Ketamine Pretreatment Exacerbated 3,4-Methylenedioxymethamphetamine-Induced Central Dopamine Toxicity

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Abstract

Currently, joint use of ketamine and 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy) represents a specific combination of polydrug abuse. Long-lasting and even aggravated central neuronal toxicity associated with mixing ketamine and MDMA use is of special concern. This study was undertaken to examine the modulating effects of ketamine treatment on later MDMA-induced dopamine and serotonin neurotoxicity. We found that repeated administration of ketamine (50 mg/kg × 7) at 1.5-h intervals did not render observable dopamine or serotonin depletion in catecholaminergic target regions examined. In contrast, three consecutive doses of MDMA (20 mg/kg each) at 2-h intervals produced long-lasting dopamine and serotonin depletions in striatum, nucleus accumbens and prefrontal cortex. More importantly, pretreatment with binge doses of ketamine (50 mg/kg × 7 at 1.5-h intervals) 12 h prior to the MDMA dosing regimen (20 mg/kg × 3 at 2-h intervals) aggravated the MDMA-induced dopaminergic toxicity. Nonetheless, such binge doses of ketamine treatment did not affect MDMA-induced serotonergic toxicity. These results, taken together, indicate that binge use of ketamine specifically enhances the MDMA-induced central dopaminergic neurotoxicity in adult mouse brain.

Key Words: ecstasy, serotonin, dopamine, anesthetic, DAT, VMAT-2

Introduction

Ketamine, 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy), flunitrazepam, d-lysergic acid diethylamide, methamphetamine and gamma-hydroxybutyrate, in together, have become the frequently-reported combined drug use of choice in US (23). Joint abuse of ketamine and MDMA is increasing throughout the world, especially at all-night dance/rave parties. Club-going young adults were especially predictive of conjunctive ketamine and MDMA use (11). Street-involved youths who used MDMA were more likely to also use ketamine (13). Thus, combined ketamine and MDMA use represents one of the most popular combinations for polydrug abuse. Long-lasting and aggravated central neuronal toxicity associated with their conjunctive binge use were of concern in this regard.

Cumulative doses of MDMA have been well documented to produce central dopaminergic and serotonergic neurotoxicity (1, 3, 6, 8, 12, 24). Single dose of ketamine (75 mg/kg) or four cumulative doses of ketamine (25 mg/kg) did not cause any neuronal degeneration, while seven consecutive doses of ketamine (25 mg/kg each) produced profound neuronal

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degeneration in neonatal rats (10). Likewise, another report confirmed that cumulative dosing protocol of ketamine (20 mg/kg each for seven doses) caused significant neuronal degeneration in neonatal rats (18). In an attempt to mimic binge use of ketamine in humans, we adopted a multiple dosing protocol of ketamine with an elevated dose of 50 mg/kg in adult mice. Binge doses of ketamine produced acute neuronal apoptotic death by a direct NMDA blockade and late-onset excitotoxic neurodegeneration *via* a compensatory up-regulation of NMDA receptors during the developmental period of synaptogenesis (16, 21). In adult brains, especially corticolimbic regions, administration of large doses of ketamine results in hydroxyl radical generation, oxidative stress and structural damages (15, 16, 20). Nonetheless, toxic effects produced by binge use of ketamine specifically on central dopaminergic and serotonergic neurons remain unknown. It was of interest to note that co-administering low doses of ketamine or MK-801, both noncompetitive antagonists for glutamate NMDA receptor, and methamphetamine, another dopamine and serotonin neurotoxin, mitigated methamphetamine-induced central neurotoxicity (5, 17, 22). Since NMDA antagonists and methamphetamine were essentially administered at the same time in these studies, complicated pharmacokinetics derived from these two drugs' interaction unavoidably hampered the prediction for the neurotoxic effects by mixing ketamine and MDMA.

Polydrug mixing can be categorized, in terms of the timing frame for multiple drug use, as simultaneous, alternating and sequential mixing within hours, days, weeks or longer (19). In this study, we determined to examine the long-lasting dopaminergic and serotonergic neurotoxicity produced by sequential mixing of binge doses of ketamine followed by MDMA.

Materials and Methods

Animals

Male C57BL/6J mice, aged 7-8 weeks, were housed in a facility located at National Cheng Kung University Laboratory Animal Center (Tainan, Taiwan, ROC) with free access to food (Purina Mouse Chow, Richmond, IN, USA) and tap water. The colony room was temperature- and humidity-controlled and maintained on a 12 h light/dark cycle (lights on at 0700). All experiments were conducted in a laboratory with temperature maintained at 24 ±1 °C. This study was performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All procedures were approved by the local Animal Care Committee at National Cheng Kung University College of Medicine.

Drug Dosing Regimens

Ketamine hydrochloride was purchased from Pfizer (Los Angeles, CA, USA). MDMA was obtained from the Ministry of Justice in Taiwan. Mice received 7 cumulative injections of ketamine (50 mg/kg for each injection, intraperitoneally) or an equivalent volume of saline injections at 1.5-h intervals. Twelve hours after the last dose of ketamine, mice received 3 cumulative, intraperitoneal doses of MDMA (20 mg/kg/dose) or a comparable volume of saline injections at 2-h intervals.

Neurochemical Determination

Following a 2-wk recovery period after the MDMA dosing regimen, mice were sacrificed by rapid decapitation and the brain was then removed within 20-30 sec and placed on the dorsal surface on a glass dish sitting on crushed ice. Prefrontal cortex, nucleus accumbens and striatum samples were dissected. Prefrontal cortex sample was obtained from the front chunk of the brain divided by the first coronal section taken approximately in the midline of the olfactory tubercle. The second coronal section was undertaken right next to the anterior border of the hypothalamus. Nucleus accumbens sample was punched out (a circle around the anterior commissure) from the slice between these two sections with a 1-ml pipetman tip cutting to an internal diameter of 1.1 mm. From the same slice, striatal sample was then removed from the caudal part, including the tissue dorsal to the punched circle, ventral to the corpus callosum and medial to the external capsule. Tissue samples were stored in liquid nitrogen until assayed by HPLC with an LC-4C amperometric detector (BAS, West Lafayette, IN, USA) for measuring DA, its main metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA). Tissue was homogenized and centrifuged at 14,000 × g for 20 min at 4°C. The supernatant was filtered and delivered through a high-pressure valve fitted with a 20-µl loop onto a Phase-II ODS column (3 µl, 100 × 3.2 mm), and oxidized with a +0.72-V potential between the glassy carbon electrode and the Ag/AgCl reference electrode. The mobile phase consisted of 0.1 M sodium phosphate dibasic, 0.1 M citric acid, 5 mg EDTA, and 7% methanol delivered at a 0.6-ml/min flow rate.

Western Blot Analysis for DAT and VMAT-2 Level

Animals were sacrificed 2 weeks after the MDMA dosing regimen. Western blotting was employed to semi-quantify their relative DAT and VMAT-2 levels. β-actin was used as an internal control to calibrate the loading amount of total protein (50 µg). Brain tissue was homogenized in PBS containing proteinase inhibitor

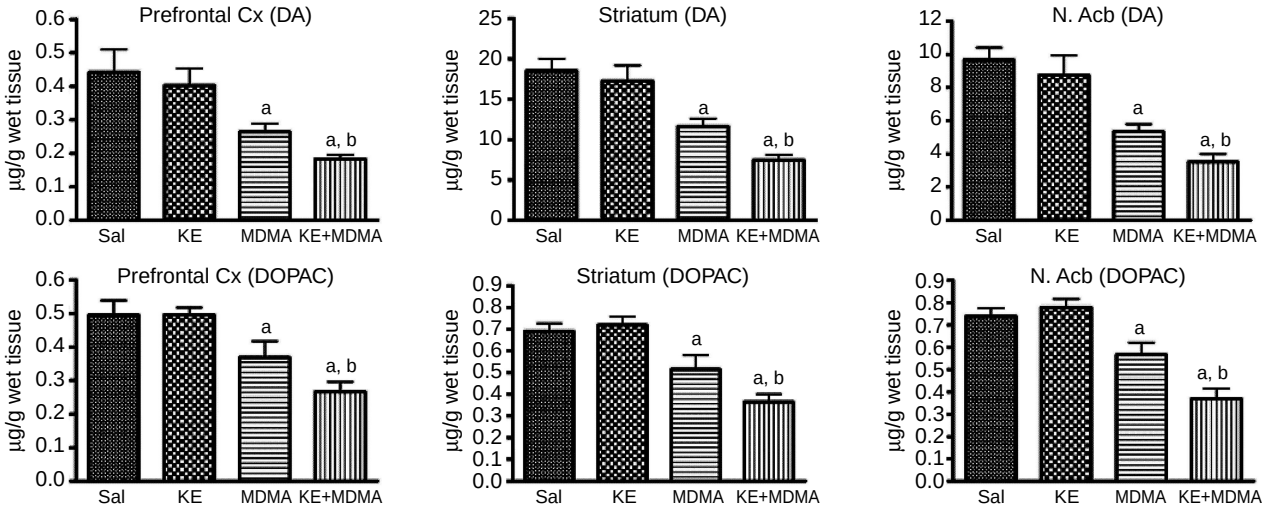

Fig. 1. Binge doses of ketamine pretreatment exacerbate MDMA-induced DA depletions in dopaminergic terminal regions. Three cumulative doses of MDMA (20 mg/kg/dose) decrease DA and DOPAC contents in prefrontal cortex (Cx), striatum and nucleus accumbens (N. Acb). Seven consecutive injections of ketamine (50 mg/kg/injection) alone do not produce obvious DA or DOPAC depletions in these regions. Pretreatment with ketamine aggravates later MDMA-induced DA and DOPAC depletions. Data are expressed as mean ± SEM. n = 6 for each group. [a]Significantly lower than Sal and KE groups. [b]Significantly lower than the other three groups. KE: a short for ketamine.

cocktail. Homogenates were centrifuged at 15,000 × g for 30 min at 4°C and the protein concentrations of supernatants were adjusted (Bio-Rad protein assay kit, Hercules, CA, USA) prior to SDS-PAGE. Rat DAT, VMAT-2 polyclonal antibodies and anti-rat immunoglobulins were purchased from Chemicon (Temecula, CA, USA), whereas mouse β-actin antibody and horseradish peroxidase conjugated anti-mouse immunoglobulin were obtained from Sigma. The electrophoretically separated proteins were transferred onto PVDF membranes and blocked with 5% non-fat milk in Tris buffer saline, containing 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, and 0.5% Tween-20. The blots were developed with the Chemiluminescent Detection System kit (Amersham Biosciences, Buckinghamshire, UK) and the relative intensities of the bands were measured on a Gel Documentation System (Bio-Rad).

Statistical Analysis

ANOVA was performed to determine group differences on DA, DOPAC, 5-HT, 5-HIAA content levels in tissues and the relative intensity of DAT and VMAT-2 levels, followed by Tukey post hoc tests if appropriate. Results were considered significant when *P*-values were < 0.05, using a two-tailed test.

Results

Seven cumulative injections of ketamine (a total of 350 mg/kg) did not produce DA, DOPAC, 5-HT or 5-HIAA depletions in primary dopamine and serotonin terminal regions that we examined (including prefrontal cortex, striatum and nucleus accumbens) (Figs. 1 and 2). In contrast, three consecutive doses of MDMA (a total of 60 mg/kg) caused drastic DA ($F(3,20) = 7.30$, $P = 0.0017$ for prefrontal Cx, $F(3,20) = 15.21$, $P = 0.0001$ for striatum, $F(3,20) = 14.34$, $P = 0.0001$ for N. Acb) and DOPAC ($F(3,20) = 8.75$, $P = 0.0007$ for prefrontal Cx, $F(3,20) = 12.89$, $P = 0.0001$ for striatum, $F(3,20) = 18.22$, $P = 0.0001$ for N. Acb) depletions in all these terminal regions (Fig. 1). Among all treatment groups, mice receiving binge doses of ketamine followed by MDMA treatment demonstrated the greatest magnitude of DA and DOPAC depletions in prefrontal cortex, striatum and nucleus accumbens (Fig. 1). We confirmed ketamine's potentiation of the MDMA-induced striatal dopaminergic toxicity by utilizing Western immunoblotting for DAT, a dopaminergic terminal marker, and VMAT-2, a protein for vesicular dopamine uptake. Cumulative MDMA injections significantly decreased DAT ($F(3,12) = 96.43$, $P = 0.0001$), but did not alter VMAT-2 expressions in striatal tissue samples (Figs. 3 and 4). Pretreatment with binge doses of ketamine followed by the MDMA dosing regimen resulted in lower DAT and VMAT-2 expressions ($F(3,12) = 5.59$, $P = 0.0123$) in striatum than those of MDMA treatment alone (Figs. 3 and 4). MDMA treatment (a total dose of 60 mg/kg) produced significant 5-HT and 5-HIAA depletions in prefrontal cortex ($F(3,20) = 7.80$, $P = 0.0012$; $F(3,20) = 3.85$, $P = 0.0253$), striatum ($F(3,20) = 4.90$, $P = 0.0103$; $F(3,20) = 6.69$, $P =$

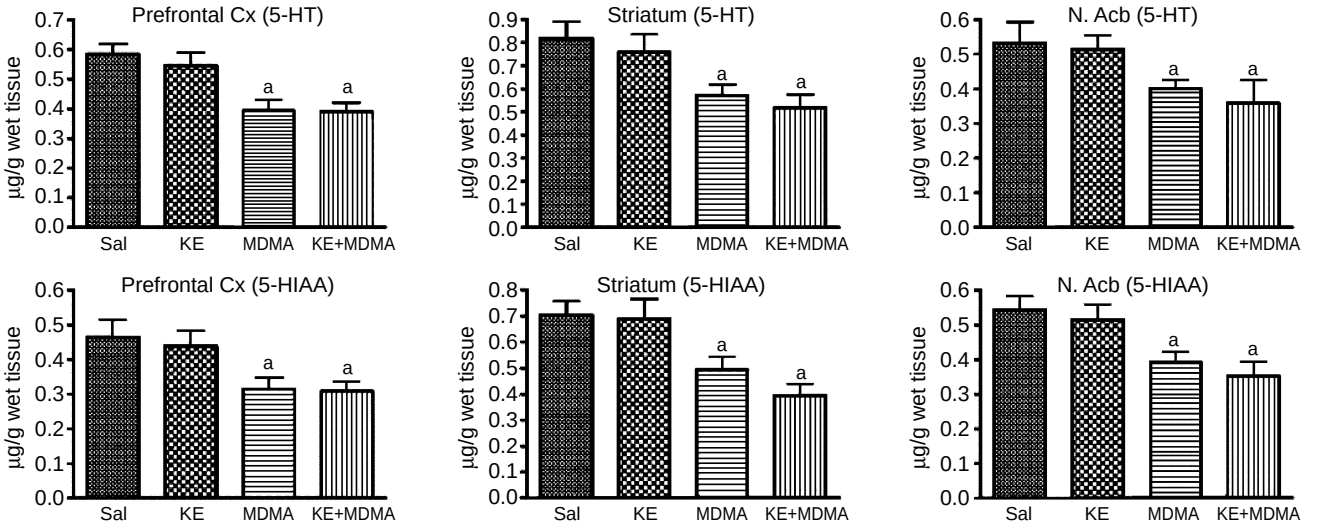

Fig. 2. Pretreatment with binge doses of ketamine does not affect MDMA-induced 5-HT depletions. Two weeks following the MDMA dosing regimen (20 mg/kg/dose × 3), MDMA significantly decreases 5-HT and 5-HIAA content levels in prefrontal cortex (Cx), striatum and nucleus accumbens (N. Acb). Seven consecutive doses (50 mg/kg for each dose) of ketamine (KE) injection do not produce obvious 5-HT or 5-HIAA depletions. Pretreatment with KE does not affect later MDMA-induced 5-HT or 5-HIAA depletions. Data are expressed as mean ± SEM. n = 6 for each group. [a]Significantly lower than Sal and KE groups.

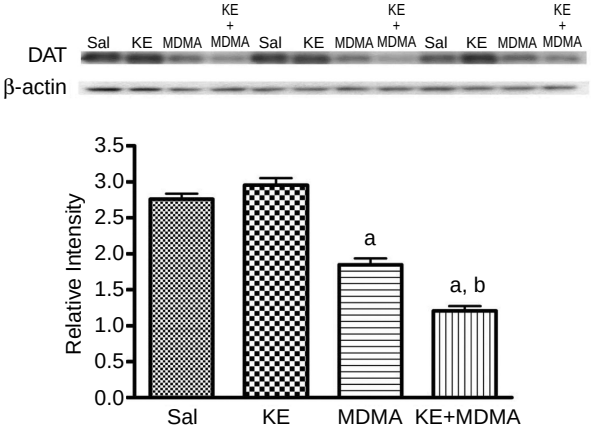

Fig. 3. Binge doses of ketamine pretreatment exacerbate MDMA-induced dopamine transporter decrease in striatum. Three cumulative doses of MDMA (20 mg/kg/dose) decrease dopamine transporter (DAT) expression in striatum two weeks after the drug treatment. Seven consecutive injections of ketamine (KE, 50 mg/kg/injection) alone do not affect DAT expression in striatum. Pretreatment with KE exacerbates later MDMA-induced DAT decrease. Data are expressed as mean ± SEM. n = 4 for each group. [a]Significantly lower than Sal and KE groups. [b]Significantly lower than the other three groups.

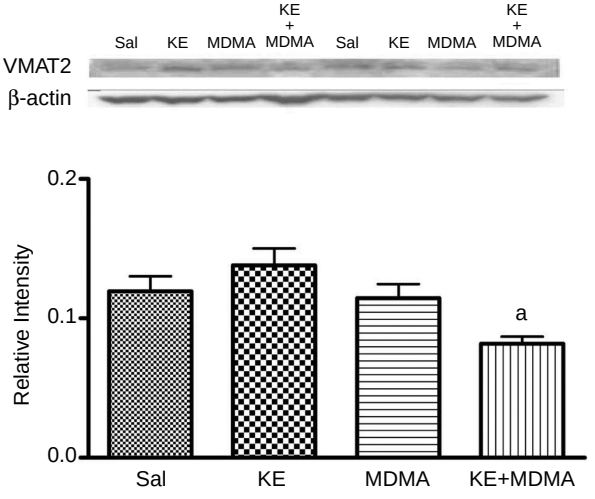

Fig. 4. Binge doses of ketamine pretreatment exacerbate MDMA-induced VMAT-2 decrease in striatum. Three cumulative doses of MDMA (20 mg/kg/dose) decrease VMAT-2 expression in striatum two weeks after the drug treatment. Seven consecutive injections of ketamine (KE, 50 mg/kg/injection) alone do not affect VMAT-2 expression in striatum. Pretreatment with KE exacerbates later MDMA-induced DAT decrease. Data are expressed as mean ± SEM. n = 4 for each group. [a]Significantly lower than Sal and KE groups.

0.0026) and nucleus accumbens (F(3,20) = 5.15, P = 0.0084; F(3,20) = 5.46, P = 0.0066) (Fig. 2). Nonetheless, pretreatment with binge doses of ketamine (50 mg/kg each) did not affect MDMA-induced serotonergic neurotoxicity in these regions (Fig. 2).

Discussion

Three consecutive doses (60 mg/kg in total) of MDMA produced long-lasting central dopaminergic neurotoxicity, as evidenced by decreased DA and DOPAC

contents in frontal cortex, striatum and nucleus accumbens as well as decreased DAT and VMAT-2 expressions in striatum. Likewise, such MDMA dosing regimen produced long-lasting central serotonergic neurotoxicity in the aforementioned regions. Although seven cumulative doses of ketamine (totally 350 mg/kg) alone did not cause dopaminergic or serotonergic depletions in these regions that we examined, pretreatment with binge doses of ketamine was found to aggravate MDMA-induced central dopamine neurotoxicity. In contrast, pretreatment with ketamine did not affect MDMA-induced serotonergic neurotoxicity.

To date, combined use of ketamine and MDMA represents a growing concern of issues in polydrug abuse. We now report that binge use of ketamine aggravates the subsequent MDMA-induced central neurotoxicity, specifically on dopaminergic systems. Such exacerbated central dopaminergic toxicities were considered long-lasting since the neurotoxic effects were examined two weeks after the drug administration in this study. Due to the fact that striatum is a relay for the extrapyramidal motor system, sequential binge use of ketamine and MDMA may cause long-lasting motor dysfunction in human subjects.

Many lines of evidence support the notion that binge doses of ketamine produced acute neuronal apoptotic death by a direct NMDA blockade and late-onset excitotoxic neurodegeneration *via* up-regulation of NMDA receptors (16, 21). Multiple doses of ketamine treatment may aggravate later MDMA-induced dopaminergic neurotoxicity by NMDA over-expression and subsequent NMDA-mediated neuronal apoptosis. Previously, a similar ketamine dosing regimen as we used was documented to cause wide-spread neurotoxicity in developing brains (10, 18). In adult brains, we found that such ketamine treatment aggravated the MDMA-induced central dopaminergic neurotoxicity, while did not affect MDMA-induced central serotonergic neurotoxicity. A non-anesthetic dose of ketamine as we used in this study was reported to enhance dopamine release in a dopaminergic terminal region (14). Thus, the specificity for ketamine's potentiation on MDMA-induced dopaminergic neurotoxicity could be attributed to conjunctive MDMA and ketamine-stimulated dopamine release and subsequent oxidative stress and free radical generation. Moreover, a recent study indicated that MDMA may produce striatal serotonergic neurotoxicity *via* dysregulation of energy metabolism since local perfusion of nicotinamide or ubiquinone, substrates of energy metabolism, was found to attenuate MDMA-induced serotonergic depletion in the striatum (7). Binge doses of ketamine pretreatment as we used in this study did not seem to ameliorate such energy metabolism in mitochondria.

Multiple doses of MDMA administration rapidly decreased DAT-mediated plasmalemmal dopamine uptake in rat striatal synaptosomes, while such deficit was completely reversed one day later (9). In contrast, MDMA treatment rapidly decreased VMAT-2-mediated vesicular dopamine transport in rat striatal vesicles and this decrease partially recovered one day later (9). In our study, we found that multiple doses of MDMA treatment produced a long-lasting DAT decrease but spared VMAT-2 expression in mice. The discrepancy of MDMA-modulated DAT expression between these studies may arise from differential sensitivity of species to MDMA treatment due to the fact that MDMA treatment barely produced striatal dopaminergic toxicity in rats, but caused profound dopaminergic neurotoxicity in mice.

It has been demonstrated that ambient temperature and core temperature are associated with the MDMA-induced serotonergic neurotoxicity (4). Lately, amiloride and its analogs were found to aggravate methamphetamine-induced dopaminergic toxicity without altering methamphetamine-associated hyperthermia in both rats and mice (2). In contrast, amiloride ameliorated MDMA-induced serotonergic toxicity and potentiated MDMA-induced hyperthermia in rats (7). Although the notion that MDMA-induced central neurotoxicity is proportional to the drug-induced hyperthermia remains to be determined, we can not rule out the possibility that pretreatment with binge doses of ketamine aggravates MDMA-induced dopaminergic toxicity *via* potentiating MDMA-induced hyperthermia.

In conclusion, our results suggest that continuous binge doses of ketamine and MDMA exacerbate MDMA-induced central dopaminergic neurotoxicity in adult mouse brains.

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