Activity in projection neurons from prelimbic cortex to the PVT is necessary for retrieval of morphine withdrawal memory

Authors
Lanfang Yu, Chenshan Chu, Yu Yuan, ..., Dongyang Cui, Bin Lai, Ping Zheng

Correspondence
laibin@fudan.edu.cn (B.L.), pzheng@shmu.edu.cn (P.Z.)

In brief
Yu et al. show that the activity of PrL-PVT projection neurons is necessary for the retrieval of morphine withdrawal memory, and a conditioned context causes a plastic change in the activity in these projection neurons during the withdrawal memory retrieval.

Highlights
- Conditioned context activates PrL-PVT projection neurons in morphine-withdrawn mice
- Silencing of PrL-PVT projection neurons inhibits withdrawal memory retrieval
- Context induces an increase in neural plasticity in PrL-PVT projection neurons
Activity in projection neurons from prelimbic cortex to the PVT is necessary for retrieval of morphine withdrawal memory

Lanfang Yu,1,3 Chenshan Chu,1,3 Yu Yuan,1 Xinli Guo,1 Chao Lei,1 Huan Sheng,1 Li Yang,1 Dongyang Cui,1 Bin Lai,1,* and Ping Zheng1,2,4,*

1State Key Laboratory of Medical Neurobiology, Institutes of Brain Science, Fudan University, Shanghai 200032, China
2Department of Neurology of Zhongshan Hospital, MOE Frontier Center for Brain Science, School of Basic Medical Sciences, Shanghai, China
3These authors contributed equally
4Lead contact
*Correspondence: laibin@fudan.edu.cn (B.L.), pzheng@shmu.edu.cn (P.Z.)
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SUMMARY

Previous work has shown that the paraventricular nucleus of the thalamus (PVT) is an important region that is involved in the conditioned context-induced retrieval of morphine withdrawal memory. However, the upstream neural circuits that activate the PVT to participate in the conditioned context-induced retrieval of morphine withdrawal memory remain unknown. In the present work, we find that the conditioned context activates projection neurons from the prelimbic cortex (PrL) to the PVT, and the inhibition of PrL-PVT projection neurons inhibits the conditioned context-induced retrieval of morphine withdrawal memory; the conditioned context induces an increase in Arc expression, intrinsic excitability, and glutamate output in PrL-PVT projection neurons in morphine-withdrawn mice. These results suggest that the activity of PrL-PVT projection neurons is necessary for the retrieval of morphine withdrawal memory, and the conditioned context causes a plastic change in the activity in these projection neurons during the withdrawal memory retrieval.

INTRODUCTION

Drug addiction is a chronic brain disorder characterized by drug relapses (Leshner, 1997). The presentation of environmental context that is previously associated with drug withdrawal symptoms can induce the retrieval of drug withdrawal memory, leading to the relapse. The study of neural basis underlying the conditioned context-induced retrieval of drug withdrawal memory would help in developing new therapeutic approaches to prevent drug relapse.

Previous studies have examined the brain regions that contribute to the conditioned context-induced retrieval of drug withdrawal memory. Among them, the paraventricular nucleus of the thalamus (PVT) is an important region that is involved in the conditioned context-induced retrieval of drug withdrawal memory. Zhu et al. (2016) reported that after the inhibition of projection neurons from the PVT to the nucleus accumbens (NAc) using the optogenetic method, the conditioned context-induced retrieval of morphine withdrawal memory was significantly inhibited, and the optogenetic activation of projection neurons from the PVT to the NAc evoked an aversive response. However, the upstream neural circuits that activate the PVT to participate in the conditioned context-induced retrieval of morphine withdrawal memory remain incompletely studied.

It has been shown that the PVT receives a complex set of cortical and subcortical afferents (Millan et al., 2017; Zhou and Zhu, 2019). Among them, neurons of the PVT receive a dense innervation from orexinergic neurons in the lateral hypothalamus (LH) and glutamatergic neurons in the prelimbic cortex (PrL) (Li et al., 2011; Otis et al., 2019; Li and Kirouac, 2012). The possible role of orexinergic projection neurons from the LH to the PVT in the conditioned context-induced retrieval of morphine withdrawal memory has been studied previously (Li et al., 2011). However, the role of glutamatergic projection neurons from the PrL to the PVT, which is the main excitatory input to the PVT, in the conditioned context-induced retrieval of morphine withdrawal memory remains unknown. In the present work, we used the retrograde labeling method to identify PrL neurons projecting to the PVT (PrL-PVT projection neurons) and examined the influence of the conditioned context on the expression of c-Fos, a marker of neuronal activation (Fields et al., 1997), and Arc, a marker of neuronal plasticity (Tzingounis and Nicoll, 2006), in these projection neurons using immunohistochemistry, and studied the role of these projection neurons in the retrieval of morphine withdrawal memory by examining the influence of the chemogenetic inactivation of PrL-PVT projection neurons on conditioned place aversion (CPA). We also studied whether there was a change in excitability and the glutamatergic output of PrL-PVT projection neurons after exposure to the conditioned context using the retrograde labeling method in combination with whole-cell recording and the optogenetic method.
Figure 1. The influence of the conditioned context on the expression of neural activity-related c-Fos in PrL-PVT projection neurons in morphine-withdrawn mice

(A) Left: diagram of the injection of fluorogold (FG) into the PVT. Right: experimental timeline. Bottom: average CPA scores in saline + saline (n = 8), saline + naloxone (n = 11), morphine + saline (n = 6), and morphine + naloxone (n = 7) groups (2-way ANOVA, ****p < 0.0001; followed by post hoc Tukey’s test, morphine + naloxone group versus saline + saline group, p < 0.0001; morphine + naloxone group versus saline + naloxone group, p < 0.0001; morphine + naloxone group versus morphine + saline group, p < 0.0001).

(B) Left: top, FG-labeling neurons in the PrL. Magnified image shows the boxed area; center, c-Fos+ neurons in the PrL; bottom, co-labeling neurons of c-Fos and FG in the PrL. Scale bars, 100 and 50 μm. Right: top, quantification of FG+ cells per square millimeter in the PrL. Morphine + naloxone (n = 6), saline + saline (n = 8), saline + naloxone (n = 5), and morphine + saline (n = 4) groups showed no statistical significance in the PrL (1-way ANOVA, p > 0.05); center, quantification of

(legend continued on next page)
RESULTS

PrL-PVT projection neurons participate in conditioned context-induced retrieval of morphine withdrawal memory in morphine-withdrawn mice, but do not participate in conditioned context-induced retrieval of morphine reward memory

To study the role of PrL-PVT projection neurons in the conditioned context-induced retrieval of morphine withdrawal memory, we examined the influence of the conditioned context on the expression of c-Fos, a marker of neuronal activation (Fields et al., 1997), and of PrL-PVT projection neurons in morphine-withdrawn mice. The results showed that the mice in the morphine + naloxone group exhibited a strong aversion to the withdrawal-paired compartment and thus spent less time in the withdrawal-paired compartment during the post-test than during the pre-test, resulting in an increase in aversion score (CPA score), whereas mice in other groups did not exhibit a significant aversion to either compartment (two-way ANOVA, drug treatment factor, $F_{(3,28)} = 6.63$, $p = 0.0016$; test condition factor, $F_{(1,28)} = 6.82$, $p = 0.0143$; drug treatment $\times$ test condition, $F_{(3, 28)} = 12.83$, $p < 0.0001$; Figure 1A, bottom panel). After the behavioral assay, the animals were sacrificed at 1.5 h after the post-test, and the effect of the conditioned context on the expression of c-Fos in the PrL in different groups was examined. The left panels of Figure 1B are typical micrographs in different groups. From these, we can see that the expression of c-Fos and the co-labeling of c-Fos and fluorogold (FG) in the PrL were significantly increased in the morphine + naloxone group after exposure to the conditioned context, but they did not change significantly in the saline + saline or morphine + saline groups, compared to the saline + saline group. The average c-Fos$^+$ neurons/mm² in the PrL in the morphine + naloxone group were significantly higher than those in the saline + saline group, saline + naloxone group and morphine + saline group (one-way ANOVA, $F_{(3,19)} = 16.04$, $p < 0.0001$; Figure 1B, right and center panels), and the average percentage of co-labeling of c-Fos and FG relative to FG in the PrL in morphine + naloxone group was significantly higher than that in the saline + saline group, the saline + naloxone group, and the morphine + saline group (one-way ANOVA, $F_{(3, 19)} = 12.86$, $p < 0.0001$; followed by post hoc Tukey’s test, morphine + naloxone group versus saline + saline group, $p = 0.0001$; Figure 1B, right and bottom panels). This result suggests that the exposure to the conditioned context activates PrL-PVT projection neurons in morphine-withdrawn mice.

To study the role of PrL-PVT projection neurons in the conditioned context-induced retrieval of morphine withdrawal memory, we examined the influence of chemogenetic (designer receptors exclusively activated by designer drugs [DREADD]) inactivation of PrL-PVT projection neurons on conditioned context-induced place aversion in morphine-withdrawn mice. The mice with the expression of hM4Di(Gi)-mCherry were divided into two groups: one group was the saline group, in which the PVT was locally injected with saline through a cannula at 20 min before post-test (hM4Di(Gi)-mCherry-saline group); another group was the clozapine-n-oxide (CNO) group, in which the PVT was locally injected with CNO through a cannula at 20 min before the post-test (hM4Di(Gi)-mCherry-CNO group). The mice with the expression of mCherry were set as the empty vector control group (mCherry-CNO group), and the CNO was locally injected into the PVT through a cannula at 20 min before post-test to exclude the effect of CNO on the CPA. Three groups were subjected to behavioral procedures as shown in the right panel of Figure 2A. As shown in Figure 2C, the conditioned context induced a strong aversion to the morphine withdrawal-paired compartment in the hM4Di(Gi)-mCherry-saline group and the mCherry-CNO group, but it did not induce a significant aversion to the morphine withdrawal-paired compartment in the hM4Di(Gi)-mCherry-CNO group (two-way ANOVA, drug treatment factor, $F_{(2,26)} = 14.32$, $p < 0.0001$; test condition factor, $F_{(1,26)} = 28.77$, $p < 0.0001$; drug treatment $\times$ test condition, $F_{(2,26)} = 20.88$, $p < 0.0001$; Figure 2C). To ascertain that the influence of the inhibition of PL-PVT projection neurons on CPA using the chemogenetic method is due to the inhibition of the retrieval of morphine withdrawal memory, rather than the inhibition of behavior in general, we examined the influence of the inhibition of PL-PVT projection neurons on the movement of mice without drug treatment in the three-compartment place conditioning apparatus used in CPA experiment and in an open field. Our results showed that the specific silencing of PrL-PVT projection neurons did not induce mice to have a place aversion or preference in the three-compartment place conditioning apparatus (two-way ANOVA, drug treatment factor, $F_{(1,14)} = 0.15$, $p = 0.7008$; test condition factor, $F_{(1,14)} = 0.01$, $p = 0.9143$; drug treatment $\times$ test condition, $F_{(1,14)} = 0.03$, $p = 0.8828$; Figure S1C), nor did it influence the distance (two-way ANOVA, drug treatment factor, $F_{(2,22)} = 0.38$, $p = 0.6868$; test condition factor, $F_{(1,22)} = 1.17$, $p = 0.2906$; drug treatment $\times$ test condition, $F_{(2,22)} = 2.12$, $p = 0.1436$; Figure S1D, bottom and left panels) and the velocity of movement of mice in an open-field (two-way ANOVA, drug treatment factor, $F_{(2,22)} = 0.38$, $p = 0.6870$; test condition factor, $F_{(1,22)} = 1.17$, $p = 0.2908$; drug treatment $\times$ test condition, $F_{(2,22)} = 2.12$, $p = 0.1436$; Figure S1D, bottom and right panels). This evidence suggests that the activity of PrL-PVT projection neurons is necessary for the conditioned context-induced retrieval of morphine withdrawal memory in morphine-withdrawn mice.

We also studied the role of PrL-PVT projection neurons in the conditioned context-induced retrieval of morphine reward memory using an approach similar to the above morphine withdrawal memory. The result showed that the mice in the morphine group exhibited a strong preference to the morphine-paired compartment and thus spent more time in the morphine-paired compartment. The mice in the saline and mCherry-CNO groups did not exhibit a place preference in the three-compartment place conditioning apparatus used in CPA experiment and in an open field. Our results showed that the specific silencing of PrL-PVT projection neurons did not induce mice to have a place preference or preference in the three-compartment place conditioning apparatus (two-way ANOVA, drug treatment factor, $F_{(1,14)} = 0.15$, $p = 0.7008$; test condition factor, $F_{(1,14)} = 0.01$, $p = 0.9143$; drug treatment $\times$ test condition, $F_{(1,14)} = 0.03$, $p = 0.8828$; Figure S1C), nor did it influence the distance (two-way ANOVA, drug treatment factor, $F_{(2,22)} = 0.38$, $p = 0.6868$; test condition factor, $F_{(1,22)} = 1.17$, $p = 0.2906$; drug treatment $\times$ test condition, $F_{(2,22)} = 2.12$, $p = 0.1436$; Figure S1D, bottom and right panels). This evidence suggests that the activity of PrL-PVT projection neurons is necessary for the conditioned context-induced retrieval of morphine withdrawal memory in morphine-withdrawn mice.

Means ± SEMs.
compartment during the post-test than they did during the pre-test, resulting in an increase in "preference score" (conditioned place preference [CPP] score), whereas mice in the saline group did not exhibit a significant preference to either compartment (two-way ANOVA, drug treatment factor, F(1, 16) = 17.32, p = 0.0007; test condition factor, F(1, 16) = 62.61, p < 0.0001; drug treatment x test condition, F(1, 16) = 23.76, p = 0.0002; Figure S2A, right panel). The average percentage of the co-labeling of c-Fos and FG relative to FG in the PrL in the morphine group showed no significant difference than those in the saline group (unpaired t test, p = 0.6224; Figure S2B, right panel). This result suggests that the conditioned context associated with morphine reward has no significant influence on the expression of the c-Fos of PrL-PVT projection neurons in morphine-dependent mice.

We further examined the influence of the chemogenetic inactivation of PrL-PVT projection neurons on the conditioned context-induced place preference in morphine-dependent mice. As shown in Figure S3C, after the chemogenetic inactivation of PrL-PVT projection neurons, the conditioned context still could induce a strong preference to the morphine-paired compartment in the hM4Di(Gi)-eGFP-CNO group (two-way ANOVA, drug treatment factor, F(2, 20) = 0.01, p = 0.9990; test condition factor, F(1, 20) = 143.70, p < 0.0001; drug treatment x test condition, F(2, 20) = 0.24, p = 0.7896; Figure S3C). This evidence suggests that PrL-PVT projection neurons do not participate in the conditioned context-induced retrieval of morphine reward memory in morphine-dependent mice.

**Conditioned context induces an increase in Arc expression, intrinsic excitability, and glutamate output in PrL-PVT projection neurons in morphine-withdrawn mice**

To examine whether the conditioned context induced a plastic change in PrL-PVT projection neurons in morphine-withdrawn mice, we studied the effect of the conditioned context on the expression of Arc, a marker of neural plasticity (Tzingounis and Nicoll, 2006), in PrL-PVT projection neurons in morphine-withdrawn mice. After the behavioral assay (two-way ANOVA, drug treatment factor, F(3, 28) = 6.63, p = 0.0016; test condition factor, F(1, 28) = 6.82, p = 0.0143; drug treatment x test condition, F(3, 28) = 12.83, p < 0.0001; Figure 1A, bottom panel), the animals were sacrificed and the effect of the conditioned context on the expression of Arc in the PrL was examined. We could see that the expression of Arc and the co-labeling of Arc and FG in the PrL was significantly increased in the morphine + naloxone group after the exposure to the conditioned context, but it did not change significantly in the saline + saline, saline + naloxone or morphine + saline groups (Figure 3B, left panels). The average Arc+ neurons/mm² (one-way ANOVA, F(3, 20) = 14.59, p < 0.0001; Figure 3B, right and center panels) and the average percentage of the co-labeling of Arc and FG relative to FG (one-way ANOVA, F(3, 20) = 11.26, p = 0.0002; Figure 3B, right and bottom panels) in the PrL in the morphine + naloxone group were significantly higher than those in other groups. This result suggests that the conditioned...
context increases the expression of plasticity-related Arc in PrL-PVT projection neurons in morphine-withdrawn mice.

To further study how PrL-PVT projection neurons were involved in the conditioned context-induced retrieval of morphine withdrawal memory, we examined the conditioned context-induced changes in the firing frequency of the action potentials (APs) of PrL-PVT projection neurons in morphine-withdrawn mice. The mice were randomly divided into three groups:
none-drug group, non-retrieval group, and retrieval group. The mice of the none-drug group were subjected to the behavioral process, as illustrated in right panel of Figure 4A, with saline as control. The mice of the non-retrieval and retrieval groups were subjected to the behavioral process, as illustrated in the right panel of Figure 4A, with both groups having post-test on the 1st day after conditioning, but only the mice of the retrieval group having post-test on the 2nd day after conditioning. Based on this, after 24 h, the mice of the retrieval group were subjected to post-test to have the conditioned context-induced retrieval of morphine withdrawal memory, whereas the mice of the non-retrieval group did not. The result showed that on the 1st day after conditioning, the mice in the non-retrieval and retrieval groups exhibited a strong aversion to withdrawal-paired compartment and thus spent less time in the withdrawal-paired compartment during the post-test than during the pre-test, resulting in an increase in CPA score (paired t test, pre-test versus post-test, non-retrieval, p = 0.0168; retrieval, p = 0.0014; Figure 4B). This result suggests that after conditioning training, the association of environmental context and morphine withdrawal memory has been established in these two groups. Then, on the 2nd day after conditioning, the mice in the retrieval group (paired t test, pre-test versus post-test, p = 0.0037; Figure 4B) were subjected to a post-test to have the conditioned context-induced retrieval of withdrawal memory and then were sacrificed at 30 min after the post-test to prepare slices for whole-cell patch-clamp recording. Mice in the none-retrieval group were sacrificed without the post-test to prepare slices for whole-cell patch-clamp recording. We measured the frequency of AP firing in response to increasing current steps using whole-cell current-clamp recordings (holding current: 0 pA; current steps: −100 to 450 pA in 50-pA increments) in these microsphere-labeled neurons in the PrL in the none-drug group, the non-retrieval group, and the retrieval group at 30 min after the post-test. Figure 4D showed typical AP traces in response to 100, 200, and 400 pA depolarizing currents in the none-drug group, the non-retrieval group, and the retrieval group, respectively. From these raw traces, we could see that in response to these depolarizing currents, neurons in the retrieval group fired more APs than those in the non-retrieval group and the none-drug group at 30 min after the post-test. The average frequency of AP firing in the neurons of the retrieval group in response to 100, 200, and 400 pA depolarizing currents increased significantly more than those in the non-retrieval group (unpaired t test, 100 pA, p = 0.0003; 200 pA, p < 0.0001; 400 pA, p < 0.0001; Figure 4E) and in the none-drug group (unpaired t test, 100 pA, p = 0.0162; 200 pA, p = 0.0124; 400 pA, p = 0.0134; Figure 4F) at 30 min after the post-test. This result suggests that the conditioned context induces an increase in the firing frequency of APs in the PrL-PVT projection neurons in morphine-withdrawn mice. We also examined whether there still was an increase in the neural excitability of the PrL-PVT projection neurons in morphine withdrawal mice at 2 h after the post-test. From the raw traces shown in Figure 4D, we could see that in response to these depolarizing currents, the neurons in the retrieval group still fired more APs than those in the non-retrieval group and the none-drug group at 2 h after the post-test. The average frequency of AP firing in the neurons of the retrieval group in response to 100, 200, and 400 pA depolarizing currents increased significantly more than those in the non-retrieval group (unpaired t test, 100 pA, p < 0.0001; 200 pA, p < 0.0001; 400 pA, p < 0.0001; Figure 4F) and in the none-drug group (unpaired t test, 100 pA, p = 0.0028; 200 pA, p = 0.0011; 400 pA, p = 0.0010; Figure 4F) at 2 h after the post-test. This result suggests that there still is an increase in the neural excitability of PrL-PVT projection neurons in morphine-withdrawn mice at 2 h after the post-test.

However, at 24 h after the post-test, the increase in the excitability of PrL-PVT projection neurons in morphine-withdrawn mice disappeared because at 24 h after the post-test, the frequency of AP firing in the neurons of the non-retrieval group did not change significantly compared with those in the none-drug group. From these raw traces shown in Figure 4D, we could see that in response to these depolarizing currents, neurons in the none-drug group did not fire more APs than those in the none-drug group at 24 h after the post-test. The average frequency of AP firing in the neurons of the non-retrieval group in response to 100-, 200-, and 400-pA depolarizing currents did not undergo a more significant change than for those in the none-drug group (unpaired t test, 100 pA, p = 0.1118; 200 pA,

Figure 4. The influence of the conditioned context on the excitability of PrL-PVT projection neurons in morphine-withdrawn mice
(A) Left: diagram of the injection of fluorescent microsphere into the PVT. Right: experimental timeline.
(B) Average CPA scores of the different groups (paired t test, pre-test versus post-test; non-drug group: 1 day, p > 0.05, and 2 days, p > 0.05; non-retrieval group: 1 day, p < 0.05; retrieval group: 1 day, **p < 0.01, and 2 days, ***p < 0.001; retrieval + 2 h: 1 day, **p < 0.01, and 2 days, ***p < 0.001).
(C) Left: the injection site of the fluorescent microsphere (green) in the PVT. Scale bar, 100 μm. Right and top: neurons in the PrL retrograde labeled (green) by fluorescent microsphere from the PVT. Right and bottom: the fluorescent image of PrL-PVT projection neurons labeled with fluorescent microsphere retrogradely transported from the PVT in a brain slice and its image under infrared differential interference contrast (IR-DIC) microscopy. Scale bar, 25 μm.
(D) Typical action potential (AP) traces in response to 100, 200, and 400 pA depolarizing currents in non-drug, non-retrieval, and retrieval + 2 h group, respectively. Scale bar, 20 mV, 100 ms.
(E) Example traces showing the voltage response to a 500-ms depolarizing current step used to elicit a mAHP (spikes are truncated). The mAHP is quantified as the peak of hyperpolarization. Scale bar, 5 mV, 20 ms.
(F) Quantification of the AP frequency in the neurons of the retrieval group (n = 8 cells from 5 animals) and the retrieval + 2 h group (n = 12 cells from 6 animals), compared with the non-drug group (n = 6 cells from 6 animals; unpaired t test, *p < 0.05, **p < 0.01) or the non-retrieval group (n = 8 cells from 5 animals; unpaired t test, ***p < 0.001, ****p < 0.0001) in response to 100, 200, and 400 pA depolarizing currents.
(G) Quantification of the amplitude of mAHP in the neurons of the retrieval group (n = 8 cells from 5 animals) and the retrieval + 2 h group (n = 11 cells from 6 animals), compared with the non-drug group (n = 6 cells from 6 animals; unpaired t test, **p < 0.01, ***p < 0.001) or the non-retrieval group (n = 9 cells from 5 animals; unpaired t test, *p < 0.05, **p < 0.01, ***p < 0.001) in response to 100, 200, and 400 pA depolarizing currents.

Means ± SEMs.
It is known that the afterhyperpolarization (AHP) that follows an AP emission is a principal feedback mechanism in the control of the firing frequency of APs (Dumênio et al., 2015). Three phases of AHP were recognized: the fast AHP-regulated AP firing at the onset of a burst, and the medium and slow AHPs, which suppressed AP firing over hundreds of milliseconds and seconds (Church et al., 2019). Among them, the decrease in the medium AHP (mAHP) was able to increase AP firing (Church et al., 2019; Stocker et al., 1999). Therefore, to confirm the influence of the conditioned context on the firing frequency of APs in PrL–PVT projection neurons, we examined whether there was a decrease in mAHP, which contributed to the increase in AP firing after the exposure to the conditioned context in morphine-withdrawn mice.

Figure 4E shows typical mAHP in response to 100, 200, and 400 pA depolarizing currents in the non-drug control, non-retrieval, retrieval, and retrieval + 2-h groups. We could see that the mAHP in response to 100, 200, and 400 pA depolarizing currents in the retrieval group was lower than that in the non-drug and the non-retrieval groups. The average mAHP in response to 100, 200, and 400 pA depolarizing currents in the retrieval group was significantly lower than that in the non-drug group (unpaired t test, 100 pA, p = 0.0002; 200 pA, p < 0.0001; 400 pA, p = 0.0002; Figure 4G) and the non-retrieval group (unpaired t test, 100 pA, p = 0.0020; 200 pA, p = 0.0007; 400 pA, p = 0.0023; Figure 4G). This result suggests that the conditioned context induces a decrease in mAHP in PrL–PVT projection neurons in morphine-withdrawn mice.

In the neuron, the mAHP is usually mediated by three different ion channel subtypes: small-conductance calcium-activated K⁺ (SK) channels, voltage-dependent M channels (KV7/KCNQ), and the hyperpolarization-activated cyclic nucleotide-gated (HCN) channel (Church et al., 2019). Among these, SK channels are the major channel because mAHP can be blocked by apamin, which has a high specificity for SK channels (Adelman, 2016). Therefore, to examine whether there was a decrease in the current of apamin-sensitive SK channels (I_{SK}), which contributed to the decrease in the mAHP after exposure to the conditioned context in morphine-withdrawn mice, we injected fluorescent microsphere into the PVT to retrograde label PrL–PVT projection neurons (Figure 5A, left panel). After recovery from the surgery, the mice were randomly divided into three groups: non-drug, retrieval, and non-retrieval. The mice were subjected to the behavioral process as illustrated in the right panel of Figure 5A and post-tested on 1st day after conditioning. The result showed that the mice in the retrieval group and the non-retrieval group exhibited a strong aversion to withdrawal-paired compartment and thus spent less time in the withdrawal-paired compartment during the post-test than that during the pre-test, resulting in an increase in CPA score on the 1st day after conditioning (paired t test, pre-test versus post-test, p < 0.0001; Figure 5B). Then, on the 2nd day after conditioning, the mice in the non-drug group and the retrieval group were subjected to the post-test and sacrificed at 30 min after the post-test to prepare slices for whole-cell patch-clamp recording, whereas the mice in the non-retrieval group were sacrificed at 30 min without the post-test to prepare slices for whole-cell patch-clamp recording. I_{SK} was isolated with a combination of pharmacological tools, appropriate holding potentials, and current subtraction protocols. Apamin-sensitive I_{SK} at different holding voltages was obtained by subtracting the traces of apamin treatment data from that of the control data. The representative traces of I_{SK} before and after the perfusion of apamin are shown in the left panels of Figure 5C. The I–V curves of the outward current before and after the perfusion of apamin in the non-drug, non-retrieval, and retrieval groups were recorded in the presence of 10 μM tetrodotoxin (TTX), cyanquixaline (CNOX) 10 μM, and 100 μM picrotixin to block Na⁺ channels, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and GABA_A receptors, respectively (Figure 5D). The I–V curves of apamin-sensitive I_{SK} current in the non-drug, non-retrieval, and retrieval groups are shown in Figure 5E. The result showed that the amplitude of the apamin-sensitive I_{SK} current of PrL–PVT projection neurons in the retrieval group was much lower than that of the non-drug and non-retrieval groups under the holding potential, from +20 to +40 mV (holding potential: +40 mV; retrieval group: 486.1 ± 71.1 mV; non-retrieval: 742.0 ± 60.6 mV; non-drug: 740.4 ± 70.7 mV, p = 0.0120, p = 0.0216; Figure 5E, right panel). This result suggests that the conditioned context induces a decrease in the apamin-sensitive I_{SK} current in PrL–PVT projection neurons in morphine-withdrawn mice. In addition, we examined the expression of the mRNA of the SK channel genes (KCNN1, KCNN2, and KCNN3) in the PrL–PVT projection neurons separated by fluorescence-activated cell sorting (FACS) using qPCR. The result showed that the expression of KCNN1 and KCNN3 mRNA was significantly reduced in PrL–PVT projection neurons collected from the retrieval group animals relative to the non-drug control.
weaken. We noticed that a large number of PrL projection neurons did not show co-labeling with c-Fos in Figure 1. The reasons that the conditioned context activated only a part of the PrL projection neurons remain unknown. One reason may be that there are different populations of neurons such as glutamatergic and GABAergic that are projecting from the PrL to the PVT, so they have different responses to the conditioned context. However, up to now, we only know there are glutamatergic neurons that are projecting from the PrL to the PVT. Another possibility may be that there are different subpopulations of glutamatergic neurons that are projecting from the PrL to the PVT, so they have different responses to the conditioned context, but we still have no evidence to support this theory. In addition, the phenomenon that a large number of PrL projection neurons did not demonstrate co-labeling with c-Fos in PrL-PVT projection neurons also was observed in a fear memory study by Do-Monte et al. (2015).

We also examined whether the PrL-PVT projection neurons participated in the conditioned context-induced retrieval of morphine reward memory. The result showed that they did not. This statement is consistent with the results that the inactivation of the PrL-PVT projection neurons had no effect on the conditioned context or the cue-induced retrieval of sucrose reward memory (Otis et al., 2017; Giannotti et al., 2018). However, it appeared that it was contradictory to that reported by Giannotti et al. (2018), because their result showed that the inactivation of the PrL-PVT projection neurons decreased the conditioned context retrieval of cocaine reward memory. The reason for this contradiction remains unknown. One possible reason may be that the retrieval time in our experiment is on the 1st day after the training, whereas the retrieval time in the Giannotti et al. experiment is on the 6th day after the training, so it appeared that the PrL-PVT projection neurons did not participate in the conditioned context-induced retrieval of morphine reward memory at an early stage after the formation of reward memory, but it did later.

The conditioned context-induced retrieval of morphine withdrawal memory is based on a learned association of a previous neutral context and morphine withdrawal symptoms. Thus, the ability of the conditioned context to induce the retrieval of morphine withdrawal memory depends on the intensity of this association. Therefore, it has been proposed that synaptic strengthening is crucial for memory retrieval (Tonogawa et al., 2015). This statement is supported by the evidence that optogenetically induced depression of the synaptic transmission of rat amygdala cells impaired existing conditioned-induced fear responses, whereas subsequent optogenetically induced synaptic strengthening of the same cells restored optogenetic cue-evoked recall of the fear memory (Nabavi et al., 2014). To examine whether there was synaptic strengthening of the PrL-PVT projection neurons during the retrieval of morphine withdrawal memory, we studied the influence of the conditioned context on Arc expression. Arc has been proposed to be a marker of synaptic strengthening (Lymberis et al., 1995). Therefore, our result that the conditioned context could induce an increase in the expression of Arc suggests that there may be synaptic strengthening of PrL-PVT projection neurons during the retrieval of morphine withdrawal memory.

DISCUSSION

One finding of the present study is that projection neurons from the PrL to the PVT may be some of the upstream neurons that activate PVT-NAc projection neurons to evoke the conditioned context-induced retrieval of morphine withdrawal memory. This statement is consistent with the result that the PrL provides glutamatergic input to the PVT-NAc projection neurons (Otis et al., 2019). However, here, we only used c-Fos as a marker of neuronal activity to examine the activation of PrL-PVT projection neurons by the conditioned context. Measurement of the activation of PrL-PVT projection neurons by the conditioned context using intracellular calcium as another marker of neuronal activity is needed to confirm this statement.
Another finding of the present study is that after exposure to the conditioned context, the excitability of the PrL-PVT projection neurons increases. It has been proposed that the excitability state may contribute to memory formation and memory retrieval (Pignatelli et al., 2019; Disterhoft and Oh, 2006). Therefore, the present result that the conditioned context can increase the excitability of the PrL-PVT projection neurons suggests that the increase in the excitability of the PrL-PVT projection neurons...
may contribute to the conditioned context-induced retrieval of morphine withdrawal memory.

The mechanism underlying the conditioned context-induced increase in neural excitability in the PrL-PVT projection neurons may be due to changes in ionic channels that are the basis of the alteration of AP firing. Among a number of ionic channels that can tune AP firing (Vacher et al., 2008), small modulation in SK channels can lead to dramatic changes in the AP firing (Adelman et al., 2012). Therefore, we examined the change in the current of this channel ($I_{SK}$) after the exposure to the conditioned context. The result showed that $I_{SK}$ decreased after the exposure to the conditioned context in morphine-withdrawn mice. Moreover, this decrease accompanied a decrease in the medium AHP, which could increase the AP firing frequency (Stocker et al., 1999).

SK channels are composed of SK1, SK2, and SK3 subunits, which were coded by KCNN1, KCNN2, and KCNN3 genes, respectively (Adelman et al., 2012). We examined the expression of mRNA of KCNN1, KCNN2, and KCNN3 in the PrL and found that all of these mRNAs were expressed in the PrL in the saline group, suggesting that SK1, SK2, and SK3 may be expressed in the PrL under normal conditions. However, in the non-retrieval group, which experienced the treatment of morphine and the coupling of the context with morphine withdrawal, the mRNA expression of KCNN1 and KCNN2 was significantly inhibited, without a change in the mRNA expression of KCNN3. Interestingly, in this situation, the current of $I_{SK}$ still did not change significantly, suggesting that the decrease of $I_{SK}$ in the non-retrieval group may be mediated by SK channels constituted by SK3 subunits. However, in the retrieval group, the mRNA expression of KCNN3 was also inhibited, being accompanied by a decrease in the current of $I_{SK}$, suggesting that a decrease in SK channels constituted by SK3 subunits may be the reason for the decrease in the current of $I_{SK}$ in the retrieval group.

Enhanced neural excitability of the PrL-PVT projection neurons should lead to an increased glutamate release at the axonal terminals of these projection neurons because the frequency of the AP firing is closely related to glutamate release (Kaczmarek and Zhang, 2017). To confirm this hypothesis, we need to measure the glutamate release from the axonal terminals of the PrL-PVT projection neurons. Since this glutamate release is from specific projection neurons, the optogenetic method is an appropriate method to measure it. Therefore, we used the PPR of the light-evoked EPSCs as the index of the presynaptic glutamate release to study the change in the glutamate release from the axonal terminals of the PrL-PVT projection neurons after the exposure to the conditioned context in morphine-withdrawn mice. Our result confirms that the enhanced neural excitability of the PrL-PVT projection neurons leads to an increased glutamate release at their terminals.

In conclusion, our results suggest that the activity of the PrL-PVT projection neurons is necessary for the retrieval of morphine withdrawal memory, and the conditioned context causes a plastic change in the activity in these projection neurons during the withdrawal memory retrieval.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:


**STAR METHODS**

**KEY RESOURCES TABLE**

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<th>REAGENT or RESOURCE</th>
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**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Ping Zheng (pzheng@shmu.edu.cn).

**Materials availability**
This study did not generate new unique reagents.

**Data and code availability**
This study did not generate any unique datasets or code.
**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

Male adult (8-12 weeks) C57BL/6J mice 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.

**METHOD DETAILS**

**Surgery**

Mice were anesthetized with ketamine and xylazine (160 mg/kg and 12 mg/kg body weight, respectively) before the stereotaxic surgery was performed. For retrograde labeling experiments, mice received injections of FG (0.2 μl, 4% dissolved in PBS; Fluorochrome, USA) into the PVT (anteroposterior (AP), –1.46 mm; mediolateral (ML), ± 0.10 mm; dorsoventral (DV), –3.30 mm, with a 45° angle toward the midline). For in vivo chemogenetic (DREADD) inhibition in CPA experiments, mice were injected with the AAV-hSyn-hM4Di-mCherry virus or the same viral vectors carrying mCherry alone (2.45 × 10^12 vector genomes/ml; Neuron Biotech Company, China) bilaterally into the PrL (AP: 2.10 mm; ML: 0.5 mm; DV: –2.40 mm) at a volume of 0.5 μl. For in vivo chemogenetic inhibition in CPP experiments and open-field task, mice were injected with the AAV2/9-hSyn-DIO-hM4D(Gi)-eGFP or the same viral vectors carrying eGFP bilaterally into the PrL at a volume of 0.5 μl, and pAAV-hSyn-mCherry-P2A-NLS-Cre-WPRE into the PVT at a volume of 0.5 μl. For optogenetic method, mice were injected with the AAV-CaMKIIα-ChR2-mCherry virus (2.45 × 10^12 vector genomes/ml; Neuron Biotech Company, China) bilaterally into the PrL at least 4 weeks before patch recording. For all the above stereotaxic injections, the needle was withdrawn 10 min after the end of the injection. For mice involved in behavioral experiments, a cannula was placed at least 500 μM above the PrL or PVT. The cannulae were secured to the skull with two anchoring screws and dental cement. After surgery, a dummy was inserted to keep the guide cannula from getting clogged. Mice were allowed at least 2 weeks to recover and to express the virus before behavioral training.

**Chronic morphine treatment**

Mice were treated with morphine (Shenyang No.1 Pharmaceutical Factory, China), as described before (Song et al., 2019). Morphine dependence was induced in mice by repeated intraperitoneal injections of morphine twice daily at 08.00 AM and 19.00 PM. Morphine doses were progressively increased from 10 mg/kg to 40 mg/kg: first day 2 × 10 mg/kg, second day 2 × 20 mg/kg, third day 2 × 30 mg/kg, fourth and fifth days 2 × 40 mg/kg. Control mice were treated with saline following the same procedure.

**Conditioned place aversion**

CPA was conducted with a three-compartment place conditioning apparatus (Med Associates, USA) with distinct visual and tactile context, and the procedure was similar to that described previously (Li et al., 2009; Chen et al., 2019). The animals were given a pre-conditioning test (Pre-test) before behavioral procedures. The animals were placed in the middle neutral area and were allowed to freely access both sides of the apparatus for 15 min. Mice with a strong preference (> 80%) for any compartment were discarded. Before conditioning, morphine dependence was induced by twice daily intraperitoneal injections of morphine at 08.00 AM and 18.00 PM, as described above. On day 1 of conditioning parts, two hours after 40 mg/kg morphine administration, mice were confined to compartment (preferred more during pre-test) for 20 min immediately after the subcutaneous injection of naloxone (0.1 mg/kg). On day 2 of conditioning parts, mice were confined to the opposite compartment for 20 min after the subcutaneous injection of saline. The entire procedure of conditioning was repeated at the next two days. The post-test was conducted 24 hours after conditioning of day 4. The mice were allowed to freely exploring three compartments for 15 min and CPA score was calculated as difference between the time spent in the saline-paired compartment and the time spent in the naloxone-paired compartment (the time in the naloxone-paired compartment minus the time in the saline-paired compartment).

**Conditioned place preference**

CPP was conducted with a three-compartment place conditioning apparatus with distinct visual and tactile context as same as CPA. The animals were given a pre-conditioning test (Pre-test) before behavioral procedures. The animals were placed in the middle neutral area and were allowed to freely access both sides of the apparatus for 15 min. Mice with a strong preference (> 80%) for any compartment were discarded. For conditioning parts, the mice were confined to compartment (preferred less during pre-test) for 30 min immediately after the subcutaneous injection of morphine (10 mg per kg) on morning, after 6 hours on afternoon, mice were confined to compartment (preferred more during pre-test) for 30 min immediately after the subcutaneous injection of saline (4 mL per kg). The entire procedure of conditioning was repeated for four days. The post-test was conducted 24 hours after conditioning of day 4. For chemogenetic inactivation part, mice were intraperitoneally injected with CNO (1mg/kg) or saline (same dose as CNO) 45min preceding the post-test. The mice were allowed to freely exploring three compartments for 15 min and CPP score was calculated as difference between the time spent in the saline-paired compartment
and the time spent in the morphine-paired compartment (the time in the saline-paired compartment minus the time in the morphine-paired compartment).

**Three-compartment place conditioning**

Mice infected with AAV2/9-hSyn-DIO-hM4D(Gi)-eGFP were allowed to freely explore both sides of a custom-made CPA training apparatus for 15 min to assess their baseline place preference. On day 1 of conditioning parts, 45 min after intraperitoneal injection of CNO (1mg/kg) or saline (same dose as CNO), mice were confined to compartment (preferred more during pre-test) for 30 min. On day 2 of conditioning parts, mice were confined to the opposite compartment for 30 min after the intraperitoneal injection of saline. The entire procedure of conditioning was repeated at the next two days. The post-test was conducted 24 hours after conditioning of day 4. The mice were allowed to freely exploring three compartments for 15 min and CPA score was calculated (the time in the compartment (preferred more during pre-test) minus the time in the opposite compartment).

**Open-field task**

For mice infected with AAV2/9-hSyn-DIO-hM4D(Gi) or -eGFP, spontaneous motor activity was measured in an open-field arena (40 x 40 cm²) for 10 min as pre-test. Then the post-test was conducted 24 hours after. Mice were confined to open-field arena 45 min after intraperitoneal injection of CNO (1mg/kg) or saline (same dose as CNO) for 10 min. Tests were conducted with 25 lux luminance in the chamber. The apparatus was cleaned before and between trials. The distance traveled in the arena was quantified using an automated detection system (TopScan, Clever Sys) (Zhou et al., 2019).

**Immunohistochemistry and imaging**

Mice were 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 overnight. The brains were cut in 40 μm coronal sections using a vibratome (Leica, USA) and collected in PBS. To do immunohistochemistry experiments, brain sections were first washed in PBS (3 x 10 min), then blocked at 4°C with 10% normal goat serum 0.3% Triton X-100 (PBS) and then incubated with primary anti-c-Fos (1:500 dilution; Cat. No. 226003, Synaptic Systems, USA) or anti-Arc antibody (1:1000 dilution; Cat. No. 156003, Synaptic Systems, Germany) for 24 hours. Brain sections were washed in PBS (3 x 10 min), followed by incubation for 2 h with fluorophore-conjugated secondary antibody (for c-Fos: 1:1,800; for Arc: 1:200 dilution in 10% normal goat serum PBS; Jackson Immunoresearch Laboratory, USA). Images were obtained by confocal microscopy with a 20 x immersion lens and collected at a resolution of 1024 x 1024 pixels. Quantification of c-Fos, FG, Arc or c-Fos + FG (Arc + FG) labeled neurons was performed with ImageJ software with the same threshold. The positive cells were defined with staining above basal background. Counts collected from at least 4 sections from each mice were averaged to produce a value.

**Fluorescence-activated cell sorting**

Mice were sacrificed at 30 min after the post-test to prepare slices for Fluorescence-activated cell sorting. Ex vivo slices were prepared using a vibratome (Leica), PrL were microdissected. The brain tissues were digested using Tissue dissociation enzyme With Neural Tissue Dissociation Kit (T) (Miltenyi, Germany) on gentleMACS Octo Dissociator with Heaters (Miltenyi, Germany). The brain tissue homogenates were filtered with a 70 μm mesh screens, and the digestion tubes were flushed twice with HBSS, then homogenates were transferred to 100% Percoll, mixed to 30% Percoll, and 70% Percoll was injected into the bottom of 30% Percoll using a puncture needle. The tubes were centrifuged at 800 rcf for 10 min, followed by incubation for 2 h with fluorophore-conjugated secondary antibody (for c-Fos: 1:1,800; for Arc: 1:200 dilution in 10% normal goat serum PBS; Jackson Immunoresearch Laboratory, USA). Images were obtained by confocal microscopy with a 20 x immersion lens and collected at a resolution of 1024 x 1024 pixels. Quantification of c-Fos, FG, Arc or c-Fos + FG (Arc + FG) labeled neurons was performed with ImageJ software with the same threshold. The positive cells were defined with staining above basal background. Counts collected from at least 4 sections from each mice were averaged to produce a value.

**Single cell sequence amplification and qPCR**

Single Cell Sequence Specific Amplification Kit (Vazyme, China) was used to perform amplification on a PCR machine (Applied Biosystems, USA). The cycling protocol consisted of 50 C for 60 min and 95°C for 3 min followed by 20 cycles at 95°C for 15 s and 60°C for 15 min, cDNAs were then frozen at 80°C until used. Desalted primers were custom synthesized (Tsingke Biological Technology, China) and intron spanning whenever possible. qPCRs were run in triplicate using Hieff qPCR SYBR Green Master Mix (Yeasen, China) on a qPCR instrument (Roche Applied Science, USA). Negative controls for contamination from extraneous and genomic DNA were run for every gene target. The cycling protocol consisted of 95°C for 5 min followed by 40 cycles at 95°C for 10 s, 60°C for 20 s, and 72°C for 20 s. The PCR cycle threshold (CT) values were determined by the maxima of the second derivative of the fluorescence response curves. Melting curves were performed to verify the amplification of single PCR products. A weighted CT was calculated from genes for each sample based on their expression stability for normalization. The mRNA levels in each group of samples were characterized by their median values.
The following primers (Tsingke Biological Technology, China) were used for PCR amplification:

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<th>Reverse Sequence</th>
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**Electrophysiological recording**

Coronal 300 μM slices containing the PrL or PVT were prepared using a vibratome (Leica). Slices were incubated in 32°C oxygenated artificial cerebrospinal fluid (in mM: 125 NaCl, 2 KCl, 2 CaCl₂, 1.3 MgSO₄·7H₂O, 1.3 NaH₂PO₄, 12 glucose and 26 NaHCO₃) for at least 1 h before recording. Slices were transferred to a recording chamber and superfused with 2 mL min⁻¹ artificial cerebrospinal fluid. Whole-cell current- and voltage-clamp recordings were made using an EPC10 amplifier and PatchMaster 2.54 software (HEKA, Lambrecht, Germany). Electrodes had a resistance of 3–4 MΩ when filled with the patch pipette solution. Cells were held at −70 mV under a voltage-clamp mode to record sEPSCs or evoked EPSC. The internal pipette solution contained 140 mM K-Gluconate, 0.1 mM CaCl₂, 2 mM MgCl₂, 1 mM EGTA, 2 mM K₂-ATP, 0.1 mM Na₃-ATP, 10 mM HEPES, 290 mM osm kg⁻¹, adjusted to pH 7.3 with KOH. Action potential firing in response to increasing current steps using whole-cell current-clamp recordings (Holding current: 0 pA; current steps: −100 to 450 pA in 50 pA increment) in microsphere-labeled neurons in the PrL in retrieval and non-retrieval group. In Retrieval group, brain slices were prepared at 30 min after the Post-test. InSK was isolated with a combination of pharmacological tools, appropriate holding potentials, and current subtraction protocols. Outward currents were recorded in PrL-to-PVT projection neurons in the two groups and were activated by 600 ms depolarizing steps from −40 mV to 40 mV in 10 mV increment. Recordings were made in the presence of 10 μM TTX, CNQX 10 μM and 100 μM picrotoxin to block the Na⁺ channels, AMPA and GABAᵦ receptors, respectively. Apamin-sensitive I₅K currents at different holding voltages obtained by subtracting the traces of apamin (100 nM) treatment data from that of control data. For the optical stimulation of PrL-to-PVT- specific glutamatergic terminals, the channelrhodopsin-2 (ChR2) expressed in the glutamatergic terminals was stimulated by flashing 470 nm light (5 ms pulses, 0.1 Hz), which delivered via an optical fiber (core diameter 200 μm, N.A. = 0.39, ThorLabs, USA) coupled to a LED light source (Mightex, USA) 500 μm above the recording cell. Cells of the PrL projecting to PVT were retrograde labeled by injected fluorescent microspheres into the PVT and visualized using infrared differential interference contrast and fluorescent microscopy.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical significance was determined using Student’s t test for comparisons between two groups or analyses of variance (ANOVARs) for comparisons among three or more groups. One-way ANOVA and two-way ANOVA were followed by post hoc Tukey’s test (treatment with different drugs as the between-subject factors and test condition (preconditioning and postconditioning) as the within-subjects factor). All of the statistical details of experiments can be found in the figure legends. In all cases, n refers to the number of cells or animals. Offline data analysis was performed using a PatchMaster (HEKA, SCR_000034). Graphpad Prism 8.4 was used to process and analyze data and make statistical graphs. Data are presented as mean ± s.e.m..