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Rhynchophylline Down-regulates NR2B Expression in Cortex and Hippocampal CA1 Area of Amphetamine-induced Conditioned Place Preference Rat

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N-methyl-D-aspartate receptor 2B subunit (NR2B) has an important role in the development of conditioned place preference (CPP) and psychostimulant abuse. Rhynchophylline is presently used to treat central nervous systems diseases and has a non-competitive antagonistic effect on NMDA receptors. In this study, amphetamine was administered in rats (2 mg/kg, s.c., once each day for 4 consecutive days), during which they were treated with rhynchophylline (60 mg/kg, i.p., once each day for the next 3 days). NR2B mRNA and protein expression were examined by *in situ* hybridization and immunohistochemistry. CPP was induced by amphetamine (2 mg/kg, s.c.) by 4th day in rats. Rhynchophylline effectively reversed the expression of amphetamine-induced CPP and itself did not produce a CPP. Amphetamine-CPP rats showed a significantly increased NR2B mRNA and protein expression in medial prefrontal cortex and hippocampal CA1 areas as compared to the control group. Rhynchophylline reversed NR2B mRNA and protein levels induced by amphetamine but rhynchophylline by itself had no effect on NR2B expression in control rats. These results indicate that rhynchophylline inhibits the expression of amphetamine-induced rewarding effect, and this action might be related to down-regulation of NR2B expression in medial prefrontal cortex and hippocampal CA1 area.

Key words: Rhynchophylline, Amphetamine, NMDA receptor 2B subunit, Medial prefrontal cortex, Hippocampal CA1 area, Conditioned place preference

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INTRODUCTION

The recreational drug, amphetamine and its analogues are popular among young people due to its energy and mood enhancing properties (Jones and Simpson, 1999) although its abuse is associated with distressing psychiatric adverse effects (Cajetan Luna, 2001). The variety of responses emerging from amphetamine has raised contradictive view points among researchers regarding the degree of the harm caused by this drug (Check, 2004; Maurer et al., 2004). Many studies were conducted with a focus on the neuroadaptive responses of amphetamine in the glutamate and dopamine systems (White et al., 1995; Bowyer et al., 2004; Liao, 2008). Glutamate is a principal excitatory amino acid neurotransmitter in the central nervous system. It activates ionotropic glutamate receptors such as *N*-methyl-D-aspartate (NMDA) and kainic acid receptors and metabotropic glutamate receptors (Hollmann and Heinemann, 1994). NMDA receptors are composed of two classes of subunits: one subunit is NMDA receptor 1 (NR1) composed of at least eight splice variants and additionally NMDA receptor 2 (NR2A-D) made of four subunits. NR1 is

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essential to form functional NMDA receptor channels, while NR2 subunits are the molecular determinants for the functional diversity in these receptors. In rats and humans, the NR2B subunit is primarily expressed in forebrain structures, such as the cortex, hippocampus and striatum (Wang et al., 1995; Laurie et al., 1997; Goebel and Poosch, 1999). The medial prefrontal cortex and hippocampal CA1 area have important roles in drug-induced conditioned place preference (CPP) (Karami et al., 2002; Hsu and Packard, 2008).

Psychostimulants such as amphetamine act by increasing monoamine transmission, thus initiating a cascade of cellular events that in turn modifies behavior. Repeated drug administration produces many changes of the regular brain functions. CPP paradigm allows the rewarding properties of a treatment to be inferred by assessing approach behavior to environmental cues previously paired with the affective consequences of the treatment. Amphetamine-induced CPP in rats is a popular model of drug-mediated associative learning in humans. Conditioned rats demonstrate a preference for the environment previously paired with amphetamine in a drug-free state (Bardo and Bevins, 2000). NMDA receptor is implicated in mediating the biological actions of drug abuse, especially the psychostimulants (Jones et al., 1999). Numerous studies have reported that NR2B plays an important role in the development of CPP (Narita et al., 2000; Ma et al., 2007) and in alcohol, cocaine, and opioid abuse (Loftis and Janowsky, 2003).

Rhynchophylline is an important active tetracyclic oxindole alkaloid isolated from *Uncaria rhynchophylla* which is a traditional Chinese medicine and often used to treat central nervous system illnesses such as lightheadedness, convulsions, numbness etc. and cardiovascular diseases like hypertension (Shi et al., 2003). In previously conducted vitro studies, rhynchophylline reduced glutamate induced Ca^{2+} influx and protected against glutamate-induced neuronal death in cultured cerebellar granule cells (Shimada et al., 1999). The protective effect of rhynchophylline against glutamate-induced excitotoxicity involved an inhibition of the NMDA receptors (Kang et al., 2002), further indicating that blockade of NMDA-type glutamate receptors by rhynchophylline is involved in the neuroprotective action of *Uncaria rhynchophylla*.

The present study was therefore designed to investigate the effects of rhynchophylline on expression of amphetamine-induced CPP in rats and NR2B receptor subunit, mRNA and protein expression changes in the CPP-related medial prefrontal cortex and hippocampal CA1 area.

MATERIALS AND METHODS

Animals

Male Wistar rats, weighing 200 - 260 g, were provided by Experimental Animal Center of First Military Medical University. All animals were adapted to the experimental conditions (temperature: $20 \pm 2^{o}C$, humidity: $60 \pm 5\%$ and 12 h dark/light cycle) for one week. All rats had free access to tap water and chow diet. The ethical aspects of the research plan and experimental procedures had been approved by Science and Technological Committee and the Animal Use and Care Committee of First Military Medical University.

Drugs and reagents

Rhynchophylline (No. H1I2, purity 99.7%) was brought from Matsuura Ykugyo Co. Amphetamine sulphate (No. 1211-9301) was purchased from National Institute for the Control of Pharmaceutical and Biological Products. Both drugs were dissolved in physiological saline to final concentrations and injected in a volume of 10 mL/kg. The kits of NR2B *in situ* hybridization (biotinylated rabbit anti-rat digoxigenin antibody), SABC-AP immunohistochemistry and polyclonal rabbit anti-NR2B antibody, and 3, 3-diaminobenzidine were obtained from Boster Biotechnology Co. Diethylpyrocarbonate (No. 10977-015) was purchased from Sigma Chemicals. All the other chemicals used in this experiment were reagent grade from commercial source.

CPP

The CPP apparatus consisted of two equal-sized compartments ($30 \times 30 \times 30$ cm), one with a white interior and the other a black interior separated by a wall with a sliding door. For testing, the sliding door was raised 12 cm above the floor to allow the rat free access to both sides of the box.

A biased procedure was used to conduct CPP test, which consisted of three phases: pre-conditioning, conditioning and post-conditioning. The CPP paradigm took place on 8 consecutive days. For the pre-conditioning phase (day 1-3), the rat was placed under the door which was left open to allow free access to the entire box for 15 min each day. On day 2 and day 3, the time spent on each compartment was measured by seconds-counter over a 15-min period as an indicator of reinforcing properties and averaged to use as the pre-conditioning time of each animal. There was significant difference between the time spent on the black compartment (not drug-paired side) (778.7 ± 37.0, $n = 32$) and the time spent on the white compartment (drug-paired side) (121.3 ± 37.0, $n = 32$) before drug

conditioning ($p < 0.01$), which indicated the used CPP apparatus was of a biased design (Tzschentke, 2007). The rats that showed place preference for the white box before conditioning (3 of the 35 rats) were excluded from further analysis. During the conditioning phase (day 4-7), the door was shut so that two compartments were separated. The rats were divided into amphetamine-paired group and saline-paired group, and underwent two conditioning sessions each day. The first session was performed in the morning, when the rats received amphetamine (2 mg/kg, s.c.) in amphetamine-paired group or sterile physiological saline (10 mL/kg, s.c.) in saline-paired group, and were immediately confined to the white compartment for 1 h. After an interval of 6 h, the second session of the day began. All the rats received saline and were confined to the black compartment immediately for 1 h during the second session. Twenty-four (24) h after the last drug-paired conditioning trial, the post-conditioning phase (day 8) was carried out, and was exactly the same as the pre-conditioning phase. The time that the rats spent on the drug-paired side (the white compartment) was recorded for the 15-min trial.

Experimental design

In order to assess the effect of rhynchophylline on the expression of amphetamine-induced CPP, the amphetamine-paired rats conditioned as described above to establish CPP to amphetamine were divided into two groups randomly. They were given an i.p. injection of vehicle 10 mL/kg (sterile physiological saline) and 60 mg/kg rhynchophylline for 3 days, 12 h after amphetamine injection on day 5, day 6 and day 7. Place preference was tested for 15 min and the time spent on the drug-paired side (the white compartment) was calculated. An additional group was designed to see whether rhynchophylline by itself induces CPP. The rats of rhynchophylline-paired group received rhynchophylline (60 mg/kg, i.p.) instead of amphetamine (2 mg/kg, s.c.) during the conditioning phase and received the vehicle (10 mL/kg, i.p.) 12 h later. Saline-paired rats was injected with the vehicle (10 mL/kg, i.p.) under the same schedule and served as a saline-paired control. On the basis of our preliminary test result (doses of 10, 20 and 60 mg/kg) and other experimental designs by several investigators who used natural sources for drug addiction therapy (normal dose ranges: 10-100 mg/kg), 60 mg/kg was chosen as a suitable dose for this experiment (Blackburn and Szumlinski, 1997; Glick et al., 1994, 2006). Each experimental group as well as each rat in different groups were orderly handled throughout the experiment to minimize the time impact on the result. Our preliminary test result showed that 20 and 60 mg/kg rhynchophylline both were efficient doses, 60 mg/kg was chosen to test whether rhynchophylline produced CPP.

Tissue preparation

After the post-conditioning test, rats are deeply anesthetized with sodium pentobarbital (60 mg/kg, i.p.) until the animal becomes unresponsive to pinching of the tail or hindlimb with forceps and through the left ventricle via the ascending aorta they were perfused with 200 mL of 0.9% sodium chloride solution contained 0.1% diethylpyrocarbonate at 25°C, at a flow rate of 50 mL/min for 3-4 min, followed by 500 mL of 4°C 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) contained 0.1% diethylpyrocarbonate at the same flow rate for 3-4 min and then at a flow rate of 4 mL/min for 1 h. The brains were removed from the skull and then were sliced at about 3 mm in thickness in accordance to the atlas of Paxinos and Watson (1998) and those tissue blocks containing medial prefrontal cortex were postfixed in cold 4% paraformaldehyde for 1 h at 4°C and kept in 70% ethanol overnight at 4°C. Tissues were dehydrated through graded ethanol series and made transparent with xylene. This was immediately followed by paraffin embedding using an automated embedding station. Tissues were cut into 7 μm thick serial sections on a microtome (Leica RM3025) and mounted on slides coated with 3-aminopropyltriethoxysilane. The sections were prepared for *in situ* hybridization and immunohistochemistry. To avoid RNase contamination, care was taken to always wear clean, powder-free gloves when manipulating the slides.

***In situ* hybridization for NR2B mRNA**

Seven-μm-thick tissue sections were deparaffinized by 3×5 min incubations in xylene and taken through a graded series of ethanol solutions contained 0.1% diethylpyrocarbonate (first in 100% followed by 95, 70 and finally 50%; 3 min each) to diethylpyrocarbonate H_2O. Sections were then incubated in pepsin buffer (3% sodium citrate, pH 9.0) for 20 min, washed in phosphate buffered saline. Prehybridization solution were added to sections for 4 h at 37°C and then hybridization was carried out in hybridization solution overnight at 37°C. Following hybridization, sections were stringently washed at 37°C for 5 min in 2 × saline-sodium citrate twice, 15 min in 0.5 × saline-sodium citrate three times, and then in 0.2 × saline-sodium citrate once. Sections were incubated in blocking solution for 30 min at 37°C, and then in biotinylated rabbit anti-rat digoxigenin antibody (5'-GGAAG CTTGA TGCAT TCATC TATGA TGCAG-3')

for 1 h at 60°C, then washed in phosphate buffered saline for 5 min four times. Sections were incubated in streptavidin-biotin complex for 20 min at 37°C, washed in phosphate buffered saline for 5 min three times. After incubated in biotinylated peroxydase for 20 min at 37°C, sections were washed in phosphate buffered saline for 5 min four times. Staining was developed with 3, 3-diaminobenzidine under the microscope in order to monitor the intensity of chromogen deposition and background labeling. Sections were washed and counterstained with light Mayer hematoxylin. The sections were taken through a graded series of alcohol, cleared in two changes of xylene, and coverslipped. Negative controls to test nonspecific hybridization composed of sections where hybridization solution and biotinylated anti-rat DIG antibody were replaced with phosphate buffered saline.

Immunohistochemistry for NR2B protein

Immunohistochemistry for NR2B protein was performed in 7-μm-thick tissue sections over 2 days, and carried out at 25°C unless otherwise stated. This procedure was strictly accordance with the study protocol. After deparaffinization in xylene, rehydration in ethanols to water, quenching for endogenous peroxidase activity using 3% hydrogen peroxide in methanol for 10 min, and antigen retrieval by microwaving in 10 mmol/L citrate buffer solution (pH 6.0) on high for 10 min, left to cool for 10 min, washed in phosphate buffered saline and then proceeded to immunostaining. Immunostaining was performed using a polyclonal rabbit antibody to NR2B (Boster Biotechnology Co.). Tissue sections were incubated in normal goat serum blocking solution for 20 min to suppress nonspecific binding of immunoglobulin and then in NR2B antibody (diluted 1:100) overnight at 4°C. Sections were washed in phosphate buffered saline, incubated in biotinylated rabbit anti-goat immunoglobulin G as secondary antibody for 20 min, and then in streptavidin-biotin complex (SABC-AP kit; Boster Biotechnology Co.), and the color was seen to develop following the addition of 3, 3-diaminobenzidine. Sections were then washed and counterstained with light Mayer hematoxylin. The sections were then dehydrated in a series of ethanol, in xylene, covered with DPX, and coverslips were placed. For negative control in the immunohistochemistry procedures performed, phosphate buffered saline replaced the primary NR2B antibody. After the tissue sections of *in situ* hybridization and immunohistochemistry, they were observed under an inverted microscope (DMIRB, Leica), images were obtained using a microscopy camera (MPS60, Leica) at a magnification of ×400.

Quantification of in situ hybridization and immunohistochemistry

Immunoreactivity was quantified by optical density of NR2B mRNA and protein immunoreactivity in medial prefrontal cortex. The optical measurements were blinded with respect to the animal groups. Optical density analysis was performed on high resolution and analyzed with Image-Pro Plus 6.0 (Media Cybernetics). A computer software was used to obtain the integrated optical density (IOD) of the region. The value is calculated as the sum of the optical densities of all pixels in the region divided by number of pixels. Background values were obtained from the neighboring white matter. Four randomly selected sections from each subject were evaluated and the average value was used for statistical analysis.

Data analyses

All the data were expressed as mean ± S.D. from 8 animals in each group. The pretreatment and after treatment time spent in white compartment in each group were analyzed by independent-samples t tests and other data were analyzed by one-way analysis of variance (ANOVA) for multiple comparisons, followed by Tukey's test where appropriate. The accepted level of significance was $p < 0.05$. All statistical analyses were done using software SPSS, version 13.0.

RESULTS

Effect of rhynchophylline on CPP

Effect of rhynchophylline on CCP was shown in

Table I. Effect of rhynchophylline on the time spent in white compartment

Group	Pretreatment	After treatment
Saline	104.3 ± 28.11	129.3 ± 36.72
Saline+rhynchophylline	119.2 ± 32.61	98.4 ± 21.25
Amphetamine	149.1 ± 37.87	277.8 ± 61.37*$^{\Delta}$
Amphetamine+rhynchophylline	112.4 ± 38.41	111.0 ± 38.85$^{\#}$

Data are mean ± S.D. ($n = 8$). *$p < 0.01$, saline+rhynchophylline group and amphetamine group compared with saline group; $^{\#}p < 0.01$, amphetamine+rhynchophylline group compared with amphetamine group. $^{\Delta}p < 0.01$, compared with pretreatment.

Table I. Amphetamine significant increased the time spent on the drug-paired side (the white compartment) compared with that of the control rats administered with saline ($F = 5.846$; $p < 0.01$) and also compared to pretreatment group ($t = 4.974$; $p < 0.01$). Rhynchophylline reduced the time spent on white compartment comparable to the control group ($F = 6.275$; $p < 0.01$). While rhynchophylline itself had no effect on the time spent on white compartment of control rats.

Effect of rhynchophylline on NR2B mRNA expression in medial prefrontal cortex

NR2B mRNA expression in medial prefrontal cortex are shown in Fig. 1 and Table II. The NR2B mRNA expression significantly increased in medial prefrontal cortex of amphetamine-induced rats (Fig. 1B) as compared to control rats (Fig. 1A) ($F = 6.473$; $p < 0.01$). Rhynchophylline (Fig. 1C) reversed the increase in NR2B mRNA expressions to near values of control group ($F = 6.059$; $p < 0.01$). NR2B mRNA expression in rhynchophylline-treated control rats (Fig. 1D) was not different from that of control ones (Fig. 1A). Brain sections incubated without antibody showed no staining.

Effect of rhynchophylline on NR2B protein expression in medial prefrontal cortex

NR2B protein expression in medial prefrontal cortex are shown in Fig. 2 and Table II. Compared to control rats (Fig. 2A), the NR2B protein expression showed a significant increase in medial prefrontal cortex of

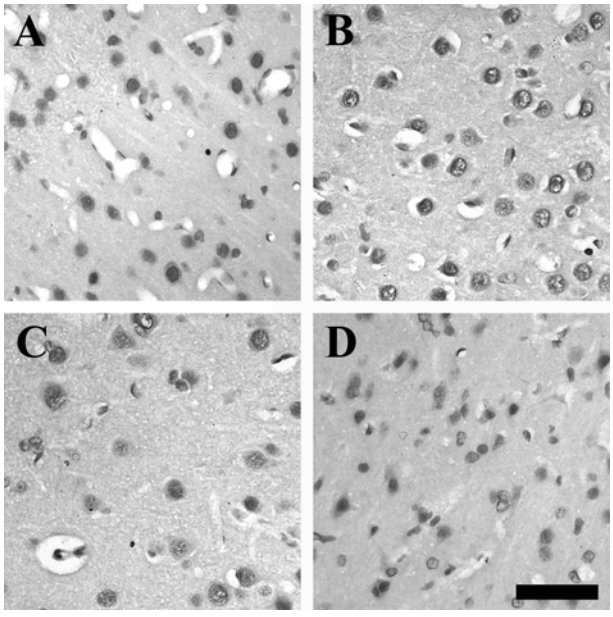

Fig. 1. Effect of rhynchophylline on NR2B mRNA expression in medial prefrontal cortex of amphetamine-CPP rat. (**A**) saline-treated control rats; (**B**) amphetamine-induced CPP rats; (**C**) amphetamine+rhynchophylline-treated rats; (**D**) saline+rhynchophylline-treated rats. Brown or yellow stains in Fig. 1B were stronger than those in Fig. 1A, C and D. Scale bar: 100 μm (in D, for A-C).

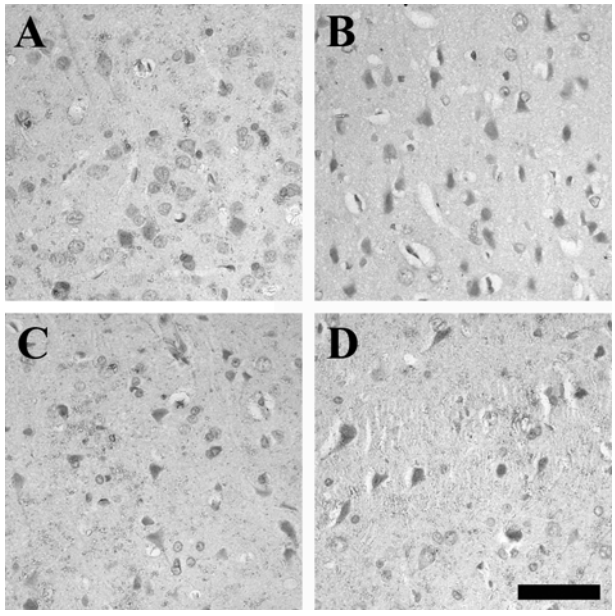

Fig. 2. Effect of rhynchophylline on NR2B protein expression in medial prefrontal cortex of amphetamine-CPP rat. (**A**) saline-treated control rats; (**B**) amphetamine-induced CPP rats; (**C**) amphetamine+rhynchophylline-treated rats; (**D**) saline+rhynchophylline-treated rats. Brown or yellow stains in Fig. 2B were stronger than those in Fig. 2A, C and D. Scale bar: 100 μm (in D, for A-C).

Table II. Effects of rhynchophylline on NR2B mRNA and protein expression (IOD) in medial prefrontal cortex of CPP rats

Group	NR2B mRNA	NR2B protein
Saline	0.98 ± 0.11	0.45 ± 0.09
Saline+rhynchophylline	1.01 ± 0.09	0.46 ± 0.04
Amphetamine	1.45 ± 0.18*	0.78 ± 0.09*
Amphetamine+rhynchophylline	1.02 ± 0.12 #	0.47 ± 0.07 #

IOD: Integrated Optical Density. Data are mean ± S.D. ($n = 8$). *$p < 0.01$, saline+rhynchophylline group and amphetamine group compared with saline group; #$p < 0.01$, amphetamine+rhynchophylline group compared with amphetamine group.

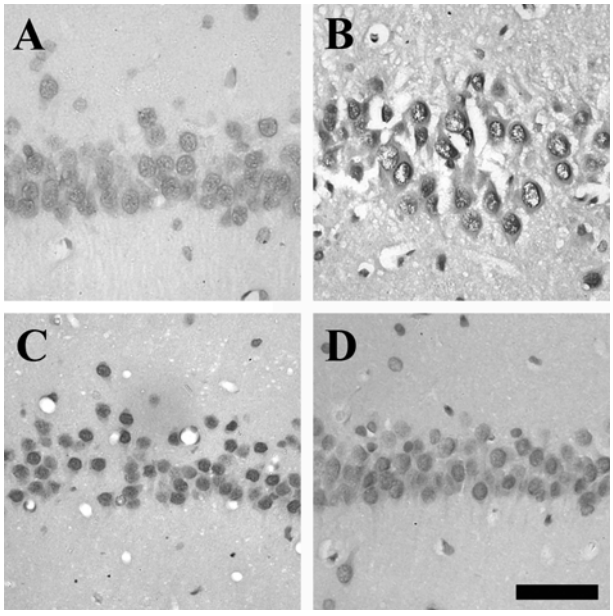

Fig. 3. Effect of rhynchophylline on NR2B mRNA expression in hippocampal CA1 area of amphetamine-CPP rat. (**A**) saline-treated control rats; (**B**) amphetamine-induced CPP rats; (**C**) amphetamine+rhynchophylline-treated rats; (**D**) saline+rhynchophylline-treated rats. Brown or yellow stains in Fig. 3B were stronger than those in Fig. 3A, C and D. Scale bar: 100 μm (in D, for A-C).

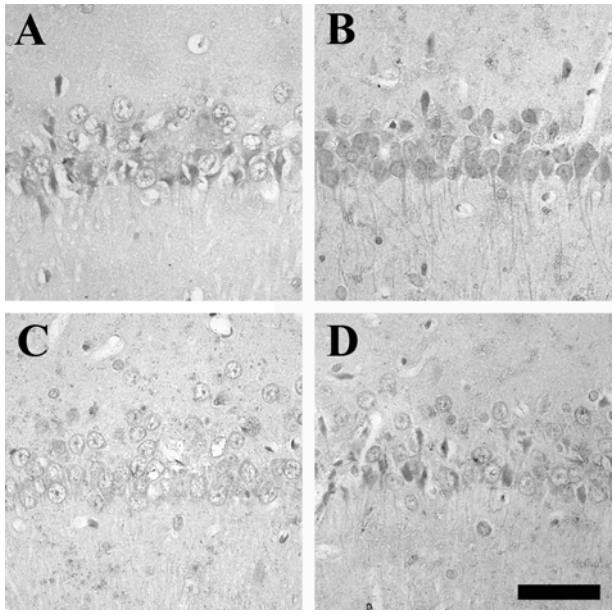

Fig. 4. Effect of rhynchophylline on NR2B protein expression in hippocampal CA1 area of amphetamine-CPP rat. (**A**) saline-treated control rats; (**B**) amphetamine-induced CPP rats; (**C**) amphetamine + rhynchophylline-treated rats; (**D**) saline + rhynchophylline-treated rats. Brown or yellow stains in Fig. 4B were stronger than those in Fig. 4A, C and D. Scale bar: 100 μm (in D, for A-C)

amphetamine-treated rats ($F = 6.199$; $p < 0.01$) (Fig. 2B). Rhynchophylline (Fig. 2C) effectively reversed the increased NR2B expression to near control values ($F = 6.882$; $p < 0.01$). Rhynchophylline had no effect on NR2B expression in control rats (Fig. 2D). It was also observed that there was no staining noted in brain sections without the primary antibody incubation.

Effect of rhynchophylline on NR2B mRNA expression in hippocampal CA1 area

NR2B mRNA expression in CA1 area are shown in Fig. 3 and Table III. In CA1 area, amphetamine-induced CPP rats (Fig. 3B) had a significantly higher NR2B mRNA expression than in control group ($F = 7.439$; $p < 0.01$) (Fig. 3A). Rhynchophylline (Fig. 3C) brought back the up-regulated NR2B mRNA expression to control values ($F = 4.003$; $p < 0.01$). Rhynchophylline-treated control rats (Fig. 3D) showed no difference in NR2B mRNA expression when compared to controls (Fig. 3A). Brain sections incubated without antibody showed no staining.

Effect of rhynchophylline on NR2B protein expression in hippocampal CA1 area

NR2B protein expressions in hippocampal CA1 area are shown in Fig. 4 and Table III. Compared to control rats (Fig. 4A), the NR2B protein expression was significantly up-regulated in CA1 area of CPP rats ($F = 6.585$; $p < 0.01$) (Fig. 4B). Rhynchophylline (Fig. 4C) restored the increased NR2B expression to near control levels ($F = 3.959$; $p < 0.01$). Rhynchophylline itself did not affect the NR2B expression in control rats (Fig. 4D). Brain sections showed no staining without the primary antibody incubation.

Table III. Effects of rhynchophylline on NR2B mRNA and protein expression (IOD) in hippocampal CA1 area in CPP rats

Group	NR2B mRNA	NR2B protein
Saline	0.76 ± 0.14	0.32 ± 0.06
Saline+rhynchophylline	0.77 ± 0.13	0.33 ± 0.08
Amphetamine	1.03 ± 0.10*	0.57 ± 0.13*
Amphetamine+rhynchophylline	0.79 ± 0.15#	0.34 ± 0.06#

IOD: Integrated Optical Density. Data are mean ± S.D. ($n = 8$). *p<0.01, saline+rhynchophylline group and amphetamine group compared with saline group; #p<0.01, amphetamine+rhynchophylline group compared with amphetamine group.

DISCUSSION

The major finding of this study is a reversible down-regulation of NR2B mRNA and protein level in medial prefrontal cortex and hippocampal CA1 area of rat brain that followed treatment with rhynchophylline, tetracyclic oxindole alkaloid components of *Uncaria* species such as *Uncaria rhynchophylla* (MIQ) Jackson. The result showed an up-regulation of NR2B mRNA and protein expressions not only in medial prefrontal cortex but also in CA1 area of amphetamine-induced CPP rats. But rhynchophylline itself did not produce a CPP effect and affect NR2B expression in the CPP-related brain areas. The present study is in agreement with previous reports on an increase of NR2B expression treated with amphetamine analogue (Kindlundh-Hogberg et al., 2008). Our preliminary test result showed that treatment with 40 and 80 mg/kg rhynchophylline reduced locomotor activity, inhibited hyperactivity, and suppressed the development and expression of amphetamine-induced sensitization in mice. Thus in this study, the locomotor activity and sensitization of rhynchophylline during conditioning and test sessions were not tested and a dose of 60 mg/kg was decided as the tested dose.

CPP is thought to be related to and predictive of the positive reinforcing effects of drugs since many drugs that serve as positive reinforcers under other conditions (e.g., maintain iv self-administration responding) can be used to establish CPP (Bardo and Bevins, 2000). Rats show CPP for a variety of drugs abused by humans (Tyhon et al., 2008; Yamada, 2008). Cocaine, amphetamines and opiates produce robust place preferences (Carr and White, 1986; Nomikos and Spyraki, 1988). When amphetamine administration is repeatedly associated with a distinct environment, the environment serves as a cue and can elicit positive subjective feelings even in the absence of drug. In the present CPP paradigm, 2 mg/kg of amphetamine increased the time spent on the drug-paired compartment and induced place preference, but only a weak increase in preference for the drug-paired chamber. These findings agree with previous studies, in which amphetamine induced CPP in the biased design of conditioning effect (Tzschentke and Schmidt, 1998). This behavioral response to reward-related stimuli (amphetamine) has been suggested to be associated with neuronal adaptations that lead to drug dependence (Robinson and Berridge, 1993).

Rhynchophylline has a non-competitive antagonistic effect on the NMDA-type ionotropic glutamate receptors (Kang et al., 2002). Rhynchophylline exerts its protective action against ischemia-induced neuronal damage primarily by preventing NMDA, muscarinic M_1, and 5-HT_2 receptors-mediated neurotoxicity during ischemia (Kang et al., 2004). In this study, rhynchophylline decreased the elevated NR2B mRNA and protein expressions induced by amphetamine, but rhynchophylline had no effect on NR2B expression in control rats. Hippocampus is important for the regulation of cognitive functions and memory. Memory loss in response to amphetamine analogue is previously reported to be caused by prevention of learning specific increase in NR1 expression (Moyano et al., 2005). Most NMDA receptors in the brain are heteromeric complexes consisting of constructive NR1 and functional NR2 (A-D) subunits (Monyer H, 1994). NR1, NR2A and NR2B, but not NR2C and NR2D, are consistently seen distributed in the brain areas that relate to drug abuse (Mori and Mishina, 1995).

Since the dopamine receptor blocker is protective for amphetamine induced amino acid neurotransmitters release (Mora and Porras, 1993) and the selective serotonin uptake inhibitor is protective for amphetamine induced 5-HT depletion (Schmidt, 1987), the current study showed amphetamine-induced enhancement in mRNA levels of glutamatergic NMDA receptor subunits may contribute to amphetamine mediated neurotoxic effects. Furthermore, there are similarities between the immediate effects of amphetamine analogue administration and other drugs of abuse. Ethanol treatment up-regulates NR2B expression in both the cortex and hippocampus. At 48 h of withdrawal, the expression of NR2B returns to almost control levels. When ethanol is repeatedly administered to rats for 8 days and then withdrawn for 24 h, changes in NR2B expression are also evident (Kalluri et al., 1998). Cocaine-induced decreases in NR2B expression were observed in the nucleus accumbens shell after 24 h of withdrawal, whereas increases in NR2B expression were found in medial cortical areas (Loftis and Janowsky, 2000). A significant increase in NR2B protein levels is apparent 24 h after the last conditioning trial in the limbic forebrain obtained from morphine-conditioned mice (Narita et al., 2000).

In conclusion, up-regulation of NR2B subunit both in medial prefrontal cortex and hippocampal CA1 area might be important for amphetamine-induced CPP. Rhynchophylline eliminated the expression of amphetamine CPP and down-regulated the increased NR2B mRNA and protein expression in medial prefrontal cortex and CA1 area in CPP rats. The medial prefrontal cortex and hippocampal CA1 area are important brain areas that are involved in the inhibitory effect of rhynchophylline on amphetamine-induced CPP. But rhynchophylline itself had no effect on expression of

CPP and NR2B in medial prefrontal cortex and CA1 area of control rats. It is likely that rhynchophylline exerts this antiamphetamine dependent action primarily by down-regulation of NR2B expression in rat cortex and hippocampus.

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