Potential for Dependence on Lisdexamfetamine - *In vivo* and *In vitro* Aspects

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Abstract

Although lisdexamfetamine is used as a recreational drug, little research exists regarding its potential for dependence or its precise mechanisms of action. This study aims to evaluate the psychoactivity and dependence profile of lisdexamfetamine using conditioned place preference and self-administration paradigms in rodents. Additionally, biochemical techniques are used to assess alterations in the dopamine levels in striatal synaptosomes following administration of lisdexamfetamine. Lisdexamfetamine increased both conditioned place preference and self-administration. Moreover, after administration of the lisdexamfetamine, dopamine levels in the striatal synaptosomes were significantly increased. Although some modifications should be made to the analytical methods, performing high performance liquid chromatography studies on synaptosomes can aid in predicting dependence liability when studying new psychoactive substances in the future. Collectively, lisdexamfetamine has potential for dependence possible via dopaminergic pathway.

Key Words: Lisdexamfetamine, Dependence, Conditioned place preference, Self-administration, Synaptosome, Dopamine

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

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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; Ivannikov *et al*., 2013). While neurotransmission in the synaptosome has been analyzed using isotopes in several previous studies (Whittaker, 1965; Ivannikov *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: 1401MFDS15).

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, 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 μM 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-β-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
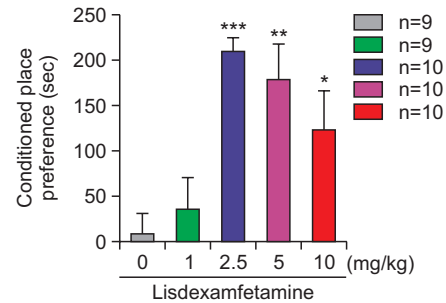
Fig. 1. Effects of lisdexamfetamine treatment on the behavior of mice in the conditioned place preference test. Mice were treated with lisdexamfetamine (1, 2.5, 5, and 10 mg/kg, P.O) and then conditioned by being placed in the white chamber for 60 min every other day for 8 days. Data are expressed as the mean ± standard error of 8-10 animals per group. *p<0.05, **p<0.01, ***p<0.001 compared to the vehicle-treated group (one-way ANOVA followed by a Newman-Keuls post-hoc test).

of 6.9 g NaH$_2$PO$_4$, 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 Chromeleon™ 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).
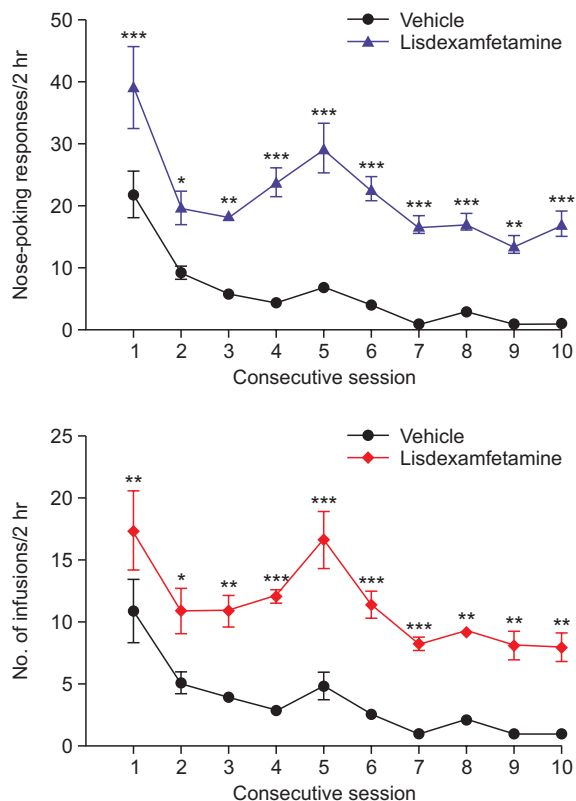
Fig. 2. Acquisition of lisdexamfetamine (125 μg/kg/infusion) self-administration behavior in Sprague-Dawley rats as assessed via the hole-poking response. The rats had jugular vein surgery and were allowed to recover for 7 days prior to the start of experiments. Doses of the tested substances were determined 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
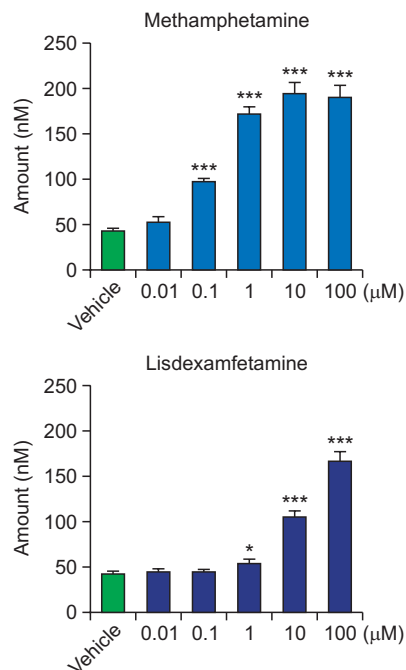
Fig. 3. Changes in dopamine levels induced by administration of lisdexamfetamine. Dopamine levels in the striatal synaptosomes were analyzed and compared between the vehicle-treated group and the drug-treated group. The vehicle (5% dimethyl sulfoxide) served as a negative control and methamphetamine served as a positive control. Methamphetamine and lisdexamfetamine were administered in doses of 0.01, 0.1, 1, 10, and 100 μM. HPLC analysis was performed after synaptosome cultivation at 37°C for 15 min using an HPLC-ECD detector (flow rate: 500 μL/min, ECD voltage: 250 mV, sample amount: 10 μL). *$p<0.05$, ***$p<0.001$ compared to the vehicle-treated group (Student's *t*-test).

provided by the kit's manufacturer (data not shown).

Before measuring dopamine changes followed by treatment of the lisdexamfetamine, the extracted synaptosomes were treated with KCl (4.8 and 50 mM), and dopamine levels, which increased dose-dependently, were measured by HPLC to confirm whether the extracted synaptosomes worked properly (data not shown). Methamphetamine (0.01, 0.1, 1, 10, and 100 μM) was used as a positive control and vehicle (5% dimethyl sulfoxide) was used as a negative control. Methamphetamine and lisdexamfetamine significantly increased dopamine release in a dose-dependent manner. Dopamine levels were increased at doses of 0.1, 1, 10, and 100 μM methamphetamine and 1, 10, and 100 μM lisdexamfetamine. The rate of increase was sigmoidal (Fig. 3). The increased dopamine levels followed by the treatment of lisdexamfetamine indicate that dopamine may have a role in the behavioral changes.

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

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

Lisdexamfetamine reportedly increases the extraneuronal concentrations of dopamine and noradrenaline in the prefrontal 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 lisdexamfetamine, the present study may be the first to use the CPP paradigm to show the rewarding effects of lisdexamfetamine.

Moreover, lisdexamfetamine facilitated self-administration in rats, suggesting that lisdexamfetamine may have both rewarding and reinforcing effects. The CPP data in the present 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-amphetamine 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 results suggest that lisdexamfetamine may not have reinforcing properties, Heal *et al*. (2013) stated that some individual animals 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 always 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 region is consistent with the results of previous studies, but further studies analyzing neurotransmitters other than dopamine are needed to confirm the mechanism of action of lisdexamfetamine.

Our study has a limitation in that the experiment investigating changes in the dopamine levels in striatal synaptosomes used only a single administration of the tested compounds, whereas repeated treatments are generally used when assessing 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 dopamine levels in striatal synaptosomes that were established in the present study could, after data on various other psychoactive substances accumulates, be applied to predict dopamine alterations in the central nervous system when screening new psychoactive substances.

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