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Effect of 5-aza-2-deoxycytidine microinjecting into hippocampus and prelimbic cortex on acquisition and retrieval of cocaine-induced place preference in C57BL/6 mice

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ABSTRACT

The long lasting addiction-related abnormal memory is one of the most important foundations for relapse. DNA methylation may be a possible mechanism for persistence of such memory. Here we injected the DNA methyltransferases (DNMTs) inhibitor, 5-aza-2-deoxycytidine (5-aza) into hippocampus CA1 area and prelimbic cortex during the stages of acquisition and expression of cocaine-induced place preference in C57BL/6 mice. Results showed that in CA1 DNA methylation inhibitors could restrain acquisition but had no impact on expression of the cocaine-induced conditioned place preference (CPP). On the contrary, in prelimbic cortex, 5-aza had no effect on acquisition but blocked expression. Our results indicated that DNA methylation in hippocampus is required for learning; while DNA methylation in prelimbic cortex is necessary for memory retrieval. The present finding is consistent with the role of the hippocampus as a structure contributing to *cocaine-induced memory acquisition*, and prelimbic cortex, a part of prefrontal cortex as an area responsible for *cocaine-induced memory retrieval*. In conclusion, DNA methylation does play an important role in drug-induced learning and memory although the detailed effect still calls for further research.

1. Introduction

Drug addiction is debilitating chronic disease characteristic of persistent craving even long after drug abstinence, abnormal learning and memory associated with drug taking is one of the potential key contributor for the persistent psychic dependence. When environmental cues and drugs abusing have been repeatedly paired, it can lead to rapid and intensive association between contextual cues and drug-taking behaviors (Dong et al., 2006). When re-exposed to the context associated with drug, memory retrieval will trigger the motivation for drug seeking and taking. Many evidences indicated that the hippocampus and the medial prefrontal cortex are implicated in the encoding and retrieval of drug-related memories (Krasnova et al., 2008; Robbins et al., 2008).

The neuroplasticity of hippocampus and prefrontal cortex can be abnormally altered by addictive drug such as cocaine through regulation of gene expression (Krasnova et al., 2008; Marie-Claire et al., 2003). Chromatin remodeling may be a key regulatory mechanism underlying cocaine-induced neural and behavioral plasticity (Kumar et al., 2005). DNA methylation as an epigenetic mechanism which can remodel chromatin, is dynamically regulated in the adult nervous system (Miller et al., 2008; Miller and Sweatt, 2007) and may play an important role in regulating gene expression in various stage of drug abuse (Renthal and Nestler, 2008; Tsankova et al., 2007). DNA methyltransferases (DNMTs) inhibitor can inhibit DNA methylation in postmitotic neuronal cells or adult mouse brains (Endres et al., 2000; Qiang et al., 2010), result in activity-dependent demethylation of genomic DNA in hippocampal neurons cultures (Nelson et al., 2008) and change the methylation of specific genes in rat brains (Miller et al., 2008; Miller and Sweatt, 2007). The DNMTs inhibitors resulted in an immediate and significant diminution of memory formation after contextual fear conditioning (Levenson et al., 2006; Miller and Sweatt, 2007).

The existing results showed that addictive drugs could also change DNA methylation. For example, methamphetamine could alert expression of DNA methyltransferase 1 mRNA and repeated injection of cocaine induced significant expression of methyl-CpG-binding protein 2, as well as the methyl-CpG-binding protein MBD1 in rat brain (Cassel et al., 2006; Numachi et al., 2007). Furthermore, modification of histone deacetylation alters locomotor and rewarding responses to cocaine (Kumar et al., 2005). The mechanisms of DNA methylation induced by drugs are unknown and it remains unclear

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whether DNA methylation is involved into the process of learning and memory retrieval associated with addiction.

The specific objectives of the present experiment were: (1) to determine whether the inhibitor of DNMTs could disturb the processing of drug-induced learning and memory retrieval; (2) to find out whether DNA methylation played a role in different stages of learning and memory in certain brain locations (hippocampus and prelimbic cortex). We sought to provide evidence of the DNA methylation in addiction of drugs.

2. Materials and methods

2.1. Subjects

Male C57BL/6 mice 25–30 g, Laboratory Animal Center of the Academy of Military Medical Sciences, Beijing, China were group housed in a colony room on a 12 h/12 h light/dark cycle (lights on at 07:00) with controlled temperature (20–24 °C) and humidity (40–70%). Food and water were available ad libitum except during the experiment. Mice were gently handled daily for 3 days before the behavioral procedure to be acclimatized to handling. All experiments were conducted in the light phase (08:00–18:00). The experimental protocol and procedures were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985) and the Regulations for the Administration of Affairs Concerning Experimental Animals (China, 1988). The experimental protocol was approved by Research Ethics Review Board of Institute of Psychology, Chinese Academy of Sciences.

2.2. Surgery

Mice were anesthetized with 10% chloral hydrate (3.0–4.0 ml/kg, i.p.) and placed in a stereotaxic apparatus (stoelting company, USA). The stainless steel guide cannulae were implanted bilaterally into the CA1 of hippocampus (1.9 mm posterior to the bregma, 1.5 mm lateral to midline, and 1.0 mm ventral to the skull) and prelimbic cortex (part of mPFC) (2.4 mm posterior to the bregma, 0.3 mm lateral to midline, and 1.4 mm ventral to the skull), respectively. The coordinates are based on the Paxinos and Franklin stereotaxic atlas (The mouse Brain in Srereotaxic Cocodinates, 2001). Guide cannulae were implanted 0.5 mm above the targeted areas. A stylet was inserted into the guide cannula to prevent occlusion and all mice were treated with penicillin (80,000 units) after surgery to prevent infection. The mice were allowed to recover for 7–10 days and were handled every other day to reduce stress associated with handling at the time of testing.

2.3. Drugs and microinjections

Cocaine Hydrochloride (China National Drug Lab 1210-920) were dissolved and diluted with 0.9% saline to the concentration of 20.0 mg/ml, and injected intraperitoneally (i.p.) at a volume of 10.0 ml/kg body weight (20 mg/kg body weight). 5-aza-2-deoxycytidine (5-aza, Sigma, USA), an inhibitor of DNA methyltransferases (DNMTs), was dissolved and diluted with 0.8% acetic acid at the concentration of 0.5 μg/μl and 5.0 μg/μl (Miller and Sweatt, 2007). The drug or vehicle (0.2 μl each side) was injected through an injector cannula which protrudes 0.5 mm below the guide cannula. Infusions were delivered with a 1.0 μl Hamilton microsyringe and the injection was done at the rate of 0.2 μl/min over 1 min with the injector cannula remaining in the guide cannula for another minute to prevent backflow. In order to get animals adapted to the injection procedures, they were given to four mock injections in the days preceding the experimental phase. Intracranial injections were conducted 15 min prior to the intraperitoneal injection of either cocaine or saline during the conditioning phase.

2.4. Behavioral tests

The conditioned place preference (CPP) paradigm has been extensively used to investigate the anatomical and neurochemical substrates of rewarding, learning and memory, especially in drug addiction research (Bardo and Bevins, 2000; Tzschentke and Schmidt, 1998).

2.4.1. Apparatus

The place conditioning apparatus consisted of a rectangular three-compartment plastic chamber with two side compartments (16 cm × 20 cm × 20 cm) and one center compartment (5.5 cm × 20 cm × 20 cm). The side compartments have different visual and tactile cues with grey wall and dot floor on one side, and two black stripes on the grey wall and grid floor on the other side. The testing apparatus was placed in a room with dim light provided by three incandescent bulbs (15 W). The location and movement of mice were monitored by a video camera (about 1.5 m above the test arena) and the video were analyzed for time and distance in each compartment by professional Software (Taiji Software Company, Beijing).

2.4.2. Conditioned place preference (CPP)

The conditioned place preference procedure was based on a previous study with minor modifications (Maldonado et al., 2007; Peakman et al., 2003) which consisted of four different phases: adaptation (day 1), pretest (baseline, day 2), conditioning (days 3–6), and posttest (day 7). On adaptation day, the animals were given 5 min to access the apparatus to reduce the stress and novelty. On the next day, the initial preference was assessed, each mouse was placed in the central compartment and allowed to freely explore the apparatus for 20 min, and the time spent each compartment was recorded and the preference score was calculated (preference score = time spent in the to be drug-paired side − time spent in the to be saline-paired side) as the baseline preference score (pretest). During the cocaine conditioning periods, an unbiased CPP design was used in which half of the animals in each group were assigned to receive cocaine (20.0 mg/kg i.p.) in the non-preferred compartment, and the other half in that group received cocaine in the preferred compartment. The mice received two conditioning sessions (20 min each) a day. The cocaine and saline training sessions were counterbalanced in the morning or afternoon in the following 4 days. A 20-min posttest session was performed on day 7 after the conditioning, during which the mice were placed in the apparatus with free access to the three compartments in a drug-free state with the guillotine door removed. The time spent in each compartment was recorded and preference score was calculated (posttest). The shift score (shift score = preference score in the posttest − preference score in the pretest) was used to assess cocaine conditioning preference. In retrieval experiment, the mice that did not develop cocaine CPP (shift score is less than 100) received two more cocaine conditioning.

2.4.2.1. Experiment 1: the effect of microinjection of DNMTs inhibitor into the hippocampus on the acquisition and retrieval of cocaine CPP. Two different concentrations of 5-aza (0.2 μg and 2.0 μg) or vehicle were infused into the hippocampus 15 min prior to cocaine conditioning session to investigate the effect of DNMTs inhibitor on the acquisition of cocaine CPP. Another three groups of animals were used to examine the effect of DNMTs inhibitor on retrieval of cocaine CPP. The mice that developed obvious CPP (shift score is more than 100) after 4 to 6 days cocaine conditioning received different dose of 5-aza (0, 0.2 and 2.0 μg) 15 min before the retrieval test. The doses of the drug and the time interval before the behavioral testing were based on previous studies (Endres et al., 2000; Miller and Sweatt, 2007; Momparler et al., 1985).

2.4.2.2. Experiment 2: the effect of microinjection of DNMTs inhibitor into the prelimbic cortex on the acquisition and retrieval of cocaine CPP.

In the first experiment we observed that there were no differences on the acquisition and retrieval of CPP between the high and low dose of 5-aza. In this experiment, only the low dose of 5-aza (0.2 μg) was used to examine the effect of microinjection of DNMTs inhibitor into the prelimbic cortex on acquisition and retrieval as described above.

2.5. Cannula verification

At the end of the experiment, all the mice were deeply anesthetized with chloral hydrate (40 mg/kg), perfused transcardially with saline followed by 4.0% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Coronal sections from the injection site were obtained at 60 μm using a freezing microtome. The brain slices were processed for cresyl violet staining according to standard Nissl-staining procedures (Meyer et al., 2008).

2.6. Statistical analysis

The shift score were analyzed using one-way ANOVA in experiment 1 (data are shown as mean ± S.E.M), and two-way ANOVA in experiment 2 with group (saline, cocaine) and treatment (saline, 5-aza) as between factors. Post-hoc Least Significant Difference LSD analysis was used to determine if differences between groups were significant. Results were considered significant when p-values were <0.05, using a two-tailed test.

3. Results

3.1. Cannula verification

Correct cannulae placement was determined with postmortem histological verification. The injector cannulae were targeted towards the middle of the CA1 of the hippocampus (Fig. 1A) and the inferior part of the prelimbic cortex (Fig. 1B). The mice with cannula placements within the following coordinate scopes were used in the statistical analysis: 1.7 mm–2.06 mm posterior to the bregma, 1.2 mm–1.5 mm lateral to midline, and 1.4 mm–1.5 mm ventral to the skull for the CA1 of the hippocampus, and 2.46 mm–2.80 mm posterior to the bregma, 0.2 mm–0.3 mm lateral to midline, and 1.6 mm–1.9 mm ventral to the skull for the prelimbic cortex. Seven mice were removed because the placements were outside the targeted areas.

3.2. Microinjection of 5-aza into the hippocampus impaired the acquisition but not the retrieval of cocaine CPP

One-way ANOVA revealed that there were significant differences among the groups ($F_{(2,\ 21)}=7.87$, $P<0.01$; $n=7$–11) in the acquisition test, post-hoc analysis showed that the mice received low (0.2 μg, $n=8$) and high (2.0 μg, $n=9$) dose of 5-aza significantly decreased the time spent in the cocaine-paired side compared to the saline ($n=7$) group ($P<0.01$, $P<0.01$) (figures are not shown), but no difference was observed between the low and high dose ($P=0.82$). In the retrieval test, one-way ANOVA showed that there was no significant difference between groups ($F_{(2,\ 19)}=0.157$, $P>0.05$; $n=7$–10). The data suggested that microinjection of 5-aza into the hippocampus impaired the acquisition but not the retrieval of cocaine CPP.

Since there is no difference between the low and high dose of 5-aza, we combined the data in the two groups and a two-way ANOVA analysis was used. In the acquisition test, the ANOVA revealed a significant interaction effect of group by treatment on shift score ($F_{(1,\ 38)}=5.98$, $P<0.05$; Fig. 2A). In the cocaine-treated mice (cocaine + veh $n=7$), microinjection of 5-aza ($n=17$) significantly decreased the time spent in cocaine-paired side ($F_{(1,\ 39)}=4.30$, $P<0.05$; Fig. 2A); However, in the saline-treated animals, infusion of 5-aza ($n=11$) had no effect on shift score ($F_{(1,\ 39)}=0.03$, $P>0.05$; Fig. 2A). These results suggested that

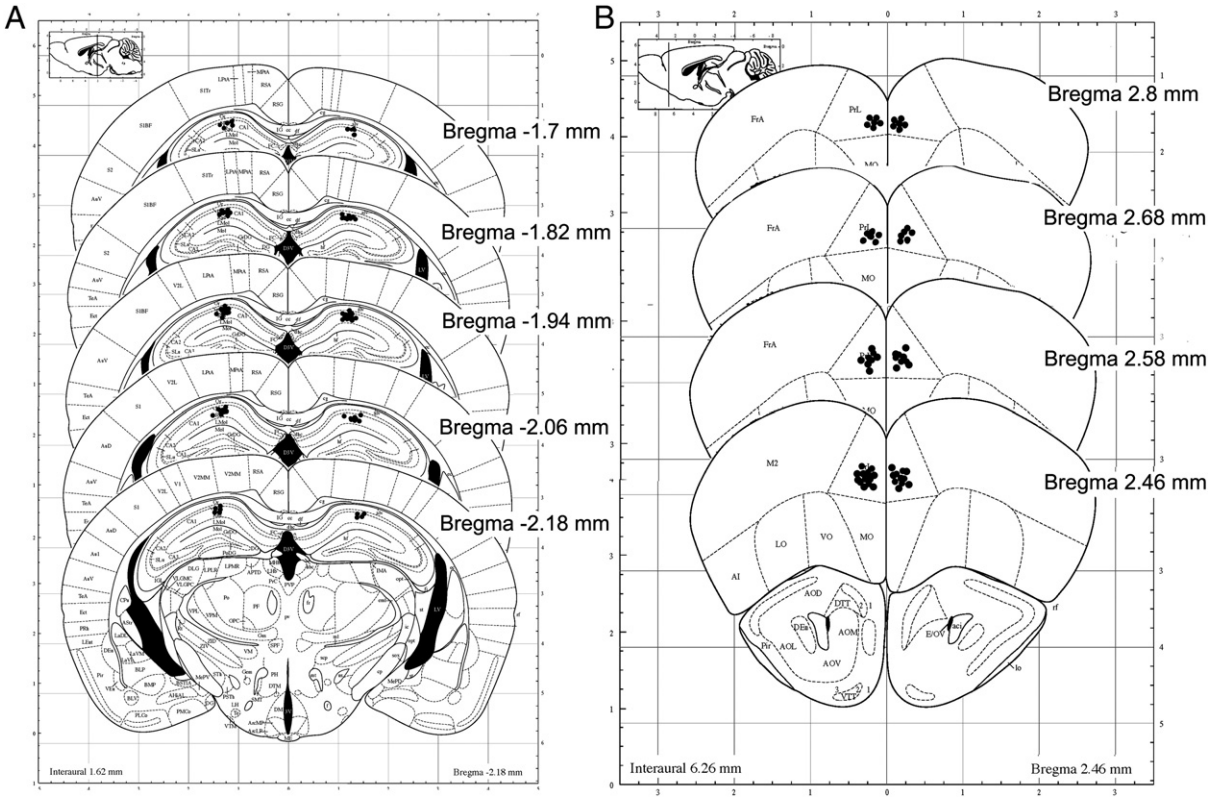

Fig. 1. The figure shows all cannula placements in corresponding levels of dorsal hippocampus (A) and prelimbic cortex (B). Numbers on right indicate the approximate rostrocaudal plane posterior to bregma. Black dots show the location of the tip of the injector cannula for the mice.
The figure is adapted from diagrams of a stereotaxic atlas of the mouse brain (The Mouse Brain in Srereotaxic Cocodinates, 2001, the second edition).

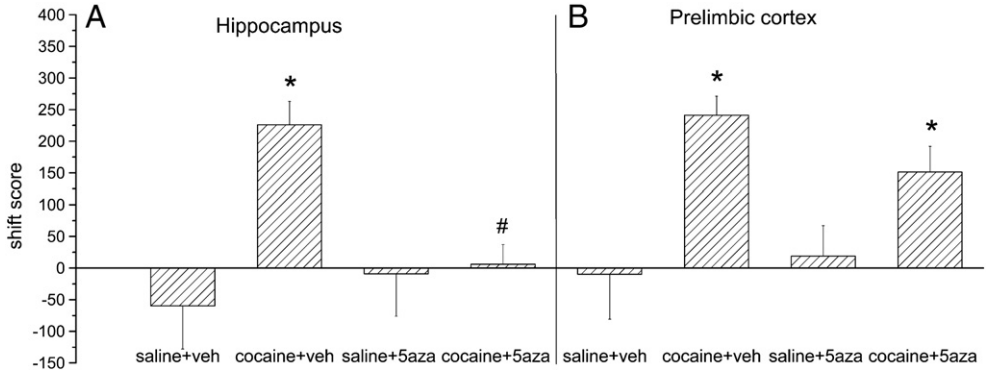

Fig. 2. Effect of microinjection of 5-aza-2-deoxycytidine (5-aza) into (A) CA1 of hippocampus and (B) prelimbic cortex on acquisition of cocaine induced conditioned place preference. * P<0.05 vs. saline + veh group; # P<0.05 vs. cocaine + veh group. Groups of hippocampus: saline + veh n = 7, cocaine + veh n = 7, saline + 5-aza n = 11, cocaine + 5-aza 0.2 μg n = 9, 2.0 μg n = 8. Groups of prelimbic cortex: saline + veh n = 11, cocaine + veh n = 7, saline + 5-aza n = 7, cocaine + 5-aza n = 8.

microinjection of 5-aza into the hippocampus impaired the acquisition of cocaine CPP and did not cause any aversive effect. In the retrieval test, the ANOVA revealed that there was significant main effect of group ($F_{(1, 34)} = 20.69$, $P<0.01$; Fig. 3A), but there was no significant main effect of treatment or interaction effect of group by treatment ($F_{(1, 39)} = 0.171$, $P>0.05$; $F_{(1, 39)} = 0.024$, $P>0.05$, respectively; Fig. 3A), which suggested that microinjection of 5-aza into the hippocampus had no effect on retrieval of cocaine CPP.

3.3. Microinjection of 5-aza into the prelimbic cortex inhibited the retrieval but not the acquisition of cocaine CPP

Two-way ANOVA analysis was used to examine the effect of microinjection of 5-aza into the prelimbic cortex on the acquisition and retrieval of cocaine CPP (Figs. 2 and 3). In the acquisition test, the ANOVA showed significant main effect of group on shift score ($F_{(1, 35)} = 11.95$, $P<0.01$; Fig. 2B; n = 7–11), but no significant main effect of treatment ($F_{(1, 35)} = 0.25$, $P>0.05$) or interaction effect of group by treatment ($F_{(1, 35)} = 1.24$, $P>0.05$; Fig. 2B). The results implicated that microinjection of 5-aza into the prelimbic cortex had no effect on cocaine CPP acquisition. In the retrieval test, two-way ANOVA showed significant interaction effect of group by treatment on shift score ($F_{(1, 29)} = 2.99$, $P<0.05$; Fig. 3B; n = 6–10) as in the cocaine-treated animals, microinjection of 5-aza (n = 6) into the prelimbic cortex significantly decreased the time spent in the cocaine-paired side compared to the vehicle (n = 6) group ($F_{(1, 30)} = 5.22$, $P<0.05$; Fig. 3B), while in the saline-treated animals, infusion of 5-aza into prelimbic cortex had no effect on shift score ($F_{(1, 35)} = 0.01$, $P>0.05$; Fig. 3B). The results suggested that DNA methylation inhibitor 5-aza restrained cocaine CPP retrieval.

4. Discussion

Drug associated learning and memory play a key role in drug craving and psychological dependence. The conditioned place preference paradigm has been used extensively in drug addiction research to investigate reward-related learning and memory as well as the motivation of drug seeking (Bardo and Bevins, 2000; Tzschentke and Schmidt, 1998). During conditioning, animals are trained in one environment paired with drug and the other environment paired with saline. On the test day, animals are given free access to both environments in a drug-free state and their preferences for drug- versus saline-paired environments are assessed, the expression test is thought to be a measure of memory retrieval (Bardo and Bevins, 2000; Markou et al., 1993). In the present study, our results demonstrated that DNA methylation is involved in the course of addiction-related memory acquisition and retrieval. Interfering DNA methylation with DNMTs inhibitor in the hippocampus could inhibit cocaine CPP acquisition, whereas inhibiting the effect of DNMTs in prelimbic cortex blocked the retrieval of drug-associated memory.

Neural circuits underlying associative learning may include several brain regions, such as hippocampus (Kuo et al., 2007; Meyers et al., 2006) and basolateral amygdala (Feltenstein and See, 2007). Previous studies suggested that molecular changes in the hippocampus might participate in acquisition of cocaine-related memory (Krasnova et al., 2008), and the medial prefrontal cortex is involved into the retrieval

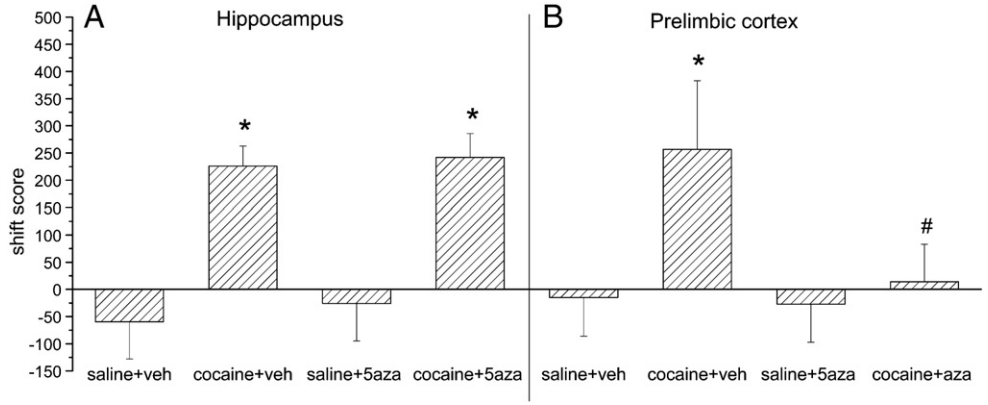

Fig. 3. Effect of microinjection of 5-aza-2-deoxycytidine (5-aza) into (A) CA1 of hippocampus and (B) prelimbic cortex on retrieval of cocaine induced conditioned place preference. * P<0.05 vs. saline + veh group; # P<0.05 vs. cocaine + veh group. Groups of hippocampus: saline + veh n = 7, cocaine + veh n = 7, saline + 5-aza n = 10, cocaine + 5-aza 0.2 μg n = 7, 2.0 μg n = 7. Groups of prelimbic cortex: saline + veh n = 11, cocaine + veh n = 6, saline + 5-aza n = 10, cocaine + 5-aza n = 6.

of drug-related memories which lead to drug craving and drug use (Robbins et al., 2008). However, there are some controversies over the role of prelimbic cortex in cocaine CPP acquisition (Tzschentke and Schmidt, 1998, 1999; Zavala et al., 2003). Our findings are consistent with previous research on the effect of hippocampus on cocaine CPP acquisition rather than retrieval, by showing DNMTs in hippocampus is contributing to memory encoding but not retrieval. A set of research shows that lesions of the hippocampus or intra-hippocampus microinjections of dopamine D1 or D2 receptor antagonists prior to testing impair the expression of context-elicited cocaine-seeking behavior (Fuchs et al., 2005), or retrieval of cocaine (Meyers et al., 2006) and morphine CPP(Rezayof et al., 2003), which is inconsistent with our finding. In our study, microinjections of 5-aza into hippocampus prior to retrieval test did not disrupt memory recall, suggesting inhibition of DNMTs may not impair recall of previous contextual CS-US associations in hippocampus. The mechanism of changes in gene transcriptions and expressions involved in hippocampus is not clear, although some studies reported the differences in types and quantity of genes in some brain regions (Krasnova et al., 2008) or transcript peptides (Marie-Claire et al., 2003) in signaling cascade. Our results extended previous studies suggesting the role of DNMTs inhibitor in regulating learning but not memory recall in hippocampus.

We also found a dissociation effect of microinjection of 5-aza into prelimbic cortex on acquisition and retrieval of cocaine-related memory and the activity of DNMTs in prelimbic cortex was required for memory retrieval. Functional brain imaging results indicate that there is a time-dependent reorganization of the neuronal circuitry underlying long-term memory storage, in which a transitory interaction between the hippocampal formation and the neocortex would mediate the establishment of long-lived cortical memory representations (Bontempi et al., 1999). Prelimbic cortex is one of the subareas in the medial prefrontal cortex (mPFC) for permanent memory storage (Frankland and Bontempi, 2006; Frankland et al., 2006). For example, after recalling a preference for the cocaine-paired environment, rats show elevated levels of immediate early genes (IEGs; e.g. c-fos) in the prelimbic cortex (Miller and Marshall, 2005). Prefrontal cortex activation has also been observed in brain imaging studies of explicit retrieval (Bontempi et al., 1999; Ungerleider, 1995). Other researchers found that cocaine-induced CPP was abolished by the pretreatment of anisomycin in prefrontal cortex (Kuo et al., 2007). In summary, our results confirmed the role of prelimbic cortex in memory recall, and extended that DNA methylation plays a critical role in the retrieval of addictive memory.

Traditionally, DNA methylation is known as a static process, but surprisingly high level of DNMTs mRNA was observed in adult neurons (Feng et al., 2006). The permanent molecular and cellular changes may account for the life-long behavioral changes associated with addiction or normal memory. For example, exposure of slices to DNMTs inhibitors (Zeb or 5-aza-2-deoxycytidine) resulted in an immediate and significant diminution of long-term potentiation (LTP) without any deleterious effect on synaptic transmission, short term plasticity, NMDA receptor function, or postsynaptic response to the theta-burst stimulation which induced LTP (Levenson et al., 2006). The deficit in synaptic plasticity produced by 5-aza-2-deoxycytidine can be overcome by enhancing levels of histone acetylation (Miller et al., 2008). Some evidence implicated that DNA methylation can have rapid and dynamic changes in mature neurons (Levenson et al., 2006; Miller and Sweatt, 2007; Nelson et al., 2008). Our results showed that DNMTs is rapid enough to affect learning or memory retrieval process but the detailed mechanism need further experiments to identify. Our experiment used only behavioral pharmacological method to test the effect of 5-aza in different phases of drug-induced CPP, and we did not use molecular test to confirm the effect of the 5-aza on DNMTs and changes of methylation induced by behavioral training. However, the suppressing effect of 5-aza on DNMTs has been established in previous studies (Endres et al., 2000; Miller and Sweatt, 2007; Qiang et al., 2010). The reduction in DNA methyltransferase activity in the activated and depolarized neuron could contribute to the enhanced intensity and multiplicity of gene expression frequently reported (Sharma et al., 2008). Some existing researches confirmed the role of DNA methylation in learning-induced changes in regional-specific global cytosine methylation or DNA methyltransferase expression (Brown et al., 2008) or particular genes such as gene pp1, reelin in the adult hippocampus (Miller and Sweatt, 2007) or brain-derived neurotrophic factor (BDNF) (Lubin et al., 2008) etc.

Our research for the first time demonstrated the behavioral evidence of DNMTs contribution to cocaine-related learning and memory. We also found the dissociation efforts of microinjection of 5-aza into hippocampus and prelimbic cortex in different phases of CPP although the detail effect still calls for further research.

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