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Supplementary Materials for

Crucial role of feedback signals from prelimbic cortex to basolateral amygdala in the retrieval of morphine withdrawal memory

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Supplementary Materials and Methods

***In vivo* chemical-genetic approach for CPA.** For *in vivo* chemical-genetic inhibition of cell bodies of BLA neurons projecting to the PrL in CPA experiments, mice were injected with AAV-hSyn-mCherry-IRES-WGA-Cre virus (4.28×10^{12} vector genomes/ml, Neuron Biotech Company, China) bilaterally into the PrL and AAV-hSyn-DIO-hM4Di(Gi)-mCherry virus (3.44×10^{12} vector genomes/ml, Neuron Biotech Company, China) bilaterally into the BLA at a volume of 0.3 μl for 10 min. For *in vivo* chemical-genetic inhibition of cell bodies of BLA neurons projecting to the infralimbic (IL) in CPA experiments, mice were injected with AAV-hSyn-mCherry-IRES-WGA-Cre virus (4.28×10^{12} vector genomes/ml, Neuron Biotech Company, China) bilaterally into the IL (AP, +1.78 mm; ML, ±0.30 mm; DV, -3.00 mm) and AAV-hSyn-DIO-hM4Di(Gi)-mCherry virus (3.44×10^{12} vector genomes/ml, Neuron Biotech Company, China) bilaterally into the BLA at a volume of 0.3 μl for 10 min. For all above stereotaxic injections, the needle was retained in place for an additional 10 min to allow diffusion of the injected solutions.

After injected with virus for a minimum of 4 weeks, mice were subjected to CPA behavioral training. As shown in left panel of fig. S1B, the pre-test (day 1), drug treatment (days 2-6) and conditioning (days 7-10) phases were similar described above. On post-test day (day 11), mice were divided into two groups: clozapine-n-oxide (CNO) and saline. For mice in CNO group, CNO (10 mg/kg, i.p.) were injected 60 min before the post-test. For mice in saline group, saline (0.1 ml, i.p.)

were injected 60 min before the post-test.

Two-step virus injection approach. To trace the feedback circuit from the PrL to the BLA, mice were firstly injected with pAAV-hSyn-Cre-EGFP virus (1.13×10^{13} vector genomes/ml, Obio Technology Company, China) bilaterally into the BLA (60 nl) for 10 min. One week later, a second injection of pAAV-Ef1α-DIO-hChR2-mCherry virus (2.27×10^{13} vector genomes/ml, Obio Technology Company, China) bilaterally into the PrL (60 nl) for 10 min. Mice were allowed to examine the expression of virus for at least four weeks following the second injection.

Immunohistochemistry and imaging. After the end of CPA testing, all mice were perfused with 0.9% saline followed by ice-cold solution of 4% paraformaldehyde in phosphate buffer (PFA, pH 7.4). The brains were removed and fixed in 4% PFA overnight. The brains were cut in 50 μm coronal sections using a vibratome (Leica, USA) and collected in PBS. To examine the injection site and the expression of each virus, brain slices were rinsed in PBS for three times (5 min for each wash) and incubated with DAPI (C1002, Beyotime Biotechnology, USA) diluted into 0.5 μg/ml for 10 min at room temperature. To do the immunohistochemistry experiments, brain slices were rinsed in PBS for three times (5 min for each wash) and incubated with blocking solution containing 10% normal goat serum and 0.3% Triton X-100 in PBS for 2 h at 4 °C. For the analysis of Arc expression after *in vivo* chemical-genetic inhibition of cell bodies of BLA neurons projecting to the PrL, brain slices were

incubated with rabbit anti-Arc/Arg 3.1 antibody (#I56003, Synaptic Systems, Germany) diluted 1:1000 overnight at 4 °C. This antibody was dissolved into 10% normal goat serum and 0.3% Triton X-100 in PBS. Subsequently, they were rinsed in PBS for three times (5 min for each wash) and incubated with goat anti-rabbit IgG antibody (Vector, US) diluted 1:200 for 1 h at 37 °C followed by 488-conjugated streptavidin (Jackson Immuno Research Laboratory, USA) diluted 1:1000 for 1 h at 37 °C. This antibody was dissolved into 10% normal goat serum in PBS. Finally, immunolabeled sections were rinsed in PBS for three times (5 min for each wash) and mounted on glass slides and were imaged by confocal microscopy (Nikon AIR-MP, Japan).

Figure S1

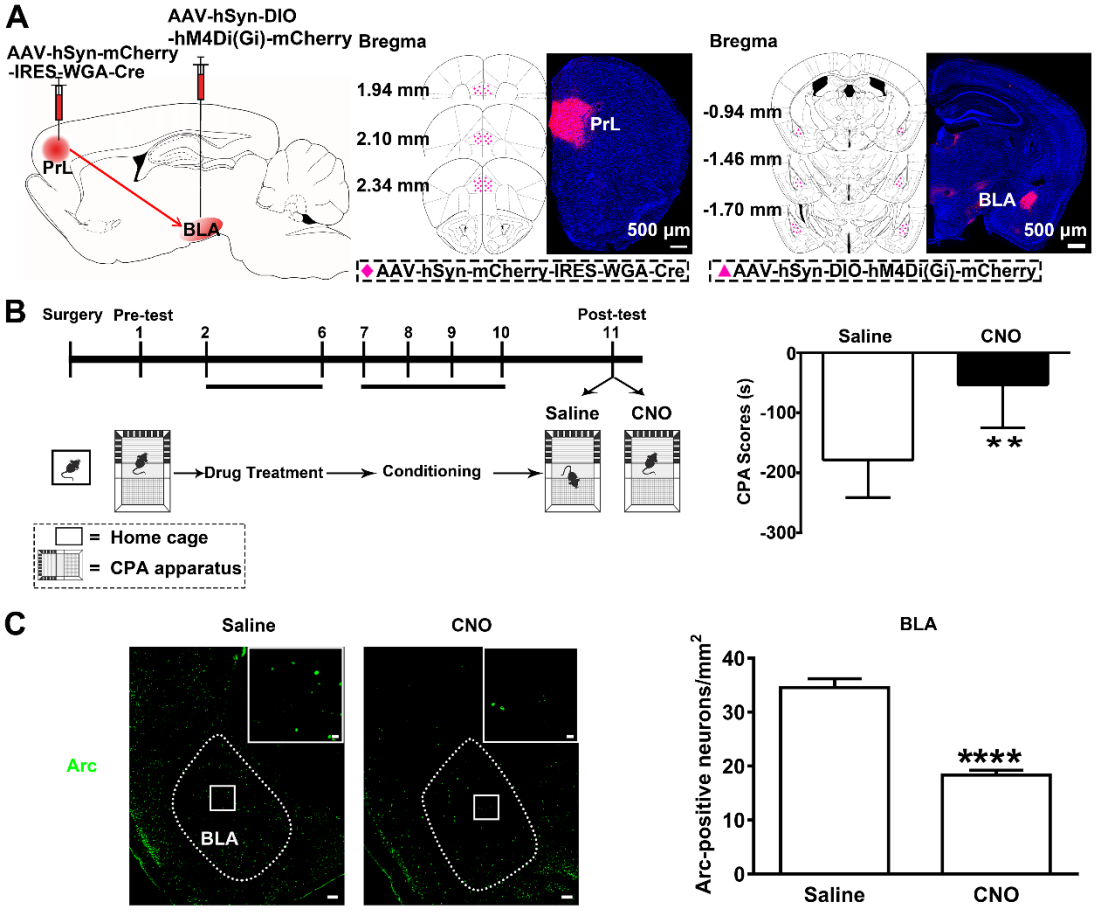

Fig. S1. Influence of in vivo chemical-genetic inhibition of cell bodies of BLA neurons projecting to the PrL on conditioned context–induced place aversion in morphine withdrawal mice. (A) Left panel: diagram of virus injection site in the BLA and the PrL. Middle panel: image of coronal brain slice showing the expression of mCherry-WGA-Cre (red-colored) 4 weeks after virus injection into the PrL. Numbers indicate coordinates relative to bregma. Scale bar = 500 μm. Right panel: image of coronal brain slice showing the expression of hM4Di(Gi)-mCherry (red-colored) 4 weeks after virus injection into the BLA. Numbers indicate coordinates relative to bregma. Scale bar = 500 μm. (B) Left panel: experimental timeline for the CPA procedure. Right panel: average CPA score in saline and CNO groups (*n* = 6 mice in

each group, unpaired *t* test, ***P* < 0.01). (C) Left panel: Arc positive neurons in the BLA in saline and CNO groups (green-colored). Scale bar = 100 μm. BLA regions enclosed by white boxes were shown in a higher magnification in upper right square images (scale bar = 20 μm). Right panel: average Arc positive neurons/mm^2 in the BLA in saline and CNO groups (*n* = 6 mice in each group, unpaired *t* test, *****P* < 0.01). Data are shown as the mean ± s.e.m.

Figure S2

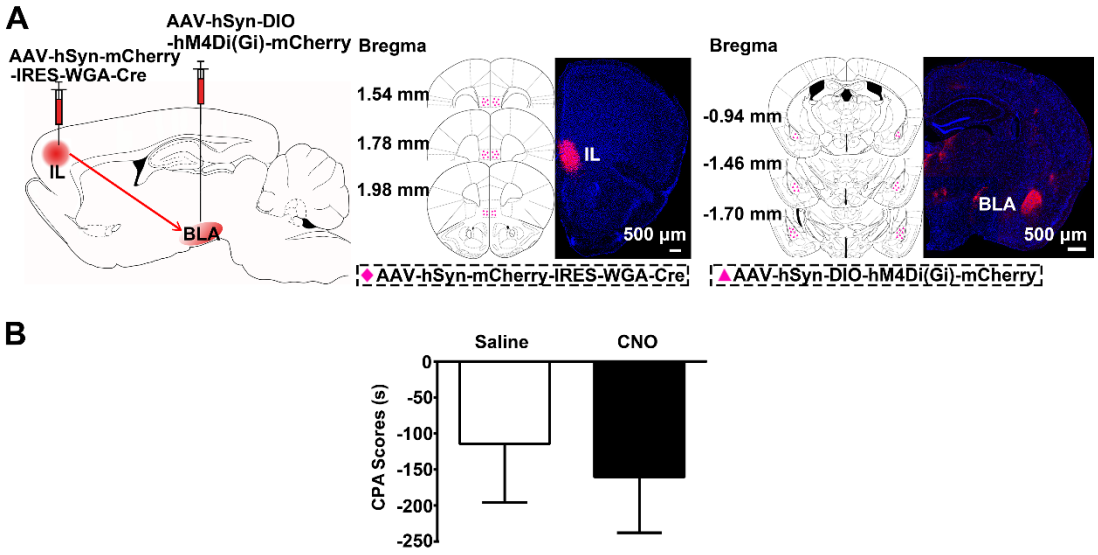

Fig. S2. Influence of in vivo chemical-genetic inhibition of cell bodies of BLA neurons projecting to the IL on conditioned context–induced place aversion in morphine withdrawal mice. (A) Left panel: diagram of virus injection site in the BLA and the IL. Middle panel: image of coronal brain slice showing the expression of mCherry-WGA-Cre (red-colored) 4 weeks after virus injection into the IL. Numbers indicate coordinates relative to bregma. Scale bar = 500 μm. Right panel: image of coronal brain slice showing the expression of hM4Di(Gi)-mCherry (red-colored) 4 weeks after virus injection into the BLA. Numbers indicate coordinates relative to bregma. Scale bar = 500 μm. (B) Average CPA score in saline and CNO groups (n = 6 mice in each group, unpaired t test, $P > 0.05$). Data are shown as the mean ± s.e.m.

Figure S3

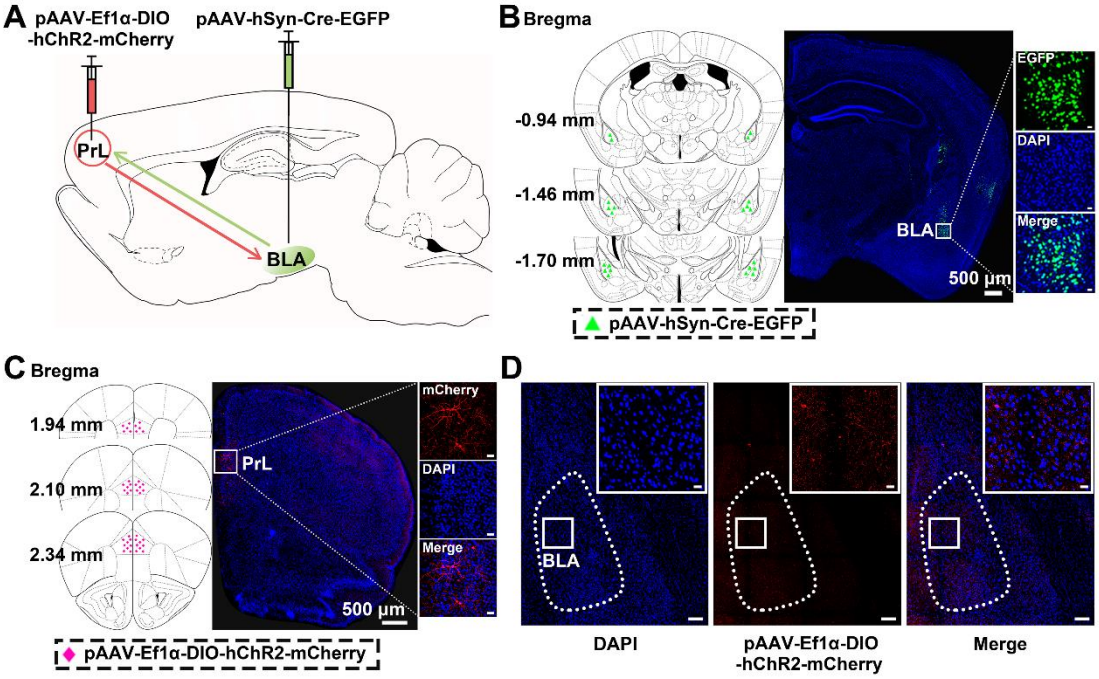

Fig. S3. Tracing the feedback circuit from the PrL to the BLA. (A) Diagram of two-step viral injection sites in the BLA and the PrL. (B) Images of coronal brain slice showing the expression of Cre-EGFP (green-colored) 1 week after the first virus injection into the BLA. Numbers indicate coordinates relative to bregma. Scale bar = 500 μm. BLA region enclosed by white box was shown in a higher magnification in right images (scale bar = 50 μm). Top right image: EGFP-labeling neurons in the BLA (green-colored). Middle right image: DAPI positive neurons in the BLA (blue-colored). Bottom right image: co-labeling neurons of EGFP and DAPI in the BLA (cyan-colored). (C) Images of coronal brain slice showing the expression of hChR2-mCherry (red-colored) 4 weeks after the second virus injection into the PrL. Numbers indicate coordinates relative to bregma. Scale bar = 500 μm. PrL region enclosed by white box was shown in a higher magnification in right images (scale bar = 50 μm). Top right

image: mCherry-labeling neurons in the PrL (red-colored). Middle right image: DAPI positive neurons in the PrL (blue-colored). Bottom right image: co-labeling neurons of mCherry and DAPI in the PrL (purple-colored). (D) Images of coronal brain slices showing the hChR2-mCherry-expressed axonal terminals (red-colored) of PrL neurons in the BLA 4 weeks after the two-step viral injection. Left panel: DAPI positive neurons in the BLA (blue-colored). Middle panel: mCherry-positive fibers in the BLA (red-colored). Right panel: merge image of DAPI positive neurons (blue-colored) and mCherry-positive fibers (red-colored) in the BLA. Numbers indicate coordinates relative to bregma. Scale bar = 100 μm. BLA regions enclosed by white boxes were shown in a higher magnification in upper right square images (scale bar = 20 μm).

Figure S4

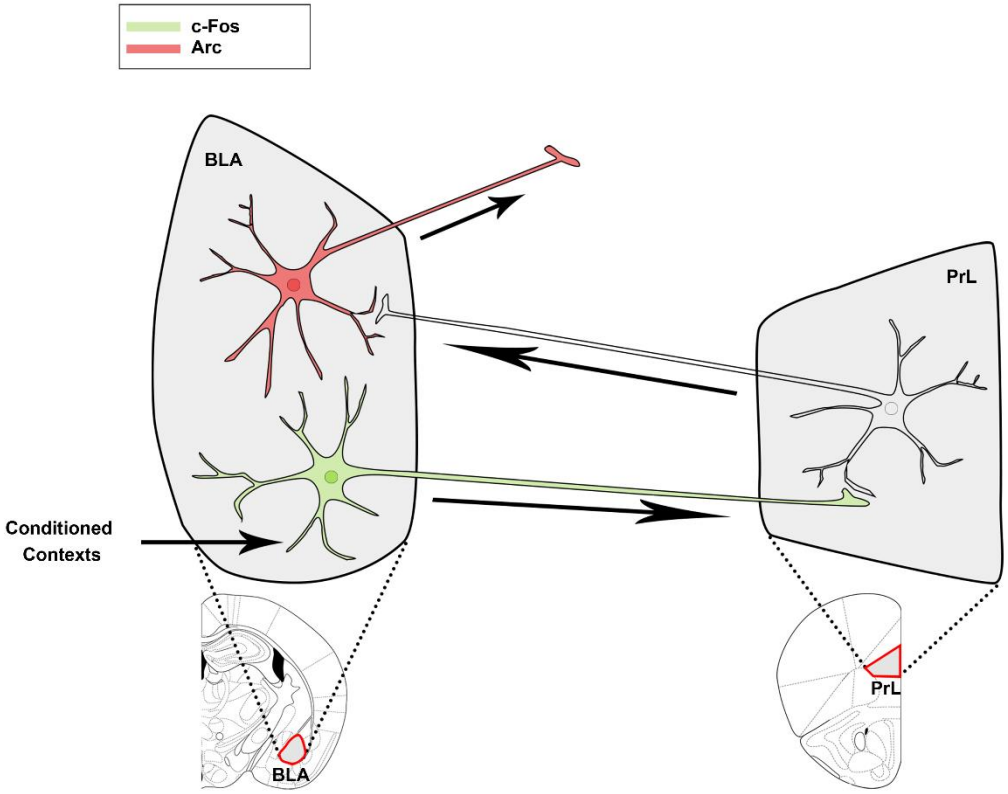

Fig. S4. Diagram of the roles of BLA-PrL-BLA neuronal circuit in conditioned context–induced Arc activation in the BLA and the retrieval of morphine withdrawal memory.