Individual and Combined Effects of Methamphetamine and Ketamine on Conditioned Place Preference and NR1 Receptor Phosphorylation in Rats

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Abstract
Methamphetamine (MA), a commonly abused psychostimulant, induces the drug dependence by enhancing the dopamine-mediated neurotransmission. Ketamine (KET) is a non-competitive N-methyl-D-aspartate receptor antagonist, which can be actually mixed with MA for polydrug abuse. In the present study, the individual and combined effects of KET (10 mg/kg, i.p.) and MA (1 mg/kg, i.p.) on conditioned place preference in rats were investigated. The alterations of serine 897 phosphorylations of NR1 receptors in the striatum and ventral tegmental area of after-conditioning rats were measured immunohistochemically. The results showed repeated administrations of MA, KET and their combination, at the doses studied, all could induce psychological dependences evaluated by conditioned place preference. KET was not able to suppress the MA-induced place preference. The modulations of NR1 phosphorylations in basal ganglia were partly responsible to place preference. Although the alterations induced by KET were not significant in most areas we studied, MA showed a significant increase in the ventral tegmental area but a marked decrease in caudate putamen and nucleus accumbens. Such alterations were much more significant when KET and MA were combined. These results have important implications for public awareness of harm with combined drug abuse. Further investigations toward the specific interaction of the two drugs are necessary.

Introduction
Methamphetamine (MA) belongs to amphetamine-type psychostimulant. The incidence of MA abuse is constantly increasing [1]. MA is an active ingredient in a variety of tablets illegally sold for recreational purposes. The tablets make users feel a short yet intense ‘rush’ when the drug is initially administered and become addicted quickly, needing higher doses and more often. The tablets of abuse usually contain one or more ingredients other than MA, such as 3,4-methylenedioxymethamphetamine, amphetamine and ketamine (KET) [2]. Amid these ingredients which are usually taken together, MDMA and amphetamine are structurally and pharmacologically similar to MA. KET is pharmacologically
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classified as a non-competitive \( N \)-methyl-D-aspartate (NMDA) receptor antagonist and is also a common drug of abuse [3]. Although regarded as an addictive substance, KET was reported to have the ability to suppress the morphine-induced place preference, a paradigm for assessing rewarding or reinforcing effects of drugs [4]. Hence it is necessary that we should not only evaluate the individual addictive potential of MA and KET, but also assess the combined effect of MA and KET. The results would add to our knowledge of these commonly abused drugs.

One of the most important targets in brain of MA dependence is the basal ganglia, a group of nuclei in the brain interconnected with the cerebral cortex, thalamus and brain stem [5]. Three crucial areas in the basal ganglia, i.e., the ventral tegmental area (VTA), nucleus accumbens (NAc, namely ventral striatum) and caudate putamen (CPu, namely dorsal striatum), were investigated in the present study. The VTA-NAc system is implicated in the stimulant and reinforcing properties of drugs of abuse. Many of the neuroadaptations induced by the drugs reside in a circuit that includes the dopamine projections from the VTA to NAc, as well as the corticofugal excitatory projections from the prefrontal and limbic cortices to the mesoaccumbens projection [for review, see 6]. So far, it is well known that the primary molecular site of action for MA-induced behaviors and cellular adaptations is the loss of dopamine transporters, which results in an increase in extracellular dopamine content in the NAc [7]. Non-competitive NMDA receptor antagonists were found to increase dopamine release in NAc by activating dopaminergic neurons whose cell bodies were found in the VTA [8, 9]. Meanwhile, the VTA-NAc system has been proved to be a target of the action of KET [10]. Other than the ventral striatum-NAc, the dorsal striatum-CPu is also involved in drug dependence. MA binge altered the levels of multiform transmitters in CPu [11–13]. The CPu region is also relevant to the action of KET, though without sufficient detailed evidence [14].

Within the basal ganglia, glutamate receptors play a major role in the transmitter balance. There are two basic types of glutamate receptors: ionotropic receptors (mainly NMDA receptors; \( \alpha \)-amino-3-hydroxy-5-methylisoxazole-4-proponic acid (AMPA) receptors) and metabotropic glutamate receptors [15–17]. Previous results have implied that NMDA receptors, but not the AMPA and the metabotropic glutamate receptors, are the main groups of receptors that affect addictive behaviors [for review, see 18]. Accordingly, we preferred to focus the research on the alterations of NMDA receptors. NMDA receptors are heteromeric assemblies of NMDA receptor (NR) sub-units. There are three distinct classes of NR subunits: obligatory NR1 subunits, modulatory NR2A-2D subunits, and NR3 [16, 19, 20]. Protein phosphorylation has been established as an important mechanism for the regulation of NMDA receptor function [21]. NR1 shows phosphorylation at three distinct serine sites (897, 896, and 890) in the carboxyl tail region (intracellular domain). The previous study found that phosphorylated NR1 at serine 897 (pNR1S897) was expressed in the brains of the normal rats at the highest level amid three phosphorylated NR1 subunits (pNR1S897, pNR1S896, pNR1S890) [22]. It is an appropriate way to investigate the phosphorylation of NR1 serine 897 following the repeated drug exposure [22], but the phosphorylation status of the NR1 has not been measured in the major regions (VTA and striatum) of reward system after chronic drug exposure. Therefore, we investigated the alterations of pNR1S897 expressions in the rat brains after repeated MA or KET administration. The effects on pNR1S897 of their combination were examined at the same time.

In this research, conditioned place preference (CPP) was employed for behavioral tests. CPP is a classic model for evaluating the psychological dependence [23]. Drugs of abuse display a differential ability to produce CPP. In general, psychostimulants lead to rodent CPP over a wide range of experimental conditions [for review, see 24]. It was shown that MA could induce a significant CPP [25–27]. KET was also confirmed to be able to produce place preference at the sub-anesthetic dose [28, 29]. Furthermore, the psychological dependence evaluated by CPP was well documented to be influenced by the manipulations of NMDA receptors in basal ganglia [for review, see 29]. The present study therefore attempted to evaluate psychological dependence in rats and then histochemically examine the relevant alterations of NMDA receptors in basal ganglia.

Materials and Methods

Experimental Animals

Adult male Wistar rats (200–230 g, 8 rats per group) were obtained from Laboratory Animal Research Center, Southern Medical University (Guangzhou, PR China). The rats were bred in closed colony. The rats were housed at a temperature of 22 ± 1°C with a 12-hour light-dark cycle (light on 07:00–19:00 h). Food and water were available ad libitum.

Drug Administration and Place Conditioning

All animal experiments were approved by the Laboratory Animal Research Committee, Southern Medical University. The place conditioning experiments were performed in accordance...
with a previous study [23]. There were three phases of behavioral test protocols: pre-conditioning, conditioning and post-conditioning. Place conditioning was conducted according to a biased procedure. The apparatus consisted of a shuttlebox (30 × 60 × 30 cm; w × l × h). The box was divided into two compartments of equal size by means of a sliding partition. One compartment was black with a textured floor, the other was white with a smooth floor. For conditioning, rats were immediately confined to the white compartment following drug injection and to the dark compartment following vehicle injection.

During the pre-conditioning phase (1 day), the baseline preference of rats was determined. The partition of the shuttlebox separating the two compartments was removed. Each rat was placed in the shuttlebox and allowed to explore the two compartments for 15 min. The time spent by each rat in the white compartment was recorded. The position of the rat was defined by the position of its head. All the trials were conducted under conditions of dim illumination and masking white noise.

Conditioning sessions were conducted once daily for 8 consecutive days. The rats were injected with the drug (MA racemate, 1 mg/kg, i.p., National Laboratory of Narcotic Drugs, PR China, dissolved in sterile 0.9% NaCl solution; KET racemate, 10 mg/kg, i.p., Jiangsu Hengrui Medicine Co., Ltd, PR China, dissolved in sterile 0.9% NaCl solution; or normal saline, 1 ml/200 g) and placed into the white compartment on the first day. On the next day, rats were injected with saline and placed into the black compartment. From day 3, this cycle (session) for the conditioning was repeated three times, each session was 60 min in duration. In a combination study, the rats were pretreated with KET (10 mg/kg, i.p.) 30 min prior to each MA injection (1 mg/kg, i.p.).

During the post-conditioning phase (next day), the time spent in the white compartment during a 15-min session was measured again.

Data Analysis
All the data in the test are expressed as the mean ± SEM. Site preference, namely the time spent in the white compartment of each different group of rats, was tested. The statistical analysis was carried out by one-way ANOVA, followed by the least significant different test. Two-sided probability values of p < 0.05 were considered to be statistically significant.

Immunohistochemistry
Four rats of each group after the conditioning test were deeply anesthetized with chloral hydrate (350 mg/kg, i.p.) as a 3.5% solution in 0.9% NaCl solution. The rats were perfused transcardially with 50–100 ml of saline followed by 200 ml of fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4), and the brains were hardened by post-fixation in the paraformaldehyde solutions at 4°C overnight. The brains were sectioned using a vibrating microtome. Sections (70 μm) obtained with a vibrating microtome were collected in phosphate-buffered saline (PBS; pH 7.4).

Immunofluorescence was performed as described in our previous studies [30, 31]. The sections of the rat brains in all the four groups were incubated and reacted in one immunofluorescence reaction for comparison. The sections were incubated in primary antibodies against pNR1S897 (rabbit polyclonal; 1:4,000 in Tris-buffered saline (TBS, pH 7.6) supplemented with 0.03% Triton X-100 (PBS-Triton) + 10% normal goat serum (NGS); Chemicon) at room temperature overnight. The sections were washed with TBS and incubated in goat anti-rabbit secondary antibody conjugated to Alexa 488 (1:500 dilution in TBS-Triton; Molecular Probes) for 2 h at room temperature in the dark. The sections were then mounted on clean slides with mounting medium (Dako) and observed in a laser scan confocal microscope (Zeiss LSM 510, excitation at 488 nm). Digital images showing immunoreactivity of different groups were captured under the same parameters in the confocal microscope.

Control for Immunofluorescence
A series of control experiments were first conducted to evaluate the selectivity of antibodies used in the present study. Controls for the immunofluorescence were performed by omission of either the primary or secondary antibodies in turn in the reaction sequences of the labeling experiments. The sections were also observed in the laser scan confocal microscope. Specificity of the anti-pNR1S897 was characterized by a previous study [32].

Digital Image Analyses and Statistics
In the single-labeling study, quantifications of immunoreactivity were carried out by counting the total numbers and determining the relative intensities of neurons which were immunoreactive to pNR1S897 in different areas as follows: (i) five selected areas [dorsolateral (DL), dorsomedial (DM), ventrolateral (VL), ventromedial (VM), and central subdivisions] in the dorsal striatum (namely CPu) [22] (see fig. 1A); (ii) the ventral striatum (namely NAc) histochemically subdivided into a core region and a shell region for measurement [33] (see fig. 1B), and (iii) VTA.

The random views for each subdivided regions of a rat brain section were used for semiquantitative analyses at low (using a 10× lens) or high (using a 63× lens) magnifications, respectively. At low magnification, the numbers of immunopositive neurons in each photomicrograph were counted regardless of size or shape. The data were expressed as the total number of labeled cells in each of areas. At high magnification, the fluorescence intensities of positive neurons in the confocal microscope images were determined by using an image analyzing software (Image-Pro Plus). The immunofluorescence in the neuropilar elements and immunonegative nuclei were excluded. Only the presumed medium spiny neurons in the striatum were analyzed. In each region, statistical comparisons between the groups were made by one-way ANOVA, followed with Bonferroni comparison using SPSS software. The probability levels of p < 0.05 were considered statistically significant.

Results
Effects of KET on MA-Induced Place Preference
On the pre-conditioning day, the rats spent more time in the black compartment than in the white compartment. The number of seconds that each rat spent in the drug-associated (white) compartment was tested. Initially, these site preferences were not significantly different between the groups (F(3, 31) = 0.610, p > 0.05).
In the analysis of post-conditioning time, one-way ANOVA indicated significant difference between the drug treatment groups \((F(3, 31) = 5.232, p < 0.01)\). After conditioning, the times spent in drug-associated place of normal saline group, MA, KET group and their combination are 307.77 ± 39.86, 477.11 ± 32.94, 424.33 ± 24.09, and 440.29 ± 28.92 s, respectively. As shown in figure 2, MA at the dose of 1 mg/kg and KET at the dose of 10 mg/kg both induced significant place preference versus normal saline (\(p < 0.01/p < 0.05\)). In the combination study of MA and KET, pretreatment of 10 mg/kg KET cooperated with 1 mg/kg MA to the acquisition of place preference. In terms of post-conditioning time, the difference between the combination group and normal saline group was remarkably significant \((p < 0.01)\).

**Expression of pNR1S897 in the Striatum (CPu and NAc) and VTA**

Control for Immunofluorescence
When either the primary or secondary antibodies were omitted, no immunofluorescence was detected in the sections of neostriatum or VTA (data not shown).

Changes of pNR1S897 Expression in the Neostriatum (CPu and NAc)

In the single-labeling study, pNR1S897 showed a high level of immunoreactivity in the entire CPu. The regional distribution of pNR1S897-immunoreactive neurons seemed heterogeneous in terms of staining intensities and the numbers of positive cells in the different subdivisions of the CPu area, and individual cells showed different levels of immunostaining. Cellurally, pNR1S897 was observed over a large number of medium-sized striatal neurons, intense staining on cell bodies with little or no fiber staining in normal rats. In all the staining neurons, the nuclei of neurons are immunonegative (fig. 3A: b, d, f, h). At low magnification \((10\times)\), the numbers of pNR1S897-positive medium spiny neurons in the subdivided regions of the CPu were examined. Statistical analyses showed that there was no significant difference of...
Fig. 3. Effects of repeated administration of MA, KET, and their combination on the expression of phospho-NR1 serine 897 in CPu. A Fluorescence photomicrographs illustrate the effects of saline, MA (1 mg/kg, i.p.), KET (10 mg/kg, i.p.), their combination on pNR1S897 immunoreactivity in CPu at low magnification (a, c, e, g) and at high magnification (b, d, f, h). Scale bars: 200 μm (in g for a, c, and e); 20 μm (in h for b, d and f). B Bars represent the average fluorescence intensity ± SEM of pNR1S897-positive neurons in the CPu observed at higher magnification.

* p < 0.05; ** p < 0.01, versus normal saline group. *** p < 0.001, versus MA-alone group. @@@ p < 0.001, versus KET-alone group.
the numbers of pNR1S897-labeled cells between drug treatment groups in DL, DM, VL, VM, and central regions of CPu. At high magnification (63×), the intensities of pNR1S897-positive medium spiny neurons in the subdivided regions of CPu were determined. As shown in figure 3B, in the DL region there was no statistically significant difference of groups (F(3, 159) = 2.055, p > 0.05). In the CPu central and VL region, pNR1S897 fluorescence intensity was not altered by the injection of MA or KET (p > 0.05 vs. control). However, pretreatment of 10

Fig. 4. Effects of repeated administration of MA, KET, and their combination on the expression of phospho-NR1 serine 897 in NAc shell. A Fluorescence photomicrographs illustrate the effects of saline, MA (1 mg/kg, i.p.), KET (10 mg/kg, i.p.), combination of MA and KET on pNR1S897 immunoreactivity in the NAc shell at low magnification (a, c, e, g) and at high magnification (b, d, f, h). Scale bars: 200 μm (in g for a, c, and e); 20 μm (in h for b, d and f). B Bars represent the average number ± SEM of pNR1S897-positive neurons in NAc observed at lower magnification. C Bars represent the average fluorescence intensity ± SEM of pNR1S897-positive neurons in NAc observed at higher magnification. ** p < 0.01; *** p < 0.001, versus normal saline group. b@ p < 0.01, versus KET-alone group.
mg/kg KET before the administration of 1 mg/kg MA markedly decreased pNR1S897 expression (p < 0.001 vs. control; p < 0.001 vs. MA alone; p < 0.001 vs. KET alone). In the medial area of CPu, the intensity of pNR1S897 staining was significantly weaker in the MA group than in saline control (p < 0.001), and the fluorescence intensity in the MA+KET groups was remarkably lower than in the control group (p < 0.001), also than in the MA-alone group and KET-alone group (p < 0.001 vs. control; p < 0.01 vs. MA alone; p < 0.001 vs. KET alone). In the dorsal part of the medial CPu, the KET group showed significantly low pNR1S897 fluorescence intensity (p < 0.05 vs. control).

The expression of pNR1S897-immunoreactive neurons in the ventral striatum (NAc) was not as strong as in the dorsal striatum (CPu) in one section under the same parameters on the whole. The pNR1S897-immunoreactive neurons clustered more in the core than in the shell of the NAc, but the intensities of the two parts appeared homogeneous. MA apparently decreased the number of...
pNR1S897-immunopositive neurons in the NAc shell and core regions, albeit the decrease was not significant statistically (see fig. 4B). In the analysis of fluorescence intensities in pNR1S897-positive neurons, MA significantly decreased intensity compared with control in the NAc shell region (p < 0.05), but there is no significant difference between the MA group and control in the NAc core region (p > 0.05) (see fig. 4C). As shown in figure 4B and C, obviously in both the NAc shell and NAc core, the analyses of intensities and numbers in pNR1S897-positive neurons showed that KET cooperated with MA in decreasing pNR1S897 expression significantly. In the analysis of pNR1S897-positive number, there was a significant difference between the MA+KET group and saline control (NAc shell: p < 0.01/NAc core: p < 0.001). Also, the number of the MA+KET group was significantly less than the KET group in NAc (p < 0.01).

In the analysis of fluorescence intensity, there was a significant difference between the MA+KET group and control (NAc shell: p < 0.001/NAc core: p < 0.001), and the intensity of the MA+KET group was significantly weaker than the MA group in the NAc core (p < 0.001). The significant difference was shown between the combination group and KET-alone group in NAc (NAc shell: p < 0.001/NAc core: p < 0.001).

Changes of pNR1S897 Expression in VTA

In contrast to in CPu and NAc, in VTA the pNR1S897 immunoreactivity of the normal rats was more intensive. As is seen in figure 5A (a, c, e, g), immunostaining appeared heterogeneous in the VTA. The immunonegative neurons were more clustered adjacent to the bottom of the substantia nigra. At the cellular lever, the cell bodies of neurons were immunonegative but the nuclei parts were immunonegative (fig. 5A: b, d, f, h). There was no significant difference of fluorescence intensities between groups (p > 0.05), but the data from counting positive neurons showed the significant difference between groups according to one-way ANOVA (F(3, 22) = 5.645, p < 0.01). The average immunoreactive numbers of saline control, MA, KET, and MA+KET were 73.33 ± 7.14, 92.33 ± 3.31, 81.50 ± 3.96, and 106.83 ± 8.44, respectively. Shown in the post-hoc analysis, MA (1 mg/kg, i.p.) significantly increased pNR1S897 immunoreactive neuron number (p < 0.05 vs. saline control); whereas there was no significant difference between the KET and control group (p > 0.05). The increase induced by the combination of MA and KET was significant compared with saline control (p < 0.01) and KET alone (p < 0.01).

Discussion

The CPP paradigm is one of the most widely used procedures to assess rewarding or reinforcing effects of drugs in experimental animals. It relies on the capacity of drug stimuli to elicit approach responses and maintenance of contact as a measure of rewarding effects [34]. MA was reported that it could induce a positive CPP [25–27], a drug-seeking behavior. In the present study, the rats, administered with MA, spent significantly more time in the drug-associated compartment than did those administered with normal saline. The phenomenon verified that the repeated MA administration could lead to serious psychological dependence.

KET, as a NMDA receptor antagonist, has a wide range of effects on human, including analgesia, anesthesia, hallucinations, arterial hypertension, and so on. In clinic, it is primarily used as an anesthetic, usually in combination with some sedative drugs [for review, see 35], while KET use in medical and veterinary settings is well documented and has a good safety record. However, the increase in its unregulated use outside such controlled environments is supposed to be concerned. In low- to upper-middle dosages, KET produces hallucinatory effects similar to other dissociative anesthetics [36]. Little has been reported about the assessment of KET in terms of CPP paradigm. We found KET, at dose of 10 mg/kg, produced a place preference phenomenon in rats, which was consistent with a previous research [4]. Suzuki et al. [4] also revealed that KET suppressed the place preference elicited by morphine in mice albeit KET induced CPP when used alone. However, our investigation demonstrated that pretreatment of KET before MA administration did not interfere with MA-induced CPP. The arising divergence occurred mainly due to the distinct mechanisms underlying MA and morphine dependence. The reversal effect of KET on morphine-induced CPP probably could be explained with the alteration of opioid receptors [37]. In contrast, KET even at relatively high dose could not attenuate MA-induced CPP. This is one of the rational explanations for illicit use of the combination, which can enhance addictive potential. Considering these, more attention should be paid to the control of KET, and more addictive assessments for their combination should be performed clinically.

Protein phosphorylation is a major mechanism for regulation of NMDA receptor function. The phosphorylation of NR1 subunits determines the activation of NMDA receptors. It is well known that the rewarding properties of various abused drugs are mediated by me-
solimbic dopaminergic neurons that terminate into the ventral striatum-NAc, and the neurons could be modulated by NMDA receptors in the VTA and neostriatum of the brain [6]. The alterations of pNR1S897 are quite distinct in the VTA and neostriatum under exposure of MA, KET or their combination.

In our study, the dopaminergic neurons, which play an important part in the reward system in the VTA, showed an increase in the phosphorylation levels of the NR1 subunit of the NMDA receptor at serine 897 following MA repeated exposure, and the pretreatment of KET did not block, but facilitated the increase of pNR1S897 in the VTA induced by MA. According to the addictive theory, in the VTA, chronic drug exposure activates NMDA receptors which, by elevating Ca$^{2+}$ influx, leads to a higher extracellular signal-regulated kinase activity and tyrosine hydroxylase expression. Tyrosine hydroxylase, the rate-limiting enzyme of dopamine synthesis increased in the VTA in the drug-dependent state, leads to enhanced dopamine release [for review, see 6]. It was demonstrated that MA could increase dopamine release in the NAc [38], but the specific mechanism is not clear. In terms of the results, the increase in pNR1S897 phosphorylation of VTA neurons played a role in the MA dependence. Although KET is a non-competitive NMDA receptor antagonist, it could indeed indirectly intensify MA-induced pNR1S897 expression. The mechanisms underlying the serine 897 phosphorylation is not very clear. Protein kinase A was found to phosphorylate NR1 at serine 897 [21, 39]. Thus the increase of pNR1S897 in the VTA was probably due to the enhancement of protein kinase A activity.

In contrast to the VTA, the present results showed a significant decrease of pNR1S897 after MA exposure in the NAc shell. KET did not alter the phosphorylation levels of NR1 at serine 897 both in the shell and in the core areas of NAc, but KET facilitated the decrease of pNR1S897 induced by MA in the NAc-containing shell and core regions. The principal neuronal cell type in the NAc is medium GABAergic spiny neurons. After drug exposure, the decrease of phosphorylation levels on NR1 receptors played an important role in the release of neurotransmitters. The neurotransmitter produced by these neurons is γ-aminobutyric acid (GABA), one of the main inhibitory neurotransmitters of the central nervous system. The reduced GABA release in the brain may indirectly boost up drug dependence. If MA and KET are combined, the severer impairment could occur in NAc.

The caudate nucleus is highly innervated by dopamine neurons. These neurons originate mainly from the VTA and the substantia nigra pars compacta. There are also additional inputs from various association cortices. MA brought out the down-regulated phosphorylation of NR1 at the serine 897 site in the medial region of CPu, whereas in most of the CPu subdivisions, the down-regulations of pNR1S897 were not significant in the KET treatment group. However, MA combined with KET resulted in a greater decrease of pNR1S897 than MA alone. It also indicated that KET could aggravate the alteration of pNR1S897 induced by MA, which mediated changes in the rewarding system.

Taken together, the results showed repeated administrations of MA, KET and in their combined mode, at the doses studied, could all induce psychological dependencies evaluated by CPP. KET was not able to suppress the MA-induced place preference. The modulations of NR1 phosphorylations in basal ganglia were partly responsible to place preference. Although the alterations induced by KET were not significant in most of the areas we studied, MA showed a significant increase in the VTA but a marked decrease in striatum containing CPus, the core and shell regions of NAc. Such alterations were much more significant when KET and MA were combined. These results have important implications for public awareness of the harm with combined drug abuse. Further investigations toward the specific interactive effects of the two drugs are necessary.

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