Parvalbumin (PV) interneurons of Central Amygdala Regulates the Negative Affective States and the Expression of Corticotrophin-Releasing Hormone during Morphine Withdrawal

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Significant Statement

The central nucleus of amygdala (CeA) mediates aversive emotions, including the negative emotion during withdrawal from the addictive drugs. However, the regulation of the neurobiological interconnectivity in the CeA, and its potential functions in the negative reinforcement of drug dependents are poorly understood. Here, we found chronic morphine withdrawal upregulated the activity of CeA PV⁺ interneurons, and optogenetic inhibition of the activity of CeA PV⁺ interneurons attenuated morphine withdrawal-induced negative affective states, while optogenetic activation of CeA PV⁺ interneurons was sufficient to trigger those withdrawal-like affective behaviors. Optogenetic inhibition of the CeA PV⁺ interneurons during the morphine withdrawal also significantly attenuated the elevated level of corticotropin-releasing hormone (CRH) mRNA of CeA. These results indicate that the activation of PV⁺ interneurons of CeA during morphine withdrawal is crucial for the induction of the negative emotion and the upregulated expression of CRH, a hormone critically involved in the regulation of emotion.
Abstract

Background: The central nucleus of amygdala (CeA) is a crucial component of the neuronal circuitry mediating aversively emotion. Its role in the negative affective states during drug withdrawal includes changes in opioidergic, GABAergic, and CRF neurotransmission, etc. However, the modulation of the neurobiological interconnectivity in the CeA, and the effects in the negative reinforcement of drug dependents are poorly understood.

Method: We performed electrophysiological recordings to assess the membrane excitability of parvalbumin (PV)⁺ interneurons in the CeA during chronic morphine withdrawal, and tested the morphine withdrawal-induced negative affective states, such as the aversive (assessed by CPA), anxiety (assessed by EPM), and anhedonic-like (assessed by SPT) behaviors, as well as the mRNA level of corticotropin-releasing hormone (CRH) when optogenetic inhibiting or activating PV⁺ interneurons in the CeA.

Result: Chronic morphine withdrawal increased the firing rate of CeA PV⁺ interneurons. Optogenetic inhibition of the activity of CeA PV⁺ interneurons attenuated the morphine withdrawal-induced negative affective states, such as the aversive, anxiety, and anhedonic-like behaviors, while direct activation of CeA PV⁺ interneurons could trigger those negative affective-like behaviors. Optogenetic inhibiting the CeA PV⁺ interneurons during the morphine withdrawal significantly attenuated the elevated CRH mRNA level in the CeA.

Conclusion: The activity of PV⁺ interneurons in the CeA was upregulated during the chronic morphine withdrawal. The activation of PV⁺ interneurons during morphine withdrawal was crucial for the induction of the negative emotion and the upregulation of CRH mRNA level in
the CeA.

**Key words:** morphine withdrawal; negative affective states; PV; the central amygdala; CRH
**Introduction**

Drug addiction is defined as compulsive use of drugs (Hyman et al., 2006), and relapse is the primary problem in treating drug abuse (O'Brien, 1997). Addiction to drugs such as opiates depends not only on their positive reinforcing and hedonic effects, but also on avoidance of the negative, aversive consequences of withdrawal (Solomon and Corbit, 1974; Koob et al., 1989). A severe opiate withdrawal syndrome in opiate addicts, is composed of influenza-like somatic signs and negative affective symptoms, such as anxiety, dysphoria and anhedonia (American Psychiatric Association., 2000). The negative affective consequences of opiate withdrawal can enhance the incentive value of the drug and contribute to the maintenance of drug seeking behavior (Hutcheson et al., 2001; Koob and Le Moal, 2005; Kenny et al., 2006). Thus relief of opiate withdrawal-induced negative affective states might play an important role in alleviating the relapse of opioid addiction, and elucidating the neuronal mechanism modulating the negative affective states during drug withdrawal should be an important obstacle in solving the relapse of drug addiction.

The central nucleus of amygdala (CeA) is a functionally interconnected region of the extended amygdala that integrates emotional, learning, motivational, nociceptive, and decision-making information (Sirohi et al., 2012), and may represent a common anatomical substrate to produce the negative emotional states that promote negative reinforcement mechanisms associated with the development of addiction (Weiskrantz, 1956; LeDoux, 2000; Phelps and LeDoux, 2005). CeA consists of a dense of interneurons as well as mostly GABAergic projection neurons (McDonald, 1982). The complex interconnectivity of CeA which plays prominent roles in fear and anxiety (Rodrigues et al., 2004; Ciocchi et al., 2010;
Davis et al., 2010; Haubensak et al., 2010; Tye et al., 2011) have been discovered recently by the emerging optogenetic technique. CeA has been indicated to play a critical role in opioid withdrawal-induced negative affective states, as well as the stress-induced relapse. A role for the CeA in the aversive effects of drug withdrawal includes changes of opioidergic, GABAergic, and CRH neurotransmission in the CeA. The CRH system in the CeA is activated during acute cocaine, alcohol, opioid, and nicotine withdrawal as measured by in vivo microdialysis and neuropharmacological probes (Contarino and Papaleo, 2005; Papaleo et al., 2007).

As one of the biggest interneuron population in the BLA (McDonald and Mascagni, 2001; Bocchio et al., 2015), the PV⁺ interneurons play an important role in control of the emotional behavior by regulating the activity of principle neurons through providing the inhibitory postsynaptic potential (Wilson et al., 1994; Povysheva et al., 2006), and is thought to be involved in the control of fear by targeting the perisomatic region of principle neurons (Bienvenu et al., 2012), inhibiting and synchronizing their firing (Popescu and Pare, 2011). Although PV⁺ interneurons are not the biggest interneuron population in the CeA, its function in the CeA has been indicated to be significantly related with the anxiety-like behavior (Ravenelle et al., 2014). However, whether the plasticity of PV⁺ interneurons of CeA can be modulated during drug withdrawal, and its function and neurophysiological mechanism to regulate negative affective symptoms during withdrawal are largely unknown.

In the present study, we investigated the influence of chronic morphine exposure on the activity of PV⁺ interneurons in the CeA, and then optogenetically manipulated the activity of PV⁺ interneurons in the CeA to explore its role in withdrawal-induced negative affective
behaviors, such as dysphoria, depressed mood and anhedonia, etc. When the activity of PV$^+$ interneurons in the CeA was inhibited by the optogenetic approach during the morphine-withdrawal stage, we also explored the changes of corticotropin-releasing hormone (CRH) in the CeA, which is not only a key mediator of the behavior responses to stressors, but also the mostly-related emotional hormone (Koob, 2010).
Materials and Methods

Animals and Housing

Parvalbumin (PV)-Cre (#008069), CRH<sup>tm1(cre)Zjh</sup> (#012704), Gt(ROSA)26Sor<sup>tm27.1(CAG-COP4</sup><sup>*H134R/hdTomato)Hez</sup> (#012567) and Gt(ROSA)26Sor<sup>tm35.1(CAG-aop3/GFP)</sup> (#012735) mice were purchased from the Jackson Laboratory. All mice were bred onto C57BL/6J genetic background. We generated the conditional expression mice: 1. To label and excite the PV<sup>+</sup> interneurons by the 473nm laser stimulation, we generated the PV-Cre; Gt(ROSA)26Sor<sup>tm27.1(CAG-COP4</sup><sup>*H134R/hdTomato)Hez</sup> (simplified as PV; ChR2-tdTomato<sup>(+/-)</sup>) mice by crossing of PV-Cre with Gt(ROSA)26Sor<sup>tm27.1(CAG-COP4</sup><sup>*H134R/hdTomato)Hez</sup> mice; 2. To label and inhibit the PV<sup>+</sup> interneurons by the 594nm laser stimulation, we generated PV-Cre; Gt(ROSA)26Sor<sup>tm35.1(CAG-aop3/GFP)</sup> Hez (simplified as PV; Arch-GFP<sup>(+/-)</sup>) mice by crossing of PV-Cre with Gt(ROSA)26Sor<sup>tm35.1(CAG-aop3/GFP)Hez</sup> mice; 3. To label the CRH<sup>+</sup> neurons, we generated CRH<sup>tm1(cre)Zjh</sup>; Gt(ROSA)26Sor<sup>tm35.1(CAG-aop3/GFP)Hez</sup> (simplified as CRH; Arch-GFP<sup>(+/-)</sup>) mice by crossing of CRH<sup>tm1(cre)Hez</sup> with Gt(ROSA)26Sor<sup>tm35.1(CAG-aop3/GFP)Hez</sup> mice. The male offspring were used in experiments, and the PV; ChR2-tdTomato<sup>(+/-)</sup>, PV; Arch-GFP<sup>(+/-)</sup> littermates were used as control mice, respectively (see Fig. S1a-c for breeding scheme). Genotypes were determined by PCR of mouse tail DNA samples (Zhao et al., 2011). Mice were housed in groups on a 12h light/dark cycle with food and water available ad libitum. All animal treatments were strictly in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and Use of Laboratory Animals and were approved by Animal Care and Use
Committee of School of Basic Medical Sciences of Fudan University. The male mice with 8–10 week age were used for all behavioral tests.

**Stereotaxic Surgery and Laser Stimulation**

Male mice of 8-12 week age anesthetized with choral hydrate (40 mg/kg, i.p.) were placed in a stereotactic instrument, and followed by a craniotomy, cannula guides (Plastics one, Roanoke, VA, USA) were implanted to the brain dorsal to bilateral CeA and secured with dental cement. The intended stereotaxic coordinates were: AP -1.1 mm; ML ± 2.4 mm; DV – 4.1 mm (Tye et al., 2011; Li et al., 2013). All mice were given at least 14 days to recover before behavioral experiments. To manipulate neuronal activity during behavioral experiments, two 200 μm diameter optical fibers were inserted through the cannula to deliver laser to the CeA (DV -4.4 mm). Optical fibers were attached through an FC/PC adaptor to a 473 nm blue laser or 594nm yellow laser diode (Brain-King, Beijing, China), light pulses were generated through a Master-8 pulse stimulator (AMPI, Jerusalem, Israel) to deliver light trains at 20 Hz, 5 ms pulse-width (Zhao et al., 2011) for 473 nm light, and constant light for 594 nm light (Huff et al., 2013) in the experiments. The light intensity of 473 nm and 594nm laser at the fiber tip was 8~10 mW, measured using a light sensor (Thorlabs, Newton, NJ, USA) before implantation. After the behavior tests, the mice were sacrificed, and the optical tracts and the cannula placements were confirmed by histology (Fig. S2a). The behavior data from the mice in which the tracts were in the accurate position were used.

**Morphine Withdrawal-induced Conditioned Place Aversion**
Conditioned place Aversion (CPA) is a recognized paradigm of negative affective learning. The CPA procedure induces place aversion when the mouse pairs the equipment environment with the negative effects of morphine withdrawal syndrome. The conditioned place aversion (CPA) apparatus consists of a two-chamber apparatus (Med-Associates, St. Albans, VT, USA) with distinct tactile environments to maximize contextual differences. One chamber of the box has a wire mesh floor while the other chamber has a grid rod floor. During the test sessions, the opened door (7 × 5 cm) in the central partition allows the mice to enter both sides of the apparatus, whereas during the conditioning trials the individual compartments are closed off from each other. The mice were put on food and water restriction for 1 hour before each session. On day 1, each mouse is allowed to explore freely in the entire CPA apparatus for 20 min and time spent in each of the two compartments are measured (pre-test). Within each genotype, mice are divided into two groups. One group is assigned to receive saline and the other receive increasing morphine doses progressively (20–80mg/kg). Every 12 h (10:00 am; 22:00 pm) the mice are treated with saline or morphine according to the following protocol: day 2: 10mg/kg, day 3: 20mg/kg, day 4: 40mg/kg, day 5: 60mg/kg, day 6: 80mg/kg, and day 7: 80mg/kg (only one injection in the morning). CPA conditioning trials take place on days 4–7 when morphine-treated mice are in an opiate withdrawal state. For this purpose, 10 h after the evening injection while the drug is almost cleared in the body and induce the maximal expression of somatic opiate withdrawal signs, mice are confined for 30 min into their preferred compartment of the CPA apparatus, determined during the preconditioning test. Post-conditioning tests for 20 min with 8–10 mW of constant 594 nm light take place 6 days after the last conditioning trial (day 13), when somatic opiate withdrawal signs have largely dissipated in both genotypes (Papaleo et al., 2008; Ingallinesi et al., 2012).

Optogenetic activation-induced Conditioned Place Aversion
Conditioned place aversion-induced by laser stimulating CeA PV+ interneurons was also assessed using the two-chamber apparatus (Med-Associates, St. Albans, VT, USA). A manual guillotine door separates the two chambers. On day 1, mice were placed in one of the chambers and allowed to freely explore the entire apparatus for 20 min (pre-test). On days 2-4, mice were given a 473 nm laser stimulation (20Hz frequency, 5 ms duration delivered at 5 min duration with 5 min interval) through the embedded cannula in the CeA in the morning when confined to one chambers for 20 min, and in the afternoon they were inserted an equivalent false optical fiber without laser then confined to the other chamber for 20 min. On day 5, mice were allowed to freely explore the entire apparatus for 20 min (test). The time spent in each chamber was recorded during the pre-test and test sections. The CPA score was defined as the time (in seconds) spent in the 473nm laser-paired chamber minus the time spent in the no laser-paired chamber.

Elevated Plus Maze

6 days after the last morphine injection (day 13) when somatic opiate withdrawal signs have largely dissipated in both genotypes, we first use the elevated plus maze (EPM) to test the morphine withdrawal-induced anxiety-like behavior. The mice were put on food and water restriction for 1 hour before the test. The elevated plus maze consisted of four arms (34.5 cm length × 6.3 cm width × 19.5 cm height) elevated 75 cm above the floor. Two of the arms had 20 cm high dark walls (closed arms), and two had 0.8 cm high ledges (open arms). The arms were angled at 90° to each other. The apparatus was placed in a quiet and dimmed room. The mice were placed in the center of the maze facing the open arm and were allowed to explore the equipment freely for 5 min. The apparatus was wiped with water and dried between tests to ensure the absence of olfactory cues. EthoVision XT 8.5 video tracking program (Leesburg, VA, USA) was used to track mouse location, velocity and movement of head, body and tail.
Laser stimulation protocols are specified by groups. Bilateral illumination of PV<sup>+</sup> interneurons in the CeA delivered at 5 min duration with constant 594 nm light of 8-10 mW at the tip of the fiber.

Another group of mice without morphine injection and withdrawal were used to test the function of CeA PV<sup>+</sup> interneurons in the anxiety when directly giving a bilateral 473 nm laser with 20Hz frequency, 5 ms duration delivered at 3 min duration after 3 min laser off.

**Saccharin Preference Test**

Morphine withdrawal-induced decrease in the preference for saccharin is thought to reflect anhedonia-like states. This experiment had training and testing sessions. During training, mice were restricted from food and water for 12 h and trained to consume 1% (w/v) saccharin solution before the first morphine injection. Saccharin preference was tested 1 day after the EPM or CPA test (7 days after the last morphine injection, day 14). Mice were again deprived of food and water for 12 h before the 30 min testing session, mice could select between two pre-weighed bottles, one with 1% (w/v) saccharin solution and the other with tap water. The saccharin preference was calculated as the following formula: saccharin preference = (saccharin solution intake (g) × 100) / (saccharin solution intake(g) + water intake (g)).

Bilateral constant light for the 594 nm laser was delivered during the 30 min testing session.

Another group mice without morphine injection and withdrawal were used to test the function of CeA PV<sup>+</sup> interneurons in the anhedonia when directly given bilateral 473 nm laser (with 20Hz frequency, 5 ms duration delivered at 5 min duration with 5 min interval) during the 30 minutes saccharin preference test.

**Immunohistochemistry**
Mice were anesthetized with choral hydrate and perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The brains were removed, fixed in 4% paraformaldehyde overnight and subjected to dehydration in increasing saccharin solutions (20%–30%) at 4°C. The frozen coronal slices of 20 μm thicknesses were prepared and stored at -20°C in 20% ethanediol PBS solution containing 20% saccharin. Brain sections were incubated in 3% normal goat serum and 0.2% Triton-X for 1 h. Then they were incubated in Rabbit anti-Parvalbumin (Swant, Bellinzona, Ticino, Switzerland) or mouse anti-CRF (Abcam, Cambridge, England) antibody overnight at 4°C. Slices were rinsed in PBS then incubated in donkey anti-rabbit Cy3, or Alex488 or DyLight 647, or donkey anti-mouse Cy3 (Jackson Immunoresearch, West Grove, PA, USA) for 1 h and DAPI for 10 min, then mounted after rinsing. Images were acquired on a microscope using a 20× air objective, a 40× objective (IX-83; Olympus, Japan) or a 63× oil immersion objective (Zeiss 510; Carl Zeiss Jena, Germany).

**Electrophysiology**

6 days after the last morphine injection (Day 13), coronal sections (300 μm) containing CeA were cut from PV;ChR2 -tdTomato (+/-) mice and slices were prepared as previously described (Zhao et al., 2011). Briefly, the mice were deeply anesthetized by intra-peritoneal injection of chloral hydrate (400 mg/kg, i.p.) and then transcardially perfused with cold protective ACSF (92 mM N-methyl-D-glucamine (NMDG), 2.5 mM KCl, 1.25 mM NaH2PO4, 30 mM NaHCO3, 20 mM HEPES, 25 mM D-glucose, 2 mM thiourea, 5 mM Na-ascorbate, 3 mM Na-pyruvate, 0.5 mM CaCl2, and 10 mM MgCl2) and then initially recovered at 32-34 °C for 10-15 min. The slices were transferred into a holding chamber containing room temperature carbogenated ACSF (119 mM NaCl, 2.5 mM KCl, 1.25 mM NaH2PO4, 26 mM NaHCO3, 12.5 mM glucose, 2 mM CaCl2, 2 mM MgCl2, 2 mM thiourea, 5 mM Na-ascorbate, and 3
mM Na-pyruvate), and slices were stored for 0.5-3 hours prior to transferring to the recording chamber for use. All solutions were saturated with 95% O₂, 5% CO₂, and the slices were used within 6 hours after preparation.

Whole-cell current-clamp recordings were performed in ACSF at room temperature from PV-positive neurons in CeA with an EPC-10 amplifier and Pulse v8.78 software (HEKA Elektronik, Lambrecht/Pfalz, Germany). Intracellular solution composition was: 126 mM K-gluconate, 4 mM KCl, 10 mM HEPES, 4 mM ATP-Mg, 0.3 mM GTP-Na₂, 10 mM creatine phosphate (pH 7.2, 290-300 mOsm). At least 5 min after achieving whole cell configuration, a current-step protocol (from -200 to +200 pA, with a 10 pA increment) was run and repeated. The after-hyperpolarization potential (AHP) was sampled following the first single action potential spike. Data were filtered at 300 Hz and were analyzed by Mini Analysis Program (Synaptosoft Inc., Fort Lee, NJ, USA). Spontaneous miniature excitatory postsynaptic current (mEPSC) events were recorded in presence of ACSF containing 2 μM TTX, 50 μM bicuculline (Tocris Bioscience, Bristol, UK) at a holding potential of -70 mV. Data were filtered at 300 Hz and were analyzed by Mini Analysis Program (Synaptosoft Inc., Fort Lee, NJ, USA). All electrophysiological recordings were performed and analyzed blind to genotype.

**RNA extraction and real-time PCR analysis**

Mice which were given 30 min 594 nm laser stimulation on day 14 after the EPM or CPA test (7 days after the last morphine injection) were sacrificed and the brains were removed immediately. The central amygdala (CeA) was dissected within 5 min at 0 °C by using Microm HM 650V (Thermo Fisher Scientific Inc, Waltham, MA, USA) according to stereotaxis coordinates from Bregma −0.9 mm to −1.7 mm, rinsed in PBS, frozen in TRIzol® Reagent (Thermo Fisher Scientific Inc, Waltham, MA, USA) and stored at −70 °C until
extraction. Total RNA was extracted from tissues using the TRIzol® Reagent according to the manufacturer’s instructions with the inclusion of a DNase digestion step. The Superscript First-Strand Synthesis System for RT-PCR (TAKARA, Shiga, Japan) was used with random primers for reverse transcription. Quantitative RT-PCR amplification of the cDNA was performed on samples in triplicate with Power SYBR Green PCR Master Mix (TAKARA, Shiga, Japan) using the Eppendorf Mastercycler ep gradient S PCR System (Eppendorf, Germany). CRH mRNA expression was normalized to the internal control GAPDH.

Statistical analysis

Data are expressed as means ± SEM, and analyzed by GraphPad Prism (GraphPad Software, Inc, USA). A Mann-Whitney U Test was used for the AP initiation, the frequency and amplitude of mEPSCs, the travel distance, velocity, saccharin preference and EPM tests of the different genotype or treatment. Two-way RM ANOVA was used for assess the spike number within each genotype, the effects of laser stimulation or genotype on CPA scores within each genotype and treatment (saline or morphine). Two-way ANOVA is used to examine the CRH mRNA level within each genotype. Bonferroni’s post-hoc test will used. The accepted value for significance is *P< 0.05, **P< 0.01, ***P< 0.001.
Results

CeA PV⁺ interneurons are activated during the withdrawal of chronic morphine exposure.

The membrane excitability during chronic morphine withdrawal was examined first. We recorded evoked action potential (AP) of the PV⁺ interneurons of CeA (Fig. 1a) in PV;ChR2-tdTomato (+/-) transgenic mice which had been consecutively withdrew from the chronic morphine injection for 6 days (Ingallinesi et al., 2012). Compared with the saline controls, the membrane excitability was increased in PV⁺, but not PV⁻ interneurons (Fig. 1b). The number of spikes recorded from PV⁺ interneurons in the CeA was increased relative to that from the saline control (Fig. 1c, treatment x current: F (1, 56) = 17.742, P< 0.001). PV⁺ interneurons exhibited lower threshold for AP initiation (Fig. 1d, p=0.025), suggesting that the membrane excitability of PV⁺ interneurons was increased during chronic morphine withdrawal. No significant changes were detected in the after-hyperpolarization (AHP), spike amplitude, and half-width of PV⁺ interneurons (Fig. S1d-f, d: p=0.360; e: P= 0.289; f: P= 0.429), suggesting that morphine-withdrawal enhanced membrane excitability without changing membrane properties of fast-spiking PV⁺ GABAergic interneurons (Hu et al., 2014).

To assess the effect of chronic morphine withdrawal on the synaptic transmission of PV⁺ interneurons of CeA, pharmacologically isolated spontaneous miniature excitatory postsynaptic currents (mEPSCs) of PV⁺ interneurons were recorded and analyzed (Fig. 1e-g). We found that the frequency (Fig. 1f) and amplitude (Fig. 1g) of mEPSCs recorded from PV⁺ interneurons of the morphine withdrawal mice significantly increased relative to that from the saline control, indicating an up-regulation of excitatory synaptic transmission of PV⁺ interneurons of CeA during the chronic morphine withdrawal phase. These results suggested the PV⁺ interneurons in the CeA were activated during the chronic morphine withdrawal.
phase, accompanied with the negative affective states.

**Optogenetic inhibition of PV⁺ interneurons activity in the CeA during the morphine withdrawal attenuates negative affective states.**

We inhibited the activity of CeA PV⁺ interneurons by the 594 nm laser stimulation in the *PV:Arch-GFP(+/−)* mice, of which Arch-GFP fusion protein is selectively expressed in PV⁺ interneurons. The expression of Arch-GFP in the CeA was shown by immunofluorescence images in Fig. 2a. As compared with LA/BLA, the CeA does not have as much PV⁺ soma indicated by the PV antibody staining, but has a large amount of dendritic protrusions of PV⁺ interneurons (Fig. 2a) indicated by the Arch-eGFP expression. The localization of Arch-eGFP in PV⁺ cells in the CeA confirmed that the CeA has moderate level of PV⁺ interneurons.

Opiate withdrawal-induced affective-like states are composed of dysphoria, anxiety, and anhedonia. An intermittent and progressively-increasing-dose morphine injection procedure was given to the mice to mimic the drug intake patterns of opiate addicts. Previous studies showed that 6 days after the last morphine injection, mice suffered from the morphine withdrawal-induced negative affective states such as aversive, increased anxiety, and anhedonia (Papaleo et al., 2007; Ingallinesi et al., 2012). To examine the possible role of the CeA PV⁺ interneurons in the morphine withdrawal-induced negative affective states, conditioned place aversion (CPA), elevated plus maze (EPM) and saccharin preference test (SPT) experiments were carried out, and the activity of PV⁺ interneurons in the CeA of *PV:Arch-GFP(+/−)* mice was inhibited by the bilateral 594 nm laser stimulation during these tests.
We used the elevated plus-maze (EPM) to assess the anxiety-like behavior after 6-days withdrawal from morphine (Zanos et al., 2014). We found that the $PV;Arch-GFP^{+/+}$ mice with 594 nm laser stimulation (laser on) spent increased time in the open-arm of the EPM, when compared with the $PV;Arch-GFP^{+/+}$ mice without laser stimulation (laser-off) or the laser-stimulated $PV;Arch-GFP^{+/−}$ group (Fig. 2c), indicating that inhibiting the activity of CeA PV$^+$ interneurons could attenuate the morphine withdrawal-increased anxiety level.

The saccharin preference test (SPT) was used as a depressive-like behavior to investigate the anhedonic state of morphine withdrawal (Casarotto and Andreatini, 2007; Pisu et al., 2011; Fadaei et al., 2015). After the 7-days morphine withdrawal, the $PV;Arch-GFP^{+/−}$ mice showed the increased saccharin preference during the 594 nm laser stimulation, as compared with the $PV;Arch-GFP^{+/+}$ mice without laser stimulation and the laser-stimulated $PV;Arch-GFP^{+/−}$ mice (Fig. 2d). These results suggested that inhibiting the activity of CeA PV$^+$ interneurons could attenuate the morphine withdrawal-induced anhedonic-like behavior.

The conditioned place aversion (CPA) is a typical paradigm of negative affective states associated with opiate withdrawal (Contarino and Papaleo, 2005). In the pretest session of CPA experiment, both of the $PV;Arch-GFP^{+/−}$ mice and the control littermates spent similar time in the two compartments of CPA apparatus. After 4 sessions of morphine withdrawal conditioning, mice were given the 594 nm laser stimulation in the CeA during the test session (Fig. 2e). We found that laser-stimulated $PV;Arch-GFP^{+/−}$ mice and the $PV;Arch-GFP^{+/+}$ mice without laser stimulation avoided and spent less time in the apparatus previously paired with the morphine withdrawal, however, the laser-stimulated $PV;Arch-GFP^{+/−}$ mice did not show any aversion to the environmental cues paired with morphine withdrawal (Fig 2f. laser
×treatment interaction effect: $F_{1,12}= 4.60$, $P<0.05$, and genotype ×treatment interaction effect: $F_{1,10}= 11.37$, $P<0.05$). Optogenetic inhibiting the activity of PV$^+$ interneurons in CeA alone did not have any effect on travelling velocity (Fig. S2b) and distance travelled (Fig. S2c). These results suggested that inhibiting the activity of PV$^+$ interneurons in CeA abolished the expression of aversion when exposure in the environmental cues paired with the dysphoria state induced by morphine withdrawal, as well as alleviated the morphine withdrawal-induced anxiety, and anhedonic-like behaviors.

**Optogenetic activation of CeA PV$^+$ interneurons can induce similar negative affective states as morphine withdrawal.**

Since inhibiting the activity of CeA PV$^+$ interneurons during morphine withdrawal could alleviate the morphine withdrawal-induced affective states, we speculated that direct activation of the CeA PV$^+$ interneurons might trigger the similar negative emotions as the morphine withdrawal. Therefore we activated the CeA PV$^+$ interneurons by the 473 nm laser stimulation in the $PV; ChR2$-$tdTomato^{+/+}$ mice, of which the ChR2(H134R)-tdTomato fusion protein was selectively expressed in the PV$^+$ interneurons (Fig. 3a), and found the 473 nm laser stimulation in the CeA of $PV; ChR2$-$tdTomato^{+/+}$ mice triggered dramatically increased c-fos$^+$ cells in the CeA, but not the LA/BLA (Fig. S3a-b).

Our results (Fig. 3c) showed that in $PV; ChR2$-$tdTomato^{+/+}$ mice, morphine withdrawal could reduce the time spent on the open-arm in the EPM (Fig. 3c, left panel), and reduce the saccharin preference (Fig. 3c, right panel) as compared with the saline group, indicating morphine withdrawal could increase the anxiety level and anhedonia-like behavior. To assess
to which extent artificial optogenetic activation of PV⁺ interneurons in CeA can mimic the
effect of morphine withdrawal, we directly activated the CeA PV⁺ interneurons during the
EPM and SPT tests. The results showed that 473 nm laser stimulation of CeA in
PV;ChR2-tdTomato(+/−) mice decreased their time spent on the open-arm and their saccharin
preference, as compared with the PV;ChR2-tdTomato(+/−) mice without laser stimulation, or
with the laser-stimulated PV;ChR2-tdTomato(+/−) mice (Fig. 3d). Activation of PV⁺ interneurons
in CeA did not have any effect on the velocity (Fig. S2d, P= 0.31) and distance travelled (Fig.
S2e, P= 0.13) of the mice._

The morphine withdrawal induced CPA test showed that the PV;ChR2-tdTomato(+/−) mice
avoided and spent less time in the apparatus previously paired with the morphine withdrawal
in the test session (Fig.3e, genotype × treatment interaction effect: F1, 14= 6.96, P< 0.05) after
4 sessions of morphine withdrawal conditioning. To assess to whether optogenetic activation
of PV⁺ interneurons in CeA can induced CPA as morphine withdrawal, we directly activated
PV⁺ interneurons in CeA during the conditioning. During the 20-min pretest session (day 1),
time spent in the two compartments of the CPA apparatus was similar between
PV;ChR2-tdTomato(+/−) or PV;ChR2-tdTomato(+/−) mice. During the conditioning sessions
(day2-4), one chamber of the apparatus was paired with or without the 473 nm laser
stimulation for 20 min. On the test day (day 5), the time spent on exploring the laser-paired
chamber of the PV;ChR2-tdTomato(+/−) mice was significantly decreased, however, the
PV;ChR2-tdTomato(+/−) mice without laser stimulation during the conditioning sessions
(Fig.3f , Laser × treatment interaction effect: F1, 16= 6.06, P< 0.05), and the laser-stimulated
PV;ChR2-tdTomato(+/−) mice (Fig. 3f, genotype × treatment interaction effect: F1, 12= 13.12, P<
0.05) did not develop the CPA, indicating the $PV; ChR2$-tdTomato$^{+/−}$ mice developed an aversion for the laser-paired chamber. These results indicated the optogenetic-activating PV$^+$ interneurons in CeA could trigger the similar extend of negative affective states as morphine withdrawal, such as increased anxiety level, anhedonic-like, and the aversive behaviors.

**Optogenetic inhibition of CeA PV$^+$ interneurons during morphine withdrawal down-regulates the CRH mRNA level in the CeA.**

The corticotropin-releasing factor (CRF) (also known as corticotropin-releasing hormone, CRH) system coordinates not only neuroendocrine and autonomic responses to stressors (Rivier et al., 1982; Koob, 1999), but also to substance dependence (Rouibi and Contarino, 2013). The CRH receptor antagonists can ameliorate the negative affective-like states associated with alcohol, cocaine, or opiate withdrawal (Sarnyai et al., 1995; Valdez et al., 2002; Contarino and Papaleo, 2005; Stinus et al., 2005; Papaleo et al., 2008). Upregulation of the CRH brain stress system in the extended amygdala have been observed in rodents, nonhuman primates and humans during abstinence from drugs of abuse, including tobacco (Sarnyai et al., 2001; Koob and Volkow, 2010). The nicotine withdrawal-induced increase of CRH release in the CeA leads to negative affective states which contributes to increased nicotine intake (George et al., 2007; Bruijnzeel et al., 2009; Bruijnzeel et al., 2012; Qi et al., 2014; Cohen et al., 2015).

Here, we generated $CRH; Arch-GFP^{+/−}$ mice which expressed the Arch-GFP fusion protein specifically directed by the mouse CRH promoter/enhancer (Fig. 4a). We found that the CeA had a dense of Arch-GFP fluorescence signal (Fig. 4a, left), and this Arch-GFP fusion protein
localized with CRH$^+$ cells as indicated by CRH antibody staining (Fig. 4a, middle and right), indicating there is a large amount of CRH$^+$ neurons in the CeA. Furthermore, we found that the PV$^+$ and CRH$^+$ neurons had the close connection in the CeA (Fig. 4b), and the cell body of PV$^+$ interneurons were circled or crossed by the dendrites of CRH$^+$ neurons (Fig. 4b, middle and right).

To assess whether the modulation of morphine withdrawal-induced negative affective states by the CeA PV$^+$ interneurons might involve the CRH system of CeA, we carried out the real-time PCR assay to measure the effect of optogenetic inhibiting the activity of CeA PV$^+$ interneurons on the CRH mRNA level of the CeA during the morphine withdrawal (Fig. 4c). We found in the saline treatment groups, there was no significant difference of the CRH mRNA level between the laser stimulated $PV;Arch-GFP^{+/+}$ and the $PV;Arch-GFP^{+/−}$ control mice. However, in the chronic morphine treated groups, the CRH mRNA of the control mice was markedly increased as compared with the saline group, while optogenetic inhibition of the activity of CeA PV$^+$ interneurons in the $PV;Arch-GFP^{+/+}$ mice significantly decreased the CRH mRNA level in CeA (Fig. 4d, genotype × treatment interaction effect: $F(1, 20)= 44.95, P< 0.001$) as compare with the control mice, suggesting that the activation of CeA PV$^+$ interneurons may be involved in regulation of the expression level of corticotropin-releasing hormone during morphine withdrawal.

**Discussion**

In this study, we used an optogenetic approach to examine the role of CeA PV$^+$ interneurons in the regulation of the morphine withdrawal-induced negative affective states. We found that optogenetic inhibition of the CeA PV$^+$ interneurons activity during morphine withdrawal was
sufficient to impair the morphine withdrawal-induced aversion, anxiety, and anhedonic-like behavior. Instead, direct optogenetic activation of the CeA PV+ interneurons of PV;ChR2-tdTomato(+/−) mice could mimic several morphine withdrawal like affective states. These results suggest that the CeA PV+ interneurons positively regulate morphine withdrawal-induced negative affective states. Optogenetic inhibition of CeA PV+ interneurons activity within morphine withdrawal could down-regulate the CRH mRNA level, which was upregulated and correlated with the negative affective-like states during opiate withdrawal, in the CeA. Therefore the activation of CeA PV+ interneurons might mediate the morphine withdrawal-induced negative affective states, and related with upregulation of CRH system in the CeA.

Although the CeA has a few soma of PV+ interneurons, PV+ interneurons might extend a large amount of dendrites or/and axons which may form an extensive synaptic connections with other neurons in the CeA. It had been reported few PV+ interneurons were observed in the CeA by immunoreactive method in the rat (Zahm et al., 2003; Ravenelle et al., 2014). From the immunohistochemical images of Zahm et al, there is a small quantity cell body of PV+ interneurons in the CeA, which is consistent with our immunofluorescence results using PV antibody. Fluorescent fusion protein Arch-eYFP and ChR2-tdTomato expressed on the cell membrane and could label the dendritic structure clearly, and we saw large amount of dendrites or/and axons of PV+ interneurons in the CeA of PV;Arch-GFP(+/−) and PV;ChR2-tdTomato(+/−) reporter mice. These data hint the limited PV+ interneurons of CeA might have extensive connections with other neurons in the CeA, and regulate the activity of other neurons through providing the inhibitory postsynaptic potential. It has been reported
that PV⁺ interneurons of striatum (Cowan et al., 1990; Kawaguchi, 1993) could affect overall input–output functions in a manner disproportionate to its relatively small numbers (Zahm et al., 2003). The anatomical and electrophysiological evidence have showed that PV⁺ interneurons are electronically coupled (Kita et al., 1990; Koos and Tepper, 1999) and activation of a single PV⁺ interneuron may exert this robust inhibitory influence on up to 400 projection neurons (Koos and Tepper, 1999). Therefore it is no wonder that the limited PV⁺ interneurons in the CeA might also have strong effect in the morphine withdrawal-induced negative affective-like states.

It has been widely reported that the relationship of CRH and the negative affective-like states associated with alcohol, cocaine, or opiate withdrawal. CRH⁺ neurons in the CeA send dense projections to the locus coeruleus (LC) and stimulation of CRH⁺ terminals in the LC induced aversive and anxiogenic behaviours (McCall et al., 2015). Here we found that a large amount of CRH⁺ interneurons existed in the CeA and had the close connection with the PV⁺ interneurons, thus we explored the change of CRH mRNA level in the CeA when optogenetic inhibiting the CeA PV⁺ interneurons during the morphine withdrawal. Optogenetic inhibition of CeA PV⁺ interneurons down-regulated the CRH mRNA level and morphine withdrawal-induced negative affective states. Inhibition of the activity of PV⁺ interneurons in the CeA has no effect on the CRH mRNA level in the saline group, indicating the activity of PV⁺ interneurons might be low in the physiological states and is dysregulated in the morphine withdrawal state.

Besides the PV⁺ and CRH⁺ neurons in the CeA, there are also a large amount of somatostatin-positive (SOM⁺) interneurons (Li et al., 2013), PKC-δ⁺ neurons (Haubensak et
al., 2010) and GABAergic medium spiny neurons. It is quite possible that PV⁺ interneurons influence the activity of CRH⁺ neurons through the indirect way. The PKC-δ⁺ neurons, which is a subpopulation of GABA-containing neurons and located in the lateral subdivision of the central amygdala (CEl), gates CEm output to control the level of conditioned freezing and anxiety (Haubensak et al., 2010). Whether the PV⁺ interneurons in CeA regulate the morphine withdrawal-induced negative affective states through PKC-δ⁺ neurons will be assessed in future.

Therefore, our study discovered that the CeA PV⁺ interneurons were activated during the withdrawal of chronic morphine exposure, and optogenetic inhibition of the PV⁺ interneurons in the CeA attenuated the morphine withdrawal-induced negative affective states as well as the elevated CRH mRNA level. The precise mechanisms how CeA PV⁺ interneurons modulate the activity of CRH⁺ neurons in the CeA during morphine withdrawal and how they ultimately regulate morphine withdrawal-induced affective-like behaviors need to be further investigated.

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The authors declare no conflict of interest.
Statement of interest
None.

Authors’ contribution
LM and FW were responsible for the conception and design of the study and analysis of the data. LW, MS, and CJ contributed to the acquisition of animal data. MS carried out the electrophysiology experiment. LW and MS assisted with data analysis.
Figure Legends

Figure 1. Morphine withdrawal increases the membrane excitability of PV+ interneurons in CeA.

(a) Left: Representative images of a PV+ neuron which has been patched. Middle: The black zoom in the left was enlarged. Right: the PV+ neuron filled with Alexa Fluor-568 dye through the patch pipette after recording. Scale bar, 100 µm (left) and 20 µm (right). (b) Example of AP trains upon current injections from PV− neurons and PV+ interneurons in the CeA of PV;ChR2-tdTomato(+/−) transgenic mice on the 6th day from the last saline or morphine exposure. (c) Spike number of PV+ interneurons in the CeA after morphine withdrawal. Two-way RM ANOVA, ***P< 0.001. (d) PV+ interneurons in the CeA exhibited significant decrease in AP threshold after withdrawal from morphine exposure. (e) Representative traces of mEPSCs recorded from PV+ interneurons in the CeA 6 days after morphine withdrawal (right) or saline control (left). (f-g) Average frequency and amplitude recorded from PV+ interneurons in the CeA after morphine withdrawal or saline control. Mann-Whitney U Test, *P< 0.05, ***P< 0.001. Data are presented as mean ± s.e.m.

Figure 2. Optogenetic inhibition of the PV+ interneurons in the CeA during the morphine withdrawal attenuates its-induced negative affective states.

(a) Presentative image of the amygdala of mice staining with the PV antibody. White dashes labeled the CeA and LA/BLA structure. White box was enlarged on the right. Arrows indicated the containing of PV antibody with the Arch-GFP+ neurons. Green: GFP; Red: PV; Blue: DAPI. Scale bar, 200 µm (Left), 20 µm (Right). (b-f) PV;Arch-GFP(+/−) and PV;Arch-GFP(+/−) mice were administrated with increasing morphine dose for 7 days and
withdrawal for 6-7 days. (b) The schematic of experimental schedule of the EPM and SPT tests. (c) Time spent in the open arms of the 5-min EPM test in the $PV; Arch-GFP^{+/−}$ mice with or without the 594 nm laser stimulation, and $PV; Arch-GFP^{+/−}$ with the 594 nm laser stimulation. *P< 0.05, ***P< 0.001, Mann-Whitney U Test. (d) Saccharin preference test in the $PV; Arch-GFP^{+/−}$ mice with or without the 594 nm laser stimulation, and in the $PV; Arch-GFP^{+/−}$ mice with the 594 nm laser stimulation. *P< 0.05, ***P< 0.001, Mann-Whitney U Test. (e) The schematic of experimental schedule of the CPA test. (f) Place Aversion scores in the $PV; Arch-GFP^{+/−}$ mice with or without the 594 nm laser stimulation, and in the $PV; Arch-GFP^{+/−}$ mice with the 594 nm laser stimulation. *P< 0.05, Two-Way RM ANOVA with Bonferroni’s post-hoc test. Data are presented as mean ± s.e.m.

Figure 3 Optogenetic activation of the PV$^{+}$ interneurons in the CeA mimics negative affective states during morphine withdrawal.

(a) Presentative image of the amygdala of $PV; ChR2-tdTomato^{+/−}$ mice staining with the PV antibody. White dashes labeled the CeA and LA/BLA structure. White box was enlarged on the right. Arrows indicated the colocalized of PV antibody with the ChR2-tdTomato$^+$ neurons. Red: tdTomato, Green: PV, Blue: DAPI. Scale bar: 200 µm (Left), 20 µm (Right). (b-d) The EPM and SPT tests. (b) The schematic of experimental schedule of the EPM and SPT tests during morphine withdrawal group. (c) The time spent in the open arms of the EPM (Left) or the Saccharin preference (Right) in the $PV; ChR2-tdTomato^{−/−}$ mice in morphine withdrawal or saline group. **P< 0.01, ***P< 0.001, Mann-Whitney U Test. (d) The time spent in the open arms of the EPM (Left) or the saccharin preference (Right) in the $PV; ChR2-tdTomato^{+/−}$
mice with or without the 473nm laser stimulation, and in the \( PV;ChR2-tdTomato^{+/—} \) mice with the 473 nm laser stimulation. *P< 0.05, ***P< 0.001, Mann-Whitney U Test. (e) The CPA test of the \( PV;ChR2-tdTomato^{+/—} \) mice in the morphine withdrawal or saline group. (f) The CPA test of the \( PV;ChR2-tdTomato^{+/—} \) mice with or without the 473 nm laser stimulation, and of the \( PV;ChR2-tdTomato^{+/—} \) mice with the 473 nm laser stimulation during the conditioning sessions. Upper panel was the schematic of experimental schedule. *P< 0.05, Two-Way RM ANOVA with Bonferroni's post-hoc test. Data are presented as mean ± s.e.m.

Figure 4. CRH+ neurons localize in the CeA and the CRH mRNA level is regulated by the activity of PV+ interneurons.

(a) Presentative image of the amygdala of CRH; Arch-GFP\(^{+/—}\) mice staining with the CRH antibody. White dashes labeled the CeA and LA/BLA structure. White box was enlarged on the right. Arrows indicated the containing of CRH antibody with the Arch-GFP+ neurons. Green: GFP, Red: CRH, Blue: DAPI. Scale bar: 100 µm (Left), 10 µm (Middle), 5 µm (Right).

(b) Presentative image of the amygdala of CRH; Arch-GFP\(^{+/—}\) mice staining with the PV antibody. White dashes labeled the CeA and LA/BLA structure. White box was enlarged on the right. Arrows indicated the colocalization of PV antibody with the Arch-GFP fluorescent. Green: GFP; Cyan: PV, Blue: DAPI. Scale bar: 200 µm (Left), 20 µm (Middle), 20 µm (Right). (c) The schematic of experimental schedule. The CeA was dissected from \( PV;Arch-GFP^{+/—} \) and control mice after the 30-min 594 nm laser stimulation on the 7th day after the last saline or morphine exposure. (d) Quantification of the Expression of the CRH mRNA level in the CeA of the \( PV;Arch-GFP^{+/—} \) and \( PV;Arch-GFP^{−/−} \) mice both with the
30-min laser stimulation on the 7th day after saline and morphine exposure. ***$P < 0.001$, Two-Way ANOVA with Bonferroni’s post-hoc test. Data are presented as mean ± s.e.m.
References


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**Fig. 1**

(a) PV cell

(b) Saline

Withdrawal

40 pA

200 pA

PV* cell

Withdrawal

20.0 mV

100 ms

(c) Spike number vs. Current Injected (pA)

(d) AP threshold (mV)

31

28

(e) Saline

Withdrawal

10 pA

5 s

0.5 s

(f) Frequency (Hz)

30

30

(g) Amplitude (pA)

30

30
Fig. 2

a

PV:Arch-GFP (+/−) 
LA/BLA 
CeA 

b

Day 1 2 3 4 5 6 7 13 14
Morphine treatment
EPM SPT Laser

C

Time in Open Arms (s)

350
300
250
200
150
100
50
0

*** *

PV:Arch-GFP (+/−) Laser off
PV:Arch-GFP (+/−) Laser on
PV:Arch-GFP (+/−) Laser on

D

0.1% Saccharin Preference (%)

100
90
80
70
60
50
40
30
20
10
0

*** *

PV:Arch-GFP (+/−) Laser off
PV:Arch-GFP (+/−) Laser on
PV:Arch-GFP (+/−) Laser on

E

Pre-test Conditioning Test Laser

Day 1 2 3 4 5 6 7 13
Morphine treatment

F

Place Aversion Score (s)

400
300
200
100
0

* *

PV:Arch-GFP (+/−) Laser off
PV:Arch-GFP (+/−) Laser on
PV:Arch-GFP (+/−) Laser on

Pre-test Test
Fig. 4

a

b

C

R

H, A

rch-

GFP(+/−)

LA/BLA

CeA

CRH, Arch-GFP(+/−)

LA/BLA

CeA

GFP

Merge

CRH

Merge

PV

PV, ArchGFP(+/−)

Laser

Sacrifice

↑ Morphine / Saline treatment

↓ Morphine / Saline treatment

Randomized Control

Relative CRH mRNA Level

Saline

Withdrawal

***