Ultrasonic vocalization

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effects of a substance for a long time (Mucha et al., 1982; Gorelick et al., 2004). Recently, ultrasonic vocalization (USV) using rodents has been introduced as a putative index feature to measure emotional states (Simola et al., 2012; Burgdorf et al., 2000, 2007; Portfors, 2007). Typically, USVs can be subdivided into two distinct categories based on average frequencies (22 and 50 kHz) in rats (Simola et al., 2012). Moreover, the mesolimbic DA system, the brain circuitry implicated in rewarding effects, is the major component responsible for specific frequencies of USV emissions. For example, rats would emit increased rates of 50 kHz USVs upon delivery of electrical brain stimulation to several reward-associated brain regions (Burgdorf et al., 2000, 2007). In contrast to rats, adult mice do not produce USVs indicative for negative or positive emotional states. Rather, they tend to emit USVs exclusively to facilitate or inhibit social interactions, particularly during mating behaviors (Portfors, 2007).

As stated earlier, dependence is related to mesolimbic dopaminergic pathway in the CNS. Hence, DA levels in CNS can be indicative for predicting the dependence liability of a substance. Several previous investigations have measured neurotransmitter levels using brain slices or synaptosomes (Messripour and Clark, 1982, 1985; Perez et al., 2007).

Regarding dopaminergic effects induced by psychoactive substances, a few biomarkers have been identified. Among them, SGK1 and PER2 are of interest since they are known to be altered in relation with psychological and/or physical dependence induced by psychoactive substances. According to previous studies, SGK1 transcript levels are enhanced by psychostimulant amphetamine and hallucinogenic drug LSD in striatal or neuronal regions (Lang et al., 2006, 2010). Additionally, several previous investigations have shown that opiate withdrawal affects transcriptional rhythms of clock genes PER1 (period circadian protein homolog 1) and PER2 in rat brains (Li et al., 2009, 2010). Especially, PER2 expression appears to be altered in limbic forebrain of rats due to abrupt discontinuation of morphine after chronic administration of the substance (Hood et al., 2011).

The objective of the present study was to characterize rewarding effects of 25I−NBOMe through CPP and SA paradigms. CPP test can evaluate hedonic value (rewarding or aversive) of a drug while SA test can measure motivational/reinforcing effects (Schippenberg and Koob, 2002). USVs were then measured to determine whether such measurement could be used as one of indications for rewarding state of mice. Furthermore, changes of DA levels induced by 25I−NBOMe treatment were measured via high performance liquid chromatography (HPLC) to elucidate the relationship between dopaminergic changes and rewarding effects induced by 25I−NBOMe treatment. Lastly, expression level changes of two known biomarkers, SGK1 and PER 2, were analyzed using extracts of the striatal region to investigate mechanisms of action of 25I−NBOMe at molecular level.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice (age: 7–8 weeks; weight: 25–30 g) were obtained from Central Lab Animal Inc. (Seoul, Korea). For self-administration test, male Sprague Dawley (SD) rats were purchased from Orient Bio Co., Ltd (Seoul, Korea). These rats were housed alone. They weighed 300–400 g at the time of experiments. They were provided free access to food and water except during food training. Both mice and rats were maintained in a temperature (23 ± 1 °C) and humidity controlled (55 ± 5%) room with 12-h light/dark cycle (lights on from 07:00 to 19:00). Laboratory mouse chow and water were provided *ad libitum*. Handling occurred only during the light cycle. All animal experiments in the present study were approved by the National Institute of Food and Drug Safety Evaluation/Ministry of Food and Drug Safety Animal Ethics Board (Approval number: MFDS-1601-11).

2.2. Chemicals

25I−NBOMe (purity > 98%) was synthesized by Kyung Hee University (Seoul, Korea). Methamphetamine hydrochloride (purity > 98%) was purchased from Samung Industry (Seoul, Korea). They were dissolved in vehicle of saline: DMSO: Tween80 (18: 1: 1). Dimethyl sulfoxide (DMSO) and polyoxyethylene sorbitan mono-oleate (Tween 80) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Heparin was obtained from JW-Pharma Co., Ltd (Seoul, Korea).

2.3. Apparatus

The CPP apparatus was manufactured by Saeronbio Inc. (Uiwang, Korea). It consisted of two distinct compartments (black and white) separated by guillotine doors. Dimensions of each compartment of the white and black boxes were 15 × 17 × 15.5 cm. These compartments were illuminated by dim light (12 Lux). The duration of time spent by animals in each compartment for conditioning was recorded by infrared detection via a sensor controller (Saeronbio Inc., Uiwang, Korea). The SA test apparatus was purchased from Med Associates Inc. (St. Albans, VT, USA). The size was 28 × 26 × 20 cm. Its chambers contained two holes: 1) an active hole to deliver a substance through catheter connected to a jugular vein, and 2) an inactive hole not connected to the animal. Infusion pumps were placed outside the chamber and connected to a 10 ml syringe. A computerized system (Med-PC) (Med Associates Inc., Fairfax, VT, USA) was connected to record data. USVs were recorded in a sound-attenuating chamber (30 × 60 × 30 cm) using a pair of high-sensitivity SONOTRACK ultrasonic microphones (Metris B.V., KA Hoofddrop, The Netherlands).

2.4. CPP test

Each test was composed of five phases: (1) Habituation: for the first two days (day 1 and 2), mice were allowed to access both compartments of the apparatus for 15 min once a day; (2) Pre-conditioning: on the third day, mice were allowed to access both compartments for 15 min without substance treatment. Time spent (s) in each compartment was recorded. These values were used as baseline. Accordingly, mice showed preference for a specific compartment (black or white) within the scope of the mean (10–15%). In other words, mice that did not show preference for a certain compartment were selected for further experiments and divided into five groups; (3) Conditioning: from the 4th to the 13th day, the guillotine door was closed. During this period, mice were injected with vehicle or 25I−NBOMe (0.3, 1, 2 mg/kg, i.p.) and placed in the white compartment for 40 min. On the next day (day 5), they received vehicle. They were placed in the black compartment for 40 min. This protocol was repeated for 5 times (10 days); (4) Post-conditioning: on the 14th day, mice were allowed to access both compartments for 15 min. The time spent (s) in each compartment was recorded and results were used as test values; (5) Scoring: Results were calculated based on difference in post-conditioning (test values) and pre-conditioning (baseline values). The CPP system was validated using methamphetamine (1 mg/kg, i.p.) as positive control before test with 25I−NBOMe.

2.5. SA test

To facilitate the acquisition of operant response, rats were initially trained to press a lever to obtain 45 mg of food pellets (Bio-Serv, Frenchtown, NJ, USA) until desired criteria were achieved (100 food pellets over three consecutive days) in 3-h daily sessions. Rats were returned to *ad libitum* feeding conditions after completing food training. For surgeries, rats (n = 5) were anesthetized with pentobarbital sodium (50 mg/kg; Entobar®, Hanlim pharmaceuticals, Seoul, South korea). Briefly, a catheter (0.3 mm inner diameter; 0.64 mm outer diameter; Dow Corning, Midland, TX, USA) was inserted into each rat's right

jugular vein and exited at the rat's shoulder. Catheters were flushed with 0.2 ml of antibiotic gentamicin sulfate (0.32 mg/ml; Shin Poong Pharm Co., Ltd, Seoul, Korea) in heparinized saline (30 IU/ml). The rat then received an intramuscular injection of procillin (20000 IU/ml) in saline followed by 0.2 ml of heparinized saline alone daily during the experimental period. After the surgery, each rat was allowed to recover in a controlled cage for at least 7 days. Then rats self-administered substances at the dose referred to the dose which presented the highest value in the CPP test or vehicle (DMSO: Tween80: saline = 1: 1: 18, 0.1 ml/infusion) for 5 s during a 2-h session on a fixed-ratio 1 schedule.

Testing procedures were as follows. At the start of a session, two response levers were placed into the chamber. Pressing the right lever resulted in delivery of 0.1 ml of a drug solution over 4 s. During an injection, a stimulus light above the active lever was illuminated and lasted during the time-out period (20 s) that followed each injection. Pressing the left lever was counted. However, it had no programmed consequences. Sessions were ended by withdrawal of levers. The SA test system was validated using methamphetamine (0.05 mg/kg/infusion, i.v.) before testing 25I−NBOMe.

2.6. Measurement of USVs

Animals were tested following chronic injection (i.p.) of vehicle, 1 mg/kg amphetamine, and 0.3 mg/kg 25I−NBOMe to naïve mice under the CPP test schedule. On the day of testing, animals were acclimated to the test apparatus which consisted of Metris Chambers each over-fitted with a pair of high-sensitivity SONOTRACK ultrasonic microphones (Metris B.V., KA Hoofddrop, The Netherlands). The inside of the chamber was shielded by lining the floor and side walls with sound insulation materials. After 10 min in the chamber, the apparatus was activated for 30 min in order to record any baseline USVs or detect potential interfering noise. These animals were then carefully injected with substances (vehicle, methamphetamine, or 25I−NBOMe) and placed back into the chamber. The SONOTRACK system was then simultaneously activated and monitored concurrently. Drug-induced USVs frequencies were continuously monitored for up to 30 min.

2.7. Measurement of DA levels in synaptosomal fractions

Synaptosomes were prepared as previously described (Messripour and Messripour, 2013; Birch and Fillenz, 1985; Messripour and Clark, 1989) with slight modifications. Briefly, untreated male C57BL/6 mice were killed by cervical dislocation and decapitation. The striatal region of each brain was quickly removed (n = 3). The striatum was homogenized in 10 vol of ice-cold 0.32 M sucrose using a Dounce tissue grinder (Kontes, USA). Lysates were then centrifuged at 3000 × *g* for 10 min at 4 °C. The supernatant (S1) containing crude synaptosomal fraction was transferred to a new tube. The S1 lysate was added and diluted with Krebs-Hepes buffer pH 7.4 (117 mM NaCl, 4.8 mM KCl, 2.5 mM $MgCl_2$, and 25 mM Hepes) at 1:1 and then centrifuged at 10,000 × *g* for 20 min at 4 °C to obtain pellet (P1).

Protein concentrations of the cytosol, synaptosome, and brain tissue were determined with Smart BCA Protein Assay Kit (iNtRON Biotechnology, Seongnam-si, South Korea). Proteins (10–60 μg protein per lane) were separated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and then electrophoretically transferred to polyvinylidene difluoride membranes (Invitrogen, Carlsbad, CA, USA) for 2 h at 200 mA. These membranes were blocked with 5% skim milk prior to incubation with primary antibody overnight at 4 °C. After three washes with Tris-buffered saline containing 0.1% Tween-20 (TBST), membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature and then washed three times with TBST. The following primary and secondary antibodies were used: rabbit anti-N-Methyl-D-aspartic acid receptor 2A (NMDAR2A; G9038, Sigma, St. Louis, MO, USA; 1:3000 dilution), mouse anti-glyceraldehyde-3′-phosphate dehydrogenase (GAPDH) (CB1001, Millipore, Billerica, MA, USA; 1:1000 dilution), anti-rabbit IgG HRP-linked antibody (7074S, Cell Signaling Technology, MA, USA; 1:2000 dilution), and anti-mouse IgG HRP-linked antibody (7076S, Cell Signaling Technology, MA, USA; 1:2000 dilution). Blots were visualized using enhanced chemiluminescence (ECL) detection method by incubating with mixture of ECL reagents provide by ECL detection kit (#32132, Thermo Fisher Scientific, Waltham, MA, USA) for 5 min at room temperature followed by exposure to chemidoc (Bio-rad, Hercules, CA, USA) for a few minutes. Intensity of bands on blots was quantified by densitometirc analysis using lab program (version 4.1, Bio-rad, Hercules, CA, USA).

Pellet P1 was mixed with 80 vol of 20 nM DA and then incubated at 37 °C for 15 min After centrifuging at 10,000 × *g* for 10 min at 4 °C, pellet P2 was obtained. Thereafter, several doses of 300 μl methamphetamine (0.01, 0.1, 1, 10, and 100 μM) and 25I−NBOMe (0.01, 0.1, 1, 10, and 100 μM) were added to the same amount of P2 prior to incubation at 37 °C for 15 min. The supernatant was obtained after the addition of 1 ml of 0.1 M perchloric acid and centrifugation at 20,000 × *g* for 3 min at 4 °C. After filtration, the supernatant was measured using a HPLC-electrochemical detector (ECD) instrument (DIONEX UltiMate 3000, Thermo Fisher Scientific, MA, USA).

DA levels in synaptosomes were detected using an Acclaim™ RSLC120 C18 column (2.2 μm 120 Å 2.1 × 50 mm; Thermo Fisher Scientific, MA, USA) and an oven temperature of 35 °C. The flow rate was at 0.5 ml/min, and the injection volume was 30 μl. The voltage of the ECD was maintained at 250 mV. Sample detection was conducted for 10.2 min. DA was separated sing a mobile phase consisting of 6.9 g NaH_2PO_4, 250 mg 1-heptanesulfonic acid sodium salt, 80 mg EDTA (pH 3.2), and 5% HPLC-grade methanol. DA levels were analyzed using Chromeleon™ 7 (Thermo Fisher Scientific, MA, USA).

2.8. Expression levels of biomarkers by quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from mouse striatum using a total RNA extraction kit (iNtRON Biotech, Daejeon, Korea). Complementary DNA (cDNA) was synthesized from total isolated RNA using a SuperScript III first-strand synthesis system (Invitrogen). Subsequent quantitative real-time PCR was performed with an iCycler iQ5 Real-Time Detection System (Bio-Rad, Hercules, CA, USA) using SYBR GreenER qPCR SuperMix Universal (Invitrogen). For polymerase activation, the initial incubation was conducted at 50 °C for 2 min followed by 95 °C for 10 min. Then 40 cycles of 95 °C for 15 s and 60 °C for 1 min were performed. cDNA was included in a 25-μL volume PCR reaction with the following components: 0.125 μL each of forward and reverse primers, 12.5 μL SYBR Green, and 0.5 μg of cDNA with sterilized water. Primers used were as follows: mouse SGK1, forward 5′- GAAAGTGATCGGAAA GGGCA -3′ and reverse 5′-ACAGAACATTCCGCTCTGAC-3'; mouse PER2, forward 5′- GCCCAGCGGAAACGA GAAC -3′ and reverse 5′-GAGTCTGAAGGCATCAG -3'; and mouse GAPDH, forward 5′-TGTCAA GCTCATTTCCTGGT-3′ and reverse 5′-CTTACTCCTTGGAGGCCATG-3'. Results were normalized to GAPDH and quantified relative to expression in vehicle samples. For relative quantification calculation, $2^{-\triangle\triangle CT}$ method was used where: $\triangle\triangle CT = (C_{T,target} - C_{T,GAPDH})$ experimental sample – $(C_{T,target} - C_{T,GAPDH})$ control sample.

2.9. Statistical analyses

All results are expressed as means and standard error of the mean (± SEM) using Prism 6.0 software (GraphPad Software, Inc.). Data from CPP test, HPLC, and molecular assays were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test. For SA and USVs tests, statistical analyses were performed using two-way ANOVA with Bonferroni post-hoc test. Statistical significance was set at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the vehicle-treated group.

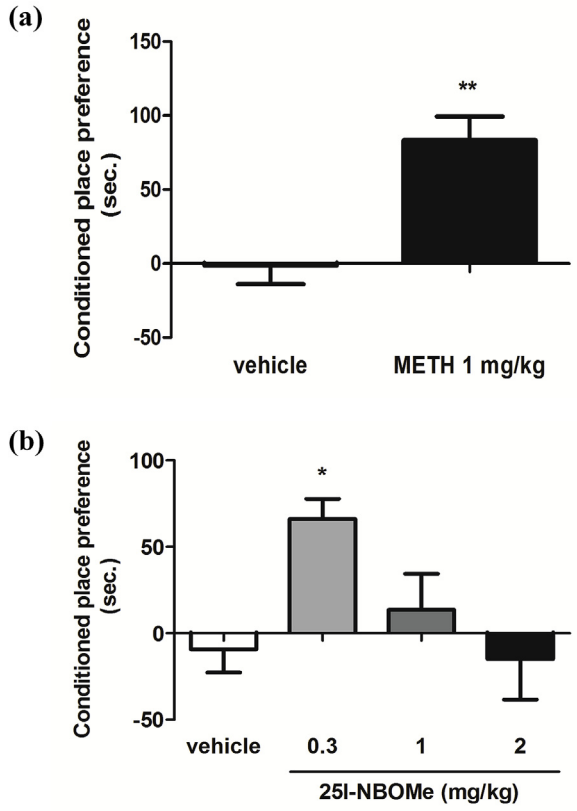

Fig. 1. Effects of intraperitoneally injected 25I−NBOMe determined by CPP test. (a) METH (b) 25I−NBOMe induced CPP in mice. Data are expressed as mean ± standard error of the mean of six animals per group. $^{*}P < 0.05$, $^{**}P < 0.01$ indicate statistical significance compared to vehicle control treated group by one-way analysis of variance followed by Bonferroni post-hoc test.

3. Results

3.1. CPP test

CPP test was conducted using unbiased method. Place preference for substance-paired compartment followed by five pairs of conditioning with 25I−NBOMe tested at three doses (0.3, 1 and 2 mg/kg, i.p.) was calculated. To verify the test system, methamphetamine (METH) at 1 mg/kg and vehicle were used as positive control and negative control, respectively (Fig. 1a). Methamphetamine is a well-known 'hard drug' of phenethylamine class in which 25I−NBOMe is included. After the validation process, three doses of 25I−NBOMe were administered to separate mice and their preference for substance-paired compartment was measured. ANOVA for each drug condition was significant ($p < 0.05$). Post-hoc tests indicated that mice treated with 25I−NBOMe at 0.3 mg/kg dose spent more time in the white drug-paired chamber compared to vehicle-injected mice paired with the black chamber. Collectively, 25I−NBOMe showed a dose response curve, with each drug producing a CPP effect (Fig. 1b).

3.2. SA test

SA test was performed for 2 h (1 session) per day with a fixed ratio 1 schedule. The effect of METH (0.05 mg/kg/infusion) was tested to validate the SA paradigm prior to the test with 25I−NBOMe. Collectively, METH significantly increased the number of infusions on days 2, 4, and 7 and significantly increased the number of active lever presses on days 2, 4, 5, and 7 of SA compared to the vehicled-treated group (Bonferroni post-hoc test) (Fig. 2a and e). The number of infusions and active lever presses for 25I−NBOMe (0.03 mg/kg/infusion) were not significantly different compared to the vehicle-treated group according to a two-way ANOVA (Fig. 2c and g).

Fig. 2i and k describe the number of presses to the inactive lever, which was not different between either of the NPS-treated groups and the vehicle-treated group on any of the days.

Fig. 2b, d, 2f and 2h represent the mean number of infusions and mean number of active lever presses from days 5–7. According to a two-way ANOVA, rats in the METH-treated group SA significantly more infusions and active lever presses than did the vehicle-treated group (Fig. 2b and f). However, 25I−NBOMe-treated group SA was tended to be higher than that in the vehicle-treated group (Fig. 2d and h). Also, mean number of inactive lever presses from days 5–7 was no significant difference between groups (Fig. 2j, l).

3.3. USV behavior

To investigate whether emotional states induced by psychoactive substances could change USV frequencies in mice, SONOTRACK system was used. Methamphetamine and 25I−NBOMe significantly produced higher fundamental frequencies than vehicle ($p < 0.05$ and $p < 0.01$, respectively, Fig. 3b and c). However, the total number of calls was not changed (Fig. 3a), suggesting that frequency might be more likely to be associated with the emotional state due to administration of psychoactive substances.

3.4. DA releases in synaptosomes

To confirm the presence of synaptosomes, $NMDA_{2A}$ receptor levels were assessed via western blotting. Expression levels of $NMDA_{2A}$ receptors in extracted synaptosomes were significantly higher than those in the cytosol (Fig. 4a). Extracted synaptosomes were then treated with test substance, with methamphetamine as positive control and vehicle (Krebs-HEPES buffer) as negative control, and then changes in DA levels were measured using HPLC-ECD. Results showed that 25I−NBOMe and methamphetamine significantly increased DA levels at doses of 0.1, 10, and 100 μM (Fig. 4b).

3.5. Expression levels of SGK1 and PER2 genes in the striatum

Following the last test of 25I−NBOMe CPP, gene activities in the vehicle control group and 25I−NBOMe 0.3 mg/kg group were evaluated by real-time PCR analysis. Results showed that 0.3 mg/kg 25I−NBOMe significantly increased SGK-1 levels but decreased PER2 levels in striatal region ($p < 0.05$, Fig. 5a and b). Considering changes in gene expression related to drug dependence, it could be concluded that 25I−NBOMe can act on the CNS that is liable for its dependence.

4. Discussion

25I−NBOMe is a powerful hallucinogen with effects similar to LSD. It continues to pose a danger to the society (Lawn et al., 2014). A previous research has shown that 25I−NBOMe produces locomotor activities similar to other phenethylamine hallucinogens (Halberstadt, 2017). Another study has reported that 25I−NBOMe can produce robust and potent head-twitch behavioral responses (HTR) in mice (Hanks and González-Maeso, 2013). Although these articles have implied that 25I−NBOMe can act on the CNS, information on its dependence liability is limited.

In the present study, two of widely used animal models of drug addiction were employed: CPP test and SA test (Torres et al., 2008; Shram and Lê, 2010). CPP results of the present study showed that 25I−NBOMe produced place preference, similar to methamphetamine, the positive control, indicating that the rewarding effect of 25I−NBOMe would be comparable to that of methamphetamine. Many review articles have suggested that a lot of new psychoactive substances could

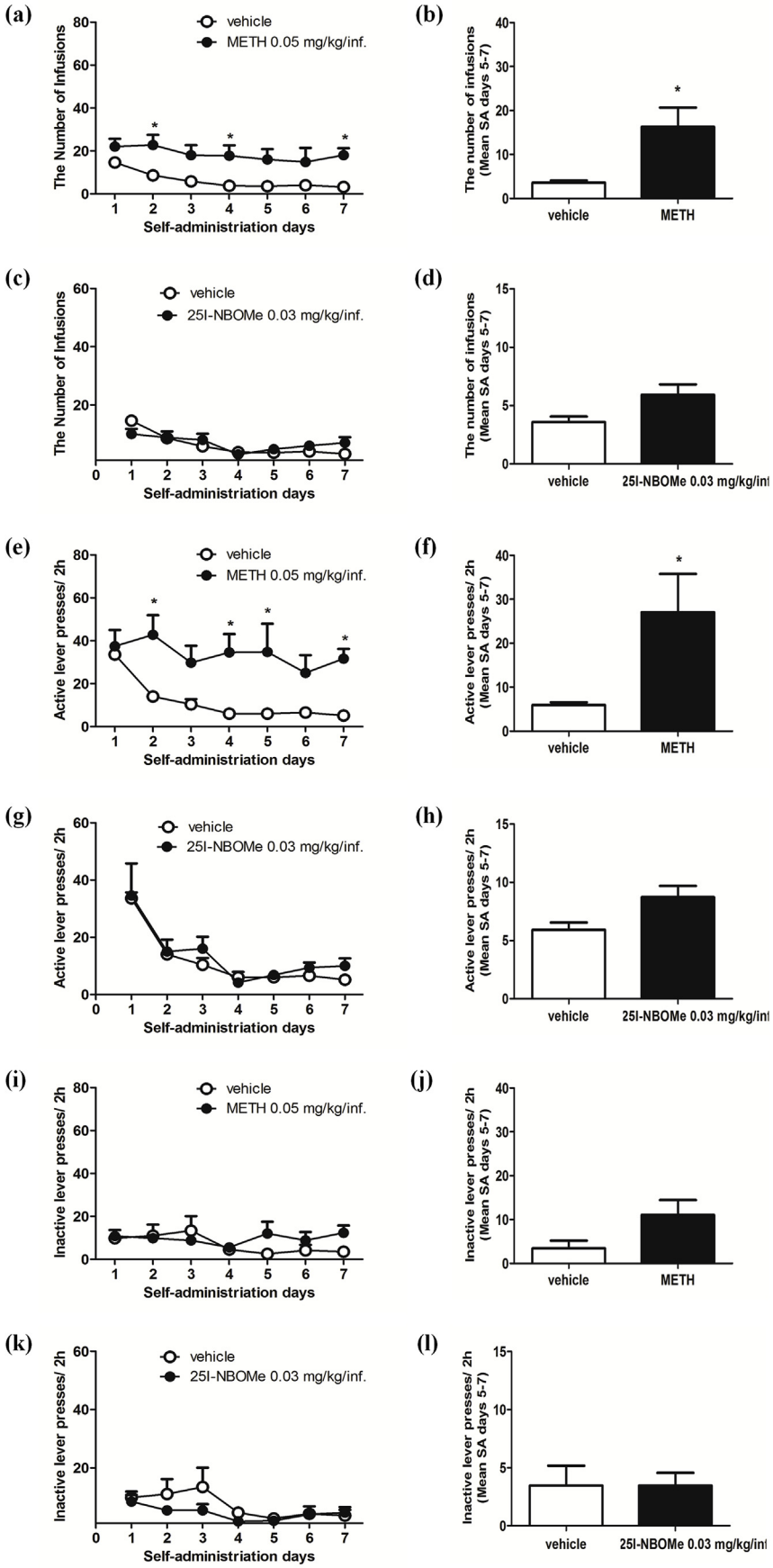

Fig. 2. Acquisition of METH (upper panel) and 25I−NBOMe (lower panel) SA in SD rats with drug infusions (a, c), lever pressing responses (e, g), and inactive lever pressing responses (i, k) during 7 days (one 2-h SA session per day) under the FR 1 schedule. Open symbols indicate vehicle SA results while filled symbols show SA results for the drug. The mean number of infusions (b, d) and lever pressing responses (f, h), and inactive lever pressing response during stable periods (5th to 7th day) of SA are also presented. Data represent the mean ± standard error of the mean of five animals per group. $^{*}P < 0.05$ indicated statistical significance compared to vehicle control treated group, two-way analysis of variance followed by Bonferroni post-hoc test.

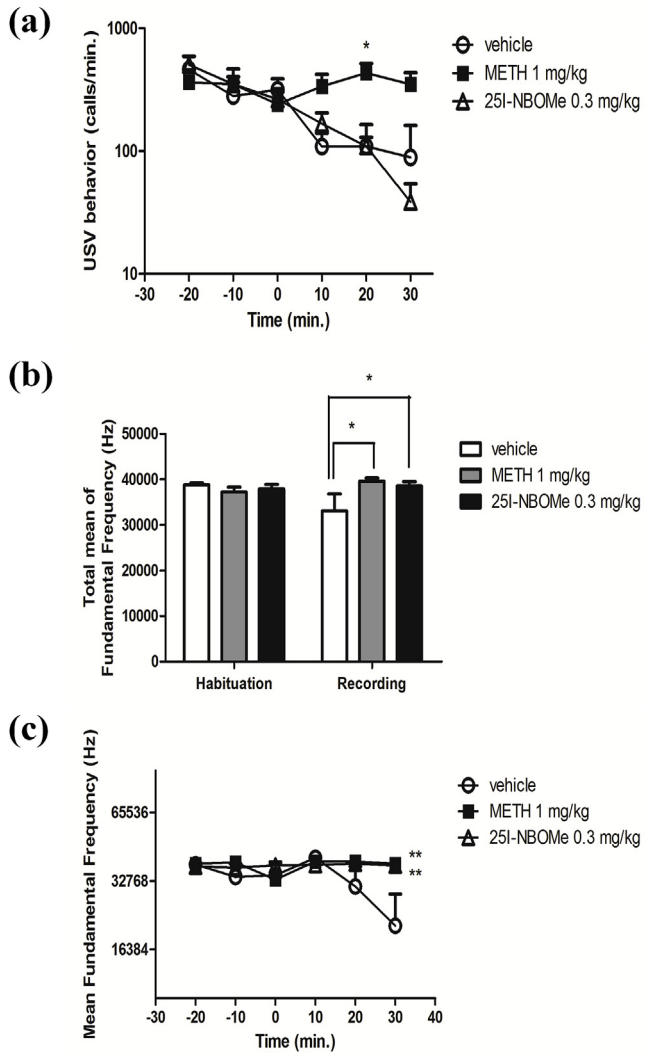

Fig. 3. Effects of administration on 25I−NBOMe-induced USVs. (a) Total numbers of USVs calls, (b) Mean of total fundamental frequencies (FF), and (c) Mean of time interval fundamental frequencies (Hz) by repeated 25I−NBOMe administration. Each bar represents mean ± standard error of the mean of 7–8 animals per group.

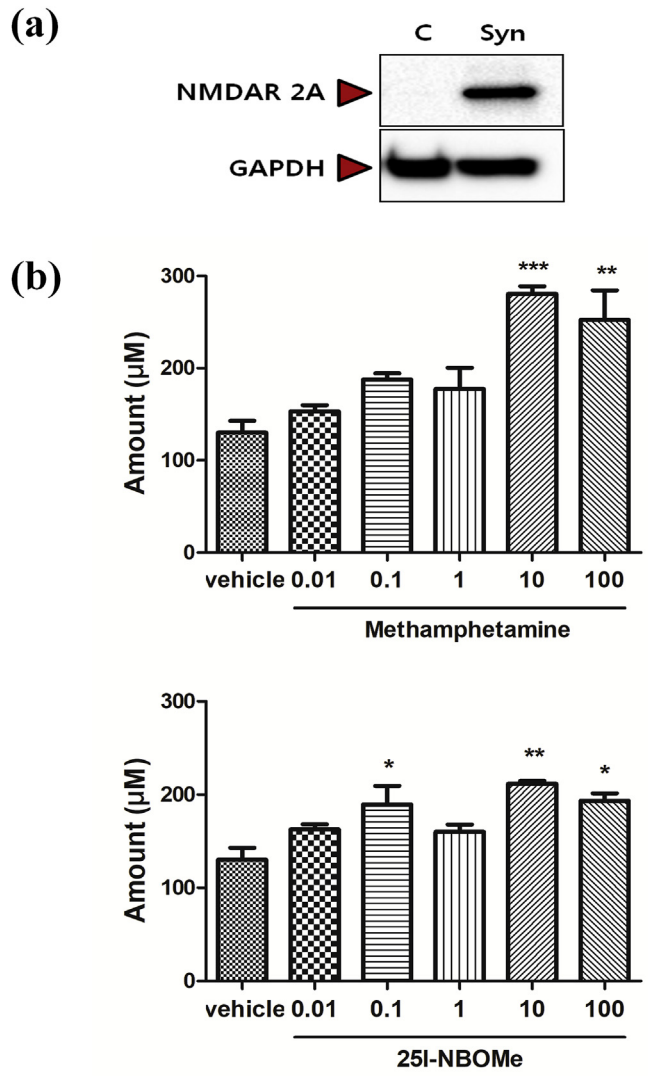

Fig. 4. Changes in DA levels induced by administration of 25I−NBOMe. (a) Protein expression in striatal synaptosomes of mice. A total of 10 μg of proteins obtained from mouse brain tissue cytosol (C) and synaptosome (Syn) were analyzed by western blotting. $NMDA_{2A}$ receptor was used as a synaptic marker whereas GAPDH was used as an internal control for western blotting. An analysis of blots was performed via digital image analysis with chemidoc (Bio-rad). (b) Changes in DA levels induced by administration of methamphetamine and 25I−NBOMe. DA levels were dose-dependently increased in striatal synaptosomes from mice based on high-performance liquid chromatography-electrochemical detector (ECD) method (flow rate: 500 μl/min, ECD voltage: 250 mV, sample injection: 40 μl). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ indicate statistical significance compared to vehicle control treated group, one-way analysis of variance, followed by Bonferroni post-hoc test.

cause ill effects comparable to 'hard drugs' such as methamphetamine, cocaine, and some opioids (Elliott and Evans, 2014; Home Office, 2014; King and Kicman, 2011). 25I−NBOMe produced relatively lower level of overall SA compared to that of CPP in the present study. Whether food deprivation before the test is necessary has been a matter of debate. This means that food factor has certain effects on SA responses (Cadoni et al., 2003; Stafford et al., 2015). Although food can influence SA results at some extent, food deprivation is considered to be effective in increasing sensitivity (O'connor et al., 2011). However, this factor should be considered carefully. In this regard, data during days 5–7 in the present study would be meaningful as lever responses in the first few days could be affected by residual memory of food training or 12 h of food deprivation before the test.

Psychostimulant agents known to stimulate the mesolimbic system also can elicit spontaneous emission of 50 kHz ultrasonic vocalizations in rats (Williams and Undieh, 2016). Knutson et al. have previously reported that amphetamine injection in the nucleus accumbens (NAc) of the ventral striatum can selectively evoke 50-kHz USVs in rats, supporting the notion that elevated DA levels within the mesolimbic region may unconditionally elicit a state of reward anticipation (Knutson et al., 2002). However, information of USVs in adult mice during positive or negative emotional state is considerably limited. In the present study, the frequency range was significantly increased due to administration of 25I−NBOMe at a dose that the highest CPP score was produced. The range was similar to the case of methamphetamine. However, the number of calls was not changed by these substances. Results of USVs measurement suggested that the frequency range could be indicative to substance-induced rewarding state of mice. The present study could be meaningful in that this was the first attempt to investigate USVs in mice in light of emotional state. Further studies are needed to confirm this hypothesis.

It is well known that mesolimbic pathway plays a critical role in drug abuse and addiction. In particular, activity of DA neurons in the ventral tegmental area (VTA) is known to mediate the rewarding action

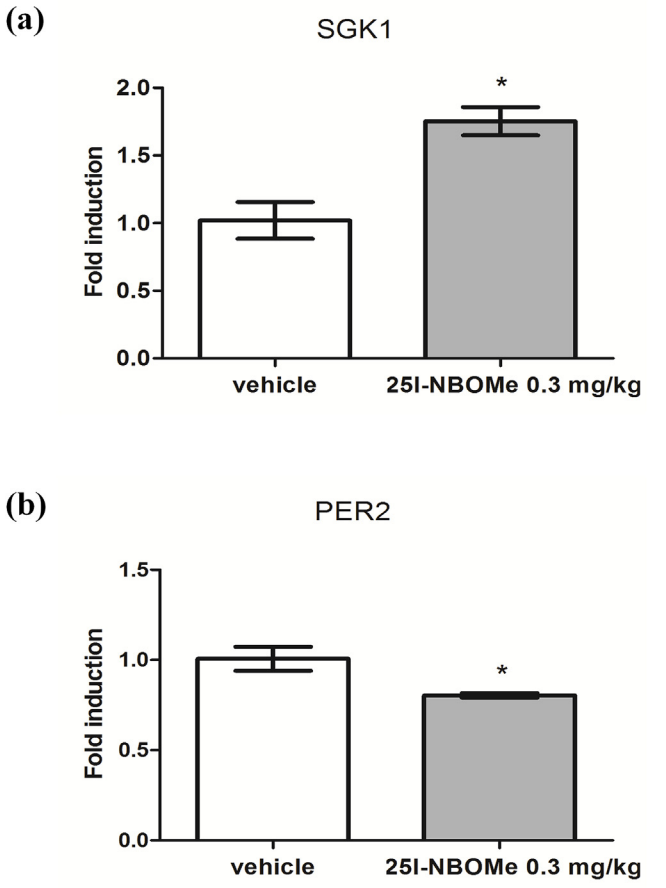

Fig. 5. Expression levels of SGK 1 and PER 2 mRNAs by qRT-PCR. Expression levels of (a) SGK 1 and (b) PER 2 mRNAs in the striatum of mice following repeated 25I−NBOMe administration. Each bar represents mean ± standard error of the mean of three animals per group. *$P < 0.05$ indicated statistical significance compared to vehicle control treated group, one-way analysis of variance followed by Bonferroni post-hoc test.

of psychostimulants, partly through DA signaling increases in the NAc (Dragomir et al., 2017; Corrigall et al., 1992). In addition, several previous studies have emphasized the role of the striatum as a whole, including both the shell and core components of the NAc, in the processes leading to drug abuse first and then leading to addiction (Robbins and Everitt, 1992; Porrino et al., 2004). The existence of synaptosomes can be confirmed through evaluating expression of $NMDA_{2A}$ receptors since it has been reported that $NMDA_{2A}$ receptors are abundant in the presynaptic region (Corlew et al., 2007). Considering these previous findings, DA levels in synaptosomes extracted from the striatal region after treatment with psychoactive substances could be used as one indicator for rewarding effects of these substances. In this regard, increased DA levels after treatment with 25I−NBOMe could indicate a rewarding effect of the substance. The dopaminergic pathway could be involved to some extent.

Recent studies have also suggested that SGK1 and PER2 are putative biomarkers for behavioral effects of drug-induced addiction. SGK1, one of genes upregulated by morphine and cocaine administration in the VTA (Heller et al., 2015), is also upregulated by administration of amphetamine, ethanol, heroin, morphine, and methamphetamine in the striatal region (Piechota et al., 2010). SGK1 expression was significantly increased in 25I−NBOMe-treated groups in the present study, thereby confirming previous findings. In terms of functions of PER2, several previous studies have shown that morphine, heroin, and cocaine can down-regulate the expression of PER2 gene, suggesting that PER2 could be related to consumption behavior (Li et al., 2009; Perreau-Lenz and Spanagel, 2008). In addition, Garmabi et al. have suggested putative relationship between PER2 and vulnerability to morphine preference in mice (Garmabi et al., 2016). PER2 has also been suggested to be associated with regulation of dopamine 2 receptor (D2R) expression in the brain. As such, it might be associated with cocaine addiction (Castañeda et al., 2004; Abarca et al., 2002). The present study showed that PER2 was down-regulated after treatment with 25I−NBOMe, corresponding to previous findings. Further studies using gene-regulated model are needed to elucidate detailed pathways involving SGK1 and PER2.

There are some limitations to be considered regarding the SA paradigm used in the present study. Since the present study used only one dose and schedule (FR1), further studies would be needed using various doses and schedules to elucidate the reinforcing effect or break point of 2-DPMP.

5. Conclusions

In conclusion, results of CPP and SA tests in the present study indicated that 25I−NBOMe had dependence liability, particularly in the aspect of rewarding effects. CNS pathways involving SGK1 and PER2 and DA level changes in the striatal region were associated with these behavioral changes. In addition, USV frequency range might be used as one of indicative factors of emotional states induced by psychoactive substances. Further studies are needed to confirm the usefulness of USV measurement in mice in the aspect of dependency and detailed function of these two putative biomarkers.

Conflicts of interest

The authors declare that they have no conflict of interest.

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