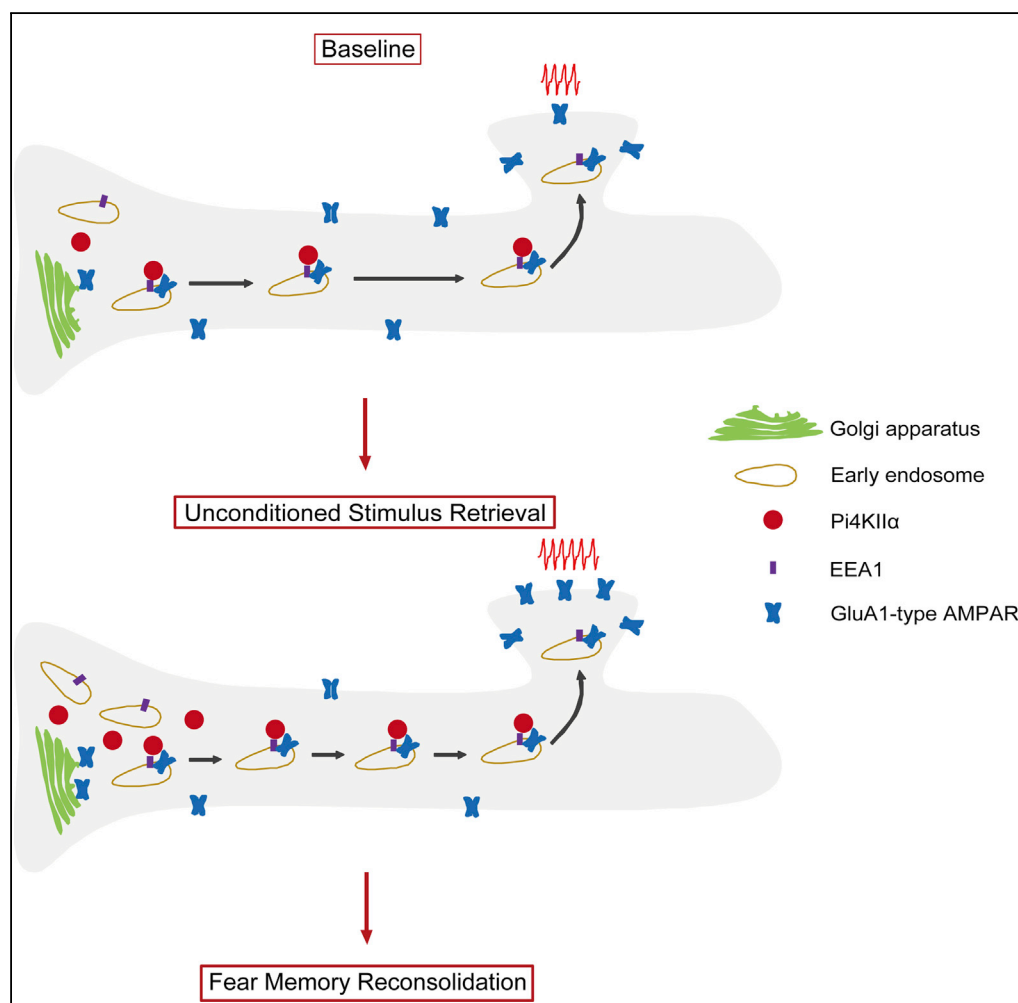


## Article

# Pi4KII $\alpha$ Regulates Unconditioned Stimulus-Retrieval-Induced Fear Memory Reconsolidation through Endosomal Trafficking of AMPA Receptors



Hongling Guo, Kai Yuan, Zhongyu Zhang, ..., Wen Zhang, Lin Lu, Ying Han

zhangw@hsc.pku.edu.cn (W.Z.)  
linlu@bjmu.edu.cn (L.L.)  
yinghan@bjmu.edu.cn (Y.H.)

## HIGHLIGHTS

Unconditioned stimulus (US) retrieval induces a transient increase in Pi4KII $\alpha$  expression

Pi4KII $\alpha$  regulates early endosomal trafficking of AMPARs during memory reconsolidation

Pi4KII $\alpha$  contributes to US-retrieval-induced synaptic enhancement in rat BLA

Pi4KII $\alpha$  inhibition after US retrieval impairs fear expression and shows long-term effects

Guo et al., iScience 23, 100895  
March 27, 2020 © 2020 The Author(s).  
<https://doi.org/10.1016/j.isci.2020.100895>

## Article

# Pi4KII $\alpha$ Regulates Unconditioned Stimulus-Retrieval-Induced Fear Memory Reconsolidation through Endosomal Trafficking of AMPA Receptors

Hongling Guo,<sup>1,4</sup> Kai Yuan,<sup>1,4</sup> Zhongyu Zhang,<sup>2</sup> Yanxue Xue,<sup>2</sup> Wei Yan,<sup>1</sup> Shiqiu Meng,<sup>2</sup> Weili Zhu,<sup>2</sup> Ping Wu,<sup>2</sup> Yanping Bao,<sup>2</sup> Jie Shi,<sup>2</sup> Wen Zhang,<sup>2,\*</sup> Lin Lu,<sup>1,3,\*</sup> and Ying Han<sup>2,5,\*</sup>

**SUMMARY**

**Targeting memory reconsolidation is an effective intervention for treating posttraumatic stress disorder (PTSD). Disrupting unconditioned stimulus (US)-retrieval-induced fear memory reconsolidation has become an effective therapeutic approach to attenuate fear memory, but the underlying molecular mechanisms remain unknown. Here, we report that US-retrieval-dependent increase in phosphatidylinositol 4-kinase II $\alpha$  (Pi4KII $\alpha$ ) promotes early endosomal trafficking of AMPA receptors, leading to the enhancement of synaptic efficacy in basolateral amygdala (BLA) neurons. The inhibition of Pi4KII $\alpha$  by an inhibitor or short hairpin RNA impaired contextual fear memory reconsolidation. This disruptive effect persisted for at least 2 weeks, which was restored by Pi4KII $\alpha$  overexpression with TAT-Pi4KII $\alpha$ . Furthermore, the blockade of early endosomal trafficking following US retrieval reduced synaptosomal membrane GluA1 levels and decreased subsequent fear expression. These data demonstrate that Pi4KII $\alpha$  in the BLA is crucial for US-retrieval-induced fear memory reconsolidation, the inhibition of which might be an effective therapeutic strategy for treating PTSD.**

**INTRODUCTION**

Posttraumatic stress disorder (PTSD) is a complex neuropsychiatric disorder that is characterized by re-experiencing the trauma, avoidance, emotional numbing, and hyperarousal (Shalev et al., 2017). Abnormalities in the learning and processing of trauma-related fear memory are believed to play a prominent role in the pathophysiology of PTSD (Fanselow and LeDoux, 1999). In laboratory studies, Pavlovian fear conditioning is widely used in both rodents and humans to investigate fear memory. Subjects are typically trained to associate a neutral conditioned stimulus (CS) with an aversive US, such as footshock. After acquisition, fear memory becomes permanent through a process called consolidation (Dudai et al., 2015). Once retrieved or reactivated, the consolidated fear memory is believed to enter a labile state, known as reconsolidation. During reconsolidation, the memory is dynamic and susceptible to modifications, thus providing an opportunity to interfere with seemingly stable memories (Beckers and Kindt, 2017; Lee et al., 2017).

Considerable rodent and human studies have revealed that pharmacological interventions following CS retrieval can disrupt memory reconsolidation and attenuate fear expression (Brunet et al., 2008; Debiec et al., 2010; Nader et al., 2000). Recent studies reported the US-exposure-specific reconsolidation of learned fear in an amygdala-dependent manner (Debiec et al., 2010; Diaz-Mataix et al., 2011). Pharmacological interventions after US retrieval disrupted more CS-US associations than CS retrieval (Huang et al., 2017). US-retrieval-based reconsolidation interventions were also shown to successfully target remote memories (Liu et al., 2014), thus suggesting a powerful noninvasive procedure to treat psychiatric disorders, such as PTSD. However, the practicality of implementing US-retrieval-based manipulations is questionable when considering the ethical aspects of reexposing PTSD patients to traumatic events, such as war, assault, or natural disasters. Perhaps one of the best ways to overcome this problem is to understand the distinct mechanisms that underlie CS-retrieval- and US-retrieval-triggered memory reconsolidation, thus developing a more practical way to clinically rewrite maladaptive memories. Cellular and molecular processes of CS-retrieval-induced memory reconsolidation have been widely studied (Johansen et al., 2011; Tronson and Taylor, 2007), but the mechanisms that underlie US-retrieval-induced memory reconsolidation remain largely unknown.

<sup>1</sup>Peking University Sixth Hospital, Peking University Institute of Mental Health, NHC Key Laboratory of Mental Health (Peking University), National Clinical Research Center for Mental Disorders (Peking University Sixth Hospital), 51 Huayuan Bei Road, Haidian District, Beijing 100191, China

<sup>2</sup>National Institute on Drug Dependence and Beijing Key Laboratory of Drug Dependence, Peking University, 38 Xueyuan Road, Haidian District, Beijing 100191, China

<sup>3</sup>Peking-Tsinghua Center for Life Sciences and PKU-IDG/McGovern Institute for Brain Research, Peking University, Beijing 100871, China

<sup>4</sup>These authors contributed equally

<sup>5</sup>Lead Contact

\*Correspondence:

zhangw@hsc.pku.edu.cn (W.Z.),

linlu@bjmu.edu.cn (L.L.),

yinghan@bjmu.edu.cn (Y.H.)

<https://doi.org/10.1016/j.isci.2020.100895>



Accumulating evidence indicates that  $\alpha$ -amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid receptors (AMPA) play an important role in synaptic plasticity, learning, and memory. The exocytosis and endocytosis of AMPARs are regulated by complex molecular and cellular mechanisms, including AMPAR binding proteins, posttranslational modifications, and endosomal trafficking (Anggono and Huganir, 2012; Jacobi and von Engelhardt, 2018). Alterations of AMPAR surface expression have been shown to be involved in fear memory reconsolidation in the amygdala (Clem and Huganir, 2010; Hong et al., 2013; Zhou et al., 2015). Our recent work indicated a possible role for AMPAR trafficking in US-retrieval-induced reconsolidation (Liu et al., 2014; Luo et al., 2015; Yuan et al., 2019). These findings strongly support a role for AMPAR trafficking in fear memory reconsolidation, but the underlying molecular mechanisms remain unexplored.

Phosphatidylinositol 4-kinase II $\alpha$  (Pi4KII $\alpha$ ) is the dominant lipid kinase that generates Pi4-phosphate. Pi4KII $\alpha$  is mainly localized in the *trans*-Golginetwork (TGN) and endosomes (Clayton et al., 2013; Minogue, 2018). Pi4KII $\alpha$  is involved in many different cell pathways, including membrane trafficking, ion channel regulation, and vesicle trafficking (Guo et al., 2003; Minogue et al., 2006; Pan et al., 2008; Salazar et al., 2005; Wang et al., 2003, 2007). Pi4KII $\alpha$  regulates the trafficking of cargo proteins, including transferrin, epidermal growth factor receptor, lysosome membrane protein 2, glucocerebrosidase, and vesicle-associated membrane protein 3 (Jovic et al., 2012, 2014; Minogue et al., 2006). Pi4KII $\alpha$  was recently reported to play a role in the surface expression of GluA1 in hippocampal neurons (Robinson et al., 2014), suggesting its potential involvement in AMPAR trafficking. Pi4KII $\alpha$  also regulates receptor sorting at early endosomes, and Pi4KII $\alpha$  knockdown decreases the efficiency of sorting from early endosomes (Henmi et al., 2016). However, the role of Pi4KII $\alpha$  in the early endosomal trafficking of AMPARs and US-retrieval-induced fear memory reconsolidation remains unclear.

Based on these previous findings, we hypothesized that Pi4KII $\alpha$  plays a critical role in US-retrieval-induced fear memory reconsolidation by regulating the early endosomal trafficking of AMPARs. Using a combined behavioral, biochemical, molecular biological, and electrophysiological approach, we found that US retrieval resulted in the rapid and transient upregulation of Pi4KII $\alpha$  expression, which in turn increased the recruitment of early endosomes, promoted synaptic AMPAR incorporation, and enhanced synaptic transmission in the basolateral amygdala (BLA). These molecular and synaptic alterations ultimately contributed to the US-retrieval-induced reconsolidation of fear memory.

## RESULTS

### Pi4KII $\alpha$ Localizes to Early Endosomes in BLA Neurons and Colocalizes with AMPARs

Previous studies have shown that Pi4KII $\alpha$  is expressed in neurons and astrocytes throughout the brain (Larimore et al., 2011; Simons et al., 2009). In the present study, we used a specific antibody to examine the localization of Pi4KII $\alpha$  in the rat brain. Pi4KII $\alpha$  expression was enriched in learning- and memory-related brain areas, including the prelimbic cortex (PrL), infralimbic cortex (IL), nucleus accumbens (NAc) core, NAc shell, dorsal hippocampus (DH), ventral hippocampus (VH), central nucleus of the amygdala (CeA), and BLA (Figure S1A).

We then examined the subcellular distribution of endogenous Pi4KII $\alpha$  in the BLA by Western blot and immunostaining (Figures S1B–S1E). Consistent with previous findings (Guo et al., 2003), we found that Pi4KII $\alpha$  was enriched in the cytosolic fraction but not in the synaptosomal membrane fraction and presented no colocalization with postsynaptic density protein 95 (PSD95), a postsynaptic marker (Figures S1C and S1E). Immunostaining revealed the partial colocalization of Pi4KII $\alpha$  with the somatodendritic marker mitogen-activated protein 2 (Figure S1E). Pi4KII $\alpha$  was also localized to early endosomes that were labeled with early endosome antigen 1 (EEA1) in BLA neurons (Figure S1D). We also found the colocalization of Pi4KII $\alpha$  with GluA1 and GluA2 in BLA neurons (Figure S1D).

Given the colocalization of Pi4KII $\alpha$ , EEA1, and GluA1, we performed co-immunoprecipitation using rat BLA homogenates to detect whether endogenous Pi4KII $\alpha$  directly binds EEA1 and GluA1. We found that an antibody against Pi4KII $\alpha$  co-immunoprecipitated EEA1 and GluA1 (Figure S1F). In addition, an antibody against EEA1 co-immunoprecipitated Pi4KII $\alpha$  and GluA1 (Figure S1G). These results indicate that Pi4KII $\alpha$  is a binding partner of EEA1 and GluA1 *in vivo*.

### Unconditioned Stimulus Retrieval Transiently Upregulates Pi4KII $\alpha$ Levels and Promotes Synaptic AMPAR Incorporation in the Rat BLA

We first determined the appropriate US intensity to reactivate fear memory in rats. Six groups of rats were trained for contextual fear conditioning. Twenty-four hours later, the rats underwent different retrieval

patterns (no retrieval [NoR], weak US retrieval [USR], or strong USR) immediately followed by bilateral infusions of anisomycin (62.5  $\mu\text{g}/\mu\text{L}$ ) or vehicle in the BLA. A freezing test was performed 24 h later (Figure S2A). The two-way analysis of variance (ANOVA) of fear expression revealed a significant retrieval  $\times$  treatment interaction ( $F_{2, 39} = 4.96$ ,  $p = 0.012$ ). The *post hoc* analysis showed that fear responses in rats that underwent weak US retrieval and received an anisomycin injection significantly decreased compared with rats that underwent weak US retrieval and received a vehicle injection ( $p = 0.0093$ , Figure S2B). These results indicate that exposure to a weak electric shock triggered US-specific memory reconsolidation in rats.

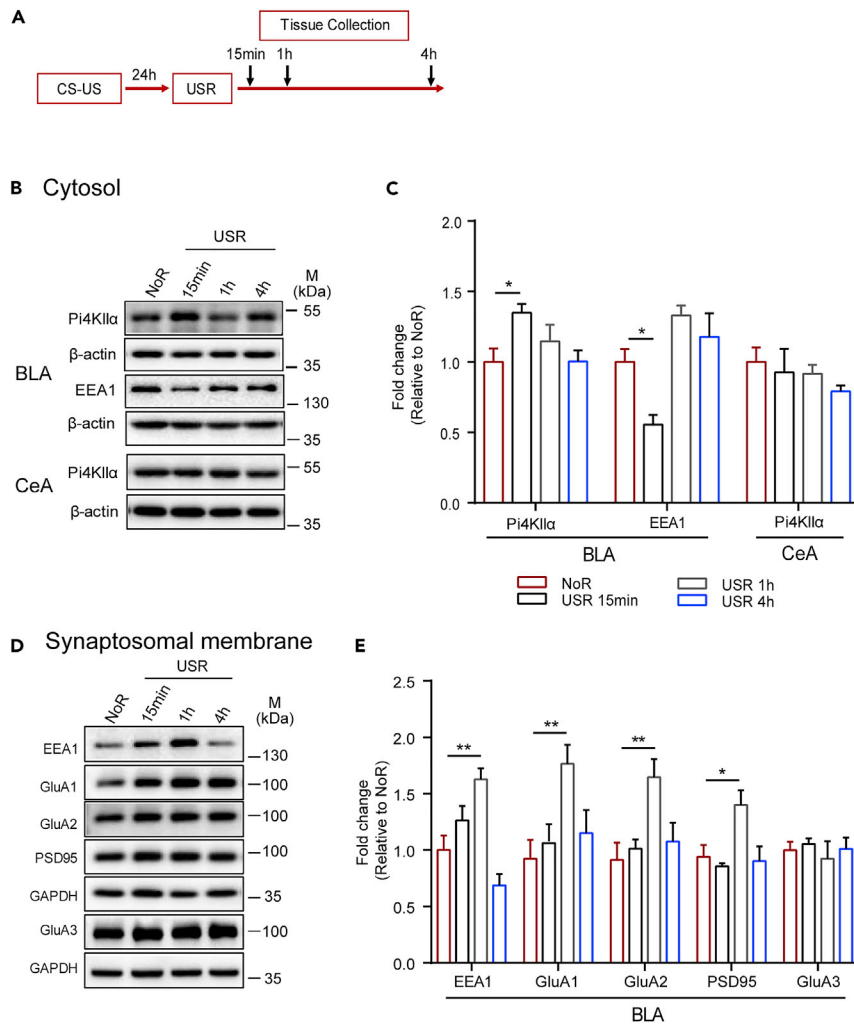
To examine whether Pi4KII $\alpha$  is activated after US retrieval, four groups of rats were trained for contextual fear conditioning and underwent US retrieval one day later. Brain tissues were then collected 15 min, 1 h, or 4 h later, time points within the reconsolidation window (Figure 1A). Pi4KII $\alpha$  levels increased 15 min after US retrieval in the BLA but not CeA and returned to baseline levels at 1 h (one-way ANOVA; BLA,  $F_{3, 20} = 3.217$ ,  $p = 0.0448$ ; *post hoc*, USR 15 min versus NoR,  $p = 0.0362$ ; Figures 1B and 1C). These results suggest the transient regulation of Pi4KII $\alpha$  in US-retrieval-induced contextual fear memory reconsolidation.

Pi4KII $\alpha$  is known to play an important role in endosomal trafficking (Minogue, 2018). We examined whether US retrieval alters the levels of endosomal compartments. We found a significant reduction of cytosolic EEA1 levels 15 min after US retrieval but a significant increase in the synaptosomal membrane fraction 1 h after retrieval (one-way ANOVA; cytosol,  $F_{3, 20} = 9.666$ ,  $p = 0.0004$ ; *post hoc*, USR 15 min versus NoR,  $p = 0.0227$ ; membrane,  $F_{3, 20} = 12.11$ ,  $p < 0.0001$ ; *post hoc*, USR 1h versus NoR,  $p = 0.0026$ ; Figures 1B–1E). This shift suggests the dynamic regulation of EEA1 levels in memory reconsolidation.

Fear memory retrieval induces transient changes in AMPAR surface expression (Hong et al., 2013; Liu et al., 2014; Rao-Ruiz et al., 2011; Zhou et al., 2015). We found that US retrieval significantly increased the levels of GluA1 (one-way ANOVA;  $F_{3, 18} = 4.27$ ,  $p = 0.0192$ , *post hoc*, USR 1 h versus NoR,  $p = 0.0092$ ), GluA2 (one-way ANOVA;  $F_{3, 18} = 5.16$ ,  $p = 0.0095$ , *post hoc*, USR 1 h versus NoR,  $p = 0.005$ ), and PSD95 (one-way ANOVA;  $F_{3, 16} = 4.899$ ,  $p = 0.0133$ , *post hoc*, USR 1 h versus NoR,  $p = 0.0217$ ) in the synaptosomal membrane fraction of the BLA 1 h after US retrieval (Figures 1D and 1E). These data indicate that US retrieval after fear conditioning enhanced AMPAR trafficking and synaptic potentiation.

### Unconditioned Stimulus Retrieval Increases Dendritic Spine Density and Synaptic Activity in Rat BLA Neurons

Alterations of synaptic morphology contribute to fear memory acquisition and cocaine relapse (Gipson et al., 2013; Roberts et al., 2010). However, unclear is whether BLA neurons undergo a similar process during memory reconsolidation. We investigated spine density alterations during memory reconsolidation in the BLA using Golgi staining. Four groups of rats underwent contextual fear conditioning. The next day, the rats underwent a US retrieval procedure. Brain tissues were then collected 15 min, 1 h, or 4 h later and subjected to Golgi staining (Figure 2A). The spine densities of BLA neurons in rats in the US retrieval group significantly increased at all three time points compared with the NoR group (one-way ANOVA;  $F_{3, 53} = 44.12$ ,  $p < 0.0001$ ; *post hoc*, all time points versus NoR, 15 min,  $p = 0.0001$ ; 1 h,  $p = 0.0001$ ; 4 h,  $p = 0.0001$ ; Figure 2B). We then classified dendrite spines into several types, including stubby, thin, mushroom, branched, and filopodia (Harris et al., 1992; Lippman and Dunaevsky, 2005; Zagrebelsky et al., 2005). Golgi staining revealed that US retrieval increased the density of mushroom and thin-shaped spines at the 15 min, 1 h, and 4 h time points, whereas the density of filopodia-shaped spines increased only 4 h after US retrieval (one-way ANOVA; mushroom,  $F_{3, 53} = 39.04$ ,  $p < 0.0001$ ; *post hoc*, all time points versus NoR, 15 min,  $p = 0.0017$ ; 1 h,  $p = 0.0001$ ; 4 h,  $p = 0.0001$ ; thin,  $F_{3, 53} = 14.37$ ,  $p < 0.0001$ ; *post hoc*, all time points versus NoR, 15 min,  $p = 0.0101$ ; 1 h,  $p = 0.0001$ ; 4 h,  $p = 0.0006$ ; filopodia,  $F_{3, 53} = 5.381$ ,  $p = 0.0026$ ; *post hoc*, USR 4 h versus NoR, 4 h,  $p = 0.0038$ ; Figure 2C). The density of stubby and branched spines remained unchanged (Figure 2C). We also measured miniature excitatory postsynaptic current (mEPSC) frequency and amplitude 1 h after US retrieval, the time point identical to the changes in synaptosomal membrane AMPARs (Figure 2D). Consistent with the increases in spine density and synaptosomal membrane GluA1 and GluA2 levels in the BLA, US retrieval significantly increased mEPSC frequency (unpaired *t* test;  $t = 3.032$ ,  $p = 0.005$ ) but had no effect on mEPSC amplitude in BLA neurons (Figures 2E–2G). These data suggest that US retrieval after fear conditioning enhances neuronal connectivity in the BLA.



**Figure 1. Unconditioned Stimulus Retrieval Transiently Upregulates Pi4KII $\alpha$  Levels and Promotes Synaptic AMPAR Incorporation in the Rat BLA**

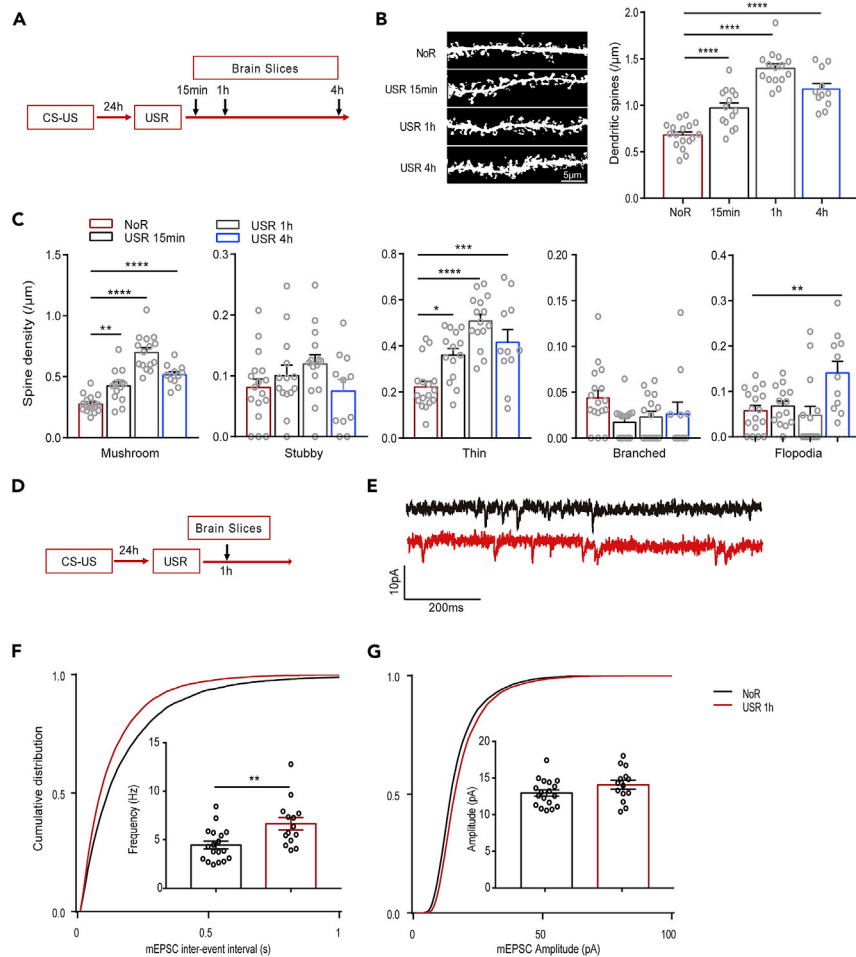
(A) Experimental timeline.

(B and C) (B) Representative Western blots and (C) protein levels of Pi4KII $\alpha$  and EEA1 in the basolateral amygdala (BLA) and central nucleus of the amygdala after US retrieval. Cytosolic Pi4KII $\alpha$  levels increased in the BLA but not CeA 15 min after US retrieval. Cytosolic EEA1 levels in the BLA decreased 15 min after US retrieval ( $n = 6$  rats/group).

(D and E) (D) Representative Western blots and (E) protein levels of EEA1, GluA1-3, and PSD95 in the BLA after US retrieval. Unconditioned stimulus retrieval increased the synaptosomal membrane expression of EEA1, GluA1, GluA2, and PSD95 but not GluA3 ( $n = 4-6$  rats/group). Data are reported as mean  $\pm$  SEM. One-way ANOVA followed by Dunnett's multiple-comparison *post hoc* test. \* $p < 0.05$  and \*\* $p < 0.01$ . NoR, no retrieval; USR, unconditioned stimulus retrieval.

### Pharmacological Inhibition of Pi4KII $\alpha$ in the BLA after US Retrieval Impairs Contextual Fear Memory Reconsolidation, and This Effect Is Long Lasting

We next tested whether Pi4KII $\alpha$  is necessary for the US retrieval-induced reconsolidation process. The intra-BLA infusion of phenylarsine oxide (PAO), an inhibitor of Pi4KII $\alpha$  (Boura and Nencka, 2015), was performed immediately after retrieval. As shown in Figure 3A, four groups of rats underwent contextual fear conditioning. On the next day, the rats were given different doses of PAO (0, 50, 100, and 200  $\mu$ M/side) bilaterally in the BLA immediately after US retrieval. A freezing test was conducted 24 h later. Rats that received 200  $\mu$ M PAO after US retrieval exhibited a significant reduction of fear expression (one-way ANOVA;  $F_{3, 28} = 3.56$ ,  $p = 0.0267$ ; *post hoc*, vehicle versus 200  $\mu$ M PAO,  $p = 0.0139$ ; Figure 3B), suggesting that the PAO injection in the BLA impaired the reconsolidation of contextual fear memory.



**Figure 2. Unconditioned Stimulus Retrieval Increases Spine Density and Synaptic Activity in the Rat BLA**

(A) Experimental timeline.

(B) Representative images of Golgi-stained sections (*left*) and quantitative results (*right*). Total dendritic spine density increased 15 min, 1 h, and 4 h after US retrieval. NoR, n = 17 neurons, 12 slices from 3 rats; USR 15 min, n = 14 neurons, 12 slices from 3 rats; USR 1 h, n = 15 neurons, 12 slices from 3 rats; USR 4 h, n = 11 neurons, 10 slices from 3 rats. Scale bar = 5  $\mu$ m.

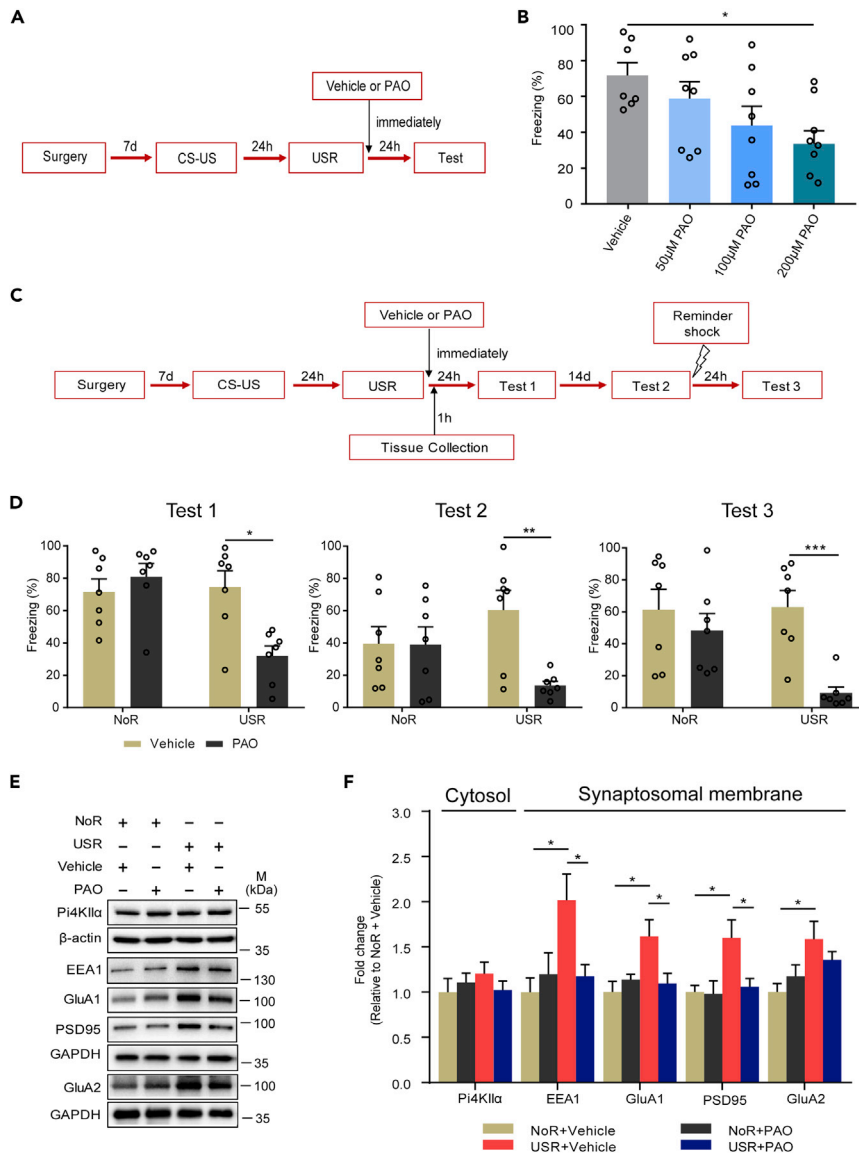
(C) Quantification of five types of dendritic spines. The data are reported as mean  $\pm$  SEM. One-way ANOVA followed by Dunnett's multiple-comparison *post hoc* test. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 and \*\*\*\*p < 0.0005.

(D) Experimental timeline.

(E) Example mEPSC traces from BLA neurons in the NoR group (n = 18 neurons, 8 slices from 5 rats) and USR 1 h group (n = 14 neurons, 7 slices from 4 rats).

(F and G) Cumulative distribution of mEPSC interevent intervals and average frequency (F), or mEPSC amplitude (G) of neurons in the NoR group and USR group. Data are reported as mean  $\pm$  SEM. Unpaired t test. \*\*p < 0.01. NoR, no retrieval; USR, unconditioned stimulus retrieval; mEPSC, miniature excitatory postsynaptic current.

We then tested whether PAO affects contextual fear memory acquisition, consolidation, and retrieval. Two groups of rats were microinjected with PAO or vehicle in the BLA before fear conditioning, and a freezing test was performed 1 h after conditioning (Figure S3A). No differences in fear expression were found between rats that received PAO and rats that received vehicle (unpaired t test;  $t = 0.561$ ,  $p = 0.586$ ; Figure S3B). We used another two groups of rats to determine the effect of a PAO injection in the BLA on fear memory consolidation. Immediately after contextual fear conditioning, the rats were microinjected with PAO or vehicle in the BLA, and a freezing test was performed 24 h later (Figure S3C). No differences in fear expression were found between rats that received PAO and rats that received vehicle (unpaired t test;  $t = 0.011$ ,  $p = 0.991$ ; Figure S3D). Another two groups of rats were used to determine the effect of a PAO injection in the BLA on memory retrieval. Twenty-four hours after contextual fear conditioning, the rats were



**Figure 3. Pharmacological inhibition of Pi4KIIα after US Retrieval Impairs Contextual Fear Memory Reconsolidation and Exerts a Long-Lasting Effect**

(A) Experimental timeline.

(B) Tests of fear expression in rats that were injected with PAO (0, 50, 100, and 200 µM/side) in the BLA immediately after US retrieval. USR + Vehicle, n = 7 rats; USR + 50 µM PAO, n = 8 rats; USR + 100 µM PAO, n = 8 rats; USR + 200 µM PAO, n = 9 rats. PAO, phenylarsine oxide. Data represent the mean ± SEM. One-way ANOVA followed by Dunnett’s multiple-comparison *post hoc* test. \*p < 0.05.

(C) Experimental timeline.

(D) The inhibitory effect of Pi4KIIα inhibition in the BLA after US retrieval on fear expression lasted at least 2 weeks and was not restored by a reminder footshock. NoR + Vehicle, n = 7 rats; NoR + PAO, n = 7 rats; USR + Vehicle, n = 7 rats; USR + PAO, n = 7 rats; Data represent the mean ± SEM. Repeated measures two-way ANOVA followed by Tukey’s multiple-comparison *post hoc* test. \*p < 0.05, \*\*p < 0.01, and \*\*\*p < 0.001.

(E and F) (E) Representative Western blots and (F) protein levels of Pi4KIIα, EEA1, GluA1, GluA2, and PSD95 in the BLA in rats that were injected with PAO 1 h after US retrieval (n = 4–6 rats/group). The inhibition of Pi4KIIα activity in the BLA immediately after US retrieval blocked the increases in synaptosomal membrane EEA1, GluA1, and PSD95 levels that were induced by US retrieval. Data are reported as mean ± SEM. Two-way ANOVA followed by Tukey’s multiple-comparison *post hoc* test. \*p < 0.05. NoR, no retrieval; USR, unconditioned stimulus retrieval.



microinjected with PAO or vehicle in the BLA, and a freezing test was performed 1 h later (Figure S3E). We found no difference in fear expression between PAO- and vehicle-injected rats (unpaired t test;  $t = 0.397$ ,  $p = 0.699$ ; Figure S3F). These results indicated that the inhibition of Pi4KII $\alpha$  with PAO in the BLA had no effect on fear memory acquisition, consolidation, or retrieval. Additionally, the inhibition of Pi4KII $\alpha$  in the BLA had no effect on CS-retrieval-induced fear memory reconsolidation (Figure S4). The PAO injection in the BLA also had no effect on locomotor activity, anxiety-like behavior, or depression-related behavior (Figure S5).

We next tested the long-lasting effect of Pi4KII $\alpha$  inhibition after US retrieval on contextual fear memory reconsolidation. As shown in Figure 3C, on day 1, four groups of rats underwent contextual fear conditioning. Twenty-four hours later, two groups of rats were microinjected with PAO or vehicle in the BLA immediately after the US retrieval procedure. Another two groups of rats received PAO or vehicle without retrieval. On day 3, all of the rats underwent a freezing test (Test 1). Fourteen days later, freezing behavior was assessed in all of the rats (Test 2). A strong electric footshock (1.0 mA) was then given immediately after Test 2. Twenty-four hours later, the reinstatement test (Test 3) was performed to evaluate fear expression. The two-way repeated-measures ANOVA of fear expression revealed main effects of test ( $F_{2, 48} = 10.91$ ,  $p = 0.0001$ ) and treatment ( $F_{3, 24} = 10.73$ ,  $p = 0.0001$ ). The *post hoc* analysis revealed that fear expression in the group that underwent US retrieval and received the PAO injection in the BLA significantly decreased compared with the group that underwent US retrieval and received vehicle in Test 1 ( $p = 0.0108$ ), Test 2 ( $p = 0.0041$ ), and Test 3 ( $p = 0.0007$ ; Figure 3D).

We then used four groups of rats to determine the effect of PAO administration in the BLA on the expression of AMPAR subunits. As shown in Figure 3C, tissues were collected 1 h after retrieval for Western blot analysis. The PAO injection in the BLA immediately after US retrieval decreased the levels of EEA1 (two-way ANOVA; group  $\times$  treatment interaction:  $F_{1, 19} = 6.006$ ,  $p = 0.0241$ ; *post hoc*, USR-vehicle versus USR-PAO,  $p = 0.0446$ ), GluA1 (two-way ANOVA; group  $\times$  treatment interaction:  $F_{1, 19} = 6.215$ ,  $p = 0.0221$ ; *post hoc*, USR-vehicle versus USR-PAO,  $p = 0.0456$ ), and PSD95 (two-way ANOVA; group  $\times$  treatment interaction:  $F_{1, 19} = 3.662$ ,  $p = 0.0709$ ; *post hoc*, USR-vehicle versus USR-PAO,  $p = 0.0436$ ), whereas the expression of Pi4KII $\alpha$  (two-way ANOVA; group  $\times$  treatment interaction:  $F_{1, 12} = 1.376$ ,  $p = 0.264$ ; *post hoc*, USR-vehicle versus USR-PAO,  $p = 0.736$ ) and GluA2 (two-way ANOVA; group  $\times$  treatment interaction:  $F_{1, 12} = 2.374$ ,  $p = 0.1493$ ; *post hoc*, USR-vehicle versus USR-PAO,  $p = 0.616$ ) remained unchanged (Figures 3E and 3F). We also found no difference in Pi4KII $\alpha$  levels between vehicle- and PAO-injected rats 24 h after US retrieval (unpaired t test;  $t = 0.731$ ,  $p = 0.493$ ; Figure S6).

Altogether, these results indicate that the pharmacological inhibition of Pi4KII $\alpha$  in the BLA after US retrieval disrupted contextual fear memory reconsolidation, which lasted for at least two weeks, and fear memory did not recover after a reminder footshock.

### Pharmacological Inhibition of Pi4KII $\alpha$ in the BLA without Exposure or 6 h after Exposure to a US Has No Effect on the Expression of Contextual Fear Memory

We further tested whether the effect of PAO on subsequent fear expression is retrieval dependent and temporally specific. Two groups of rats were trained for contextual fear conditioning and then received a microinjection of vehicle (1.0  $\mu$ L/side) or PAO (200  $\mu$ M/side) bilaterally in the BLA without undergoing a US retrieval trial (Figure S7A). The PAO injection in the BLA had no effect on the subsequent expression of contextual fear memory in the absence of US retrieval (Figure S7B). Another two groups of rats underwent contextual fear conditioning and then received a microinjection of vehicle (1.0  $\mu$ L/side) or PAO (200  $\mu$ M/side) bilaterally in the BLA 6 h after US retrieval, and a freezing test was performed 24 h later (Figure S7C). No differences in fear expression were observed between vehicle- and PAO-injected rats (unpaired t test;  $t = 0.0822$ ,  $p = 0.936$ ; Figure S7D), indicating that the effect of PAO on contextual fear memory reconsolidation was temporally limited.

### Genetic Knockdown of Pi4KII $\alpha$ Disrupts the Reconsolidation of Contextual Fear Memory and Inhibits Synaptic Transmission in the BLA

The microinjection of PAO in the BLA after US retrieval significantly impaired contextual fear memory reconsolidation. In addition to inhibiting Pi4KII $\alpha$ , PAO has also been shown to inhibit neurotransmitter release and phosphatase activity (Searl and Silinsky, 2000; Zhang et al., 1992), which may explain the effect on memory reconsolidation that was observed herein. To further reveal the role of Pi4KII $\alpha$  in the



reconsolidation of contextual fear memory, we constructed an adeno-associated virus (AAV) to knockdown endogenous Pi4KII $\alpha$ . We first evaluated the efficiency of the viral vector and found that it specifically decreased Pi4KII $\alpha$  levels in the BLA (unpaired t test;  $t = 8.326$ ,  $p = 0.0002$ ; Figures 4A–4C).

To test whether Pi4KII $\alpha$  downregulation affects the acquisition and consolidation of contextual fear memory, rats were injected Pi4KII $\alpha$  short hairpin RNA (shRNA; shPi4KII $\alpha$ ) or scrambled shRNA (SCR) and underwent a training session followed by a freezing test 1 or 24 h later (Figure 4D). No differences in freezing were found between the shPi4KII $\alpha$  and SCR groups (unpaired t test; 1 h,  $t = 0.363$ ,  $p = 0.723$ ; 24 h,  $t = 1.041$ ,  $p = 0.318$ ; Figure 4E), suggesting that Pi4KII $\alpha$  knockdown had no effect on the acquisition or consolidation of contextual fear memory. Rats with Pi4KII $\alpha$  knockdown were also tested for anxiety- and depression-like behavior, and no significant effects were observed (Figure S8).

To further evaluate the role of Pi4KII $\alpha$  in the US-retrieval-induced reconsolidation of contextual fear memory, we prepared TAT-Pi4KII $\alpha$  proteins that were microinjected in the BLA in Pi4KII $\alpha$  knockdown rats to mimic normal Pi4KII $\alpha$  expression patterns. We first evaluated the transduction efficacy of TAT-Pi4KII $\alpha$  by assessing protein levels using Western blot (Figure S9A). Pi4KII $\alpha$  levels in the BLA significantly increased 1 h after the TAT-Pi4KII $\alpha$  microinjection (unpaired t test;  $t = 2.624$ ,  $p = 0.0394$ ; Figures S9B and S9C).

Four groups of rats received microinjections of shPi4KII $\alpha$  or SCR in the BLA 21 days before contextual fear conditioning. Twenty-three hours after training, the rats were microinjected with TAT-Pi4KII $\alpha$  or saline and then underwent US retrieval trials 1 h later. One day later, all of the rats underwent a freezing test (Test 1). Fourteen days later, freezing expression was assessed in all of the rats (Test 2), and then a strong electric footshock (1.0 mA) was given immediately after Test 2. Twenty-four hours later, a reinstatement test (Test 3) was performed to evaluate long-lasting inhibitory effects (Figure 4F). The two-way repeated-measures ANOVA of fear expression revealed main effects of test ( $F_{2, 58} = 32.2$ ,  $p < 0.0001$ ) and treatment ( $F_{3, 29} = 13.09$ ,  $p < 0.0001$ ; Figure 4G). The *post hoc* analysis revealed that Pi4KII $\alpha$  knockdown in the BLA significantly reduced fear expression compared with SCR-injected rats (Test 1,  $p = 0.0144$ ; Test 2,  $p = 0.0027$ ; Test 3,  $p = 0.0002$ ; Figure 4G). The TAT-Pi4KII $\alpha$  microinjection in the BLA restored fear expression in Pi4KII $\alpha$  knockdown rats (Test 1,  $p = 0.0230$ ; Test 2,  $p = 0.0128$ ; Test 3,  $p = 0.0415$ ; Figure 4G).

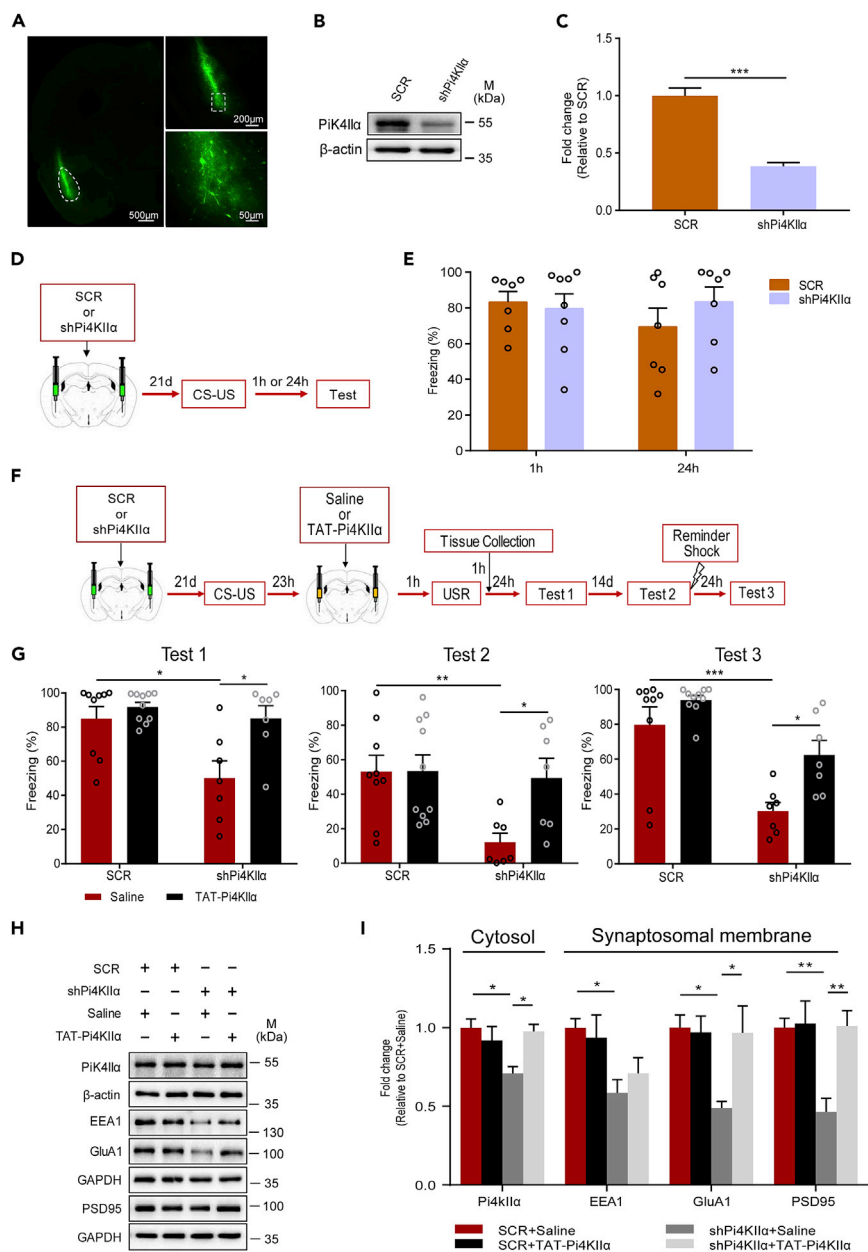
We then examined protein expression 1 h after US retrieval in shPi4KII $\alpha$ - and TAT-Pi4KII $\alpha$ -injected rats (Figures 4H and 4I). We found that shPi4KII $\alpha$  microinjections in the BLA significantly decreased the levels of Pi4KII $\alpha$  (two-way ANOVA; AAV  $\times$  treatment interaction:  $F_{1, 12} = 8.08$ ,  $p = 0.0148$ ; *post hoc*,  $p = 0.0259$ ), EEA1 (two-way ANOVA; main effect of AAV:  $F_{1, 20} = 9.823$ ,  $p = 0.0052$ ; *post hoc*,  $p = 0.0432$ ), GluA1 (two-way ANOVA; AAV  $\times$  treatment interaction:  $F_{1, 20} = 5.345$ ,  $p = 0.0316$ ; *post hoc*,  $p = 0.0179$ ), and PSD95 (two-way ANOVA; AAV  $\times$  treatment interaction:  $F_{1, 20} = 6.511$ ,  $p = 0.0190$ ; *post hoc*,  $p = 0.0069$ ) after US retrieval (Figures 4H and 4I). TAT-Pi4KII $\alpha$  microinjections restored the decrease in the levels of Pi4KII $\alpha$  ( $p = 0.0407$ ), GluA1 ( $p = 0.0279$ ), and PSD95 ( $p = 0.0058$ ) in shPi4KII $\alpha$ -injected rats (Figures 4H and 4I).

To investigate the effect of Pi4KII $\alpha$  knockdown on synaptic activity in the BLA, we performed whole-cell voltage-clamp recordings in SCR- and shPi4KII $\alpha$ -infected BLA neurons in acute brain slices from untrained rats (Figure 5A). Pi4KII $\alpha$  knockdown did not significantly alter mEPSC frequency or amplitude (Figures 5B–5D). We then examined whether Pi4KII $\alpha$  knockdown in the BLA affects the US-retrieval-induced increase in synaptic strength. Pi4KII $\alpha$  knockdown significantly decreased mEPSC frequency (unpaired t test;  $t = 2.503$ ,  $p = 0.0202$ ) 1 h after US retrieval but had no effect on mEPSC amplitude (Figures 5E–5H).

Altogether, these results demonstrate a critical role for Pi4KII $\alpha$  in US-retrieval-induced fear reconsolidation, likely via the regulation of AMPAR trafficking and synaptic transmission in the BLA.

### Preventing Early Endosomal Trafficking in the BLA after US Retrieval Impairs the Reconsolidation of Contextual Fear Memory and Exerts a Long-Lasting Effect

Early endosomal sorting has been shown to be involved in AMPAR trafficking (Parkinson and Hanley, 2018). We found that EEA1 functions as a binding partner of Pi4KII $\alpha$  (Figures S1F and S1G). Dynasore is used to block early endosome transport (Mesaki et al., 2011). We tested the effect of local dynasore infusion (400  $\mu$ M/side) in the BLA immediately after US retrieval on the reconsolidation of contextual fear memory. As shown in Figure 6A, four groups of rats underwent contextual fear conditioning. Twenty-three hours later, the rats were microinjected with TAT-Pi4KII $\alpha$  or saline. One hour later, the rats were microinjected



**Figure 4. Knockdown of Pi4KII $\alpha$  in the BLA Disrupts the Reconsolidation of Contextual Fear Memory**

(A) Representative infusion site in the rat BLA.

(B and C) (B) Representative Western blots and (C) Pi4KII $\alpha$  expression in the BLA 21 days after the injection of AAV-expressing Pi4KII $\alpha$  shRNA (shPi4KII $\alpha$ ) or scramble shRNA (SCR; n = 4 rats/group). Data represent the mean  $\pm$  SEM. Unpaired t test. \*\*\*p < 0.001.

(D) Experimental timeline.

(E) shPi4KII $\alpha$ -injected rats and SCR-injected rats exhibited comparable fear expression. 1 h: SCR, n = 7 rats; shPi4KII $\alpha$ , n = 8 rats; 24 h: SCR, n = 7 rats; shPi4KII $\alpha$ , n = 7 rats. Data are reported as mean  $\pm$  SEM. Unpaired t test.

(F) Experimental timeline.

(G) shPi4KII $\alpha$ -injected rats exhibited impairments in fear reconsolidation, which lasted at least two weeks and were not reinstated by a reminder footshock. The TAT-Pi4KII $\alpha$  injection restored these behavioral effects. SCR + Saline, n = 9 rats; SCR + TAT-Pi4KII $\alpha$ , n = 10 rats; shPi4KII $\alpha$  + Saline, n = 7 rats; shPi4KII $\alpha$  + TAT-Pi4KII $\alpha$ , n = 7 rats. Repeated measures two-way ANOVA followed by Tukey's multiple-comparison *post hoc* test. \*p < 0.05, \*\*p < 0.01, and \*\*\*p < 0.001.

(H and I) (H) Representative Western blots and (I) protein levels of Pi4KII $\alpha$ , EEA1, GluA1, and PSD95 in the BLA in shPi4KII $\alpha$ -injected rats 1 h after US retrieval. Pi4KII $\alpha$ , EEA1, GluA1, and PSD95 levels decreased in the BLA 1 h after US

**Figure 4. Continued**

retrieval. The TAT-Pi4KII $\alpha$  injection reversed the expression of Pi4KII $\alpha$ , GluA1, and PSD95 ( $n = 4-6$  rats/group). Data are reported as mean  $\pm$  SEM. Two-way ANOVA followed by Tukey's multiple-comparison *post hoc* test. \* $p < 0.05$  and \*\* $p < 0.01$ . USR, unconditioned stimulus retrieval.

with dynasore or vehicle in the BLA immediately after US retrieval. On day 3, all of the rats underwent a freezing test (Test 1). Fourteen days later, fear expression was assessed in all of the rats (Test 2), and then a strong electric footshock (1.0 mA) was given immediately after Test 2. Twenty-four hours later, the reinstatement test (Test 3) was performed to evaluate fear expression. The repeated-measures ANOVA of the percentage of freezing revealed main effects of test ( $F_{2, 58} = 13.09$ ,  $p < 0.0001$ ) and treatment ( $F_{3, 29} = 12.06$ ,  $p < 0.0001$ ; Figure 6B). The *post hoc* analysis revealed that the dynasore microinjection in the BLA immediately after US retrieval significantly decreased fear expression in Test 1 ( $p = 0.0038$ ), Test 2 ( $p = 0.0126$ ), and Test 3 ( $p = 0.0160$ ), which was not restored by TAT-Pi4KII $\alpha$  treatment (Figure 6B).

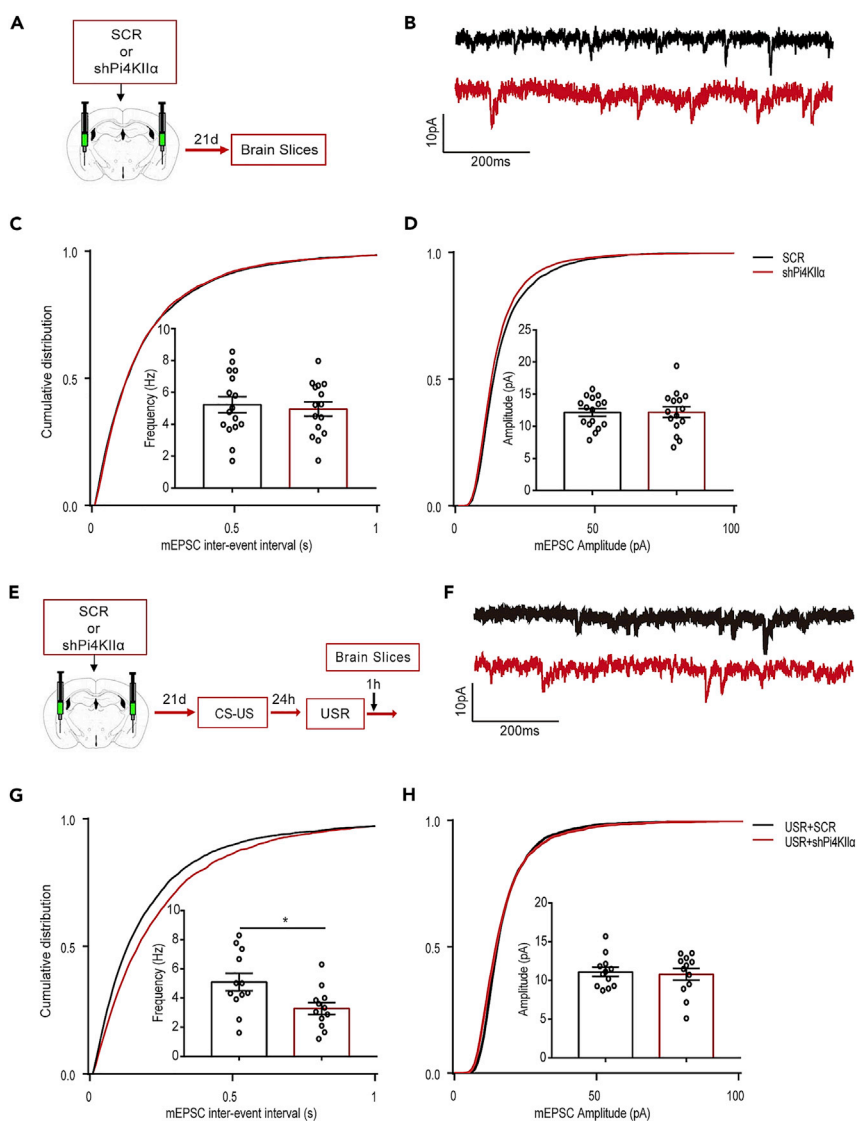
Finally, we examined the effect of dynasore and TAT-Pi4KII $\alpha$  infusions in the BLA on protein expression levels 1 h after US retrieval (Figures 6C and 6D). The dynasore microinjection after US retrieval significantly decreased the levels of EEA1 (two-way ANOVA; main effect of drug [dynasore or vehicle]:  $F_{1, 20} = 9.552$ ,  $p = 0.0058$ ; *post hoc*,  $p = 0.0331$ ) and GluA1 (two-way ANOVA; main effect of drug [dynasore or vehicle]:  $F_{1, 20} = 47.1$ ,  $p < 0.0001$ ; *post hoc*,  $p = 0.0005$ ; Figures 6C and 6D). TAT-Pi4KII $\alpha$  treatment did not reverse the decrease in the expression of EEA1 ( $p = 0.232$ ) or GluA1 ( $p = 0.232$ ) in dynasore-injected rats (Figures 6C and 6D). These results suggest that Pi4KII $\alpha$  may regulate US-retrieval-induced fear memory reconsolidation via early endosomal trafficking.

**DISCUSSION**

In the present study, we found that US retrieval significantly increased cytosolic Pi4KII $\alpha$  levels and synaptosomal membrane EEA1 and GluA1 levels and decreased cytosolic EEA1 levels in the BLA in rats. The intra-BLA infusion of PAO and knockdown of Pi4KII $\alpha$  disrupted contextual fear memory reconsolidation and decreased subsequent fear expression. This disruptive effect persisted for at least two weeks and was not reversed by a reminder footshock. We also found that US retrieval increased spine density and synaptic efficacy in BLA neurons. The genetic knockdown of Pi4KII $\alpha$  abolished the US-retrieval-induced increase in synaptic transmission in the BLA. Furthermore, we found that Pi4KII $\alpha$  bound to EEA1 and GluA1, and the pharmacological inhibition of early endosomal trafficking after US retrieval impaired the surface expression of GluA1 and subsequent fear expression. Altogether, these results indicate that Pi4KII $\alpha$  plays a critical role in US-retrieval-induced fear reconsolidation, likely via the regulation of early endosomal sorting and AMPAR trafficking.

Posttraumatic stress disorder causes great suffering and is associated with high social and economic costs. Accumulating evidence suggests that disruption of the reconsolidation of an activated fear memory prevents subsequent fear expression. Our understanding of the cellular and molecular mechanisms of memory reconsolidation is still at an early stage. Several neurotransmitter systems and intracellular signaling cascades have been shown to be required for memory consolidation and reconsolidation (Johansen et al., 2011). However, the nonspecific involvement of some key molecules in multiple memory processes may limit their preclinical to clinical translation. In the present study, we found that Pi4KII $\alpha$  inhibition did not affect contextual fear memory acquisition, consolidation, or retrieval. Interference with Pi4KII $\alpha$  outside the time window of reconsolidation had no effect on the subsequent expression of fear, indicating that Pi4KII $\alpha$  specifically affects the reconsolidation of fear memory.

Unconditioned stimuli serve as powerful reminders to trigger reconsolidation (Debiec et al., 2010). A previous study reported that the second learning occurred independently of dorsal hippocampal N-methyl-D-aspartate (NMDA) receptors in rats that were previously exposed to a similar fear conditioning procedure (Finnie et al., 2018). However, the single exposure to a weak footshock that was used in the present study may not be sufficient to induce the second learning. Indeed, we found that weak US retrieval, followed by an anisomycin injection in the BLA, decreased the fear response, indicating that exposure to the US triggered the reconsolidation process. Additionally, although interventions that target US-retrieval-induced reconsolidation are associated with a better treatment outcome than CS exposure (Huang et al., 2017), the molecular and cellular mechanisms that underlie these differences remain unclear. A recent study showed that US retrieval induced significant CREB activation in almost whole amygdala and hippocampus,



**Figure 5. Unconditioned-Stimulus-Retrieval-Dependent Alterations of Synaptic Efficacy Depend on Pi4KII $\alpha$**

(A) Experimental timeline.

(B) Example mEPSC traces from BLA neurons in the shPi4KII $\alpha$  group (n = 15 neurons, 7 slices from 4 rats) and SCR group (n = 16 neurons, 5 slices from 4 rats).

(C and D) Cumulative distribution of mEPSC interevent intervals and average frequency (C) or mEPSC amplitude (D) of neurons in the shPi4KII $\alpha$  group and SCR group.

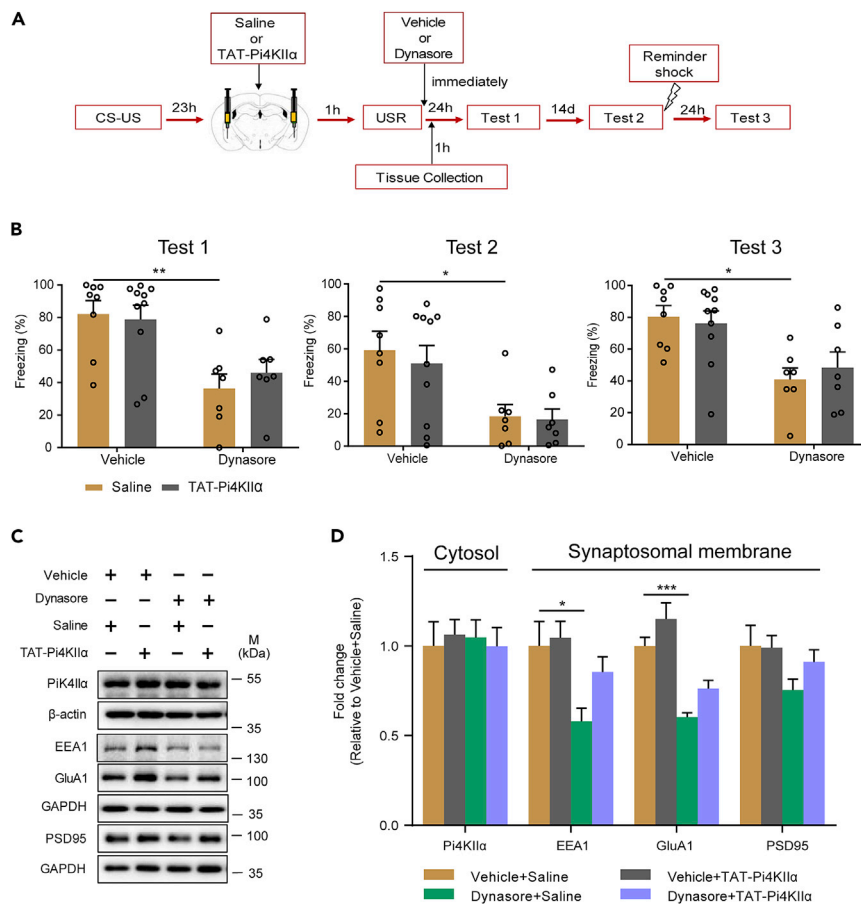
(E) Experimental timeline.

(F) Example mEPSC traces from BLA neurons in the shPi4KII $\alpha$  group (n = 12 neurons, 7 slices from 5 rats) and SCR group (n = 12 neurons, 4 slices from 3 rats) 1 h after US retrieval.

(G and H) Cumulative distribution of mEPSC inter-event interval and average frequency (G), or mEPSC amplitude (H) of neurons in the shPi4KII $\alpha$  group and SCR group 1 h after US retrieval. Data are reported as mean  $\pm$  SEM. Unpaired t test.

\*p < 0.05. SCR, scramble; USR, unconditioned stimulus retrieval; mEPSC, miniature excitatory postsynaptic current.

whereas CS retrieval only stimulated CREB activation in the lateral amygdala and the CA3 (Huang et al., 2017). In the present study, we found that Pi4KII $\alpha$  levels in the BLA increased 15 min after US retrieval and decreased to baseline levels 1 h after US retrieval. Additionally, Pi4KII $\alpha$  inhibition in the BLA had no effect on CS retrieval-induced memory reconsolidation but significantly influenced US-retrieval-induced memory reconsolidation. The dysfunction of Pi4KII $\alpha$  contributes to Alzheimer disease, Gaucher disease, Hermansky-Pudlak syndrome, and X-linked centronuclear myopathy (Jovic et al., 2012; Kang et al., 2013;



**Figure 6. Preventing Early Endosomal Trafficking in the BLA after US Retrieval Impairs the Reconsolidation of Contextual Fear Memory and Exerts a Long-Lasting Effect**

(A) Experimental timeline.

(B) Rats that were injected with dynasore exhibited an impairment in fear expression, which lasted at least two weeks and was not reinstated by a reminder footshock. The TAT-Pi4KII $\alpha$  injection within the reconsolidation time window did not restore fear expression. Vehicle + Saline, n = 8 rats; Vehicle + TAT-Pi4KII $\alpha$ , n = 10 rats; Dynasore + Saline, n = 7 rats; Dynasore + TAT-Pi4KII $\alpha$ , n = 8 rats. Repeated measures two-way ANOVA followed by Tukey's multiple-comparison *post hoc* test. \*p < 0.05 and \*\*p < 0.01.

(C and D) (C) Representative Western blots and (D) protein levels of Pi4KII $\alpha$ , EEA1, GluA1, and PSD95 in the BLA in rats that were injected with dynasore 1 h after US retrieval. The dynasore injection decreased synaptosomal membrane EEA1 and GluA1 levels in the BLA 1 h after US retrieval. The TAT-Pi4KII $\alpha$  injection did not reverse the protein expression of EEA1 or GluA1 (n = 6 rats/group). Data are reported as mean  $\pm$  SEM. Two-way ANOVA followed by Tukey's multiple-comparison *post hoc* test. \*p < 0.05 and \*\*\*p < 0.001. USR, unconditioned stimulus retrieval.

Ketel et al., 2016; Salazar et al., 2005, 2009; Wu et al., 2004). The present study identified a functional role for Pi4KII $\alpha$  in learning and memory, suggesting that it may be a promising molecular target for erasing aversive emotional memories.

Memory retrieval and reconsolidation are associated with glutamate receptor expression and activity. AMPAR trafficking is essential for the retrieval and subsequent reconsolidation of fear memory in the amygdala (Clem and Huganir, 2010; Hong et al., 2013). In the present study, we found that US retrieval increased GluA1 and GluA2 levels in synaptosomal membrane fractions of the BLA. We also found that US retrieval increased dendritic spine density and synaptic strength in the BLA. The higher expression of PSD95 and GluA1 after US retrieval may be a structural basis for the enhancement of synaptic transmission in the BLA. Consistent with our findings, previous studies revealed that fear memory reactivation promoted the surface expression of GluA1 subunits and excitatory synaptic transmission in the lateral amygdala (Zhou et al., 2015). Fear memory retrieval also induces a transient increase in engram cell excitability

(Pignatelli et al., 2019). However, some studies found that fear memory retrieval caused the endocytosis of AMPARs (Hong et al., 2013; Rao-Ruiz et al., 2011). This discrepancy may reflect distinct retrieval paradigms. Indeed, US retrieval has been shown to activate more memory traces and stronger protein alterations than CS retrieval (Liu et al., 2014; Xue et al., 2017; Yuan et al., 2019). Furthermore, we found that the pharmacological inhibition of Pi4KII $\alpha$  in the BLA decreased GluA1 levels 1 h after US retrieval, and the genetic knock-down of Pi4KII $\alpha$  impaired the enhancement of mEPSC frequency that was induced by US retrieval, suggesting a regulatory role for Pi4KII $\alpha$  in GluA1 surface expression and synaptic efficacy in the rat BLA.

Pi4KII $\alpha$  is extensively expressed in both the central and peripheral nervous systems, especially in the cerebellum, dorsal root ganglion, and spinal cord (Simons et al., 2009). In the present study, we found that Pi4KII $\alpha$  was also expressed in many brain areas that are related to learning and memory, including the DH and BLA. In neurons, Pi4KII $\alpha$  localizes to dendrites, endosomes, the Golgi apparatus, and synaptic vesicles (Clayton et al., 2013; Minogue, 2018). Similarly, we found that Pi4KII $\alpha$  colocalized with EEA1 in rat BLA neurons. Pi4KII $\alpha$  regulates receptor sorting at early endosomes, and Pi4KII $\alpha$  depletion by siRNA impairs receptor sorting from early endosomes (Henmi et al., 2016). In the present study, EEA1 levels decreased in the cytosolic fraction 15 min after US retrieval and increased in the synaptosomal membrane fraction 1 h after US retrieval. Such changes suggest an interaction between Pi4KII $\alpha$  and EEA1. Indeed, the co-immunoprecipitation results showed that EEA1 was a binding partner of Pi4KII $\alpha$ . The pharmacological blockade of early endosome transport with dynasore inhibited subsequent fear expression, indicating a role for early endosome trafficking in US-retrieval-induced fear memory reconsolidation. The TAT-Pi4KII $\alpha$  microinjection did not restore the lower expression of EEA1 or GluA1 and did not reverse behavioral inhibition that was induced by the blockade of early endosome trafficking, indicating that Pi4KII $\alpha$  may serve as an upstream regulator of early endosome sorting and AMPAR trafficking. Interestingly, the TAT-Pi4KII $\alpha$  microinjection increased Pi4KII $\alpha$  levels in untrained rats but had no effect on Pi4KII $\alpha$  expression in trained rats that received the SCR or dynasore injection. This discrepancy may be attributed to the distinct sampling time points and influence of fear conditioning and US retrieval.

In neurons, endosomes have been shown to translocate into spines to promote the insertion of AMPARs (Esteves da Silva et al., 2015; Park et al., 2006). Early endosomes are found throughout the soma and dendrites and play a crucial role in the sorting of AMPARs (Parkinson and Hanley, 2018). AMPARs on early endosomes can recycle back to the dendritic plasma membrane through a mechanism that involves the recruitment of recycling endosomes (Anggono and Huganir, 2012). In the present study, US retrieval decreased cytosolic EEA1 levels and increased EEA1 and GluA1 levels in the synaptosomal membrane fraction of the BLA. The pharmacological inhibition of early endosome trafficking in the BLA after US retrieval decreased GluA1 levels in the synaptosomal membrane fraction. These results support a role for early endosomes in the membrane trafficking of AMPARs.

In summary, the present study demonstrated that Pi4KII $\alpha$  contributes to the early endosomal trafficking of GluA1-containing AMPARs during US retrieval-induced contextual fear memory reconsolidation. Our findings may contribute to the development of effective therapeutic strategies that target Pi4KII $\alpha$  to alleviate the return of fear in PTSD.

### Limitations of the Study

The present study demonstrated important roles for Pi4KII $\alpha$  in the early endosomal trafficking of GluA1-containing AMPARs, synaptic enhancement, and US-retrieval-induced contextual fear memory reconsolidation. Further studies are needed to examine whether Pi4KII $\alpha$  also plays a role in remote memory reconsolidation and whether recycling endosomes also participate in US-retrieval-induced synaptic AMPAR incorporation.

### METHODS

All methods can be found in the accompanying [Transparent Methods supplemental file](#).

### DATA AND CODE AVAILABILITY

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

## SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.isci.2020.100895>.

## ACKNOWLEDGMENTS

This work was supported in part by the National Natural Science Foundation of China (no. 81521063, 81701312, and 91732109), National Basic Research Program of China (no.2015CB856400 and 2015CB553503), National Key Research and Development Program of China (2019YFC0118604 and 2019YFA0706201), and Peking University (no.BMU20160555).

## AUTHOR CONTRIBUTIONS

Conceptualization: H.G., W.Z., K.Y., Y.H., and L.L.; Investigation: H.G., W.Z., K.Y., Y.H., and L.L.; Electrophysiology: H.G., W.Z., and Z.Z.; Behavior: H.G., W.Y., and Y.H.; Vector design: S.M and H.G.; Stereotactic surgery: H.G., W.Z., and Y.H.; Confocal analysis: H.G., P.W., and W.Z.; Data analysis: H.G., W.Z., Z.Z., and Y.H.; Data interpretation and manuscript writing: H.G., Y.H., and L.L.; Manuscript review and editing, H.G., Y.H., W.Z., Z.Z., Y.X., W.Y., K.Y., S.M, W.Z., P.W., Y.B., J.S., and L.L.; Funding acquisition, W.Z., Y.H., and L.L.

## DECLARATION OF INTERESTS

The authors declare no conflict of interest.

Received: September 7, 2019

Revised: January 6, 2020

Accepted: February 4, 2020

Published: March 27, 2020

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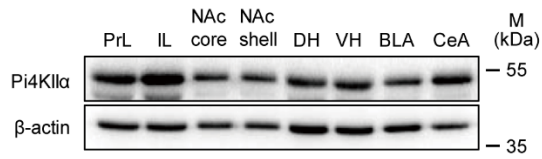
**Supplemental Information**

**Pi4KII $\alpha$  Regulates Unconditioned Stimulus-  
Retrieval-Induced Fear Memory Reconsolidation  
through Endosomal Trafficking of AMPA Receptors**

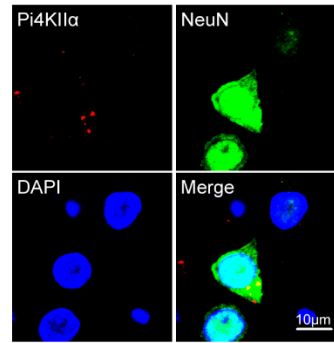
**Hongling Guo, Kai Yuan, Zhongyu Zhang, Yanxue Xue, Wei Yan, Shiqiu Meng, Weili Zhu, Ping Wu, Yanping Bao, Jie Shi, Wen Zhang, Lin Lu, and Ying Han**

## Supplemental Information

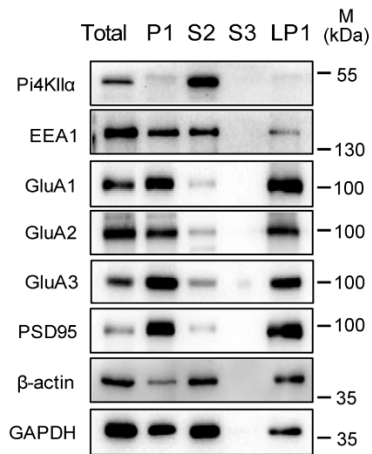
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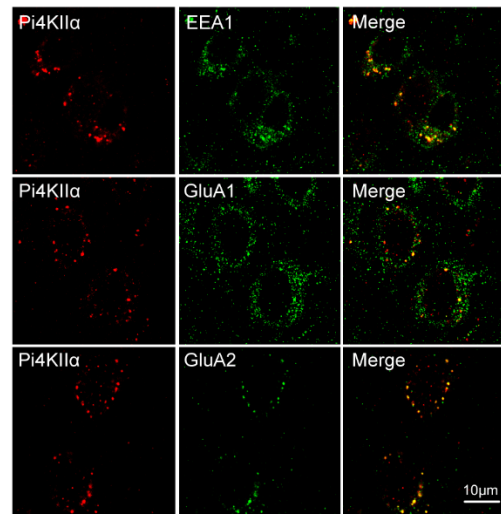
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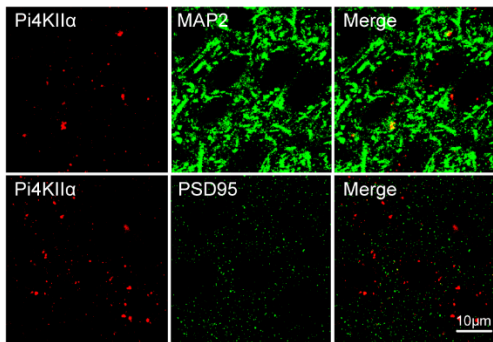
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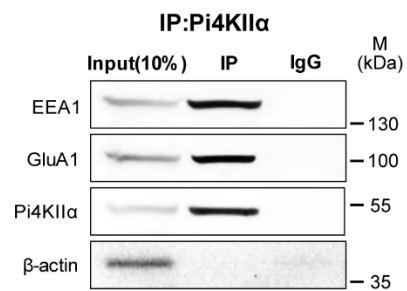
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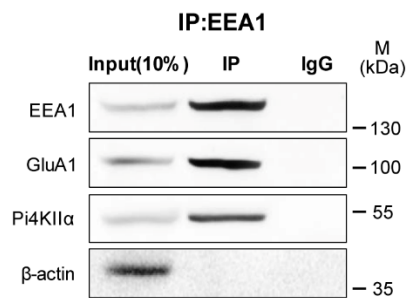
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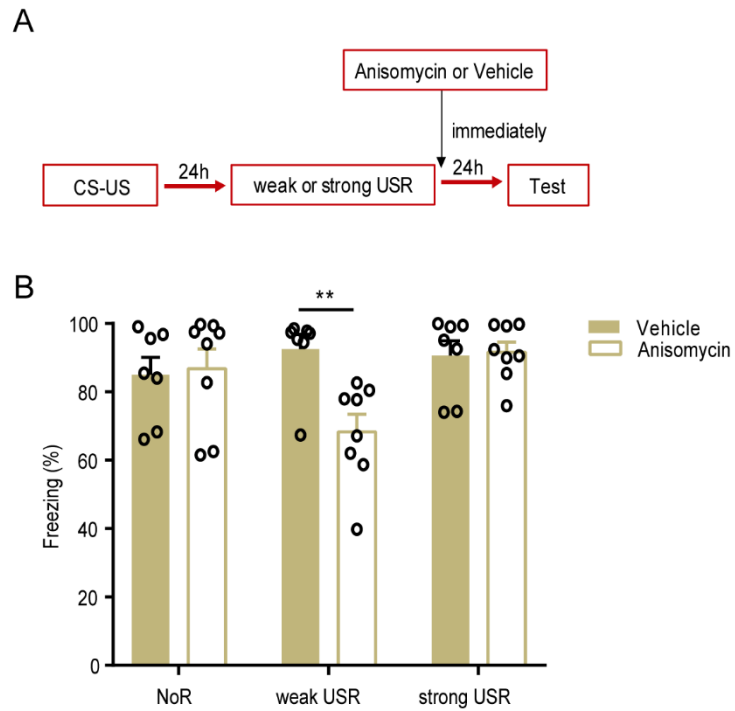
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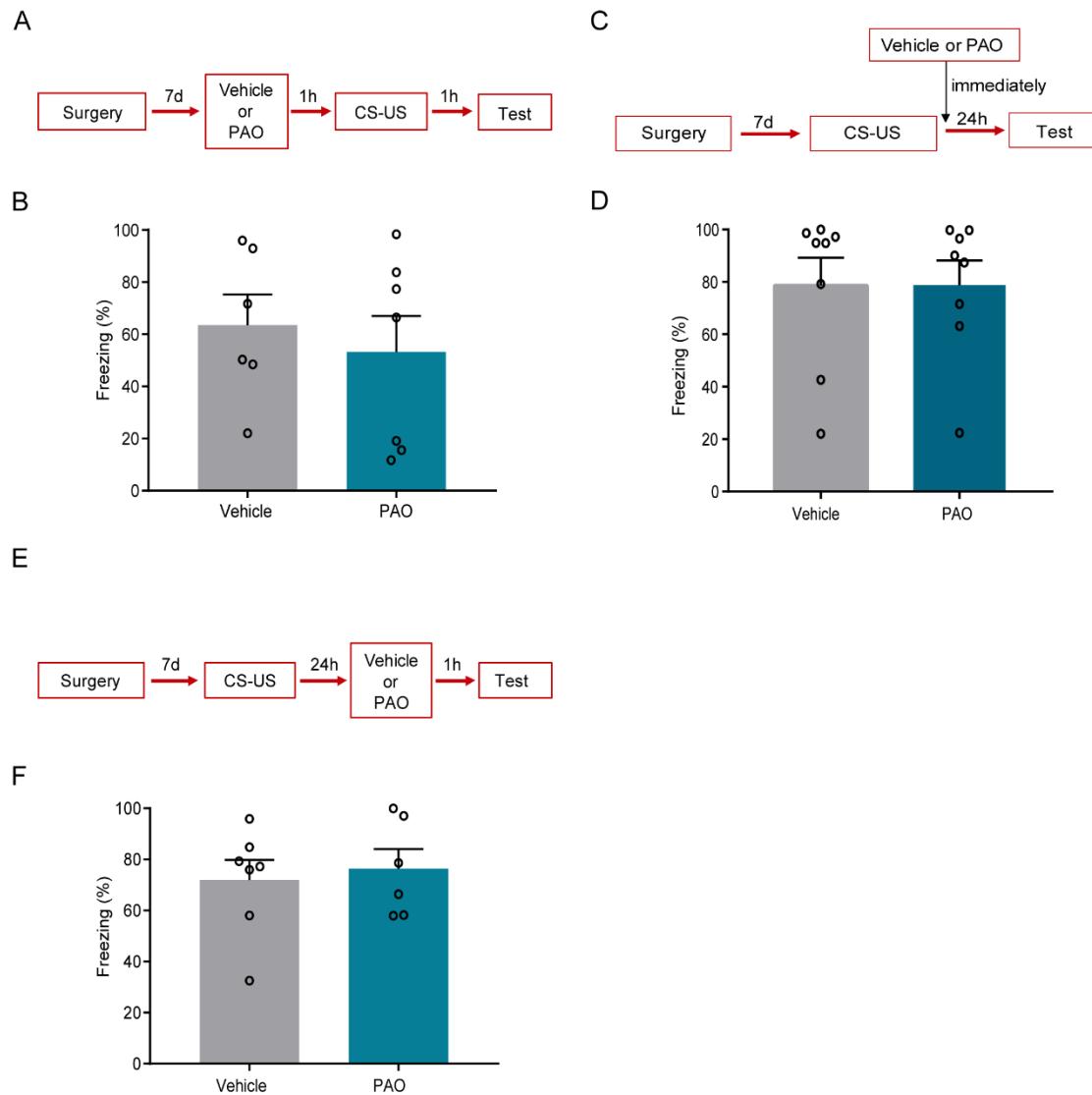
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**Figure S1. Pi4KII $\alpha$  localizes to early endosomes in basolateral amygdala neurons and colocalizes with AMPARs, Related to Figure 1.** (A) Pi4KII $\alpha$  was enriched in the PrL, IL, NAc core, NAc shell, DH, VH, BLA, and CeA. PrL, prelimbic cortex; IL, infralimbic cortex; NAc, nucleus accumbens; DH, dorsal hippocampus; VH, ventral hippocampus; BLA, basolateral amygdala; CeA, central nucleus of the amygdala. (B) Pi4KII $\alpha$  immunoreactivity was detected in rat BLA neurons. Scale bar = 10  $\mu$ m. (C) Subcellular fractions of the rat BLA were probed for Pi4KII $\alpha$ , the early endosome marker EEA1, AMPAR subunits GluA1-3, and postsynaptic marker PSD95. Total, homogenates; P1, nuclei and large cellular debris; S2, cytosolic fraction; S3, supernatant after high-speed centrifugation at 25,000  $\times$  g; LP1, synaptosomal membrane fraction. (D) Pi4KII $\alpha$  colocalized with EEA1, GluA1, and GluA2. Scale bar = 10  $\mu$ m. (E) Double immunostaining showed that Pi4KII $\alpha$  colocalized with MAP2 but not PSD95. Scale bar = 10  $\mu$ m. (F and G) Pi4KII $\alpha$  interacted with EEA1 and GluA1. Extracts from the adult rat BLA were immunoprecipitated with antibodies against Pi4KII $\alpha$  (F) and EEA1 (G) and blotted with corresponding antibodies.  $\beta$ -actin was used as a negative control.

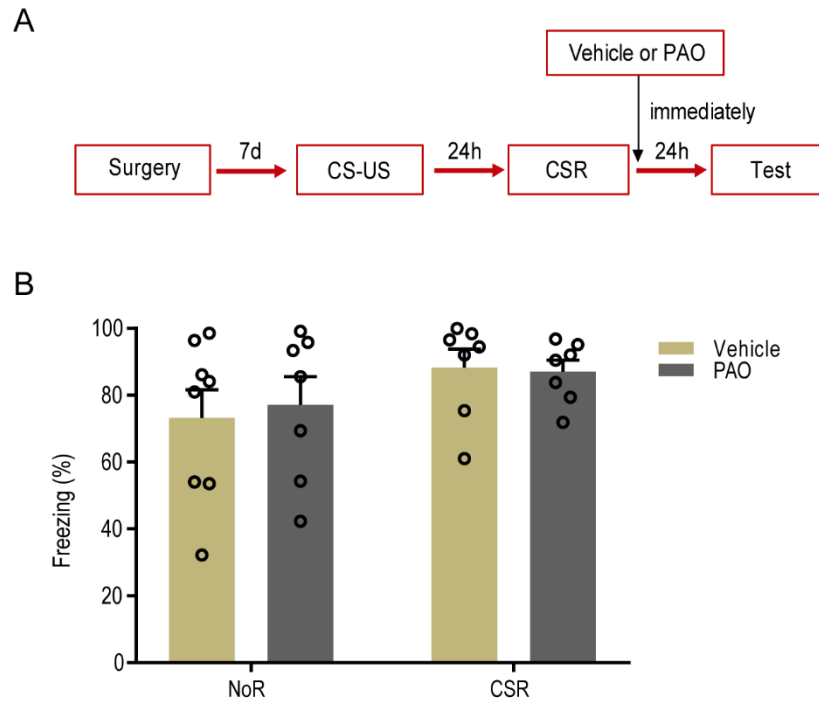


**Figure S2. Weak US exposure followed by an anisomycin injection impaired contextual fear memory reconsolidation in rats, Related to Figure 1.** (A) Experimental timeline. (B) Rats that received an anisomycin injection after weak US retrieval (weak USR: 1 s, 0.3 mA) exhibited lower fear responses compared with rats that underwent weak US retrieval and received vehicle injection. NoR + Vehicle, n = 7 rats; NoR + Anisomycin, n = 8 rats; weak USR + Vehicle, n = 7 rats; weak USR + Anisomycin, n = 8 rats; strong USR + Vehicle, n = 7 rats; strong USR + Anisomycin, n = 8 rats. Data represent the mean  $\pm$  SEM. Two-way ANOVA followed by Tukey's multiple-comparison *post hoc* test. \*\* $p < 0.01$ . NoR, no retrieval; USR, unconditioned stimulus retrieval.

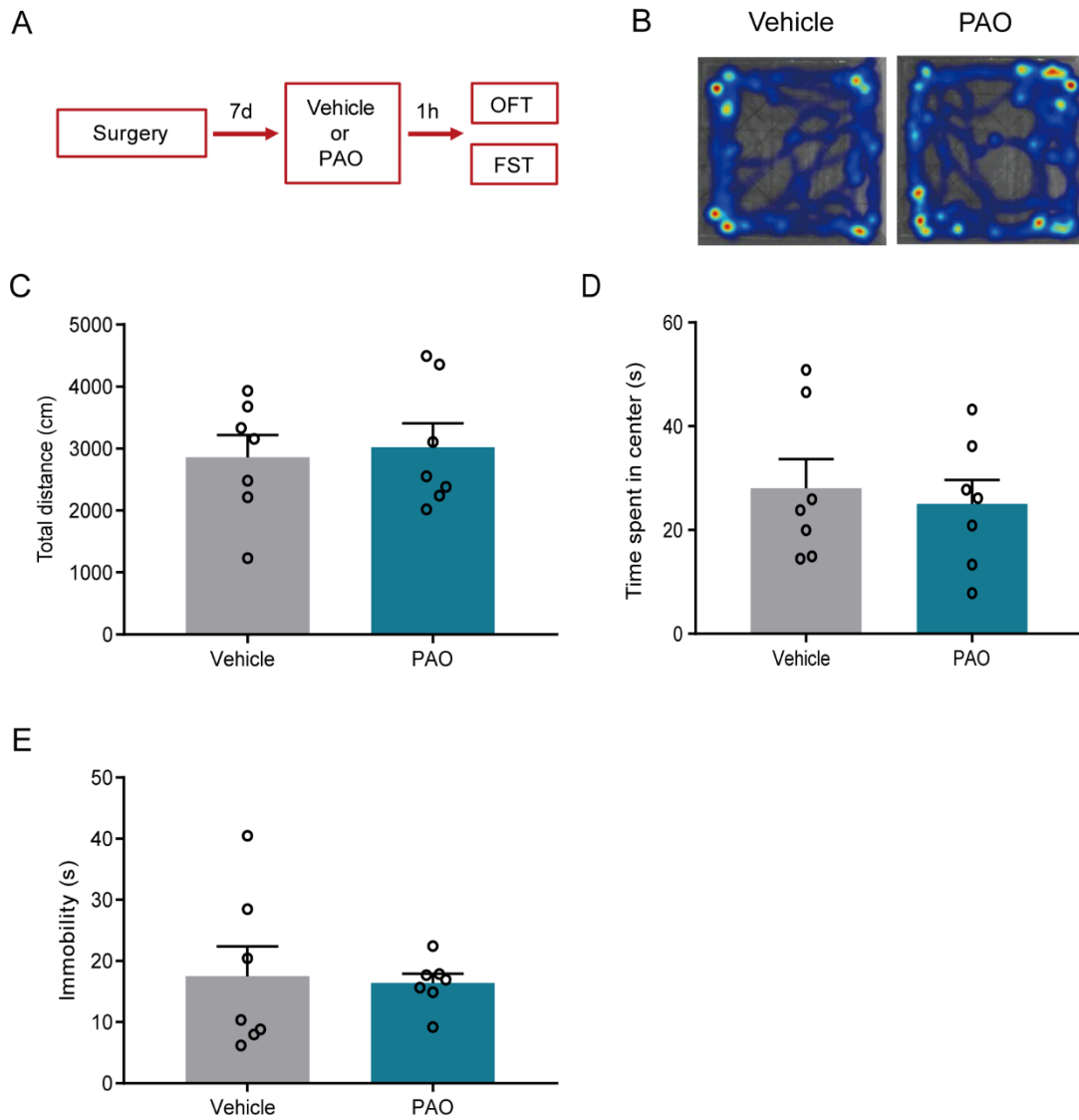


**Figure S3. Inhibition of  $Pi4KII\alpha$  in the BLA has no effect on contextual fear memory acquisition, consolidation, or retrieval, Related to Figure 3.** (A) Experimental timeline. (B) The microinjection of the  $Pi4KII\alpha$  inhibitor PAO before conditioning had no effect on contextual fear memory acquisition. Vehicle,  $n = 6$  rats; PAO,  $n = 7$  rats. (C) Experimental timeline. (D) The microinjection of PAO immediately after fear conditioning had no effect on memory consolidation. Vehicle,  $n = 8$  rats; PAO,  $n = 8$  rats. Data are reported as mean  $\pm$  SEM. Unpaired t test. (E) Experimental timeline. (F) The microinjection of PAO before test had no effect on the retrieval of contextual fear memory. Vehicle,  $n = 7$  rats; PAO,  $n = 6$  rats. Data are reported as mean  $\pm$  SEM. Unpaired t test.

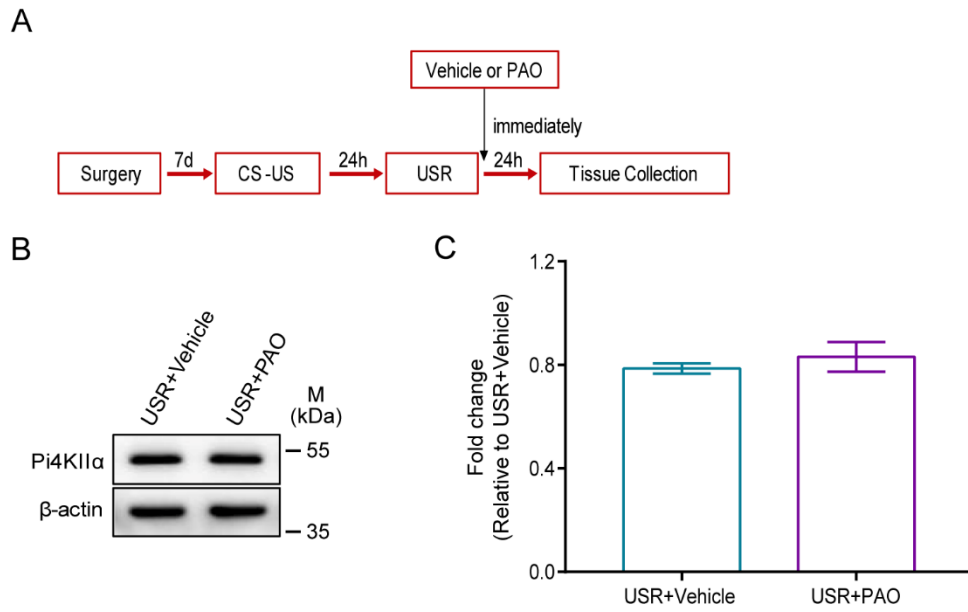




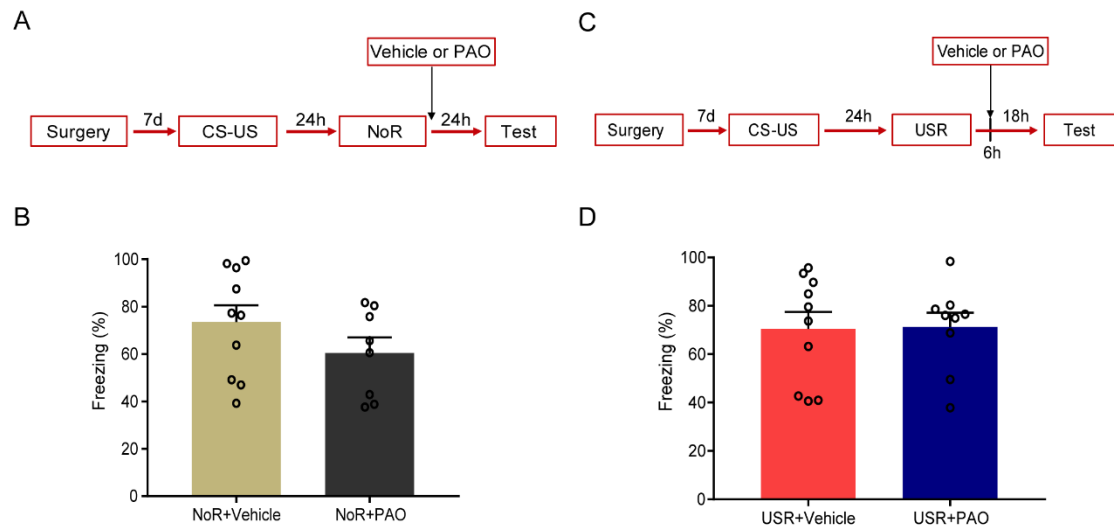
**Figure S4. Inhibition of  $Pi4KII\alpha$  in the BLA has no effect on CS retrieval-induced memory reconsolidation, Related to Figure 3.** (A) Experimental timeline. (B) Freezing test in rats that underwent CS retrieval followed by PAO or vehicle injection in the BLA. NoR + Vehicle,  $n = 8$  rats; NoR + PAO,  $n = 7$  rats; CSR + Vehicle,  $n = 7$  rats; CSR + PAO,  $n = 7$  rats. Data are reported as mean  $\pm$  SEM and were analyzed by two-way ANOVA followed by Tukey's multiple-comparison *post hoc* test. NoR, no retrieval; CSR, conditioned stimulus retrieval.



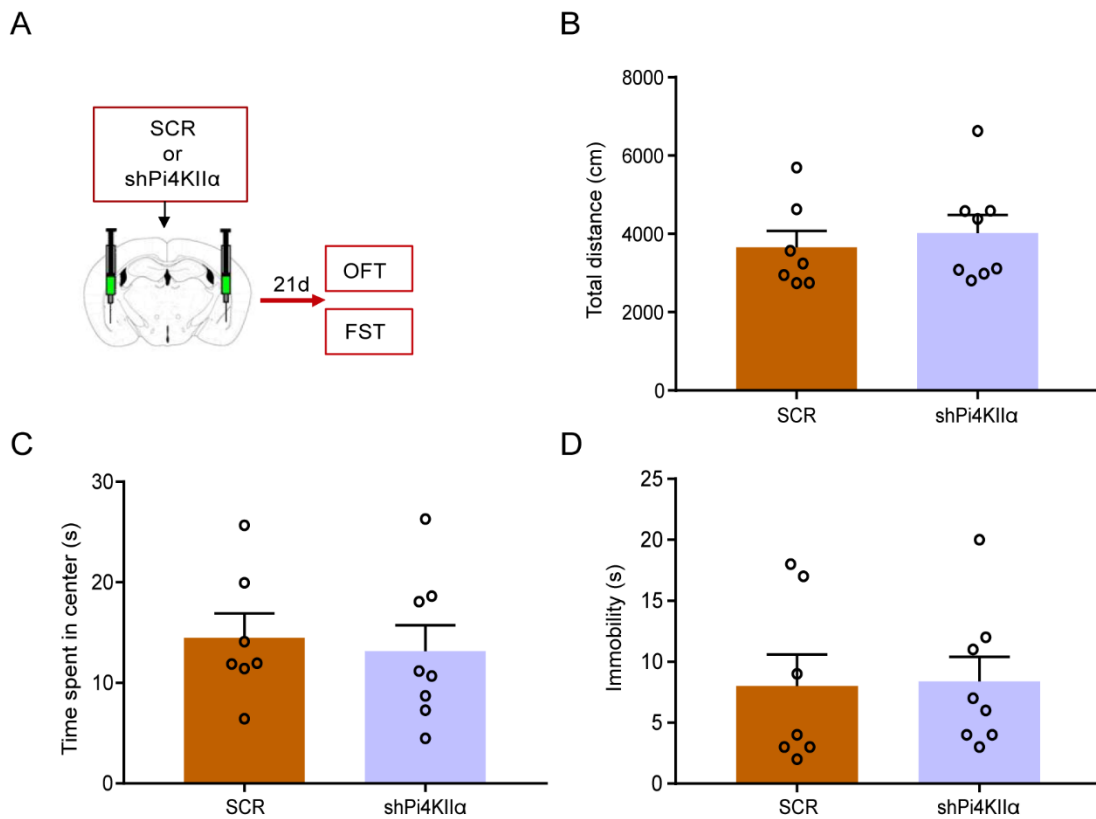
**Figure S5. Inhibition of  $Pi4KII\alpha$  in the BLA has no effect on anxiety- or depressive-like behavior in rats, Related to Figure 3.** (A) Experimental timeline. (B) Representative exploration tracks in the open field test (OFT) in rats that were injected with PAO or vehicle. (C) No significant difference in the total distance travelled was found between vehicle- and PAO-treated rats. (D) No significant difference in the time spent in the central area was found between vehicle- and PAO-treated rats. (E) No significant difference in immobility time in the forced swim test (FST) was found between vehicle- and PAO-treated rats. Vehicle,  $n = 7$  rats; PAO,  $n = 7$  rats. Data are reported as mean  $\pm$  SEM. Unpaired  $t$  test.



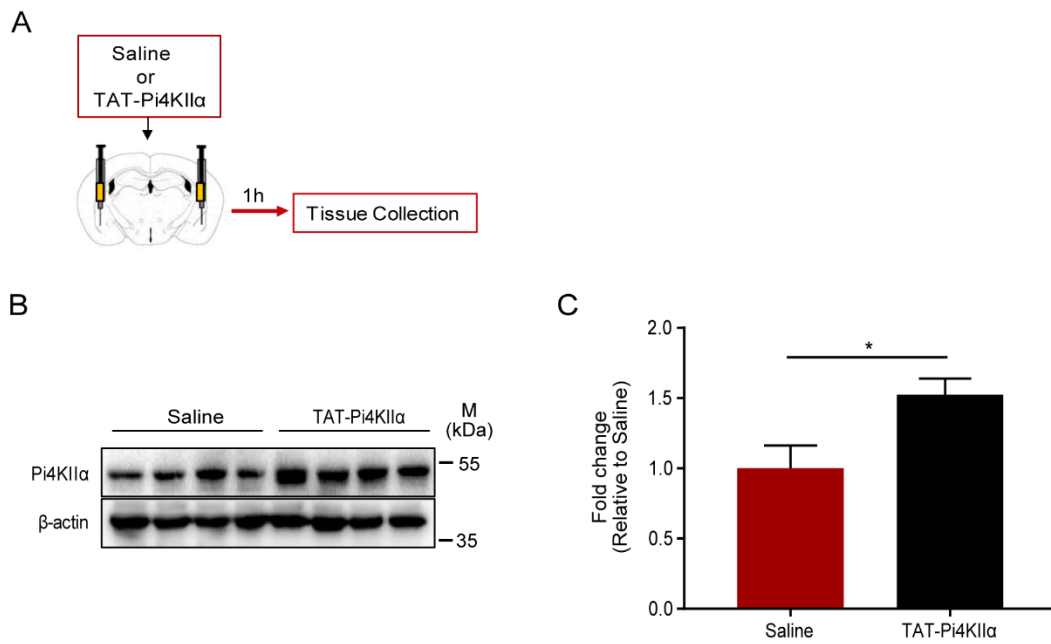
**Figure S6. PAO injection in the BLA has no effect on Pi4KII $\alpha$  expression 24 h after US exposure, Related to Figure 3.** (A) Experimental timeline. (B) Representative Western blots and (C) protein levels of Pi4KII $\alpha$  in the BLA 24 h after US retrieval ( $n = 4$  rats/group). Data are reported as mean  $\pm$  SEM. Unpaired t test. USR, unconditioned stimulus retrieval.



**Figure S7. Pharmacological inhibition of  $Pi4KII\alpha$  in the BLA without US exposure or 6 h after US exposure has no effect on the expression of fear memory, Related to Figure 3.** (A) Experimental timeline. (B) Freezing test in rats that were injected with vehicle (1.0  $\mu$ l/side) or PAO (200  $\mu$ M/side) in the BLA without the US retrieval procedure. NoR + Vehicle, n = 10 rats; NoR + PAO, n = 8 rats. (C) Experimental timeline. (D) Freezing test in rats that were injected with vehicle (1.0  $\mu$ l/side) or PAO (200  $\mu$ M/side) in the BLA 6 h after US retrieval. USR + Vehicle, n = 10 rats; USR + PAO, n = 9 rats. Data are reported as mean  $\pm$  SEM. Unpaired t test. NoR, no retrieval; USR, unconditioned stimulus retrieval.



**Figure S8. Genetic knockdown of Pi4KII $\alpha$  in the BLA has no effect on anxiety- or depressive-like behavior in rats, Related to Figure 4.** (A) Experimental timeline. (B) No significant difference in the total distance travelled in the open field test (OFT) was found between shPi4KII $\alpha$ - and SCR-injected rats. (C) No significant difference in the time spent in the central area in the OFT was found between shPi4KII $\alpha$ - and SCR-injected rats. (D) No significant difference in immobility time in the forced swim test (FST) was found between shPi4KII $\alpha$ - and SCR-injected rats. SCR, n = 7 rats; shPi4KII $\alpha$ , n = 8 rats. Data are reported as mean  $\pm$  SEM. Unpaired t test. SCR, scramble.



**Figure S9. Efficacy of TAT-Pi4KII $\alpha$ , Related to Figure 4.** (A) Experimental timeline. (B) Representative Western blots and (C) protein levels of Pi4KII $\alpha$  in the BLA 1h after the TAT-Pi4KII $\alpha$  injection. The TAT-Pi4KII $\alpha$  injection significantly increased Pi4KII $\alpha$  levels in the rat BLA (n = 4 rats/group). Data are reported as mean  $\pm$  SEM. Unpaired t test. \*p < 0.05.

## Transparent Methods

### Animals

Male Sprague-Dawley rats (240-260 g) were obtained from the Laboratory Animal Center, Peking University Health Science Center. The rats were housed in groups of five in a temperature ( $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ) and humidity ( $50\% \pm 5\%$ ) controlled animal facility and maintained on a 12 h/12 h light/dark cycle. Food and water were provided *ad libitum* throughout the experiment. The rats were acclimated to this environment for 1 week before beginning the experiments. All of the procedures complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Biomedical Ethics Committee for Animal Use and Protection of Peking University.

### Construction, expression, and purification of TAT-Pi4KII $\alpha$

The construction, expression, and purification of recombinant TAT-Pi4KII $\alpha$  infusion protein were performed according to (Holz et al., 2019; Nagel et al., 2008) with a slight modification. Briefly, the DNA sequence encoding full-length rat Pi4KII $\alpha$  was amplified by PCR and cloned into a modified pcDNA3.1 vector containing the N-terminus PTD of TAT protein and HA sequences. The accuracy of the construct was confirmed by DNA sequencing. The plasmid was transformed into *E. coli* BL21 (DE3) to produce  $6 \times$  His-tagged TAT-Pi4KII $\alpha$ . A selected single colony was grown in 50 ml LB medium containing 50 mg/L kanamycin for 8 h at  $37^{\circ}\text{C}$ . These cultures were diluted 50-fold with fresh LB media and cultured at  $37^{\circ}\text{C}$  to  $\text{OD}_{600} = 0.8$ . The expression of TAT-Pi4KII $\alpha$  was induced by the addition of 0.5 mM isopropyl- $\beta$ -dthiogalactoside (IPTG) for 10 h at  $28^{\circ}\text{C}$ . The bacterial cells were harvested by centrifugation at  $12,000 \times g$  and then resuspended in pre-cooled binding buffer (20 mM Tris-HCl, 500 mM NaCl, and 20 mM imidazole, pH 7.5) and lysed by sonication on ice. The supernatant that contained target protein was collected by centrifugation at  $12,000 \times g$  for 20 min at  $4^{\circ}\text{C}$  and loaded onto a Ni-NTA resin column (Invitrogen, Shanghai, China) that was pre-equilibrated with binding buffer. The proteins were then eluted by a stepwise imidazole gradient in elution buffers (20 mM  $\text{Na}_3\text{PO}_4$ , 500 mM NaCl, and 50-500 mM imidazole, pH 7.5). Eluted fractions were analyzed by 12% (wt/vol) SDS/PAGE, followed by Coomassie blue staining. The purified proteins were lyophilized and kept at  $280^{\circ}\text{C}$ . The proteins were dissolved in normal saline, and protein concentrations were estimated by the BCA assay (Applygen Technologies, Beijing, China) before use. Protein purity was evaluated by SDS-PAGE. Purified recombinant proteins were confirmed by Western blot.

### Adeno-associated virus construction and injections

Short-hairpin RNAs were designed specifically for Pi4KII $\alpha$  (shPi4KII $\alpha$ , 5'-GCCAGTTCATAAGCAGATTG-3'; scramble, 5'-TTCTCCGAACGTGTCACGT-3'). The U6 promoter and shPi4KII $\alpha$  sequence were inserted into an adeno-associated virus 9 (AAV9) vector (Vigenebio, Jinan, China). All of the vectors contained the enhanced green fluorescence protein (eGFP) coding sequence. The rats were anesthetized with 2% isoflurane. shPi4KII $\alpha$  ( $0.6 \mu\text{l}/\text{side}$ ;  $1.16 \times 10^{14}$  vg/ml) or SCR ( $0.6 \mu\text{l}/\text{side}$ ;  $2.38 \times 10^{13}$  vg/ml) was bilaterally injected in the BLA using Hamilton syringes that were connected to 30-gauge injectors (Plastics One, Roanoke, VA, USA). After the 10-min infusion period, the infusion needle was kept in place for an additional 5 min to allow for diffusion.

### Surgery

All of the rats weighed 300-320 g on the day of surgery. Surgical anesthesia was induced with 4% isoflurane that was delivered in an air/oxygen mixture. The rats were intubated and secured in a stereotaxic frame. The rats were maintained at a surgical level with 1.5% isoflurane and carrier gases during surgery. Three jeweler screws were implanted in the skull using a Kopf stereotax, and then two 23-gauge stainless-steel cannulas were inserted bilaterally in the BLA (anterior/posterior, -2.9 mm; medial/lateral,  $\pm 5.0$  mm; dorsal/ventral, -8.0 mm). Dental cement was applied to stabilize the implants. Obturators were inserted in the guide cannulas to prevent clogging. The rats were then allowed 7 days to recover from surgery and handled daily during the recovery period.

### Intracranial injections

Anisomycin (AG Scientific) was dissolved in equimolar HCl, diluted with artificial cerebrospinal fluid (aCSF), and adjusted to pH 7.4 with NaOH. Phenylarsine oxide (PAO; J&K Scientific, Beijing, China) was dissolved in dimethylsulfoxide (DMSO) and diluted to a final vehicle of 0.5% DMSO in saline. Dynasore (MedChemExpress, Shanghai, China) was dissolved in 0.9% saline and stored at  $4^{\circ}\text{C}$  until use. Drugs and vehicles were microinjected bilaterally in the BLA at a rate of  $0.25 \mu\text{l}/\text{min}$  using Hamilton syringes that were connected to 30-gauge injectors (Plastics One, Roanoke, VA, USA) that extended 1 mm below the guide cannula 1 h before or immediately after the US retrieval procedure. The infusion



volume was 1.0  $\mu$ l/side. The needle was kept in place for an additional 1 min to allow for drug diffusion.

### **Contextual fear conditioning, memory reactivation, and freezing test**

The Startle and Fear-conditioning system (Panlab, Barcelona, Spain) was used for fear conditioning during all the experiments. Training was performed in an experiment chamber that consisted of black methacrylate walls and a transparent front door. On the training day, each rat was placed in the chamber and allowed to explore it for 2 min, after which the rat received a 1 s, 1.0 mA electric footshock (US). Two additional identical footshocks were given with an interval of 2 min between each shock. Following the last footshock, the rat was allowed to explore the chamber for an additional 1 min before being returned to its home cage. Conditioned stimulus retrieval (CSR) was performed 24 h after fear conditioning by re-exposing the rats to the conditioning context for 3 min in the absence of footshock. Unconditioned stimulus retrieval (USR) was performed 24 h after fear conditioning by exposing the rat to a weak footshock (1 s, 0.3 mA, weak USR) or a strong footshock (1 s, 1.0 mA, strong USR) in a novel context. The walls, cover, and floor of the retrieval chamber were different from the training chamber. The freezing test was performed in the same training chamber for 5 min without footshock, and freezing behavior was analyzed using Packwin 2.0 software (Panlab, Barcelona, Spain).

### **Open field test**

The rats were placed in a corner of a square wooden open field box (75 cm  $\times$  75 cm  $\times$  40 cm). The surface area was divided into 16 equally sized squares, and the center square was designated the central area. The time spent in the central area and total distance travelled were monitored for 5 min and assessed using EthoVision XT 10.1 software.

### **Forced swim test**

The rats were placed in a Plexiglas cylinder (20 cm diameter  $\times$  50 cm height) that was filled with 25°C  $\pm$  1°C water to a depth of 35 cm. Each rat was allowed to swim for 6 min, and the duration of immobility during the last 5 min of the test was recorded. Immobility was analyzed using EthoVision XT 10.1 software.

### **Tissue preparation**

Tissue preparation was performed according to (Liu et al., 2014). The BLA samples were homogenized in RIPA buffer (Applygen Technologies, Beijing, China) with phosphatase inhibitor and protease inhibitor cocktails (Applygen Technologies, Beijing, China). The homogenates were then centrifuged at 10,000  $\times$  g for 20 min to obtain the supernatant (total protein, T). Subcellular fractions were prepared according to (Liu et al., 2014). Bilateral tissue punches of the BLA were homogenized in ice-cold homogenization buffer (0.32 M sucrose, 4 mM HEPES, 1 mM EDTA, 1 mM EGTA, and protease/phosphatase inhibitor cocktail, pH 7.4) to obtain the homogenate fraction, which was centrifuged at 1000  $\times$  g for 10 min at 4°C to obtain the supernatant (S1) and pellet (P1). S1 was again centrifuged again at 10,000  $\times$  g for 30 min at 4°C to generate a crude synaptosomal fraction (P2) and supernatant (S2). P2 was lysed hypo-osmotically and centrifuged at 25,000  $\times$  g for 30 min at 4°C to generate the synaptosomal membrane fraction (LP1) and supernatant (S3). LP1 was resuspended in HEPES-lysis buffer (50 mM HEPES, 1 mM EDTA, 1 mM EGTA, and protease/phosphatase inhibitor cocktail, pH 7.4). The protein concentrations of S2 (cytosolic lysates) and LP1 were determined using the BCA kit (Applygen Technologies, Beijing, China). HEPES-lysis buffer was used to equalize the protein concentrations. Five-times loading buffer (16% glycerol, 20% mercaptoethanol, 2% SDS, and 0.05% bromophenol blue) was added to each sample (4:1, sample:loading buffer). The protein samples were boiled for 5 min before loading.

### **Western blot**

The Western blot procedures were performed according to (Liu et al., 2014; Xue et al., 2015). Equal amounts of total proteins were subjected to SDS-PAGE for ~40 min at 80 V in stacking gel and ~1 h at 120 V in resolving gel. The separated proteins were then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) at 250 mA for 1-2.5 h. The membranes were blocked for 1 h at room temperature with 5% bovine serum albumin (BSA) in TBST (Tris-buffered saline plus 0.05% Tween-20, pH 7.4) and incubated with one of the following primary antibodies: Pi4KII $\alpha$  (1:500; catalog no. sc-390026, Santa Cruz Biotechnology, Dallas, Texas, USA), EEA1 (1:1000; catalog no. 3288S, Cell Signaling Technology, Danvers, USA), GluA1 (1:1000; catalog no. ab109450, Abcam, Cambridge, UK), GluA2 (1:1000; catalog no. ab133477, Abcam, Cambridge, UK), GluA3 (1:1000; catalog no. ab40845, Abcam, Cambridge, UK), PSD95 (1:1000; catalog no. ab2723, Abcam, Cambridge, UK),  $\beta$ -actin (1:1000; catalog no. TA-09, ZSGB-BIO, Beijing, China), and GAPDH (1:1000; catalog no. TA-08, ZSGB-BIO,

Beijing, China). After four 5-min washes in TBST, the membranes were incubated in horseradish peroxidase (HRP)-conjugated secondary antibody for 50 min at room temperature. The membranes were then washed four times for 5 min each with TBST. The HRP signals were visualized with Immobilon Western Chemilum HRP Substrate (Detection Reagents 1 and 2, 1:1 ratio, Merck Millipore, Darmstadt, Germany) and measured using the ChemiDoc MP System (Bio-Rad, Hercules, CA, USA). The intensity of each band was quantified using ImageJ software. The representative Western blots of cytosolic Pi4KII $\alpha$  and EEA1 were cropped and are presented along their corresponding control  $\beta$ -actin in the figures in the main text. The representative Western blots of synaptosomal membrane EEA1, GluA1-3, and PSD95 were cropped and are presented along with their corresponding control GAPDH in the figures in the main text.

### **Immunohistochemistry**

The rats were deeply anesthetized with 10% chloral hydrate and perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.2 M phosphate buffer. Brains were postfixed overnight in the same 4% paraformaldehyde solution and cryoprotected by immersion in 30% sucrose in 0.2 M phosphate buffer. For eGFP in AAV-injected rats, consecutive 40  $\mu$ m coronal brain sections were examined using an Olympus BX53 fluorescence microscope. Rats with misplaced cannulas were excluded. To assess Pi4KII $\alpha$  localization and colocalization in the BLA, frozen brains were sectioned at 20  $\mu$ m. Free-floating slices were incubated in blocking solution for 2 h at room temperature, followed by incubation with primary antibody in blocking solution overnight at 4°C. The following antibodies were used: Pi4KII $\alpha$  (1:100; catalog no. sc-390026, Santa Cruz Biotechnology, Dallas, Texas, USA), EEA1 (1:200; catalog no. 3288S, Cell Signaling Technology, Danvers, USA), GluA1 (1:200; catalog no. ab109450, Abcam, Cambridge, UK), GluA2 (1:200; catalog no. ab133477, Abcam, Cambridge, UK), MAP2 (1:500; catalog no. ab183830, Abcam, Cambridge, UK), PSD95 (1:500; catalog no. ab18258, Abcam, Cambridge, UK), and NeuN (1:1000; catalog no. ab177487, Abcam, Cambridge, UK). The secondary antibody was then incubated for 2 h at room temperature with slow shaking. The following secondary antibodies were used: AlexaFluor goat anti-rabbit 488 (1:500) and AlexaFluor goat anti-mouse 594 (1:500, Waltham, MA USA). Fluorescent image acquisition was performed using a TCS-SP8 confocal microscope.

### **Co-immunoprecipitation**

The co-immunoprecipitation assay was performed according to (Chiu et al., 2017) with minor modification. Basolateral amygdala tissues were lysed in ice-cold lysis buffer that contained 1% NP-40, 200 mM NaCl, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 20 mM HEPES (pH 7.4), and protease inhibitors. After clearing cellular debris by centrifugation at 14,000  $\times$  g at 4°C, the protein concentration of the extracts was determined using the BCA assay kit (Applygen Technology, Beijing, China). Extracts that contained 300-500  $\mu$ g total protein were incubated with nonspecific IgG (2  $\mu$ g), Pi4KII $\alpha$  (2-5  $\mu$ g), or EEA1 (2-5  $\mu$ g) overnight at 4°C, followed by the addition of 40  $\mu$ l of protein A or G beads for 3 h at 4°C. Immunoprecipitates were subjected to immunoblotting analysis using an anti-Pi4KII $\alpha$  antibody, anti-EEA1 antibody, or anti-GluA1 antibody. The bands were examined using a ZF-90D Dark Box Ultraviolet Analyzer (Shanghai Guanghao Analytical Instrument Co., Ltd., Shanghai, China).

### **Golgi staining**

The rats were deeply anesthetized with 10% chloral hydrate (i.p.) 15 min, 1 h, or 4 h after US retrieval and then perfused with saline. Brains were removed and immersed in Golgi-Cox solution for 3 days in the dark at 37°C, after which they were immersed in 30% sucrose in 0.1 M phosphate buffer. Coronal sections were cut at 120  $\mu$ m using a ZQP-86 tissue slicer (Zhixin Co., Ltd., Shanghai, China), collected on poly-L-lysine-coated microscope slides, and immersed in ammonium hydroxide for 30 min in the dark. The slides were then rinsed in distilled water, dehydrated in an ascending series of ethanol concentrations (50% for 1 min, 75% for 1 min, 90% for 1 min, and 100% twice for 5 min) and dimethylbenzene (twice for 15 min). The slides were then mounted with neutral balsam. Four to six BLA principle neurons in three animals per group were examined using a microscope with a 60  $\times$  oil-immersion objective. The number of dendritic spines was counted using ImageJ software. The average number of spines per 1.0  $\mu$ m of dendrite was calculated. Dendritic spine morphology was classified according to previous studies, including mushroom spines, stubby spines, thin spines, branched spines, and filopodia (Harris et al., 1992; Lippman and Dunaevsky, 2005; Zagrebelsky et al., 2005).

### **Whole-cell electrophysiology**

For whole-cell voltage-clamp electrophysiological recordings, 300  $\mu$ m horizontal BLA slices were

prepared in cutting solution that contained 87 mM NaCl, 3.0 mM KCl, 1.5 mM CaCl<sub>2</sub>, 1.3 mM MgCl<sub>2</sub>, 1.0 mM NaH<sub>2</sub>PO<sub>4</sub>, 26 mM NaHCO<sub>3</sub>, 20 mM D-glucose, and 75 mM sucrose. After incubation for 30 min at 32°C, the slices were recovered at room temperature for > 30 min in aCSF solution (124 mM NaCl, 3.0 mM KCl, 2 mM CaCl<sub>2</sub>, 1.3 mM MgCl<sub>2</sub>, 1.0 mM NaH<sub>2</sub>PO<sub>4</sub>, 26 mM NaHCO<sub>3</sub>, and 20 mM D-glucose). The slices were transferred to a recording chamber and maintained at 30-32°C and a flow rate of 2 ml/min, and BLA neurons were held at -70 mV and recorded using a pipette solution that contained the following: 115 mM Cs methylsulfate, 5 mM CsCl, 4 mM ATP-Mg, 0.5 mM GTP-Na, 0.5 mM EGTA, 10 mM HEPES, and 4.0 mM QX-314 [pH 7.2, 280-290 mOsm with sucrose]. For mEPSC recordings, 1.0 μM tetrodotoxin (Chengdu Must Bio-Technology, Chengdu, China) and 100 μM picrotoxin (Sigma-Aldrich, St. Louis, MO, USA) were bath-applied. The recording electrodes had resistances of 2.5-4 MΩ. The series resistance, usually between 8 and 14 MΩ, was uncompensated. The series resistance was monitored regularly during the experiments. Data were discarded when the series resistance was > 16 MΩ or the fluctuation of series resistance was > 15% during the recordings. Whole-cell recordings were performed using a Multiclamp 700B amplifier (Molecular Devices, San Jose, CA, USA) and Axon Digidata 1440A (Molecular Devices, San Jose, CA, USA) that was controlled by pClamp 10 software (Molecular Devices, San Jose, CA, USA). The data were filtered at 4 kHz and digitized at 20 kHz. The data were analyzed offline using Axograph X and Igor Pro software (Wavemetrics).

### Statistical analysis

Data were presented as mean ± SEM. All of the statistical analyses were performed using GraphPad Prism 7.0 software. Rats with misplaced injection sites were excluded from the analyses of the behavioral, biochemical, and electrophysiological data. The investigators were blinded to group allocation during the experiment. Data collection and processing were both randomized. Animals were selected randomly for all of the tests. The data were analyzed using unpaired two-tailed Student's *t*-test, one-way analysis of variance (ANOVA) followed by Dunnett's multiple-comparison *post hoc* test, two-way ANOVA followed by Tukey's *post hoc* test, or two-way repeated-measures ANOVA followed by Tukey's *post hoc* test. For all of the tests, values of *p* < 0.05 were considered statistically significant.

### Supplemental References

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