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Original Article

Effect of social interaction on learning and memory of morphine withdrawal mice

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Abstract: Drug abuse is characterized by its long term persistence which has physical and psychological dependence. Clinical treatment, psychological counselling and social intervention can help patients have a certain withdrawal from physical dependence, but psychological addiction remains a serious problem leading over 90% of relapse. Influence of social interaction on drug effects has a close relationship with psychological dependence. In order to study the enduring changes in long-term memory in brains of drug-withdrawal mice, we established a social interaction model to compared hippocampal and prefrontal cortex gene expression in drug abuse mice. Two groups of physical morphine-withdrawal mice were caged in morphine abuse mice and saline-treated mice for four weeks, respectively. Morris' water maze was used for the detection of learning and memory, magnetic resonance spectroscopy (MRS) was used for the integrity of neurons in the brains between two groups. Expression $GABA_A$ and PKC_α which mediated neurotransmission of GABA and glutamate in hippocampus and the prefrontal cortex were determined by immunohistochemistry and Western blot. The results showed that compared with morphine-withdrawal mice that social interacted with saline treated mice, the ones who lived with morphine abuse mice had a decreasing learning and memory ability and much more damaged neurons. Moreover, overexpression of mRNA and protein levels of $GABA_A$ and PKC_α reveals the upregulation of GABA and downregulation of glutamate in neurons, enhancing the learning and memory in morphine withdrawal mice to trigger the reward pathway in morphine addiction at first. However, over activation of GABA and glutamate inside or outside of neurons had a negative feedback in learning and memory function. In conclusion, drug-withdrawal mice that social interacted with drug abuse mice had an increasing memory caused by environmental stimulus, but the over simulations may lead to neuron damages and a drop in functions of learning and memory through the over expression of $GABA_A$ and PKC_α.

Keywords: Morphine, social interaction, $GABA_A$, PKC_α

Introduction

Opiate addiction is not only a chronic tolerance endangering people's physical and mental health, the mortality caused by drug abuse is very high even after cardiovascular diseases and malignant tumors in some countries. One significant characteristic of opiate addiction is the physical and psychological dependence [1-3]. Clinical treatment, psychological counselling and social intervention can help patients have a certain withdrawal from physical dependence, but psychological addiction is still a worldwide problem. In the field of neuroscience, brain function in addictive patient is altered in certain areas rather than nonaddicted brain, as the chronic administration of opiate is involved in regulation of gene expression of genes in receptor regulation, signaling and transcription [4-6]. "Reward pathway" which including the ventral midbrain, nucleus accumbens and prefrontal cortex in brain is the key in the development of addiction [7, 8]. Opiate inhibits the neural activity of GABA in midbrain ventral tegmental area to activate the DA neurons, therefore accelerates the release of dopamine in the nucleus accumbens to establish the reward circuits [9, 10]. Hippocampus, another region of brain, associates with the effects of reward pathway in long time drug abuse and may be one factor in psychological dependence [11, 12]. Psychological addition in drug abuse causes the relapse rate in detoxificated patients over 90% due to the long lasting memory of

"reward pathway". Therefore, how to withdrawal from psychological addiction and help patients return to normal life has been the key and difficulty in the study of drug withdrawal.

GABAergic neurons occupy about one fifth of the central nervous system neurons and play a decisive role in the process of brain development [13]. As a kind of important inhibitory neurons, some mental disorders are often occurred by the impairment of GABAergic neurons with the excitability [10]. In the mammalian brain development, $GABA_A$ receptor is widely distributed in hippocampus, prefrontal cortex and striatum, mediated with most of the GABAergic neuron activity [14, 15].

Protein kinase C (PKC) belongs to the multifunctional serine and threonine kinase, encoded by multi-gene families, molecular weight is about 67-83 KD [16]. It is widely distributed in a variety of tissues, organs and cells in mammals. PKC pathways play central roles in changes of neuronal physiology that involved in learning and cognition [17]. PKC_α is a kind of calcium dependent PKC sub-types, report shows that the over activation of it can inhibit the intake of glutamine by neurons to lead impairment of learning and memory [18, 19].

A number of animal studies showed that early psychologically harmful events can alter the normal development of brain, suggested the social environmental effects on brain function. Moreover, social context influenced the affective valence of drug abuse such as alcohol [20, 21]. The goal of this study was to find whether the social factor was involved in the psychological dependence in drug relapse by establishing a social interaction model. In this model, mice in the period of morphine withdrawal were performed, and the environmental stimuli was to live with groups of mice that had morphine administration and saline as control. The experiments examined the psychological response in learning and memory to social interaction and the possible mechanisms involved in changes of $GABA_A$ and PKC_α in prefrontal cortex and hippocampus, to provide a new aspect on drug addiction treatment.

Methods and materials

Animals

Male Bal B/C mice weighing 20-22 g was used in the study. Animals were housed in plastic cages with free access to food and water under certain conditions (12 h/12 h light-dark cycle and temperature of 22±2°C). Experimental procedures were approved by the Laboratorial Animal Care Committee of Sichuan University.

Animal procedure

40 mice were randomly divided into morphine abuse group (MA, n = 20) and morphine control group (MC, n = 20). Both MA and MC were exposed to consistent eight days of morphine injection. Morphine hydrochloride (Simopharm Group Sichuan Medicines Co., Ltd, Sichuan, China) dissolved in physiological saline was injected in constantly increasing doses according to Sukhotina (Day 1: 10 mg/kg; Day 2: 20 mg/kg; Day 3: 30 mg/kg till Day 8: 80 mg/kg) [22]. Physical morphine withdrawal was carried lasting for ten days since Day 9 in both groups. Another 40 mice were randomly divided intosocial interaction group (SI, n = 20) and social control group (SC, n = 20). SI and SC were treated with 50 mg/kg morphine and same amount of saline water from Day 19 to Day 46, respectively. On Day 19, MA started to live with SI (in ratio of 1:1) and MC with SC (in ratio of 1:1) for 4 weeks until Day 46.

Morris' water maze

The alternation of learning and memory in MA and MC mice was examined by Morris' water maze (Chengdu Technology & Market Co., China) according to the procedure on day 47 and finished on day 53. The apparatus was a circular pool (120 cm diameter) fulfilled with opaque water (24±1°C) added with milk. A circular transparent platform around 12 cm diameter was submerged 1 cm under the surface of water in the third quadrant for testing learning and memory. Each mouse was placing into the tank at the edge of each quadrant and the latency was recorded for searching and locating the platform. It would be guided to the platform and stayed for 10 s when 120 s of searching time had elapsed without locating. Three trials were conducted every day in seven consecutive days. Probe trial was taken when the platform was removed. Percentage of times spent in the 3rd quarter and times of swimming across the platform in 60 s were recorded. The activities were monitored by a camera 140 cm above the tank and analyzed by software (MT-200 MWM video tracking analyze system, Chengdu Technology & Market Co., China).

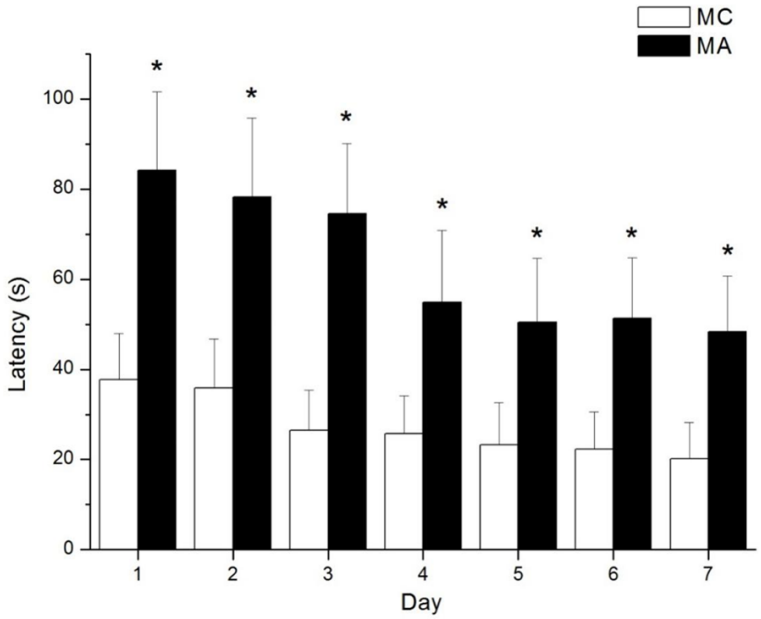

Figure 1. Time spend (latency) in acquisition experiment between MC and MA group in Morris' Water Maze. Note: Compared with MC mice, *P<0.05 in t-test.

Table 1. Probe trial results between MC and MA group in Morris' water maze ($\bar{x}\pm s$)

Groups	n	Time spent in Q3 (%)	Times across platform
MC	20	43.06±8.46	3.406±1.13
MA	20	28.48±9.21*	1.748±0.85*

Note: Compared with MC mice, *P<0.05 in t-test.

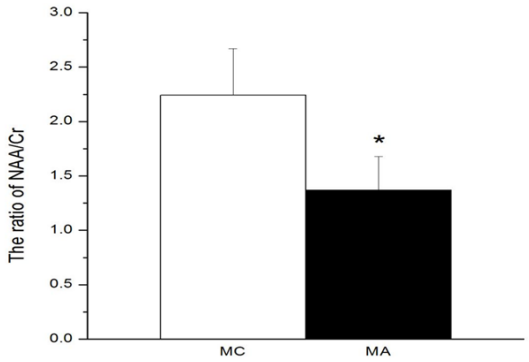

Figure 2. The ratio of NAA/Cr between MC and MA group in MRS experiment. Note: Compared with MC mice, *P<0.05 in t-test.

MRS examination

Proton Magnetic resonance spectroscopy (1H-MRS) was used to detect the integrity of neurons in the brains on Day 54. The experiment was taken at Nanchong Central Hospital using a 1.5 T Signa scanner (GE Medical systems, USA).

Immunohistochemistry

Tenmice in MA and MC group were used for immunohistochemistry. Mice were anaesthetized with 4% chloralic hydras (350 mg/kg), perfused with 0.1 mol/L phosphate-buffered saline (PBS, pH = 7.4) and then fixed with 4% paraformaldehyde (PFA) in 0.1 mol/L PBS. The brains were removed and stored overnight at 4°C. Subsequently, the tissues were given to College of Public Health (West China Center of Medical Sciences, Sichuan University) for immunohistochemistry. The antibodies used were rabbit anti-GABA antibody (Santa Cruz Biotechnology, Inc, USA, 1:400) and rabbit anti-PKC_{α} (Boster, Wuhan, China, 1:200), followed by the addition of goat anti-rabbit IgG secondary antibody (Boster, Wuhan, China, 1:200). Images were acquired from randomly four sections in different ratios (40×, 100×, 400×) and analyzed with Image Software (Nikon NIS-Elements D) by an observer blind to samples. Four grades were made according to the degree of expression as negative (-), lightly positive (+), positive (++), strongly positive (+++).

Brain collection and Western blot

Another Ten mice in each group were sacrificed and different parts of brains (the prefrontal cortex and hippocampus according to a mouse brain atlas) were dissected out immediately and frozen in liquid nitrogen for subsequent experiments. The dissected and frozen parts of brains (prefrontal cortex, hippocampus) in different mice groups were thawed and homogenized in RIPA lysis buffer (1 mmol/L PMSF) according to the manufactures' instruction (Beyotime Institute of Biotechnology, Jiangsu, China). Lysates were centrifuged at 14,000 g for 5 min, and the supernatant was collected. Protein concentrations were quantified with BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, China) at 562 nm. Sub-

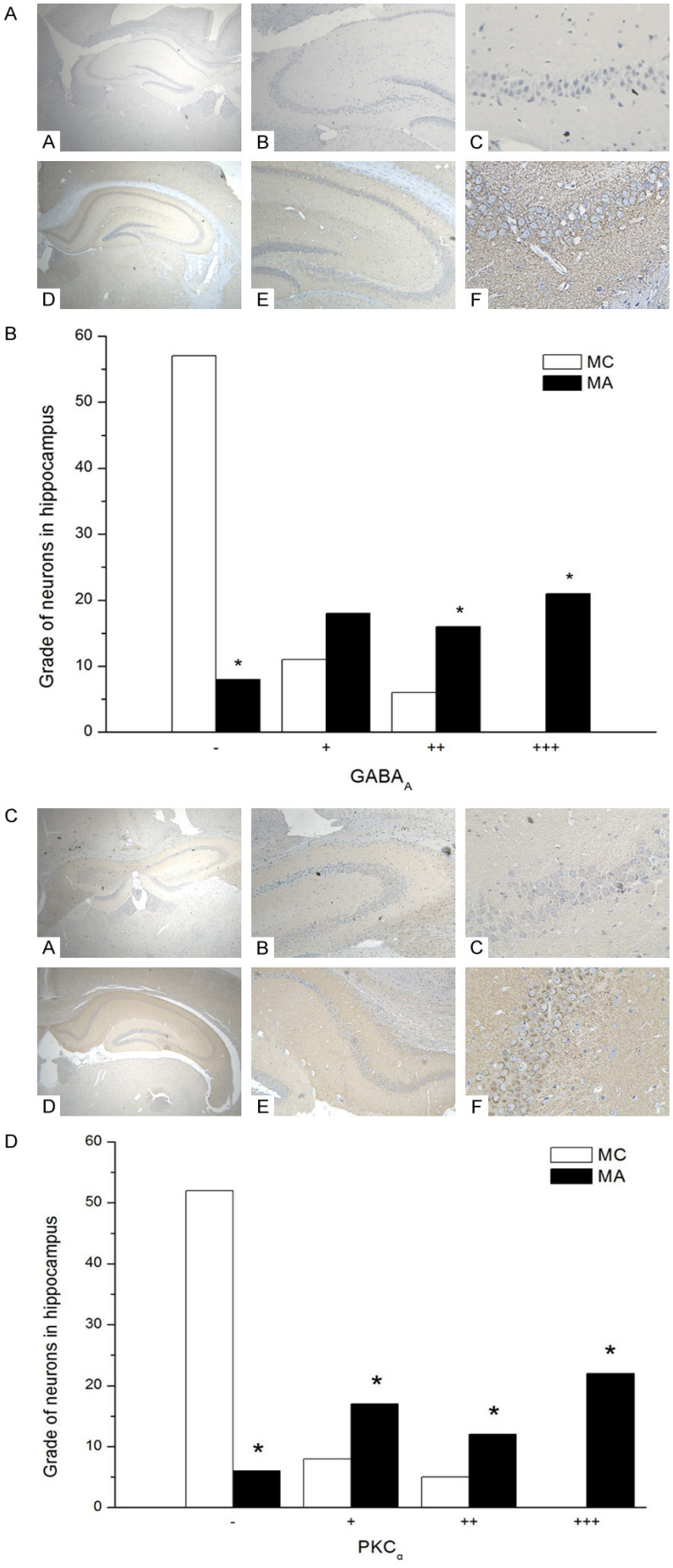

Figure 3. A: Immunohistochemical detection of $GABA_A$ in hippocampus. Representative photomicrographs of hippocampus from MC and MA mice with different multiple are shown: A = MC group (40×); B = MC group (100×); C = MC group (400×); D = MA group (40×); E = MA group (100×); F = MA group (400×). B. The expression of $GABA_A$ in hippocampus of mice between MC and MA mice. C. Immunohistochemical detection of PKC_α in hippocampus. Representative photomicrographs of hippocampus from MC and MA mice with different multiple are shown: A = MC group (40×); B = MC group(100×); C = MC group (400×); D = MA group (40×); E = MA group (100×); F = MA group (400×). D. The expression of PKC_α in hippocampus of mice between MC and MA group.

sequently, the protein samples were prepared by mixing with loading buffer (0.1 mol/L Tris-HCl, pH = 6.8; 2% mercaptoethanol; 2% SDS; 0.01% bromophenol blue and 10% glycerol) followed by denaturation at 95°C water for 5 min and cooled to room temperature. Each sample was separated by 15% SDS-PAGE electrophoresis using 150 V for 50 min. The durations of protein transferring to PVDF membrane at 200 mA were different ($GABA_A$-105 min; PKC_α-150 min) depended on the size of proteins. The membranes were blocked with 5% milk (TBST buffer: 10 mmol/L Tris-HCl, pH 7.5; 0.2 mol/L NaCl; 0.01% Tween) for 1 h at room temperature followed by incubation overnight at 4°C with primary antibodies. The dilutions of primary antibodies were: $GABA_A$ (1:1000) (Santa Cruz Biotechnology, Inc, USA); PKC_α (1:500) (Boster, Wuhan, China); β-actin (1:500) (Beijing Biosynthesis Biotechnology Co., LTD). The membranes were washed 3 times with TBST buffer before 1 h incubati-

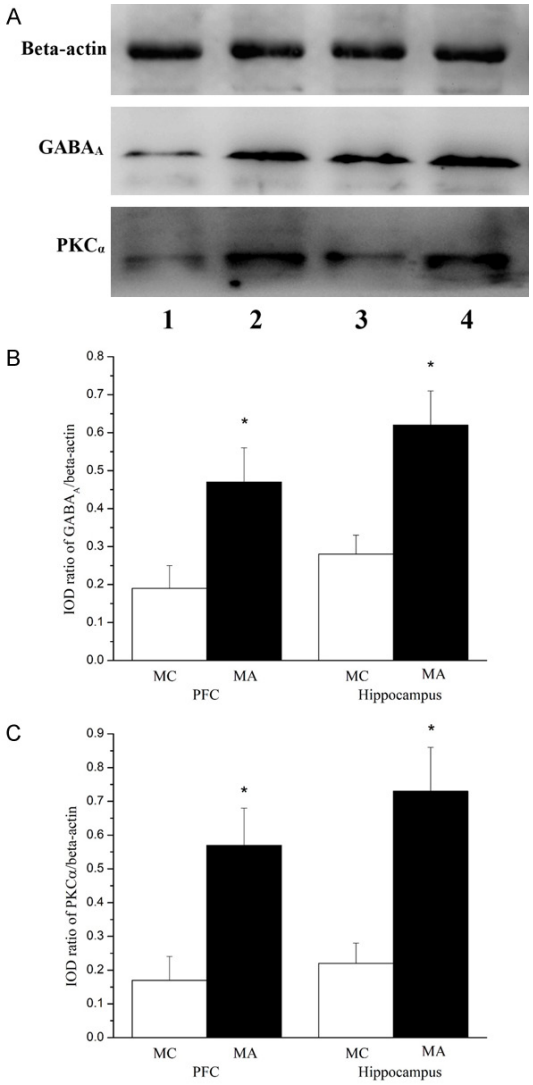

Figure 4. A. Electrophoresis of Western blotting products of beta-actin, $GABA_A$ and PKC_α in hippocampus and PFC between MC and MA mice. Templates are total protein isolated from brain of BAL/B/C mice. Lane 1-isolated from PFC of MC mice, Lane 2-isolated from PFC of MA mice, Lane 3-isolated from Hippocampus of MC mice, Lane 4-isolated from Hippocampus of MA mice. B. IOD ratio of western blotting products of $GABA_A$ and beta-actin in PFC between MC and MA mice. (*P<0.05). Note: Compared with MC mice, **P*<0.05 in t-test. C. IOD ratio of western blotting products of PKC_α and beta-actin in the PFC and hippocampus between MC and MA mice. (*P<0.05). Note: Compared with MC mice, **P*<0.05 in t-test.

on with conjugated goat anti-rabbit IgG secondary antibody (Boster, Wuhan, China; 1:20,000) and goat anti-rabbit β-actin (Beijing Biosynthesis Biotechnology Co., LTD; 1:20000), respectively. The blots were visualized by chemiluminescence using EasySee Western Blot kit (TransGen Biotech, Beijing, China) under X-ray and analyzed with Image Lab (Bio-Rad, USA).

Statistical analysis

Data was expressed as mean ± SEM and analyzed by T-test to find the differences between two groups (SPSS 16.0).

Results

MA mice had worse performance on learning and memory

In Morris' water maze, the results demonstrated that the ability of learning and memory of the mice could be altered by the different living environments. Compared with MC, MA had a significant worse performance on learning and memory (**Figure 1**; **Table 1**). The time spent in water maze searching for the platform in MA was much longer than that of MC at Morris' water maze experimental at Day 1 to Day 7 (P<0.05, n = 20). The integrity of neurons in brains between MA and MC showed a difference (**Figure 2**). In the MRS examination, NAA (n-acetyl-L-aspartic acid) is an amino acid located in neurons and its concentration correlates with neuronal mitochondrial function [23, 24]. The reduction of NAA concentration reflects decline of both neuronal density and integrity of neuronal mitochondria in several psychiatric disorders. Creatine is an important neurometabolite with dietary supplementation and used as a biomarker correlated with NAAin MRS. In the experiment, the ratio of NAA and Cr in MA was declined compared with that with MC, which had a more damaged in the integrity of neurons (P<0.05, n = 20) [25].

Expression of $GABA_A$ in MA mice was increased than MC mice

After living with drug-abuse mice, the level of $GABA_A$ in MA mice was significantly increased in both prefrontal cortex and hippocampus (**Figures 3A**, **3B**, **4A**, **4B**). In the immunohistochemistry experiment, the expression of hippocampal $GABA_A$ showed a much stronger expression in MA than MC (P<0.05, n = 10). According to the blind test, the MC mice had more negative expression (-) than MA, and less positive (++) and strongly positive (+++). In western blot, the ratio of $GABA_A$/beta-actin in hippocampus was higher in MA rather than MC (P<0.05, n =

10). The expression of was also $GABA_A$ in MA was also higher in prefrontal cortex ($P<0.05$, n = 10).

Expression of PKC_α in MA mice was increased than MC mice

The level of PKC_α protein in morphine withdrawal mice showed a significant decrease compared with morphine control group in prefrontal cortex and hippocampus (**Figures 3C**, **3D**, **4A**, **4C**). The expression of hippocampal PKC_α showed a much stronger expression in MA than MC in the immunohistochemistry experiment ($P< 0.05$, n = 10). According to the blind test, the MC mice had more negative expression (-) than MA, and much less slightly positive (+), positive (++) and strongly positive (+++). In western blot, the ratio of PKC_α/beta-actin in hippocampus was higher in MA rather than MC ($P<0.05$, n = 10). The expression of was also PKC_α in MA was also higher in prefrontal cortex ($P<0.05$, n = 10).

Discussion

Many studies have suggested that the regulation of gene expression was involved in morphine administration and biological mechanisms were specified in drug dependence for the treatment of drug addiction [26-28]. However, few studies have been carried the treatment after the drug withdrawal, which over 90% of patients would have drug relapse in the next six months. Social environment was considered when the patient returned in the society after treatment. One characteristic of opiate addiction is long term memory consolidation that lead a strong desire for drug [29]. In this study, we established an animal model that mice had physical withdrawal treatment after acute morphine addiction in eight days, followed by a social interaction with mice which had morphine administration every day. Whether this stimulation of surrounding environmental factor would alter the functions of brain, the memory system in hippocampus and the reward pathway were examined.

The ability of learning and the formation of a stable spatial memory was established in Morris' water maze, which is an experimental study of the conditioned reflex. Studies have shown that it was mainly involved in the regulation of neurons, neurotransmitters and receptors in regions of hippocampus, striatum, the basal forebrain and cerebellum. The modulation in coordination of spatial learning ability in brain regions leads to different searching latency. For example, the damage in hippocampus showed a longer time in exploring platform than control. In this study, result suggested that compared with the MC group, the learning and memory ability in MA mice had reduced after social interaction. This was different from that social interaction of morphine abuse mice stimulated the reward pathway of MA mice to sensation of morphine addiction. Reports have proved that even some exogenous non-drug dependence stimulus (such as food, sex) activated the midbrain dopamine reward pathway, but the relative amount of dopamine release was very small, compared with drug addiction, and short duration [30]. We suspect that the environmental interaction stimulates the reward system which modulated in morphine administration period. However, the four weeks repeated social stimulation may lead to a chronic adaptive changes in nervous system and cause impairment of learning and memory. Further experiment in MRS also support the more serious damage in neurons in MA mice rather than MC mice. NAA (n-acetyl-L-aspartic acid) which mainly exists in the brain neurons and axons, is a biochemical indicator of neuron injury severity [24]. A study shows the decline of NAA in ischemic brain tissue indicates a novel metabolism in neuron system [23]. Creatine (Cr) is often used as a reference in the MRS examination for its relative stable concentration in brain tissues [31, 32]. Therefore, the lower ratio of NAA and Cr in MA mice brains showed the relative neural functional damage compared with MC mice [25].

In our results, the morphine withdrawal and drug abuse social interaction caused an upregulation in the expression of $GABA_A$ protein in both prefrontal cortex and hippocampus. Type A receptor belongs to Cl^- ion channel receptor, mediating the inhibitory effect on synaptic transmission, which is highly involved in anxiety and depression [33, 34]. Dysfunction of GABA neurotransmission in PFC has been reported in mental disorders [14, 35, 36]. For example, the inhibition of GABAergic in PFC leads the decreased activity in depression. On the other hand, the GABAergic inhibition is over suppressed in anxiety [14]. Prefrontal cortex is an active region in reward pathway which is traditionally

considered as one of the main factors in drug addiction. Hippocampus plays an important role in drug addiction since the consolidation of long-term memory for drug seeking and taking. The overexpression of $GABA_A$ in prefrontal cortex and hippocampus observed by western blot illustrated the social interaction with drug abuse mice increased the level of $GABA_A$ to alter the activities of reward pathway indrug seeking.

Glutamic acid (Glu) has been proved in promotion of learning and memory function [37, 38]. Glu and NMDA receptors causing its Ca^{2+} channels open, results in an increases of Ca^{2+} concentration in the postsynaptic membrane, which in turn makes the membrane of the nature of the changes, cause long time history synaptic strengthening (long-term potentiation, LTP). LTP is an important indicator in learning and memory function at the synaptic level reflects the process of information storage, involved in the process of memory consolidation and the physiological activities of the neurons [11, 39]. Brain injection of Glu agonists also has the effect of memory enhancement [40]. Therefore, this experiment, PKC_α protein level in prefrontal cortex and hippocampus of MA mice were significantly increased, not only can prompt the inhibitory neurons on the absorption of Glu, lead to abnormal high concentration of Glu to form pathological LTP, therefore the disorder of learning and memory. Moreover, by combination of the expression of $GABA_A$, high ratio of Glu/GABA in MA mice showed that a decreased cognition in morphine withdrawal mice social interacted with morphine abuse mice.

Disclosure of conflict of interest

None.

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