**Limonene Inhibits Methamphetamine-Induced Sensitizations via the Regulation of Dopamine Receptor Supersensitivity**

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**Abstract**

Limonene is a cyclic terpene found in citrus essential oils and inhibits methamphetamine-induced locomotor activity. Drug dependence is a severe neuropsychiatric condition that depends in part on changes in neurotransmission and neuroadaptation, induced by exposure to recreational drugs such as morphine and methamphetamine. In this study, we investigated the effects of limonene on the psychological dependence induced by drug abuse. The development of sensitization, dopamine receptor supersensitivity, and conditioned place preferences in rats was measured following administration of limonene (10 or 20 mg/kg) and methamphetamine (1 mg/kg) for 4 days. Limonene inhibits methamphetamine-induced sensitization to locomotor activity. Expression of dopamine receptor supersensitivity induced by apomorphine, a dopamine receptor agonist, was significantly reduced in limonene-pretreated rats. However, there was no significant difference in methamphetamine-induced conditioned place preferences between the limonene and control groups. These results suggest that limonene may ameliorate drug addiction-related behaviors by regulating postsynaptic dopamine receptor supersensitivity.

**Key Words:** Dopamine receptor supersensitivity, Methamphetamine, Sensitization, Limonene

**INTRODUCTION**

Limonene is a common terpene found in citrus fruits. This monoterpenoid is widely used as a flavor and fragrance and is listed to be generally recognized as safe in food by the Food and Drug Administration (Flamm and Lehman-McKeeman, 1991). Limonene has been shown to exert anxiolytic effects, regulatory effects on neurotransmitters, and antinociceptive effects (do Amaral et al., 2007; Zhou et al., 2009; de Almeida et al., 2012; Lima et al., 2013). Recently, we have reported that limonene inhibits an acute single methamphetamine-induced hyperlocomotion in rats by regulating dopamine levels in the nucleus accumbens (Yun, 2014). However, the potential for limonene in the treatment of drug dependence is largely unknown.

Drug dependence is a condition that involves a cluster of behavioral, cognitive, and physiological symptoms that can develop following repeated substance use. Preclinical models have been shown to be useful in identifying many molecular and cellular targets of drug dependence. In rodents, acute administration of stimulants results in hyperactivity, whereas repeated administration results in progressive, enhanced locomotor activity (Shimosato and Ohkuma, 2000; Filip et al., 2006; Fukushima et al., 2007). This phenomenon is also known as context-dependent behavioral sensitization, and this may play a role in the development of compulsive drug-seeking behaviors (Hooks et al., 1993; Mattingly et al., 2000; Shen et al., 2006). It has been suggested that enhanced mesolimbic dopaminergic neuronal transmission is responsible for the development of behavioral sensitization to an abused drug (Pak et al., 2006; Bello et al., 2011); this is a model for studying the psychotoxicity of dependence-liable drugs (Allen and Young, 1978; Robinson and Becker, 1986). It has also been reported that chronic abuse of drugs can cause the development of postsynaptic dopamine receptor supersensitivity in the central nervous system (CNS) (Martin and Takemori, 1986; Ujike et al., 1990; Kim et al., 1999). This increased sensitivity can be detected as a hypersensitivity to direct-acting dopamine agonists and as an increase in the affinity of dopamine receptors (Martin and Takemori, 1986; Woo et al., 2001).
Animals and drugs

Male Sprague-Dawley rats (all male, weight range: 180-220 g) were obtained from the Daehan Bio Link (DBL, Chungbuk, Korea) and were housed in groups of 2 rats in a temperature-controlled room (22 ± 2°C) with a 12-h light/dark cycle (lights on 08:00 from 20:00). The rats were given a solid diet and tap water, ad libitum. All animals were treated in Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited facilities, operating according to the Guide for the Care and Use of Laboratory Animals. All experiments were approved by the Institutional Animal Care and Use Committee of Chungbuk National University. The following agents were used in this study: methamphetamine-HCl, (R)-(+) limonene, and apomorphine hydrochloride, all obtained from Sigma (St. Louis, MO, USA). Morphine hydrochloride was purchased from Guju Pharmaceutical Co (Seoul, Korea). All drugs were dissolved in distilled water (0.9% NaCl) immediately prior to the experiments, except for the (R)-(+) limonene, which was dissolved in water containing 4% Tween 80 and for the apomorphine, which was dissolved in water containing 0.1% ascorbic acid and intraperitoneally (i.p.) injected at a volume of 1 ml/kg.

Locomotor activity

To induce sensitization, 1 or 5 mg/kg of methamphetamine was administered once a day, for 4 days. To test the degree of sensitization development, all groups were challenged with methamphetamines on day 5. Each rat was placed in an activity chamber (43.2 cm × 43.2 cm × 30.5 cm, ENV-515, Med Associates Inc.) and, after an adaptation period of 10 min, 1 mg/kg of methamphetamine was i.p. administered. The locomotor activity of the rats was measured using a photo-beam (infrared) activity chamber for 1 h, immediately after the injection of methamphetamine. The development and inhibition of sensitization was evidenced by enhanced and reduced response to methamphetamine, respectively.

Postsynaptic dopamine receptor supersensitivity

Postsynaptic dopamine receptor supersensitivity induced by methamphetamine (1 mg/kg) was demonstrated using 0.5 mg/kg of apomorphine (Fig. 1). Additional groups of rats, which underwent the same chronic methamphetamine and limonene treatment, were used to determine the effects of these treatments on the development of postsynaptic Dopamine receptor supersensitivity. Treatment involved methamphetamine administration (1 mg/kg), once daily, for 4 days, and limonene administration (10 or 20 mg/kg), once a day, 40 min before the injection of methamphetamine, for 4 days. The degree of methamphetamine-induced postsynaptic dopamine receptor supersensitivity developed was determined by measuring the enhanced locomotor activity induced by apomorphine on day 5, 24 h after the final injection of methamphetamine. Each rat was placed in an activity chamber and, after an adaptation period of 10 min, received 0.5 mg/kg of apomorphine (i.p.). The locomotor activity of the rats was measured using an infrared photo-beam activity chamber for 1 h, from immediately after the injection of apomorphine.

CPP

The apparatus and procedure used for the CPP test have been published previously (Kim et al., 1998), but were modified slightly. The CPP test apparatus used in the present study consisted of three compartments, and included the ENV-013 IR Infrared Sensor Package (Med Associates Inc.). The two-
Fig. 2. Inhibitory effect of limonene on methamphetamine-induced sensitization of locomotion. The total distance on (A) day 1 and (B) day 5 is shown. On day 5, all groups were administered methamphetamine (1 mg/kg, intraperitoneal [i.p.]). Values indicate the mean ± standard error (SE) (n=8). * p<0.05, ** p<0.01 vs. vehicle/saline group, * p<0.05, # p<0.01 vs. vehicle/METH group (two-way repeated-measures ANOVA followed by Bonferroni’s test). METH, methamphetamine; LIM, limonene.

Fig. 3. Inhibitory effect of limonene on methamphetamine-induced postsynaptic dopamine receptor supersensitivity. On day 5, all groups were administered apomorphine (0.5 mg/kg, i.p.). Values indicate the mean ± standard error (SE) (n=8). ** p<0.01 vs. vehicle/METH group (one-way ANOVA, followed by Bonferroni’s test). METH, methamphetamine; APO, apomorphine; LIM, limonene.

Western blotting

Limonene was administered once a day for 4 days and the striatum was sampled 24 hours after last injection. The tissues were homogenized with 1x lysis buffer (RIPA buffer, Thermo Scientific, MA, USA) including 1x protease inhibitor (Thermo Scientific) and incubated on ice for 30 min, and centrifuged at 13,000 rpm for 150 min at 4°C. An equal amount of total protein (30 μg) was resolved on 12% sodium dodecyl sulfate polyacrylamide gel and then transferred to a PVDF membrane (Immobilon-P; pore size 0.45 μm, EMD Millipore, Burlington, MA, USA). The membranes were blocked for 1 h in 5% skim milk solution and incubated for overnight at 4°C with glutamate decarboxylase 67 (Gad67; 1:1000, Sigma Aldrich, USA) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:1000, EMD Millipore); the blots were then incubated with secondary antibody; goat anti-mouse IgG-horseradish peroxidase (HRP) (1:5000, Sigma Aldrich, St. Louis, MO, USA). Immunoreactive proteins were detected with an enhanced chemiluminescence Western blotting detection system. The relative density of the protein bands was scanned and quantified by ImageJ (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Analyses were carried out using one-way analysis of variance (ANOVA), followed by Bonferroni’s test, two-way ANOVA, or repeated-measures ANOVA with Bonferroni’s test, for multi-group comparisons.

RESULTS

Effect of methamphetamine on methamphetamine-induced sensitization and apomorphine-induced postsynaptic dopamine receptor supersensitivity

Locomotor sensitization, induced by repeated administra-
Inhibitory effect of methamphetamine on postsynaptic dopamine receptor supersensitivity

Methamphetamine-induced CPP and locomotion sensitization are associated with an increase in mesolimbic dopaminergic neurotransmission (Das et al., 2011). We observed that methamphetamine administration (1 or 5 mg/kg, i.p.) for 4 days induced the development of sensitization to locomotor activity in rats (Fig. 1A). Furthermore, apomorphine (0.5 mg/kg) administration triggered the demonstration of postsynaptic dopamine receptor supersensitivity in methamphetamine-pretreated rats (Fig. 1B). In this study, we used 1 mg/kg of methamphetamine as a submaximal dose in locomotor sensitization and 0.5 mg/kg of apomorphine for expression of supersensitivity.

Inhibitory effect of limonene on the development of sensitization to methamphetamine-induced hyperactivity

To explore the effect of limonene on methamphetamine-induced sensitization, we pretreated the rats with limonene (10 or 20 mg/kg, i.p.) prior to every methamphetamine injection, for 4 days. Limonene treatment did not inhibit hyperactivity induced by a single methamphetamine injection on day 1 (Fig. 2A). However, the locomotor sensitization induced by methamphetamine on day 5 was reduced in the rats pretreated with methamphetamine and limonene (Fig. 2B). These results suggest that limonene inhibits methamphetamine-induced behavioral sensitization.

Inhibitory effect of limonene on the development of postsynaptic dopamine receptor supersensitivity in methamphetamine-induced sensitized rats

The rats that received the same chronic administration of methamphetamine (1 mg/kg) as used in the sensitization test demonstrated enhanced locomotor activity in response to apomorphine (0.5 mg/kg) treatment, as compared to the vehicle group, suggesting the development of postsynaptic dopamine receptor supersensitivity in methamphetamine-induced sensitized rats (Fig. 3). However, 10 or 20 mg/kg of limonene administered 40 min before the methamphetamine injection was found to reduce the locomotor activity of apomorphine, as compared to the methamphetamine group. These results suggest that limonene inhibits the development of postsynaptic dopamine receptor supersensitivity in methamphetamine-induced sensitized rats.

**Fig. 4.** Effect of limonene on CPP induced by methamphetamine. The preferences were calculated from the changes of the testing phase (15 min) and the pre-testing phase (15 min) in the white phase (15 min) and the pre-testing phase (15 min) in the white compartment. Values indicate the mean ± standard error (SE) (n=10–14). *p<0.05 vs. vehicle (one-way ANOVA, followed by Bonferroni’s test). METH, methamphetamine; LIM, limonene.

**Fig. 5.** Effect of limonene on protein expression of Gad67. The expression of Gad67 was detected by Western blotting using specific antibodies in striatum. Each blot is representative of three experiments. Values indicate the mean ± standard error (SE) (n=8). **p<0.05 vs. vehicle (one-way ANOVA, followed by Bonferroni’s test).

**Effect of limonene on methamphetamine-induced CPP**

The methamphetamine group showed a significant increase in CPP score, as compared with the vehicle group (Fig. 4). The limonene (20 mg/kg) group showed a decreasing trend in CPP score, but this was not significant (Fig. 4). These results suggest that a dose of 20 mg/kg of limonene inhibits the development of sensitization, although it did not have a significant effect on the CPP induced by methamphetamine.

**Effect of limonene on protein expression of Gad67 in striatum**

Because it has been reported that limonene regulates GABA expression, we measured expression levels of Gad67 protein which is involved in synthesis of GABA in striatum. In this study, limonene (10 or 20 mg/kg) inhibited the development of locomotor sensitization, therefore, we examined whether a lower dose of limonene (10 mg/kg) has effects on the Gad67 expression. Administration of limonene significantly increased the expression levels of Gad67 (Fig. 5). This observation suggest that the increased level of Gad67 protein induced by limonene play a role in the reduced sensitization.

**DISCUSSION**

We demonstrated that limonene may have therapeutic potential for the treatment of methamphetamine dependence. We have previously reported that limonene inhibits methamphetamine-induced hyperactivity (Yun, 2014); in the present study, limonene administration also reduced the development of sensitization to methamphetamine-induced locomotor activity. Furthermore, the limonene-treated groups showed a decreasing trend in their place preference in the methamphetamine-induced CPP test, even though this was not significant. Sensitization to locomotor activity and CPP are associated with an increase in mesolimbic dopaminergic neurotransmission.
sion, which is a mechanism underlying drug dependence (Pak et al., 2006; Bello et al., 2011). We also previously reported that limonene may reverse methamphetamine-induced elevation of dopamine levels in the nucleus accumbens of rats, by regulating GABA levels and activating GABA-B receptors (Yun, 2014). Therefore, the results from the present study suggest that the inhibitory effect of limonene on methamphetamine-induced dopamine release may play a role in the reduction of sensitization.

It has previously been reported that an enhanced response to apomorphine, a direct-acting dopamine receptor agonist, results from the development of postsynaptic dopamine receptor supersensitivity after repeated administration of a drug of abuse (Ritzmann et al., 1979; Bhargava, 1980). Overall, evidence indicates that postsynaptic dopamine receptor supersensitivity may be associated with behavioral effects, such as the CPP and sensitization induced by morphine, cocaine, and amphetamines (Wolf et al., 1994; Kim et al., 1995, 1996a, 1996b; Henry et al., 1998). In this study, limonene inhibited the development of postsynaptic dopamine receptor supersensitivity in the methamphetamine-induced sensitized rats, although the mechanism underlying the limonene-mediated reversal of supersensitivity development remains unclear. Limonene had no effect on the acute behavioral effects of apomorphine (Supplementary Data 1) and the role of dopamine receptors in the development of methamphetamine-induced sensitization remains controversial (Reed et al., 1987; Ujike et al., 1989; Nonaka and Moroji, 1990; Yoo et al., 2010). However, other neurotransmitter systems, including GABAergic, 5-HTergic, and glutamatergic systems, are also associated with methamphetamine-induced locomotor sensitization and postsynaptic dopamine receptor supersensitivity (Ohmori et al., 1994; Kim and Jang, 1997; Yoo et al., 2010). It has been reported that GABAergic (Zhou et al., 2009) and 5-HTergic (Yun, 2014) neuronal systems may mediate the effects of limonene on the CNS. Therefore, we suggest that limonene may inhibit the development of postsynaptic dopamine receptor supersensitivity in methamphetamine-induced sensitized rats via regulation of GABAergic and serotonergic modulation of dopaminergic neuronal transmission. In another study, Zhou et al. (2009) demonstrated that limonene administration significantly increased brain GABA levels in rats, and our present study also showed that protein expression level of Gad67 significantly induced through administration of limonene in striatum. Methamphetamine-induced behavioral sensitization down-regulated Gad67 levels in the nucleus accumbens (Zhang et al., 2006). Furthermore, we observed that a dopamine agonist, apomorphine-induced stereotypy behavior was potentiated in Gad67 knock-down animal (data not shown). Pharmacologic increases in brain GABA levels have been reported to block the increase in dopamine levels elicited by morphine or cocaine injection (Kliteneck et al., 1992; Morgan and Dewey, 1998). Therefore, limonene may reduce the development of physiological dependence by regulating GABAergic and dopaminergic neuronal transmission.

In conclusion, these results suggest that limonene is a promising candidate in the treatment of drug dependence.

**CONFLICT OF INTEREST**

The authors have no conflict of interest to declare.

**ACKNOWLEDGMENTS**

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**REFERENCES**


Supplementary data 1.

Climbing behavior

Climbing behavior was measured using the modified methods described in previous study (Yun et al., 2001). Immediately after and administration of vehicle or APO (2 mg/kg, i.p.), the mice were put into cylindrical cages (diameter: 12 cm; height: 14 cm) with the floor and wall consisting of metal bars (diameter: 0.2 cm; separated by 1 cm gaps) and covered with a lid. The mice were pretreated with limonene (i.p.) 40 min before the injection of APO. After a 10 min period of exploratory activity, an observer who was blind to the drug treatment estimated the time spent in climbing behavior for 1 min at 10, 20 and 30 min after APO administration. Hence, the maximum score for climbing behavior was 180 s.

Effect of limonene on APO-induced climbing behaviors. Climbing behavior has been used as a convenient means of screening dopamine agonists or antagonists and to assess striatal dopamine activity. We confirmed that climbing behavior was no choice between administration of vehicle and limonene. Moreover, limonene had also no effect on APO-induced climbing behavior (2.0 mg/kg).