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Optogenetic Long-Term Depression Induction in the PVT-CeL Circuitry Mediates Decreased Fear Memory

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Abstract

The dysregulation of fear learning and abnormal activities of cerebral networks may contribute to the etiologies of anxiety disorders. Although it has been proposed that decreased activity in the paraventricular nucleus of the thalamus (PVT) to the lateral central nucleus of amygdala (CeL) pathway could induce an attenuation of learned fear, no study has shown the effect of the direct optogenetic activation of PVT projecting CeL neurons in vivo on unconditioned fear-related behaviors or learned fear expression. The mechanisms that control the neuronal activity of the PVT-CeL pathway involved in anxiety are rare. Here, we found that CeL neurons have varied responses to optogenetic excitation of PVT terminals in the CeL: neurons with relative high excitability(~ 30%), neurons with relative low excitability(~ 60%), and neurons with no excitability (~ 10%). We next explored the role of the PVT-CeL pathway in unconditioned and conditioned fear-related behaviors by using optogenetics and anxiety assays in freely moving mice. We observed that temporally precise optogenetic activation of the CeL-projecting PVT neurons had no effect on unconditioned fear-related behaviors on the elevated plus maze test and the open field test. But optogenetic activation of the CeL-projecting PVT neurons increased conditioned fear expression. We then found that optogenetic long-term depression (LTD) induction in the CeL receiving PVT afferents effectively exerted a persistent attenuation of learned fear. The percentage of neurons with relative high excitability was decreased by the LTD induction, and the percentage of neurons with relative low excitability was increased by the LTD induction. Taking these results together, we identify that increased activity of the PVT-CeL pathway could lead to as excessive learned fear. The CeL neurons with relative high responses to the photostimulation of PVT afferents in the CeL may be the key neurons that regulate the output of learned fear expression. Our optogenetic LTD protocol may inspire the development of novel treatments for anxiety disorders involving deep brain stimulation to induce plasticity at relevant brain areas.

Keywords The PVT-CeL circuitry · Optogenetics · LTD · Fear memory

Introduction

Learned fear represents an advantageous evolutionary adaptation: the ability to learn by experiencing some stimuli or dangerous circumstances is essential for animals to survive in the wild. The dysregulation of fear learning [1] and abnormal activities of cerebral networks [2] may contribute to the development of anxiety disorders. It is commonly believed that discovering the mechanisms underlying learned fear will also lead to improvements in the treatment of anxiety disorders [3]. In the laboratory, the classical paradigm for this process is Pavlovian fear conditioning where an initially neutral stimulus (conditioned stimulus (CS)), such as tone, is paired with a noxious unconditioned stimulus (US), typically a mild foot shock. As a result, the CS elicits conditioned fear responses (such as freezing) when the CS is presented alone [4]. The new developments highlight that the conditioned fear depends on far more complex networks than initially envisioned. Uncertainty key areas remain, particularly with respect to the connectivity of the different cell types and the functional connections between different brain regions. Filling

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these gaps in our knowledge is important because much evidence indicates that human anxiety disorders results from the abnormal regulation of fear learning [4].

The central amygdale (CeA) is a critical component of the neural circuitry underlying fear learning [4]. The CeA is commonly divided in lateral (CeL) and medial (CeM) sectors. In the mammalian brain, the paraventricular nucleus of the thalamus (PVT) is one putative stress sensor that is readily activated by both physical and psychological stressors [5–7]. Previous studies showed that selective inactivation of PVT–CeL pathway prevented fear expression 24 h after fear acquisition, an effect that can be accounted for by an impairment in fear conditioning-induced synaptic potentiation onto somatostatin-expressing (SOM+) CeL neurons [8]. Another study found that silencing the PVT → CeL projection at late (7 days), but not early(6 h), times induced a persistent attenuation of fear [9]. However, there are no evidences showing the effect of optogenetic activation of PVT-CeL pathway on fear memory. Besides, although the CeL for unconditioned fear has been optogenetically dissected [10], the function of PVT-CeL pathway on unconditioned fear have not yet been investigated, pointing to the need for optogenetic exploration of PVT-CeL circuitry in innate fear.

Previous studies showed that high frequency photostimulation of PVT afferents in CeL induced a slow inward current exclusively in CeL SOM+ neurons, of which the amplitudes were no more than 10 pA [8]. But other studies found that the amplitudes of photo-evoked EPSCs recorded from the PVT-projecting neurons in the nucleus accumbens (NAc) were more than 200 pA [11]. We then decided to explore if there were any CeL neurons with larger input currents involving in the function of PVT-CeL pathway that were not found by the previous studies.

The LTD is an activity-dependent reduction in the efficacy of neuronal synapses lasting hours or longer following a relative long-patterned stimulus. The LTD is impaired at the dorsal intercalated cell mass (ITC) of animals exhibiting PTSD-like behaviors [12]. The optogenetic LTD protocol may inspire the development of novel treatments for anxiety disorders involving plasticity at relevant synapses. We therefore tried to prove if optogenetic LTD induction in the PVT-CeL circuitry will also regulate fear memory expression.

Methods and Materials

Subjects

Adult male C57 BL/6 mice weighing 20–25 g and aged 12–13 weeks were housed (four to five per cage) in standard laboratory cages on a 12-h light/dark cycle (lights on at 8:00 A.M.) in a temperature-controlled room (21–25 °C). The mice were housed with free access to food and water. Behavioral testing was performed during the light cycle between 10:00 A.M. and 4:00 P.M. All procedures were in accordance with the Chinese Council on Animal Care Guidelines [13, 14]. All experimental protocols were also conducted in accordance with the guidelines set by the Wuhan University and approved by the Committee on Animal Care and Use of the Basic Medical Sciences at the Wuhan University. Efforts were made to minimize mouse suffering and reduce the number of mice sacrificed.

All chemicals were from Sigma-Aldrich. Dose selections for these drugs were based on previously published studies [13–15].

Surgery and Viral Injection

Viral vectors were injected into the PVT as previously described [11]. Mice were anesthetized and placed in a stereotaxic frame (Stoelting, USA). The mouse scalp was removed, and small burr holes were drilled into the skull. A small volume of concentrated virus solution was injected into the PVT (0.2-μl AAV, bregma − 1.4 mm; lateral ± 0.1 mm; ventral 3.0 mm, with a 4° angle toward the midline) with a pulled glass capillary at a slow rate (0.1 μl/min) using a pressure micro-injector (Micro 4 system, World Precision Instruments). The needle rested in position for 10 min post-injection. For mice involved in behavioral experiments, a fiber-optic cannula (plastic one) was placed at least 500 μm above the medial shell of the CeL (− 1.4 mm posterior to the bregma, ± 2.5 mm lateral to the midline, and − 4 mm the from the pial surface) and cemented onto the skull using dental cement (Lang Dental Manufacturing). Mice were allowed 3–4 weeks to recover and express the virus before behavioral analysis. The AAVs used in this study were produced by BrainVTA Co., Ltd.: rAAV-hSyn-hChR2-EGFP-WPRE-pA (titer 2×10^{12} vg/ml); rAAV-hSyn-EGFP-WPRE-pA (titer 2×10^{12} vg/ml). For optical stimulation, the fiber was connected to a 473-nm laser (MBL F473; Opto Engine) controlled by a function generator (33220A; Agilent Technologies). For optogenetic activation of the CeL, mice received 5-ms light pulses at 20 Hz for all light-on conditions. For optogenetic LTD induction, mice were photostimulated for 15 min with trains of 473-nm light (1 Hz, 4 ms, 900 pulses, 7 mW per mm^2 at the CeL). Mice with properly placed optical fibers were included in this study (Fig. S1). Mice without properly placed optical fibers were excluded from this study.

Behavioral Tests

Open Field Test

As previously described [13–16], the open field testing chamber was illuminated by halogen bulbs (200 lx, 200 cm above the field). The open field chamber (50 × 50 × 40 cm) was

divided into a central field (center, 23 × 23 cm) and an outer field (periphery). Mice were gently placed into one corner of the chamber and were allowed 5 min of free movement, which was monitored by an automated video tracking system. Images of the activities were automatically analyzed using the DigBehv animal behavior analysis program.

Elevated Plus Maze Test

As previously described [13–15], the test was performed on an elevated, plus-sign-shaped runway that was ~ 40 cm above the floor, with two opposing closed arms (10 × 50 × 40 cm), two open arms (10 × 50 cm), and one intersection (10 × 10 cm). It was illuminated by halogen bulbs (200 lx). Before the test, mice were allowed to acclimate to the testing room for 30 min. At the time of the test, each mouse was placed at the center of the EPM facing the closed arm and was videotaped for 5 min. The time spent in the closed and open arms was quantified autonomously by the DigBehv animal behavior analysis software.

Fear Conditioning

As previously described [17–20], two kinds of fear conditioning shock chambers (chamber A: 25 × 25 × 31 cm, plexiglass front and back with black-and-white cross-stripes pattern and aluminum side walls, grid-floor connected to a scrambled shocker; chamber B: 25 25 31 cm, square plastic chamber surrounded with white walls, plastic floor with sani-chips) and multi-parameter activity monitors (JLBehv-LG Instrument) were used. The mice were handled twice a day for 3 consecutive days before the experiments. They were habituated to the manipulation. The CS used was a 70-dB sound, and the US was one time-continuous scrambled foot shock at 0.6 mA for 1 s. On the conditioning day, mice were transported from the housing room and individually placed in fear conditioning chamber A. Animals were left undisturbed for a 3-min acclimation period, followed by four CS (70 dB; 30-s duration; 80-s inter-shock interval); each session was terminated with a US (0.6 mA; 1-s duration). Mice remained in the chamber for an additional 2 min, immediately brought back to their home cages and returned to the colony room.

To test the tone-cued fear memory, the mice were put in chamber B and monitored for 3 min (pre-tone freezing) and then subjected to 3 min of CS exposure (tone-cued freezing). The total freezing time in each period, expressed as the percentage of total time, was quantified by DigBehv animal behavior analysis software.

Histological Verification

Histological verification was carried out as described previously [15, 21], to determine the accuracy of fiber implantation and the extent of viral expression. Three to four weeks after virus injection, mice were anesthetized with ketamine/xylazine (Sigma, 100/20 mg/kg, respectively, i.p.) and perfused transcardially with 4% paraformaldehyde (PFA) in PBS and tissues were post-fixed in 4% PFA at 4 °C for 24 h. Brain tissues were then incubated in 20% sucrose (20 g/100 ml) in PBS at 4 °C for 48 h. Brain tissues were frozen in OCT and frozen brain blocks were cut into 30-μm-thick sections by cryostat (Thermo Scientific, HM 550). After washing with PBS for three times, samples were mounted with Vectashield mounting medium (Vector) and images were taken by the Olympus BX51 fluorescence microscope.

Electrophysiological Analysis

As previously described [22, 23] mice were anesthetized with ketamine/xylazine (Sigma, 100/20 mg/kg, respectively, i.p.), brains were quickly removed and chilled in ice-cold modified artificial cerebrospinal fluid (ACSF) containing (in mM): 250 glycerol, 2 KCl, 10 $MgSO_4$, 0.2 $CaCl_2$, 1.3 NaH_2PO_4, 26 $NaHCO_3$, and 10 glucose. Amygdala slices (300 μm) were cut using a VT-1000S vibratome (Leica, Germany) and transferred to a chamber containing regular ACSF (in mM, 126 NaCl, 3 KCl, 1 $MgSO_4$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 glucose) at 34 °C for 30 min and at 25 ± 1 °C for additional 1 h before recording. All solutions were saturated with 95% O_2/5% CO_2 (vol/vol). The recording chamber was superfused (2 ml/min) with ACSF at 32–34 °C. Recording pipettes (3–5 MΩ) contained (in mM): 140 K-gluconate, 10 NaCl, 10 Hepes, 0.2 EGTA, 0.5 $MgCl_2$, 4 Mg-ATP, 0.4 Na-GTP, and 10 phosphocreatine (pH 7.4, 285 mOsm). Voltage-clamp recordings were made at both − 70 mV, to isolate EPSCs, and at 0 mV, to isolate IPSCs. The action potentials were recorded under the I-clamp condition with currents (1-s duration, 0 pA or 100 pA) injected into the neuron. A blue LED (470 nm, Thorlabs) controlled by digital commands from the Digidata 1440A was coupled to the microscope with a dual lamp house adapter (5-UL180, Olympus) to deliver photostimulation.

Statistical Analyses

The number of experimental animals is indicated by "*n*." All data met the assumption of normality. For paired comparisons, an independent *t* test or a two-way ANOVA followed by a repeated measure ANOVA was used. For multiple comparisons, an SNK test was used when equal variances were assumed, and Dunnett's test was used when equal variances were not assumed. Throughout the study, statistical analyses were performed using SPSS software (SPSS, Inc.). All data are expressed as the mean ± SEM. Values of $P < 0.05$ were considered significant.

Result

Projection-Specific Excitation of PVT Terminals in the BLA Does Not Change the Firing of BLA Neurons

To examine if PVT projects to and regulates the excitability of other amygdala nuclei, such as the basolateral amygdala (BLA), we bilaterally injected AAV-ChR2 into the PVT. Three to four weeks after the virus injection, weakly ChR2-GFP-labeled fibers were observed throughout the BLA in acute slices prepared from these mice (Fig. 1a), confirming a weak PVT → BLA projection. High frequency photo-stimulation of PVT afferents in the BLA also evoked very minor excitatory synaptic transmission and little net inhibition in BLA neurons (Fig. 1b). Though the EPSC amplitudes were increased by high frequency photo-stimulation of PVT afferents in the BLA [8.53 ± 2.56 pA; $t(28) = 2.148$, $P = 0.046$; Fig. 1d], we did not find any significant differences in the IPSC amplitudes between the light-on group and light-off group [$t(28) = 0.706$, $P = 0.382$; Fig. 1d]. To our surprise, high frequency photo-stimulation of PVT afferents in the BLA failed to evoke any detectable changes of action potentials in the recorded BLA neurons (Fig. 1b). The firing frequencies of action potentials induced by 100-pA injection current were not different between the GFP group and ChR2 group [$t(28) = -0.597$, $P = 0.556$; Fig. 1f]. These results suggest that though there are glutamatergic projections from the PVT to the BLA, the PVT → BLA projections are too weak to change the firing of the BLA neurons.

CeL Neurons Have Varied Responses to Projection-Specific Excitation of PVT Terminals in the CeL

Previous studies showed photo-stimulation of PVT afferents in CeL induced a slow inward current exclusively in CeL neurons, of which the amplitudes were no more than 10 pA [8]. But other studies found that the amplitudes of photo-evoked EPSCs recorded from the PVT-projecting neurons in the nucleus accumbens (NAc) were more than 200 pA [11]. We then decided to explore if there were any CeL neurons with larger input currents from PVT projecting neurons and involving in the function of PVT-CeL pathway that were not found by the previous studies. We then bilaterally injected

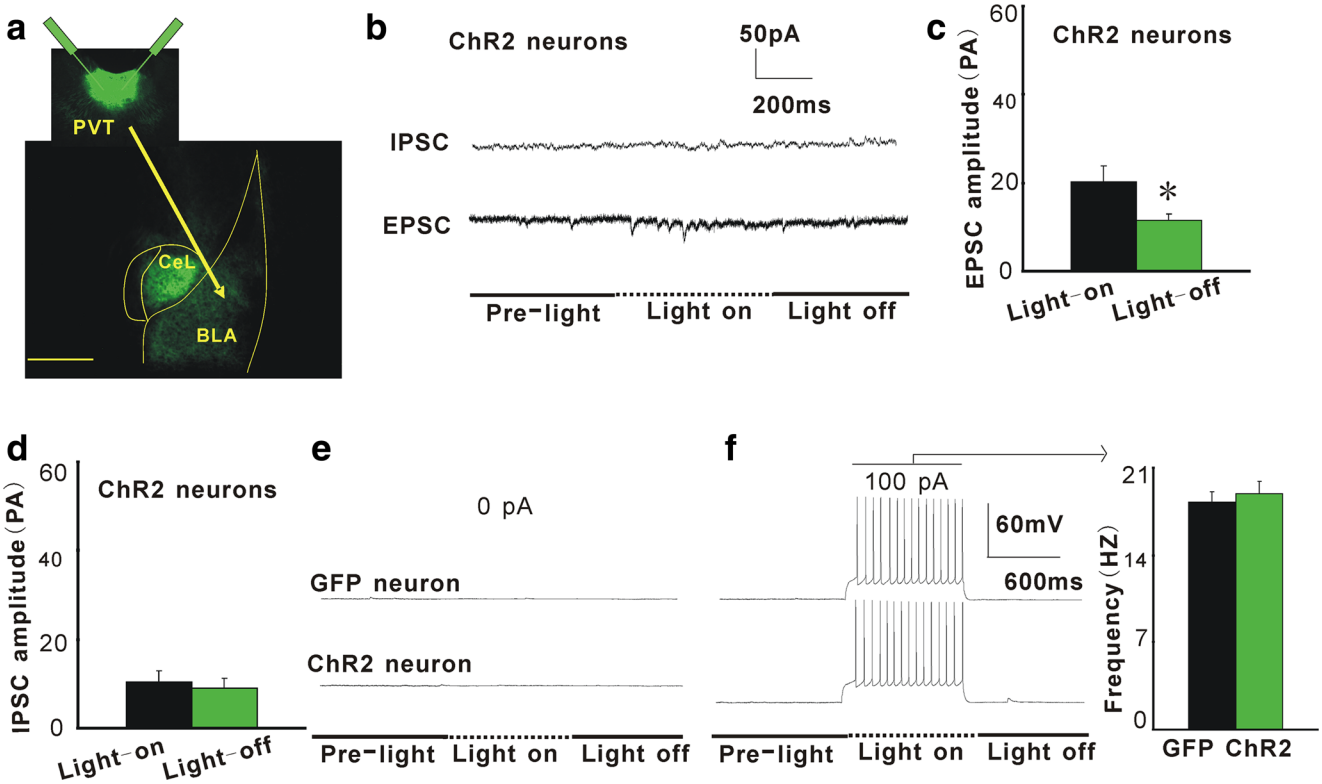
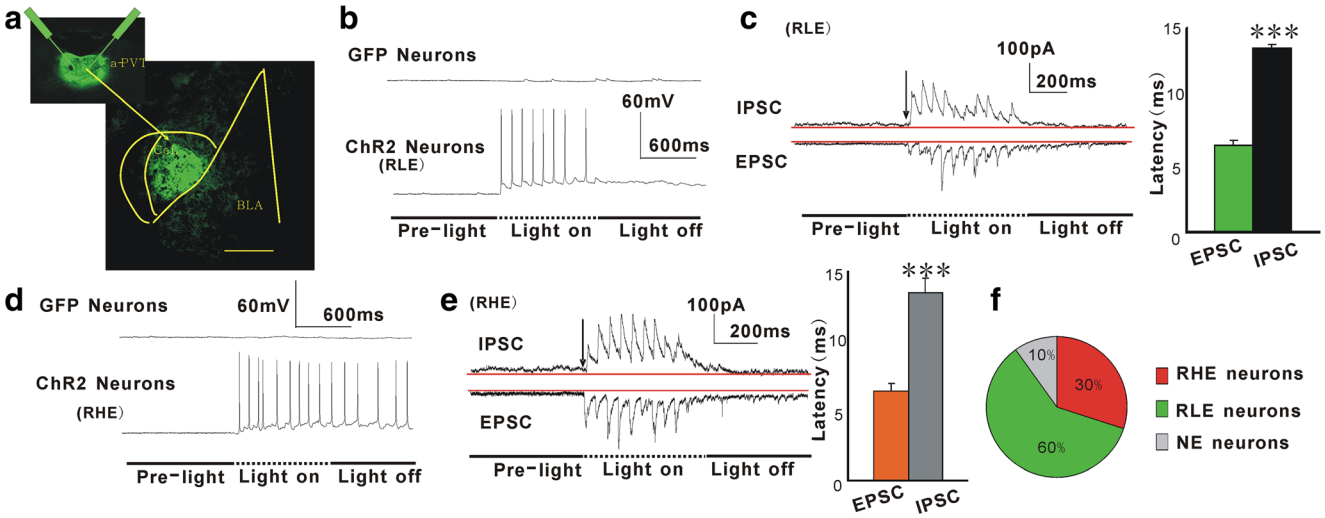


Fig. 1 Projection-specific excitation of PVT terminals in the BLA does not change the firing of BLA neurons. **a** A representative image shows relatively few ChR2-GFP expressing terminals from the PVT in the BLA. Inset, injection site in the PVT. Scale bar: 500 μm. **b** On illumination of the BLA, the same BLA ChR2 neurons showed low net excitation and little net inhibition under voltage-clamp conditions. **c** The analysis results of EPSC amplitudes for ChR2 neurons with or without light stimulation. **d** The analysis results of IPSC amplitudes for ChR2 neurons with or without light stimulation. **e**, **f** Representative traces from BLA neurons in the terminal field of PVT projection neurons. **e** Sub-threshold excitatory responses on photostimulation (injection current: 0 pA, light pulse: 20 Hz). **f** Supra-threshold excitatory responses on photostimulation (injection current: 100 pA, light pulse: 20 Hz) and the analysis results of firing frequency (inset right). $n = 15$/ group, *$P<0.05$, two-tailed t test

AAV-ChR2 into the PVT. Bright ChR2-GFP-labeled fibers were readily observed throughout the CeL in brain slices prepared from these mice (Fig. 2a), confirming a strong PVT → CeL projection. Optogenetic stimulation of the PVT–CeL pathway promotes intra-CeL excitation. Figure 2b, d shows representative traces from GFP neurons and ChR2 neurons on or after 20-Hz illumination of PVT terminals in the CeL. CeL-ChR2 neurons with relative low excitability (RLE) showed increased excitability upon high frequency photo-stimulation of PVT afferents in the CeL (Fig. 2b, g). But these neurons did not have any neuronal spike responses after the photo-stimulation of PVT afferents (Fig. 2b, g). By contrast, CeL-ChR2 neurons with relative high excitability (RHE) showed increased neuronal spike responses both upon or after high frequency photo-stimulation of PVT afferents in the CeL (Fig. 2d, g).

High frequency photo-stimulation of PVT afferents in the CeL reliably evoked both inhibitory and excitatory synaptic transmission in CeL neurons (Fig. 2c, e). The EPSC amplitudes of RHE neurons were larger than that of RLE neurons [$t(25) = 2.25$, $P = 0.033$; Fig. 2g]. Interestingly, high frequency photo-stimulation of PVT afferents in CeL induced a slow recovery of inward current during the light-off phase exclusively in RHE neurons (Fig. 2e). But the IPSC amplitudes did not differ between the RHE neurons and RLE neurons [$t(25) = 0.161$, $P = 0.873$; Fig. 2g]. The onset latency for photo-evoked IPSCs was significantly longer than that of EPSCs [$t(25) = -7.882$ and -6.209, respectively; both $P < 0.001$; Fig. 2c, e]. There were some other CeL-ChR2 neurons that showed no changes of excitability upon high frequency photo-stimulation of PVT afferents in the CeL (Fig. 2g). We did find some CeL neurons with larger input currents from PVT projecting neurons and involving in the function of PVT-CeL pathway that were not found by the previous studies. The percentages of neurons with relative different excitability are as follows: RHE neurons are about 30%, RLE neurons are about 60%, and neurons with no excitability (NE) are about 10% (Fig. 2f).

These results suggest that CeL neurons have varied responses to projection-specific excitation of PVT terminals in



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	Firing frequency (HZ)		EPSC Amplitude (pA)		IPSC Amplitude (pA)	
	Light on	light off	Light on	Light off	Light on	Light off
RHE neurons	12. 11 ± 0. 71	6. 55 ± 0. 55	164. 11 ± 15. 16	37 ± 5. 86	136. 11 ± 13. 06	22. 67 ± 1. 69
RLE neurons	6. 94 ± 0. 72	0	119. 39 ± 11. 81	19. 55 ± 2. 24	132. 83 ± 12. 75	16. 83 ± 1. 81
NE neurons	0	0	8. 33 ± 1. 67	8 ± 2. 33	7 ± 2. 52	6 ± 2. 51

Fig. 2 CeL neurons have varied responses to projection-specific excitation of PVT terminals in the CeL. **a** A representative image shows considerable ChR2-GFP expressing terminals from the PVT in the CeL. Inset, injection site in the PVT. Scale bar: 500 μm. **b** Representative traces from GFP neurons and ChR2 neurons (RLE neurons) in response to a current step (0 pA) upon or after 20 Hz illumination of PVT terminals in the CeL. **c** Inset left, on illumination of the RLE neurons, the same RLE neurons showed net excitation or net inhibition under voltage-clamp conditions. Inset right, quantification of the onset latency for photo-evoked IPSCs and EPSCs in the same neuron. **d** Representative traces from GFP neurons and ChR2 neurons (RHE neurons) in response to a current step (0 pA) upon or after 20-Hz illumination of PVT terminals in the CeL. **e** Inset left, on illumination of the RHE neurons, the same RHE neurons showed net excitation or net inhibition under voltage-clamp conditions. The sample trace of EPSCs following photo-stimulation of pPVT afferents expressing ChR2 showed slow recovery after stimulus offset. Inset right, quantification of the onset latency for photo-evoked IPSCs and EPSCs in the same neuron. **f** The percentage of neurons with different excitability. $n = 30$ for total numbers. **g** The analysis results of firing frequencies, EPSC amplitudes, and IPSC amplitudes for neurons with different excitability. RHE, relative high excitability; RLE, relative low excitability; NE, no excitability ***P<0.001, two-tailed t test

the CeL. The excitatory PVT inputs could activate both the RHE neurons and the RLE neurons and elicit feed-forward inhibition onto these neurons. The RHE neurons may be the key neurons that regulate the output of fear expression.

In Vivo Optical Activation of the PVT → CeL Pathway Increases Fear Memory Expression

To determine whether optogenetic activation of the PVT-CeL pathway regulate innate fear and learned fear, C57 mice were randomly allocated into two groups ($n = 8$/group) according to the intra-PVT treatment (AAV-GFP or AAV-ChR2). Three to four weeks after bilateral virus injections, mice underwent two well-validated unconditioned fear assays: the elevated plus maze (EPM) test and the open field test. Mice underwent the unconditioned fear tests with CeL-specific optogenetic photostimulation. Compared with photostimulation of the CeL of GFP mice, photostimulation of the CeL of ChR2 mice did not change the open-arm time [$t(25) = 0.759$, $P = 0.461$; Fig. 3a], or the closed-arm time on the EPM test [$t(25) = 0.34$, $P = 0.739$; Fig. 3b]. Due to the different time spent in the intersection of the EPM, the total time spent in both closed arms and open arms was different between the GFP group the ChR2 group. Photostimulation of the CeL of ChR2 mice did not change the center time [$t(25) = 1.04$, $P = 0.316$; Fig. 3c] and locomotion [$t(25) = -0.457$, $P = 0.654$; Fig. 3d] on the open field test.

Mice were trained in the fear conditioning box 1 week after the innate fear tests. We did not find significant difference in the freezing percentage between the GFP group and the ChR2 group during the fear training process without optogenetic stimulation ($F_{1,16} = 0.1$, $P = 0.756$; Fig. 3e). Average tone-cued freezing during fear memory expression was measured

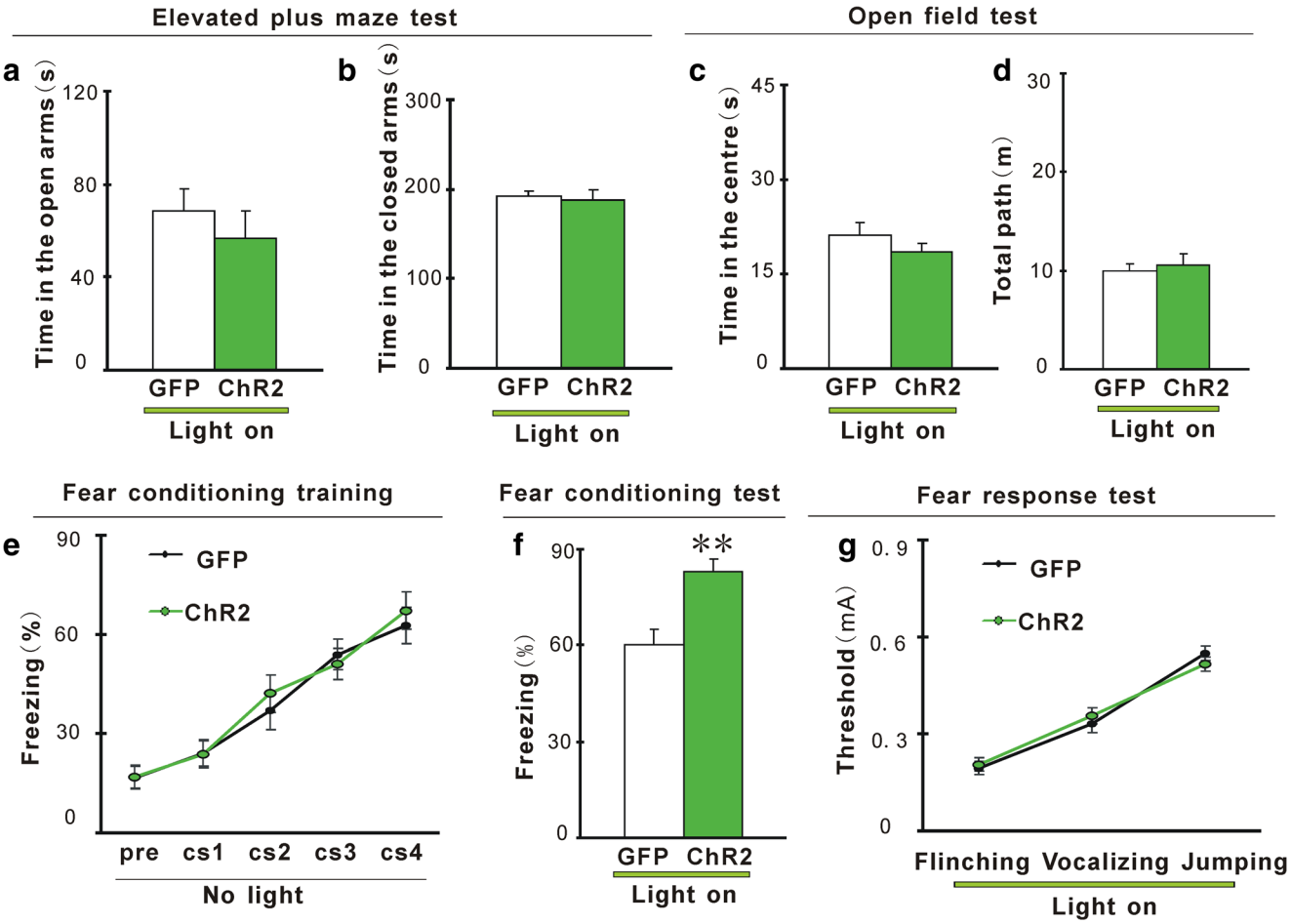

Fig. 3 In vivo optical activation of the PVT → CeL pathway increases fear memory expression. **a**, **b** On the elevated plus maze test, the ChR2 group spent similar time in the open arms and in the closed arms, compared with the GFP group. **c** The locomotor activity was not different among the groups on the open field test. **d** The two group spent similar time in the center arena on the open field test. **e** There was no difference in the freezing percentage between the GFP group and the ChR2 group during the fear training process without optogenetic stimulation. **f** 20-Hz illumination of PVT terminals expressing ChR2 in the CeL increased fear memory expression 24 h after fear training. **g** The liminal current intensities to elicit flinching, jumping, and vocalizing were not changed by the optogenetic stimulation. $n = 8$/group, $^{**}P < 0.01$, two-tailed t test

24 h after fear training (Fig. 3e). Compared with photostimulation of the CeL of GFP mice, photostimulation of the CeL of the ChR2 mice significantly increased fear responses measured as freezing in the fear retrieval test [$t(25) = -3.664$, $P = 0.003$; Fig. 3e]. These results are consistent with previous findings that long-term (≧ 24 h), but not short-term (0.5 and 6 h) fear memories are susceptible to PVT manipulations [8, 9].

To test whether the significantly different freezing responses was due to the impairment of fear cognition by optical activation of the PVT → CeL pathway, the liminal current intensities to elicit flinching, jumping and vocalizing were comparable among all groups. The liminal current intensities to elicit flinching, jumping, and vocalizing were not changed by the optogenetic stimulation ($F_{1,\ 16} = 0.06$, $P = 0.938$; Fig. 3g). The above results suggest that the PVT → CeL pathway did not regulate innate fear, but specifically involves in regulation of learned fear.

In Vivo Optogenetic LTD Induction Inhibits Neuronal Transmission at PVT → CeL Synapses and Suppresses Fear Expression

Based on the above results suggesting that the optogenetic activation of the PVT-CeL pathway increased fear expression, we hypothesized that optogenetic LTD induction in the CeL may produce the opposite effect. To test this, we bilaterally injected AAV-ChR2 into the PVT of C57 mice. Mice were randomly allocated into two groups according to intra-CeL treatment (ChR2-No LTD, or ChR-LTD). One day after fear conditioning, mice were given optogenetic LTD induction light in the CeL. Forty-five minutes after LTD induction, mice were tested. Optogenetic induction of LTD in the CeL of ChR2 mice decreased fear memory expression in tone-cued fear expression 1 day after the fear training [$t(14) = 4.535$, $P < 0.001$; Fig. 4a]. Freezing remained reduced 7 days following LTD induction [$t(14) = 5.167$, $P < 0.001$; Fig. 4b].

The above results suggest that the CeL neurons with relative high responses to the high frequency photo-stimulation of PVT afferents in the CeL may be the key neurons that regulate the output of fear expression. To test it, we next measured the neuronal excitability and transmission of CeL neurons receiving PVT projecting synapses after the above fear tests. On illumination of the ChR2-No LTD neurons, the same CeL neurons showed net excitation or net inhibition under voltage-clamp conditions (Fig. 4c). However, on illumination of the ChR2-LTD neurons, the CeL neurons showed much smaller net excitation or much smaller net inhibition under voltage-clamp conditions (Fig. 4d). Both the amplitudes of EPSCs and IPSCs were decreased by LTD induction [$t(39) = 5.346$, $P < 0.001$, Fig. 4e; $t(39) = 3.769$, $P = 0.001$, Fig. 4f]. The onset latency for photo-evoked IPSCs was significantly longer than that of EPSCs [$t(39) = -8.334$ and -5.547, respectively; both $P < 0.001$; Fig. 4c, d].

Figure 4g shows the representative traces from ChR2-No LTD neurons and ChR2-LTD neurons. The ChR2-LTD neurons showed decreased firing frequencies upon or after high frequency photo-stimulation of PVT afferents in the CeL ($F_{1,\ 39} = 46.221$ and 6.146, respectively; $P < 0.001$ and $P = 0.018$, respectively; Fig. 4h). The analysis results showed that the percentage of RHE neurons was decreased by the LTD induction, and the percentage of RLE neurons was increased by the LTD induction (Fig. 4i). These results support the hypothesis that the CeL neurons with relative high responses to the high frequency photo-stimulation of PVT afferents in the CeL may be the key neurons that regulate the output of fear expression.

Optogenetic LTD Induction in the CeL Receiving PVT Afferents Reversed the Effects of Chronic Stress on Learned Fear

We next explored whether optogenetic LTD induction in the CeL could reverse the effects of chronic stress on learned fear. To test this, C57 mice were randomly allocated into three groups 3 weeks after bilateral virus injection (the control group, the stress group, or the stress + LTD group). Mice were placed in a restraint chamber for 30 min for 7 consecutive days. Control mice experienced a similar degree of daily handling, but were not restrained. Both the stress group and the LTD + stress group showed increased fear memory acquisition in fear training model compared with the control group ($F_{2,\ 24} = 8.683$, $P = 0.003$ and 0.007, respectively, Fig. 5b). One day after fear conditioning, the stress + LTD mice were given optogenetic LTD induction light in the CeL. Forty-five minutes after LTD induction, mice were tested for fear expression. Repeated restraint stress increased fear memory expression in tone-cued fear expression model compared with the control group ($F_{2,\ 24} = 26.390$, $P = 0.019$, Fig. 5c). Optogenetic LTD induction in the CeL reversed the effect of repeated restraint stress on fear expression. Compared with the stress group, the LTD + stress group showed decreased fear expression in tone-cued fear expression model ($F_{2,\ 24} = 26.390$, $P < 0.001$; Fig. 5c). Seven days after fear conditioning, repeated restraint stress still showed increased freezing percentage in tone-cued fear test model compared with the control group ($F_{2,\ 24} = 28.488$, $P = 0.029$, Fig. 5d). Optogenetic LTD induction in the CeL reversed the effect of repeated restraint stress on fear freezing. Compared with the stress group, the LTD + stress group showed decreased freezing percentage in tone-cued fear test model ($F_{2,\ 24} = 28.488$, $P < 0.001$; Fig. 5d). Taken together, these data suggest that optogenetic LTD induction in the CeL receiving PVT afferents reversed the effect of chronic stress on learned fear.

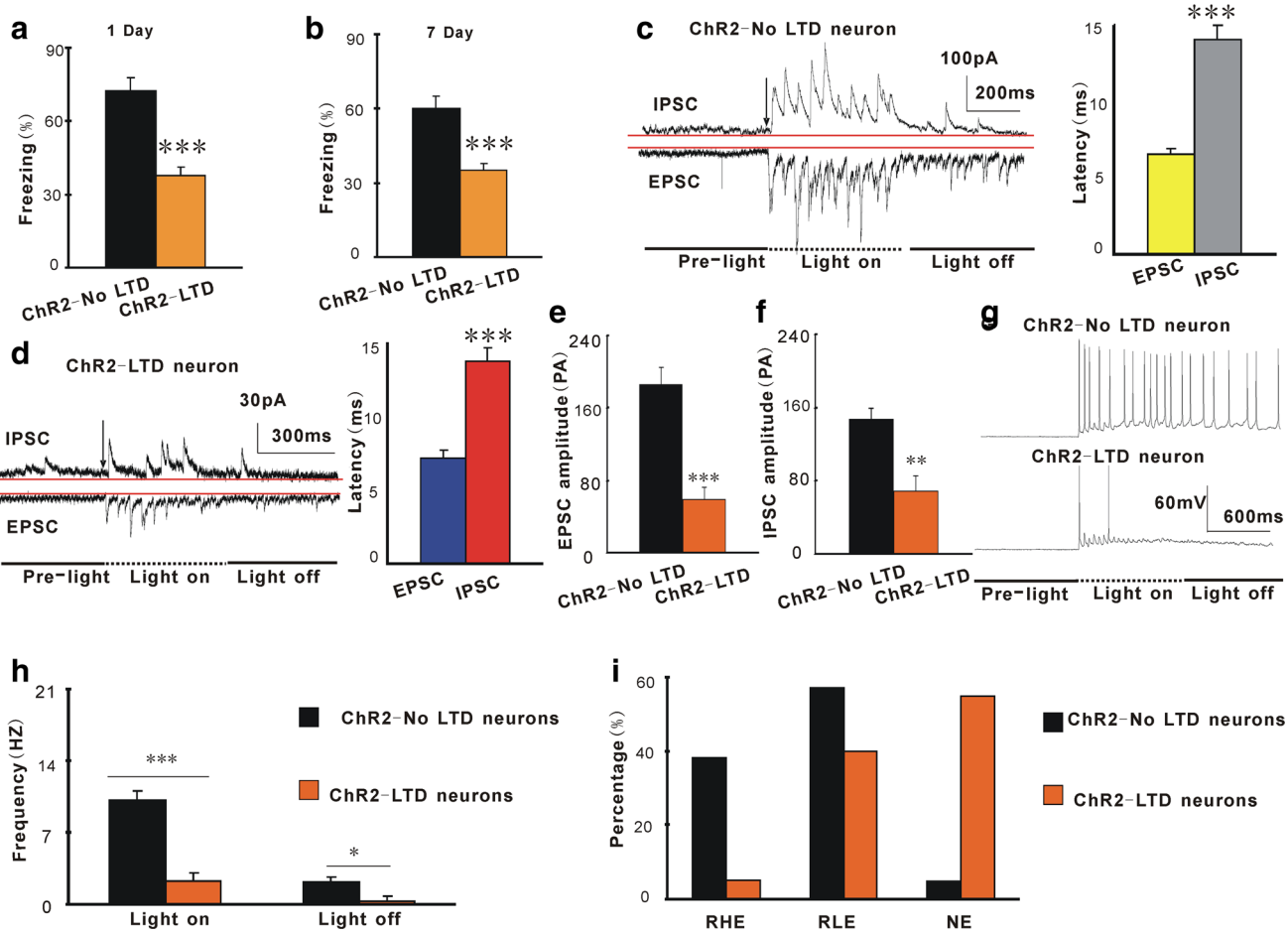

Fig. 4 In vivo optogenetic LTD induction inhibits neuronal transmission at PVT → CeL synapses and suppresses fear expression. **a** 1 day after the fear training, LTD induction in the CeL was delivered. 45 min after LTD induction, ChR2-LTD mice showed decreased fear expression. **b** 7 days after LTD induction, ChR2-LTD mice showed less fear expression too ($n = 8$/group). After the above behavioral test, mice were sacrificed for slice recording. **c** Inset left, on illumination of the ChR2-No LTD neurons, the same CeL neurons showed net excitation or net inhibition under voltage-clamp conditions. The sample trace of EPSCs following photo-stimulation of pPVT afferents expressing ChR2 showed slow recovery after stimulus offset. Inset right, quantification of the onset latency for photo-evoked IPSCs and EPSCs in the same neuron. **d** Inset left, on illumination of the ChR2-LTD neurons, the same CeL neurons showed much smaller net excitation or much smaller net inhibition under voltage-clamp conditions. Inset right, quantification of the onset latency for photo-evoked IPSCs and EPSCs in the same neuron. **e**, **f** The amplitudes of EPSCs and IPSCs were decreased by LTD induction. **g** Inset upper panel, representative trace from ChR2-No LTD neurons in response to a current step (0 pA) and promotion of spiking on 20-Hz illumination of PVT terminals in the CeL. Inset bottom panel, representative trace from ChR2-LTD neurons in response to a current step (0 pA) and promotion of spiking on 20-Hz illumination of PVT terminals in the CeL. **h** The spike frequencies were significantly reduced by LTD induction. **i** The percentage of neurons with different excitability. ChR2-No LTD neurons, $n = 21$; ChR2- LTD neurons, $n = 20$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, two-tailed t test for 1A, 1B, 1E, and 1F, two-way ANOVA test with one factor as repeated measure for 1H

Discussion

Previous studies showed photo-stimulation of PVT afferents in CeL induced a slow inward current exclusively in somatostatin-expressing (SOM+) CeL neurons, of which the amplitudes were no more than 10 pA [8]. We found that there were some CeL neurons with larger fast input EPSCs from PVT projecting neurons (> 100 pA) and involving in the function of PVT-CeL pathway that were not found by the previous studies. The percentages of CeL neurons with relative different excitability activated by PVT projections are as follows: RHE neurons are about 30%, RLE neurons are about 60%, and NE neurons are about 10% (Fig. 2f). These results suggest that CeL neurons have varied responses to projection-specific excitation of PVT terminals in the CeL. We also examined if PVT projects to and regulates the excitability of other amygdala nuclei, such as the BLA. We found the PVT → BLA projection is too weak to change the firing frequency of the BLA neurons.

Behavioral studies have shown that the CeL is involved in the regulation of conditioned and unconditioned fear [10, 24, 25]. Silencing the PVT-CeL projection at late (≧ 24 h), but not early (6 h), times induced a attenuation of learned fear [8, 9]. However, no study has shown the effect of direct optogenetic

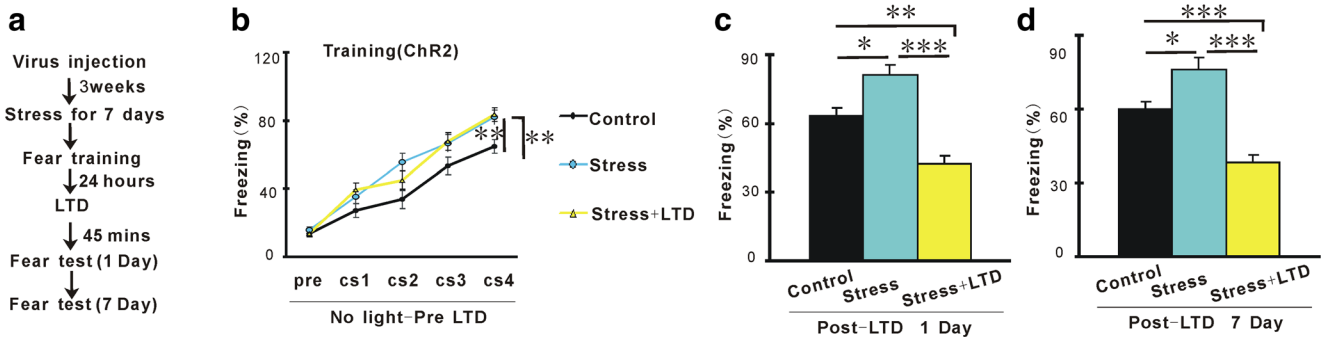

Fig. 5 Optogenetic LTD induction in the CeL receiving PVT afferents reversed the effects of chronic stress on learned fear. Mice were placed in a restraint chamber for 30 min for 7 consecutive days. Control mice experienced a similar degree of daily handling, but were not restrained. **a** Experimental protocol. **b** Repeated restraint stress increased freezing percentage during fear training in the stress group and the stress + LTD group. **c** Mice in the stress group showed increased tone-cued fear expression relative to the control mice and the stress + LTD mice on the first day test. **c** Mice in the stress group showed increased tone-cued fear expression relative to the control mice and the stress + LTD mice on the seventh day test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $n = 9$/group, repeated measures for 5B, one-way ANOVA test for 5C

activation of the PVT-CeL pathway in vivo on the learned fear expression or innate fear-related behaviors. Here, we selectively activated the PVT-CeL pathway and identified the PVT-CeL pathway as a critical neural circuitry for modulation of the conditioned fear expression, but not for the regulation of innate fear. Based on the result that the optogenetic activation of the PVT-CeL pathway increased fear expression, we hypothesized that optogenetic LTD induction in the CeL may produce the opposite effect. We then found that optogenetic induction of LTD in the CeL of ChR2 mice decreased fear memory expression in tone-cued fear expression 1 day after the fear training, which lasted even at post-LTD stimulus day 7.

The CeA is the primary output region of the amygdala, which mediates autonomic and behavioral responses associated with fear and anxiety [26–28]. Previous studies showed that two populations of neurons may exist in the CeL, one having inhibitory (CeL-Off) and the other excitatory (CeL-On) responses to the conditioned stimulus after fear conditioning [10, 29]. CeL-On and CeL-Off neurons both project to CeM and reciprocally inhibit each other [29, 30]. However, according to these studies it is not clear how one population could become dominant as a result of fear conditioning. We then found two populations of neurons in the CeL which were activated by the PVT projections: RLE neurons and RHE neurons. The increased learned fear was accompanied by the increased excitability of RHE neurons and RLE neurons. The RLE neurons only had neuronal spike responses during the photo-stimulation of PVT afferents. In contrast, the RHE neurons had neuronal spike responses even after the photo-stimulation of PVT afferents. These findings led to the hypothesis that the HLE neurons may become dominant as a result of fear conditioning. We also found that the percentage of RHE neurons was decreased by the LTD induction, and the percentage of HLE neurons was increased by the LTD induction (Fig. 4i). These results support the hypothesis that the CeL neurons with relative high responses to the high frequency photo-stimulation of PVT afferents in the CeL may be the key neurons that regulate the output of fear expression. Our results could provide new neuronal mechanism for the PVT-CeL pathway involved in learned fear.

In contrast to the RLE neurons, the RHE neurons had neuronal spike responses even after the photo-stimulation of PVT afferents. Given that stimulation of PVT afferents in CeL evoked fast synaptic responses on both RHE and RLE neurons during the light-on phase and a slow recovery of inward current exclusively on RHE neurons during the light-off phase, it is likely that the PVT-CeL transmission is mediated by both neurotransmitter and neuromodulator. The fast excitatory synaptic responses were responsible for the increased firing frequencies of both RHE neurons and RLE neurons during the light-on phase. The slow recovery of inward current led to the spike responses of RHE neurons during the light-off phase. Previous studies showed that the brain-derived neurotrophic factor (BDNF) might be one modulator of slow inward synaptic currents [8]. They found that BDNF mainly regulated the slow inward currents of SOM+ neurons in the CeL. They also showed stimulation of PVT afferents in CeL only evoked slow inward currents, rather than canonical fast synaptic responses in SOM+ neurons, so the RHE neurons and RLE neurons which had fast synaptic responses to photo-stimulation of PVT terminals in the CeL could not be the SOM+ neurons. Further investigations should be done to verify whether BDNF regulates the neurotransmission of RHE neurons, or to find out the new candidate for the synaptic modulator of RHE neurons in the CeL. So far, we firstly find that there were some CeL neurons with both fast input EPSCs from PVT projecting neurons and slow recovery of synaptic responses involving in the function of PVT-CeL pathway. Our studies might trigger new explorations of neuromodulator of PVT-CeL synaptic function and provide evidences for such kind of studies.

It is traditionally thought that the BLA is the main input nuclei of the amygdala, and the CeA is the main output nuclei of the amygdala. The information could be passed from the BLA to the CeA [31, 32]. One previous study found that optogenetic stimulation of the pathway between the BLA and CeA can decrease unconditioned fear-related behavior and that the projection-specific excitation of BLA terminals in the CeA activates CeL neurons and elicits feed-forward inhibition of CeM neurons [16]. However, direct evidence for such serial information processing in fear conditioning has been lacking. On the other hand, some other studies have found functions of the CeA—including its involvement in attention or alerting processes—that are independent of the BLA [33, 34], suggesting that the two nuclei are not simply organized in series. Here, we show that the PVT-CeL pathway is a critical neural circuitry for modulation of the conditioned fear expression, which is also independent of the BLA. It could be the case that different inputs to the CeL target different cell types in the CeL and that, depending on stimulation parameters, different behavioral responses could be induced.

Although the cellular and molecular mechanisms leading to anxiety disorders are just beginning to be explored, anxiety disorders are thought to be due to a dysfunction in the suppression of learned fear [12, 35, 36]. We demonstrated that optogenetic LTD induction in the CeL-projecting PVT neurons effectively decreased fear memory. This leads us to suggest that a maladaptation of GABAergic signaling and the resultant LTD impairment at the CeL-projecting PVT neurons may contribute, at least in part, to endophenotypes of anxiety disorders, such as heightened fear responses. Our optogenetic LTD protocol may inspire the development of novel treatments for anxiety disorders involving deep brain stimulation to induce plasticity at relevant brain areas.

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