Accumbal 14-3-3 ζ Protein Downregulation Is Associated with Cocaine-Conditioned Memory

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Key Words

Conditioned place preference • Nucleus accumbens • Cocaine memory • Conditioned cues • Reinstatement

Abstract

Cocaine-conditioned memory has been known to cause cocaine craving and relapse, while its underlying mechanisms remain unclear. We explored accumbal protein candidates responsible for a cocaine-conditioned memory, cocaine-induced conditioned place preference (CPP). Two-dimensional gel electrophoresis in conjunction with liquid chromatography mass spectrometry analysis was utilized to identify accumbal protein candidates involved in the retrieval of cocaine-induced CPP. Among the identified candidate proteins, a downregulated 14-3-3 ζ protein was chosen and confirmed by Western immunoblotting. A polymer-mediated plasmid DNA delivery system was then used to overexpress 14-3-3 protein in mouse nucleus accumbens before the CPP retrieval tests. Overexpression of accumbal 14-3-3 ζ protein was found to diminish conditioned cue/context-mediated cocaine-induced CPP. In contrast, another isoform of 14-3-3 protein, 14-3-3 ϵ protein, did not affect conditioned cue/context-mediated cocaine-induced CPP. Overexpression of accumbal 14-3-3 ζ protein did not produce motor ac-

tivity-impairing effect or alter local dopamine metabolism. Moreover, overexpression of accumbal 14-3-3 ζ protein did not affect food-induced CPP. These results, taken together, indicated that overexpressed accumbal 14-3-3 ζ protein specifically decreased conditioned cue/context-mediated cocaine memory. Further understanding of the function of accumbal 14-3-3 ζ protein may shed light on the treatment of cocaine craving and relapse.

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Introduction

One of the most challenging problems for drug-dependence treatment is addicts' long-lasting vulnerability to drug craving during abstinence [1]. Aside from the drug itself, context and discrete cues associated with drug use are found to induce drug craving and conditioned emotional responses in abstinent addicts [2, 3]. Treatment of cocaine addiction frequently fails because of ineffective prevention of the conditioned cue/context-induced craving [4]. Likewise, accrued evidence demonstrates that the presence of drug-conditioned cues and context reinstates the drug taking/seeking behavior during withdrawal in many animal models [5–12]. There-

fore, it is of importance to understand the underlying mechanisms of drug-conditioned cue/context memory. Cocaine-induced conditioned place preference (CPP) serves as an excellent model for studying cocaine-conditioned cue/context memory in this regard. In this model, cocaine-conditioned cue/context induces animals' preference to the cocaine-associated place for a period of time even when cocaine is not provided any longer after conditioning. Previously, we demonstrated that newly synthesized proteins are involved in the consolidation, expression and reconsolidation of this cocaine-conditioned memory [13–15]. However, the roles of specific proteins in mediating the retrieval of this cocaine memory remain unexplored. In the present study, a proteomic approach, two-dimensional gel electrophoresis (2-DE) in conjunction with liquid chromatography mass spectrometry analysis was employed to identify the protein candidates associated with the retrieval of this cocaine memory. A polymer-mediated plasmid DNA delivery system was then used to express specific proteins to assess their roles in mediating the retrieval of such a cocaine memory.

It has been well established that cocaine may activate the mesolimbic dopamine (DA) system that normally mediates the reinforcing value of naturally occurring unconditioned stimuli. The nucleus accumbens (NAc) is one primary projection target of the mesolimbic DA system. Repeated cocaine injections are found to induce synaptic plasticity in the NAc [16, 17]. Cocaine-induced CPP training activates extracellular receptor kinase in the NAc, while intra-accumbal infusions of an extracellular receptor kinase inhibitor prevents the expression of cocaine-induced CPP [18]. Moreover, modulation of chromatin modification in the NAc facilitates cocaine-induced CPP extinction and prevents the reinstatement of this memory [19]. Most importantly, NAc is implicated in conditioned cue-elicited drug seeking behavior in many animal studies [20–23]. Taken together, these results all support the notion that NAc could be one of the memory storage sites of cocaine-induced CPP. We accordingly hypothesized that specific protein expression in the NAc may be involved in this cocaine-conditioned memory. Therefore, we decided to study the roles of accumbal protein candidates in mediating the retrieval of cocaine-induced CPP.

Materials and Methods

Animals

Male C57BL/6 mice were used at 8–9 weeks of age. Mice were housed in a facility located at National Cheng Kung University Laboratory Animal Center (Tainan, Taiwan). Mice had free access

to food (Purina Mouse Chow, Richmond, Ind., USA) and tap water throughout the experiments. The colony room was temperature- and humidity-controlled and maintained on a 12-hour light/dark cycle (lights on at 07:00). This study was performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All experimental procedures were approved by the local Animal Care Committee at National Cheng Kung University College of Medicine.

Cocaine-Induced CPP Training, Test and Cocaine-Priming Test

Pretest, cocaine-induced CPP training, test and cocaine priming test were all conducted in commercial chambers designed for mice (MedAssociates Inc., Georgia, Vt., USA) as described in our previous reports [13, 14]. Mice of the paired and unpaired groups were placed in the center of any randomly chosen chamber and their time spent in each compartment of the chamber was measured for unconditioned preference in a 15-min pretest on day 1. Mice spending <40% of the time in any one compartment or center were included in the study. For cocaine-induced CPP training (days 2–9), mice receiving an intraperitoneal injection of cocaine hydrochloride (5 mg/kg) or an equivalent volume of saline injection were immediately confined in one randomly assigned compartment of their pretest chambers for 30 min. Eight hours apart, these mice received a saline or cocaine hydrochloride (5 mg/kg) injection and were immediately confined in the other compartment for 30 min in the corresponding chambers. These procedures were repeated for another 7 consecutive days. On days 10, 17 and 25 around noon, mice were placed in the center of their corresponding chambers with guillotine doors open and started a 15-min preference test (i.e. test 1, 2 and 3) in a cocaine-free status. On day 24 around noon, mice receiving an injection of cocaine solution (2.5 mg/kg) were placed in the center of their respective chambers with guillotine doors open and started a 15-min cocaine-priming test. Durations (in seconds) for mice exploring each of the 3 compartments were recorded in tests 1, 2 and 3, and the cocaine-priming test. Cocaine-induced CPP magnitude was represented by subtracting the time spent in the saline-paired compartment from the time spent in the cocaine-paired compartment for each mouse.

2-DE and Candidate Protein Determination

In an attempt to exclude any obvious confounding caused by cocaine treatment per se, 2 groups of mice were used for candidate protein specification and identification experiment. One group (paired group) of mice received cocaine-place conditionings in a paired manner. The other group (unpaired group) of mice received cocaine injections and place conditionings in an unpaired way. That is, mice of the paired group receiving an intraperitoneal injection of cocaine hydrochloride (5 mg/kg) and an equivalent volume of saline injection were immediately confined in one randomly assigned compartment of their corresponding chambers for 30 min. In contrast, mice of the unpaired group received cocaine and vehicle injections in their home cages and were kept in their respective chambers for 30 min 2 h after injections. These procedures were repeated for another 7 consecutive days for paired and unpaired groups. Both paired and unpaired groups of mice were sacrificed 1 h after the cocaine-induced CPP test by rapid decapitation and their accumbal tissues were dissected. Since the paired group, not the unpaired group, was expected to

exhibit cocaine-induced CPP, differential expression of accumbal proteins between the paired and unpaired groups could be attributed to the retrieval of cocaine-induced CPP. Therefore, a 2-DE in conjunction with liquid chromatography mass spectrometry was employed to specify and identify protein candidates in accumbal samples from mice of the paired and unpaired groups. 2-DE was performed using isoelectric focusing as the first dimension (IPGphor, Amersham Biosciences, Uppsala, Sweden) using an immobilized pH gradient strip (IPG strip, 18 cm in length, linear pH 4–7) followed by the second dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Protein II xi cell, Bio-Rad, Calif., USA) [24]. The in-gel digestion, mass spectrometry analysis, and database search were performed as described in our previous report [24].

Mouse 14-3-3ζ and 14-3-3ε Plasmid DNA Construct

We utilized reverse transcription-polymerase chain reaction (RT-PCR) to construct the complete open reading frames (ORFs) of 14-3-3ζ and 14-3-3ε from mouse brain full-length cDNA library. In brief, the mouse 14-3-3s ORFs were amplified from male C57BL/6 mouse whole brain full-length cDNA library by RT-PCR using Pfu Turbo HotStart polymerase (Stratagene, La Jolla, Calif., USA). The 754 base-pair of 14-3-3ζ product was then cloned into pcDNA3.1/myc-His B⁺ (Invitrogen, La Jolla, Calif., USA) and the 787 base-pair of 14-3-3ε product was cloned into pcDNA3.1/myc-His A⁺ (Invitrogen) to obtain the pcDNA3.1-m14-3-3ζ-myc-His and pcDNA3.1-m14-3-3ε-myc-His constructs. These constructs were used for expression of the mouse 14-3-3ζ and 14-3-3ε proteins, respectively, in infected accumbal neurons.

Preparation of the PEI-Mediated Plasmid DNA Delivery System

Since nonviral vectors were proven to be low-toxic and long-lasting for *in vivo* gene transfer in adult mouse brain [25], a cationic polymer, polyethylenimine (PEI), was used as a nonviral plasmid DNA delivery vehicle. PEI, known to condense plasmid DNA into positively charged nanoparticles in order to interact with TManionic proteoglycans at the cell membrane, enabled plasmid DNA delivery into cytoplasm via endocytosis [26, 27]. In addition, PEI may act as a proton sponge to buffer the endosomal pH, thus protecting plasmid DNA from degradation [26, 27]. Since effective cell entry depends on the amount of cationic particles, a net cationic balance of mixing cationic PEI with anionic plasmid DNA was favored. The N/P ratio, the number of nitrogen residues of *in vivo*-jetPEITM (Polyplus Transfection, Illkirch, France) per DNA phosphate, is a measure of the ionic balance of the PEI and plasmid DNA mixture. Therefore, the N/P ratio was determined to be 6 (1 μg DNA was mixed with 0.12 μl *in vivo*-jetPEI) according to the manufacturer's instructions. For preparation of our PEI-mediated plasmid DNA delivery system, PEI (*in vivo*-jetPEI; Polyplus Transfection) was mixed with a plasmid encoding for pcDNA3.1-14-3-3ζ-myc-His, pcDNA3.1-14-3-3ε-myc-His (both were driven by a cytomegalovirus promoter) or a plasmid encoding for pcDNA3.1-myc-His (i.e. vector only).

Stereotaxic Surgery and Cannula Implantation

In an attempt to avoid anesthesia-associated interference with mouse performance, mice underwent stereotaxic surgery and 26-gauge guide cannula implantation (coordinates: anteroposterior +1.34 mm; lateral −0.70 mm; dorsoventral −4.50 mm; the dor-

soventral coordinate was measured from the skull) was done 1 week before starting the pretest. Clearance through the guide cannula was maintained with dummy cannulas. The infusion cannula, a 33-gauge dental needle, was inserted into the guide cannula and was lowered 2.0 mm below the guide cannula toward the center of the NAc. The infusion cannulas were mostly in or bordered on the NAc. Data from mice with any side of the infusion cannula position outside this range in histological checks were excluded for further analyses.

Behavioral Procedures

Although downregulation of accumbal 14-3-3ζ proteins was found to be associated with the expression of cocaine-induced CPP in the 2-DE experiment, whether accumbal 14-3-3ζ downregulation was involved in the acquisition and/or retrieval of cocaine-induced CPP remains unknown. Therefore, two behavioral procedures (procedures 1 and 2) were used to study the effect of accumbal 14-3-3ζ overexpression on cocaine-induced CPP. In both procedures, mice underwent three cocaine-free tests (tests 1, 2 and 3) following cocaine-induced CPP training. Intra-accumbal infusion of 14-3-3ζ/14-3-3ε plasmid DNA or vector was done 2 days before test 1 in procedure 1 in an attempt to determine the effect of accumbal 14-3-3ζ overexpression on the acquisition and retrieval of cocaine-induced CPP. Intra-accumbal infusion of 14-3-3ζ/14-3-3ε plasmid DNA or vector was done 2 days before test 2 in procedure 2 to specifically examine the effect of accumbal 14-3-3ζ overexpression on the retrieval of cocaine-induced CPP.

Locomotor Activity

In order to rule out the possibility that overexpression of 14-3-3ζ protein into the NAc could impair mouse motor ability, mouse locomotor activity (vertical rearing and ambulatory activity in combination) was monitored in a custom-made transparent Plexiglas chamber (41 × 41 × 30 cm) inside the Optovarimax (Columbus Instrument, Columbus, Ohio, USA). Three groups of mice were used. One group of mice received no intra-accumbal infusion and the remaining 2 groups received PEI/14-3-3ζ plasmid DNA and PEI/vector infusion, respectively. Mice were individually placed in the center of the chamber and allowed free navigation over a 30-min period (pretest). Forty-eight hours after intra-accumbal infusion of 14-3-3ζ plasmid DNA or plasmid vector, mouse locomotor activity was measured again (posttest). The vertical infrared beam break was used as an index for the vertical rearing numbers and the horizontal distance traveled was recorded as the ambulatory activity.

DA and 3,4-Dihydroxyphenylacetic Acid Determination

The DA content level in the NAc was used to test the hypothesis that 14-3-3ζ overexpression may alter local DA synthesis. Forty-eight hours after the 14-3-3ζ plasmid DNA infusion, mice were killed by rapid decapitation. The brain was then removed and placed on the dorsal surface on a glass dish sitting on crushed ice. The first coronal section was taken at the center of the olfactory tubercle. The second coronal section was made at the anterior border of the hypothalamus. An NAc sample was punched out (a circle around the anterior commissure) with a 1-ml pipetman tip cutting to an internal diameter of 1.2 mm from the slice between these two sections. Tissue samples were wrapped in tin foil and stored immediately in liquid nitrogen until assayed by HPLC

Fig. 1. Cocaine-induced CPP was established by a paired training schedule. These paired and unpaired mice were further used for the proteomic study. Paired mice received cocaine-induced CPP training (pairing cocaine/saline injection and compartment exploration), while unpaired mice received the same training procedure in an unpaired way (compartment exploration 2 h after cocaine/saline injections). Paired but not unpaired mice exhibit reliable cocaine-induced CPP in test 1. *** $p < 0.001$.

Fig. 2. A representative silver-stained 2-DE gel displays the protein spots obtained from the paired and unpaired mouse accumbal tissue samples 1 h after the test. Arrowheads point out the upregulated protein spots, while bold arrowheads point out the downregulated protein spots in the paired mouse accumbal tissue sample. Arabic number 879 represented the identified 14-3-3 protein spot.

with an LC-4C amperometric detector (BAS, West Lafayette, Ind., USA) to measure DA and its main metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC) [27]. Tissue was homogenized and centrifuged at 14,000 g for 20 min at 4°C. The supernatant was decanted, filtered and delivered through a high-pressure valve fitted with a 20- μ l loop onto a Phase-II ODS column (3 μ l, 100 $\times$ 3.2 mm), and oxidized with a +0.72-V potential between the glassy carbon electrode and the Ag/AgCl reference electrode. The

mobile phase consisted of 0.1 M sodium phosphate dibasic, 0.1 M citric acid, 5 mg EDTA, and 7% methanol delivered at a 0.6-ml/min flow rate.

Food-Induced CPP Training and Test

We decided to test whether intra-accumbal 14-3-3 ζ overexpression affected natural reward- (food) induced CPP. Pretest, food-induced CPP training and test were all conducted in com-

mercial chambers as aforementioned. Mouse unconditioned preference for the respective chamber was determined using a similar procedure and criterion as described in the cocaine-induced CPP pretest. Throughout the experiment, mice had access to water ad libitum. However, these mice were under food deprivation and their body weights were monitored as previously reported [28]. Bilateral cannula implantation was done a week before the beginning of the pretest. Three groups of mice ($n = 5$ for each group) were used. They all received 5-day food-induced CPP trainings. Each morning, mice were confined in the non-preferred compartment where 4 sucrose pellets [28] were provided in a tray for 15 min. Eight hours apart, mice were placed in the preferred compartment where an empty tray was available for 15 min. These procedures were repeated for another 4 consecutive days. In a 15-min preference test (test 1), mice were placed in the center of the chambers with guillotine doors open. No sucrose pellet was provided in the preference test. Food-induced CPP was represented by subtracting the time spent in food pellet-associated compartment from the time spent in no food pellet-associated compartment. Four hours following the preference test, 2 groups of mice received intra-accumbal 14-3-3 ζ plasmid DNA or plasmid vector infusion, while the remaining group did not receive any infusion. Two days after infusion, 3 groups of mice underwent another 15-min preference test (test 2).

Statistical Analysis

All results were indicated as the mean $\pm$ standard error of mean. Paired t tests were used to examine the establishment of cocaine-induced CPP and food-induced CPP. One-way (plasmid DNA vs. vector treatment) repeated measure ANOVAs (across various tests) were employed to test group differences in cocaine-induced CPP and food-induced CPP magnitude. ANOVAs were used to analyze group differences in locomotor activity, DA, DOPAC levels [29], and Western immunoblotting results. The levels of statistical significance were set at $p < 0.05$.

Results

Cocaine-Induced CPP Was Reliably Established in the Paired Group

In the cocaine-induced CPP test, paired t tests revealed that cocaine-induced CPP was reliably established in the paired group ($t_{34} = 5.203$, $p < 0.0001$, $n = 18$), but not in the unpaired group ($t_{36} = 0.5101$, $p = 0.6131$, $n = 19$; fig. 1). Therefore, differential expressions of accumbal proteins between paired and unpaired groups could be very likely attributed to the retrieval of such a cocaine memory.

Downregulated 14-3-3 ζ Protein in the NAc Was Identified and Confirmed in the Paired Group

One hour after the CPP test, mice were killed by rapid decapitation and their accumbal tissues were obtained. Six pairs of computer-generated images were

Fig. 3. Magnified images of the identified 14-3-3 gel spots in 6 matched (paired vs. unpaired) pairs of accumbal tissue samples were listed. 14-3-3 spot intensity in the paired group appears to be lower than that in the unpaired group. Arrows point out the 14-3-3 protein spots in both paired and unpaired groups.

Fig. 4. Western immunoblotting results confirmed downregulated 14-3-3 ζ protein in mouse nucleus accumbens of the paired group. **A** Paired but not unpaired mice exhibited reliable cocaine-induced CPP in test 1. **B** Western immunoblotting results demonstrate downregulated accumbal 14-3-3 ζ protein following the retrieval of cocaine-induced CPP. In contrast, 14-3-3 ϵ protein expression was not altered in the paired mouse nucleus accumbens following the retrieval of cocaine-induced CPP. * $p < 0.01$.

created for statistical analysis and each pair consisted of one image from the paired group and the other image from the unpaired group. In excess of 500 protein spots were detected on all of the silver-stained 2-DE gel images (fig. 2). Comparing these protein spots against the unpaired groups, five spots (dihydropyrimidinase-like 2, guanosine diphosphate dissociation inhibitor 1, ATP5b protein, thioredoxin-related protein, and an unnamed protein) were increased and six spots (14-3-3 ζ , t-complex protein 1, guanine nucleotide binding protein α o, vacuolar adenosine triphosphatase subunit D, phosphatidylinositol transfer protein α , and putative membrane-associated progesterone receptor component)

were decreased in intensity in the paired group (fig. 2). Among these proteins, only 14-3-3 protein (including two abundant isoforms, ϵ and ζ) was chosen for quantification primarily due to its highest sequence coverage score. Six pairs of amplified images for the identified 14-3-3 protein spots in the paired and unpaired groups are listed in figure 3. Another batch (paired and unpaired groups) of mice were used to confirm this 2-DE result. Not surprisingly, our training procedure established cocaine-induced CPP in the paired group but not in the unpaired group (fig. 4A). Western immunoblotting analyses indicated that the relative level (to actin) of 14-3-3 ζ protein was indeed decreased following the retrieval

Fig. 5. Mouse 14-3-3 ζ and 14-3-3 ϵ proteins were expressed in HEK293 cell line and mouse nucleus accumbens following plasmid DNA delivery. **A** The HEK293 cells were transfected with PEI and pcDNA3.1-14-3-3 ζ -myc-His plasmid DNA and pcDNA3.1-14-3-3 ϵ -myc-His plasmid DNA mixture at 80% confluence in 6-cm² cell culture dishes ($5.0 \times 10^5/60 \text{ mm}^2$) using Lipofectamine LTX reagent. Expressed myc proteins across time were quantified. Myc protein expression peaked at 48 h after PEI-mediated transfection in HEK293 cells. **B** Mouse accumbal 14-3-3 ζ RNA. **C** 14-3-3 ζ protein expression was measured following PEI-14-3-3 ζ plasmid DNA mixture infusion into mouse nucleus accumbens. 14-3-3 ζ protein expression was found to peak at 48 h after PEI-mediated transfection in accumbal tissue extracts.

al of cocaine-induced CPP in the paired group ($t_{10} = 3.379$, $p = 0.007$, $n = 6$), while the relative level of 14-3-3 ϵ protein was not altered after the retrieval of cocaine memory in the paired group ($t_{10} = 0.6107$, $p = 0.555$, $n = 6$; fig. 4B).

Mouse 14-3-3 ζ and 14-3-3 ϵ Constructs Were Delivered into the NAc Neurons

Since myc-tagged 14-3-3 ζ and 14-3-3 ϵ DNA constructs were designed, myc protein expression was used to indicate the magnitude of 14-3-3 ζ and 14-3-3 ϵ protein expression. We first assessed the latency of maximal ex-

Fig. 6. 14-3-3 ζ protein was successfully delivered in mouse nucleus accumbens. **A** Immunohistochemical methods were used to reveal the infusion site. Mice underwent cardiac perfusion with saline and 4% paraformaldehyde under sodium pentobarbital anesthesia. Their brains were removed, post-fixed and a 30- μ m coronal section slice was made within a range of Bregma +2.1 and Bregma +0.5. The mouse anti-myc antibody (1:500) was incubated with brain slice for 16 h at 4°C and incubated with HRP-conjugated goat

anti-mouse antibody (1:1,000; Jackson ImmunoResearch, West Grove, Pa., USA) at room temperature for 1 h, and then visualized using a DAB kit (Vector Laboratories, Burlingame, Calif., USA). 14-3-3 ζ -myc proteins were expressed in the nucleus accumbens. **B** Triangles represent cells with a myc protein-positive signal (brown color; refers to the online version only) in the nucleus accumbens.

pression for the 14-3-3 ζ and 14-3-3 ϵ construct transfection in HEK293 cells, human embryonic kidney cells. Myc protein expression was evident starting from 24 h and peaking at 48 h after a PEI-mediated 14-3-3 ζ and 14-3-3 ϵ construct transfection in HEK293 cells (fig. 5A). Then, a total of 1 μ l PEI and 14-3-3 ζ /14-3-3 ϵ plasmid DNA mixture (0.5 μ g plasmid DNA/ μ l mixture) was infused into each side of mouse NAc with a Hamilton syringe driven by a microdialysis pump (CMA/Microdialysis, Stockholm, Sweden) at a rate of 0.5 μ l/min. As PEI-14-3-3 ζ plasmid DNA complex was injected into mouse NAc, 14-3-3 ζ RNA expression was observed in accumbal tissue extracts (fig. 5B). 14-3-3 ζ protein expression was found to peak 48 h after PEI-mediated transfection in accumbal tissue extracts (fig. 5C). Likewise, 14-3-3 ϵ protein expression was noted to peak 48 h after PEI-mediated 14-3-3 ϵ plasmid DNA transfection in the NAc (data not shown). Based upon these results, 14-3-3 ζ and 14-3-3 ϵ constructs were delivered into NAc neurons 2 days before the cocaine-induced CPP test in the following behavioral experiments. Infusion sites for each animal were validated by immunohistochemical methods (fig. 6A). It was of importance to note that myc proteins were clearly expressed in the NAc (fig. 6B). Moreover, PEI and 14-3-3 ζ plasmid DNA complex entered a number of specified neurons (NeuN-positive cells) in the NAc (fig. 7).

Expression of Accumbal 14-3-3 ζ Protein Mitigated Conditioned Cue/Context-Mediated Cocaine Memory Retrieval in a Long-Lasting Manner

Two behavioral procedures were used to study the effect of accumbal 14-3-3 ζ overexpression on cocaine-induced CPP (fig. 8). After the cocaine-induced CPP training, mice underwent three cocaine-free tests (tests 1, 2 and 3). Intra-accumbal infusion of 14-3-3 ζ /14-3-3 ϵ plasmid DNA or vector was done 2 days before test 1 in procedure 1 and 2 days before test 2 in procedure 2 (fig. 8). Mice received a 15-min cocaine priming test 1 day before test 3 in both procedures 1 and 2. The cocaine-priming test in procedure 1 was used to specifically verify the acquisition of cocaine-induced CPP. All groups of mice exhibited reliable cocaine-induced CPP (procedure 1: $t_{42} = 4.09$, $p = 0.0002$ for the 14-3-3 ζ plasmid DNA-infused group, $n = 22$; $t_{38} = 11.49$, $p < 0.0001$ for the vector-infused group, $n = 20$; procedure 2: $t_{32} = 10.24$, $p < 0.0001$ for the 14-3-3 ζ plasmid DNA-infused group, $n = 17$; $t_{32} = 9.873$, $p < 0.0001$ for the vector-infused group, $n = 17$; fig. 9A, B). In procedure 1, a one-way repeated measure ANOVA revealed that the 14-3-3 ζ plasmid DNA-infused group had a lower CPP magnitude than that of the vector-infused group in tests 1, 2 and 3 ($F_{(1, 160)} = 80.64$, $p < 0.0001$; fig. 9A). Nonetheless, CPP magnitude was significantly increased in the cocaine-priming test for the 14-3-3 ζ

Fig. 7. 14-3-3 ζ protein was successfully expressed in accumbal neurons. Two days after intra-accumbal pcDNA3.1-14-3-3 ζ -myc-His plasmid DNA infusion, brain slices were obtained and incubated with rabbit anti-myc tag antibody (1:500; Abcam, Cambridge, UK) and NeuN antibody (1:500; Chemicon International, Temecula, Calif., USA) for double labeling purpose. **A** Bright field was detected via indirect immunofluorescence with anti-myc-tag antibodies, anti-rabbit secondary antibody (Alexa Fluor 488 con-

jugated goat anti-rabbit IgG). The pcDNA3.1-14-3-3 ζ -myc-His plasmid DNA was transfected into the accumbal region (green). **B** Double labeling (yellow) of myc-positive (green) and NeuN-positive (red) staining are shown in the nucleus accumbens. **C** A confocal laser scanning image demonstrates co-localization of myc-expressed cells and neurons (dictated by triangles). Colors refer to the online version only.

plasmid DNA-infused group ($F_{(3, 160)} = 5.745$, $p = 0.0009$; fig. 9A). In addition, there was an interactive effect of treatment and test on CPP magnitude ($F_{(3, 160)} = 4.292$, $p = 0.0061$; fig. 9A). In procedure 2, a one-way repeated measure ANOVA revealed that the 14-3-3 ζ plasmid DNA-infused group exhibited lower CPP magnitude as compared to vector-infused group in tests 2 and 3 ($F_{(1, 128)} = 24.91$, $p < 0.0001$; fig. 9B). The 14-3-3 ζ plasmid DNA-in-

fused group displayed comparable CPP magnitude as did the vector-infused group in the cocaine priming test ($F_{(3, 128)} = 11.78$, $p < 0.0001$; fig. 9B). Finally, there was an interactive effect of treatment and test on CPP magnitude ($F_{(3, 128)} = 5.825$, $p = 0.0009$; fig. 9B). Three groups of mice (14-3-3 ζ plasmid DNA-infused, vector-infused, and naïve control group (with cannula implantation but no infusion), $n = 4$ for each group) were used to test whether

Fig. 8. Two procedures were used for behavioral experiments. Mice were measured for their unconditioned preference in a 15-min free navigation pretest. Then an 8-day cocaine-induced CPP training procedure was employed. On days 10, 17 and 25 around noon, mice received a 15-min preference test (tests 1, 2 and 3) in a cocaine-free status. Intra-accumbal infusion of plasmid DNA or vector was done on day 8 for the procedure 1, while infusion was on day 15 for procedure 2. On day 24 around noon, mice received a 15-min cocaine-priming test.

accumbal 14-3-3 ζ plasmid DNA infusion impaired mouse locomotor activity. It was of importance to note that accumbal 14-3-3 ζ expression did not affect mouse locomotor activity 2 days after 14-3-3 ζ plasmid DNA infusion ($F_{(2,9)} = 0.0034$, $p = 0.99$ in the pretest; $F_{(2,9)} = 0.1241$, $p = 0.88$ in the posttest; fig. 9C). These results suggest that decreased CPP magnitude in procedure 1 or 2 was not due to the motor activity-impairing effect produced by accumbal 14-3-3 ζ protein expression. Since 14-3-3 ζ protein was documented to modulate tyrosine hydroxylase activity and DA synthesis in MN9D cells [28], accumbal DA and its primary metabolite, DOPAC, were assessed for the potential effect of 14-3-3 ζ plasmid DNA infusion on DA metabolism in mouse NAc. Three groups of mice (14-3-3 ζ plasmid DNA-infused group, $n = 5$; vector-infused group, $n = 8$; naïve control group, $n = 8$) were used. Two days after intra-accumbal infusion, mice were sacrificed and their accumbal tissues were dissected. The results show that accumbal DA and DOPAC contents are similar in 14-3-3 ζ plasmid DNA-, vector-infused and naïve control group ($F_{(2,18)} = 0.5933$, $p = 0.5624$ for DA content level; $F_{(2,18)} = 0.6414$, $p = 0.537$ for DOPAC content level; fig. 9D).

Accumbal 14-3-3 ϵ Protein Did Not Affect Conditioned Cue/Context-Mediated Cocaine Memory Retrieval

In an attempt to further specify the role of accumbal 14-3-3 ϵ protein in this cocaine memory, 14-3-3 ϵ plasmid DNA-infused mice were used to serve as negative controls. All groups of mice exhibited reliable cocaine-induced CPP (procedure 1: $t_{16} = 6.555$, $p < 0.0001$ for 14-3-3 ϵ plasmid DNA-infused group, $n = 9$; $t_{16} = 8.117$, $p < 0.0001$ for the vector-infused group, $n = 9$; procedure 2: $t_{24} = 8.98$,

$p < 0.0001$ for the 14-3-3 ϵ plasmid DNA-infused group, $n = 13$; $t_{22} = 10.81$, $p < 0.0001$ for the vector-treated group, $n = 12$; fig. 9E, F). In procedure 1, a one-way repeated measure ANOVA revealed that intra-accumbal infusion of 4-3-3 ϵ plasmid DNA did not affect CPP magnitude in tests 1, 2 and 3, or cocaine priming test ($F_{(1,64)} = 0.3148$, $p = 0.5767$; fig. 9E). Cocaine-induced CPP magnitude remained unaltered across tests in both 4-3-3 ϵ plasmid DNA- and vector-infused groups ($F_{(3,64)} = 0.4153$, $p = 0.7426$; fig. 9E). Moreover, there was no treatment and test interaction ($F_{(3,64)} = 0.0836$, $p = 0.9688$; fig. 9E). In procedure 2, a one-way repeated measure ANOVA revealed that intra-accumbal infusion of 14-3-3 ϵ plasmid DNA did not affect CPP magnitude ($F_{(1,92)} = 1.97$, $p = 0.1638$; fig. 9F). Cocaine-induced CPP magnitude was not altered across tests ($F_{(3,92)} = 0.0633$, $p = 0.9792$; fig. 9F). Finally, there was no treatment and test interaction on CPP magnitude ($F_{(3,92)} = 0.0597$, $p = 0.9808$; fig. 9F).

Expression of Accumbal 14-3-3 ζ Protein Did Not Affect Food-Induced CPP

Following the training procedure, food-induced CPP was reliably established in 3 groups of mice ($t_8 = 7.847$, $p < 0.0001$ for the 14-3-3 ζ plasmid DNA-infused group, $n = 5$; $t_8 = 7.837$, $p < 0.0001$ for the vector-infused group, $n = 5$; $t_8 = 6.132$, $p = 0.0003$ for the non-infused group, $n = 5$; fig. 10). Moreover, these 3 groups of mice exhibited comparable food-induced CPP magnitude in test 1 ($F_{(2,14)} = 0.3282$, $p = 0.7265$). Likewise, 3 groups of mice exhibited comparable food-induced CPP magnitude in test 2 approximately 48 h after the intra-accumbal infusion ($F_{(2,14)} = 1.56$, $p = 0.2499$).

Fig. 9. Over-expression of accumbal 14-3-3 ζ protein decreased conditioned cue-mediated cocaine memory retrieval. Over-expression of 14-3-3 ζ protein in the nucleus accumbens 2 days before the test 1 in procedure 1 (**A**) and 2 days before the test 2 in procedure 2 (**B**) decreased the following conditioned cue/context-mediated cocaine-induced CPP. However, over-expression of accumbal 14-3-3 ζ protein did not affect cocaine-mediated retrieval of cocaine-induced CPP in either procedure 1 (**A**) or procedure 2 (**B**). Over-expression of accumbal 14-3-3 ζ protein was done by

employing procedure 2. Two days following the expression of accumbal 14-3-3 ζ protein, mouse locomotor activity (**C**) and local DA metabolism (**D**) in the nucleus accumbens were not altered, suggesting dissociations of motor-impairing or DA-altering effect and expression of accumbal 14-3-3 ζ protein. In contrast, expression of 14-3-3 ϵ protein in the nucleus accumbens either 2 days before test 1 (**E**) or 2 days before test 2 (**F**) failed to affect conditioned cue/context- or cocaine-mediated, cocaine-induced CPP retrieval in procedure 1 or procedure 2. * $p < 0.05$.

Fig. 10. Over-expression of accumbal 14-3-3 ζ protein did not affect the retrieval of food-induced CPP. Three groups of mice ($n = 5$ for each group) all exhibited reliable food-induced CPP in test 1. Over-expression of 14-3-3 ζ protein in the nucleus accumbens did not alter mouse food-induced CPP performance in test 2.

Discussion

In this study, we found that mice exhibiting cocaine-induced CPP displayed downregulated 14-3-3 ζ protein in the NAc. In contrast, accumbal 14-3-3 ζ protein overexpression significantly decreased mouse CPP magnitude in CPP tests. Overexpression of 14-3-3 ζ protein in the NAc appeared to be negatively associated with the expression of the cocaine-induced CPP. Nonetheless, 14-3-3 ζ protein overexpression in the NAc did not decrease CPP magnitude in the cocaine priming test, suggesting a minor role of accumbal 14-3-3 ζ protein overexpression in modulating the storage of such a cocaine-conditioned memory. Although 14-3-3 ζ plasmid DNA-infused mice exhibited low cocaine-induced CPP magnitude in test 1 of procedure 1, their cocaine-induced CPP magnitude in a later cocaine-priming test was comparable with that of the vector-infused mice. Therefore, overexpression of 14-3-3 ζ protein in the NAc did not seem to affect the formation of cocaine-induced CPP. Therefore, we suggest that downregulated accumbal 14-3-3 ζ protein could be responsible for successful retrieval of cocaine-induced CPP. Most importantly, reversal of 14-3-3 ζ protein downregulation in the NAc was found to effectively decrease conditioned cue/context-mediated cocaine memory retrieval. The latter finding indicated that accumbal 14-3-3 ζ protein was a potential target for treating cocaine-conditioned

memory and thus memory-induced craving for cocaine.

Although overexpression of accumbal 14-3-3 ζ protein did not decrease cocaine-primed CPP retrieval, this treatment was effective in mitigating conditioned cue-mediated CPP retrieval after the cocaine-priming test. Moreover, overexpression of accumbal 14-3-3 ζ protein before the CPP tests reliably decreased subsequent conditioned cue-mediated CPP retrieval across a 1- to 2-week period. The implication of these results was that overexpression of 14-3-3 ζ protein in the NAc might decrease conditioned cue/context-mediated CPP retrieval for a period of time. Since addicts are vulnerable to drug memory-induced craving over an extended period of time after abstinence [1–3], it is worthwhile to develop a long-term treatment protocol against the drug-conditioned memory retrieval. Our results suggest that overexpression of 14-3-3 ζ protein in accumbal neurons exerted a long-lasting inhibitory effect on the retrieval of cocaine-conditioned memory. Therefore, using 14-3-3 ζ protein as a treatment target seemed to be a promising strategy in cocaine dependence therapy.

As a nonspecific effect associated with accumbal 14-3-3 ζ protein overexpression was assessed, we observed that accumbal 14-3-3 ζ protein overexpression did not affect mouse locomotor activity 2 days after intra-accumbal infusion. This finding ruled out the possibility that accumbal 14-3-3 ζ overexpression decreased cocaine-induced CPP magnitude by impairing mouse locomotor activity. Moreover, accumbal DA and DOPAC levels remained unaltered 2 days after the 14-3-3 ζ plasmid DNA or vector infusions, suggesting that PEI and 14-3-3 ζ plasmid DNA mixture or intra-accumbal infusion itself did not cause dopaminergic terminal deficits. A previous study documented that 14-3-3 ζ protein decreased tyrosine hydroxylase activity and subsequent DA synthesis in MN9D cells [30]. However, we here report that expression of accumbal 14-3-3 ζ protein did not affect DA and its primary metabolite, DOPAC, in the NAc. The discrepancy could arise from differences in cell types and 14-3-3 ζ expression magnitude. First, we demonstrated that a number of accumbal neurons were transfected with 14-3-3 ζ constructs. However, most of these accumbal neurons are characterized as GABAergic neurons [31]. Therefore, intra-accumbal infusion with 14-3-3 ζ constructs is not expected to decrease accumbal DA or DOPAC level in GABAergic neurons. Second, in this study we overexpressed 14-3-3 ζ protein in an attempt to reverse CPP-decreased 14-3-3 ζ protein. However, reversal of cocaine-induced CPP-decreased 14-3-3 ζ protein could be different

from overexpression of 14-3-3 ζ protein in another study [30]. Finally, 14-3-3 ζ overexpression in the NAc is less likely to produce a long-term impairing effect on accumbal function. So far many lines of evidence have implicated the NAc in the extinction and maintenance of cocaine-supported behavior [32]. If a long-term functional impairment is associated with intra-accumbal 14-3-3 ζ overexpression, it is reasonable to predict that accumbens-supported behavior should be affected. However, we found that such treatment did not affect CPP performance in a cocaine-priming condition or food-induced CPP performance.

In theory, cocaine-induced CPP can be retrieved by two means. Both presentation of an unconditioned stimulus (receiving a cocaine injection) and presentation of a conditioned stimulus (cocaine-conditioned cue/context) may provide the memory retrieval cues [33]. Although such a cocaine-conditioned memory is expected to be formed following a classical conditioning procedure, these two memory retrieval cues may be routed through different neural pathways and synapses. To date, inconsistent results between conditioned cue/context-induced and cocaine-induced memory retrieval are frequently explained by intact cocaine-conditioned memory storage but memory retrieval failure. However, another interpretation of these inconsistent results is that there are two independent sets of synapses formed following the cocaine-induced CPP training. Each set of synaptic changes is responsible for modulating unconditioned stimulus (cocaine) and conditioned stimulus- (cocaine-conditioned cue/context) induced memory retrieval (an approaching response for the cocaine-paired compartment in cocaine induced CPP). Using similar training protocols, for example, methamphetamine (1 mg/kg/conditioning) and cocaine (5 mg/kg/conditioning) produce similar CPP magnitudes in a drug-free test [34]. However, we report that single methamphetamine prim-

ing injection impairs the retrieval of methamphetamine-induced CPP, while a cocaine-priming injection does not affect, if not enhance, cocaine-induced CPP retrieval [34]. Therefore, it is reasonable to suspect that cocaine induces CPP retrieval via certain synaptic changes different from those induced by presentation of conditioned cue/context.

14-3-3 protein has been thought to function as homo- or heterodimers that bind with varieties of proteins. A previous report indicates that 14-3-3 protein may interact with varieties of proteins and subsequently affect the degradation of these proteins [35]. Lately, 14-3-3 protein is found to bind to Nedd4-2 protein and the formed complexes can modulate the ubiquitination of the epithelial sodium channel [36]. Protein ubiquitination is a critical step for 26S proteasome-mediated protein degradation. In fact, polyubiquitination and protein degradation in postsynaptic neurons are necessary to modulate the extinction of a retrieved fear memory [37]. Therefore, 14-3-3 ζ overexpression in the NAc is suspected to mitigate conditioned cue/context-mediated cocaine-induced CPP retrieval by facilitating formation of ubiquitinated proteins and/or proteasome-mediated protein degradation in the NAc. Here we provide a scenario to explain our primary finding that accumbal 14-3-3 ζ protein overexpression mitigated the retrieval of conditioned cue/context-mediated cocaine-induced CPP. We hypothesize that 14-3-3 ζ -enhanced protein degradation in the NAc could be responsible for the quick extinction of the conditioned cue/context-mediated cocaine memory in this study.

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