

Rhynchophylline Downregulates Phosphorylated cAMP Response Element Binding Protein, Nuclear Receptor-related-1, and Brain-derived Neurotrophic Factor Expression in the Hippocampus of Ketamine-induced Conditioned Place Preference Rats

Youli Guo^{1,2,†}, Chaohua Luo^{1,†}, Genghong Tu^{3,†}, Chan Li¹, Yi Liu¹, Wei Liu¹, Ken Kin Lam Yung⁴, Zhixian Mo¹

¹School of Traditional Chinese Medicine, Southern Medical University, ²Department of Pharmacy, Guangdong Provincial Corps Hospital of Chinese People's Armed Police Forces, ³Department of Pathophysiology, Guangdong Province Key Laboratory of Functional Proteomics, Southern Medical University, Guangzhou, ⁴Department of Biology, Hong Kong Baptist University, Kowloon Tong, Hong Kong, China

[†]These authors contributed equally to this work.

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ABSTRACT

Background: Addiction to ketamine is becoming a serious public health issues, for which there exists no effective treatment. Rhynchophylline (Rhy) is an alkaloid extracted from certain *Uncaria* species that is well known for both its potent anti-addictive and neuroprotective properties. Increasing evidence supports the contributions of cAMP response element binding protein (CREB), nuclear receptor-related-1 (Nurr1), and brain-derived neurotrophic factor (BDNF) in modulating neural and behavioral plasticity which was induced by addictive drugs. **Objective:** To investigate the effects of Rhy on the behavior and the levels of phosphorylated CREB (p-CREB), Nurr1, and BDNF in the hippocampus of ketamine-induced conditioned place preference (CPP) rats.

Materials and Methods: CPP paradigm was used to establish the model of ketamine-dependent rats and to evaluate the effect of Rhy on ketamine dependence. The expressions of p-CREB, Nurr1, and BDNF were tested by Western blotting and immunohistochemistry. **Results:** We observed that Rhy can reverse the behavior preference induced by ketamine CPP training. At the same time, expression of p-CREB, Nurr1, and BDNF which was significantly increased by ketamine, was restored in the Rhy-treated group. **Conclusion:** This study indicates that Rhy can reverse the reward effect induced by ketamine in rats and the mechanism can probably be related to regulate the hippocampal protein expression of p-CREB, Nurr1, and BDNF.

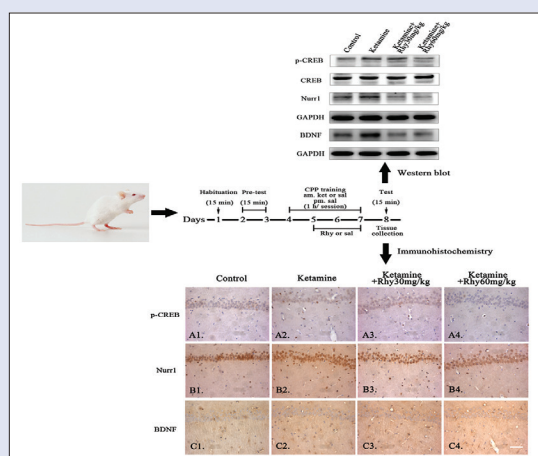
Key words: Brain-derived neurotrophic factor, conditioned place preference, ketamine, nuclear receptor-related-1, phosphorylated cAMP response element binding protein, rhynchophylline

SUMMARY

- p-CREB, Nurr1 and BDNF play an important role in the formation of ketamine-induced place preference in rats
- Rhynchophylline reversed the expression of p-CREB, Nurr1 and BDNF which was activated by ketamine in the hippocampus
- Rhynchophylline demonstrates the potential effect of mediates ketamine induced rewarding effect.

Abbreviations used: Rhy: Rhynchophylline; CREB: cAMP response element binding protein; Nurr1: Nuclear receptor-related-1; BDNF: Brain-

derived neurotrophic factor; CPP: Conditioned place preference; NMDA: N-methyl-D-aspartic acid; METH: Methamphetamine; CNS: Central nervous system; PFA: Paraformaldehyde; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; LTP: long-term potentiation.



Correspondence:

Prof. Ken Kin Lam Yung,
Department of Biology, Hong Kong Baptist University, Kowloon Tong, Hong Kong, China.
E-mail: kklyung@hkbu.edu.hk
Prof. Zhixian Mo,
School of Traditional Chinese Medicine, Southern Medical University, 1063 Shatai Road, Guangzhou, 510 515, China.
E-mail: 1021336808@qq.com
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INTRODUCTION

Ketamine is a noncompetitive antagonist of N-methyl-D-aspartic acid (NMDA) glutamate receptor that was clinically employed for an effective anesthetic for half a century. Ketamine is also a "club drug" that has been widely abused in various places of entertainment.^[1] Although potent anti-addictive properties have been reported in both experimental and clinical trials,^[2,3] a great deal of evidence has shown that ketamine does indeed have a high potential for abuse and addiction.

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Rats will appear compulsive ketamine-seeking behavioral,^[4] reverse natural behavioral preference,^[5] and increase locomotor activity, following repetitive ketamine administration.^[6] Clinical observation has shown that long-lasting and unconstrained ketamine injections in the treatment of depression can lead to drug addiction.^[7] Ketamine abuse, a chronic and complex neurological disorder as other drug addiction, can affect protein translation in multiple regions of the brain,^[8] leading to degenerative changes in the nervous system.^[9]

At present, opioid receptor agonists, opioid receptor antagonists, and nonopioid receptor agonists are the three types of medications mainly used for anti-addiction therapy. Opioid receptor agonists including methadone can detoxify the addiction rapidly but have high chance of misuse so they have restriction in use.^[10] Opioid antagonists successfully shorten the withdrawal process but fail to relieve withdrawal symptoms.^[11] In addition, clonidine, a nonopioid receptor agonist commonly utilized to relieve symptoms from the addiction substance in the clinic, has been associated with obvious adverse reactions, including sedation and hypotension.^[12] Moreover, none of the above medications is suggested to be therapeutic for addiction to newer, synthetic drugs such as ketamine. As no effective drug therapy currently exists that can alleviate ketamine addiction, more and more research aims to identify potential anti-addiction substances in plants.

Several constituents within herbs exhibit notable anti-addiction effects, but the mechanisms of these effects are poorly understood.^[13] Rhynchophylline (Rhy) [Figure 1] is a predominant tetracyclic oxindole alkaloid derived from *Uncaria rhynchophylla* that is routinely prescribed to treat symptoms related to drug addiction.^[14] Studies have shown that Rhy has various beneficial effects, being anti-addictive, anti-arrhythmic, anticonvulsant, anti-anxiety, and anti-hypertensive, as well as exhibiting sedative and neuroprotective properties in various models.^[15-18] Rhy can alleviate methamphetamine (METH)-induced neurotoxicity in rat cortical neurons^[19] and inhibit Ca^{2+} influx to prevent glutamate-induced neuronal death *in vitro*.^[20] Thus, Rhy is widely used to treat central nervous system (CNS) disorders, including convulsions, numbness, and lightheadedness, as well as cardiovascular disease, such as hypertension. Rhy is also a noncompetitive antagonist of NMDA glutamate receptor^[21] and our previous studies have demonstrated that Rhy can remarkably eliminate the rewarding effect and decrease the NR2B level in the brain of amphetamine-induced conditioned place preference (CPP) rats,^[22] mice,^[23] and zebrafish.^[24] Furthermore, recent research has shown that the decreased levels of phosphorylated cAMP response element binding protein (p-CREB) and p-Fos in the brain are responsible for the therapeutic effects of Rhy on METH-induced CPP rats.^[25] As Rhy can

relieve amphetamines-associated addiction, we hypothesized that Rhy may be anti-addictive for other drugs, such as ketamine. We therefore established a ketamine-dependent model of rats using CPP paradigm and examined behavior effect of Rhy on behavioral preference rats and then further elucidate the possible molecular mechanisms by measuring levels of p-CREB, nuclear receptor related-1, (Nurr1), and brain-derived neurotrophic factor (BDNF) in the hippocampus. Our findings indicate that the abnormally increased levels of p-CREB, Nurr1, and BDNF in the hippocampus are associated with ketamine addiction and the effect of Rhy on counteracting the ketamine CPP behavior may be involved with restoring the level of p-CREB, Nurr1, and BDNF.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats, weighing 180–220 g, were bought from Southern Medical University Animals Center (Guangzhou, China) and adapted to the housing conditions with a 12-h light/dark cycle, stable temperature ($22^{\circ}\text{C} \pm 1^{\circ}\text{C}$), controlled humidity ($60\% \pm 5\%$), food and water *ad libitum*. Rats were handled 1 week before behavioral tests. All experiments were deferred to the Guidelines of National Institutes of Health and were approved by the Animal Care and Use Committee of the Southern Medical University.

Drug and treatment

Ketamine hydrochloride (Fujian Gutian Medicine Company, China) and Rhy (purity $\geq 98\%$; National Pharmaceutical Engineering Center, China) were dissolved in physiological saline, which will hereinafter be referred to saline.^[25] All injections were administered intraperitoneally (i.p.) at a volume of 10 ml/kg.

Conditioned place preference

According to our published procedures,^[26] we used CPP experiments as the biased procedure. Training took place in an apparatus consisting of two equal size chambers ($30\text{ cm} \times 30\text{ cm} \times 30\text{ cm}$) which were separated by a sliding door. One chamber is white and rough, and the other is black and smooth. On day 1, as an adaptive operation, rats were allowed to freely access the apparatus for 15 min. On day 2 and day 3, rats were pretested 15 min/day and recorded for the time spent in each compartment to determine an innate preference. Rats showing preference to the white compartment in the pretesting phase were excluded. From day 4 to day 7, rats assigned to ketamine CPP treatment were trained two daily conditioning sessions per day. In brief, rats were confined to the white compartment for 1 h after receiving ketamine (10 mg/kg) injection and confined to black compartment for 1 h after receiving equal volume of saline with an interval of 8 h. To evaluate the effect of Rhy on the expression of ketamine-induced CPP, rats assigned to ketamine CPP training were further divided into three groups randomly with eight rats per group. Each group was administered saline or Rhy (varied from 30 to 60 mg/kg) once a day, 12 h after the ketamine injection on days 5, 6, and 7. The dose and time points of drug administration were selected based on our previous work.^[23,25] Rats assigned to saline CPP (as control) were treated the same as ketamine CPP, except that both sessions were paired with a saline injection. Twenty-four hours after the last conditioning session, rats were allowed to free access to the apparatus for 15 min in a drug-free state and measured the time spent in each compartment. The CPP score was determined by the change of activity time of rats in white compartment before and after the conditioning sessions.^[27]

Immunohistochemistry and data analysis

After the CPP test, four rats per group were randomly assigned to immunohistochemistry test. Rats were deeply anesthetized using

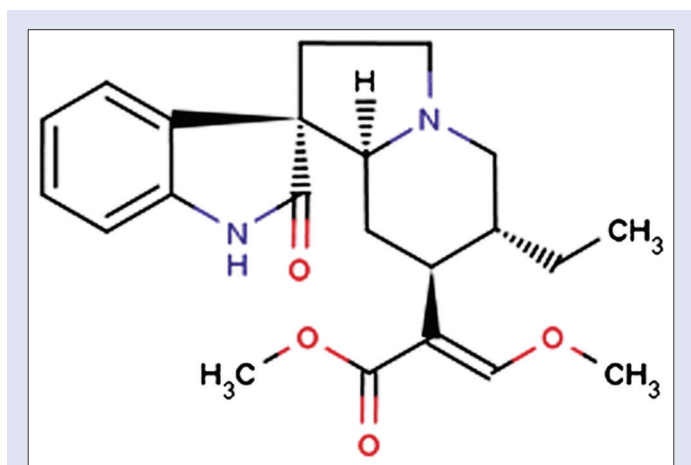


Figure 1: Molecular structure of rhynchophylline

3% sodium pentobarbital i.p (30 mg/kg) and perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) intracardially. The brains were then removed and postfixed in 4% PFA immediately. After dehydration and paraffin embedding, the hippocampus was cut into 5- μ m sections using a Leica RM2135 microtome. Immunohistochemistry was performed as described in product manual of streptavidin-biotin complex with peroxidase (Rabbit IgG) Kit (Boster, China). Antibodies for rabbit anti-p-CREB (1:150 dilution; Abcam, UK), rabbit anti-Nurr1 (1:50 dilution; Santa Cruz, USA), and rabbit anti-BDNF (1:150 dilution; Abcam, UK) were used to determine the levels of p-CREB, Nurr1, and BDNF. Five slices per rat containing hippocampus were selected, and then, three horizons of each slice were randomly observed in the light microscope ($\times 40$). Positive expression of p-CREB, Nurr1, and BDNF was defined as appearance of brown particles within the cell. We used Image-pro plus software (IPP 6.0, Media Cybernetics Inc., Silver Spring, Maryland, USA) to measure positive cells' integrated optical density, and the relative content for p-CREB, Nurr1, and BDNF was represented by the mean value of each group.

Western blotting and data analysis

After the CPP test, four rats per group were randomly assigned to Western blot test. Rats were decapitated and the hippocampus was isolated by gross dissection. To detect CREB, p-CREB, Nurr1, and BDNF, extracts were prepared from individual rats using RIPA lysis buffer (Beyotime, China) with protease inhibitors and phosphatase inhibitors. Homogenized tissue was sonicated and centrifuged at 14,000 g for 15 min at 4°C. The supernatants were collected and employed for protein determination using the bicinchoninic acid method. The protein samples were mixed with 2 $\times$ loading buffer and denatured at 95°C for 5 min. Samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis before electrophoretic transfer onto a polyvinylidene difluoride membrane (Millipore, USA). Primary antibodies for rabbit anti-CREB (1:1000; Abcam, UK), rabbit anti-p-CREB (1:1000; Abcam, UK), rabbit anti-Nurr1 (1:200; Santa Cruz), and rabbit anti-BDNF (1:1000; Abcam, UK) and horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:8000; Millipore, USA) were used to detect the expression of CREB, p-CREB, Nurr1, and BDNF and then visualized by enhanced chemiluminescent detection (Millipore, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (housekeeping gene) expression is shown as a loading control using rabbit anti-GAPDH antibody (1:3000; CWBIO, China). The results normalized for each membrane were expressed as percentages of saline-treated controls. Relative band intensities were quantified by Gel-Pro Analyzer (Media Cybernetics, USA).

Statistical analysis

Data are summarized as the mean $\pm$ standard error of mean. Statistical analysis was performed with SPSS software (version 20.0 for Windows, Chicago, IL, USA). All data were analyzed using one-way analysis of variance followed by a *post hoc* test (two-tailed) with Bonferroni correction when equal variances assumed or with Tamhane's T2 when not assumed. We considered differences significant at $P < 0.05$.

RESULTS

Rhynchophylline reversed the behavioral responses to ketamine

Given that Rhy is an NMDA receptor which can counteract to amphetamine- and METH-induced place preference,^[22,25] here, we determined whether Rhy can reverse the behavioral preference induced by ketamine. As CPP is one of the most popular experiments to assess

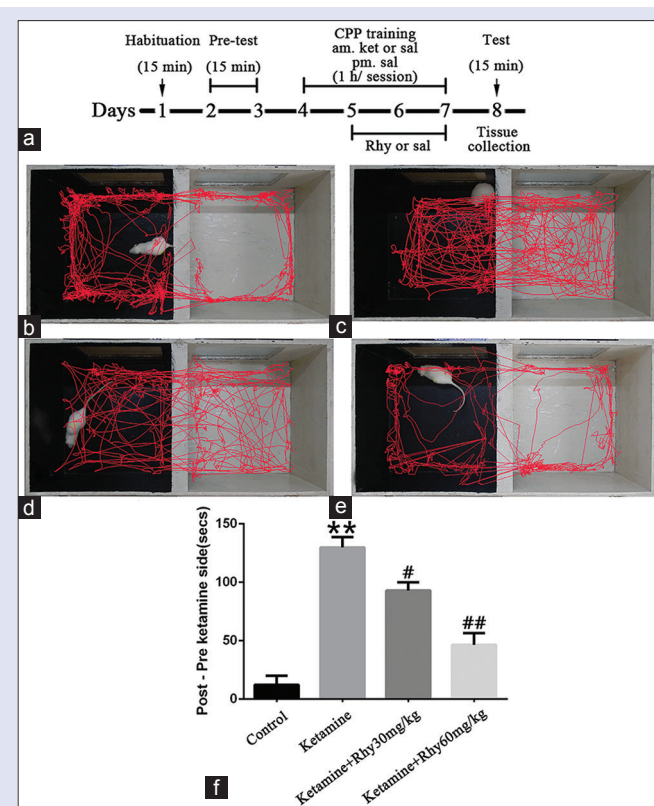


Figure 2: Rhynchophylline prevents ketamine-induced conditioned place preference. (a) The schematic of experimental design for conditioned place preference testing. (b-e) Representative running trajectory of rats in the conditioned place preference compartments recorded and analyzed with the Noldus Ethovision XT 8.5 software; b-e represent the control conditioned place preference group, ketamine conditioned place preference group, ketamine with 30 mg/kg rhynchophylline group and ketamine with 60 mg/kg rhynchophylline group, respectively. (f) Time difference between post ketamine training and pre-ketamine training. Data are expressed as mean values $\pm$ standard error of the mean for 8 rats per group. ** $P < 0.01$ versus the control conditioned place preference group; # $P < 0.05$, ## $P < 0.01$ versus the ketamine conditioned place preference group via Bonferroni *post hoc* analysis after one-way analysis of variance

the reward effects of drugs,^[28] we successfully established a ketamine addiction model of rats by four consecutive ketamine CPP training using a dose of 10 mg/kg. Compared with the control group, ketamine significantly increased the time difference in white compartments between post- and pre-ketamine CPP training ($P < 0.01$), as shown in Figure 2. Two different doses of Rhy were applied to testify the effect on ketamine addiction and find out which dose would be better. Compared with ketamine CPP group, low-dose Rhy (30 mg/kg) administration reduced the time difference induced by ketamine ($P < 0.05$), while the high dose of Rhy (60 mg/kg) reduced the time difference even more significantly ($P < 0.01$) [Figure 2].

Rhynchophylline regulated the levels of phosphorylated cAMP response element binding protein, nuclear receptor-related-1, and brain-derived neurotrophic factor to relieve the ketamine-dependent behavior

To find out the possible molecular mechanism involved the behavioral

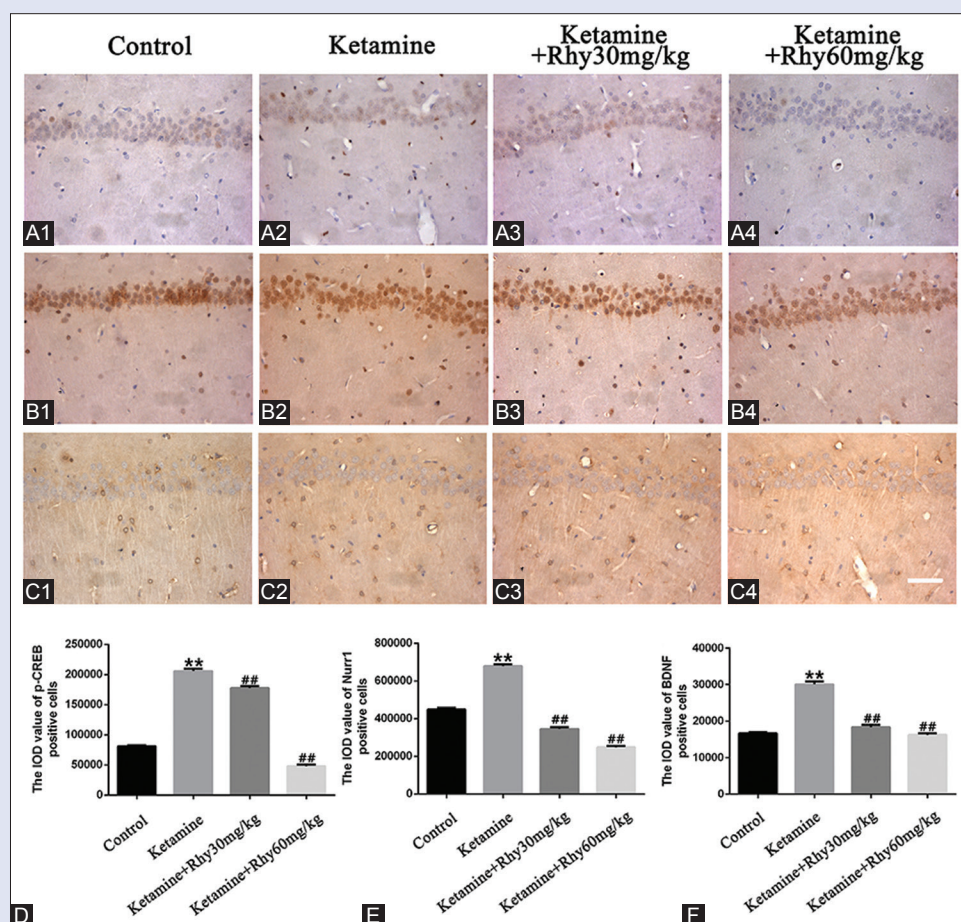


Figure 3: Expression of p-CREB, Nurr1 and BDNF in the hippocampus of rats detected by immunohistochemistry. p-CREB (A1-A4), Nurr1 (B1-B4) and BDNF (C1-C4) were detected using immunohistochemistry. Photomicrographs are representative of results from multiple brain sections obtained from four animals in each treatment group. Quantitative analysis is shown in (D-F). Scale bar: 50 μ m. Data are expressed as mean values $\pm$ standard error of mean. ** $P < 0.01$ versus the control group; ## $P < 0.01$ versus the ketamine conditioned place preference group via Bonferroni *post hoc* analysis after one-way analysis of variance. p-CREB: Phosphorylated cAMP response element binding protein, Nurr1: Nuclear receptor-related-1, BDNF: Brain-derived neurotrophic factor

changes by ketamine and Rhy, first, we used immunohistochemistry to detect the levels of Nurr1 and BDNF and the active state of CREB (p-CREB) in the hippocampus of CPP rats. As shown in Figure 3, the levels of Nurr1, BDNF, and p-CREB, which were significantly higher in ketamine CPP group than that of the control group ($P < 0.01$), were significantly downregulated after Rhy administration ($P < 0.01$).

In addition to immunohistochemistry, the expression of BDNF, Nurr1, and p-CREB in the hippocampus rats was also assessed using Western blot analysis. In line with immunohistochemical results, ketamine significantly upregulated the expression of BDNF, Nurr1, and p-CREB ($P < 0.01$). Both 30 mg/kg Rhy and 60 mg/kg Rhy significantly reduced BDNF and Nurr1 levels ($P < 0.01$) in the hippocampus of ketamine-induced CPP rats, but only 60 mg/kg Rhy treatment reversed the level of p-CREB significantly ($P < 0.01$) [Figure 4].

DISCUSSION

There are three neural circuits involved in motivation and reward: mesocortical pathway, mesolimbic pathway, and nigrostriatal pathway.^[29] Drugs of abuse “hijack” these natural reward circuitries, leading to abnormal craving and compulsive drug-seeking behavior. The fact that drug-associated stimuli can trigger relapse even if abstinence

is achieved suggests that it may be an aberrant form of learning and memory.^[30] A common mechanism in drug addiction such as cocaine which is related to long-term potentiation (LTP). More and more proof indicates that the reason of addiction is the stubborn persistence of addiction memory.^[31] Hence, we decided to focus our investigation on the hippocampus which is the main region to engage learning and plays a vital part in processing drug-related memory.^[32] Rats after ketamine CPP training established connection between drug and the context of white compartment, spent more time in white compartment, indicating drug-associated memory triggered the rats’ craving behavior.

Drug addiction is a complicated brain disease that results in large-scale protein and/or gene expression changes, making addictive behavior extraordinarily steady and enduring.^[33] As a cellular transcription factor, CREB is central to learning and memory storage.^[34] p-CREB transcribes a host of genes and activates a cascade of events which cause tolerance and dependence adaptations.^[35] One of the CREB’s downstream targets is Nurr1, an immediate-early gene which might be associated with learning and memory processes.^[36] It has been shown that Nurr1 is implicated in the pathogenesis of neuropsychiatric disorders, such as drug dependence, schizophrenia, and attention deficit hyperactivity disorder.^[37-40] Another downstream target of CREB is BDNF which is distributed throughout the entire CNS and expressed strongest in the hippocampus.^[41] It is considered to be a psychostimulant-regulated immediate early gene

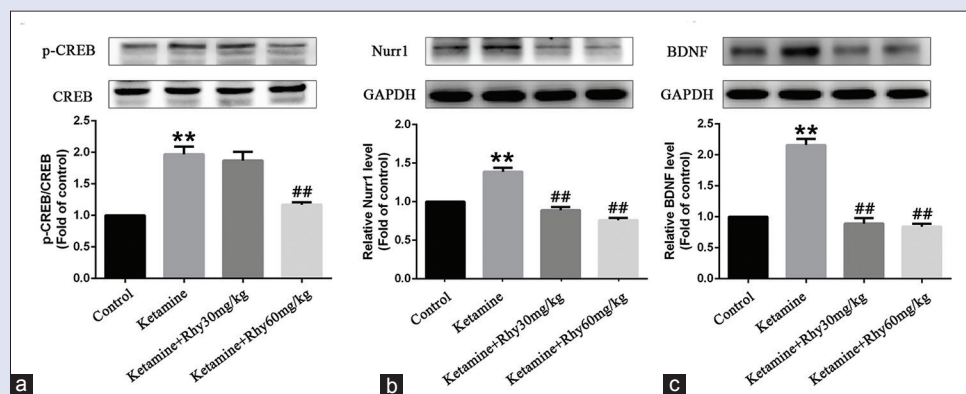


Figure 4: Expression of p-CREB, Nurr1 and BDNF in the hippocampus of rats detected by Western blotting (a-c). p-CREB was normalized to total CREB, Nurr1, and BDNF were normalized to glyceraldehyde-3-phosphate dehydrogenase. Data are shown as mean values $\pm$ standard error of mean for 4 rats per group. ** $P < 0.01$ versus the control group; ## $P < 0.01$ versus the ketamine group via Bonferroni *post hoc* analysis after one-way analysis of variance. p-CREB: Phosphorylated cAMP response element binding protein, Nurr1: Nuclear receptor-related-1, BDNF: Brain-derived neurotrophic factor

that involves in regulating synaptic plasticity and an important protein highly related to drug addiction.^[42,43] As drug addiction is considered as a maladaptive learning and memory and the critical molecules include CREB, Nurr1, and BDNF, we focused on CREB, Nurr1, and BDNF as ideal candidates for studying the mechanisms of ketamine addiction and the role of Rhy in ketamine deaddiction. Our results showed that ketamine reversed the rats' natural preference of black and upregulated p-CREB, Nurr1, and BDNF in the hippocampus. Studies have shown that the increased p-CREB may enhance BDNF transcription, leading to increased neuroplasticity and addiction.^[44] Besides, BDNF can facilitate the autophosphorylation of tyrosine protein kinase B, leading to activation of CREB.^[45] Therefore, the mutual regulation between CREB and BDNF may be involved in the mechanism of ketamine addiction. In this study, we observed that Rhy counteracts the CPP behavior and downregulates the levels of p-CREB, Nurr1, and BDNF, which were elevated by ketamine. The findings of this study indicate a critical role for Rhy in inhibiting ketamine dependence and the mechanism probably be related to regulate the hippocampal protein expression of p-CREB, Nurr1, and BDNF.

NMDA receptor, which can mediate synaptic plasticity processes such as LTP and long-term depression, is crucial in learning and memory.^[46] Evidence supports that NMDA receptor inhibition prevents tolerance, sensitization, and seeking behavior to cocaine^[47,48] and reduces locomotor activities induced by opiates such as cocaine and heroin.^[49] It is quite intriguing that, on the one hand, ketamine can inhibit NMDA receptor to disrupt cocaine self-administration,^[50] and, on the other hand, it is highly addictive. Studies indicate that CREB and BDNF are downstream targets of NMDA receptor^[51,52] and Nurr1 is a key regulator of NMDA receptor to modulate neuronal survival.^[28] The expression of p-CREB, BDNF, and Nurr1 elevated by ketamine indicates that there must be another pathway to strongly trigger these three molecules even if ketamine can counteract with NMDA receptor to downregulate them. As an NMDA receptor antagonist, Rhy may reverse the rewarding effect of ketamine by blocking NMDA receptor and the cascade signaling, downregulating the p-CREB, BDNF, and Nurr1.

CONCLUSION

The present results underscore the effect of Rhy in the inhibition of drug addiction induced by ketamine and link this action with reduced levels of p-CREB, Nurr1 and BDNF in the hippocampus. Although these results broaden our understanding of Rhy on ketamine addiction, it is

still not intact. The exact mechanisms of ketamine addiction and Rhy anti-addiction are needed to illuminate in the future research.

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Conflicts of interest

There are no conflicts of interest.

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