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5-(2-Aminopropyl)benzofuran and phenazepam demonstrate the possibility of dependence by increasing dopamine levels in the brain

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ABSTRACT

Although 5-(2-aminopropyl)benzofuran (5-APB) and 7-bromo-5-(2-chlorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one (phenazepam) are being used as recreational drugs, research on their dependence liability or mechanisms of action is lacking. The present study aimed to evaluate the behavioral effects and dependence liability of these drugs using conditioned place preference and self-administration paradigms in rodents. Additionally, biochemical techniques were used to assess the substance-induced alterations in synaptosome-released dopamine. While both of the tested substances elicited increases in conditioned place preference and dopamine, neither of them facilitated self-administration, suggesting that 5-APB and phenazepam have rewarding effects, rather than reinforcing effects.

1. Introduction

In our society, drugs that are prescribed for medicinal purposes are increasingly being abused. New psychoactive substances are also problematic owing to their harmful effects on humans; further, the chemical structures of these substances can be easily modified, thus the clandestine manufacturing of these substances has increased dramatically. Two such abused psychoactive substances are 5-(2-aminopropyl)benzofuran (5-APB) and 7-bromo-5-(2-chlorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one (phenazepam).

5-APB was first synthesized as an inhibitor of norepinephrine (NE), dopamine (DA), and serotonin (5-HT) transport. The chemical structure of 5-APB is similar to the structures of two other psychoactive substances, namely 3,4-methylenedioxymethamphetamine (ecstasy) and 3,4-methylenedioxyamphetamine (MDA). Moreover, 5-APB reportedly induces DA release in the nucleus accumbens region of the brain in rats (Rickli et al., 2015) and induces NE and 5-HT release *in vitro* (Dawson et al., 2014).

Phenazepam is a benzodiazepine derivative (Schoch et al., 1985; Voronina et al., 1997) that was developed to treat neurological disorders, epilepsy, and alcohol withdrawal syndrome (Ashhar et al., 2015; Corkery et al., 2012). Although a few countries have approved phenazepam for therapeutic purposes, it is often used as a recreational drug, since it can help reduce the actions of other stimulating substances (Gjerde et al., 2011; Lillsunde and Gunnar, 2005). Moreover, it has been suggested that both of the above mentioned substances act on DA neurotransmission, either directly or indirectly, as they produce "euphoria" in the user, and evidence supports a relationship between DA and positive rewarding effects (Meyer and Quenzer, 2013).

Since the existing data on the abuse liabilities of new psychoactive substances were mostly derived from anecdotal reports, in the present study, using rodents, we evaluated the dependence liabilities of 5-APB and phenazepam. The dependence potentials of these substances were evaluated *in vivo* using conditioned place preference (CPP) and self-administration tests, which are often used in the field (Gorelick et al., 2004; Koob, 1992; Meyer and Quenzer, 2013; Mucha et al., 1982; Taylor, 2002). In addition, *in vitro* examinations of synaptosomes were performed since synaptosomes are known to be a useful tool for studying the structure-function relationships of synaptic release (Abekawa et al., 1994; Ivannikov et al., 2013). Specifically, to investigate the dependence mechanisms of the test substances, we extracted synaptosomes from the striatal regions of the rat brain and evaluated the

Abbreviations: 5-APB, 5-(2-aminopropyl)benzofuran; 5-HT, serotonin; ANOVA, analysis of variance; CPP, conditioned place preference; DA, dopamine; ECD, electrochemical detector; GABA, gamma aminobutyric acid; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; MDA, 3,4-methylenedioxyamphetamine; NE, norepinephrine; NMDA, *N*-Methyl-D-aspartic acid; phenazepam, 7-bromo-5-(2-chlorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one.

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substance-induced changes in the synaptosomal DA levels using high-performance liquid chromatography (HPLC).

2. Materials and methods

2.1. Animals

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). Animal care procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Center, 2001). 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).

2.2. Substances

5-APB and phenazepam were 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 to 6 doses of the test substances were intraperitoneally administered to ICR mice (5-APB: 0, 0.5, 1, 2, 4 mg/kg; phenazepam: 0, 0.1, 0.5, 1, 2, 4 mg/kg). For the self-administration test, 250 μg/(kg·infusion) of cocaine, 50 μg/(kg·infusion) of 5-APB, and 100 μg/(kg·infusion) of phenazepam were intravenously administered to Sprague-Dawley rats. For the HPLC analyses, six concentrations of methamphetamine (0, 0.01, 0.1, 1, 10, 100 μM) and six concentrations of the test substances (5-APB: 0, 0.01, 0.1, 1, 10, 100 μM; phenazepam: 0, 1, 10, 100, 150, 180 μM) were administered to synaptosomes that were extracted from the striatal regions of the rat brain.

2.3. Apparatus

The CPP test apparatus used in the present study consisted of three compartments (overall inside dimensions: 15.8 × 17 × 15.5 cm) and included the ENV-013 IR Infrared Sensor Package (Med Associates, St. Albans, VT, USA). The self-administration test chamber for rats (29 × 21 × 24 cm) was purchased from Med Associates. The chamber contained two holes: an active hole, which delivered a drug through a catheter to the jugular vein when the rat poked its nose into the hole, and an inactive hole, which was not connected to the experimental animal. The chamber was connected to a computer, which recorded the test data and controlled the experimental processes.

2.4. CPP test

Before starting the experiment, the mice (n = 8–10) were acclimated to the experimental apparatus and handled for 6 days. The procedures were similar to those described previously (Narita et al., 2004) and consisted of three steps: (1) pre-conditioning: the mice were allowed free access to both compartments of the apparatus for 20 min per day for 2 days. On day 2, the amount of time the mice spent in each compartment was recorded and served as a baseline. The mice that showed a preference for the black compartment were selected for the remaining experiments and were divided into two groups; (2) conditioning: conditioning was conducted for 8 days. On day 3, one of the groups was treated with the test substances (5-APB: 0.5, 1, 2, 4 mg/kg; phenazepam: 0.1, 0.5, 1, 2, 4 mg/kg) and placed in the non-preferred compartment (white) for 45 min (5-APB, phenazepam). The other group of mice was treated with saline and placed in the preferred compartment (black) for the same duration. The groups were switched daily and the same procedure was conducted; and (3) post-conditioning: the mice were allowed free access to both compartments of the apparatus for 20 min. The amount of time the mice spent in each compartment was recorded, with these values serving as a test line.

2.5. Self-administration test

For the surgeries, the rats (n = 8–9) were anesthetized with pentobarbital sodium (50 mg/kg; Entobar®, Hanlim pharmaceuticals, Seoul, South Korea). Briefly, a catheter was inserted into the right jugular vein of each rat. The rats received heparin each day during the experimental periods. After the surgery, each rat was allowed to recover in a controlled cage for at least 7 days.

For the test, the rats self-administered the substances at the dose that showed the highest value in the CPP test or a negative control substance was administered (vehicle, DMSO:Tween80:saline = 1:1:18 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 the rats inserted their nose into the active hole, they received a dose of the test substance *via* catheter injection. As mentioned above, the self-administration chamber contained two holes linked to a computer program that recorded the data. The test was performed for 10 days.

2.6. Preparation of synaptosomes

Several preparation methods for synaptosomes exist (Kamat et al., 2014; McKenna et al., 1991). In the present study, the entire striatal region was obtained by sectioning the brains of untreated rats (n = 4), and then homogenizing them in 2.7 mL of ice-cold 0.32 M sucrose using a homogenizer (KINEMATICA, Luzern, Switzerland). The homogenized striatum was 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 $CaCl_2$, 2.5 mM $MgCl_2$, 25 mM HEPES, 10 μM pargyline). The supernatant was mixed thoroughly and centrifuged at 10,000 ×*g* for 20 min to obtain the pellet.

2.7. Western blot analysis

The protein concentration of synaptosomes was determined using the Smart BCA Protein Assay kit (iNtRON Biotechnology, Seongnam-si, South Korea). The protein (10 μg) was resolved on a sodium dodecyl sulfate-polyacrylamide gel followed by transfer of the resolved proteins 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, Waltham, MA, 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). The protein expression level was detected by an image analyzer (Kodak, Rochester, NY, USA).

2.8. Preparation of samples

The pellet (P1) obtained from the homogenized striatum was mixed in 4 mL of 1× Krebs-HEPES buffer, and then the synaptosomes were loaded with DA (4 μL of 20 μM) 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 different concentrations of methamphetamine (0.01, 0.1, 1, 10, 100 μM), 5-APB (0.01, 0.1, 1, 10, 100 μM), and phenazepam (0.01, 0.1, 1, 150, 180 μM) were added to the same amount of synaptosome