Inhibition by ketamine and amphetamine analogs of the neurogenic nitrergic vasodilations in porcine basilar arteries

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ABSTRACT

The abuse of ketamine and amphetamine analogs is associated with incidence of hypertension and strokes involving activation of sympathetic activities. Large cerebral arteries at the base of the brain from several species receive dense sympathetic innervation which upon activation causes parasympathetic-nitrergic vasodilation with increased regional blood flow via axo-axonal interaction mechanism, serving as a protective mechanism to meet O_2 demand in an acutely stressful situation. The present study was designed to examine effects of ketamine and amphetamine analogs on axo-axonal interaction-mediated neurogenic nitrergic vasodilation in porcine basilar arteries using techniques of blood-vessel myography, patch clamp and two-electrode voltage clamp, and calcium imaging. In U46619-contracted basilar arterial rings, nicotine (100 μM) and electrical depolarization of nitrergic nerves by transmural nerve stimulation (TNS, 8 Hz) elicited neurogenic nitrergic vasodilations. Ketamine and amphetamine analogs concentration-dependently inhibited nicotine-induced parasympathetic-nitrergic vasodilation without affecting that induced by TNS, nitroprusside or isoproterenol. Ketamine and amphetamine analogs also concentration-dependently blocked nicotine-induced inward currents in Xenopus oocytes expressing α3β2-nicotinic acetylcholine receptors (nAChRs), and nicotine-induced inward currents as well as calcium influxes in rat superior cervical ganglion neurons. The potency in inhibiting both inward-currents and calcium influxes is ketamine > methamphetamine > hydroxyamphetamine. These results indicate that ketamine and amphetamine analogs, by blocking nAChRs located on cerebral perivascular sympathetic nerves, reduce nicotine-induced, axo-axonal interaction mechanism-mediated neurogenic dilation of the basilar arteries. Chronic abuse of these drugs, therefore, may interfere with normal sympathetic-parasympathetic interaction mechanism resulting in diminished neurogenic vasodilation and, possibly, normal blood flow in the brainstem.

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1. Introduction

The large arteries at the base of the brain such as the basilar artery irrigating the brainstem are densely innervated by sympathetic and parasympathetic nerves (Lee et al., 1982; Kimura et al., 1997; Yu et al., 1998). Nitric oxide (NO) released from the parasympathetic nerve terminals is the major neurotransmitter which dilates the cerebral arteries (Kimura et al., 1997; Yu et al., 1998; Liu and Lee, 1999). In these arteries, the sympathetic-parasympathetic interaction plays a role in regulating neurogenic nitrergic vasodilation (Zhang et al., 1998; Si and Lee, 2002; Lee et al., 2011). According to this hypothesis, upon activation of nicotinic acetylcholine receptor (nAChR) on cerebral perivascular sympathetic nerves, norepinephrine (NE) released from the sympathetic nerves acts on β2-adrenoceptor located on parasympathetic nitrergic nerves, causing NO release. The released NO subsequently activates guanylyl cyclase and cyclic guanosine monophosphate (cGMP) production in smooth muscle cells, leading to vasodilation with increased blood flow (Chang et al., 2012). These findings indicate that activation of cerebral perivascular sympathetic

nerves to the basilar artery causes dilation, but not constriction, of the basilar arteries via the axo-axonal or sympathetic- parasympathetic interaction mechanism. These results reveal that autonomic regulation of the arteries at the base of the brain is different from that of the cortical vessels where the close apposition of the sympathetic and parasympathetic nerve terminals is not found, and therefore the axo-axonal interaction mechanism is less obvious (Lee and Saito, 1984). The physiological significance of this axo-axonal interaction mechanism in the large arteries at the base of the brain is supported by the findings that the axo-axonal interaction mechanism-mediated nitrergic vasodilation is diminished in hypertension (Chang et al., 2012). Although the exact reasons underlying this change in hypertension remain to be examined, it has been reported that the parasympathetic nerve terminals significantly decrease while the sympathetic nerve terminals increase or decrease depending on type of hypertension (Lee and Saito, 1984; Saito and Lee, 1985). These morphological alterations in hypertension obviously diminish anatomical basis for the axo-axonal interaction leading to decreased neurogenic nitrergic vasodilation in hypertension (Lee and Saito, 1984; Saito and Lee, 1985; Chang et al., 2012). Accordingly, the axo-axonal interaction mechanism-mediated neurogenic vasodilation may play an important role, especially, as a protective mechanism in increasing brainstem blood flow to meet the O_2 demand when facing an acutely stressful situation (Chang et al., 2012).

Ketamine, a dissociated antagonist of NMDA receptors, is a popularly abused drug among teenagers (Gunduz-Bruce, 2009). Overdosed ketamine is known to cause dysfunctions of the nervous, urinary and cardiovascular systems. In the cerebrovascular system, low dose S(+)-ketamine shifted the autoregulatory curve toward higher mean arterial pressure (Engelhard et al., 1997), suggesting its possible activation of sympathetic tone. In addition, ketamine in concentrations higher than 5 μM has been reported to reduce the active muscle tone of cerebral arteries induced by vasoconstrictors (Wendling et al., 1996; Kamel et al., 2008). The exact mechanism of action of ketamine in the neurogenic regulation of large arteries at the base of the brain is not elucidated.

Methamphetamine and its analogs, D-/L-amphetamines and 3,4-methylenedioxymethamphetamine (MDMA), also are popular club drugs among young adults (Smith et al., 2002). These illicit drugs are powerful sympathetic stimulants, causing physical and psychological abnormalities, such as cardiovascular dysfunctions, hyperactivity, impaired memory, anxiety and stroke (Smith et al., 2002). Furthermore, acute and chronic treatment of methamphetamine induced striatal hypoxia and dopamine reduction, indicating a possible regulation of neuronal functions via a vascular mechanism (Kousik et al., 2011). The exact mechanism in the regulation of arterial tone by methamphetamine and its analogs are not well elucidated.

Ketamine has been reported to block the nAChR-mediated increases of intracellular calcium in rat intracardiac ganglionic neurons (Weber et al., 2005) and the nAChR-mediated inward currents in SH-SY5Y, PC12 cells, and Xenopus oocytes exogenously expressing α4β2-, α4β4 and α7-nAChRs (Friederich et al., 2000, Sasaki et al., 2000, Yamakura et al., 2000). Both methamphetamine and MDMA also are shown to bind the nAChRs in PC12 cells, indicating a direct interaction between amphetamine derivatives and nAChRs (Garcia-Rates et al., 2007). It is therefore important to examine if ketamine and amphetamine analogs inhibited the nAChRs located on the perivascular sympathetic nerves, leading to diminished neurogenic dilation of the arteries at the base of the brain. Positive results may provide significant clinical information that chronic uses of these club drugs may impair the neurogenic vasodilation mechanism and the decreased blood flow may precipitate brainstem stroke and dysfunctions. Using isolated porcine basilar arteries and the circle of Willis in the present study, we demonstrated that ketamine and amphetamine analogs blocked nAChRs located on the perivascular sympathetic nerves and inhibited nicotine-induced neurogenic nitrergic vasodilation.

2. Materials and methods

2.1. Tissue and cell preparation

For porcine cerebral arteries, pig heads were collected from Fong-lin packing company in Fong-lin, Hualien, Taiwan. Entire brains, with pial arteries attached, were carefully removed and placed in Krebs' bicarbonate solution equilibrated with 95% O_2 and 5% CO_2 at room temperature. For culture of superior cervical ganglion (SCG) neurons, the dissected SCGs of the Sprague Dawley rats were immediately transferred to the Hank's balanced salt solution (HBSS) and cut into small pieces. The HBSS containing collagenase D was added to the SCG fragments with final concentrations of 1–2 mg/ml. After incubation at 30–35 °C for 30 min, cells were dissociated by gentle trituration, and precipitated by centrifugation at 1500 rpm. Cells were resuspended in the neurobasal medium containing B27 (1:50 dilution), L-glutamine (0.5 mM), nerve growth factor (50 ng/ml), and antibiotics (streptomycin 100 U/ml and penicillin 100 U/ml). The cell suspension was loaded into a 4-well dish in which coverslips precoated with 0.1 mg/ml poly-D-lysine were placed in advance, and then incubated with humidified air containing 5% CO_2 at 37 °C. For the heterologous expression of nAChRs, the oocytes in stages V and VI from Xenopus laevis were harvested, and intracellularly injected with the α3β2-nAChR RNA through a nanoinjector (Drummond, Broomall, PA). The oocytes were maintained at 18 °C. All experimental procedures were conducted according to "Guide for the care and use of laboratory animals" from the United States Public Health Service, and also approved by the Institutional Animal Care and Use Committee of Tzu Chi general hospital and Tzu Chi University.

2.2. In vitro tissue bath techniques for recording tension in arterial rings

The basilar arteries and circle of Willis of the porcine brain were carefully removed and dissected into 4 mm-long segments. The ring segments were cannulated with a stainless steel rod and a short piece of platinum wire, and mounted in an isolated tissue bath containing 9 ml of Krebs' bicarbonate solution (NaCl 96.87 mM, KCl 5.16 mM, $MgSO_4$ 1.22 mM, $NaHCO_3$ 25.56 mM, $CaCl_2$ 1.33 mM, L-ascorbic acid 0.11 mM, ethylenediaminetetraacetic acid (EDTA) 0.34 mM, and glucose 11.0 mM, pH 7.4). The Krebs' bicarbonate solution was maintained at 37 °C and equilibrated with 95% 0_2 and 5% CO_2. Resting tension of the artery was adjusted to 0.7 g. A period of 1 h was allowed for equilibration. A pair of stimulating electrodes, one on either side of the artery, was arranged for transmural nerve stimulation (TNS) which was delivered across the electrodes via a stimulator (Grass S88, Astro-Med. Inc., RI, USA). Stimulation parameters were trains of 200 biphasic square wave pulses of 0.6 ms, 200 mA. Active muscle tone was induced by vasoactive agent, U46619. The effect of drugs on the degree of vasodilation was expressed as a percentage of papaverine (300 μM)-induced maximum relaxation (Si and Lee, 2002; Lee et al., 2000).

For denudation of the endothelium, the endothelium was mechanically removed by using syringe needle (size from gauge 28–24) equivalent to the outer diameter of the arteries according to our previous report (Lee, 1982). The complete denudation was verified by lack of N-nitro-L-arginine (NLA)-induced further constriction of U46619-precontracted arterial rings (Lee et al., 1982; Si and Lee, 2002).

2.3. Whole-cell patch clamp technique on dissociated SCG neurons

A coverslip with dissociated neurons was placed in the recording chamber on the stage of an inverted phase-contrast microscope (Leica, Wetzlar, Germany) and perfused with the extracellular solution (NaCl 140 mM, KCl 2.5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 2 mM, HEPES 10 mM, and glucose 10 mM, pH 7.45). Whole-cell recording from neurons were obtained according to our previous reports (Si and Lee, 2002; Long et al., 2006;
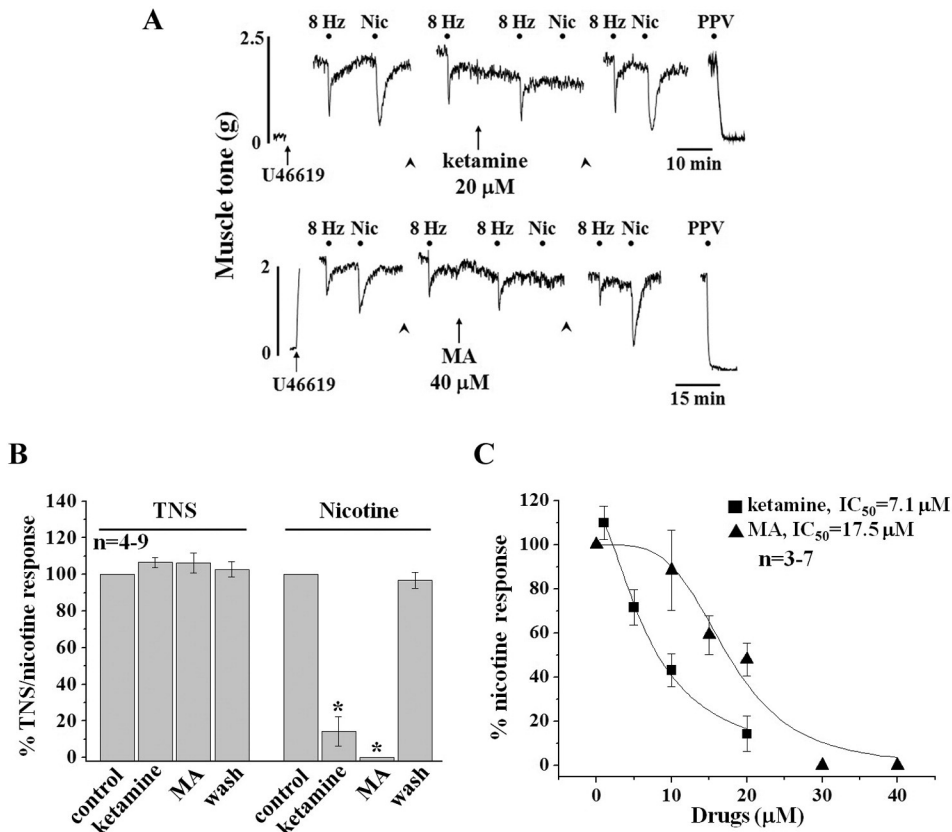
Fig. 1. Effect of ketamine and methamphetamine on TNS- and nicotine-induced relaxation of porcine basilar arteries and circles of Willis. Representative tracings in panel A showing that ketamine and methamphetamine (MA) inhibited relaxations induced by nicotine (100 μM) and TNS at 8 Hz in the endothelium-denuded cerebral arteries in the presence of active muscle tone induced by U46619 (0.1–0.2 μM). Ketamine (20 μM) and MA (40 μM) almost abolished the nicotine-induced relaxation without affecting that elicited by TNS (8 Hz). These results were summarized in a bar graph in panel B. Data points were normalized to their respective control. Panel C showing concentration-dependent inhibition of nicotine-induced vasodilations by ketamine (■) and MA (▲). The IC_{50} values for ketamine and MA are 7.1 μM and 17.5 μM, respectively. Complete inhibition was estimated for curve fitting. Arrowheads in A indicate repeated washings. Values are means ± SEM. n = number of experiments. *P < 0.05 indicates significantly different from the respective control.
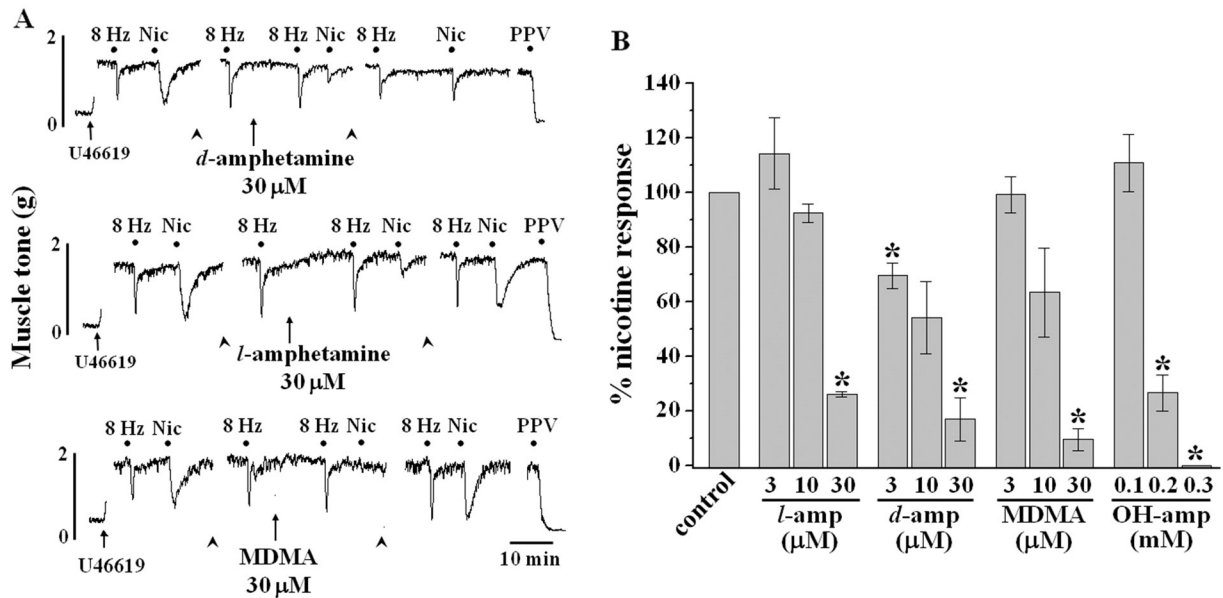

Fig. 2. Effects of ᴅ-amphetamine (ᴅ-amp), ʟ-amphetamine (ʟ-amp), MDMA and hydroxyamphetamine (OH-amp) on nicotine- and TNS-induced relaxations of basilar arteries. Representative tracings showing that ᴅ-Amphetamine, ʟ-amphetamine and MDMA at 30 μM almost abolished the relaxation induced by nicotine without affecting that elicited by TNS in basilar arteries in the presence of active muscle tone induced by U46619. Arrowheads indicate repeated washings. These results are summarized in panel B. The extent of nicotine-induced relaxations was normalized to their own controls. Values are means ± SEM. n = 3–6. *P < 0.05 indicates significantly different from respective controls.

Chen et al., 2012) and those by others (Hamill et al., 1981). The recording electrodes pulled by a P-97 microelectrode puller (Sutter, Novato, CA) were filled with the intracellular solution composed of (in mM) K-gluconate 145, KCl 10, EGTA 1, and HEPES 10, at pH 7.4, and had resistance of 8–18 MΩ. For drug application, nicotine, ketamine, and amphetamine analogs were filled in individual tubes which were connected to a three-barreled glass perfusion pipett controlled by a fast exchange system SF-77 (Warner, Hamden, CT). Drugs dissolved in the extracellular solution were applied onto neurons by gravity flow. The extracellular solution was allowed to perfuse the recording chamber continuously.

2.4. Two-electrode voltage clamp on oocytes expressing α3β2-nAChR

Two-electrode voltage clamp for the whole oocyte recording (Chen and Chen, 2001; Mozayan et al., 2006) was performed at room temperature by using an OC-725C amplifier (Warner, Hamden, CT). The borosilicate glass capillaries (1.5 mm, outer diameter) (World Precision Instruments, FL, USA) were pulled using a P-97 microelectrode puller (Sutter, Navato, CA). When filled with 3 M KCl, the electrodes had 0.3–1 MΩ resistance. During recording, the oocytes were perfused with ND96 solution (NaCl 96 mM, KCl 2 mM, MgCl$_2$ 1 mM, CaCl$_2$ 1.8 mM, and HEPES 5 mM, pH 7.5) at a rate of 10 ml/min.

Data acquisition and analysis for electrophysiological recordings: the membrane potential was held at −60 mV. Data acquisition and analysis were performed with pClamp 9.0 and Digidata 1322 A (Axon instruments, Union city, CA). The traces were filtered at 1 KHz and sampled at 2 KHz. The maximum inward current was determined as the current amplitude. To compensate for differences in nAChR expression levels, the data were normalized as percent of the nicotine-induced response. The IC$_{50}$ values were obtained by fitting the means with a logistic equation.

2.5. Intracellular calcium imaging

This was carried out according to our previous report (Si and Lee, 2002; Mozayan et al., 2006; Chen et al., 2012). Briefly, the cultured SCG neurons were loaded with 5 μM fluo-4 AM in physiological buffer containing (in mM, pH 7.3) NaCl 130, KCl 5, glucose 5, CaCl$_2$ 2, MgCl$_2$ 2, HEPES 10, and incubated at 37 °C for 45 min. The cells were washed with calcium indicator-free buffer to remove any dye that was non-specifically associated with the cell surface, and then incubated for a further 30 min to allow complete de-esterification of intracellular AM esters. Calcium fluorescence images and real-time changes of the fluorescence intensity were examined and analyzed by using an inverted fluorescence microscope (Leica, Wetzlar, Germany) and the MetaFluor software (Nihon Molecular device, Tokyo, Japan). Drugs were applied as described in whole-cell patch clamping.

2.6. Statistical analysis

Data were summarized and presented as means ± SEM. Paired t-test were used for the statistics. To compare the SNP- or isoproterenol-induced vasodilation in the presence and absence of drugs, two-way ANOVA was used. A P < 0.05 was considered as significant.

3. Results

3.1. Ketamine and amphetamine analogs inhibited nicotine-induced neurogenic vasodilation of porcine cerebral arterial rings

In the presence of active muscle tone induced by U46619, isolated endothelium-denuded basilar arteries and the circle of Willis relaxed upon electrical depolarization of perivascular nerves by TNS (8 Hz) and applications of nicotine (100 μM). In consistent to our previous reports (Zhang et al., 1998; Si and Lee, 2002), the vasorelaxation induced

by TNS and nicotine was significantly blocked by tetrodotoxin (blockade of 100% of TNS-induced and 63% of nicotine-induced relaxations; n = 7 for each) and N-nitro-L-arginine (blockade of 100% of both TNS- and nicotine-induced relaxations; n = 4 for each). Nicotine- induced vasodilations were blocked by ketamine and methamphetamine in a concentration-dependent manner with the IC$_{50}$ values of 7.1 μM (n = 5–7) and 17.5 μM (n = 3–6), respectively (Fig. 1C). The maximum inhibition of nicotine-induced neurogenic vasodilation by ketamine at 20 μM and methamphetamine at 40 μM was greater than 80% (Figs. 1A & B), and 100% inhibition was applied for the curve fitting (Fig. 1C). Both drugs in similar concentrations, however, did not affect TNS-induced neurogenic vasodilations (Figs 1A&B). The inhibition by ketamine and methamphetamine of nicotine-induced relaxations was fully recovered after washing off both drugs (Fig. 1A).

Furthermore, similar results as those for methamphetamine were obtained for D-amphetamine, L-amphetamine and MDMA. These amphetamine analogs in a concentration-dependent manner inhibited nicotine-induced vasodilations (Fig. 2B) without affecting TNS-induced vasorelaxations (Fig. 2A). At 30 μM, a maximal inhibition was observed for D-amphetamine, L-amphetamine and MDMA (Fig. 2A & B). Hydroxyamphetamine did not affect nicotine-induced relaxations until its concentration was greater than 0.1 mM (Fig. 2B), which has a significantly lower potency (IC$_{50}$ = 154.9 μM, n = 4–6) than that of D-amphetamine (IC$_{50}$ = 15.1 μM, n = 3), L-amphetamine (IC$_{50}$ = 19.7 μM, n = 3–4) and MDMA (IC$_{50}$ = 12.7 μM, n = 3–4).

3.2. Ketamine and methamphetamine did not affect sodium nitroprusside (SNP)- or isoproterenol-induced relaxations of isolated cerebral arteries

Since β$_2$-adrenoceptors on parasympathetic nerves mediates norepinephrine transmission causing NO release (Si and Lee, 2002), we examined whether inhibition by ketamine and methamphetamine of nicotine-induced vasodilations is resulted from blockade of β$_2$-adrenoceptors and/or the NO-cGMP pathway. In the presence of active muscle tone induced by U46619, endothelium denuded cerebral
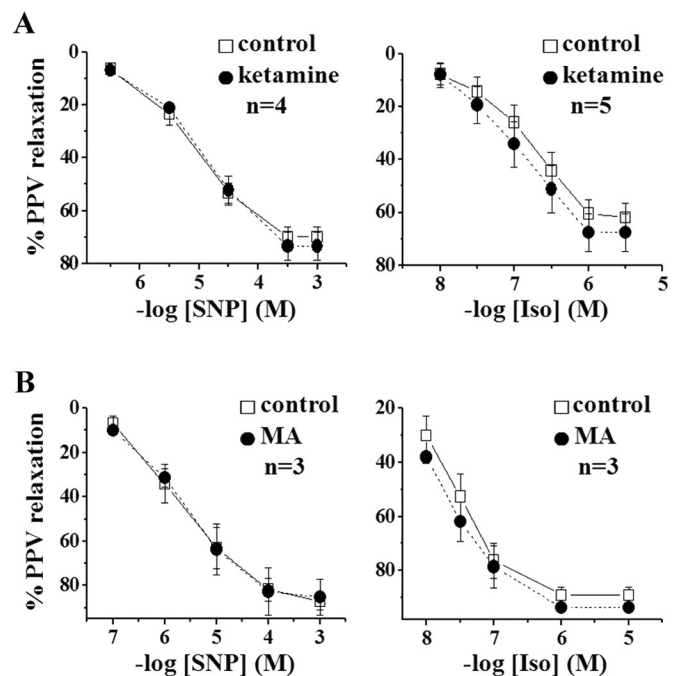

Fig. 3. Ketamine and MA did not affect sodium nitroprusside (SNP)- or isoproterenol (ISO)-induced relaxations of basilar arteries and circles of Willis. SNP and isoproterenol were cumulatively added to the endothelium-denuded cerebral arteries in the presence of active muscle tone induced by U46619 (0.1–0.2 μM). The magnitude of relaxations was estimated as a percentage of papaverine/PPV-elicited maximum relaxation. Values are means ± SEM. n = number of experiments.

arteries relaxed upon application of SNP and isoproterenol in a concentration-dependent manner. The relaxation induced by both vasodilators was not appreciably affected by ketamine or methampetamine (Fig. 3A & B). Similar results were also obtained for other amephetamine analogs (see Supplementary information Fig. S1).

3.3. The nAChRs-mediated inward currents were reduced by ketamine and methamphetamine in dissociated neuronal cells of the SCG

The cerebral perivascular sympathetic nerves originate in the SCG (Lee et al., 2011). To elucidate whether ketamine and methamphetamine blocked native nAChRs, effects of these drugs on nicotine-induced inward currents in dissociated rat SCG neurons were examined. Nicotine at 30 μM elicited inward currents in neurons, which deactivate slowly (Fig. 4A). In the presence of ketamine and methamphetamine (100 μM), the amplitudes of nicotine-induced inward currents were reduced. This reduction by both drugs was concentration-dependent (Fig. 4B) with the IC_{50} values of 12.7 and 35.6 μM for ketamine and methamphetamine, respectively (n = 3–7, Fig. 4B). The inhibitory potency of *d*-amphetamine (IC_{50} = 14.6 μM, n = 4–6) was very close to that of ketamine (Fig. 4B). For comparison, hydroxyamphetamine which had much less effect on nicotine-induced nurogenic vasodilation

(Fig. 2B), had less effects on nicotine-induced currents on the SCG neurons with the IC_{50} value of 220.3 μM (n = 3; Fig. 4B). The potency in blocking nicotine-induced inward currents was ketamine > d-ampetamine > methamphetamine > hydroxyamphetamine (Fig. 4B). Similar potency rank of ketamine and amphetamine analogs in blocking nicotine-elicited inward current in α3β2-nAChRs heterologously expressing Xenopus oocytes was obtained (see Supplementary information Fig. S2).

3.4. Ketamine and methamphetamine inhibited nAChR-mediated calcium influxes of dissociated rodent SCG neurons

Since the calcium ion plays a critical role in triggering the release of the neurotransmitter norepinephrine, we further examined whether nicotine-initiated calcium influxes were inhibited by ketamine and/or amphetamine analogs. Nicotine at 100 μM drastically elevated the fluorescence intensity of fluo-4, an indicator of the internal calcium level, and this elevation was significantly reduced by ketamine and methamphetamine in a concentration-dependent manner (Fig. 4C). Ketamine also was more potent than methamphetamine in inhibiting nicotine-induced calcium influx. D-Amphetamine at slightly lower concentrations than methamphetamine also inhibited calcium influx to similar degree
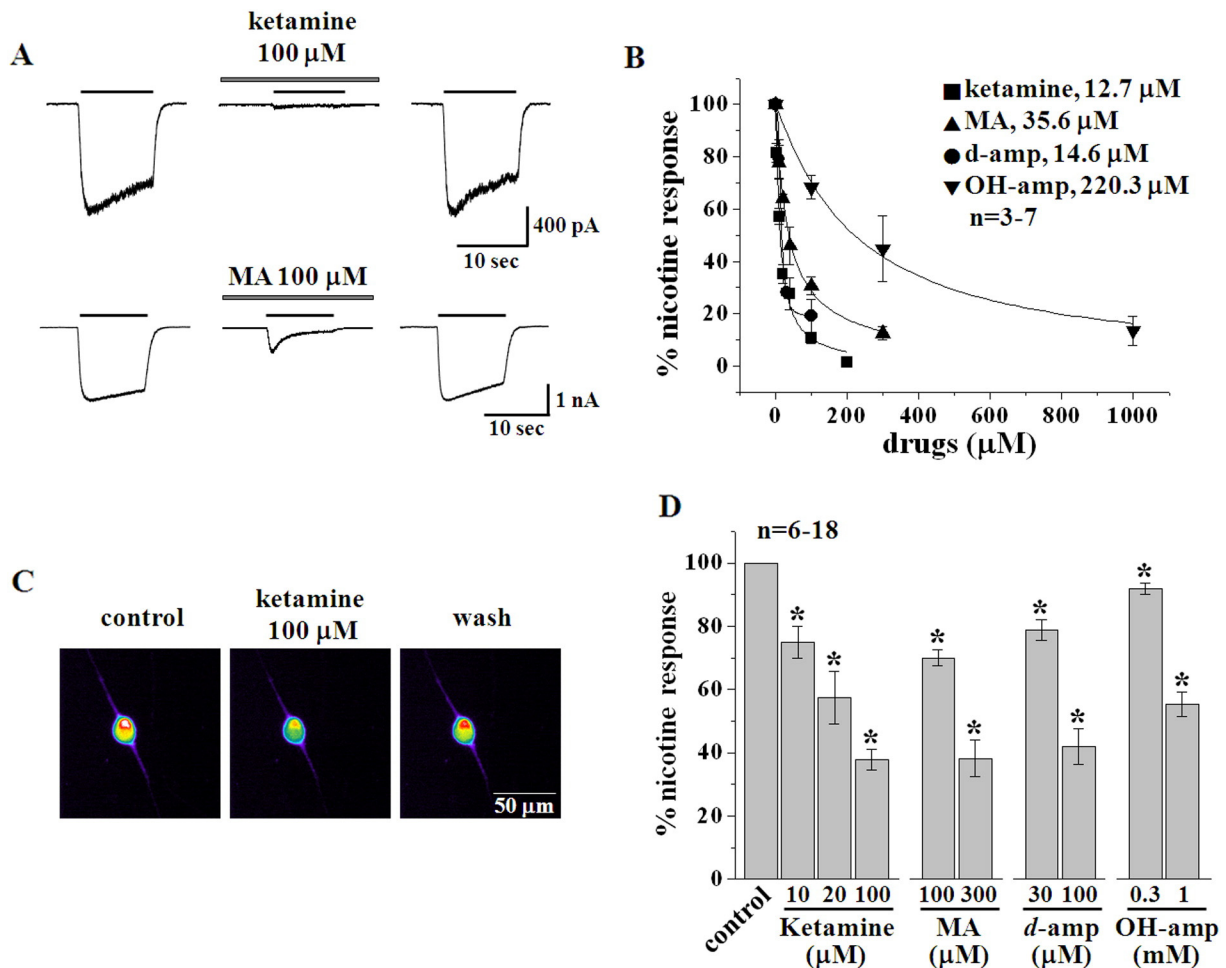

Fig. 4. Effects of ketamine and methamphetamine on nAChR-mediated inward currents and calcium influxes in the rat SCG neurons. Representative tracings in panel A show that ketamine and methamphetamine (MA) at 100 μM markedly decreased nAChR-mediated inward currents induced by nicotine (30 μM). Thin line bars and the gray color-filled bar indicate applications of nicotine and drugs, respectively. The concentration-response relationships for ketamine and MA inhibition of nicotine-induced inward currents are shown in panel B. The current amplitudes in the presence of ketamine (■) and MA (▲) were normalized to those prior to drug application in the same SCG neurons. The IC_{50} values for ketamine and methamphetamine were 12.7 and 35.6 μM, respectively. n = 3–7. In panel C, representative images showing changes in intracellular calcium levels stimulated by 100 μM nicotine in the presence or absence of 100 μM ketamine. Pseudocolors are used to indicate the intensity of Fluo-4 fluorescence. Inhibition of different concentrations of ketamine, methamphetaminae (MA), D-amphetamine (D-amp) and hydroxyamphetamine (OH-amp) on nicotine-induced increases in intracellular calcium levels is summarized in panel D. Nicotinic responses in the presence of drugs were normalized to their own controls. n = 7–22. Data were means ± S.E.M. *P < 0.01 indicates significant difference from the respective controls.
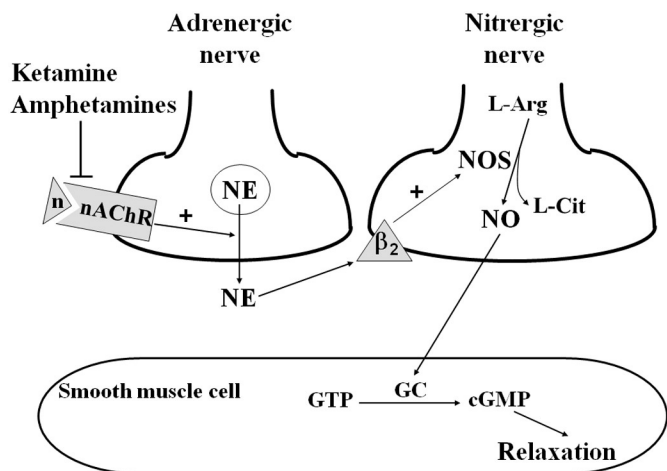
Fig 5. Ketamine and amphetamine analogs inhibit nicotine-induced cerebral nitrergic neurogenic vasodilation via blockade of nAChRs on the sympathetic nerves. A diagram depicts the close apposition of an adrenergic nerve terminal and a cholinergic-nitrergic nerve terminal, and their relationship to a smooth muscle cell in the porcine cerebral artery. The binding of nicotine or acetylcholine to the nAChR on the adrenergic nerve terminal causes calcium influx, facilitating NE release. NE subsequently acts on β_2-adrenoceptors located on the nitrergic nerve terminal resulting in activation of NO-cGMP pathway and relaxation of the smooth muscle cell. NO is the predominant vasodilator in cerebral arteries and is produced by converting L-arginine (L-Arg) to L-citrulline (L-Cit) via NO synthase (NOS) (Chen and Lee, 1995). Ketamine and amphetamine analogs do not block NO synthesis or its neurovascular transmission, rather these drugs block nAChRs on perivascular sympathetic nerves, leading to decreased nicotine-induced axo-axonal interaction-mediated cerebral neurogenic nitrergic vasodilations.

(Fig. 4D). In consistent to its effects on nicotine-induced relaxations of porcine cerebral arteries and inward currents in the rat SCG neurons, hydroxyamphetamine in μM concentrations only slightly affected nicotine-elicited calcium influxes in the SCG neurons (Fig. 4D).

4. Discussion

The presence of dense sympathetic adrenergic and parasympathetic nitrergic nerves in large arteries at the base of the brain of many species is well established (Toda, 1981; Edvinsson et al., 1982; Lee et al., 1982; Kimura et al., 1997; Yu et al., 1998). Electrical depolarization of these perivascular nerves and application of nicotine elicit exclusive nitrergic neurogenic dilation of porcine cerebral arteries (Zhang et al., 1998; Lee et al., 2000). The present studies demonstrated that ketamine and amphetamine analogs concentration-dependently inhibited nicotine-induced neurogenic dilations of the porcine isolated basilar and circle of Willis arteries without affecting TNS-induced neurogenic nitrergic vasodilation of these arteries. In parallel, these drugs in similar potency order inhibited calcium influxes and nAChRs-mediated inward currents in dissociated rat SCG neurons and the α3β2-nAChR-expressing oocytes. These results indicate that ketamine and amphetamine analogs (particularly, methamphetamine, D-amphetamine, L-amphetamine and MDMA) inhibit nAChRs on the sympathetic neurons, leading to diminished sympathetic-parasympathetic interaction-mediated nitrergic neurogenic vasodilations of arteries at the base of the brain (Fig. 5).

Ketamine is known to act primarily on the N-methyl-D-aspartate (NMDA) glutamate receptors to block the permeation of these receptors (Kamel et al., 2008). It also binds several subtypes of nAChRs including the α3β4-, α4β2-, α7-, or mixed types of nAChRs on primary neurons (Flood and Krasowski, 2000; Friederich et al., 2000; Weber et al., 2005). The present findings further indicated that ketamine blocked the α3β2-nAChR and its mediated neurogenic dilation of the basilar artery. The IC$_{50}$ values for ketamine inhibition of nicotine-induced cerebral neurogenic vasodilation are close to the clinical EC$_{50}$ doses during anesthesia, and are much lower than those of the abused doses (Friederich and Urban, 1999; Yamakura et al., 2000). These results

reveal possible clinical significance of the present findings that decreased neurogenic vasodilation by administration of ketamine may lead to diminished brainstem blood flow, and subsequent brainstem dysfunctions due to ischemia. It should be noted that high concentration of ketamine (5 μM to 1 mM) has been shown to induce relaxations of isolated canine basilar arteries (Fukuda et al., 1983) and bovine middle cerebral arteries (Wendling et al., 1996) by blocking calcium influxes into arterial smooth muscle cells. Inhibition of KCl- or serotonin-induced muscle contractions of canine basilar arteries by ketamine at maximum concentrations, however, accounted only for about 10% (Fukuda et al., 1983). In the present study in porcine basilar arteries, ketamine at 20 μM and MA at 40 μM, which did not relax the basilar arteries, blocked greater than 80% and 90%, respectively, of nicotine-induced neurogenic vasodilation. It appears that ketamine concentrations for either clinical or abuse purposes will exert its more prominent inhibitory effects on the perivascular sympathetic nAChRs, leading to diminished neurogenic vasodilation before its possible vasorelaxation effects. Since sympathetic-parasympathetic interaction-mediated nitrergic neurogenic vasodilation with increased blood flow may play an important physiological role in coping with acutely emergency response (Chang et al., 2012), ketamine blockade of nAChR and decrease of neurogenic nitrergic dilation of the basilar artery may lead to altered auto-regulation and diminished blood perfusion in the brainstem (Engelhard et al., 1997). Since nAChRs are ubiquitously distributed in the brain, our present finding may also imply that the nAChRs-mediated neurological functions may be altered by ketamine under anesthesia or after drug abuse, which possibly contributes to the cognitive difficulties in human.

Methamphetamine is primarily metabolized to amphetamine and hydroxyamphetamine (Sakai et al., 1982). Hydroxyamphetamine was a weak inhibitor in inhibiting nicotine-induced neurogenic vasodilator, since it was effective only in mini molar concentrations. On the other hand, ethamphetamine, amphetamine and MDMA were more potent than hydroxyamphetamine, and were approximately equipotent in inhibiting nicotine-induced neurogenic nitrergic dilation of the basilar arteries. These amphetamine analogs are known to be powerful sympathetic stimulants, which induce excessive norepinephrine outflow by the monoamine transporter-mediated exchange-diffusion (Filinger and Stefano, 1981). According to the axo-axonal interaction hypothesis (Fig. 5), increased synaptic concentrations of norepinephrine will increase activation of β$_2$-adrenoceptors located on the parasympathetic nitrergic nerve terminals, resulting in release of NO and vasodilation. This phenomenon of increased neurogenic vasorelaxation, however, was not observed upon applications of full concentrations (0.3 μM–40 μM) of methamphetamine and amphetamine in the present experiments. It is possible that the concentration of increased released norepinephrine in the synapse is below that capable of significantly activating the β$_2$-adrenoceptors and mediated neurogenic vasodilation, or the enhanced vasodilation is overcome by their possible direct α-adrenoceptor-mediated smooth muscle constriction effects (Easton et al., 2007). This latter suggestion in the basilar arteries is excluded, since porcine basilar arteries are endowed, from functional aspect, predominant β$_1$-adrenoceptors (Lee et al., 1982, 2011). Furthermore, the threshold concentration of these agonists (>30 μM) for a direct constriction of smooth muscle was significantly higher than that for their inhibition of nicotine-induced vasorelaxations (our preliminary results). These findings favor the notion that lack of neurogenic vasodilation induced by amphetamine and its analogs is most likely due to their potent inhibition of the sympathetic nAChRs, and their effects on norepinephrine release in the basilar arteries are diminished. Accordingly, the nAChRs-initiated axo-axonal interaction-mediated neurogenic nitrergic vasodilation is predominantly blocked by amphetamine and its analogs (except hydroxyamphetamine).

The plasma concentrations of amphetamines in abusers are frequently in several micromolar or even 10 times higher (Anggard et al., 1970; Jones and Holmgren, 2005; Melega et al., 2007). These high concentrations of amphetamine likely will induce vasoconstriction in

addition to inhibition of nicotinic receptor-initiated axo-axonal interaction-mediated neurogenic nitrergic vasodilation. Thus, amphetamine analogs (MA, amphetamine, MDMA) abusers may be prone to suffer from chronic ischemia due to lack of protective mechanism in the brainstem, especially, during the acute stressful situation when oxygen supply is needed in order to meet the demand of activated neurons in the sympathetic nerve center in the brainstem (Fujii et al., 1991). It has been reported that reduced blood flow in basilar artery may attenuate auditory brainstem responses (Wada et al., 1988). Brainstem ischemia may lead to several neurovascular diseases including locked-in syndrome (Lacroix et al., 2012) and Alzheimer's disease (Marchesi, 2011). Thus, chronic inhibition of the neurogenic vasodilations of basilar arteries by ketamine and amphetamine analogs may reveal their possible serious toxicological consequence on the neurovascular functions in drug abusers.

Although ketamine and amphetamine analogs inhibited nicotine-induced axo-axonal interaction-mediated vasodilations in basilar arteries and the circles of Willis, these drugs did not affect the nitrergic neurogenic relaxations of these arteries induced by TNS (direct electrical depolarization of the perivascular parasympathetic nitrergic nerves), or vasorelaxation induced by sodium nitroprusside (SNP). These results imply that ketamine and amphetamines do not directly affect neuronal NO synthesis and release, or NO-cGMP signaling in the smooth muscle cells (Fig. 5). Furthermore, isoproterenol-induced vasodilation was not affected by ketamine or amphetamines either, indicating that these drugs did not block nitrergic neurogenic vasodilation by inhibiting the β_2-adrenoceptors on parasympathetic nitrergic neurons (Fig. 5). These results favor the possibility that inhibition by ketamine and amphetamine analogs of nicotine-induced, nitrergic neurogenic vasodilation is due to their blocking the nAChRs located on the perivascular postganglionic sympathetic nerves (Fig. 5). The basilar arteries, used in the present myographic study, are known to receive postganglionic sympathetic neurons without the SCG (Lee et al., 1982; Edvinsson et al., 1982). It is reasonable to propose that nicotine-induced nitrergic neurogenic vasodilation is due to nicotine activation of the nAChRs on the postganglionic sympathetic fibers. Indeed, nicotine fails to elicit any nitrergic neurogenic dilation of sympathetically denervated basilar arteries (Zhang et al., 1998; Chang et al., 2012). Together with the present findings that ketamine and amphetamine analogs block nicotine-induced inward currents and calcium influxes in the SCG which is the sole original of the cerebral perivascular sympathetic nerves, it is most likely that ketamine and amphetamine analogs inhibit nicotine-induced, axo-axonal interaction-mediated nitrergic neurogenic vasodilation by blocking the nAChRs on the postganglionic sympathetic neurons.

The hypothesis that ketamine and amphetamines blockade of sympathetic nAChRs leading to diminished neurogenic nitrergic vasodilation of the basilar arteries and the circle of Willis is further supported by results from electrophysiological and calcium imaging studies. Experiments using two-electrode voltage clamp and patch clamp techniques demonstrated that ketamine and methamphetamine concentration-dependently reduced nicotine-induced inward currents mediated by $\alpha3\beta2$-nAChRs exogenously expressed in Xenopus oocytes, and by the native ganglionic nAChRs in primarily cultured rat SCG neurons. The potency order of both agents in inhibiting nicotine-induced currents is comparable in both preparations. In addition, ketamine and methamphetamine in similar concentration ranges inhibited calcium influxes into the SCG neurons. These results provide convincing evidence that ketamine and amphetamine analogs inhibited nicotine-induced neurogenic vasodilation by directly blocking the perivascular sympathetic nAChRs. It is interesting to note that hydroxyamphetamine, which is a weak inhibitor for axo-axonal-interaction-induced neurogenic vasodilation (Figs. 2 and 5), possessed rather weak effects on blocking nicotine-induced inward currents in the SCG neurons and $\alpha3\beta2$-nAChRs expressing Xenopus oocytes, and calcium influx into the SCG neurons. This may justify its safety in clinical practice (Mughal and Longmuir, 2009).

In summary, ketamine and several amphetamine-type stimulants are common club drugs abused by young peoples. In addition to reported serious health problems (Morgan and Curran, 2012; De Silva et al., 2007; Winslow et al., 2007), chronic use of these drugs may, via blockade of nAChRs on perivascular sympathetic nerves, potentially reduce neurogenic nitrergic dilations of large arteries at the base of the brain. These effects may lead to decreased blood flow and innate protective mechanism, causing significant neurovascular dysfunctions in the brainstem and related cardiovascular diseases.

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Disclosures

None declared conflict of interest.

Transparency document

The Transparency document associated with this article can be found, in online version.

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