Experimental Neurology 311 (2019) 234–246

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Research Paper

Key determinants for morphine withdrawal conditioned context-induced increase in Arc expression in anterior cingulate cortex and withdrawal memory retrieval

Ming Chen[1], Da Shao[1], Yali Fu, Qianqian Ma, Ming Chen, Dongyang Cui, Jiaojiao Song, Huan Sheng, Li Yang, Yi Dong, Bin Lai*, Ping Zheng*

State Key Laboratory of Medical Neurobiology, Collaborative Innovation Center for Brain Science, School of Basic Medical Sciences, Institutes of Brain Science, Fudan University, Shanghai 200032, China

ARTICLE INFO

Keywords:
Addiction
Anterior cingulate cortex
Arc
Dendritic spine
CA3 projection neurons

ABSTRACT

Addiction memory is subjected to time-dependent shifts toward neocortex in remote period to promote long-term addiction memory storage, which plays an important role in drug relapse. However, how the activity and neuroplasticity of the anterior cingulate cortex (ACC) change after contextual morphine withdrawal conditioning and what factors determine this change remain to be determined. In this paper, using immunohistochemical and single-cell microinjection techniques, combining behavioral assay, we found that (1) contextual withdrawal conditioning increases the expression of c-Fos, but not Arc, in the ACC in morphine withdrawal mice; (2) at the first day after conditioning, conditioned context has no influence on the expression of c-Fos and Arc in the ACC in morphine withdrawal mice; (3) at the 14th day after conditioning, conditioned context increases the expression of both c-Fos and Arc in the ACC in morphine withdrawal mice; (4) the inhibition of dendritic spines of the ACC or projection neurons from the CA3 of the hippocampus to the ACC attenuates the conditioned context-induced increase of Arc expression in the ACC and abolishes the retrieval of withdrawal memory at the 14th day after conditioning. These results suggest that the ACC may exhibit a change in neuroplasticity at the 14th day after conditioning, and the dendritic spines of the ACC and the projection neurons from the CA3 of the hippocampus to the ACC are key determinants for conditioned context induced-increase in Arc expression in the ACC and the retrieval of withdrawal memory at the 14th day after conditioning.

1. Introduction

Drug addiction is a chronic brain disease characterized by remissions and relapses. Classical addiction theory posits that: (1) the development of dependence produces withdrawal symptoms that appear following discontinuation of drug use and (2) those withdrawal symptoms motivate drug use reinstatement (Piper, 2015). Moreover, withdrawal symptoms are transferred into long-term withdrawal memory, which can be retrieved when addicts are re-exposed to morphine withdrawal conditioned context, leading to relapse (Hyman et al., 2006). Therefore, the understanding of the process by which long-term withdrawal memory is formed in the brain represents a critical objective in the addiction field.

Previous studies examined the influence of morphine withdrawal conditioned context on the expression of c-Fos, a marker of neuronal activity (Fields et al., 1997), in a number of brain regions at the second day and 1 month after conditioning. Morgan Lucas et al. found that the conditioned context increased the expression of c-Fos in a number of brain regions, including the hippocampus, but had no influence on the expression of c-Fos in the ACC at the second day after conditioning (Lucas et al., 2012). However, at 1 month after conditioning, the conditioned context increased the expression of c-Fos in the ACC (Lucas et al., 2012). These results suggest that morphine withdrawal memory may undergo a shift from recent store regions, like the hippocampus, toward cortical areas, like the ACC. However, how the activity and neuroplasticity of the ACC change after contextual morphine withdrawal conditioning and what factors determine this change remain unknown.

In this paper, we used immunohistochemical and single-cell microinjection techniques, combining behavioral assay to study the

* Corresponding authors.
E-mail addresses: laibin@fudan.edu.cn (B. Lai), pzheng@shmu.edu.cn (P. Zheng).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.expneurol.2018.10.009
Received 6 June 2018; Received in revised form 17 October 2018; Accepted 17 October 2018
Available online 18 October 2018
0014-4886/

influence of morphine withdrawal conditioning on the expression of c-Fos, a marker of neuronal activity and Arc, a marker of neuronal plasticity (Plath et al., 2006), in the ACC and the influence of conditioned context on the expression of c-Fos and Arc in the ACC at the first day and the 14th day after conditioning. We further studied the role of dendritic spines of the ACC in the enhanced expression of Arc and related memory behavior at the 14th day after conditioning and explored the source of signals that induced neuronal plasticity change in the ACC by examining the influence of the ablation of the projection neurons from the CA3 of the hippocampus to the ACC after recent withdrawal memory formation on the expression of Arc in the ACC and related memory behavior at the 14th day after conditioning.

2. Materials and methods

2.1. Animals

Male adult (8–12 weeks) C57BL/6 J mice and male Sprague Dawley rats (220–250 g) were housed singly in a 12 h light/dark cycle in a temperature- and humidity-controlled environment with food and water freely available. All experimental procedures conformed to Fudan University as well as international guidelines on the ethical use of animals. All efforts were made to minimize animal suffering and reduce the number of animals used.

2.2. Stereotaxic surgery

Animals were anesthetized with ketamine and xylazine (160 mg/kg and 12 mg/kg body weight, respectively) before the stereotaxic surgery was performed. For the experiments of the inhibition of cortactin expression, lentiviruses carrying shRNA targeting mouse cortactin were purchased from shanghai GeneChem. The shRNA sequences are below: the cortactin (LV-Cttn-RNAi (30376-1)): 5′-TCCCAGAAAGACTACT CTA-3′; the non-targeting control (CON207): 5′-TTCTCCGAACGTGTC ACGT-3′ (Hering and Sheng, 2003). LV-Cttn-RNAi (30376-1) virus (1.0×10^9 TU/mL) was injected bilaterally into the ACC (AP, +0.6 mm; ML, ± 0.4 mm; DV, −2.0 mm) based on the atlas of Paxinos and Watson (2001) in a volume 0.5 μl for 10 min in male C57BL/6J mice. Stereotaxic injection needle was retained in place for an additional 10 min to optimize diffusion. LV-Cttn-RNAi (30376-1) virus was allowed to express for 14 days. For the experiment of the inactivation of the ACC by the intra-ACC injection of TTX, SD rats were used. The reason for the use of rats here was that the operation of embedding stainless-steel guide cannula into the ACC could result in a higher death rate in mice. Stainless-steel guide cannula was placed 1 mm above the ACC (AP, +0.2 mm; ML, ± 0.6 mm; DV, −2.6 mm) with a 21.8° lateral angle based on the atlas of Paxinos and Watson (2001) in male SD rats. Cannulas were secured to the skull with anchoring screws and dental cement. Stainless steel stylets were inserted into the cannulae to prevent the occlusion. All rats were allowed to recover for 1 week after the surgery before conditioned place aversion (CPA) testing. Injection needle inserted into cannula was connected to a 1 μl microsyringe (Hamilton) by polyethylene tube and controlled by a syringe pump (Harvard Apparatus). TTX(0.5 ng/0.5 μl)or 0.9% saline was injected bilaterally into the ACC in a volume 0.5 μl for 1 min at 30 min before post-conditioning test. Injection needle was retained in place for an additional 10 min to optimize diffusion. The number of excluded missed-site stereotaxic placement animals does not exceed 10% of the sample.

2.3. Diphtheria toxin (DT) receptor (DTR)-mediated ablation of projection neurons from the CA3 of the hippocampus to the ACC

The 8-week-old C57/BL6 male mice were anesthetized with ketamine and xylazine (160 mg/kg and 12 mg/kg body weight, respectively), and placed in stereotaxic instruments (Stoelting, Wood Dale, USA). Each side of the hippocampal CA3 (AP: −1.7 mm; ML: ± 2.1 mm; DV: −1.85 mm) was injected with 0.5 μl AAV double-floxed inverted open reading frame (DIO)-diphtheria toxin receptor (DTR)-mCherry (2.25×10^{12} vector genomes/ml, Neuron Biotech Company, China) for 10 min followed by an additional 10 min to allow the diffusion of virus. Each side of the ACC (AP: +0.6 mm; ML: ± 0.4 mm; DV: −2.0 mm) was injected with 0.5 μl AAV-hSyn-mCherry-IRES-WGA-Cre (2.05×10^{12} vector genomes/ml, Neuron Biotech Company, China). The virus was allowed to express for a minimum of 4 weeks in order to allow sufficient accumulation in the hippocampal CA3. After the 1 d post-test, diphtheria toxin (DT, 50 μg/kg) was intraperitoneally administrated to ablate the projection neurons from the hippocampal CA3 to the ACC.

2.4. Clozapine-n-oxide (CNO)-mediated inhibition of projection neurons from the CA3 of the hippocampus to the ACC

The 8-week-old C57/BL6 male mice were anesthetized with ketamine and xylazine (160 mg/kg and 12 mg/kg body weight, respectively), and placed in stereotaxic instruments (Stoelting, Wood Dale, USA). Each side of the hippocampal CA3 (AP: −1.7 mm; ML, ± 2.1 mm; DV, −1.85 mm) was injected with 0.5 μl AAV double-floxed inverted open reading frame (DIO)-Gi-coupled DREADD hM4D (Gi)-mCherry (3.44×10^{12} vector genomes/ml, Neuron Biotech Company, China) for 10 min followed by an additional 10 min to allow the diffusion of virus. Each side of the ACC (AP: +0.6 mm; ML, ± 0.4 mm; DV, −2.0 mm) was injected with 0.5 μl AAV-hSyn-mCherry-IRES-WGA-Cre (4.28×10^{12} vector genomes/ml, Neuron Biotech Company, China). The virus was allowed to express for a minimum of 4 weeks in order to allow sufficient accumulation in the hippocampal CA3. After the 1 d post-test, mice accessed to CNO-treated water instead of regular drinking water to inhibit the projection neurons from the hippocampal CA3 to the ACC. CNO was dissolved in animals' regular drinking water at a concentration of 0.25 mg/ml (Carvalho Poyraz et al., 2016). Mice had this CNO solution as their only source of drinking water for 13 days. CNO in the drinking water was freshly prepared every day.

2.5. Chronic morphine treatment

Male C57BL/6J mice (8–12 weeks) and male Sprague Dawley rats (220–250 g) were treated with morphine according to procedures described previously (Desjardins et al., 2008). Briefly, morphine dependence was induced in animals by repeated intraperitoneal (i.p.) injections of morphine twice daily at 8:00 A.M. and 7:00 P.M. Morphine dose was progressively increased from 10 mg/kg to 40 mg/kg: day 1, 2 × 10 mg/kg; day 2, 2 × 20 mg/kg; day 3, 2 × 30 mg/kg; days 4 and 5, 2 × 40 mg/kg. Control animals were treated with saline following the same procedure.

2.6. Conditioned place aversion (CPA)

The procedure for CPA was similar to that described previously (Li et al., 2009, 2011; Valverde et al., 1996). CPA took place in a three-compartment place conditioning apparatus (Med Associates) with distinct visual and tactile context, which are readily discriminated by mice. In the preconditioning phase (pre-test), mice were placed in the central neutral area of the apparatus for 2 min and then allowed to freely explore the apparatus for 15 min. Mice showing strong unconditioned aversion or preference for any compartment were discard (i.e., < 20% and ≥80% of the session time, the number of excluded animals does not exceed 10% of the sample). All mice that had no biased nature for the compartment after the criteria of assessment were randomly divided into five groups: saline, naloxone, morphine, withdrawal and conditioning (Ciccarelli et al., 2013; Frenois et al., 2005). On days 2–6, mice were treated with morphine (i.p., for morphine,

withdrawal and conditioning groups) or saline (i.p., for saline and naloxone groups) for 5 consecutive days as described above. During the conditioning phase, mice in conditioning group received a naloxone injection (precipitate morphine withdrawal) before being placed in the minor preference compartment on days 7 and 9 after a morphine injection, and on alternate days (8 and 10), they received a saline injection before being placed in the opposite compartment after a morphine injection. To isolate responses induced by contextual stimuli, mice in withdrawal group received a naloxone injection on days 7 and 9 after a morphine injection in home cage, and on alternate days (8 and 10), they received a saline injection after a morphine injection in home cage. Mice in saline group received only saline injection (in the minor preference compartment and opposite compartment). Mice in naloxone group received a naloxone injection before being placed in the minor preference compartment on days 7 and 9 after a saline injection, and on alternate days (8 and 10) they received a saline injection before being placed in the opposite compartment after a saline injection. Mice in morphine group received a saline injection before being placed in the minor preference compartment on days 7 and 9 after a morphine injection, and on alternate days (8 and 10), they received a saline injection before being placed in the opposite compartment after a morphine injection. On day 11 (1 d Retrieval) or on day 24 (14 d Retrieval), each mouse was placed in the same apparatus for 15 min to assess place aversion response (Fig. S1). CPA score was defined as the time in the minor preference compartment minus time in the opposite compartment. Since the withdrawal/aversion conditioning was performed in the minor preference compartment, the opposite compartment became more preferred compartment at retrieval after conditioning in the conditioned withdrawal group. So the CPA score was positive in the pre-test session, but was negative at retrieval in the conditioned withdrawal group.

2.7. Immunohistochemistry and imaging

After experiments, animals were anesthetized with ketamine and xylazine (160 mg/kg and 12 mg/kg body weight, respectively) and perfused with 0.9% saline followed by ice-cold solution of 4% paraformaldehyde in phosphate buffer (PFA, pH 7.4). The brains were rapidly removed and post-fixed in 4% PFA at 4 °C for 12–18 h. The brains were cut in 40 μm coronal sections using a vibratome (Leica) and collected in 0.01 M PBS. The range from Bregma that ACC slices were derived from was +0.14 ~ +1.10. Free-floating slices containing the ACC were rinsed three times in 0.01 M PBS. Subsequently, slices were incubated with blocking solution containing 10% normal goat serum and 0.3% Triton X-100 in 0.01 M PBS for 2 h at 37 °C. Slices containing the ACC were incubated with rabbit anti-c-Fos antibody (1:1000) or rabbit anti-Arc/Arg 3.1 antibody (1:1000) overnight at 4 °C. Slices were washed with 0.01 M PBS three times and incubated with biotinylated goat anti-rabbit antibody (1:200) for 1 h at 37 °C followed by Cyanine 3-conjugated streptavidin (1:1000) or 488-conjugated streptavidin (1:500) for 1 h at 37 °C. Finally, immuno-labeled slices were rinsed three times in 0.01 M PBS, and mounted on glass slides using aqua-mount mounting medium. All antibodies were dissolved in 0.01 M PBS with 10% normal goat serum and 0.3% Triton X-100.

A series of slices containing the ACC were imaged by confocal microscopy (Nikon AIR-MP) with a 20×-immersion lens and collected at a resolution of 1024 × 1024 pixels. The same laser and scanning settings were used for all confocal images within an experiment to allow for comparison across groups. Generally, coronal sections from 5 to 7 mice were used for quantitative analysis. The Arc or c-Fos positive neurons were counted in Image J software using the cell counter function. Before cell count, the boundary of ACC was drawn on the picture. All the positive neurons within the boundary were counted by different researchers and the average number of positive neurons was used to statistical analysis. The data of 5–8 slices belong to a mouse were averaged for a final value.

2.8. Western blotting

The brain tissues containing the ACC region were homogenized in a buffer containing 100 mM Tris-HCl (pH 6.7), 1% SDS, 143 mM 2-mercaptoethanol and 1% protease inhibitor. The lysate was centrifuged at 12,000 rpm for 10 min at 4 °C. The samples were treated with the SDS sample buffer at 95 °C for 5 min, loaded on a 10% SDS polyacrylamide gel and blotted to a PVDF membrane. The PVDF membrane was incubated with a rabbit anti-cortactin antibody (1:100) at 4 °C for 8 h. The membrane was washed and incubated with IRDye 800CW goat anti-rabbit secondary antibodies (1:20,000) at 37 °C for 1 h and images were acquired using a LI-COR Odyssey system.

2.9. Dendritic spine analysis

Mice were anesthetized with ketamine and xylazine (160 mg/kg and 12 mg/kg body weight, respectively) and perfused transcardially with a 4% paraformaldehyde solution containing 0.125% glutaraldehyde (pH 7.4). Brains were then removed and post-fixed for 2 h in 4% paraformaldehyde. The brains were cut in 200 μm coronal sections using a vibratome (Leica) and collected in 0.01 M PB. Coronal sections were mounted into an injection chamber, and placed on the fixed stage of an Olympus microscope equipped with a micromanipulator. The neurons in layers II/III of the ACC (Aceti et al., 2015) were loaded iontophoretically with a 5% Lucifer Yellow solution (Sigma Aldrich, St Louis, MO), using sharp micropipettes with a negative current of 3000 nA (Axon Axopatch 200B, Molecular Devices, US). Loading time per cell was 3–4 min, and 5–6 cells were injected per slice. Three slices per mouse were injected with Lucifer yellow. Images of basal and apical dendrites of pyramidal neurons in the ACC region were obtained with a Leica SP2 confocal microscope at 63× under oil immersion (NA, 1.4). To avoid interference from dendrites of other neurons, only dendritic segments that were spatially isolated from the nearest dendrites were analyzed. Spine detection and measurement were performed semi-automatically by the NeuronStudio software (Dumitriu et al., 2011; Sigler et al., 2017). Once the starting points of dendritic tracing were manually determined, dendritic shafts were automatically detected. Then, the individual spines were automatically detected. Erroneous detection, such as short dendritic branches, optical noise, and very closely neighboring spines that were not automatically separable with NeuronStudio, were manually corrected by an experimenter blind to behavioral conditions. Then, the diameter of spine head and neck and the length of spines and dendritic shafts were measured with NeuronStudio. The number of spines per micrometer along the dendritic longitudinal axis was defined as the spine density.

2.10. Drugs and materials

Lucifer yellow, sodium dodecyl sulfate (SDS), Triton X-100, Naloxone and 0.01 M PBS were purchased from Sigma, USA. Morphine was purchased from Shenyang No.1 Pharmaceutical Factory, China. TTX was made in the Research Institute of Aquatic Products of Hebei, China. Rabbit anti-c-Fos antibody (#5348) was purchased from Cell Signaling Technology, USA. Rabbit anti-Arc/Arg 3.1 (I56 003) primary antibody was purchased from Synaptic Systems, Germany. Goat serum and Alexa Fluor 594-conjugated goat anti-rabbit antibody were purchased from Jackson Immuno Research Laboratory, USA. Biotinylated anti-rabbit secondary antibody was purchased from Vector Laboratories, USA. Virus was purchased from Neuron Biotech Company, Shanghai, China. Aqua-mount mounting medium was purchased from Thermo Fisher Scientific.

2.11. Off-line data analysis

Statistical analyses for all data were performed with Graphpad Prism 6. Numerical data were expressed as the mean ± s.e.m. In all

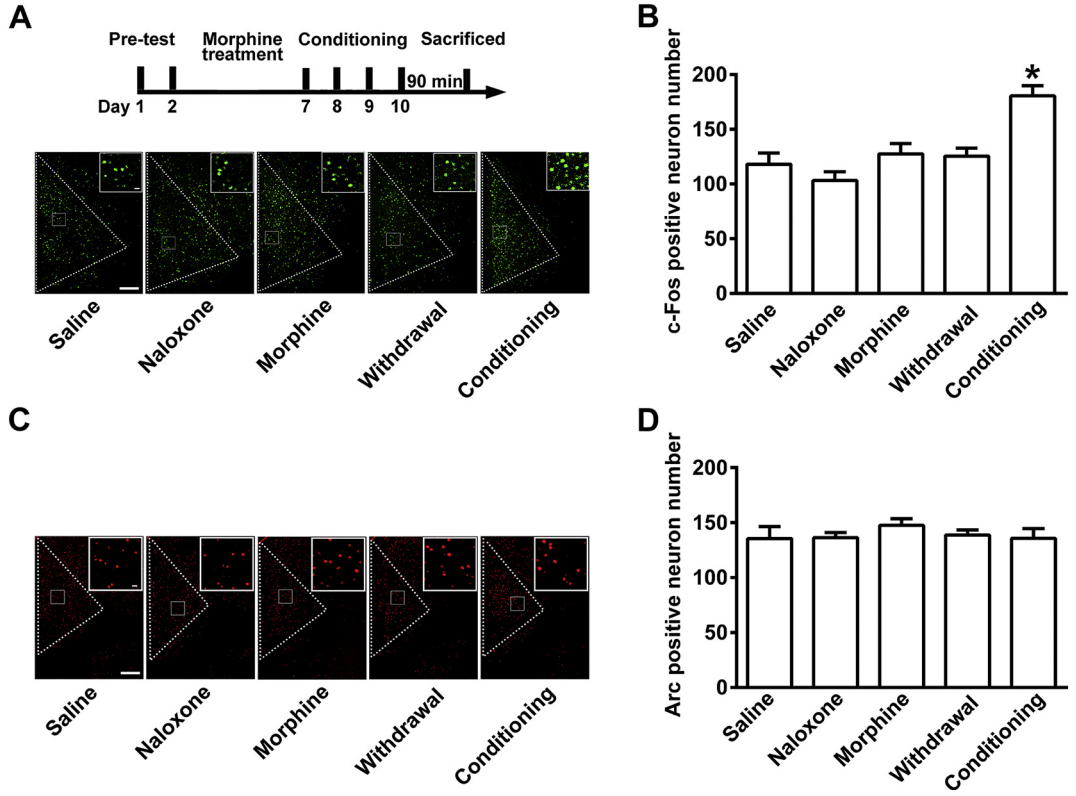


Fig. 1. Influence of the conditioning of environmental context to morphine withdrawal on the expression of c-Fos and Arc in the ACC in morphine withdrawal mice. **(A)** Top panel: experimental timeline of the behavioral procedure. Bottom panel: c-Fos positive neurons in the ACC in saline, naloxone, morphine, withdrawal and conditioning groups (green-colored). Scale bar = 100 μm. ACC regions enclosed by white boxes were shown in a higher magnification in top right square images (scale bar = 10 μm). **(B)** Average c-Fos positive neurons in the ACC in saline, naloxone, morphine, withdrawal and conditioning groups (n = 5 mice in saline and naloxone groups, n = 6 mice in morphine group, n = 7 mice in withdrawal and conditioning groups, *p < .05). **(C)** Arc positive neurons in the ACC in saline, naloxone, morphine, withdrawal and conditioning groups (red-colored). Scale bar = 100 μm. ACC regions enclosed by white boxes were shown in a higher magnification in top right square images (scale bar = 10 μm). **(D)** Average Arc positive neurons in the ACC in saline, naloxone, morphine, withdrawal and conditioning groups (n = 5 mice in saline, naloxone, morphine, withdrawal and conditioning groups). Data are represented as mean ± s.e.m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cases, n refers to the number of animals. Statistical significance was determined using Student's *t*-test for comparisons between two groups. For multi-groups of samples, the statistical significance was analyzed with one-way ANOVA following by Tukey post-hoc analysis. Two-way ANOVA with repeated measures was used to evaluate the influences of the two within-subject factors and Bonferroni post hoc test was used to detect the subgroup differences after the ANOVA comparison. For all results, $p < .05$ was accepted as being statistically significant.

3. Results

3.1. Contextual morphine withdrawal conditioning induces an increase in the expression of c-Fos, but not Arc, in the ACC in morphine withdrawal mice

To study the influence of contextual withdrawal conditioning (the conditioning of environmental context to morphine withdrawal) on the activity of the ACC, we examined the expression of c-Fos, a molecular marker of neuronal activation (Curran and Morgan, 1995), after conditioning in the ACC. Fig. 1A was the behavioral experimental timeline and the original images of c-Fos positive neurons in the ACC. Fig. 1B was the average number of c-Fos positive neurons in the ACC. We could see that the expression of c-Fos in the ACC significantly increased in conditioning group ($F_{(4,\ 163)} = 8.089$, $p < .0001$, one-way ANOVA followed by Tukey's multiple comparison test, Fig. 1B). Since mice also experienced naloxone injection, morphine treatment and withdrawal in conditioning group, we examined the change of the expression of c-Fos in the ACC in naloxone, morphine and withdrawal (only withdrawal without conditioning) groups to exclude the activate effects of drug treatment and withdrawal. The results showed that naloxone injection, morphine treatment and withdrawal had no significant influence on the expression of c-Fos in the ACC (compared to saline group, $p > .05$, one-way ANOVA followed by Tukey's multiple comparison test, Fig. 1B). These results suggest that the conditioning can activate the neurons of the ACC in morphine withdrawal mice.

We also examined the influence of contextual withdrawal conditioning on the expression of Arc, a marker of neuronal plasticity (Plath et al., 2006), in the ACC. Fig. 1C showed the original images of Arc positive neurons in the ACC and Fig. 1D was the average number of Arc positive neurons in the ACC. We could see that there was no significant change in the expression of Arc in the ACC in each group ($F_{(4,\ 20)} = 0.4694$, $p = .7575$, one-way ANOVA followed by Tukey's multiple comparison test, Fig. 1D). This result suggests that contextual withdrawal conditioning may have no influence on the neuroplasticity of the ACC.

3.2. At the first day after conditioning, conditioned context has no influence on the expression of c-Fos and Arc in the ACC in morphine withdrawal mice

To study whether the ACC was involved in the withdrawal memory retrieval at the first day after conditioning, we prepared animal model of withdrawal memory retrieval (CPA model) as described in methods. Left panel of Fig. 2A showed the behavioral experimental timeline. Right panel of Fig. 2A showed CPA score in different groups. We could

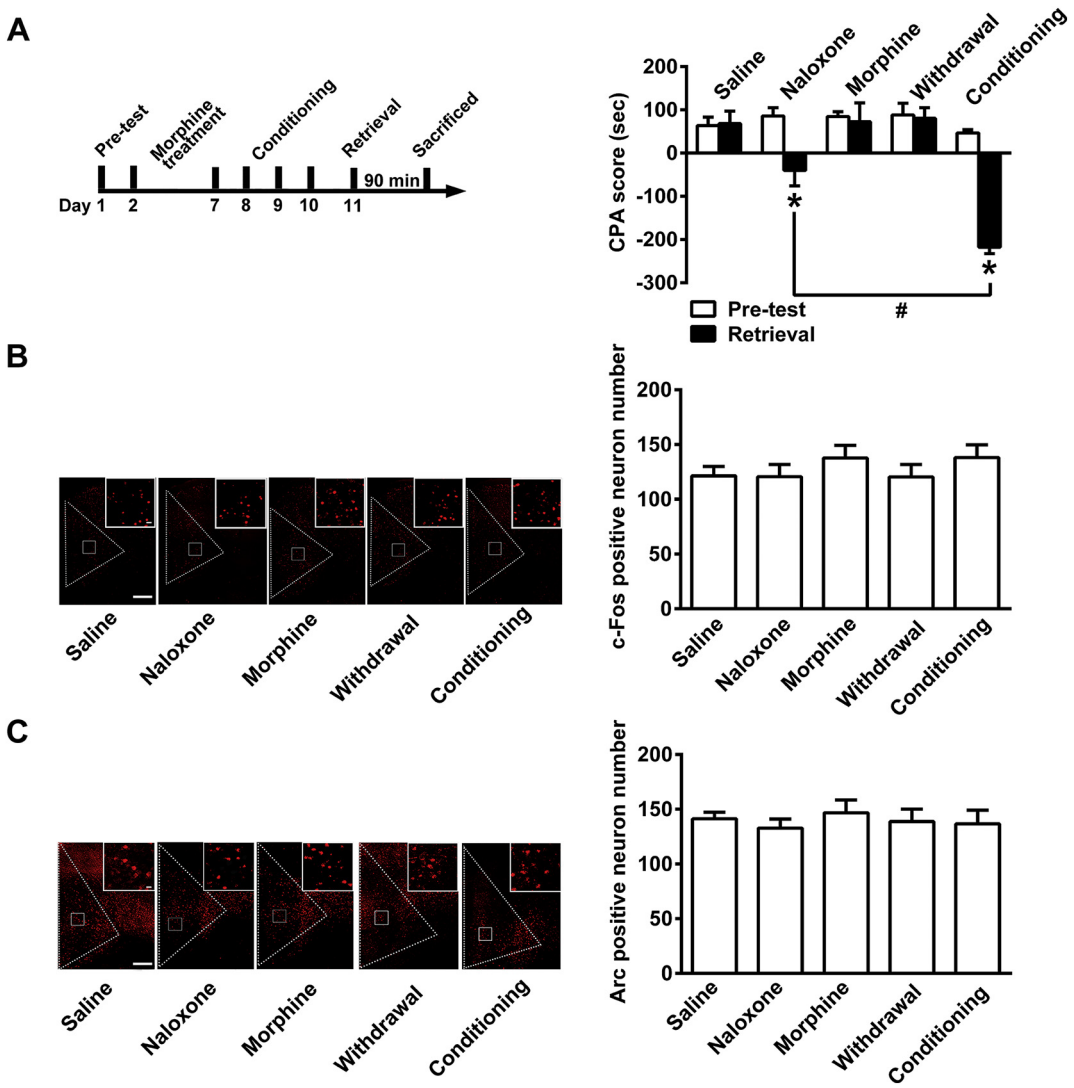
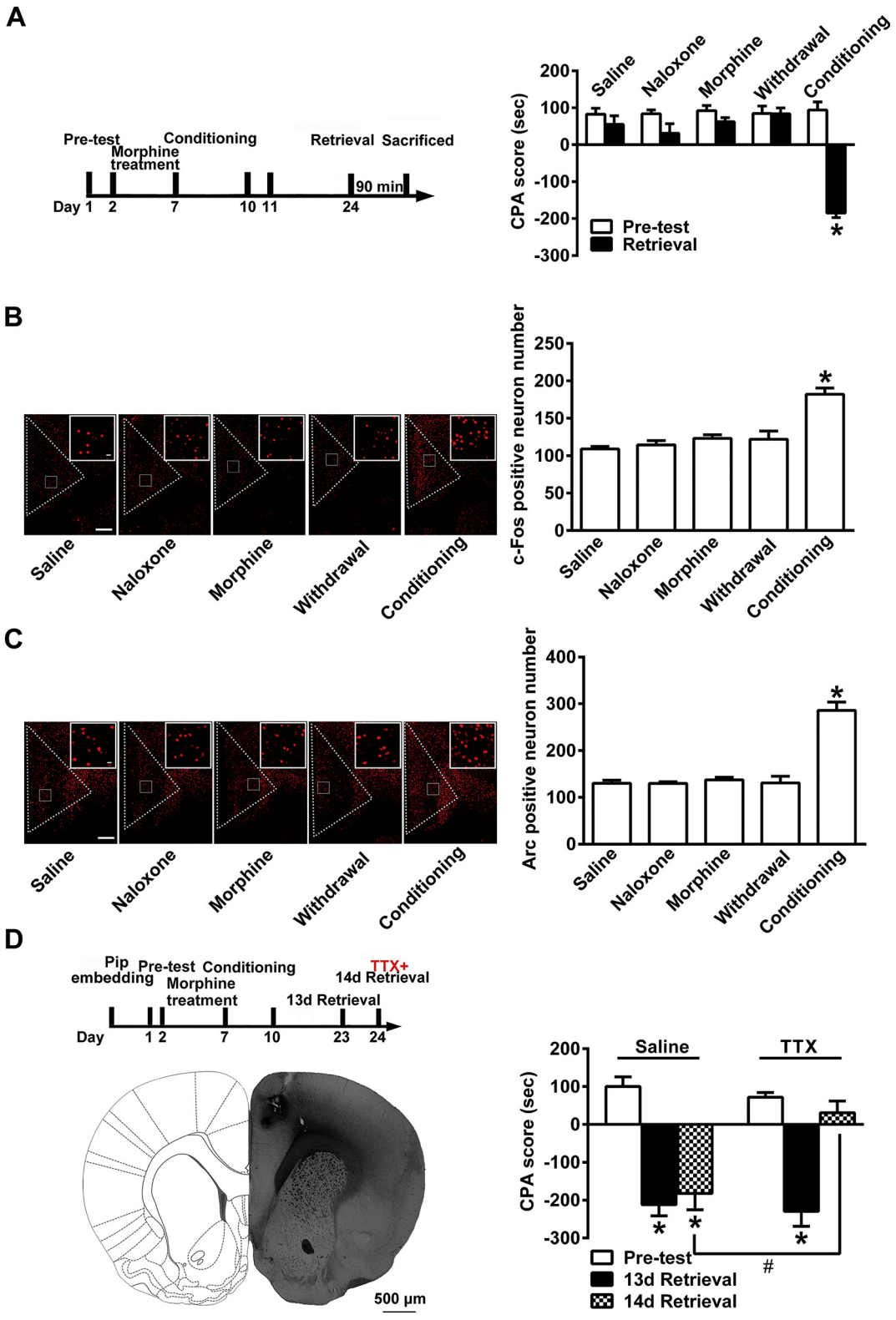


Fig. 2. Influence of conditioned context on the expression of c-Fos and Arc in the ACC at the first day after conditioning in morphine withdrawal mice. **(A)** Conditioned context-induced place aversion at the first day after conditioning in morphine withdrawal mice. Left panel: experimental timeline of the behavioral procedure. Right panel: average CPA score in saline, naloxone, morphine, withdrawal and conditioning groups (n = 8 mice in saline and morphine groups, n = 7 mice in naloxone group, n = 6 mice in withdrawal group, n = 16 mice in conditioning group, *p < .05, #p < .05). **(B)** Influence of conditioned context on the expression of c-Fos in the ACC at the first day after conditioning. Left panel: c-Fos positive neurons in the ACC in saline, naloxone, morphine, withdrawal and conditioning groups (red-colored). Scale bar = 100 μm. ACC regions enclosed by white boxes were shown in a higher magnification in top right square images (scale bar = 10 μm). Right panel: average c-Fos positive neurons in the ACC in saline, naloxone, morphine, withdrawal and conditioning groups (n = 6 mice in saline, naloxone, morphine, withdrawal and conditioning groups). **(C)** Influence of conditioned context on the expression of Arc in the ACC at the first day after conditioning. Left panel: Arc positive neurons in the ACC in saline, naloxone, morphine, withdrawal and conditioning groups (red-colored). Scale bar = 100 μm. ACC regions enclosed by white boxes were shown in a higher magnification in top right square images (scale bar = 10 μm). Right panel: average Arc positive neurons in the ACC in saline, naloxone, morphine, withdrawal and conditioning groups (n = 7 mice in saline group, n = 5 mice in naloxone, morphine and withdrawal groups, n = 6 mice in conditioning group). Data are represented as mean ± s.e.m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

see that the mice in conditioning group exhibited a strong aversion to withdrawal-paired compartment, and the mice in naloxone group exhibited a weak aversion to withdrawal-paired compartment (drug factor, $F_{(4,\ 40)} = 24.00$, $p < .0001$; test factor, $F_{(1,\ 40)} = 34.10$, $p < .0001$; drug × test, $F_{(4,\ 40)} = 20.23$, $p < .0001$; two-way ANOVA, Bonferroni post hoc analysis, right panel of Fig. 2A). Whereas the mice in other groups (saline group, morphine group and withdrawal group) did not exhibit a significant aversion to either compartment. The average CPA score of post-test in naloxone group had a weak difference compared with that of pre-test, it was previously reported by other researcher (Gomez-Milanes et al., 2012). In agreement with these studies, present result demonstrated that naloxone could induce aversive response on morphine independent animals by blocking the tonic release of endogenous opioid peptides that act through μ opioid receptors in brain regions, including VTA and NAc (Gugusheff et al., 2014). However, compared to naloxone-induced CPA in the morphine withdrawal mice, this effect was much weaker.

On this basis, we examined the influence of conditioned context on the expression of c-Fos in the ACC at the first day after conditioning. Left panel of Fig. 2B showed the original images of c-Fos positive neurons in the ACC and right panel of Fig. 2B was the average number of c-Fos positive neurons in the ACC. We could see that there was no significant change in the expression of c-Fos in the ACC in each group ($F_{(4,\ 25)} = 0.733$, $p = .5781$, one-way ANOVA followed by Tukey's multiple comparison test, right panel of Fig. 2B). This result suggests that conditioned context has no influence on the activity of the ACC at



(caption on next page)

the first day after conditioning.

We also examined the influence of conditioned context on the expression of Arc in the ACC at the first day after conditioning. Left panel of Fig. 2C showed the original images of Arc positive neurons in the ACC and right panel of Fig. 2C was the average number of Arc positive neurons in the ACC. We could see that there was no significant change in the expression of Arc in the ACC in each group ($F_{(4,\ 23)} = 0.253$, $p = .9048$, one-way ANOVA followed by Tukey's multiple comparison test, right panel of Fig. 2C). This result suggests that conditioned context has no influence on the neuroplasticity of the ACC at the first day after conditioning.

Fig. 3. Role of the ACC in morphine withdrawal memory retrieval at the 14th day after conditioning. **(A)** Conditioned context-induced place aversion at the 14th day after conditioning in morphine withdrawal mice. Left panel: experimental timeline of the behavioral procedure. Right panel: average CPA score in saline, naloxone, morphine, withdrawal and conditioning groups (n = 6 mice in saline, naloxone, morphine, withdrawal groups, n = 8 mice in conditioning group, *p < .05). **(B)** Influence of conditioned context on the expression of c-Fos in the ACC at the 14th day after conditioning. Left panel: c-Fos positive neurons in the ACC in saline, naloxone, morphine, withdrawal and conditioning groups (red-colored). Scale bar = 100 μm. ACC regions enclosed by white boxes were shown in a higher magnification in top right square images (scale bar = 10 μm). Right panel: average c-Fos positive neurons in the ACC in saline, naloxone, morphine, withdrawal and conditioning groups (n = 5 mice in saline, naloxone, morphine and withdrawal groups, n = 6 mice in conditioning group, *p < .05). **(C)** Influence of conditioned context on the expression of Arc in the ACC at the 14th day after conditioning. Left panel: Arc positive neurons in the ACC in saline, naloxone, morphine, withdrawal and conditioning groups (red-colored). Scale bar = 100 μm. ACC regions enclosed by white boxes were shown in a higher magnification in top right square images (scale bar = 10 μm). Right panel: average Arc positive neurons in the ACC in saline, naloxone, morphine, withdrawal and conditioning groups (n = 5 mice in saline, naloxone, morphine and withdrawal groups, n = 7 mice in conditioning group, *p < .05). **(D)** Influence of the inactivation of the ACC by the intra-ACC injection of TTX on the environmental context-induced conditioning place aversion in morphine-withdrawal rats at the 14th day after conditioning. Left top panel: experimental timeline of the behavioral procedure. Left bottom panel: anatomical location of injection site of TTX in the ACC. Scale bar = 500 μm. Right panel: average CPA score in saline and TTX groups. (n = 8 rats in saline group and n = 10 rats in TTX group, *p < .05, #p < .05). Data are represented as mean ± s.e.m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. At the 14th day after conditioning, conditioned context induces an increase in the expression of both c-Fos and Arc in the ACC in morphine withdrawal mice

To study whether the ACC was involved in the withdrawal memory retrieval at the 14th day after conditioning, we prepared animal model of withdrawal memory retrieval (CPA model) as described in methods and measured CPA score at the 14th day after conditioning. Left panel of Fig. 3A showed the behavioral experimental timeline. Right panel of Fig. 3A showed CPA score in different groups. We could see that the mice in conditioning group exhibited a strong aversion to withdrawal-paired compartment (drug factor, $F_{(4,\ 27)} = 17.12$, $p < .0001$; test factor, $F_{(1,\ 27)} = 56.85$, $p < .0001$; drug × test, $F_{(4,\ 27)} = 28.65$, $p < .0001$; two-way ANOVA, Bonferroni post hoc analysis, right panel of Fig. 3A). While the mice in other groups had no a strong aversion to either compartment.

On this basis, we examined the influence of conditioned context on the expression of c-Fos in the ACC at the 14th day after conditioning. Left panel of Fig. 3B showed the original images of c-Fos positive neurons in the ACC and right panel of Fig. 3B was the average number of c-Fos positive neurons in the ACC. We could see that the expression of c-Fos in the ACC significantly increased in conditioning group ($F_{(4,\ 20)} = 17.54$, $p < .0001$, one-way ANOVA followed by Tukey's multiple comparison test, right panel of Fig. 3B). This result suggests that conditioned context increases the activity of ACC neurons at the 14th day after conditioning.

We also examined the influence of conditioned context on the expression of Arc in the ACC at the 14th day after conditioning. Left panel of Fig. 3C showed the original images of Arc positive neurons in the ACC and right panel of Fig. 3C was the average number of Arc positive neurons in the ACC. We could see that the expression of Arc in the ACC significantly increased in retrieval group ($F_{(4,\ 22)} = 34.65$, $p < .0001$, one-way ANOVA followed by Tukey's multiple comparison test, right panel of Fig. 3C). This result suggests that at the 14th day after conditioning, conditioned context may induce a change in neuroplasticity of the ACC, which may be related to withdrawal memory retrieval. This statement was supported by our result that the inactivation of the ACC by the intra-ACC injection of TTX could abolish conditioned context-induced conditioning place aversion in morphine-withdrawal rats at the 14th day after conditioning (drug factor, $F_{(1,\ 16)} = 2.815$, $p = .1128$; test factor, $F_{(2,\ 32)} = 69.81$, $p < .0001$; drug x test, $F_{(2,\ 32)} = 13.84$, $p < .0001$; two-way ANOVA, Bonferroni post hoc analysis, right panel of Fig. 3D).

3.4. Inhibition of dendritic spines of the ACC attenuates the conditioned context-induced increase of Arc expression in the ACC and abolishes the retrieval of withdrawal memory at the 14th day after conditioning

To study the role of dendritic spines of the ACC in the enhanced expression of Arc at the 14th day after conditioning, we examined the influence of the inhibition of dendritic spines of the ACC on the expression of Arc in the ACC at the 14th day after conditioning. We use RNAi method to knock down the expression of endogenous cortactin, which is an F-actin binding protein in neurons and the inhibition of it can induce a decrease in dendritic spines (Hering and Sheng, 2003), to inhibit dendritic spines of the ACC. Cortactin-specific shRNAs were packaged into mCherry-lentivirus (LV-Cttn-RNAi (30376–1)) and injected in ACC. Results showed that LV-Cttn-RNAi (30376-1) could significantly inhibit cortactin protein expression and decrease the number of dendritic spine in the ACC (Fig. S2). On this basis, we examined the influence of the inhibition of dendritic spines of the ACC on the expression of Arc at the 14th day after conditioning. Mice with morphine withdrawal were divided into two groups: saline group and cortactin RNAi group. In cortactin RNAi group, LV-Cttn-RNAi (30376-1) was stereotaxically delivered into the ACC and the virus was allowed to express for 14 days before the morphine treatment in order to effectively decrease dendritic spines of the ACC. In saline group, saline was locally injected into the ACC under other conditions same to cortactin RNAi group. The expression of Arc and CPA score were examined at the 14th day after conditioning. Fig. 4A was the behavioral experimental timeline. Fig. 4B was original images of Arc positive neurons in the ACC and Fig. 4C was the average number of Arc positive neurons in the ACC. We could see that Arc positive neurons in the ACC significantly decreased in cortactin RNAi group ($t_{(8)} = 3.521$, $p = .0078$, unpaired t-test, Fig. 4C). Since the enhanced expression of Arc is closely related to neuronal plasticity change and thus to withdrawal memory, we examined the influence of the inhibition of dendritic spines of the ACC on withdrawal context-induced place aversion in morphine withdrawal mice. Fig. 4D showed the influence of the inhibition of dendritic spines of the ACC on conditioned context-induced place aversion in morphine withdrawal mice at the 14th day after conditioning. We could see that the inhibition of dendritic spines of the ACC could significantly abolish the CPA score at the 14th day after conditioning (virus factor, $F_{(1,\ 24)} = 8.891$, $p = .0065$; test factor, $F_{(2,\ 48)} = 41.45$, $p < .0001$; virus × test, $F_{(2,\ 48)} = 6.728$, $p = .0027$; two-way ANOVA, Bonferroni post hoc analysis, Fig. 4D). The average CPA score at the 14th day after conditioning had no statistically significant difference from that of pre-test, whereas the average CPA score at the 14th day after conditioning in saline group was significantly different from that of pre-test. These results suggest that the dendritic spines of the ACC play an important role in enhanced expression of Arc and conditioned context-induced place aversion at the 14th day after conditioning.

Moreover, we examined the influence of the inhibition of dendritic spines of the ACC after 1 d post-test on the expression of Arc in the ACC at the 14th day after conditioning. The morphine withdrawal mice were divided into two groups: saline group and cortactin RNAi group. Fig. 5A showed the behavioral experimental timeline. Fig. 5B was the original images of Arc positive neurons in the ACC and Fig. 5C was the average number of Arc positive neurons in the ACC. We could see that Arc positive neurons in the ACC at the 14th day after conditioning

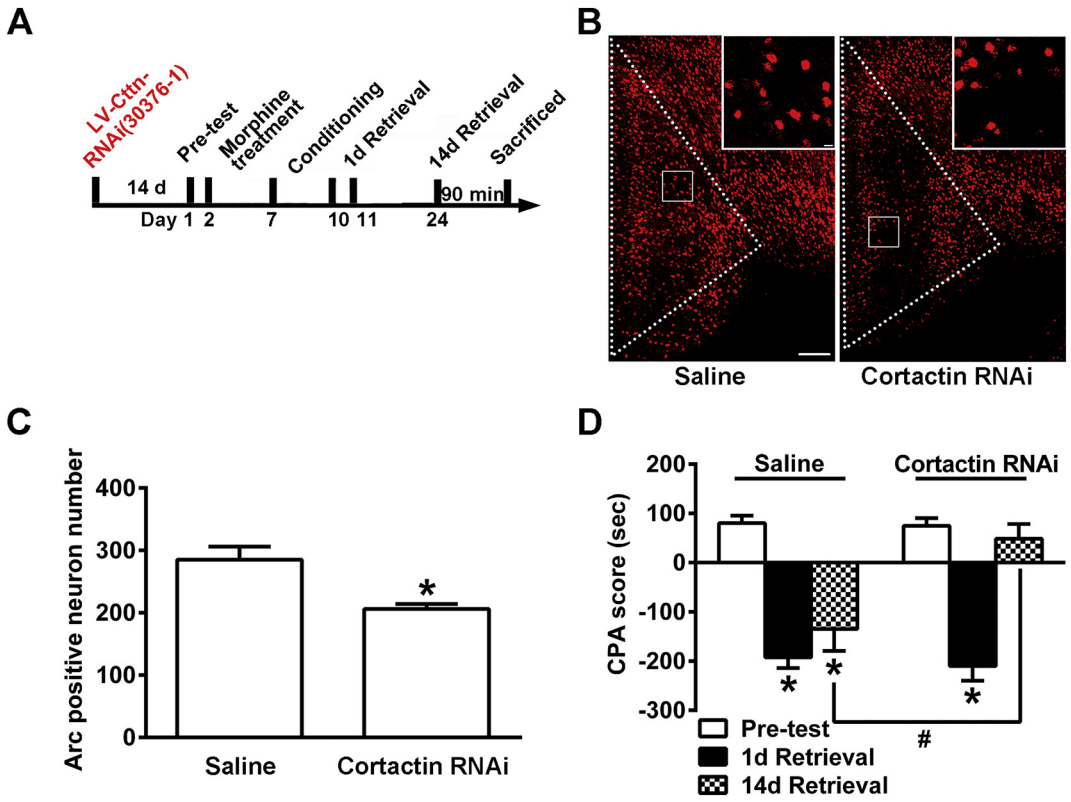


Fig. 4. Influence of the inhibition of dendritic spines of the ACC on the expression of Arc and CPA at the 14th day after conditioning in morphine withdrawal mice. **(A)** Experimental timeline of the behavioral procedure. **(B)** Arc positive neurons in the ACC in saline and cortactin RNAi groups (red-colored). Scale bar = 100 μm. ACC regions enclosed by white boxes were shown in a higher magnification in top right square images (scale bar = 10 μm). **(C)** Average Arc positive neurons in the ACC in saline and cortactin RNAi groups (n = 5 mice in saline group and cortactin RNAi group, *p < .05). **(D)** Average CPA score in saline and cortactin RNAi groups. (n = 11 mice in saline group and n = 15 mice in cortactin RNAi group, *p < .05, #p < .05). Data are represented as mean ± s.e.m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significantly decreased in cortactin RNAi group ($t_{(8)} = 3.357$, $p = .01$, unpaired t-test, Fig. 5C). Fig. 5D showed the influence of the decrease of dendritic spines of the ACC by LV-Cttn-RNAi (30376-1) on the CPA score at the 14th day after conditioning. We could see that the inhibition of dendritic spines of the ACC could significantly decrease the CPA score at the 14th day after conditioning (virus factor, $F_{(1, 14)} = 5.499$, $p = .0343$; test factor, $F_{(2, 28)} = 36.41$, $p < .0001$; virus × test, $F_{(2, 28)} = 4.399$, $p = .0218$; two-way ANOVA, Bonferroni post hoc analysis, Fig. 5D). However, in cortactin RNAi group, if LV-Cttn-RNAi (30376-1) was stereotaxically delivered into the ACC after 1 d post-test, the average CPA score at the 14th day after conditioning had no significant difference from that of pre-test and was significantly different from that at the first day after conditioning. These results suggest that the inhibition of dendritic spines of the ACC, whether before contextual withdrawal conditioning or after recent withdrawal memory formation, can attenuate the increased expression of Arc in the ACC and abolish the related memory behavior, suggesting the key role of dendritic spines of the ACC in remote withdrawal memory.

3.5. Inhibition of projection neurons from the CA3 of the hippocampus to the ACC attenuates the conditioned context-induced increase of Arc expression in the ACC and abolishes the retrieval of withdrawal memory at the 14th day after conditioning

To study the role of projection neurons from the CA3 of the hippocampus to the ACC in conditioned context-induced change in the expression of Arc in the ACC at the 14th day after conditioning, we traced projection neurons from the CA3 of the hippocampus to the ACC by the injection of the retrograde tracer Fluoro-gold (FG) into the ACC (left panel of Fig. 6A). The result showed that FG injection resulted in labeling in the CA3 of the hippocampus, indicating that CA3 neurons have direct projection to the ACC (right panel of Fig. 6A). Then, we examined the influence of the ablation of the projection neurons from the CA3 of the hippocampus to the ACC on the expression of Arc in the ACC at the 14th day after conditioning by diphtheria toxin receptor (DTR)-mediated method. AAV virus expressing DTR-mCherry was stereotaxically injected into the CA3 and AAV virus expressing WGA-Cre was injected into the ACC of mice (left top panel of Fig. 6B). Four weeks after the infection, the cre-dependent expression of DTR-mCherry was observed in the CA3 (left bottom panel of Fig. 6B). Then we prepared animal model of contextual withdrawal conditioning as described in methods. The morphine withdrawal mice were divided into two groups: saline group and DT group. At the first day after conditioning, mice were intraperitoneal injected with DT (50 μg/kg). The ablation effectiveness was evaluated by NeuN immunofluorescence method in hippocampal slice. The result showed that the DT treatment could effectively kill the projection neurons from the CA3 to the ACC (right panel of Fig. 6B). On this basis, we examined the influence of the ablation of the projection neurons from the CA3 to the ACC at the first day after conditioning on the expression of Arc in the ACC at the 14th day after conditioning. The result showed that Arc positive neurons in the ACC significantly attenuated in DT group ($t_{(9)} = 3.733$, $p = .0047$, unpaired t-test, Fig. 6E) at the 14th day after conditioning. Moreover, the ablation of projection neurons from the CA3 to the ACC by DTR-mediated method also could significantly abolish the CPA score at the 14th day after conditioning (DT factor, $F_{(1, 16)} = 5.739$, $p = .0292$; test factor, $F_{(2, 32)} = 44.31$, $p < .0001$; DT × test, $F_{(2, 32)} = 3.905$, $p = .0304$; two-way ANOVA, Bonferroni post hoc analysis, Fig. 6F). The average CPA score of post-test in the saline group was significantly different from that of pre-test, but in the DT group, the average CPA score of

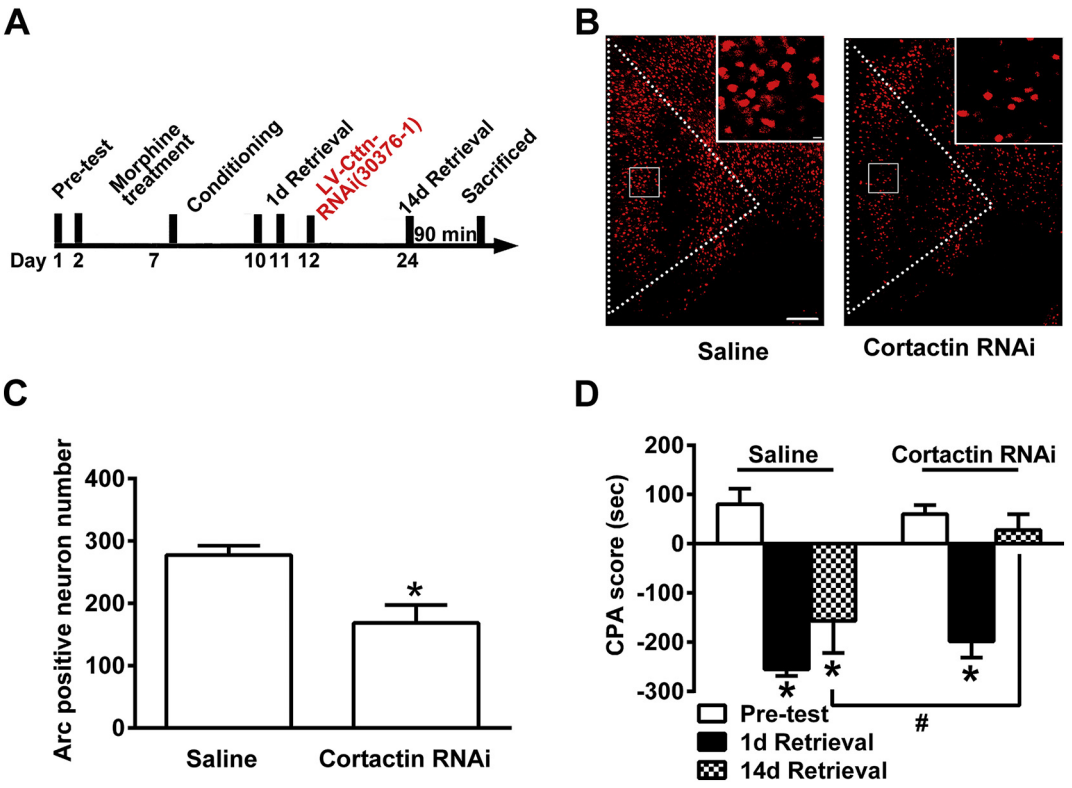


Fig. 5. Influence of the inhibition of dendritic spines of the ACC at the first day after conditioning on the expression of Arc in the ACC and CPA in morphine withdrawal mice at the 14th day after conditioning. **(A)** Experimental timeline of the behavioral procedure. **(B)** Arc positive neurons in the ACC in saline and cortactin RNAi groups (red-colored). Scale bar = 100 μm. ACC regions enclosed by white boxes were shown in a higher magnification in top right square images (scale bar = 10 μm). **(C)** Average Arc positive neurons in the ACC in saline and cortactin RNAi groups (n = 5 mice in saline group and cortactin RNAi group, *p < .05). **(D)** Average CPA score in saline and cortactin RNAi groups. (n = 8 mice in saline and cortactin RNAi groups, *p < .05, #p < .05). Data are represented as mean ± s.e.m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

post-test had no significant difference from that of pre-test. This result suggests that projection neurons from the CA3 of the hippocampus to the ACC play an important role in conditioned context-induced change in the expression of Arc in the ACC and related memory behavior at the 14th day after conditioning.

To further confirm the role of projection neurons from the CA3 of the hippocampus to the ACC in conditioned context induced change in the expression of Arc in the ACC at the 14th day after conditioning, we used chemical genetics method to examine the influence of chronically inhibition of CA3-ACC projection neurons on the expression of Arc in the ACC. AAV-hSyn-DIO-hM4D(Gi)-mCherry was stereotaxically injected into the CA3, and AAV-hSyn- IRES-WGA-Cre was injected into the ACC of mice (left panel of Fig. 7A). Four weeks after the infection, the cre-dependent expression of hM4D(Gi)-mCherry was observed in the CA3 (right panel of Fig. 7A) by retrograde transporting the WGA-Cre from the ACC to the CA3. Then behavioral experiments were performed as described in methods. The morphine withdrawal mice were divided into two groups: AAV + saline group and AAV + clozapine-n-oxide (AAV + CNO) group. We could see that Arc positive neurons in the ACC significantly attenuated in AAV + CNO group ($t_{(10)} = 3.682$, p = .0042, unpaired *t*-test, Fig. 7D) at the 14th day after conditioning. Moreover, the inhibition of projection neurons from the CA3 to the ACC by chemical genetics method also could significantly attenuate the CPA score at the 14th day after conditioning (CNO factor, $F_{(2,\ 21)} = 4.99$, p = .0169; test factor, $F_{(2,\ 42)} = 146.1$, p < .0001; CNO x test, $F_{(4,\ 42)} = 6.04$, p = .0006; two-way ANOVA, Bonferroni post hoc analysis, Fig. 7E). The average CPA score of post-test in the AAV + saline group was significantly different from that of pre-test,but in the AAV + CNO group, the average CPA score of post-test had no significant difference from that of pre-test. To exclude the effect of CNO on behavioral, we set the CNO group. The result showed that CNO (1 mg/kg) alone indeed have no significant behavioral effect in our experiment. This result confirms that projection neurons from the CA3 of the hippocampus to the ACC indeed play an important role in conditioned context-induced change in the expression of Arc in the ACC and related memory behavior at the 14th day after conditioning.

4. Discussion

The main findings of present study are that (1) associative morphine withdrawal conditioning induces an increase in the expression of c-Fos, but not Arc, in the ACC in morphine withdrawal mice; (2) at the first day after conditioning, conditioned context does not induce a change in the expression of both c-Fos and Arc in the ACC in morphine withdrawal mice; (3) at the 14th day after the condoning, conditioned context induces an increase in the expression of both c-Fos and Arc in the ACC in morphine withdrawal mice; (4) the inhibition of dendritic spines of the ACC at the first day after conditioning attenuates the conditioned context-induced increase in the expression of Arc in the ACC and CPA score at the 14th day after conditioning; (5) the inhibition of projection neurons from the CA3 of the hippocampus to the ACC at the first day after conditioning by both DTR-mediated neuron ablation and CNO-mediated inhibition of neuron function attenuates the conditioned context-induced increase in the expression of Arc in the ACC and CPA score at the 14th day after conditioning.

One finding of the present study is that associative morphine withdrawal conditioning induces an increase in the expression of c-Fos, a molecular marker of neuronal activity, in the ACC. This finding is consistent with the activation of the ACC during other forms of associative conditioning. For example, the ACC is preferentially activated

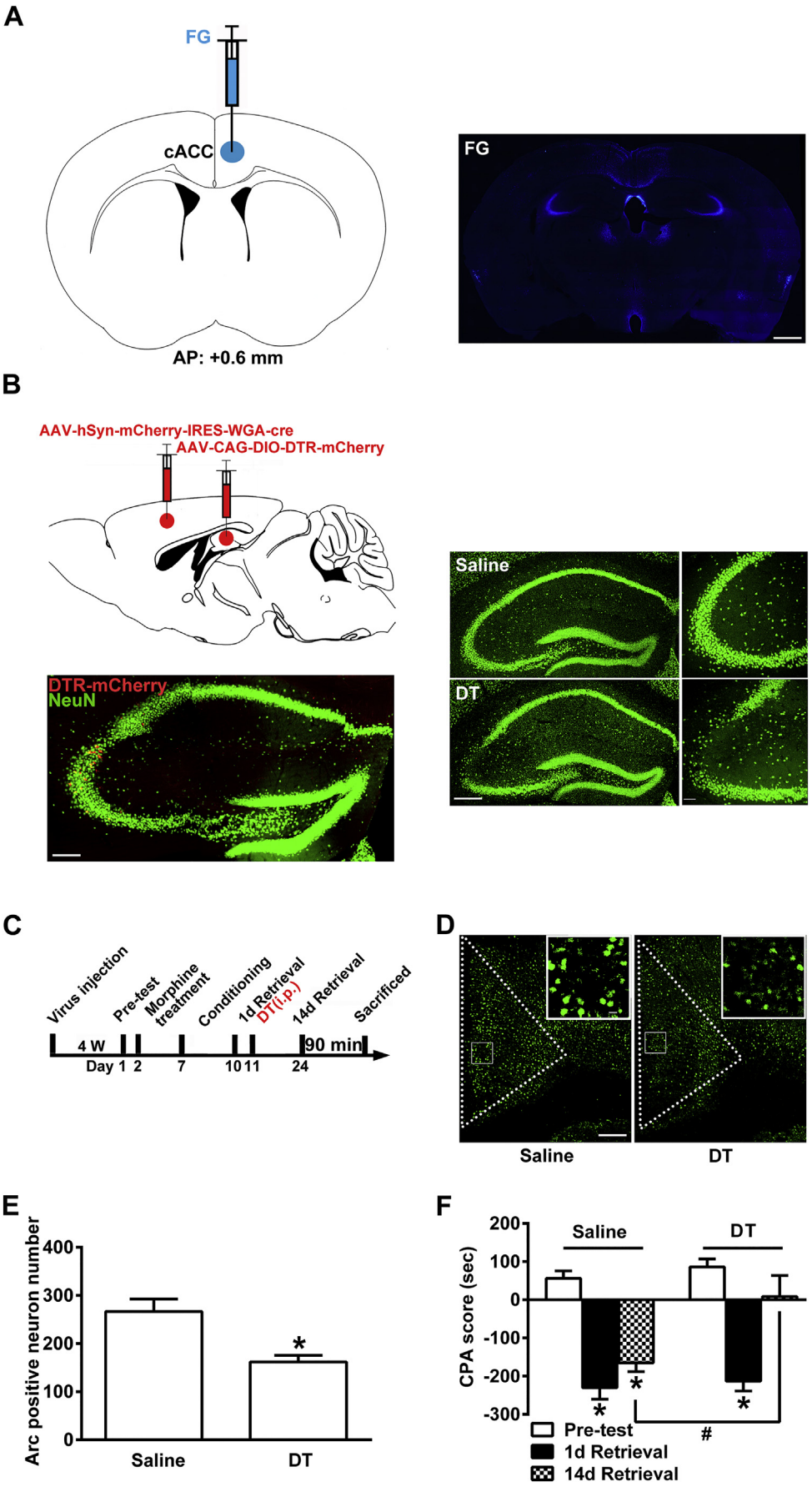


Fig. 6. Influence of the ablation of the projection neurons from the CA3 to the ACC at the first day after conditioning on the expression of Arc in the ACC and CPA in morphine withdrawal mice at the 14th day after conditioning. **(A)** Left panel: diagram of FG injection into the ACC. Right panel: sagittal section of the CA3 with FG cells (blue-colored). Scale bar = 500 μm. **(B)** Left top panel: diagram of virus injection into the ACC and the CA3. Left bottom panel: image of coronal brain slice showing expression of DTR-mCherry (red-colored), NeuN (green-colored) at the 14th day after virus injection into the CA3. Scale bar = 500 μm. Right panel: original images of the CA3 after saline or DT injection by NeuN staining. Scale bar = 500 μm. **(C)** Experimental timeline of the behavioral procedure. **(D)** Arc positive neurons in the ACC in saline and DT groups (green-colored). Scale bar = 100 μm. ACC regions enclosed by white boxes were shown in a higher magnification in top right square images (scale bar = 10 μm). **(E)** Average Arc positive neurons in the ACC in saline and DT groups (n = 5 mice in saline group and n = 6 mice in cortactin RNAi group, *p < .05). **(F)** Average CPA score in saline and DT groups. (n = 8 mice in saline group and n = 10 mice in DT group, *p < .05, $^{\#}$p < .05). Data are represented as mean ± s.e.m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

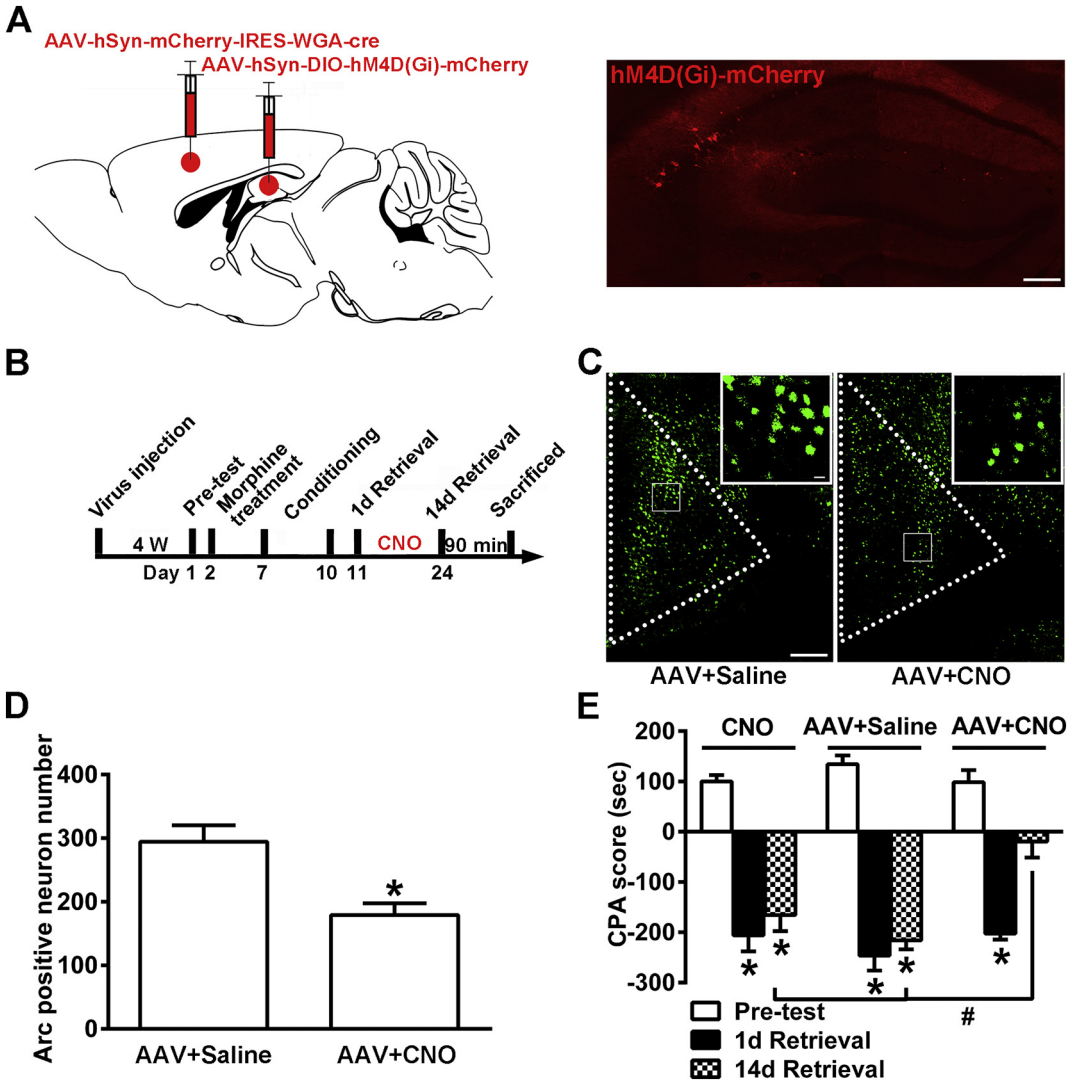


Fig. 7. Influence of the inhibition of the projection neurons from the CA3 to the ACC at the first day after conditioning on the expression of Arc in the ACC and CPA in morphine withdrawal mice at the 14th day after conditioning. **(A)** Left panel: diagram of virus injection into the ACC. Right panel: image of coronal brain slice showing expression of hM4D(Gi) (red-colored) at the 14 day after virus injection into the ACC. Scale bar = 100 μm. **(B)** Experimental timeline of the behavioral procedure. **(C)** Arc positive neurons in the ACC in AAV + saline and AAV + CNO groups (green-colored). Scale bar = 100 μm. ACC regions enclosed by white boxes were shown in a higher magnification in top right square images (scale bar = 10 μm). **(D)** Average Arc positive neurons in the ACC in AAV + saline and AAV + CNO groups (n = 6 mice in saline and CNO groups, *p < .05). **(E)** Average CPA score in CNO, AAV + saline and AAV + CNO groups. (n = 8 mice in CNO and AAV + saline groups, n = 7 mice in AAV + CNO groups, *p < .05, $^{\#}$p < .05). Data are represented as mean ± s.e.m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

during presentation of the conditional stimulus during aversive trace conditioning (Buchel et al., 1999); trace fear conditioning requires the ACC (Han et al., 2003); the ACC is critical for trace eye blink conditioning in rabbits (Kronforst-Collins and Disterhoft, 1998; Weible et al., 2000). The ACC is proposed to be an important neural substrate of attention (Lucas et al., 2008). For example, attention has been correlated with increased activity in the ACC (Bush et al., 1998, 2000, 2002; Davis et al., 2000; Devinsky et al., 1995; Jovicich et al., 2001) and the ACC is implicated in tasks requiring visual attention in rats (Bussey et al., 1997a, 1996, 1997b; Muir et al., 1996; Robbins, 2002; Rogers et al., 2001). Therefore, these evidences suggest that attention may be required during the establishment of the association of conditional stimulus and unconditional stimulus.

Prevailing theory proposes that memories are initially stored within the hippocampal circuits, and, over weeks to months, slowly consolidated within the neocortex to promote long-term memory storage. Such a two phase memory theory has been described for both spatial and emotional memories. For instance, the recall of recent spatial memories or contextual fear memories involved activation of the hippocampus, whereas the remote recall of these memories activated cortical regions (Bontempi et al., 1999; Frankland et al., 2004; Maviel et al., 2004). In addition, like other types of memories, addiction memory associated with positive drug effects or negative withdrawal states may be subjected to time-dependent shifts in the circuits that support memory at different time points. Morgan Lucas et al. examined the influence of morphine withdrawal conditioned context on the expression of c-Fos in different brain regions and found that the conditioned context could increase the expression of c-Fos in a number of brain regions, including the hippocampus, but had no influence on the expression of c-Fos in the ACC at the second day after conditioning (Lucas et al., 2012). However, at 1 month after conditioning, the conditioned context could increase the expression of c-Fos in the ACC (Lucas et al., 2012). These results suggest that affective memories linked to opiate withdrawal may undergo anatomical reorganization, with a shift from recent store regions, like the hippocampus, toward cortical areas, like the ACC. However, the change in the expression of

Arc, a marker of neuroplasticity, in the ACC after the associative morphine withdrawal conditioning remains unknown. c-Fos is an immediate early gene (IEGs), which is responsive to a number of extrinsic cellular stimuli and couples action potential firing to gene expression (Fields et al., 1997; Sheng et al., 1993). The signal transduction cascade by which membrane depolarization activates c-Fos gene transcription has been characterized in great detail (Sheng et al., 1993). In a quiescent neuron, c-Fos expression is extremely low, but when excitatory synaptic inputs increase, depolarization causes an influx of extracellular calcium. In response to elevated intracellular calcium, a calcium response element binding protein (CREB) is rapidly phosphorylated, so that CREB and its DNA binding site, the Ca/CRE, can function as a regulatory element that activates the c-Fos gene (Dilgen et al., 2013). Arc is an immediate early gene that not only participate in memory formation (Tzingounis and Nicoll, 2006), but also is involved in the retrieval of memory (Tzingounis and Nicoll, 2006). Arc knockout mice failed to form long-lasting memories (Plath et al., 2006) and the inhibition of Arc expression disrupted the retrieval of memory (Nakayama et al., 2015). So the expression of Arc has widely been used to examine neuronal plasticity change that is closely related to memory. The present study showed that at the first day after the associative morphine withdrawal conditioning, conditioned context had no influence on the expression of Arc in the ACC, but increased the expression of Arc in the ACC at the 14th day after conditioning. This evidence further support the statement of affective memories linked to opiate withdrawal may undergo anatomical reorganization, with a shift from recent store regions toward the ACC.

We further studied how the expression of Arc in the ACC increased at the 14th day after conditioning. One possible manner is that it depends on synaptic inputs into the ACC. To test this hypothesis, we examined the influence of the inhibition of the dendritic spines of the ACC on the expression of Arc at the 14th day after conditioning. Dendritic spines are membranous protrusions from dendrites that receive input from presynaptic axonal terminals and the inhibition of dendritic spines can achieve the inhibition of synaptic inputs. Our result showed that the inhibition of dendritic spines of the ACC, whether before associative morphine withdrawal conditioning or after recent withdrawal memory formation at the first day after conditioning could inhibit the increased expression of Arc in the ACC and CPA behavior at the 14th day after conditioning, suggesting that increased expression of Arc in the ACC and related memory at the 14th day after conditioning required dendritic spines of the ACC and synaptic input.

It has been known that the ACC receives projections from the locus credulous (Gompf et al., 2010), the medial prefrontal cortex(Hoover and Vertes, 2007) and para-hippocampal structures (Jones and Witter, 2007). Among them, the standard model of two phase memory hypothesizes that remote memory is formed in the cortex by a slow transfer of hippocampal memory (Kitamura et al., 2017). Moreover, in fear memory studies, it has been reported that fear memory learning can induce the activation of CA3 neurons of the hippocampus (Rajasethupathy et al., 2015) and the CA3 output is crucial for the consolidation of fear memory (Nakashiba et al., 2009). In addition, using FG retrograde tracing method, our result showed the CA3 area of the hippocampus had direct projections to the ACC. This is consistent to the above statement. However, whether withdrawal memory signals in the ACC in remote period is also from CA3 neurons of the hippocampus remains unknown. Our result that the inhibition of projection neurons from the CA3 of the hippocampus to the ACC by both DTR-mediated neuron ablation and CNO-mediated inhibition of neuron function attenuates the increased expression of Arc in the ACC and related withdrawal memory in remote period strongly suggests that like fear memory, projection neurons from the CA3 of the hippocampus to the ACC also have a distinctive role in the formation of remote withdrawal memory.

A shift of morphine withdrawal memory from recent store regions toward cortical areas such as the ACC has been proposed to be an important mechanism underlying the long-term addiction memory, which is closed related to addiction remissions and relapses. A deeper understanding of the mechanism by which long-term addiction memory are formed in cortical areas represents a critical objective in the addiction field. The present finding that the dendritic spines in the ACC and projection neurons from CA3 neurons of the hippocampus to the ACC are key determinants for Arc expression in the ACC and CPA behavior at the 14th day after conditioning (remote memory retrieval) provides a new strategy for the interference of withdrawal memory in cortical areas like the ACC.

Acknowledgments

This study was supported by the National Program of Basic Research sponsored by the Ministry of Science and Technology of China (2009CB52201 and 2013CB835100), Science and Technology Program of Yunnan Province (2013GA003) and Project of Foundation of National Natural Science of China (31121061, 91332204, 81371466 and 31070932).

Author contributions

M.C. and D.S., conception and design, acquisition of data, analysis and interpretation of data, drafting or revising the article; Y.L.F., Q.Q.M., M.C., D.Y.C., J.J.S., H.S., L.Y., Y.D., acquisition of data, analysis and interpretation of data; P.Z., B.L., conception and design, analysis and interpretation of data, drafting or revising the article.

Compliance with ethical standards

The authors declare that they have no conflict of interest. All experimental procedures performed in studies conformed to Fudan University as well as international guidelines on the ethical use of animals and all efforts were made to minimize the number of animals used and their suffering. This article does not contain any studies with human participants by any of the authors. The work has not been submitted elsewhere for publication, and all the authors listed have approved the manuscript that is enclosed.

The following are the supplementary data related to this article.

References

Aceti, M., Vetere, G., Novembre, G., Restivo, L., Ammassari-Teule, M., 2015. Progression of activity and structural changes in the anterior cingulate cortex during remote memory formation. Neurobiol. Learn. Mem. 123, 67–71.

Bontempi, B., Laurent-Demir, C., Destrade, C., Jaffard, R., 1999. Time-dependent reorganization of brain circuitry underlying long-term memory storage. Nature 400, 671–675.

Buchel, C., Dolan, R.J., Armony, J.L., Friston, K.J., 1999. Amygdala-hippocampal involvement in human aversive trace conditioning revealed through event-related functional magnetic resonance imaging. J. Neurosci. 19, 10869–10876.

Bush, G., Whalen, P.J., Rosen, B.R., Jenike, M.A., McInerney, S.C., Rauch, S.L., 1998. The counting Stroop: an interference task specialized for functional neuroimaging–validation study with functional MRI. Hum. Brain Mapp. 6, 270–282.

Bush, G., Luu, P., Posner, M.I., 2000. Cognitive and emotional influences in anterior cingulate cortex. Trends Cogn. Sci. 4, 215–222.

Bush, G., Vogt, B.A., Holmes, J., Dale, A.M., Greve, D., Jenike, M.A., Rosen, B.R., 2002. Dorsal anterior cingulate cortex: a role in reward-based decision making. Proc. Natl. Acad. Sci. U. S. A. 99, 523–528.

Bussey, T.J., Muir, J.L., Everitt, B.J., Robbins, T.W., 1996. Dissociable effects of anterior and posterior cingulate cortex lesions on the acquisition of a conditional visual discrimination: facilitation of early learning vs. impairment of late learning. Behav. Brain Res. 82, 45–56.

Bussey, T.J., Everitt, B.J., Robbins, T.W., 1997a. Dissociable effects of cingulate and medial frontal cortex lesions on stimulus-reward learning using a novel Pavlovian autoshaping procedure for the rat: implications for the neurobiology of emotion. Behav. Neurosci. 111, 908–919.

Bussey, T.J., Muir, J.L., Everitt, B.J., Robbins, T.W., 1997b. Triple dissociation of anterior cingulate, posterior cingulate, and medial frontal cortices on visual discrimination tasks using a touchscreen testing procedure for the rat. Behav. Neurosci. 111, 920–936.

Carvalho Poyraz, F., Holzner, E., Bailey, M.R., Meszaros, J., Kenney, L., Kheirbek, M.A., Balsam, P.D., Kellendonk, C., 2016. Decreasing striatopallidal pathway function

enhances motivation by energizing the initiation of goal-directed action. J. Neurosci. 36, 5988–6001.
Ciccarelli, A., Calza, A., Santoru, F., Grasso, F., Concas, A., Sassoe-Pognetto, M., Giustetto, M., 2013. Morphine withdrawal produces ERK-dependent and ERK-independent epigenetic marks in neurons of the nucleus accumbens and lateral septum. Neuropharmacology 70, 168–179.
Curran, T., Morgan, J.I., 1995. Fos: an immediate-early transcription factor in neurons. J. Neurobiol. 26, 403–412.
Davis, K.D., Hutchison, W.D., Lozano, A.M., Tasker, R.R., Dostrovsky, J.O., 2000. Human anterior cingulate cortex neurons modulated by attention-demanding tasks. J. Neurophysiol. 83, 3575–3577.
Desjardins, S., Belkai, E., Crete, D., Cordonnier, L., Scherrmann, J.M., Noble, F., Marie-Claire, C., 2008. Effects of chronic morphine and morphine withdrawal on gene expression in rat peripheral blood mononuclear cells. Neuropharmacology 55, 1347–1354.
Devinsky, O., Morrell, M.J., Vogt, B.A., 1995. Contributions of anterior cingulate cortex to behaviour. Brain 118, 279–306 Pt 1.
Dilgen, J., Tejeda, H.A., O'Donnell, P., 2013. Amygdala inputs drive feedforward inhibition in the medial prefrontal cortex. J. Neurophysiol. 110, 221–229.
Dumitriu, D., Rodriguez, A., Morrison, J.H., 2011. High-throughput, detailed, cell-specific neuroanatomy of dendritic spines using microinjection and confocal microscopy. Nat. Protoc. 6, 1391–1411.
Fields, R.D., Eshete, F., Stevens, B., Itoh, K., 1997. Action potential-dependent regulation of gene expression: temporal specificity in ca2+, cAMP-responsive element binding proteins, and mitogen-activated protein kinase signaling. J. Neurosci. 17, 7252–7266.
Frankland, P.W., Bontempi, B., Talton, L.E., Kaczmarek, L., Silva, A.J., 2004. The involvement of the anterior cingulate cortex in remote contextual fear memory. Science 304, 881–883.
Frenois, F., Stinus, L., Di Blasi, F., Cador, M., Le Moine, C., 2005. A specific limbic circuit underlies opiate withdrawal memories. J. Neurosci. 25, 1366–1374.
Gomez-Milanes, I., Almela, P., Garcia-Carmona, J.A., Garcia-Gutierrez, M.S., Aracil-Fernandez, A., Manzanares, J., Milanes Maquilon, M.V., Laorden, M.L., 2012. Accumbal dopamine, noradrenaline and serotonin activity after naloxone-conditioned place aversion in morphine-dependent mice. Neurochem. Int. 61, 433–440.
Gompf, H.S., Mathai, C., Fuller, P.M., Wood, D.A., Pedersen, N.P., Saper, C.B., Lu, J., 2010. Locus ceruleus and anterior cingulate cortex sustain wakefulness in a novel environment. J. Neurosci. 30, 14543–14551.
Gugusheff, J.R., Ong, Z.Y., Muhlhausler, B.S., 2014. Naloxone treatment alters gene expression in the mesolimbic reward system in 'junk food' exposed offspring in a sex-specific manner but does not affect food preferences in adulthood. Physiol. Behav. 133, 14–21.
Han, C.J., O'Tuathaigh, C.M., van Trigt, L., Quinn, J.J., Fanselow, M.S., Mongeau, R., Koch, C., Anderson, D.J., 2003. Trace but not delay fear conditioning requires attention and the anterior cingulate cortex. Proc. Natl. Acad. Sci. U. S. A. 100, 13087–13092.
Hering, H., Sheng, M., 2003. Activity-dependent redistribution and essential role of cortactin in dendritic spine morphogenesis. J. Neurosci. 23, 11759–11769.
Hoover, W.B., Vertes, R.P., 2007. Anatomical analysis of afferent projections to the medial prefrontal cortex in the rat. Brain Struct. Funct. 212, 149–179.
Hyman, S.E., Malenka, R.C., Nestler, E.J., 2006. Neural mechanisms of addiction: the role of reward-related learning and memory. Annu. Rev. Neurosci. 29, 565–598.
Jones, B.F., Witter, M.P., 2007. Cingulate cortex projections to the parahippocampal region and hippocampal formation in the rat. Hippocampus 17, 957–976.
Jovicich, J., Peters, R.J., Koch, C., Braun, J., Chang, L., Ernst, T., 2001. Brain areas specific for attentional load in a motion-tracking task. J. Cogn. Neurosci. 13, 1048–1058.
Kitamura, T., Ogawa, S.K., Roy, D.S., Okuyama, T., Morrissey, M.D., Smith, L.M., Redondo, R.L., Tonegawa, S., 2017. Engrams and circuits crucial for systems consolidation of a memory. Science 356, 73–78.
Kronforst-Collins, M.A., Disterhoft, J.F., 1998. Lesions of the caudal area of rabbit medial prefrontal cortex impair trace eyeblink conditioning. Neurobiol. Learn. Mem. 69, 147–162.
Li, M., Hou, Y.Y., Lu, B., Chen, J., Chi, Z.Q., Liu, J.G., 2009. Expression pattern of neural synaptic plasticity marker-Arc in different brain regions induced by conditioned drug withdrawal from acute morphine-dependent rats. Acta Pharmacol. Sin. 30, 282–290.
Li, Z., Luan, W., Chen, Y., Chen, M., Dong, Y., Lai, B., Ma, L., Zheng, P., 2011. Chronic morphine treatment switches the effect of dopamine on excitatory synaptic transmission from inhibition to excitation in pyramidal cells of the basolateral amygdala. J. Neurosci. 31, 17527–17536.
Lucas, M., Frenois, F., Vouillac, C., Stinus, L., Cador, M., Le Moine, C., 2008. Reactivity and plasticity in the amygdala nuclei during opiate withdrawal conditioning: differential expression of c-fos and arc immediate early genes. Neuroscience 154, 1021–1033.
Lucas, M., Frenois, F., Cador, M., Le Moine, C., 2012. Remodeling of the neuronal circuits underlying opiate-withdrawal memories following remote retrieval. Neurobiol. Learn. Mem. 97, 47–53.
Maviel, T., Durkin, T.P., Menzaghi, F., Bontempi, B., 2004. Sites of neocortical reorganization critical for remote spatial memory. Science 305, 96–99.
Muir, J.L., Everitt, B.J., Robbins, T.W., 1996. The cerebral cortex of the rat and visual attentional function: dissociable effects of mediofrontal, cingulate, anterior dorsolateral, and parietal cortex lesions on a five-choice serial reaction time task. Cereb. Cortex 6, 470–481.
Nakashiba, T., Buhl, D.L., McHugh, T.J., Tonegawa, S., 2009. Hippocampal CA3 output is crucial for ripple-associated reactivation and consolidation of memory. Neuron 62, 781–787.
Nakayama, D., Iwata, H., Teshirogi, C., Ikegaya, Y., Matsuki, N., Nomura, H., 2015. Long-delayed expression of the immediate early gene Arc/Arg3.1 refines neuronal circuits to perpetuate fear memory. J. Neurosci. 35, 819–830.
Paxinos, G., Watson, C., 2001. The mouse brain in stereotaxic coordinates, 2nd ed. Academic Press, San Diego, CA.
Piper, M.E., 2015. Withdrawal: Expanding a Key Addiction Construct. Nicotine Tob. Res. 17, 1405–1415.
Plath, N., Ohana, O., Dammermann, B., Errington, M.L., Schmitz, D., Gross, C., Mao, X., Engelsberg, A., Mahlke, C., Welzl, H., Kobalz, U., Stawrakakis, A., Fernandez, E., Waltereit, R., Bick-Sander, A., Therstappen, E., Cooke, S.F., Blanquet, V., Wurst, W., Salmen, B., Bosl, M.R., Lipp, H.P., Grant, S.G., Bliss, T.V., Wolfer, D.P., Kuhl, D., 2006. Arc/Arg3.1 is essential for the consolidation of synaptic plasticity and memories. Neuron 52, 437–444.
Rajasethupathy, P., Sankaran, S., Marshel, J.H., Kim, C.K., Ferenczi, E., Lee, S.Y., Berndt, A., Ramakrishnan, C., Jaffe, A., Lo, M., Liston, C., Deisseroth, K., 2015. Projections from neocortex mediate top-down control of memory retrieval. Nature 526, 653–659.
Robbins, T.W., 2002. The 5-choice serial reaction time task: behavioural pharmacology and functional neurochemistry. Psychopharmacology 163, 362–380.
Rogers, R.D., Baunez, C., Everitt, B.J., Robbins, T.W., 2001. Lesions of the medial and lateral striatum in the rat produce differential deficits in attentional performance. Behav. Neurosci. 115, 799–811.
Sheng, H.Z., Fields, R.D., Nelson, P.G., 1993. Specific regulation of immediate early genes by patterned neuronal activity. J. Neurosci. Res. 35, 459–467.
Sigler, A., Oh, W.C., Imig, C., Altas, B., Kawabe, H., Cooper, B.H., Kwon, H.B., Rhee, J.S., Brose, N., 2017. Formation and maintenance of functional spines in the absence of presynaptic glutamate release. Neuron 94 (304–311), e304.
Tzingounis, A.V., Nicoll, R.A., 2006. Arc/Arg3.1: linking gene expression to synaptic plasticity and memory. Neuron 52, 403–407.
Valverde, O., Tzavara, E., Hanoune, J., Roques, B.P., Maldonado, R., 1996. Protein kinases in the rat nucleus accumbens are involved in the aversive component of opiate withdrawal. Eur. J. Neurosci. 8, 2671–2678.
Weible, A.P., McEchron, M.D., Disterhoft, J.F., 2000. Cortical involvement in acquisition and extinction of trace eyeblink conditioning. Behav. Neurosci. 114, 1058–1067.