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Original Article

Formation of aversive memories associated with conditioned drug withdrawal requires BDNF expression in the amygdala in acute morphine-dependent rats

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Aim: Brain-derived neurotrophic factor (BDNF) plays an important role in learning and memory in multiple brain areas. In the present study, we investigated the roles of BDNF in aversive memories associated with conditioned drug withdrawal in acute morphine-dependent rats.
Methods: Conditioned place aversion (CPA) was induced in male SD rats exposed to a single dose of morphine (10 mg/kg, sc) followed by naloxone (0.3 mg/kg, sc). In some rats, BDNF receptor antagonist K252a (8.5 ng per side) or BDNF scavenger TrkB-FC (0.65 μg per side) was bilaterally microinjected into amygdala before naloxone injection. BDNF mRNA and protein expression levels in amygdala were detected after the behavior testing.
Results: CPA behavior was induced in rats by the naloxone-precipitated morphine withdrawal, which was accompanied by significantly increased levels of BDNF mRNA and protein in the amygdala. Bilateral microinjection of TrkB-FC or K252a into the amygdala completely blocked CPA behavior in the rats.
Conclusion: Formation of aversive memories associated with conditioned drug withdrawal in acute morphine-dependent rats requires BDNF expression in the amygdala.

Keywords: conditioned place aversion; morphine; naloxone; aversive memory; amygdala; BDNF; TrkB-FC; K252a

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Introduction

Opiate addiction is a chronic and relapsing disorder characterized by compulsive drug consumption and has been shown to induce molecular alterations in different brain regions, particularly the mesolimbic dopamine system[1-5]. Opiate drugs not only elicit positive rewards in users (positive reinforcement) but also generate withdrawal symptoms such as aversive memories (negative reinforcement) and are critical for the induction of a motivational state that leads to drug-seeking behavior[6-8].

Conditioned place aversion (CPA) is a classic Pavlov conditioned reflex and the main model for the study of negative memories associated with drug withdrawal[9, 10]. In this model, trained animals associate an unconditioned stimulus (*ie*, aversive memories induced by withdrawal) with the apparatus context, resulting in a conditioned stimulus. When the animals are re-exposed to the apparatus, they exhibit significant aversive motivation. Therefore, the CPA model is a very sensitive method for measuring the aversive motivation in drug withdrawal[11-13].

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family of growth factors, regulates neuron synaptic plasticity and is an important mediator of the induction of LTP; thus, BDNF plays a vital role in learning and memory in multiple brain areas[14-18]. Previous work has demonstrated that BDNF and its receptor TrkB are involved in amygdala-dependent fear conditioning and prefrontal cortex-dependent extinction of conditioned fear[19, 20]. In addition, cocaine self-administration results in increases in BDNF expression in the medial prefrontal cortex[21] and the nucleus accumbens[22]. However, another study has found that exogenous BDNF infusions into the medial prefrontal cortex can suppress cocaine seeking in rats[23], which indicates that the BDNF signaling pathway not only participates in drug addiction but also plays different roles in different brain areas.

Our previous studies have revealed that the extinction of

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aversive memories associated with morphine withdrawal requires BDNF transcription in the rat ventromedial prefrontal cortex[24]. Because the amygdala is important for emotion-associated learning and memory, the aim of the present study was to explore whether BDNF expression in the rat amygdala was involved in the formation of aversive memories associated with conditioned morphine withdrawal.

Materials and methods

Animals

Male Sprague-Dawley rats weighing 250–280 g were obtained from the Animal Center of Fudan University (Shanghai, China). The rats were housed three per cage and maintained on a 12-h light/dark cycle with access to food and water *ad libitum*. All experimental procedures were conducted in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Conditioned place aversion

Apparatus

The CPA apparatus [55 cm (length)×30 cm (width)×30 cm (height)] was divided into two equal-sized compartments separated by a removable board (10 cm×10 cm) that allowed the rats to have free access to each compartment. The two compartments were distinguished by visual and tactile cues; one compartment had a black wall and smooth floor, whereas the other compartment had a white wall and textured floor. A camera was placed above the middle of the apparatus to record animal activity and transfer the recorded data to a computer.

Procedures

The CPA model was established in the following three phases: pre-conditioning, conditioning, and testing. In the pre-conditioning phase, the rats were allowed to freely explore the entire apparatus for 15 min, and the time spent in each compartment were recorded. This phase usually took 2 or 3 days, and we calculated the average time that the rats spent in each compartment. If the time that the rats spent in either compartment greater than 480 s, we considered this compartment to be the drug-paired compartment. The rats that exhibited strong unconditioned aversions (<180 s) toward either compartment were eliminated from the study. Conditioning occurred over the next 2 days. On the first day, the rats were injected with saline (1 mL/kg, sc) and then returned to their home cages. Four hours later, the rats were given saline again and then confined to the compartment opposite the drug-paired compartment for 30 min. On the second day, the rats were injected with either morphine (10 mg/kg, sc) or saline (1 mL/kg, sc) and then returned to their home cages. Four hours later, the rats were injected with either naloxone (0.3 mg/kg, sc) or saline and then confined to the drug-paired side for 30 min. Twenty-four hours after conditioning, each rat was allowed to freely explore the entire apparatus for 15 min, and the time spent in each compartment was recorded. The CPA score (aversion score) was calculated as the time spent in the drug-paired compartment during the testing phase minus the time spent in the drug-paired compartment during the pre-conditioning phase.

Surgery

The rats (weighing 280–300 g at the time of surgery) were anesthetized with sodium pentobarbital (55 mg/kg, ip), treated with atropine sulfate (0.2 mg/kg, ip) and placed in a stereotaxic apparatus (Narishige, Tokyo, Japan). The rats were implanted bilaterally with guide cannulae in the amygdala [anteroposterior (AP), -2.2 mm; mediolateral (ML), ±4.5 mm; dorsoventral (DV), -6.0 mm]. The cannulae were anchored to the skulls with stainless steel screws and dental cement. A stainless steel blocker was inserted into each cannula to ensure patency and prevent infection. The rats were allowed to recover from surgery and received norfloxacin injections for 3–5 days.

Preparation of protein samples

The rats were anesthetized with pentobarbital sodium and sacrificed by decapitation. Coronal brain sections (0.5 mm thick) were obtained using a rat brain slicer (Braintree Scientific Inc, Braintree, MA, USA). Both sides of each amygdala were punched from brain slices using a blunt-end, 17-gauge syringe needle (1-mm inner diameter). In all subsequent procedures, the tissues were maintained at 4 °C. The cytoplasm protein fraction was isolated as follows: briefly, the tissue was homogenized with a Teflon pestle in a glass homogenate tube with 250 μL of RIPA lysate. The homogenate was then transferred to a new 1.5 mL tube and centrifuged at 1000×*g* for 10 min. The supernatant was transferred to a new tube to which 4× loading buffer was added at 95 °C for 10 min, and the pellet was discarded.

Drugs and antibodies

Morphine hydrochloride was purchased from Qinghai Pharmaceutical General Factory (Xining, Qinghai, China). Sigma Aldrich (St Louis, MO, USA) supplied the naloxone hydrochloride.

The BDNF antibody was purchased from Santa Cruz (Cat No sc-546) and diluted to 1:500 for the Western blot analyses, and the actin-specific antibody was acquired from Sigma Aldrich (Cat No A5441) and diluted to 1:10 000 for the Western blot analyses.

Intracerebral microinjection

Each infusion volume was 0.5 μL per side, and the infusion rate was 0.25 μL/min. Bilateral microinfusions were performed through 31-gauge injection cannulae (2.0 mm beyond the tip of the guide cannulae) connected to 10-μL microsyringes mounted in a microinfusion pump (Harvard Apparatus, Cambridge, MA, USA). The drugs were infused into the amygdala over 2 min and allowed an additional 1 min for drug diffusion.

K252a (Sigma, Cat No 05288) was first dissolved in DMSO to a concentration of 25 μg/μL and subsequently diluted with PBS to a final concentration of 35.7 μmol/L (approximately 17 ng/μL). Ten minutes before naloxone pairing, K252a was

microinjected into the amygdala bilaterally.

TrkB-FC (Sigma, Cat No T8694) was dissolved in PBS to a final concentration of 1.3 μg/μL and was microinjected into the amygdala bilaterally 30 min before naloxone conditioning pairing.

The doses of K252a and TrkB-FC were chosen based on pilot experiments and previous studies[24, 25].

Real-time PCR

We first used RNeasy Plus Mini Kits (Cat No 74134, Qiagen, Dusseldorf, Germany) to extract the total RNA from the rat amygdala tissues (approximately 5 mg). The mRNA was then reversely transcribed into first strand cDNA by using an Omniscript RT kit (Qiagen, Cat No 205111). Finally, we adopted real-time PCR for 40 cycles to detect target gene mRNA levels (ComWin Biotech Cat No CW0596). All procedures were performed in accordance with the corresponding kit instructions.

The real-time PCR primers were designed as follows: BDNF, forward primer 5′-TCATACTTCGGTTGCATGAAGG-3′ and reverse primer 5′-AGACCTCTCGAACCTGCCC-3′; GAPDH, forward primer 5′-AACGACCCCTTCATTGAC-3′ and reverse primer 5′-TCCACGACATACTCAGCAC-3′. The BDNF mRNA levels of each well were normalized to GAPDH mRNA levels. Moreover, the specificity of the PCR was verified by melting curve and agarose gel analyses of the PCR products.

Nissl staining

After behavior testing, the rats were deeply anesthetized with sodium pentobarbital and perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde in phosphate-buffered saline (PBS). The brains were removed and stored in a 30% sucrose/PBS solution for 3 days. Coronal sections (30 μm thick) were cut on a cryostat (Leica) and stained with cresyl violet. Briefly, the brain slices were successively immersed in dimethylbenzene, 100% ethanol, 95% ethanol, 70% ethanol, cresyl violet, 70% ethanol, 95% ethanol, 100% ethanol and dimethylbenzene. The brain slices were immersed in each solution for 5 min. The brain slices were then dried and examined under a light microscope to identify the injection sites.

Data analysis

The data were analyzed with either two-tailed Student's *t* tests or a one-way ANOVA followed by Newman-Keuls *post hoc* tests when appropriate. Differences of $P<0.05$ were considered statistically significant. The results are presented as the mean±SEM.

Results

Conditioned place aversion was induced by conditioned naloxone-precipitated drug withdrawal in rats exposed to a single dose of morphine

We first established a stable rat CPA model. Previous studies have found that significant place aversion can be conditioned by naloxone-precipitated withdrawal after either acute or chronic morphine injections[26-28]. In our study, we used a training paradigm in which CPA was induced by naloxone (0.3 mg/kg, sc) 4 h after a single exposure to morphine (10 mg/kg, sc), and the rats were subsequently confined to the drug-paired compartment for 30 min. Consistent with previous studies, conditioned morphine withdrawal (CMW) produced significant aversion score compared with the saline-paired control group (aversion scores: Con, -38.67±58.39 s, n=8; CMW, -298.7±33.23 s, n=13) as shown in Figure 1. Two-tailed Student's *t*-tests showed that there was a significant difference between the two groups ($t_{(19)}$=4.186, $P<0.01$). Our previous studies have also found that rats pretreated with morphine and subsequently exposed to a single pairing with saline (mor/sal) and rats pretreated with saline and subsequently exposed to a single pairing with naloxone (sal/nal) do not exhibit significant place aversions compared with saline-saline paired control groups; these findings indicate that morphine or naloxone injections alone cannot induce CPA[10]. Thus, we explored the molecular mechanism underlying the aversive memories of acute drug withdrawal on the basis of this CPA paradigm.

Increases in BDNF mRNA and protein expression were detected in the rat amygdala in the CPA model.

BDNF is an important neurotrophic factor that is widely distributed in the central nervous system and regulates neuronal survival, differentiation and synaptic growth; thus, BDNF plays a vital role in diverse forms of learning and memory[18, 29, 30]. Recent studies have also found that BDNF is involved in the process of drug addiction but may play different roles in different brain regions[22, 23]. Therefore, we sought to explore whether BDNF participated in the formation of aversive memories associated with drug withdrawal.

After saline or naloxone pairing, the rats were decapitated at different time and the amygdala tissues were separated to extract either mRNA or protein samples as described in the Materials and methods. As shown in Figure 2A, the BDNF

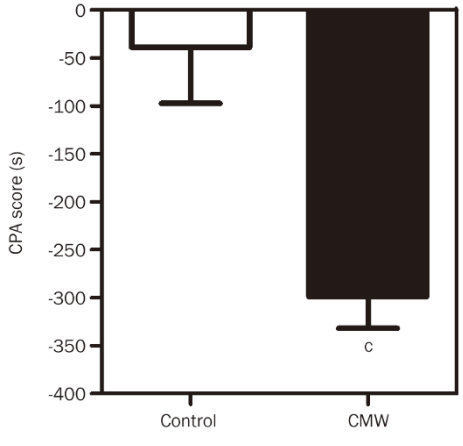

Figure 1. Place aversion was induced by conditioned naloxone-precipitated drug withdrawal in the rats exposed to a single dose of morphine. Mean±SEM. $^cP<0.01$ *vs* the saline-treated control group. Two-tailed Student's *t*-test. CMW: conditioned morphine withdrawal.

mRNA level increased after the formation of CPA behavior, and one-way ANOVA analysis indicated that there were significant differences between the saline-paired control group and the other groups (Con: 100%±15.37%, n=14; 0.5 h/CMW: 226.7%±48.25%, n=7; 1 h/CMW: 183.5%±29.96%, n=7; 0.5 h/UMW: 110.2%±25.88%, n=7; $F_{(3,31)}$=4.696, P=0.0081, Figure 2A). Subsequent Newman-Keuls *post hoc* tests revealed a significant difference between the control and CPA 0.5 h groups (P<0.05). However, we did not detect increases in the BDNF mRNA level in the unconditioned morphine withdrawal (UMW) rats (these rats were injected with only morphine and naloxone without pairing and were sacrificed 0.5 h after naloxone injection). These results indicated that the change in the BDNF mRNA level was associated with conditioned morphine withdrawal but not with unconditioned morphine withdrawal.

Next, we sought to test whether the level of BDNF protein also changed, using Western blotting. As expected, BDNF expression increased in the CPA 0.5 h group, peaked in the CPA 1 h group, and then decreased to baseline in the CPA 2 h group. The one-way ANOVA analysis revealed a significant difference between the control and CPA groups (Con: 100%±13.59%, n=5; 0.5 h/CMW: 143.6%±17.47%, n=6; 1 h/CMW: 171.2%±14.15%, n=10; 2 h/CMW: 114.9%±25.48%, n=6; $F_{(3,23)}$=3.29, P=0.0387, Figure 2B). Subsequent Newman-Keuls *post hoc* tests revealed that there was a significant difference between the control and CPA 1 h groups (P<0.05). These data revealed that BDNF is involved in the formation of aversive memories of conditioned drug withdrawal in the acute morphine-dependent rats.

CPA behavior can be inhibited by intra-amygdala micro-injection of the Trk inhibitor K252a

To validate the role of BDNF, we used the BDNF receptor TrkB inhibitor K252a, which is a tyrosine kinase inhibitor with a high affinity for all Trk receptors, to block BDNF signaling and to observe its effect on the formation of CPA behavior. Ten minutes before naloxone pairing, either K252a (8.5 ng/0.5 μL per side) or vehicle was micro-injected into the rats' amygdala. As shown in Figure 3A, K252a significantly blocked the formation of CPA behavior compared with the solvent-injected control group. Two-tailed Student's t-test analyses revealed that there was a significant difference between the vehicle and K252a groups (Figure 3A, aversion scores: Vehicle, -331.7±84.45 s, n=6; K252a, -111.8±26.81 s, n=6; $t_{(10)}$=2.482, P<0.05). Figure 3B illustrates the location of the microinjection tips in the rat amygdala in the present experiment. These data indicated that the BDNF signaling pathway indeed participates in aversive memory formation.

CPA behavior can also be inhibited by the intra-amygdala micro-injection of the BDNF scavenger TrkB-FC

Because K252a has a high affinity for all Trk receptors and is not specific for TrkB receptors, we also used the BDNF scavenger TrkB-FC to examine its effect on the formation of CPA behavior. Similarly, 30 min before naloxone conditioning pairing, either TrkB-FC (0.65 μg per side) or the corresponding solvent was injected into the amygdala; as expected, TrkB-FC

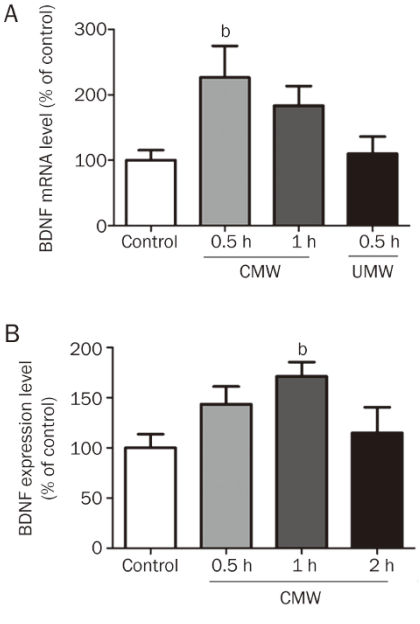

Figure 2. Increases in BDNF mRNA level and protein expression could be detected in rat amygdala of CPA model. (A) BDNF mRNA level increased at CPA 0.5 h in rat amygdala, while we did not detect any change of BDNF mRNA in unconditioned morphine withdrawal (UMW) rats. (B) BDNF expression increased at CPA 1 h and decreased to basal level at CPA 2 h. Values are expressed as mean±SEM. ^bP<0.05 compared with saline-paired control. One-way ANOVA with a Newman-Keuls *post hoc* test. CMW: conditioned morphine withdrawal; UMW, unconditioned morphine withdrawal.

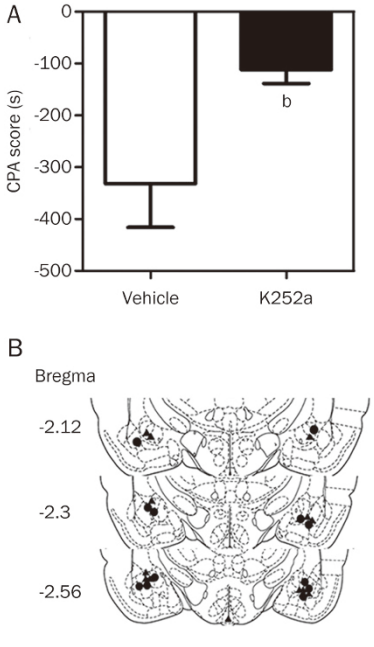

Figure 3. CPA behavior can be inhibited by intra-amygdala micro-injection of TrkB inhibitor K252a. (A) Intra-amygdala microinjection of K252a blocked the formation of CPA behavior. (B) Schematic representation of injection sites of rats used in the experiment. ▲Vehicle; ●K252a. Mean±SEM. ^bP<0.05 *vs* vehicle. Two-tailed Student's t-test.

pretreatment also blocked the formation of rat CPA behavior (Figure 4A, aversion scores: Vehicle, -311.2±53.14 s, n=8; TrkB-FC, -146.6±49.14 s, n=8; $t_{(14)}$=2.275, P<0.05). Figure 4B also illustrates the locations of the microinjection tips in the amygdala. Together, these results demonstrated that the BDNF signaling pathway is required for the formation of CPA behavior.

Histology

The injection sites for all of the rats used in the above experiments were examined with Nissl staining, and the results are illustrated in Figure 5. The animals were sacrificed after behavioral testing. There were no significant signs of damage in the amygdala of the rats in any of the three groups. All injection sites were located in the amygdala. The injection sites of the vehicle, k252a and TrkB-FC were essentially identical.

Discussion

Negative memories of drug withdrawal are critical for the induction of a motivational state that leads to drug seeking and consumption[8, 31, 32]. Conditioned place aversion is a very sensitive model for measuring the aversive motivation of drug withdrawal. Multiple lines of evidence have demonstrated that BDNF plays an important role in learning and memory in different brain regions[14, 15, 33]. Moreover, recent studies have found that the BDNF signaling pathway contributes to cocaine seeking[22, 23, 25, 34, 35]. Whether the BDNF signaling pathway participates in the formation of aversive memories associated with morphine withdrawal remains unknown.

In our present study, we found that the BDNF mRNA level in the rat amygdala significantly increased 0.5 h after conditioned morphine withdrawal, but we did not detect this change in the rats that underwent unconditioned morphine withdrawal; this finding indicated that the increase in the BDNF mRNA level is related to aversive memories associated with conditioned morphine withdrawal. In addition, we found that BDNF expression increased 1 h after conditioned morphine withdrawal and decreased to a basal level 2 h after naloxone pairing. These results suggested that BDNF in the rat amygdala plays an important role in aversive memories associated with conditioned morphine withdrawal. To further explore the role of BDNF, we used the BNDF receptor TrkB inhibitor K252a and the BDNF scavenger TrkB-FC to test their effects on the formation of CPA behaviors. The results demonstrated that intra-amygdala micro-injections of either K252a or TrkB-FC before naloxone pairing significantly blocked the formation of CPA behavior. Given the above results, our work provides the perspective that formation of aversive memories associated with conditioned morphine withdrawal requires BDNF signaling pathway in the rat amygdala.

However, the molecular mechanism by which BDNF elicits the formation of aversive memories associated with conditioned morphine withdrawal remains poorly understood. Our previous studies have revealed that actin rearrangement occurs in the amygdala and hippocampus during conditioned morphine withdrawal in rats[10]. Another study has demonstrated that BDNF regulates actin polymerization in the inferior temporal cortex of the monkey during the formation of visual pair-association memory[36]. Moreover, BDNF has been reported to regulate axonal and dendritic morphogenesis[16, 37]. Therefore, we suggest that the upregulation of BDNF expression within the amygdala in rats treated with conditioned morphine withdrawal may contribute to actin rearrangement to a large extent and thus induce the formation of aversive memo-

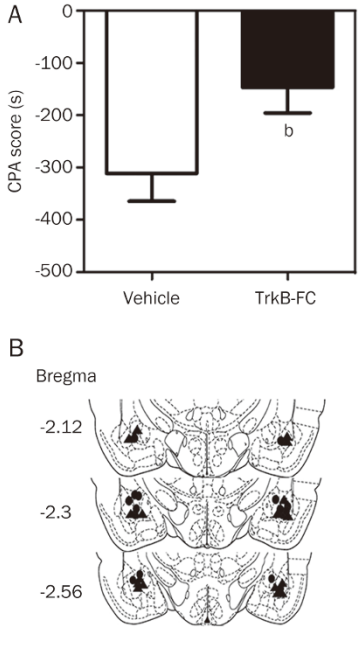

Figure 4. CPA behavior can also be inhibited by intra-amygdala microinjection of BDNF scavenger TrkB-FC. (A) Intra-amygdala microinjection of TrkB-FC blocked the formation of CPA behavior. (B) Schematic representation of injection sites of rats used in the experiment. ▲Vehicle; ●TrkB-FC. Mean±SEM. ^{b}P<0.05 *vs* vehicle. Two-tailed Student's *t*-test.

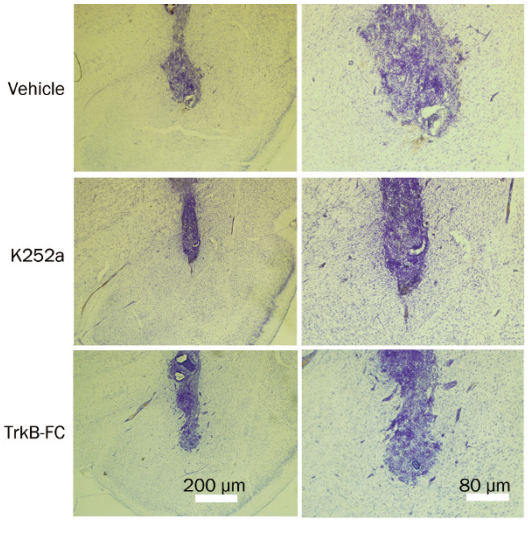

Figure 5. Nissl staining in the amygdala for all rats included in the study. All injection sites were located in the amygdala. Injection sites for the vehicle, k252a and TrkB-FC were basically the same.

ries. However, this hypothesis requires further verification.

The amygdala nucleus plays an important role in the processing of memory, particularly emotional memory[38, 39]. The amygdala consists of several nuclei, including the central amygdala (CeA), medial amygdala (MeA), and basolateral amygdala (BLA), which may play functionally distinct roles in amygdala-dependent behavioral tests[40, 41]. Evidence has shown that these nuclei indeed have different types of neuronal populations and different responses[42]. In our present study, we did not distinguish which sub-nuclei of the amygdala participated in the formation of aversive memories related to conditioned morphine withdrawal because our previous study had indicated that there were no obvious differences in the expression of some proteins among these nuclei. Therefore, we studied the amygdala as a whole. However, we have observed that intra-CeA or intra-BLA injections of a specific lentivirus may result in different behavioral outcomes. Thus, it is very important to study the specific functions of each of the nuclei of the amygdala in the formation of aversive memories. This issue will be the subject of our subsequent work.

Drug addiction-associated memories are quite different from normal memories. Although both forms of memory activate the BDNF signaling pathway, we still do not know why aversive memories associated with drug withdrawal last for such long periods or why these memories are difficult to extinguish. Recent studies have demonstrated that epigenetic regulation plays an important role in drug addiction[43-46]. Additionally, our previous study has revealed that the extinction of aversive memories of conditioned morphine withdrawal requires epigenetic regulation of BDNF gene transcription in the vmPFC, which is indicative of the important role of epigenetic regulation in aversive memory extinction[24]. Our preliminary results (data not shown) indicate that the BDNF signaling pathway may be regulated by DNA methylation. More work must be performed to address such questions; future research may help to understanding the persistence of aversive memories and provide new insights into the treatment of drug addiction and relapse.

Acknowledgements

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Author contribution

Jing-gen LIU, Yun-yue JU designed the research work; Yun-yun JU, Jian-dong LONG, and Yao LIU performed the experiments; Yun-yun JU analysed the data and wrote the manuscript; Jing-gen LIU revised the manuscript.

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