INTRODUCTION

Substance dependence is defined as loss of control when using a substance or compulsive seeking and taking of a substance despite adverse consequences (Koob, 1996). The symptoms of substance dependence include the need to use increased doses of the substance, withdrawal symptoms, unsuccessful attempts to decrease substance use, and continued use in spite of negative consequences (Nestler, 2013).

Recently, new psychoactive substances, such as synthetic cannabinoids and synthetic cathinones, have become problematic worldwide since their chemical structures can be easily modified to avoid detection in drug screens. Additionally, drugs prescribed for medicinal purposes (e.g., for weight loss, attention deficit/hyperactivity disorder [ADHD], and sedation) are increasingly being abused. In the present study, using rodents, we evaluated the potential for dependence on lisdexamfetamine, an emerging psychoactive substance.

Lisdexamfetamine dimesylate, which consists of L-lysine covalently bound to D-amphetamine, is a prodrug used to treat ADHD (Hurd and Ungerstedt, 1989). The metabolic route of conversion for lisdexamfetamine is unusual: after absorption into the bloodstream, it is metabolized by red blood cells to yield D-amphetamine and the natural amino acid L-lysine by rate-limited, enzymatic hydrolysis (Pennick, 2010). It has been reported that lisdexamfetamine has the ability to elicit stimulant pharmacological effects in humans when given at high doses (Mantle et al., 1976; Heal and Pierce, 2006; Heal et al., 2009; Jasinski and Krishnan, 2009). Furthermore, lisdexamfetamine seems to act on dopamine neurotransmission, either directly or indirectly, since individuals given the substance experience feelings of euphoria.

In this regard, lisdexamfetamine likely has some potential for abuse; however, information on lisdexamfetamine dependence comes mainly from anecdotal or case reports, rather than from scientific data. Therefore, we evaluated the abuse potential of lisdexamfetamine using experimental animals in the present study. Psychological dependence was evaluated with the conditioned place preference (CPP) and self-administration tests, which are often used in the field (Mucha et al., 1982; Gorelick et al., 2004). The CPP test is used when determining a substance’s rewarding effect, i.e., whether it
gives a positive sensation such as pleasure, whereas the self-administration test is used to evaluate a substance’s reinforcing effect (Koob, 1992; Taylor, 2002). The two paradigms are related to the dopamine pathways in the brain, especially the mesolimbic pathway, which flows from the ventral tegmental area to the nucleus accumbens, amygdala, hippocampus, and other areas (Meyer and Quenzer, 2013).

Changes in dopamine levels induced by the lisdexamfetamine were analyzed using high-performance liquid chromatography (HPLC) after synaptosomes were extracted from striatal regions in order to investigate the mechanisms of dependence of the lisdexamfetamine. Since neurotransmitters interact with their receptors, and the receptors for neurotransmitters are expressed abundantly in synapses, synaptosomes can be identified along with well-expressed receptor proteins. The synaptosome was first introduced as a “nerve ending particle” in 1964 (Whittaker et al., 1964; Whittaker, 1965) and is known to be a useful tool for studying the structure-function relationships of synaptic release (Abekawa et al., 1994; Ivanikov et al., 2013). While neurotransmission in the synaptosome has been analyzed using isotopes in several previous studies (Whittaker, 1965; Ivanikov et al., 2013), HPLC has rarely been used to measure neurotransmitter levels in striatal synaptosomes.

Here, in order to elucidate the potential for psychological dependence of lisdexamfetamine, we first investigated the rewarding and reinforcing properties of the substance. Next, we analyzed the dopamine levels associated with lisdexamfetamine administration, as identified by Western blots, using HPLC on striatal tissue from rat brains. The data indicate the possibility of using this method as an in vitro testing system to predict the effects of an unknown substance on the pleasure circuits in the brain.

MATERIALS AND METHODS

Animals and substances

Male Sprague-Dawley rats (weighing 250-300 g) and male ICR mice (weighing 22-23 g) were obtained from the Ministry of Food and Drug Safety (AAALAC member, Osong, Korea) and were housed (temperature: 23 ± 1°C, humidity: 55 ± 5%) in a room with a 12 h light/dark cycle (lights on from 08:00 to 20:00). The animals received a solid diet and tap water ad libitum, and husbandry conformed to the Guide for the Care and Use of Laboratory Animals (National Research Center, 2001). We performed all experiments between 09:00 and 18:00. All animal experiments in the present study were approved by the National Institution of Food and Drug Safety Evaluation/Ministry of Food and Drug Safety Animal Ethics Board (approval number: 1401MDFS15).

Lisdexamfetamine was purchased from Cayman Chemical (Ann Arbor, MI, USA), while methamphetamine HCl and cocaine were purchased from Sigma (St. Louis, MO, USA). For the CPP test, 15 mg/kg of cocaine and 5 doses of the test substance (lisdexamfetamine [0, 1, 2.5, 5, and 10 mg/kg]) were intraperitoneally administered to ICR mice. For the self-administration test, 250 µg/(kg·infusion) of cocaine, and 125 µg/(kg·infusion) of lisdexamfetamine were intravenously administered to Sprague-Dawley rats. For the HPLC analyses, six doses of methamphetamine and lisdexamfetamine (0.01, 0.1, 1, 10, and 100 µM) were administered to the striatal synaptosomes in the brains of rats.

Apparatus

The CPP test apparatus for mice has three distinct compartments (white, black, and gray), which are separated by automatic doors. Infrared photo-beam detectors were added to automate data collection. The overall inside dimensions of the apparatus are 15.8×17×15.5 cm. The manufacturer provided the mounting holes for the ENV-013 IR Infrared Sensor Package (Med Associates, St. Albans, VT, USA). The self-administration test chamber for rats was purchased from Med Associates and measures 29×21×24 cm. The chamber contains two holes: an active hole used to deliver a dose of a test drug via the jugular vein through a catheter when a rat pokes its nose into the hole, and an inactive hole, which is not connected to the experimental animal. Infusion pumps were placed outside the chamber and connected to a 10 mL syringe. We connected the chamber to a computer to record the test data and to control the experimental processes.

Methods

CPP test: For 6 days prior to the beginning of the experiment, the mice (n=8-10) were acclimated to the experimental apparatus and to being handled. The procedure was similar to that described previously (Bozarth, 1987; Narita et al., 2004).

Each experiment consisted of three phases, as follows. (1) Pre-conditioning: for 2 days (days 1 and 2), the mice were allowed free access to both compartments of the apparatus for 20 min (1200 s) each day. On day 2, the time the mice spent in each compartment was recorded and served as a baseline. The mice showing a preference for the black compartment were selected for further experiments and were divided into two groups. (2) Conditioning: conditioning was conducted for 8 days (days 3 to 10), for one session per day. On day 3, one group of the selected mice was treated with lisdexamfetamine (1, 2.5, 5, or 10 mg/kg), and placed in the non-preferred compartment (white) for 60 min. The other group of mice was treated with saline and placed in the preferred compartment (black) for 60 min. The groups were switched daily and the same procedure was conducted. (3) Post-conditioning: on day 11, the mice were allowed free access to both compartments of the apparatus for 20 min (1200 s). The time the mice spent in each compartment was recorded, and these values served as experimental data.

Self-administration test: Prior to undergoing surgery, rats (n=8-9) were anesthetized with pentobarbital sodium (50 mg/kg; Entobar®, Hanlim pharmaceuticals, Seoul, South Korea). Briefly, a catheter was inserted into each rat’s right jugular vein. The catheter exited the rat’s shoulder. The rats received heparin each day during the experimental period. After surgery, each rat recovered for at least 7 days.

During the test, the rats self-administered the substances at the dose that showed the highest value in the CPP test or administered a negative control substance (vehicle, 0.1 mL/infusion) for 5 s during a 2 h session on a fixed-ratio 1 reinforcement schedule. The time-out period was 10 s. When a rat inserted its nose into the active hole, it received a dose of the test substance via catheter injection. The self-administration chamber contained two holes, which were linked to a computer program that recorded the data. The test was performed over the course of 10 days.
Preparation of striatal synaptosomes: Many preparation methods for synaptosomes exist; for instance, the ficoll-sucrose method (Gonatas et al., 1971; Wislet-Gendebien et al., 2008), percoll-sucrose method (Nagy and Delgado-Escueta, 1984; Sherman, 1989), and sucrose method (McKenna et al., 1991; Kamat et al., 2014). In the present study, the sucrose method was used. The striatal region was obtained through sectioning the brains of untreated rats (n=4), which were then homogenized in 2.7 mL of ice-cold 0.32 M sucrose using a homogenizer (KINEMATICA, Luzern, Switzerland). The homogenized striatums were centrifuged at 3000×g for 10 min and the supernatant, containing the crude synaptosomal fraction, was gently decanted and diluted 1:1 with Krebs-Hepes buffer (117 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl2, 2.5 mM MgCl2, 25 mM Hepes, 10 mM pargyline). The supernatant was mixed thoroughly and centrifuged at 10,000×g for 20 min to obtain a pellet containing the synaptosomes.

Western blot analysis: The protein concentration of the striatal synaptosomes was determined using the Smart BCA Protein Assay kit (INtRON Biotechnology, Seongnam, Korea). Proteins (10 µg) were resolved on a sodium dodecyl sulfate-polyacrylamide gel followed and then transferred to a polyvinylidene difluoride membrane (Invitrogen, Carlsbad, CA, USA). The membrane was blocked with 3% bovine serum albumin, incubated with primary antibodies overnight at 4°C, treated with a horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature, and then washed. Bands were visualized with a Western blotting luminol reagent (Thermo Fisher Scientific, Southfield, MI, USA). We used the following primary and secondary antibodies: rabbit monoclonal anti-N-Methyl-D-aspartic acid (NMDA)R2B (Invitrogen), anti-NMDA1 receptor (Invitrogen), polyclonal anti-NMDAR2A (Cell Signaling Technology, Danvers, MA, USA), anti-β2 actin (Cell Signaling Technology), goat HRP-conjugated anti-mouse immunoglobulin G, and goat HRP-conjugated anti-rabbit immunoglobulin G (Cell Signaling Technology). Protein expression level was measured with an image analyzer (Kodak, Rochester, NY, USA).

Preparation of samples: The pellet (P1) obtained from the homogenized striata was mixed in 4 mL of 1× Krebs-Hepes buffer, and then treated with 4 µL of 20 µM dopamine at 37°C for 15 min. The supernatant was removed after 10 min of centrifugation at 10,000×g. Then, the pellet (P2) was mixed with 2× Krebs-Hepes buffer. After obtaining the pellet, 150 µL of several doses of methamphetamine (0.01, 0.1, 1, 10, and 100 µM) and lisdexamfetamine (0.01, 0.1, 1, 10, and 100 µM) were added in the same amounts used for synaptosome extraction at 37°C for 15 min. The supernatant was collected after centrifugation at 10,000×g for 3 min.

HPLC- electrochemical detector (ECD) instrumentation and methods: A method for analyzing the synaptosomal release of neurotransmitters using HPLC was reported previously (Janowsky et al., 2001). Dopamine levels in the synaptosomes were detected using an HPLC (DIONEX UltiMate 3000, Thermo Fisher Scientific, Waltham, MA, USA) ECD. The column used for the analysis was an Acclaim® RSLC120 C18 (2.2 µm, 120Å, 2.1×50 mm, Thermo Fisher Scientific), at a temperature of 35°C. The flow rate was 0.5 mL/min, and the injection volume was 10 µL. The mobile phase was composed of 6.9 g NaH2PO4, 250 mg 1-heptanesulfonic acid sodium salt, 80 mg ethylenediaminetetraacetic acid, and 5% HPLC-grade methanol, at a pH of 3.2. The voltage of the ECD was 250 mV, and samples were eluted for 3.5 min. The changes in dopamine levels induced by treatment with different substances were analyzed by Chromleon™ 7 (Thermo Fisher Scientific).

Statistics: For the CPP test, separate one-way ANOVAs assessing the differences in the time the mice spent in the white chamber after treatment with each substance were performed. Newman-Keuls multiple comparisons tests were used to identify the doses of drugs that induced significant changes when compared to saline treatment. For the self-administration test, separate 2 hole (no injection vs. injection)×10 session ANOVAs were performed for each drug on the frequency of nose poking. When appropriate, Bonferroni post-hoc tests were used to assess individual mean differences. For the HPLC data, the dopamine peak areas were analyzed by Student’s t-tests. p-values of <0.05 were considered statistically significant.

RESULTS

CPP test
The CPP test was conducted in a biased manner to evaluate the possibility of rewarding effects. To verify the test system, cocaine (15 mg/kg, intraperitoneal injection) was used as a positive control. The mice treated with cocaine showed statistically significant CPP compared to saline-treated mice (data not shown). Then, five doses of lisdexamfetamine (0, 1, 2.5, 5, and 10 mg/kg, intraperitoneal injection) were administered, and the CPP of the mice was assessed. The ANOVAs for differences between each of the doses were significant (p<0.05). Post-hoc tests indicated that, compared to saline-injected mice, who preferred the black chamber, mice treated with lisdexamfetamine at 2.5, 5, and 10 mg/kg spent more time in the white, drug-paired chamber. Collectively, all of the doses gave a dose-response curve, with each dose of the drug producing a different CPP effect, meaning that lisdexamfetamine may have rewarding effect (Fig. 1).
Fig. 2. Acquisition of lisdexamfetamine (125 µg/kg/infusion) self-administration behavior in Sprague-Dawley rats as assessed via the hole-poking response. The rats had jugular vein surgery and were allowed to recover for 7 days prior to the start of experiments. Doses of the tested substances were determined considering the results of the conditioned place preference test (the highest preferred dose was used). The experiment was performed for at least 10 days. Data represent the mean ± standard error of 8-9 rats per group. *p<0.05, **p<0.01, ***p<0.001 compared to the vehicle-treated group (repeated measures two-way ANOVA followed by Bonferroni post-hoc tests).

Self-administration test

The self-administration test was performed for 2 h (1 session) per day by a fixed-ratio 1 schedule. Cocaine (250 µg/kg/infusion, intravenous injection) was used as a positive control, and vehicle (dimethyl sulfoxide:Tween 80:saline=1:1:18) was used as a negative control. The responses of cocaine-treated rats to the active hole were significantly increased compared to the responses of vehicle-treated rats (Fig. 2, upper). Rats showed increased nose-poking behavior in the lisdexamfetamine (125 µg/kg/infusion, intravenous injection)-associated hole than in the control hole (p<0.05) (Fig. 2, lower). The result suggests possibility of reinforcing effect of lisdexamfetamine.

HPLC analysis

Synaptosomes extracted from the striatal region of experimental animals were used to detect dopaminergic changes related to the administration of lisdexamfetamine. To identify the synaptosomes, we used Western blotting with NMDA receptors (NMDA 1, NMDA 2A, and NMDA 2B). The expression levels of the selected NMDA receptors in the extracted synaptosomes were greater than in an extract from a non-synaptosomal region, which is in accordance with the manual

DISCUSSION

In the present study, the dependence potential of lisdexamfetamine was evaluated using the CPP and self-administration tests in rodents. Lisdexamfetamine showed statistically increased place preference for the conditioned compartment, and the frequency of nose poking in the active hole in the self-administration test. The two behavioral changes indicate that

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the tested substance may possess both the aspects of addic-
tion; rewarding and reinforcing effects. In order to explore the
mechanisms of these behavioral properties, changes in neu-
rotransmitter levels were analyzed in striatal synaptosomes
using HPLC. In the present study, only dopamine levels were
analyzed, because dopamine is related with dependence pri-
marily. The dopamine levels in the synaptosomes increased in
a dose-dependent manner following treatment with lisdexam-
fatamine, which means lisdexamfetamine let the brain release
dopamine, and the released dopamine may be responsible for
the behavioral changes.

Lisdexamfetamine reportedly increases the extraneuronal
concentrations of dopamine and noradrenaline in the pre-
frontal cortex, affecting both catecholamine neurotransmitters
equally (Heal et al., 2013). Our results from the CPP test gave
behavioral evidence for the reported dopaminergic effects of
lisdexamfetamine. Given the previous lack of CPP data on lis-
dexamfetamine, the present study may be the first to use the
CPP paradigm to show the rewarding effects of lisdexamfet-
amine.

Moreover, lisdexamfetamine facilitated self-administration
in rats, suggesting that lisdexamfetamine may have both re-
warding and reinforcing effects. The CPP data in the pres-
ent study supports this suggestion, as lisdexamfetamine
treatment was associated with the longest time spent in the
conditioned chamber. Additionally, a previous study utilizing
a drug-discrimination procedure in rats trained with d-amphet-
amine revealed that lisdexamfetamine could fully substitute
for d-amphetamine at certain doses (Heal et al., 2013). In
the same study, a self-administration test was performed, but no
significant positive effects were observed. Although those re-
sults suggest that lisdexamfetamine may not have reinforcing
properties, Heal et al. (2013) stated that some individual ani-
imals showed increased self-administration when treated with
certain doses of lisdexamfetamine. In this respect, our results
for the self-administration test partially coincide with those of
Heal et al. (2013). Moreover, a previous report suggested that
the results from CPP and self-administration tests may not al-
ways coincide, especially in studies using CPP, as animals do
not voluntarily self-administer the drugs for CPP, and it is thus
a separate issue whether animals will differ with regard to drug
self-administration (Ward et al., 1996). According to a previous
study, lisdexamfetamine produces substantial motor activation
in rats at high doses (Rowley et al., 2014), suggesting that
lisdexamfetamine stimulates dopamine release in the brain.
Moreover, one previous report showed amphetamine-induced
dopamine release using a bioimaging technique (Laruelle et al.,
1995), while another report demonstrated that euphoria
was correlated with dopamine release (Drevets et al., 2001).
Since lisdexamfetamine is converted to amphetamine when
ingested, it likely induces dopamine release in the brain, which
in turn may produce euphoria. Our result showing dopamine
level changes in the striatal synaptosomes in the striatal re-

geon is consistent with the results of previous studies, but fur-
ther studies analyzing neurotransmitters other than dopamine
are needed to confirm the mechanism of action of lisdexam-
fetamine.

Our study has a limitation in that the experiment investigat-
ings changes in the dopamine levels in striatal synaptosomes
used only a single administration of the tested compounds,
whereas repeated treatments are generally used when as-
sessing behavioral or biochemical changes, especially when
investigating dependence potential. Thus, our HPLC results
simply indicate that after a single exposure to the substances,
dopaminergic alterations occur in the striatal synaptosomes.
In conclusion, lisdexamfetamine showed the possibility for
psychological dependence along with dopaminergic changes.
Additionally, the HPLC methods used to analyze the dopa-
mine levels in striatal synaptosomes that were established in
the present study could, after data on various other psychoac-
tive substances accumulates, be applied to predict dopamine
alterations in the central nervous system when screening new
psychoactive substances.

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