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# Okadaic acid blocks the effects of 5-aza-2-deoxycytidine on consolidation, acquisition and retrieval of morphine-induced place preference in rats

Jian-Jun Zhang [a], Jin Han [a, b], Nan Sui [a, *]

[a] Key Laboratory of Mental Health, Institute of Psychology, Chinese Academy of Sciences, Beijing, 100101, China
[b] University of Chinese Academy of Sciences, Beijing, China

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Recent studies indicated that epigenetic modification, especially DNA methylation, play an important role in the persistence of addiction-related memory. 5-aza-2-deoxycytidine (5-aza), an inhibitor of DNA methyltransferases, was approved for clinical treatment. However, it is not clear whether 5-aza is involved in opiate addiction. In this study, using the morphine-induced conditioned place preference (mCPP) model in rats, we injected 5-aza into hippocampus (CA1) and prelimbic cortex (PL), and tested the behavioral consequences at various stages of consolidation, acquisition and retrieval. Moreover, to test whether protein phosphatase regulates the effects of 5-aza, protein phosphatase (PP) 1/2A inhibitor okadaic acid (OA) was infused before 5-aza injection. We found that 5-aza injection into CA1 but not into PL significantly attenuated the consolidation and acquisition of mCPP, however, the inhibition of DNA methylation in PL but not in CA1 enhanced the retrieval of mCPP. All these behavioral effects were absent when OA was infused before 5-aza injection. These findings suggest that 5-aza interfere opiate-related memory, and protein phosphatase plays an important role in this process.

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## 1. Introduction

Drug addiction is a chronic, relapsing disease. The long lasting addiction-related abnormal memory is one of the most important bases for relapse even a long time after abstinence (McLellan et al., 2000). Gene transcription (Agranoff et al., 1967) and chromatin modifying (Maddox et al., 2013) have been demonstrated to participate in the formation and maintenance of long-term fear memory. Drug abuse also induces changes in the genes expression and epigenetic modification in the brain (Robison and Nestler, 2011).

DNA methylation is the best understood and the most stable epigenetic modification (Wong et al., 2011). Accumulating evidence suggests that DNA methylation plays an important role in long-term fear memory (Levenson et al., 2006; Miller and Sweatt, 2007). Acquisition, consolidation and retrieval are three main processes which memory reflects (Abel and Lattal, 2001). Recent research indicates that DNA methylation is involved in acquisition and retrieval of cocaine-induced learning and memory in mice (Han et al., 2010; LaPlant et al., 2010). However, whether DNA methylation is required in consolidation, a time-dependent process which is essential for the storage of addiction-related memory (Cervo et al., 1997), remains poorly understood.

Another key question is how DNA methylation modulated the addiction memory. Evidence suggested that phosphatases took part in this phenomenon (Anier et al., 2010). Phosphatases such as the $Ca^{2+}$/calmodulin-dependent calcineurin and protein phosphatase (PP) 1/2A are negative regulators of both long-term potentiation and long-term memory storage (Malleret et al., 2001; Mauna et al., 2011; Mulkey et al., 1994; Woo and Nguyen, 2002). PP1 and PP2A play a key role in addiction reward and memory (Zachariou et al., 2002). Recent studies showed that DNA methylation down-regulates the transcription of *PP1* gene (Anier et al., 2010; Miller and Sweatt, 2007) and the protein expression of PP1Cβ (Pol Bodetto et al., 2013). Contrary to these findings, previous study suggested that the expression of *PP1* gene was not affected in DNMT knock-out hippocampus (Feng et al., 2010). Therefore, the

*Abbreviations:* 5-aza, 5-aza-2-deoxycytidine; DNMTs, DNA methyltransferases; PL, prelimbic cortex; mCPP, morphine-induced conditioned place preference; PP, protein phosphatase; OA, okadaic acid.

* Corresponding author. Tel.: +86 10 64857369; fax: +86 10 64872070.
*E-mail address:* suin@psych.ac.cn (N. Sui).

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issue of whether PP 1/2A participate in DNA methylation mediated addiction memory needs to be addressed.

Lots of evidence indicates that hippocampus is implicated in the learning and memory for drug addiction (Isokawa, 2012; Meng et al., 2013; Robbins et al., 2008). Chronic cocaine treatment enhances the long-term potentiation (LTP) in hippocampus (Thompson et al., 2002). Pharmacological inhibition of DNA methylation blocks the LTP in hippocampus (Levenson et al., 2006). Behavioral studies suggested that DNA methylation is required for hippocampal-dependent spatial learning and fear memory formation (Feng et al., 2010; Miller and Sweatt, 2007). Together, these studies suggest that DNA methylation in the hippocampus is crucial for memory formation.

The medial prefrontal cortex (mPFC) is implicated in the encoding and retrieval of drug-related memories, which leads to drug craving and drug use (Robbins et al., 2008). In the present context, the mPFC, in particular the prelimbic area (prelimbic cortex, PL), is another target of interest. The PL is involved in regulating the expression of fear conditioning and addiction-related memory (Peters et al., 2009). PL inactivation reduces reinstatement of drug seeking (Ball and Slane, 2012; Capriles et al., 2003).

5-aza-2-deoxycytidine (5-aza) is an inhibitor of DNA methyltransferases (DNMTs) and showed activity against several types of cancers (Christman, 2002; Momparler et al., 2000). Moreover, 5-aza was FDA (Food and Drug Administration) approved for the treatment of myelodysplastic syndrome in 2006 and was used to inhibit the DNA methylation in brain (Miller and Sweatt, 2007; Pol Bodetto et al., 2013). The present study was undertaken to clarify the possible use of gene therapy to its anti-addiction effect of 5-aza and whether PP 1/2A takes part in this effect. We injected 5-aza, into the hippocampus CA1 or PL to confirm its crucial involvement in the consolidation, acquisition and retrieval of addiction-related memories using morphine-induced conditioned place preference (mCPP) paradigms, which is one of the most widely used model for addiction memory study (Brown et al., 2008; Li et al., 2011; Ren et al., 2013). Prior to 5-aza injection, PP 1/2A inhibitor okadaic acid (OA) were micro-injected into the CA1 or PL to explore the molecular mechanism underlying the effects of 5-aza on opiate-related memory.

## 2. Materials and methods

### 2.1. Animals

Male Sprague–Dawley rats (Vital River Laboratory Animal Technology Co., Beijing, China) weighing 220–250 g were housed individually in a room in a 12-h light/dark cycle (lights on at 08:00 am) with controlled temperature (20–24 °C) and humidity (40–60%). Food and water were available ad libitum. The experiments are in compliance with the guideline provided by the Regulation for the Administration of Affairs Concerning Experimental Animals (China, 1988). All procedures were approved by the Research Ethics Review Board at the Institute of Psychology, Chinese Academy of Sciences.

### 2.2. Surgery

Rats were anesthetized with 10% chloral hydrate (4.0 ml/kg, i.p.) and mounted in a stereotaxic frame (stoelting company, USA). Stainless steel cannulas (o.d. 0.6 mm, i.d. 0.35 mm) were bilaterally implanted 1 mm above the CA1 or PL according to the following coordinates (Paxinos and Watson 1998): CA1 (−3.6 mm A/P, ±1.7 mm M/L and −2.6 mm D/V) or PL (+3.2 mm A/P, ±0.8 mm M/L; −4.0 mm D/V). The guide cannulas were secured with three screws and dental cement, and an obturator was inserted into each cannula to prevent occlusion. All rats were treated with penicillin (80,000 units) to prevent infection and allowed to recover for 7–10 days.

### 2.3. Drugs and microinjections

Morphine hydrochloride (Qinghai Pharmaceutical, China) were dissolved in sterilized physiological saline (0.9% NaCl) to the concentration of 5 mg/ml, and administered subcutaneously (s.c.) 1.0 ml/kg body weight. 5-aza (Sigma–Aldrich, USA) was dissolved and diluted with 0.8% acetic acid (AcOH) to 0.2 μg/μl. OA (LC Laboratories, USA), was dissolved and diluted with 10% dimethyl sulfoxide (DMSO) to 0.8 ng/μl. The drugs or vehicles (0.5 μl/side for CA1 and PL) were injected through an injector cannula. The dose of the drugs was chosen based on previous studies (Miller and Sweatt, 2007; Yang et al., 2006). Infusions were delivered with a 5.0 μl Hamilton microsyringe and the injection was given at the rate of 0.5 μl/min over 1 min with the injector cannula remaining in the guide cannula for another two minutes to prevent backflow. Intracranial OA or DMSO injections were conducted 15 min prior to the intracranial injection of 5-aza or 0.8% AcOH.

### 2.4. Behavioral testing

The mCPP apparatus consisted of a plastic chamber divided into two 40 cm × 40 cm × 50 cm compartments by a guillotine door. One compartment with grid floor and white horizontal stripes on black walls, the other with twill floor and white vertical stripes on black walls. The apparatus was placed in a room dimly lit. The location of the rats was monitored by a video camera suspended above the apparatus. The video data were analyzed for time in each compartment by professional software (Taiji Software Company, Beijing, China).

The mCPP procedures were based on a previous study with minor modifications (Han et al., 2010). There were three different phases: pretest (day 1–2), conditioning (days 3–8) and posttest (day 9). On the pretest days, each rat was placed in one chamber and allowed to freely explore the apparatus for 15 min. The natural preference score was calculated as the average of time spent in the same compartment on these 2 days. Conditioning continued over the next 6 days. The compartments were separated by removable partitions, and rats were confined to one side for 45 min immediately after receiving injection of either morphine (5 mg/kg, s.c.) or saline (1 ml/kg, s.c.). Rats in the saline conditioning group received saline injection (1 ml/kg, s.c.) before both training sessions. A biased conditioned place preference (CPP) design was used (Davis et al., 2007; Wu et al., 2014). Rat was assigned to receive morphine in the non-preferred compartment, and saline was administered in the preferred compartment. Each rat received three morphine and three saline pairings on alternating days. Half of the rats began with morphine pairing, and the other half began with saline pairing. On day 9, posttest was performed for 15 min. Rats were placed in the apparatus and allowed to freely explore both compartments in a drug-free state. The time spent in each compartment was recorded and the preference score was calculated: preference score = time spent in the morphine-paired side during the posttest − time spent in the morphine-paired side during the pretest.

### 2.5. Experimental design

#### 2.5.1. Testing the effects of inhibiting PP 1/2A on 5-aza-induced impairments in the consolidation of mCPP

We used 8 groups of rats to examine the effects of PP 1/2A inhibitor on 5-aza-induced impairments in the consolidation of mCPP. Rats were administered either morphine (5 mg/kg) or saline before conditioning. Immediately after each conditioning session, 4 groups of rats got CA1-injection and the other 4 groups got PL-injection. Rats were divided into groups according to the different treatments: the Vehicle group got DMSO infusion followed by AcOH infusion (abbreviated as DMSO + AcOH infusion); the 5-aza group got DMSO+5-aza (0.2 μg) infusion; the OA+5-aza group got OA (0.8 ng)+5-aza infusion; the OA group got OA + AcOH infusion. The interval between two infusions was 15 min. Posttest was assessed.

#### 2.5.2. Testing the effects of inhibiting PP 1/2A on 5-aza-induced impairments in the acquisition of mCPP

Another 8 groups of rats were used to examine whether inhibiting the activity of PP 1/2A contributes to the ability of 5-aza to the acquisition of mCPP memory. Prior to every morphine conditioning session, the rats got two infusions with a 15-min interval. Rats were divided into groups according to the different treatments described above. 5-aza/AcOH was infused 15 min prior to every morphine conditioning. Posttest was assessed.

#### 2.5.3. Testing the effects of inhibiting PP 1/2A on 5-aza-induced enhancement in the retrieval of mCPP

Another 8 groups of rats were used to examine the effect of PP 1/2A inhibitor OA on 5-aza-induced enhancement in the retrieval of mCPP. The rats that developed obvious CPP (preference score was more than 100) after 6 days of morphine conditioning received different injection treatment described above 15 min prior to the retrieval test. Shift score was calculated to assess the retrieval of mCPP. The shift score = the preference score during the retrieval test − the preference score during the posttest.

### 2.6. Histology

When experiments were completed, all the rats were deeply anesthetized with chloral hydrate (40 mg/kg) and transcardially perfused with saline followed by ice-cold 4.0% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and post-fixed for 1 h, and then stored in a 20% sucrose solution at 4 °C for 24 h. Coronal sections (40-μm thick) containing the cannula tracks were cut on a cryostat and were stained with cresyl violet. Rats with cannula placement outside the area of interest were excluded from subsequent data analysis. Overall, 16 rats

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