Inputs from paraventricular nucleus of thalamus and locus coeruleus contribute to the activation of central nucleus of amygdala during context-induced retrieval of morphine withdrawal memory

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1. Introduction

Drug addiction is a chronic brain disorder characterized by compulsive drug seeking and use (Borelli, 2009). The most troublesome problem of drug addiction is a high rate of drug relapse. One major factor that contributes to the relapse is conditioned context-induced retrieval of drug-withdrawal memory. Therefore, understanding how conditioned context induces the retrieval of drug-withdrawal memory is of importance to develop new therapeutic approaches to prevent drug relapse.

Previous studies have found that conditioned context activates a number of brain regions, such as the basolateral amygdala (BLA) (Song et al., 2019), the CA1 of dorsal hippocampus and the postrhinal cortex (POR) (Ma et al., 2020), to participate in conditioned context-induced retrieval of morphine-withdrawal memory. In addition, some studies reported that conditioned context also could activate the central nucleus of the amygdala lateral division (CeL) (Baidoo et al., 2020; Li et al., 2009; Lucas et al., 2008) and the excitotoxic lesion of the CeL could inhibit conditioned context-induced retrieval of morphine-withdrawal memory (Watanabe et al., 2002). These evidences suggest that conditioned context also activates the CeL to participate in conditioned context-induced retrieval of morphine-withdrawal memory. However, what source of input that activates the CeL during conditioned context-induced retrieval of morphine-withdrawal memory remains to be elucidated.

In this paper, we used immunohistochemical method to examine the influence of conditioned context on the expression of c-Fos, a marker of neuronal activity (Dragunow and Faull, 1989), in paraventricular nucleus of thalamus (PVT), CeL and locus coeruleus (LC) neurons. After combining with retrograde labeling technology, we could also examine...
c-Fos expression in PVT-CeL and LC-CeL projection neurons. Then we studied the role of this activation in conditioned context-induced retrieval of morphine withdrawal memory by examining the influence of inactivation of these brain regions by local microinjection of γ-aminobutyric acid A receptor (GABA_A receptor) agonist muscimol to PVT, CeL and LC in morphine withdrawn mice. In addition, we applied chemogenetic method to examine the influence of inactivation of neural circuits including PVT-CeL and LC-CeL in morphine withdrawn mice. In order to eliminate a possible influence of estrogen, only male mice were chosen for the study. Our results suggest that CeL plays an important role in conditioned context-induced retrieval of morphine withdrawal memory. In addition, PVT and LC are two upstream brain regions of CeL. Inputs from these two upstream brain regions to the CeL contribute to the activation of CeL during the retrieval of conditioned context-induced morphine withdrawal memory.

2. Materials and methods

2.1. Experimental animals

Male adult (8–12 weeks) C57BL/6 J mice were housed in a 12-h light/12-h dark cycle in an environment with suitable humidity and temperature. Water and food were freely available. All experimental procedures accorded with Fudan University and international guidelines on the ethical usage of animals. We made great efforts to minimize animal suffering and reduce the number of animals sacrificed.

2.2. Animal surgery

Mice were anesthetized with ketamine and xylazine (160 mg/kg and 12 mg/kg body weight, respectively) before the stereotaxic surgery. For retrograde labeling experiments, mice were bilaterally injected cholera toxin subunit B 555 (CTB-555, USA) into the CeL [antero-posterior (AP), –1.22 mm; mediolateral (ML), ±2.9 mm; dorsoventral (DV), –4.6 mm]. We injected 0.4 μl CTB-555 in each side for 5 min. For in vivo chemogenetic inhibition in conditioned place aversion (CPA) experiments, mice were bilaterally injected AAV-hSyn-mCherry-IRESCre (2.45 × 10^12 vector genomes/ml, Taitool Bioscience, China) 0.4 μl in each side for 5 min in the CeL. Meanwhile, 0.4 μl AAV-hSyn-DIO-hM4D (GI)-EGFP (3.92 × 10^12 vector genomes/ml, Taitool Bioscience, China) was injected into PVT [AP, –1.46 mm; ML, 0 mm; DV, –3.5 mm] for 5 min. For all the above stereotaxic injection experiments, the injection needles would be remained for the additional 10 min after the injection to allow the diffusion of the injected solutions. After the virus injection, all mice will be returned to the home cages for recovery and virus expression for 4 weeks.

For the implantation of the stainless-steel guide cannulae, we embedded two stainless-steel guide cannulae into the CeL or LC [AP, –5.34 mm; ML, ±0.75 mm; DV, –3.8 mm] bilaterally or embedded a single cannula into the PVT. Generally, a cannula was embedded 1 mm above the targeted brain region. Cannulae were immobilized on the skull with anchoring screws and dental cement. To prevent occlusion, stainless-steel stylets were inserted into the cannulae. After the surgery, mice were allowed to recover for a week until the behavioral assay.

2.3. Chronic morphine treatment

Mice were treated with morphine (Shenyang No. 1 Pharmaceutical Factory, China), as described before (Desjardins et al., 2008). Briefly, repeated intraperitoneal injections of morphine twice daily could induce morphine dependence in mice. We performed morphine intraperitoneal injections in 9:00 and 19:00 respectively for 5 days. Morphine doses were progressively increased over 5 days from 10 mg/kg to 40 mg/kg. Everyday dose was specifically as follows: Day 1, 2 × 10 mg/kg; Day 2, 2 × 20 mg/kg; Day 3, 2 × 30 mg/kg; Day 4 and Day 5, 2 × 40 mg/kg. Control mice were treated with saline.

2.4. Conditioned place aversion

CPA was performed using a three-compartment place conditioning apparatus (Med Associates, USA) with distinct visual and tactile context. The procedure was similar to that described previously (Gracy et al., 2001; Jin et al., 2004; Li et al., 2009). Thorough cleaning would be performed to the chamber and the drop pan with 75% ethanol before each behavioral session. The CPA procedure triggered naloxone-precipitated morphine withdrawal aversion following chronic morphine treatment. As shown in Fig. 1A, the CPA procedure consisted of four phases: pre-test (Day 1), drug treatment (Day 2 to Day 6), conditioning (Day 7 to Day 10) and post-test (Day 11).

On pre-test day (Day 1), an initial preference test was subjected to mice to evaluate their baseline preference. Mice exhibiting strong unconditioned preference (>75% of the session time) or aversion (<25% of the session time) for any compartment would be eliminated from the study. All mice with unbiased preferences were randomly divided into six groups: saline + saline, morphine + saline, saline + naloxone, morphine + naloxone (no retrieval), morphine + naloxone (withdrawal) and morphine + naloxone.

On drug treatment days (Day 2 to Day 6), morphine treatment was performed to mice in morphine + saline, morphine + naloxone (no retrieval), morphine + naloxone (withdrawal) and morphine + naloxone groups. Mice in saline + saline and saline + naloxone groups were treated with saline.

On conditioning days (Day 7 to Day 10), for each mouse [with the exception of mice from morphine + naloxone (withdrawal) group], morphine withdrawal was paired with the compartment that it spent more time in (baseline preference side) during the pre-test period. On Day 7 and Day 9, mice in the morphine + naloxone group and morphine + naloxone (no retrieval) group were intraperitoneally injected naloxone at the dose of 0.3 mg/kg 2 h after morphine injection (40 mg/kg, intraperitoneally) to induce enhanced withdrawal and confined in their morphine withdrawal-paired compartment for 20 min. Similar treatments were applied to mice in other groups. For mice in morphine + saline group, we replaced naloxone by same dose of saline. For mice in saline + naloxone group, we replaced morphine by same dose of saline. For mice in saline + saline group, we replaced both morphine and naloxone with same doses of saline. On alternating days (Day 8 and Day 10), mice in the morphine + naloxone group, morphine + saline group and morphine + naloxone (no retrieval) group were intraperitoneally injected 0.1 ml saline 2 h after morphine injection (40 mg/kg, intraperitoneally) and confined in their saline withdrawal-paired compartment for 20 min. Mice in saline + naloxone group and saline + saline group were intraperitoneally injected 0.1 ml saline 2 h after saline injection and confined in their saline withdrawal-paired compartment for 20 min. Specially, to isolate responses induced by contextual stimuli, mice from morphine + naloxone (withdrawal) group would not be confined in either morphine withdrawal-paired compartment or saline withdrawal-paired compartment. Instead, they would be returned to their home cages after injections of naloxone on conditioning days or saline on alternating days. On post-test day (Day 11), each mouse [with the exception of mice from morphine + naloxone (no retrieval) group] was placed in the same apparatus for 15 min to evaluate place aversion response. CPA score was defined as the value of the time the mouse spent in the naloxone-paired compartment subtracted that in the saline-paired compartment. CPA score was defined as the value of the time the mouse spent in the naloxone-paired compartment subtracted that in the saline-paired compartment. CPA score was defined as the value of the time the mouse spent in the naloxone-paired compartment subtracted that in the saline-paired compartment. CPA score was defined as the value of the time the mouse spent in the naloxone-paired compartment subtracted that in the saline-paired compartment. CPA score was defined as the value of the time the mouse spent in the naloxone-paired compartment subtracted that in the saline-paired compartment.
Fig. 1. The influence of conditioned context on the expression of c-Fos in CeL neurons. (A) Experimental timeline for CPA procedure. (B) Average CPA score in saline + saline, morphine + saline, saline + naloxone, morphine + naloxone (withdrawal) and morphine + naloxone (no retrieval) groups [n = 10 mice in saline + saline, morphine + saline, saline + naloxone and morphine + naloxone (no retrieval) groups and n = 11 in morphine + naloxone group; two-way ANOVA, ***P < 0.001]. (C) c-Fos positive neurons in the CeL in saline + saline, morphine + saline, saline + naloxone, morphine + naloxone (no retrieval), morphine + naloxone (withdrawal) and morphine + naloxone groups (green colored). (D) c-Fos positive neurons/mm² in the CeL in saline + saline, morphine + saline, saline + naloxone, morphine + naloxone (no retrieval), morphine + naloxone (withdrawal) and morphine + naloxone groups [n = 5 mice in saline + saline, morphine + saline, morphine + naloxone (no retrieval), morphine + naloxone (withdrawal) and morphine + naloxone groups; n = 6 mice in saline + naloxone and morphine + naloxone (no retrieval) groups; one-way ANOVA, ***P < 0.001]. Data are means ± SEM.
(no retrieval) group were prepared for immunohistochemistry analysis instead of CPA analysis. All CPA procedures with five control groups were almost identical as described above, except drug treatment with saline + saline, saline + naloxone, or morphine + saline instead of morphine + naloxone. In addition, mice from morphine + naloxone (withdrawal) group were absent from the conditioned context while mice from morphine + naloxone (no retrieval) group were excluded from the post-test (re-exposure to the conditioned context).

2.5. In vivo chemogenetic inhibition for CPA

Four weeks after the bilateral injection of AAV-hSyn-mCherry-IRESGAGA-Cre in the CeL and the injection of AAV-hSyn-DIO-hM4D (G1) EGFPP in the PDT, mice were subjected to the behavioral assay. On the post-test day, mice in the morphine + naloxone (CNO) group were absent from the conditioned context while the (no retrieval) group were prepared for immunohistochemistry analysis.

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2.6. Microinjection through stainless-steel guide cannulae

During the microinjection, the stainless-steel stylets were removed and replaced by stainless-steel injection needles with a certain pharmacy inside. The injection needle was connected to a 1 μl microsyringe (Hamilton, USA) by a polyethylene tube containing mineral oil (Sigma, USA) and controlled by a syringe pump (kd Scientific, USA). Prazosin and propranolol were prepared as 5 mM solutions while muscimol was prepared as 0.55 mM solution. Before the post-test, 0.4 μl of these solutions or 0.9% saline were injected to targeted brain regions in one minute. The injection needle would be remained in the guide cannula for additional one minute to allow the diffusion of these pharmacies.

2.7. Immunohistochemistry and imaging

Ninety minutes after the post-test, all mice were deeply 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 (PFA) in phosphate buffered saline (PBS) (pH 7.4). The brains were removed and fixed in 4% PFA for 48 h. Afterwards, the brains were cut in 50-μm coronal sections using a vibra-tome (Leica, Germany) and collected in PBS. To do immunohistochemistry assay, brain sections were rinsed in PBS for three times. Each wash lasted for 5 min. Then these brain sections were incubated with blocking solutions which contained 10% normal goat serum and 0.3% Triton-X 100 in PBS at 37 °C for 2 h. Afterwards, brain sections were incubated with the first antibody solution in 4 °C overnight. For the analysis of c-Fos expression, the first antibody solution consisted of guinea pig anti c-Fos antibody (226–004, Synaptic Systems, Germany) which was diluted 1:500 with the blocking solution mentioned above. Subsequently, the brain sections were rinsed in PBS for three times (5 min for each wash), followed by incubation of goat antiguinea pig IgG H&L (Biotin) antibody (ab6907, abcam, USA) solution diluted 1:400 in PBS containing 10% normal goat serum. The incubation lasted for 1 h at 37 °C. Then they were rinsed in PBS for three times (5 min for each wash) and incubated with 1:1000 Streptavidin Alexa Fluor 647 (ab272190, abcam, USA) diluted with PBS containing 10% normal goat serum for 1 h at 37 °C. Finally, immunolabeled sections were rinsed in PBS for three times (5 min for each wash), mounted on glass slides, and imaged by fluorescence microscope (Olympus, Japan). A series of slices containing the PDT and CeL were imaged by fluorescence microscopy with a 20× immersion lens and collected at a resolution of 1024 × 1024 pixels. The same laser and scanning settings were applied for all fluorescence images within an experiment to allow comparison among groups. Generally, coronal sections from five to eight mice were applied for quantitative analysis. A series of images were captured from the fluorescence microscope and converted to 8-bit gray scale images. Quantification of c-Fos, CTB-555 and c-Fos + CTB-555 co-labeled neurons were measured by the Image-Pro Plus 6.0 software.

For the analysis of the co-localization of α1 adrenoreceptor and α-amino-3-hydroxy-5-methyl-4-isoxazole-propionicacid receptor (AMPA receptor), the protocol was similar to that of the analysis of c-Fos. But we used rabbit anti α1 adrenoreceptor antibody (ab137123, abcam, USA, 1:500) and mouse anti AMPA receptor antibody (sc-55,509, Santa Cruz, USA, 1:500) to respectively combine with α1 adrenoreceptor and AMPA receptor. Subsequently, goat anti rabbit IgG H&L Alexa Fluor 594 antibody (ab150080, abcam, USA, 1:500) and goat anti mouse IgG H&L Alexa Fluor 488 antibody (ab150113, abcam, USA, 1:500) were respectively applied to label α1 adrenoreceptor and AMPA receptor. In addition, slices containing the CeL were imaged by the confocal multiphoton microscope 60×/1.33 water (AIR-MP, Nikon, Japan) and collected at a resolution of 1024 × 1024 pixels. Each slice was imaged at 0.3 μm intervals over a total depth of 1.5 μm for a total of 5 optical sections. Quantification of density of co-localization of α1 adrenoreceptor and AMPA receptor were measured by the ImageJ 1.28u software.

2.8. Western blot

The brain tissues containing the CeL 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 12000 rpm for 10 min at 4 °C. The samples were treated with the SDS sample buffer at 100 °C for 10 min, loaded on a 10% SDS polyacrylamide gel and blotted to a nitrocellulose (NC) membrane. The NC membrane was cut into two pieces between the 70 kD band and 55 kD band of the protein marker. At first, these two membranes were incubated with blocking solution (Beyotime, China) for 1 h at room temperature. Subsequently, the first membrane containing proteins whose molecular weights were over 70 kD was incubated with a rabbit anti-phosphorylated AMPA receptor serum (serine 831) antibody (ab109464, abcam, USA) at 4 °C overnight. Likewise, the second membrane containing proteins with molecular weights lower than 55 kD was incubated with a mouse anti β-actin antibody (sc-47,778, Santa Cruz, USA) at 1:1000 at 4 °C overnight. Subsequently, goat anti rabbit secondary antibody (1:10000) and IRDye 800 CW goat anti mouse secondary antibody (1:10000) for 1 h at room temperature. Finally, after rinsing these two membranes for three times (5 min for each wash) with 1 × TBST, we acquired images with LI-COR Odyssey system.

2.9. Statistical analysis

All data were analyzed with GraphPad Prism 5 software and shown as mean ± SEM. Statistical significance was determined by applying Student’s t-test for comparison between two groups and analyses of variance (ANOVA) for comparison of three or more groups. One-way ANOVA was followed by Tukey’s multiple comparison test and two-way ANOVA was followed by Bonferroni post hoc test. In all cases, n refers to the number of animals in a group. For all results, P < 0.05 was considered statistically significant.

3. Results

3.1. CeL plays an important role in conditioned context-induced retrieval of morphine-withdrawal memory

To study the role of CeL in conditioned context-induced retrieval of morphine withdrawal memory, we studied whether conditioned context could activate the CeL by examining the expression of c-Fos in morphine.
withdrawal mice. Mice were randomly divided into six groups: saline + saline, saline + saline, saline + naloxone, morphine + naloxone (no retrieval), morphine + naloxone (withdrawal) and morphine + naloxone groups. At first, mice were subjected to behavioral paradigms (Fig. 1A). Results showed that mice in morphine + naloxone group spent less time in the morphine withdrawal-paired compartment than that in the saline-paired compartment during the post-test, demonstrating that mice in this group exhibited a strong aversion to the morphine withdrawal-paired compartment. Meanwhile, mice from other control groups did not exhibit significant aversion to either compartment. The average CPA score of the morphine + naloxone group was 206.86 ± 13.30 s, which was statistically different from those of the saline + saline group (143.78 ± 34.47 s), the saline + saline group (90.24 ± 25.59 s), the saline + naloxone group (−56.56 ± 17.59 s), and the morphine + naloxone (withdrawal) group (−25.79 ± 8.22 s) (two-way ANOVA, P < 0.001; Fig. 1B). After behavioral assay, animals were sacrificed and the effect of conditioned context on the expression of c-Fos was examined. It was obvious that the expression of c-Fos in the CeL was significantly increased in the morphine + naloxone group after the re-exposure to conditioned context compared with saline + saline group. However, this index did not change significantly in the morphine + saline group, the saline + naloxone group, the morphine + naloxone (no retrieval) group and the morphine + naloxone (withdrawal) group while comparing to the saline + saline group (Fig. 1C). The average c-Fos positive neurons/ mm² in the CeL was 1575 ± 198 neurons/mm² in the morphine + naloxone group, which was significantly higher than that in the saline + saline group (251 ± 30 neurons/mm²), the morphine + saline group (247 ± 114 neurons/mm²), the saline + naloxone group (257 ± 35 neurons/mm²), the morphine + naloxone (no retrieval) group (274 ± 37 neurons/mm²) and the morphine + naloxone (withdrawal) group (24 ± 15 neurons/mm²) (one-way ANOVA, P < 0.001; Fig. 1D). This result indicates that conditioned context can activate CeL neurons in morphine withdrawal mice to a great extent than that in control groups.

On this basis, we examined the influence of the inactivation of the CeL by the local injection of GABA_A receptor agonist muscimol on the CPA score. At first, we bilaterally embedded stainless-steel guide cannulae into the CeL to prepare for the injection of muscimol (Fig. 2A). After recovering for a week, mice were randomly divided into three groups: saline + saline, morphine + naloxone (vehicle) and morphine + naloxone (muscimol) groups and subjected to the behavioral paradigms. 30 min before the post test, injection needles containing muscimol were inserted into the guide cannulae to inject muscimol (Fig. 2B). Results showed that mice in morphine + naloxone (vehicle) group spent less time in the morphine withdrawal-paired compartment than that in the saline-paired compartment during the post-test. In opposite, mice in saline + saline group and morphine + naloxone (muscimol) group spent more time in the morphine withdrawal-paired compartment than that in the saline-paired compartment during the post-test (Fig. 2C). These results illustrated that the retrieval of the conditioned context-induced morphine withdrawal memory was inhibited after the injection of CeL. The average CPA score of the morphine + naloxone (muscimol) group was 198.50 ± 149.28 s, which was statistically different from that of the morphine + naloxone (vehicle) group (−105.11 ± 27.07 s, two-way ANOVA, P < 0.05). However, there was no statistical difference of CPA score between the morphine + naloxone (muscimol) group and saline + saline group (68.55 ± 27.23 s, two-way ANOVA, P > 0.05).

Fig. 2. The influence of the inactivation of CeL during the retrieval of the conditioned context-induced morphine withdrawal memory on the CPA score. (A) Diagram of muscimol injection site in the CeL. (B) Experimental timeline for CPA procedure. (C) Average CPA score in saline + saline, morphine + naloxone (vehicle) and morphine + naloxone (muscimol) groups. [n = 10 mice in each group; two-way ANOVA, *P < 0.05]. Data are means ± SEM.
These results show that CeL plays an important role in the retrieval of the conditioned context-induced morphine withdrawal memory.

3.2. PVT is one upstream brain region of the activation of CeL during conditioned context-induced retrieval of morphine-withdrawal memory

To determine whether the PVT is an upstream brain region of the activation of the CeL during the retrieval of morphine withdrawal memory, we examined the influence of the inactivation of the PVT by the local injection of muscimol on the expression of c-Fos in the CeL. In the beginning, we embedded stainless-steel guide cannulae in the PVT to prepare for the injection of muscimol (Fig. 3A). Similarly, mice were randomly divided into three groups: saline + saline, morphine + naloxone (vehicle) and morphine + naloxone (muscimol) groups with recovery lasting for a week. Afterwards, mice were subjected to the behavioral paradigms. Thirty minutes before the post test, muscimol was injected into the PVT through the injection needles (Fig. 3B). The results of c-Fos examination showed that the expression of c-Fos in the CeL after the re-exposure to the conditioned context was significantly decreased in the morphine + naloxone (muscimol) group in comparison of that of the morphine + naloxone (vehicle) group. On the other hand, there was no statistical difference between the saline + saline group and morphine + naloxone (muscimol) group in terms of the expression of c-Fos in the CeL (Fig. 3C). The average c-Fos positive neurons/mm² in the CeL was 203 ± 17 neurons/mm² in the morphine + naloxone (muscimol) group, which was significantly different from that of the morphine + naloxone (vehicle) group (388 ± 37 neurons/mm², one-way ANOVA, P < 0.01). However, it did not show statistical difference while comparing to that of the saline + saline group (239 ± 25 neurons/mm², one-way ANOVA, P > 0.05; Fig. 3D). During the post test, mice in saline + saline group spent more time in the morphine withdrawal-paired compartment than that in the saline-paired compartment. As our expectation, mice in this group did not exhibit a strong aversion to either compartment. Meanwhile, mice in morphine + naloxone (vehicle) group and morphine + naloxone (muscimol) group spent less time in the morphine withdrawal-paired compartment than that in the saline-paired compartment. Although mice in these two groups exhibited an aversion to the morphine withdrawal-paired compartment, significant difference still remained between the CPA score of the morphine + naloxone (vehicle) group and that of the morphine + naloxone (muscimol) group, demonstrating that the inhibition of PVT attenuated the aversion to the morphine withdrawal-paired compartment in a statistically different level. The average CPA score of the morphine + naloxone (muscimol) group was −69.37 ± 64.73 s, which was statistically different from that of the morphine + naloxone (vehicle) group (−213.73 ± 17.46 s, two-way ANOVA, P < 0.05). However, there was no significant difference between it and that of the saline + saline group (47.15 ± 30.20 s, two-way ANOVA, P > 0.05; Fig. 3E). These results indicated that the inhibition of PVT could cause the attenuation of the activation of CeL, the downstream of PVT.

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Fig. 3. The influence of the inactivation of PVT on the expression of c-Fos in the CeL and the CPA score during the retrieval of the conditioned context-induced morphine withdrawal memory. (A) Diagram of muscimol injection site in the PVT. (B) Experimental timeline for CPA procedure. (C) c-Fos positive neurons in the CeL in saline + saline group, morphine + naloxone (vehicle) group and morphine + naloxone (muscimol) group (green colored). Scale bars, 100 μm. CeL regions enclosed by white boxes were shown in a higher magnification in upper left square images. Scale bars, 10 μm. (D) c-Fos positive neurons/mm² in the CeL in saline + saline group, morphine + naloxone (vehicle) group and morphine + naloxone (muscimol) group. [n = 10 in saline + saline group, n = 12 in morphine + naloxone (vehicle) group and n = 13 in morphine + naloxone (muscimol) group]. One-way ANOVA, ***P < 0.01 (E) Average CPA score in saline + saline group, morphine + naloxone (vehicle) group and morphine + naloxone (muscimol) group. [n = 10 in saline + saline group, n = 11 in morphine + naloxone (vehicle) group and morphine + naloxone (muscimol) group]. Two-way ANOVA, *P < 0.05. Data are means ± SEM.
In further, to find out if PVT-CeL projection neurons were activated during the retrieval of conditioned context-induced morphine withdrawal memory, we injected CTB-555 into the CeL to retrograde label PVT-CeL projection neurons and examine the co-labeling percentage of CTB-555 and c-Fos in the PVT (Fig. 4A). Mice were randomly divided into six groups: saline + saline, morphine + saline, saline + naloxone, morphine + naloxone (withdrawal) and morphine + naloxone groups.

After recovering from the surgery of CTB-555 injection, mice were subjected to the behavioral paradigms (Fig. 4B). The results of the post test showed that the average CPA score of morphine + naloxone group was $-206.86 \pm 13.30$ s, which was statistically different from that of the saline + saline group ($143.78 \pm 34.47$ s), the morphine + saline group ($90.24 \pm 25.59$ s), the saline + naloxone group ($56.56 \pm 17.59$ s), and the morphine + naloxone (withdrawal) group ($25.79 \pm 8.22$ s) (two-

Fig. 4. The influence of conditioned context on the expression of c-Fos in PVT-CeL projection neurons. (A) Diagram of CTB-555 injection site in the CeL. (B) Experimental timeline for CPA procedure. (C) Average CPA score in saline + saline, morphine + saline, saline + naloxone, morphine + naloxone (withdrawal) and morphine + naloxone groups [$n = 10$ mice in saline + saline, morphine + saline, saline + naloxone and morphine + naloxone (no retrieval) groups and $n = 11$ in morphine + naloxone group; two-way ANOVA, **$P < 0.01$]. (C) The c-Fos positive neurons (top line, green colored), the CTB-555 labeling neurons (middle line, red colored) and the co-labeling neurons of c-Fos and CTB-555 (bottom line, yellow colored) in the PVT in saline + saline, morphine + saline, saline + naloxone, morphine + naloxone (no retrieval), morphine + naloxone (withdrawal) and morphine + naloxone groups. Scale bars, 100 µm. PVT regions enclosed by white boxes were shown in a higher magnification in upper left square images. Scale bars, 10 µm. (E) The average number of c-Fos positive neurons/mm² in the PVT in each group [$n = 5$ mice in saline + saline, morphine + saline, saline + naloxone and morphine + naloxone (no retrieval) groups; $n = 6$ mice in saline + naloxone and morphine + naloxone (no retrieval) groups; one-way ANOVA, ***$P < 0.001$]. (F) Average percentage of the coexpression of c-Fos and CTB-555 relative to CTB-555 in the PVT in each group [$n = 5$ mice in saline + saline, morphine + saline, morphine + naloxone (withdrawal) and morphine + naloxone groups; $n = 6$ mice in saline + naloxone and morphine + naloxone (no retrieval) groups; one-way ANOVA, **$P < 0.01$]. Data are means ± SEM.
way ANOVA, \( P < 0.001; \) Fig. 4C). As mice in morphine + naloxone (no retrieval) group was not involved in the post-test, the post test result of this group was not offered. Meanwhile, there was no significant difference observed among several control groups. Thus, only mice in morphine + naloxone group exhibited a strong aversion to the morphine withdrawal-paired compartment. In terms of results about immunohistochemistry, it was obvious that the expression of c-Fos and the co-expression of c-Fos and CTB-555 in the CeL was significantly increased in the morphine + naloxone group after the re-exposure to conditioned context compared with saline + saline group. However, these two above-mentioned indexes did not change significantly in the morphine + saline group, the saline + naloxone group and the morphine + naloxone (withdrawal) group while comparing to the saline + saline group (Fig. 4D). The average c-Fos positive neurons/mm\(^2\) in the PVT was 1021 \( \pm \) 114 neurons/mm\(^2\) in the morphine + naloxone group, which was significantly higher than that in the saline + saline group (484 \( \pm \) 70 neurons/mm\(^2\)), the morphine + saline group (527 \( \pm \) 44 neurons/mm\(^2\)), the saline + naloxone group (616 \( \pm \) 23 neurons/mm\(^2\)), the morphine + naloxone (no retrieval) group (398 \( \pm \) 56 neurons/mm\(^2\)), and the morphine + naloxone (withdrawal) group (50 \( \pm \) 23 neurons/mm\(^2\)) (one-way ANOVA, \( P < 0.001; \) Fig. 4E). The average percentage of the co-expression of c-Fos and CTB-555 relative to CTB-555 in the PVT in the morphine + naloxone group was 22.48 \( \pm \) 4.46\%, which was significantly higher than that in the saline + saline group (6.74 \( \pm \) 1.77\%).

### Results

To determine the role of PVT-CeL projection neurons in the CPA, we examined the effect of the inhibition of PVT-CeL projection neurons to the CPA. We applied chemogenetic inhibition to specially inhibit these PVT-CeL projection neurons. At first, we injected AAV-hSyn-DIO-hM4D (Gi)-EGFP virus into the upstream, namely the PVT and the AAV-hSyn-mCherry-IRES-WGA-Cre virus into the downstream, namely the CeL. The injection sites of these two viruses were shown on Fig. 5A. Four weeks after the virus injection, these mice were randomly divided into three groups: saline + saline, morphine + naloxone (vehicle) and morphine + naloxone (CNO) groups and subjected to behavioral paradigms (Fig. 5B). These mice were divided into three groups. The first group was saline + saline group that would be intraperitoneally injected saline 30 min before the post test. The second group was morphine + naloxone (vehicle) group that would also be intraperitoneally injected saline 30 min before the post test. The third group was morphine + naloxone (CNO) group that would be intraperitoneally injected clozapine-n-oxide (CNO) 30 min before the post test. Results showed that the conditioned context-induced a significant

### Experimental timeline for CPA procedure (Fig. 5B)

- **Surgery**
- **Pre-test**
- **Post-test**
- **Animal sacrificed**

### Diagram of virus injection sites in the PVT and CeL (Fig. 5A)

- **AAV-hSyn-DIO-hM4D (Gi)-EGFP**
- **AAV-hSyn-mCherry-IRES-WGA-Cre**

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Fig. 5. The influence of the inhibition of PVT-CeL projection neurons on the expression of c-Fos in the CeL and the CPA score during the retrieval of the conditioned context-induced morphine withdrawal memory. (A) Diagram of virus injection sites in the PVT and CeL. (B) Experimental timeline for CPA procedure. (C) c-Fos positive neurons in the CeL in saline + saline group, morphine + naloxone (vehicle) group and morphine + naloxone (CNO) group. (green colored). Scale bars, 100 \( \mu \)m. CeL regions enclosed by white boxes were shown in a higher magnification in upper left square images. Scale bars, 10 \( \mu \)m. (D) c-Fos positive neurons/mm\(^2\) in the CeL in saline + saline group, morphine + naloxone (vehicle) group and morphine + naloxone (CNO) group. \( n = 8 \) in saline + saline group, \( n = 6 \) in morphine + naloxone (vehicle) group and \( n = 8 \) in morphine + naloxone (CNO) group. One-way ANOVA, \( *P < 0.05 \) (E) Average CPA score in saline + saline group, morphine + naloxone (vehicle) group and morphine + naloxone (CNO) group. \( n = 10 \) in each group. Two-way ANOVA, \( *P < 0.05 \). Data are means \( \pm \) SEM.
increase in CeL c-Fos expression in morphine + naloxone (vehicle) group while comparing to that of saline + saline group and morphine + naloxone (CNO) group. Meanwhile, a significant difference was also observed between the saline + saline group and the morphine + naloxone (CNO) group in CeL c-Fos expression (Fig. 5C). The average c-Fos positive neurons/mm² in morphine + naloxone (CNO) group was 544 ± 75 neurons/mm², which was significantly different from that of morphine + naloxone (vehicle) group (793 ± 90 neurons/mm², one-way ANOVA, P < 0.05) and saline + saline group (277 ± 25 neurons/mm², one-way ANOVA, P < 0.01, Fig. 5D). In addition, CPA score of CNO treated morphine + naloxone mice (−68.97 ± 78.20 s) also exhibited statistical difference while comparing to that of saline treated morphine + naloxone mice (−299.76 ± 46.67 s, two-way ANOVA, P < 0.05), although CPA scores of both groups mentioned above were negative. In comparison, CPA score of saline + saline group (83.15 ± 22.69 s, Fig. 5E) was positive, as expected. These results were consistent with results of PVT inhibition. Thus, we can conclude that it is the PVT-CeL projection neurons that regulate the c-Fos expression in the CeL and the activation of CeL. Due to the important role of CeL in the regulation of the retrieval of the conditioned context-induced morphine withdrawal memory, once this neural circuit is specially inhibited, the retrieval of the conditioned context-induced morphine withdrawal memory will be partially influenced.

3.3. LC is another upstream brain region of the activation of CeL which may show synergistic effect with PVT during conditioned context-induced retrieval of morphine-withdrawal memory

To determine whether LC plays a role as another upstream brain region of CeL which contributes to the activation of CeL during conditioned context-induced retrieval of morphine-withdrawal memory, we examined the co-labeling percentage of CTB-555 (LC-CeL projection neurons) and c-Fos in the LC. As PVT-CeL projection neurons could only partially regulate the retrieval of conditioned context-induced morphine withdrawal memory, we tried to find out other relevant brain regions, neural circuits and mechanisms. LC was selected as a candidate as it plays a role as a main source of noradrenergic neurons (Aston-Jones and Waterhouse, 2016) while norepinephrine (NE) has been reported as an important regulator of emotions (Benarroch, 2018). In this research, we did not establish new animal models in consideration of animal welfare. Instead, the rest of brain sections in the research about the relationship between PVT-CeL projection neurons and the retrieval of conditioned context-induced morphine withdrawal memory were subjected to the immunohistochemistry assay. The CTB-555 injection site, behavioral paradigms and the result of CPA respectively shown in Fig. 6A, B and C were as identical as those in Fig. 4. The results of immunohistochemistry were similar to those of the former research. It was obvious that the expression of c-Fos and the co-expression of c-Fos and CTB-555 in the LC were significantly increased in the morphine + naloxone group after the re-exposure to conditioned context while comparing to those of the saline + saline group. However, these two above-mentioned indexes did not change significantly in the morphine + saline group, the saline + naloxone group and the morphine + naloxone (withdrawal) group while comparing to those of the saline + saline group (Fig. 6D). The average c-Fos positive neurons/mm² in the LC was 825 ± 83 neurons/mm² in the morphine + naloxone group, which was significantly higher than that in the saline + saline group (364 ± 67 neurons/mm²), the morphine + saline group (471 ± 35 neurons/mm²), the saline + naloxone group (375 ± 43 neurons/mm²), the morphine + naloxone (no retrieval) group (206 ± 40 neurons/mm²), and the morphine + naloxone (withdrawal) group (378 ± 60 neurons/mm²) (one-way ANOVA, P < 0.001, Fig. 6E). The average percentage of the co-expression of c-Fos and CTB-555 relative to CTB-555 in the LC in the morphine + naloxone group was 33.39 ± 3.44%, which was significantly higher than that in the saline + saline group (7.81 ± 2.82%), the morphine + saline group (7.14 ± 2.34%), the saline + naloxone group (16.38 ± 3.27%), the morphine + naloxone (no retrieval) group (8.19 ± 1.58%), and the morphine + naloxone (withdrawal) group (6.00 ± 1.98%) (one-way ANOVA, P < 0.001; Fig. 6F). This result indicates that conditioned context re-exposure can activate LC-CeL projection neurons during morphine withdrawal memory retrieval.

To find out the role of LC in the retrieval of conditioned context-induced morphine withdrawal memory and the relationship between LC and CeL in further, we examined the influence of the inhibition of LC to the retrieval of conditioned context-induced morphine withdrawal memory by locally injecting muscimol into LC. As shown in Fig. 7A, two stainless-steel cannulae were bilaterally embedded into the LC. After recovering for a week, mice were randomly divided into three groups: saline + saline, morphine + naloxone (vehicle) and morphine + naloxone (muscimol) groups and subjected to behavioral procedures (Fig. 7B). In the immunohistochemistry analysis, we evaluated the difference of c-Fos expression in the CeL among saline + saline, morphine + naloxone (vehicle) and morphine + naloxone (muscimol) groups. Results showed that c-Fos expression in the CeL was obviously increasing in morphine + naloxone (vehicle) group while comparing to that of the saline + saline group. Meanwhile, increase of c-Fos expression in the CeL was detected in morphine + naloxone (muscimol) in comparison of that of the saline + saline group (Fig. 7C). The average c-Fos positive neurons/mm² in the CeL was 563 ± 28 neurons/mm² in the morphine + naloxone (muscimol) group, which was significantly different from that in the morphine + naloxone (vehicle) group (825 ± 102 neurons/mm², one-way ANOVA, P < 0.05) and the saline + saline group (302 ± 57 neurons/mm², two-way ANOVA, P < 0.01, Fig. 7D). Likewise, the result of CPA score was almost consistent to that of immunohistochemistry mentioned above. The average CPA score of the morphine + naloxone (muscimol) group was 1.24 ± 0.92 s, which was statistically different from that of the morphine + naloxone (vehicle) group (−305.32 ± 56.46 s, two-way ANOVA, P < 0.001). However, no statistical difference was detected between the average CPA score of the morphine + naloxone (muscimol) group and that of the saline + saline group (60.94 ± 38.14 s, two-way ANOVA, P > 0.05, Fig. 7E). This result suggests that LC plays a role as an upstream of the CeL and takes part in the regulation of the activation of CeL and the retrieval of conditioned context-induced morphine withdrawal memory.

To figure out the related molecular mechanisms of the LC-CeL neural circuit and possible synergistic effects between glutamatergic neurons and noradrenergic neurons, we tried to examine the influence of the inhibition of adrenoreceptors to the retrieval of conditioned context-induced morphine withdrawal memory and phosphorylation of AMPA receptor in the CeL. At first, we hypothesized that it was the noradrenergic neurons projecting from the LC and norepinephrine released from terminals of these neurons that play a role in the regulation of CeL by cooperating with glutamate receptors. To prove this hypothesis, we tried to inhibit these adrenoreceptors and observe the result of CPA score in the beginning. It has been reported that a1 and β adrenoreceptors are excitatory types of adrenoreceptors while a2 adrenoreceptor is an inhibitory type (Otis et al., 2015). Therefore, we selected prazosin, a specific a1 adrenoreceptor antagonist (Mantsch et al., 2010), and propranolol, a specific β adrenoreceptor antagonist (Otis et al., 2015) to respectively inhibit these receptors in the CeL region. We still bilaterally embedded stainless-steel guide cannulae into the CeL to prepare for the injection of these antagonists (Fig. 8A). These mice were randomly divided into four groups, respectively saline + saline, morphine + naloxone (vehicle), morphine + naloxone (propranolol) and morphine + naloxone (prazosin) groups. In the behavioral assay (Fig. 8B), antagonists were injected into the CeL through the injection needles 60 min before the post test. The average CPA score of saline + saline group (65.05 ± 31.17 s) indicated that mice in this group did not show any strong aversion to either compartment as expectation. The average CPA score of morphine + naloxone (vehicle) group was −247.88 ± 45.78 s, which was significantly different from that of the morphine + naloxone (prazosin) group (−97.29 ± 53.28 s, two-way ANOVA, P < 0.05).
A. CTB-555

B. Experimental design diagram

C. Graph showing CPA score over time with different groups.

D. Image of brain section with c-Fos staining.

E. Graph showing c-Fos positive neurons with different groups.

F. Graph showing the percentage of c-Fos+CTB-555/CTB-555 with different groups.

(caption on next page)
Fig. 6. The influence of conditioned context on the expression of c-Fos in LC-CeL projection neurons. (A) Diagram of muscimol injection site in the LC. (B) Experimental timeline for CPA procedure. (C) Average CPA score in saline + saline, morphine + saline, saline + naloxone, morphine + naloxone (withdrawal) and morphine + naloxone (vehicle) group [n = 10 mice in saline + saline, morphine + saline, saline + naloxone and morphine + naloxone (no retrieval) and morphine + naloxone (withdrawal) groups; two-way ANOVA, **P < 0.001]. (D) The c-Fos positive neurons (top line, green colored), the CTB-555 labeling neurons (middle line, red colored) and the co-labeling neurons of c-Fos and CTB-555 (bottom line, yellow colored) in the LC in saline + saline, morphine + saline, saline + naloxone, morphine + naloxone (no retrieval), morphine + naloxone (withdrawal) and morphine + naloxone groups. Scale bars, 100 μm. LC regions enclosed by white boxes were shown in a higher magnification in upper left square images. Scale bars, 10 μm. (E) The average number of c-Fos positive neurons/mm² in the LC in each group [n = 5 mice in saline + saline, morphine + saline, morphine + naloxone (no retrieval) and morphine + naloxone (withdrawal) groups, n = 8 mice in morphine + naloxone and saline + naloxone groups; one-way ANOVA, ***P < 0.001]. Data are means ± SEM.

Fig. 7. The influence of the inhibition of LC on the expression of c-Fos in the CeL and the CPA score during the retrieval of the conditioned context-induced morphine withdrawal memory. (A) Diagram of muscimol injection site in the LC. (B) Experimental timeline for CPA procedure. (C) c-Fos positive neurons in the CeL in saline + saline group, morphine + naloxone (vehicle) group and morphine + naloxone (muscimol) group (green colored). Scale bars, 100 μm. CeL regions enclosed by white boxes were shown in a higher magnification in upper left square images. Scale bars, 10 μm. (D) c-Fos positive neurons/mm² in the CeL in saline + saline group, morphine + naloxone (vehicle) group and morphine + naloxone (muscimol) group. (n = 5 in each group. One-way ANOVA, *P < 0.05 (E) Average CPA score in saline + saline group, morphine + naloxone (vehicle) group and morphine + naloxone (muscimol) group. (n = 11 in saline + saline group, n = 10 in morphine + naloxone (vehicle) group and n = 9 in morphine + naloxone (muscimol) group. Two-way ANOVA, ***P < 0.001]. Data are means ± SEM.

However, no statistical difference was observed between the average CPA score of morphine + naloxone (vehicle) group and that of the morphine + naloxone (propranolol) group (−188.04 ± 13.98 s, two-way ANOVA, P > 0.05; Fig. 8C). This result indicated that noradrenergic neurons projecting to the CeL are likely to partially regulate CeL through α1 adrenoreceptor instead of β adrenoreceptor. Although the post test result of the morphine + naloxone (propranolol) group exhibited a tendency of attenuation of CPA, β adrenoreceptor was not considered to be effective during the retrieval of conditioned context-induced morphine withdrawal memory, as the tendency was not statistically significant. To further study the role of α1 adrenoreceptor, we performed immunohistochemistry assay. Consistent to the result of CPA, the expression of c-Fos in the CeL in morphine + naloxone (vehicle) group was significantly increased while comparing to that in saline + saline group and morphine + naloxone (prazosin) group (Fig. 8D). The c-Fos positive neurons/mm² of morphine + naloxone (vehicle) group (1813 ± 239 neurons/mm²) was significantly different from that of morphine + naloxone (prazosin) group (586 ± 122 neurons/mm², one-way ANOVA, P < 0.01). In addition, the difference between the c-Fos positive neurons/mm² of morphine + naloxone (prazosin) group and that of saline + saline group (260 ± 32 neurons/mm²) was also statistically significant (one-way ANOVA, P < 0.01; Fig. 8E). To find out the relationship between the regulation of glutamatergic neurons and that of noradrenergic neurons, we initially hypothesized that it is a certain synergistic effect that plays a role in the regulations of these two kinds of neurons to the CeL. After the post test, mice were immediately sacrificed. CeL regions were removed and subjected to protein extraction. The protein level of phosphorylated AMPAR serine-831 (p-AMPAR S831)
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**A** Prazosin/Propranolol

**B**

| Procedure | Morphine (mg per kg body weight) | Conditioning (0.3 mg per kg body weight) |
|-----------|---------------------------------|------------------------------------------|
| Surgery   | 10, 20, 30, 40, 60 min          | 24h, 2h, 20min, 24h, 60min              |
| Pre-test  |                                 |                                           |
| Post-test |                                 |                                           |

**C**

![Graph showing CPA Score](image)

- Pre-test
- Post-test

| Condition | CPA Score (S) |
|-----------|---------------|
| S+S       |                |
| M+N       |                |
| Vehicle   |                |
| Propranolol |            |
| Prazosin  |                |

**D**

![Images showing Fos expression](image)

**E**

![Graph showing c-Fos positive Neurons](image)

- S+S
- M+N
- Vehicle
- Prazosin

**F**

![Graph showing p-AMPAR S831 expression](image)

- S+S
- M+N
- Vehicle
- Prazosin

**G**

![Graph showing p-AMPAR (S831) / β-actin expression](image)

- S+S
- M+N
- Vehicle
- Prazosin

(*caption on next page*)
Fig. 8. The influence of the inhibition of α1 adrenoceptor or β adrenoceptor on the c-Fos expression in the CeL, the CPA score and the phosphorylation of AMPA receptor at Serine 831 (p-AMPAR S831) during the retrieval of the conditioned context-induced morphine withdrawal memory and (A) Diagram of prazosin/propranolol injection site in the CeL. (B) Experimental timeline for CPA procedure. (C) Average CPA score in saline + saline group, morphine + naltrexone (vehicle) group, morphine + naltrexone (propranolol) group and morphine + naltroxone (prazosin) group. [n = 10 in saline + saline group, morphine + naltrexone (vehicle) group and morphine + naltroxone (prazosin) group, n = 11 in morphine + naltrexone (propranolol) group. Two-way ANOVA, *P < 0.05]. (D) c-Fos positive neurons in the CeL in saline + saline group, morphine + naltrexone (vehicle) group and morphine + naltroxone (prazosin) group. (E) p-AMPAR S831/β-actin ratio in saline + saline group, morphine + naltroxone (vehicle) group and morphine + naltroxone (prazosin) group [n = 5 in each group]. One-way ANOVA, *P < 0.05. Data are means ± SEM.

were examined among saline + saline, morphine + naltrexone (vehicle) and morphine + naltroxone (prazosin) groups. The expression of p-AMPAR S831 was significantly increasing in morphine + naltrexone (vehicle) while comparing to the other two groups (Fig. 8F). The protein level of p-AMPAR S831 relative to that of β-actin of morphine + naltroxone (vehicle) group was 1.04 ± 0.05, which was significantly different from that of morphine + naltroxone (prazosin) group (0.70 ± 0.12, one-way ANOVA, *P < 0.05) and saline + saline group (0.58 ± 0.03, one-way ANOVA, *P < 0.001; Fig. 8G). These results indicate that noradrenergic neurons take part in the regulation of CeL in the retrieval of conditioned context-induced morphine withdrawal memory through α1 adrenoceptor instead of β α1 adrenoceptor by promoting the phosphorylation of AMPA receptor in Serine 831.

In further, to determine whether α1 adrenoceptor and AMPA receptor converge on same neurons in the CeL, we examine the density of co-localization of α1 adrenoceptor and AMPA receptor in the CeL. Mice were randomly divided into saline + saline group (control group) and morphine + naltroxone group (withdrawal group) were subjected to the behavioral assay (Fig. 9A). After CPA procedures, we performed an immunohistochemical assay to analyze the density of co-localization of α1 adrenoceptor and AMPA receptor in the CeL. The result showed that there was a baseline level of co-localization of α1 adrenoceptor and AMPA receptor in physiological status, indicating that CeL neurons were regulated by glutamatergic neurons and noradrenergic neurons at the same time. However, conditioned context caused an increase of the density of co-localization of α1 adrenoceptor and AMPA receptor significantly (Fig. 9B). The density of co-localization of α1 adrenoceptor and AMPA receptor was significantly increased in withdrawal group (17,880.8 ± 2244.3 puncta/mm2) while comparing to that in control group (10,249.5 ± 790.7 puncta/mm2, unpaired t-test, P < 0.001; Fig. 9C). Meanwhile, statistical significance was also detected between the CPA score of the control group (28.03 ± 20.2 s) and that of the withdrawal group (196.3 ± 28.1 s, two-way ANOVA, *P < 0.001; Fig. 9D). These results suggest that CeL neurons were simultaneously regulated by glutamatergic neurons and noradrenergic neurons. However, conditioned context can induce the increase of the density of co-localization of α1 adrenoceptor and AMPA receptor. Therefore, the synergistic effects of these two kinds of receptors were enhanced. In response, the retrieval of conditioned context-induced morphine withdrawal memory was promoted. Thus, we can conclude that conditioned context can induce the increase the density of co-localization of α1 adrenoceptor and AMPA receptor in the CeL. In further, enhanced synergistic effects of these two kinds of receptors contribute to the retrieval of conditioned context-induced morphine withdrawal memory.

4. Discussion

The main findings of present study are that Baidoo et al., 2020) the PVT is one upstream brain region of the activation of CeL during conditioned context-induced retrieval of morphine-withdrawal memory (Lucas et al., 2008); the LC is another upstream brain region of the activation of CeL, which may have a synergistic effect with PVT during conditioned context-induced retrieval of morphine-withdrawal memory. It has been known that the CeL receives inputs from broad brain regions, such as the bed nucleus of stria terminals, the PVT, the BLA and the LC (Bienkowski and Rinaman, 2013; Fallon et al., 1978; Jones and Yang, 1985; Li and Kiorouac, 2008; Moore and Bloom, 1979; Previtt and Herman, 1998; Uematsu et al., 2015). Here, we examined whether the inputs from the PVT and the LC contribute to the activation of the CeL during context-induced retrieval of morphine withdrawal memory.

The PVT is an important region that participated in conditioned context-induced retrieval of drug withdrawal memory. Zhu et al. reported that after the inhibition of projection neurons from the PVT to the nucleus accumbens (NAC) using optogenetic method, conditioned context-induced retrieval of morphine withdrawal memory was inhibited and the optogenetic activation of projection neurons from the PVT to the NAC induced an aversive response (Zhu et al., 2016). Here, our results showed that conditioned context could activate projection neurons from the PVT to the CeL and the inhibition of this projection neurons using chemical-genetic method also could inhibit conditioned context-induced retrieval of morphine withdrawal memory, suggesting that in addition to the NAC, the CeL was also a downstream projecting brain region from the PVT to participate in conditioned context-induced retrieval of morphine withdrawal memory.

The CeL is highly innervated by norepinephrine afferents from the LC (Asan, 1998). Conditioned context could activate neurons of the LC (Frenois et al., 2005) and increase the extracellular norepinephrine level within the CeL (Watanabe et al., 2003). The microinjection of α1 adrenoceptor antagonist prazosin into the CeL could inhibit conditioned context-induced retrieval of morphine withdrawal memory (Watanabe et al., 2003). The present results showed that the inactivation of LC using muscimol could decrease conditioned context-induced increase in c-Fos expression and conditioned context could activate projection neurons from the LC to CeL. These evidences strongly suggest that the LC is another upstream brain region of the activation of CeL during conditioned context-induced retrieval of morphine withdrawal memory. About adrenoceptor subtypes involved in this process, we also found that if β adrenoceptor antagonist propranolol was at lower dose such as 2 nmol here, it had no influence on conditioned context-induced retrieval of morphine-withdrawal memory, which was consistent with that reported by Takeshi Watanabe et al., who reported that propranolol at 10 nmol had no influence, but only at higher dose of 30 nmol, it had the influence (Watanabe et al., 2003). In addition, here we found that the microinjection of α1 adrenoceptor antagonist prazosin at 2 nmol into the CeL could inhibit conditioned context-induced retrieval of morphine withdrawal memory, suggesting that the activation of α1 adrenoceptor in the CeL by norepinephrinergic input also was involved the activation of neurons of the CeL, which was the first report about the involvement of α1 adrenoceptor in this process. Moreover, our results showed that there was a co-expression of AMPA receptor and α1 adrenoceptor in neurons of the CeL and if the microinjection of α1 adrenoceptor antagonist prazosin into the CeL could inhibit the phosphorylation of AMPA receptor in neurons of the CeL. These evidences suggest that the two inputs from the PVT and the LC may converge on same neurons in the CeL and the input of norepinephrine may have an amplified effect on AMPA receptors by phosphorylating them through α1 adrenoceptor signaling pathway. This was consistent with the statement of α1
adrenoceptor signaling pathway could phosphorylate AMPA receptors (Otis et al., 2015).

In conclusion, the present results confirm that the CeL plays an important role in conditioned context-induced retrieval of morphine-withdrawn memory and find inputs from the PVT and the LC contribute to the activation of the CeL during context-induced retrieval of morphine withdrawal memory. Moreover, the two inputs from the PVT and the LC may converge on same neurons in the CeL and the input of NE may have an amplified effect on AMPA receptors by phosphorylating them through α1 adrenoceptor signaling pathway. This statement is consistent with the conclusion that the activation of α1 adrenoceptors can phosphorylate AMPA receptors via PLC-CaMKII signaling pathway (Otis et al., 2015).

**Author contributions**

Y.W., Y.P. and Z.C., conceptualization, investigation, methodology, data curation, formal analysis, writing - original draft; C.L., X.L., D.C., Y.Y., data curation, software, methodology; P.Z., B.L., funding acquisition, validation, writing - review & editing funding.

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**Fig. 9.** The colocalization of α1 adrenoceptor and AMPA receptor in the CeL in mice on normal condition and the influence of conditioned context to the density of colocalization of α1 adrenoceptor and AMPA receptor (A) Experimental timeline for CPA procedure. (B) AMPA receptor (green colored) and α1 adrenoceptor (red colored) and their co-labeling puncta (yellow colored) in the CeL in saline + saline group and morphine + naloxone group. Scale bars, 50 μm. Regions enclosed by white boxes were shown in a higher magnification in upper left square images. Scale bars, 10 μm. (C) Co-labeling of AMPA receptor and α1 adrenoceptor puncta/mm² in CeL in saline + saline group and morphine + naloxone group [n = 7 in each group. Unpaired t-test, ***P < 0.001]. (D) Average CPA score in saline + saline group and morphine + naloxone group [n = 7 in each group. Two-way ANOVA, ***P < 0.001]. Data are means ± SEM.
Compliance with ethical standards

The authors declare that they have no conflict of interest. All experimental procedures performed in studies performed 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.

Declaration of Competing Interest

The authors declare that they have no competing financial interest.

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