Research Article Open Access

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Effects and Mechanisms of Jinniu Capsule on Methamphetamine-Induced Conditioned Place Preference in Rats

https://doi.org/10.1515/chem-2018-0074
received April 4, 2018; accepted May 16, 2018.

Abstract: The aim of this study was to determine the effects of Jinniu Capsule on methamphetamine (METH)-induced conditioned place preference (CPP) in rats and identify the underlying mechanisms. An intraperitoneal injection of 3 mg/kg METH was used for CPP training in rats. The effects of Jinniu Capsule following a single dose on rat CPP and repeat dosing on METH withdrawal were evaluated. Western Blot analysis was used to measure protein expression of the PI3K-AKT-mTOR signaling pathway to determine the mechanisms of Jinniu Capsule. A single dose of Jinniu Capsule did not influence METH-induced CPP in rats. However, repeat dosing for 7 days significantly promoted METH withdrawal. Furthermore, METH withdrawal activated the PI3K-AKT-mTOR signaling pathway phosphorylation cascade, and Jinniu Capsule partly blocked this cascade. Jinniu Capsule demonstrated potential in promoting METH withdrawal in a rat CPP model, which may be related to its influence on the PI3K-AKT-mTOR signaling pathway.

Keywords: methamphetamine; conditioned place preference; Jinniu Capsule; withdrawal; mTOR signaling pathway.

1 Introduction

Methamphetamine (METH) is a highly addictive psychostimulant drug that is easily synthesized from inexpensive and readily obtainable chemicals. METH can produce euphoria and stimulant effects, similar to those produced by other stimulants such as cocaine, and it is the second most frequently abused group of drugs worldwide. Based on the 2016 United Nations Office on Drugs and Crime World Drug Report, there are 33 million METH users worldwide. In China, there were 2.505 million drug addicts in 2016, of which 60.5% used synthetic drugs, with METH being the most widely used. METH abuse remains a global health challenge despite intense research interest in the development of pharmacological treatments. There are no pharmacological treatments specifically for METH addiction. The effective management of METH dependence remains elusive and the large majority of METH users relapse following treatment. Therefore, novel approaches to manage METH addiction are urgently needed [1-4].

Traditional Chinese medicine (TCM), which has been used to treat opiate dependence and withdrawal for over 200 years in China, offers potential alternatives to the pharmacotherapies currently used in allopathic medicine. TCM affects many body systems simultaneously, and has greater safety and fewer adverse effects, which may provide ideal treatment opportunities for drug addiction and withdrawal symptoms [5, 6]. Jinniu Capsule is composed of herbs and extracts of Hypericum Perforatum,

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Gastrodia Elata, Radix Paeoniae Alba, Calculus Bovis Artifactus, Salvia Miltiorrhiza, Pearl, Ginseng, Eleutherococcus Senticosus, and Spina Date Seed. Jinniu Capsules are safe for short- and long-term recommended dosages use in humans. The therapeutic effects of Jinniu Capsule on morphine withdrawal syndrome in rats and monkeys were observed in preclinical studies. Clinical trials demonstrated that Jinniu Capsules were safe and well-tolerated in healthy individuals with few side effects, and were effective and safe in the management of opiate withdrawal with no severe adverse events during treatment [7]. The purpose of this study was to observe the effects of Jinniu Capsule on METH-induced Conditioned Place Preference (CPP) in rats.

2 Materials and methods

2.1 Reagents

Jinniu Capsule, provided by Yiyuan Pharma Pte Ltd. (Gordon Industrial Building, Singapore), was dissolved in 1% carboxymethylcellulose sodium solution to a concentration of 100 mg/mL. METH (purity > 99%) was provided by Compulsory isolation detoxification of Shenzhen, 3 mg/mL solution was prepared with normal saline.

2.2 Animals

Sprague-Dawley rats, weighing 200–250 g, were purchased from Guangdong Medical Laboratory Animal Center (Foshan city, Guangdong, China). Animals were kept in plastic cages in a specific pathogen-free environment. A 12-h light/dark cycle, 19.2–25.1°C and 42%–59% relative humidity was maintained. Rats were acclimated for 5 days before treatment and allowed *ad libitum* access to food and water. The study was performed in accordance with the International Guidelines on the Care and Use of Animals for Scientific Purposes, and the protocol was approved by the Ethics Committee of Peking University Shenzhen Hospital.

2.3 CPP

The equipment for CPP training and testing consisted of five identical polyvinyl chloride (PVC) boxes. As shown in Figure 1 A, the box consisted of three chambers: two large side chambers with different floor textures (bar floor in left *vs* grid floor in right, respectively) separated by a smaller chamber with a smooth PVC floor. The three distinct chambers were separated by manual guillotine doors. Baseline preference (preconditioning test, Pre-C) was determined before CPP training, and the rats were initially placed in the middle chamber with the doors removed for 15 min. The times spent in the different chambers during the 15 min period were measured by a computer. Most rats showed a natural preference for the left chamber with the bar floor texture (Figure 1 B); therefore, the right chamber was set as the METH-paired chamber and the left chamber was the saline-paired chamber. Rats which showed strong unconditioned preference (> 540 s) in one of the three chambers were excluded. Conditioning was performed using an unbiased, balanced protocol. On the subsequent CPP training days, each rat was trained for 10 consecutive days with alternate injections of drug (METH 3 mg/kg, i.p.) and saline (1 mL/kg, i.p.). Rats were confined to the corresponding chambers for 45 min after drug or saline injection. The expression of METH-induced CPP (post-conditioning test, Post-C) was tested the day after the end of CPP training. The CPP score was defined as the time spent in the right chamber (METH-paired) minus that spent in the left chamber (saline-paired) [8-10].

2.4 Western Blot analysis

Rats were sacrificed 30 min after the end of the 15-min CPP test. Rat brains were removed, and the nucleus accumbens (NAc) was removed and placed in a 1.5-mL microtube containing ice-cold lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA), 1 mM phenyl methane sulfonyl fluoride (Thermo Fisher Scientific, USA), 1 mM Na_3VO_4 (Sigma-Aldrich, St. Louis, MO USA), and 20 mM NaF (Sigma-Aldrich, St. Louis, MO USA) [11, 12]. The mixture was incubated for 30 min at 4°C and then centrifuged at 12,000 g for 15 min at 4°C. Protein samples were adjusted to the same concentration based on micro-spectrophotometer measurement, then 5 × loading buffer was added, and albuminous degeneration was performed for 10 min at 98°C. Aliquots of the samples (5.0-μL) were loaded into the wells of polyacrylamide gels and separated by electrophoresis at 80–120 V, respectively. The proteins were electrophoretically transferred onto Polyvinylidene Fluoride transfer membranes (Millipore, Bedford, MA, USA) for 1.5 h at 0.33 A. Primary antibodies were incubated with the membranes overnight at 4°C. After washing with Tris-buffered saline containing Tween (three times × 10 min), horseradish peroxidase-conjugated secondary

antibody diluted to 1: 2000 in blocking buffer was added and the membranes were incubated for 1 h at room temperature on a shaker. The ECL Chemiluminescence detection kit (Millipore, Bedford, MA, USA) was used to determine protein expression using a chemiluminescence detector (Tanon 5220S; Guangzhou Ewell Bio-technology Co., Ltd., Guangzhou, Guangdong, China). Target protein levels were normalized to β-actin and analyzed with Quantity One software (version 4.4.0; Bio-Rad, Hercules, CA, USA).

Anti-phospho-phosphatidylinositol 3-kinase (PI3K) R1 antibody (cat. no. PA5-17387), anti-PI3KR1 antibody (cat. no. PA5-38904), anti-protein kinase B (AKT) antibody (cat. no. AHO1112), anti-mammalian target of rapamycin (mTOR) antibody (cat. no. PA5-34663), anti-phospho- p70 ribosomal S6 kinase (p70s6k) antibody (cat. no. 701083), anti-p70s6k antibody (cat. no. MA5-15141), anti-phospho-ribosomal protein s6 (rps6) antibody (cat. no. MA5-15140), anti-rps6 antibody (cat. no. MA5-15164), anti-phospho-eIF4E antibody (cat. no. PA5-17919) and anti-protein synthesis initiation factor 4E (eIF4E) antibody (cat. no. PA5-41751) were provided by Invitrogen Corporation (Carlsbad, CA, USA). Anti-phospho-AKT antibody (cat. no. #4060) and anti-phospho-mTOR antibody (cat. no. #5536) were purchased from Cell Signaling Technology (Danvers, MA, USA). All primary antibodies were diluted 1000 times with western blot antibody diluent.

2.5 Statistical analysis

All statistical analyses were performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Data are expressed as the means ± standard deviation (SD). One-way analysis of variance was used to compare inter-group differences. A probability (P) value of < 0.05 was considered statistically significant.

3 Results

3.1 METH-induced CPP

To assess CPP induced by METH, 20 male rats with no strong unconditioned preference (> 540 s) in each chamber were divided into 2 groups (n=10 per group). The Pre-C test was determined before CPP training. This was followed by 10 days CPP training as described above. The Post-C test was carried out the day after the end of CPP training. The time the rats spent in the left, middle and right chambers and the CPP scores in the METH and saline groups were compared (Figure 1 C).

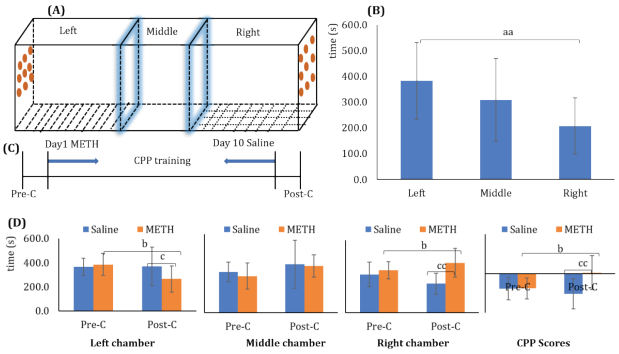

Figure 1: METH-induced CPP in rats. **(A)** CPP training and testing equipment consisted of two large side chambers with different floor textures (bar floor in the left *vs* grid floor in the right, respectively) and a smaller middle chamber with a smooth PVC floor; **(B)** Historical laboratory data of rat's natural preference, n = 630; **(C)** Protocol of CPP training and test; **(D)** Comparison of METH and saline, n = 10, the CPP score was defined as the time spent in the right chamber (METH-paired) minus that spent in the left chamber (saline-paired). Means ± SD, $^{aa}P < 0.01$ *vs* left chamber; $^{b}P < 0.05$ *vs* Pre-C test; $^{c}P < 0.05$ *vs* saline group; $^{cc}P < 0.01$ *vs* saline group. Pre-C: preconditioning test; Post-C: postconditioning test.

As shown in Figure 1 D, training with 3 mg/kg METH induced obvious CPP in male rats, and the time rats spent in the 3 chambers was not significantly different between the saline and METH group in the Pre-C test. However, after 10 days training, rats in the METH group showed CPP, the time rats in the METH group spent in the left chamber decreased compared to that in the saline group ($P < 0.05$), and significantly decreased compared with the Pre-C test ($P < 0.05$). The changes in the right chamber (METH-paired) were the reverse of the left chamber, the time spent by rats in the METH group was longer than that in the saline group ($P < 0.01$) and in the Pre-C test ($P < 0.05$). The CPP scores in the METH group were significantly higher than those in the saline group.

3.2 Effects of single dose Jinniu Capsule on METH-induced CPP

To determine the effects of Jinniu Capsule on METH-induced CPP, 30 male rats with no strong unconditioned preference were divided into three groups (n = 10 per group). The Pre-C test and CPP training were performed as described above. The Post-C test was carried out the day after the end of CPP training. Thirty min before the test, Jinniu Capsule at a dose of 0.8 g/kg was administered intragastrically to rats in the METH + Jinniu Capsule (METH+JN) group, and

the same volume of 1% Carboxymethylcellulose Sodium solution was administered to rats in the METH and control groups.

As shown in Figure 2, the time rats in the METH+JN group spent in the left and right chambers and the CPP scores were not significantly different to those in the METH group in the Pre-C and Post-C tests. These findings indicated that a single dose of Jinniu Capsule had no marked influence on METH-induced CPP.

3.3 Effects of Jinniu Capsule on METH withdrawal

To determine the effects of Jinniu Capsule on METH withdrawal, 30 male rats with no strong unconditioned preference were divided into three groups (n = 10 per group). The Pre-C test and CPP training were performed as described above. The day after the end of CPP training, Jinniu Capsule at a dose of 0.8 g/kg was administered intragastrically to rats every day in the METH + JN group, and the same volume of 1% carboxymethylcellulose sodium solution was administered to rats in the METH and control groups. The Post-C test and post treatment (Post-T) test were carried out on Day 1 and Day 7 after the end of CPP training, respectively.

As shown in Figure 3, METH-induced CPP did not change 7 days after CPP training in the METH group, as the time rats in the METH group spent in the left and right chambers and the CPP scores were not obviously different to those in the control group ($P < 0.05$, $P < 0.01$). Jinniu Capsule administered for 7 days significantly decreased the CPP scores compared with the METH group ($P < 0.01$). These findings indicated that long-term administration of Jinniu Capsule may promote METH withdrawal.

3.4 Mechanisms of Jinniu Capsule on METH withdrawal

The mTOR, a serine/threonine protein kinase that regulates cell growth and survival by controlling translation in response to nutrients and growth factors, has been demonstrated to be involved in neuronal adaptations that underlie drug addiction and learning and memory. mTOR signaling pathway in NAc were confirmed as the critical factor for cue-induced reinstatement of drug seeking in rats. To identify the mechanisms of Jinniu Capsule on METH withdrawal, all rats in Experiment 3 were sacrificed, the NAc was obtained from each rat and western blot analysis was performed as described above.

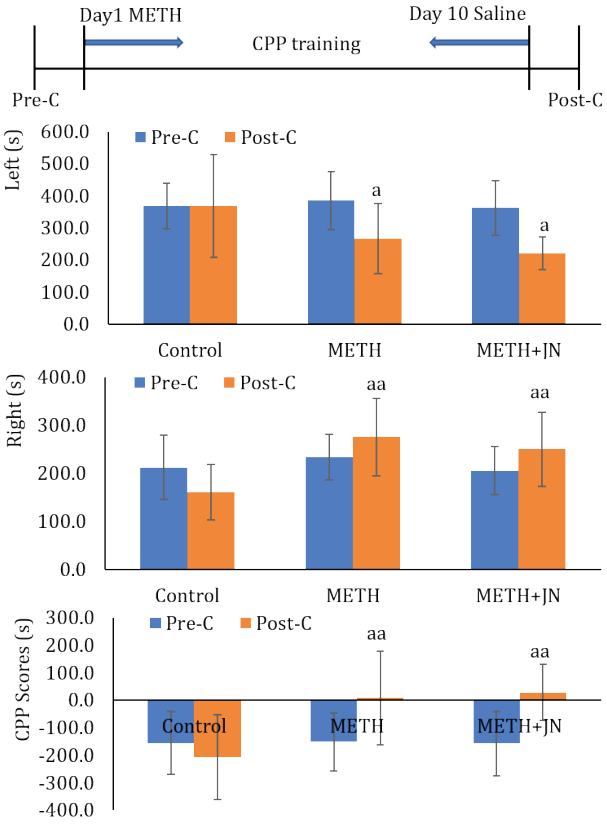

Figure 2: Effects of single dose Jinniu Capsule on METH-induced CPP. Means ± SD, n = 10, $^{a}P < 0.05$ *vs* control group; $^{aa}P < 0.01$ *vs* control group. Pre-C: preconditioning test; Post-C: postconditioning test. The doses of METH and Jinniu Capsule were 3 mg/kg and 0.8 g/kg respectively. The Pre-C and Post-C tests were performed, and Jinniu Capsule was administered 30 min before Post-C test.

The phosphorylation of PI3K, AKT, mTOR, p70s6k, rps6, and eIF4E were examined.

As shown in Figure 4, the phosphorylation cascade of PI3K, AKT, mTOR, p70s6k, rps6, and eIF4E were activated by 7 days METH withdrawal in rat NAc in the CPP tests ($P < 0.05$, $P < 0.05$). Jinniu Capsule partly blocked phosphorylation of the above proteins ($P < 0.05$, $P < 0.05$).

4 Discussion

METH is abused worldwide and there is lack of effective treatments specifically for METH addiction. TCM such as Jinniu Capsule has demonstrated great potential in treating drug addiction. In previous studies, Jinniu Capsule showed therapeutic effects on morphine withdrawal syndrome both in animals and in clinical trials [7]. However, the effects of Jinniu Capsule on METH addiction are still unknown. In this study, we found that

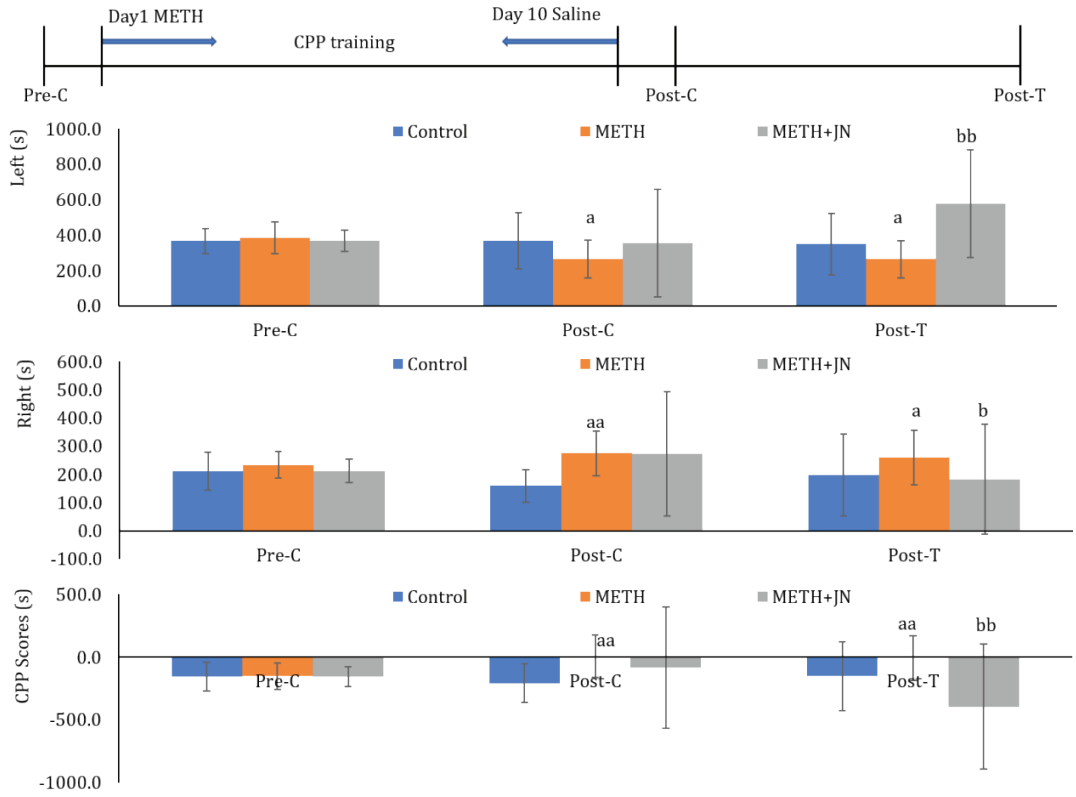

Figure 3: Effects of repeat doses of Jinniu Capsule on METH withdrawal in the CPP test. Means ± SD, $n = 10$, [a] $P < 0.05$ *vs* control group; [aa] $P < 0.01$ *vs* control group; [b] $P < 0.05$ *vs* METH group; [bb] $P < 0.01$ *vs* METH group. Pre-C: preconditioning test; Post-C: postconditioning test; Post-T: post treatment test. The doses of METH and Jinniu Capsule were 3 mg/kg and 0.8 g/kg respectively. The Pre-C, Post-C and Post-T tests were performed; Jinniu Capsule was administered every day for totally 7 days after the CPP training.

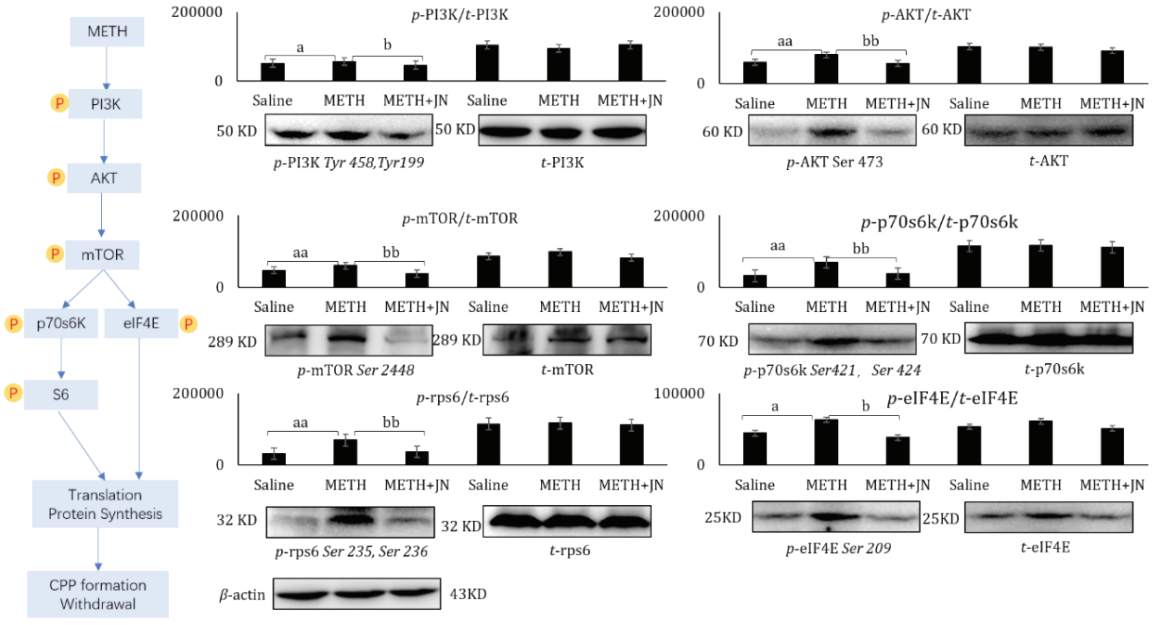

Figure 4: Influence of METH and Jinniu Capsule on the PI3K-AKT-mTOR signaling pathway phosphorylation cascade. Means ± SD, $n = 6$, [a] $P < 0.05$ *vs* saline group; [aa] $P < 0.01$ *vs* saline group; [b] $P < 0.05$ *vs* METH group; [bb] $P < 0.01$ *vs* METH group. NAc of rats was obtained and the phosphorylation of PI3K, AKT, mTOR, p70s6k, rps6, and eIF4E were examined.

the administration of Jinniu Capsule for 7 days promoted METH withdrawal in rat CPP tests.

Relapse is the main difficulty in addiction withdrawal. Cue-induced drug-seeking in rodents progressively increases after withdrawal of cocaine, heroin, METH, and alcohol. The NAc is very important in the reward pathway, memory, and relapse. To identify the mechanisms of Jinniu Capsule, we observed changes in the signaling pathways in the NAc.

mTORC1 is a serine and threonine kinase that regulates cell growth, survival and proliferation by controlling translation in response to nutrients and growth factors, and has been demonstrated to be involved in the neuronal adaptations that underlie drug-related behaviors such as reward seeking and excessive drug intake [13]. Luo [14] found that mTOR signaling in the NAc shell, but not the core, is critical for the augmentation effect of trefoil factor 3 (TFF3) on behavioral response to cocaine. James [15] examined the role of mTORC1 in cocaine-related behaviors such as cocaine taking, withdrawal, and reinstatement of cocaine seeking, and found that withdrawal from cocaine self-administration increased the indices of mTORC1 activity in the NAc. Intra-cerebroventricular rapamycin reduced p-p70s6k, Ca^{2+}/Calmodulin-dependent kinase II alpha (CAMKIIa) and AMPA receptor subunits (GluAs).

The phosphorylation of mTORC1 is regulated by the PI3K-AKT signaling pathway. Research has shown that psychoactive substances can induce cascade phosphorylation of PI3K-AKT. For example, following an intra-cerebroventricular injection of morphine, AKT was recruited to the synaptosomal membrane and activated by Thr308 and Ser473 phosphorylation [16]. Phentermine induces conditioned reward effects via activation of the PI3K-AKT signaling pathway in the NAc [17]. The PI3K inhibitors wortmannin and LY294002 decreased morphine-induced microglial migration, which is dependent on the PI3K-AKT pathway [18]. Activation of the μ-opioid receptor (OPRM1) by morphine induced time-dependent mTOR activation, and LY294002 blocked the late phase of mTOR activation [19].

In the present study, we found that after 7 days withdrawal in a METH-induced rat CPP model, the PI3K-AKT-mTORC1 phosphorylation cascade was activated in the NAc, and repeated administration of Jinniu Capsule for 7 days partly blocked the METH-induced PI3K-AKT-mTORC1 phosphorylation cascade. This may be the potential mechanisms by which Jinniu Capsule promotes METH withdrawal.

Furthermore, we observed phosphorylation downstream of mTORC1 such as phosphorylation of p70s6k, rps6 and eIF4E, which are also known to be regulatory factors in drug seeking and relapse. Similar to PI3K-AKT-mTORC1, p70s6k, rps6, and eIF4E in rat NAc were also phosphorylated after 7 days METH withdrawal in the CPP test; Jinniu Capsule also inhibited the phosphorylation of these kinases. Wang [20] found that the mTOR signaling pathway in the NAc core, but not the shell, was critical for cue-induced reinstatement of cocaine seeking in rats, and exposure to a cocaine-related cue increased p70s6k and rps6. Polakiewicz [21] found that μ-opioid agonists stimulated three different effectors of the PI3K-dependent signaling cascade; a specific agonist stimulated the activity of AKT, and upon μ-opioid receptor stimulation, p70s6k was activated and phosphorylated at Thr 389 and at Thr 421/Ser 424. The specific agonist stimulated the activity of AKT-stimulated phosphorylation of 4E-binding protein 1 and impaired its ability to bind the translation initiation factor eIF4E.

5 Conclusion

Jinniu Capsule demonstrated potential in promoting METH withdrawal in a rat CPP model, which may be related to its influence on the PI3K-AKT-mTOR signaling pathway.

Acknowledgements: We thank Health and Family Planning Commission of Shenzhen (201605018), Shenzhen Science and Technology Planning Project (JCYJ20160427185055877, ZDSYS201504301045406), Shenzhen "Sanming" project (SZSM201512026), Guangdong Bureau of Traditional Chinese Medicine Project (20171228) and Health and Family Planning Commission of Guangdong (B2018090) for financial support, and Compulsory isolation detoxification of Shenzhen for kindly providing the METH.

Disclosure Statement: The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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