Distinct eLPB\textsuperscript{ChAT} projections for methamphetamine withdrawal anxiety and primed reinstatement of conditioned place preference

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Abstract

Methamphetamine (METH) withdrawal anxiety symptom and relapse have been significant challenges for clinical practice; however, the underlying neuronal basis remains unclear. Our recent research has identified a specific subpopulation of choline acetyltransferase (ChAT\textsuperscript{+}) neurons localized in the external lateral portion of parabrachial nucleus (eLPB\textsuperscript{ChAT}), which modulates METH primed-reinstatement of conditioned place preference (CPP). Here, the anatomical structures and functional roles of eLPB\textsuperscript{ChAT} projections in METH withdrawal anxiety and primed reinstatement were further explored.

Methods: In the present study, a multifaceted approach was employed to dissect the eLPB\textsuperscript{ChAT} projections in male mice, including anterograde and retrograde tracing, acetylcholine (Ach) indicator combined with fiber photometry recording, photogenetic and chemogenetic regulation, as well as electrophysiological recording. METH withdrawal anxiety-like behaviors and METH-primed reinstatement of conditioned place preference (CPP) were assessed in male mice.

Results: We identified that eLPB\textsuperscript{ChAT} send projections to PKC\textgreekdelta-positive (PKC\textgreekdelta\textsuperscript{+}) neurons in lateral portion of central nucleus of amygdala (ICeAPKC\textgreekdelta) and oval portion of bed nucleus of the stria terminalis (ovBNSTPKC\textgreekdelta), forming eLPB\textsuperscript{ChAT}–ICeAPKC\textgreekdelta and eLPB\textsuperscript{ChAT}–ovBNSTPKC\textgreekdelta pathways. At least in part, the eLPB\textsuperscript{ChAT} neurons positively innervate ICeAPKC\textgreekdelta neurons and ovBNSTPKC\textgreekdelta neurons through regulating synaptic elements of presynaptic Ach release and postsynaptic nicotinic acetylcholine receptors (nAChRs). METH withdrawal anxiety and METH-primed reinstatement of CPP respectively recruit eLPB\textsuperscript{ChAT}–ICeAPKC\textgreekdelta pathway and eLPB\textsuperscript{ChAT}–ovBNSTPKC\textgreekdelta pathway in male mice.

Conclusion: Our findings put new insights into the complex neural networks, especially focusing on the eLPB\textsuperscript{ChAT} projections. The eLPB\textsuperscript{ChAT} is a critical node in the neural networks governing METH withdrawal anxiety and primed-reinstatement of CPP through its projections to the ICeAPKC\textgreekdelta and ovBNSTPKC\textgreekdelta, respectively.

Keywords: eLPB\textsuperscript{ChAT}; ICeAPKC\textgreekdelta; eLPB\textsuperscript{ChAT}–ovBNSTPKC\textgreekdelta; methamphetamine; anxiety; conditioned place preference

Introduction

Methamphetamine (METH) is a highly addictive and widely abused psychostimulant drug. Among individuals with METH use disorders (MUD), it has been observed that 34.3% develop withdrawal anxiety symptoms [1, 2], and up to 90% are estimated to relapse [3], posing a significant challenge for clinical practice.

The parabrachial nucleus (PBN), being located within the pons, functions as an integrator of sensory input from the surrounding environment and subsequently relays this information to forebrain structures. The majority of PBN neurons are
excitatory features [4, 5], being positively correlated with behaviors indicative of pain [6-8], itch [9, 10], fear [11], aversive [12], and emotion [10, 13]. Our recent research has identified a specific subpopulation of choline acetyltransferase (ChAT+) neurons located in the PBN, specifically within the external lateral portion of PBN (eLPBChAT) [14]. These neurons play a crucial role in modulating METH-primed reinstatement of METH conditioned place preference (CPP) in male mice. However, the precise structures and functions of eLPBChAT projections have not yet to be explored.

The LPB predominately send projections to the thalamus and limbic nuclei, such as the central nucleus of the amygdala (CeA) [6, 8, 13] and the bed nucleus of the stria terminalis (BNST) [6, 15, 16], both of which are pivotal in anxiety [13, 17] and addiction [18-20]. The activation of projections from the LPB to the CeA or BNST elicits aversive responses [6]. Given its potential as a target for anxiolytic agents, the CeA is implicated in the emotional component of the behavioral reaction to alcohol, particularly anxiety [21, 22]. Activation of LPB-CeA pathway alone was sufficient to induced anxiety-like behavior in mice [13]. It is noteworthy that social choice-induced abstinence has been found to prevent the incubation of METH craving, which was associated with the activation of protein kinase C delta-positive neurons in the CeA (CeA PKCδ) [19]. Furthermore, withdrawal from METH has been shown to increase the activity of BNST neurons [23], and BNST PKCδ neurons are also activated by aversive conditions to promote anxiety-like behavior [17]. Based on the mapping results obtained from fMOST [14], our previous findings have identified two distinct groups of eLPBChAT efferent neurons projecting to the CeA and BNST. Taken together, we aimed to gain a more comprehensive understanding of LPBChAT projections and their potential contribution to METH-related behaviors.

In the present study, a multifaceted approach was employed to dissect the LPBChAT projections in mice, including anterograde and retrograde tracing, acetylcholine (Ach) indicator combined with fiber photometry recording, as well as electrophysiological recording. We first identified the structural connectivity and physiological innervation of eLPBChAT–CeA pathway and eLPBChAT–BNST pathway in naïve male mice. Then, we explored the role of eLPBChAT–CeA pathway and eLPBChAT–BNST pathway in the anxiety-like behaviors during METH withdrawal period and METH-primed reinstatement of CPP in male mice. Our findings put new insights into the complex neural networks, especially focusing on the eLPBChAT projections, that contribute to the METH withdrawal anxiety and primed reinstatement of CPP.

Materials and methods

Animals

Male C57BL/6 wild type (WT) and ChAT-Cre male mice weighing 22-25 g (9 weeks old) were used. All animals were housed at constant humidity (40–60%) and temperature (24 ± 2°C) with a 12-hour light/dark cycle (lights on at 8 a.m.) and allowed free access to food and water. All male mice were handled for three days before onset of experiments. The naïve male mice utilized in this experiment were not subjected to any drug treatment or behavioral training, and had an initial weight range of 22-25g (9 weeks old). All procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (IACUC) at Nanjing University of Chinese Medicine, China.

Immunofluorescence

The male mice were deeply anesthetized with 10% chloral hydrate (0.2 ml, i.p.) and sequentially perfused with 0.9% saline and 4% paraformaldehyde (PFA). The brains were removed and post-fixed in 4% PFA at 4°C overnight. After dehydration of the brains with 30% (w/v) sucrose, coronal brain sections (30 μm) were cut on a cryostat (Leica, Germany) and used for immunofluorescence. The sections were incubated in 0.3% (v/v) Triton X-100 for 0.5 h, blocked with 5% donkey serum for 1.5 h at room temperature, and incubated overnight at 4°C with the following primary antibodies: goat anti-ChAT (1:200, RRID: AB_2079751, Millipore, USA), mouse anti-NeuN (1:800, RRID: AB_2247211, Millipore, USA), guinea pig anti-c-Fos (1:3000, RRID: AB_2905595, Synaptic System, USA), rabbit anti-c-Fos (1:2000, RRID: AB_2247211, Cell Signaling Technology, USA), mouse anti-c-Fos (1:1500, RRID: AB_2747772, Abcam, USA) and rabbit anti-PKCδ (1:1000, RRID: None, HuaBio, China), followed by the corresponding fluorophore-conjugated secondary antibodies for 1.5 h at room temperature. The following secondary antibodies were used here: Alexa Fluor 488-labeled donkey anti-rabbit secondary antibody (1:500, RRID: AB_2762833, Invitrogen, USA), Alexa Fluor 488-labeled donkey anti-mouse secondary antibody (1:500, RRID: AB_141607, Invitrogen, USA), Alexa Fluor 555-labeled goat anti-guinea pig secondary antibody (1:500, RRID: None, Abcam, USA), Alexa Fluor 555-labeled donkey anti-rabbit secondary antibody (1:500, RRID: AB_2762834, Invitrogen, USA), Alexa Fluor 555-labeled donkey anti-mouse...
secondary antibody (1:500, RRID: AB_2762848, Invitrogen, USA), Alexa Fluor 555-labeled donkey anti-goat secondary antibody (1:500, RRID: AB_2762839, Invitrogen, USA), Alexa Fluor 680-labeled goat anti-guinea pig secondary antibody (1:500, RRID: AB_2556678, Invitrogen, USA), Alexa Fluor 680-labeled donkey anti-rabbit secondary antibody (1:500, RRID: AB_2762836, Invitrogen, USA), Alexa Fluor 680-labeled donkey anti-mouse secondary antibody (1:500, RRID: AB_2762831, Invitrogen, USA), Alexa Fluor 680-labeled donkey anti-goat secondary antibody (1:500, RRID: AB_2762841, Invitrogen, USA). In detail of the staining for c-Fos and PKCδ in the ICeA or ovBNST, brain slices were attached to slides and placed in an oven at 60°C for 30 min, and then immersed in 4% PFA solution for 15 min in a refrigerator at 4°C for fixation. Then graded concentration alcohol dehydration (50%-70%-100%) was performed sequentially for 5 min each time. Next, antigen repair was performed by immersing the slides with brain slices attached in 10 mM PH=6.0 trisodium citrate solution and heating in a water bath at 82°C for 30 min. Normal staining steps were then followed. Fluorescence signals were captured by TCS SP8 confocal microscope (Leica, Germany), and STELLARIS 8 DIVE confocal microscope (Leica, Germany).

**Tracing virus injection**

All viruses in the present study were packaged by BrainVTA (China). The male mice were fixed in a stereotaxic frame (RWD, China) under 2% isoflurane anesthesia. A heating pad was used to maintain the body temperature of the mice at 37 °C. Unless otherwise noted, a volume of 100 nl virus was injected per side. The injections were given over 5 min at a rate of 20 nl/min by an infusion pump (Drummond, USA) and left in place for 10 min. The stereotaxic coordinates of the eLPB (AP, -5.19 mm, ML, ± 1.45 mm and DV, -3.30 mm). An optical fiber (200 μm outer diameter, 0.37 numerical aperture, Thinkerbiotech, China) was placed 100 μm above the viral injection site. After 3 weeks, the calcium-dependent fluorescence signals of Ach 3.0 sensor in the ICeA or ovBNST were recorded at homecage. The signals were obtained by stimulating cells that transfected Ach3.0 sensor using a 470 nm LED (35-40 μW at fiber tip), while calcium-independent signals were obtained by stimulating these cells with a 405 nm LED (15-20 μW at fiber tip). The LED lights of 470 nm and 405 nm were alternated at 66 fps and light emission was recorded using sCMOS camera containing the entire fiber bundle (2 m in length, NA = 0.37, 200 μm core, Thinkerbiotech, China). The analog voltage signals fluorescent was filtered at 30 Hz and digitalized at 100 Hz. The Ach3.0 signals were recorded and analyzed by ThinkerTech TrippleColor MultiChannel fiber photometry Acquisition Software and ThinkerTech TrippleColor MultiChannel fiber photometry Analysis Package (Thinkerbiotech, China), respectively. The raw heatmap data were normalized by Z-Score normalization. The formula for Z-Score is \[Z = \frac{D - \mu}{\sigma}\], where \(D\) is the raw fiber photometry signal data, \(\mu\) is the mean value of raw data and \(\sigma\) is the standard deviation of raw data. The baseline fluorescence signal which was recorded for 5 min with 1 min record and 4 min interval (1 session) prior to clozapine-N-oxide (CNO, Selleck, USA) treatment. The real-time fluorescence signal which was recorded for 60 min with a 1-min recording and 4-min interval (11 session). The raw heatmap data from one mouse was merged as a statistical point and normalized using area under the curve (AUC) normalization. The AUC represents the integral under the recording duration relative to the corresponding baseline at each trial. For the immunofluorescence experiment, another set of same mice models was utilized, the brain tissue was collected at 35 min after CNO injection.
Electrophysiology

The rAAV2/9-ChAT-CRE-P2A-EGFP-WPRE-hGH polyA (PT-0652, 5.30E+12 vg/mL, BrainVTA, China) and rAAV2/9-Ef9a-DIO-hChR2-mCherry-WPRE-hGH polyA (PT-0002, 5.05E+12 vg/mL, BrainVTA, China) virus was bilaterally injected into the eLPB of WT mice. The mice (n = 6 mice) were deeply anesthetized with isoflurane (RWD, China) and perfused with ice-cold cutting solution. Slice preparation was performed as previously described [2]. Slices containing the lCeA or ovBNST were cut at a 200 μm thickness using a vibratome in 4°C cutting solution. The slices were transferred to 37°C cutting solution for 9 min, then transferred to a holding solution to allow for recovery at room temperature for 1 h before recordings. Throughout the electrophysiological recordings, the slices were continuously perfused with oxygenated artificial CSF (aCSF) maintained at 30°C using a solution heater (TC-324C, Warner Instruments, USA).

The spontaneous excitatory postsynaptic currents (sEPSC) were recorded in voltage-clamp mode at a holding potential of -70 mV. The sEPSC baseline was recorded 5 min after breaking in. An LED fiber above the recording sites emits blue (473 nm) light to activate the terminals of the eLPBChATChAT-hM4D(Gq)-mCherry (PT-3108, 6.28E+12 vg/ml, BrainVTA, China) virus was bilaterally injected into the eLPB (AP ± 5.19 mm, ML ± 1.45 mm, DV + 3.30 mm), and stainless-steel guide cannulas (O.D. 0.41 mm, C.C 4.0 mm, Cat 62004, RWD, China) were lowered into the lCeA (AP - 1.31 mm, ML ± 2.90 mm, DV - 4.50 mm). The guide cannulas were secured in place using glass ionomer cements. Dummy cannulas (62102, RWD, China, with lengths matching the guide cannulas) were placed inside the guide cannulas to prevent occlusion. Incisions were fixed and covered with glass ionomer cement.

Conditioned place preference (CPP)

The CPP test was performed in the TopScan3D CPP apparatus (CleverSys, USA), consisting of two distinct chambers (15 × 15 × 23 cm each) with a removable guillotine door. The CPP procedure involved a conditioning test (Pre-test, Day -1), conditioning (CPP training, Day 0-6), post-conditioning test (Test, Day 7), extinction training, and a saline or METH challenge-reinstatement test. Baseline preference (pre-test) was determined by allowing male mice to freely explore both chambers of the CPP apparatus for 15 min. Conditioning was conducted in a non-drug-paired chamber paired with a saline (0.2 ml, i.p.) injection in the morning and in a drug-paired chamber paired with a METH (3 mg/kg, i.p.) injection in the afternoon for 7 consecutive days. Following each injection, the male mice were confined to one chamber (non-drug-paired chamber or drug-paired chamber) for 45 min. During the test and extinction phases, male mice had unrestricted access to both chambers without any drug treatment. For the METH-primed reinstatement test, male mice received a subthreshold dose of MET (0.5 mg/kg, i.p.) and were then allowed to freely explore both chambers for 15 min. The CPP score represents the time spent in drug-paired chamber minus that in the non-drug-paired chamber, while the ΔCPP score is calculated as the priming CPP score minus extinction CPP score.

In the chemical genetics stimulations experiment, the rAAV2/9-ChAT-hM3D(Gq)-mCherry (PT-2213, 5.54E+12 vg/ml, BrainVTA, China) or rAAV2/9-ChAT-hM4D(Gi)-mCherry (PT-3108, 6.28E+12 vg/ml, BrainVTA, China) virus was bilaterally injected into the eLPB (AP ± 5.19 mm, ML ± 1.45 mm, DV - 3.30 mm), and the cannulas were lowered into the lCeA (AP - 1.31 mm, ML ± 2.90 mm, DV - 3.0 mm) (cannula: O.D. 0.41 mm, C.C 4.0 mm, Cat 62004, RWD, China) or ovBNST (AP + 0.13 mm, ML ± 2.13 mm).
mm, DV - 2.62 mm, 15°tilt) (cannula: O.D. 0.41 mm, C 3.6 mm, Cat 62004, RWD, China).

**Designer receptors exclusively activated by designer drugs (DREADDs)**

Male mice were anesthetized with 2% isoflurane in oxygen and were fixed in a stereotactic frame (RWD, China). A heating pad was used to maintain the core body temperature of the animals at 37°C. 100 nl rAAV2/9-ChAT-hM3D(Gq)-mCherry (PT-2213, 5.54E+12 vg/ml, BrainVTA, China) or rAAV2/9-ChAT-hM4D(Gi)-mCherry (PT-3108, 6.28E+12 vg/ml, BrainVTA, China) virus was bilaterally injected into the eLPB (AP ± 5.19 mm, ML ± 1.45 mm, DV - 2.62 mm) at a rate of 1 nl/sec. After surgery, mice were allowed to recover in their homecage for one week. 10 min before the behavioral assays, CNO or saline was locally infused through the cannula at a flow rate of 0.1 µl/min. The infusion cannulas (#62204, RWD, China) were connected via polyethylene tubing (#62329, RWD, China) to 10-µl microsyringes (GAOGE, China) mounted on a microinfusion pump (RWDR462, China). For the diffusion of the drug, the infusion cannulas were kept in place for 5 min before being replaced with dummy cannulas. Male mice were injected with saline (vehicle, 0.2 ml) or CNO (2 mg/kg) 10 min before each behavioral test.

**Statistical analysis**

Statistical analysis was carried out using GraphPad Prism 8.0.2 software. All data are presented as the Mean ± SD. The data were analyzed by unpaired t-tests or two-way analysis of variance (ANOVA) with Sidak’s multiple comparisons which appropriate. All statistical significance was set as p < 0.05.

**Results**

**Anatomical dissection and functional investigation of the eLPB\textsuperscript{ChAT}–lCeA\textsuperscript{PKCδ} and eLPB\textsuperscript{ChAT}–ovBNST\textsuperscript{PKCδ} pathways**

We conducted separate retrograde and anterograde tracer experiments in different batches of mice in the current study. Consistent with our previous study [14], we observed a widespread distribution of ChAT\textsuperscript{+} neurons in the eLPB\textsuperscript{ChAT} along the anterior-posterior brain axis in naïve mice (Figures 1A, 1B and 1C). Here, employing the anterograde (Figures 1D, 1E and 1F) and retrograde (Figures 1G and 1H) virus tracing, we found that eLPB\textsuperscript{ChAT} neurons predominantly projected to lateral portion of CeA (lCeA) and the oval portion of BNST (ovBNST). Furthermore, approximately 75% and 52% of eLPB\textsuperscript{ChAT} terminals were distributed around PKCδ-positive (PKCδ\textsuperscript{+}) neurons in lCeA and ovBNST respectively, forming the eLPB\textsuperscript{ChAT}–lCeA\textsuperscript{PKCδ} pathway and eLPB\textsuperscript{ChAT}–ovBNST\textsuperscript{PKCδ} pathway.

Since cholinergic neurons have the potential to act as interneurons for local regulation [25] or innervating other nuclei through long projections [26, 27] in the brain, we explored the physiological innervation of eLPB\textsuperscript{ChAT} neurons on PKCδ\textsuperscript{+} neurons in lCeA and ovBNST. First, to observe the real-time Ach signals from eLPB\textsuperscript{ChAT} terminals within the lCeA and ovBNST, DREADDs method and fiber photometry were employed in male naïve mice (Figures 2A, 2B and 2I). As shown in Figure S1B, systemic administration of CNO efficiently activates eLPB\textsuperscript{ChAT} neurons. In parallel, the Ach release was significantly increased in lCeA from 15 to 40 min (Figure 2C), and in ovBNST at 35 min following CNO injection (Figure 2D) in free-moving male mice compared to vehicle controls. In another set of identical mouse models, we collected brain tissue at 35 min after CNO injection and found that both lCeA\textsuperscript{PKCδ} neurons (Figure 2E) and ovBNST\textsuperscript{PKCδ} neurons (Figure 2F) were evoked by activating eLPB\textsuperscript{ChAT} neurons, as indicated by increased c-Fos\textsuperscript{+} PKCδ\textsuperscript{+} neurons. To further characterize the potential postsynaptic elements involved in the eLPB\textsuperscript{ChAT}–lCeA/ovBNST pathway, we employed optogenetic activation strategies combined with patch-clamp recordings on acutely prepared slices (Figures 2G and 2H). Blue light (473 nm) was utilized to activate the eLPB\textsuperscript{ChAT} terminals within either lCeA or ovBNST. As shown in Figures 2I and 2J, the frequency of sEPSC in the lCeA and ovBNST terminals was increased when activating LPB\textsuperscript{ChAT} terminals. Inhibition of nAChRs with mecamylamine incubation during optogenetic activation of eLPB\textsuperscript{ChAT} terminals resulted in a blockade of the triggered sEPSC frequency in slices of lCeA and ovBNST. Notably, no significant differences were observed in the amplitude of sEPSC between the lCeA and ovBNST neurons throughout the entire process. Collectively, these findings suggest that eLPB\textsuperscript{ChAT} neurons positively modulate lCeA\textsuperscript{PKCδ} and ovBNST\textsuperscript{PKCδ} neurons, at least partially through synaptic elements of presynaptic Ach release and postsynaptic nAChRs.

**Inhibiting eLPB\textsuperscript{ChAT}–lCeA\textsuperscript{PKCδ} pathway alleviates anxiety-like behaviors in METH-withdrawn male mice**

The involvement of LPB–CeA and LPB–BNST pathways in anxiety has been previously reported [15, 16, 28, 29]. Here, mice were subjected to daily intraperitoneal injections of METH or saline for 7 consecutive days, followed by a 14-day withdrawal period. Anxiety-like behaviors were assessed using EPM on Day 22 (Figure 3A).
In comparison with the saline-withdrawn mice, METH-withdrawn mice exhibited anxiety-like behaviors characterized by reduced time spent and fewer entries into the open arms (Figure 3B). Additionally, both eLPB\textsuperscript{ChAT} neurons and lCeA\textsuperscript{PKC\delta} neurons, but not ovBNST\textsuperscript{PKC\delta} neurons, showed significantly activation in METH-withdrawn male mice (Figure 3C).

To investigate the role of eLPB\textsuperscript{ChAT}–lCeA \textsuperscript{PKC\delta} pathway in METH withdrawal-induced anxiety-like behavior, rAAV2/9-ChAT-hM4D(Gi)-mCherry virus was injected into the eLPB, and cannulas were placed in the lCeA. 10 min before EPM test, CNO or vehicle was locally infused through the cannula to selectively inhibit eLPB\textsuperscript{ChAT} terminals within the lCeA or not (Figures 4A and 4B). Results showed that inhibiting eLPB\textsuperscript{ChAT}–lCeA \textsuperscript{PKC\delta} pathway decreased the activity of PKC\delta\textsuperscript{+} neurons without affecting PKC\delta\textsuperscript{-} neurons of the lCeA in both METH-withdrawn and control mice (Figure 4C). Additionally, inhibition of eLPB\textsuperscript{ChAT}–lCeA \textsuperscript{PKC\delta} pathway efficiently attenuated anxiety-like behaviors specifically in METH-withdrawn male mice, while having no impact on related behaviors in controls (Figure 4D). Conversely, when we activated eLPB\textsuperscript{ChAT} terminals within the lCeA using similar viral tools (Figures 4E and 4F), there was an increased activation of PKC\delta\textsuperscript{+} neurons but not PKC\delta\textsuperscript{-} neurons in the lCeA (Figure 4G). As depicted in Figure 4H, activating eLPB\textsuperscript{ChAT}–lCeA \textsuperscript{PKC\delta} pathway induced heightened anxiety-like behavior specifically in METH-withdrawn mice, while having no influence on such behavior in control mice.
Taken together, these findings suggest that the eLPB<sup>ChAT</sup>-lCeA<sup>PKCδ</sup> pathway is involved in encoding METH withdrawal anxiety, and has no influence on anxiety-like behavior in saline withdrawal mice.

**Activating eLPB<sup>ChAT</sup>-ovBNST<sup>PKCδ</sup> pathway suppresses METH-primed reinstatement of CPP in male METH-exposed mice**

Recently, emerging evidence demonstrate that PKCδ<sup>+</sup> GABAergic neurons might involve in encoding relapse to drugs [19, 30]. The METH-primed reinstatement of CPP procedure was established in male mice (Figure 5A). Our findings revealed that a single METH challenge (0.5 mg/kg) effectively induced the reinstatement of CPP in METH-exposed mice rather than those exposed to saline (Figures 5B and S2A). For the METH extinction mice, METH challenge resulted in heightened activity levels in eLPBChAT, lCeA PKCδ and ovBNST PKCδ neurons (Figure 5C). Further, METH priming induced a more pronounced increase in eLPBChAT and ovBNSTPKCδ neurons, with no discernible impact on lCeA PKCδ neurons in METH-exposed mice compared to saline-exposed mice. These findings suggest that ovBNST<sup>PKCδ</sup> neurons play a more prominent role in mediating METH-primed reinstatement of METH CPP.

Based on the neuronal activation phenotypes observed in METH-primed mice in the present...
study, we speculate that precise inhibition of the eLPBChAT–lCeA PKCδ or eLPBChAT–ovBNST PKCδ pathway may attenuate the METH-primed reinstatement of CPP in METH-exposed mice. As shown in Figures 6A-6C, utilizing DREADDs methodology, we successfully achieved inhibition of eLPBChAT terminals in the ICeA PKCδ in METH-exposed mice. Contrary to our initial expectations, inhibiting the eLPBChAT–ICeA PKCδ pathway did not exert any influence on METH-primed reinstatement of CPP in mice (Figure 6D). Furthermore, there were no significant differences in ΔCPP score between the vehicle-treated and CNO-treated groups in saline-challenged METH-exposed mice (Figure 6D), suggesting that inhibiting eLPBChAT terminals in ICeA PKCδ without administrating METH challenge cannot facilitate the reinstatement of CPP in mice. Previously, our research showed that activating eLPBChAT neurons or CeA-projecting eLPBChAT neurons, rather than suppressing them, could prevent METH-primed reinstatement of CPP in mice [14]. Therefore, we conducted local activation of eLPBChAT terminals in the ICeA PKCδ in METH-exposed mice (Figures 6E and 6F). Unexpectedly, activating the eLPBChAT–ICeA PKCδ pathway also failed to elicit any impact on METH-primed reinstatement of METH CPP in mice (Figure 6G), indicating that this pathway does not regulate METH-primed reinstatement behavior. These findings raise questions regarding other potential pathways involving eLPBChAT neurons responsible for modulating METH-primed reinstatement.

Figure 3. METH withdrawal enhances anxiety-like behaviors and triggers excitability of eLPBChAT and ICeA PKCδ neurons in male mice. A Experimental design and timeline. B EPM test. Two-tailed unpaired t test. S group, n = 9 mice; M group, n = 7 mice. Time in open arms, t (14) = 4.060, p = 0.0012. Entries into open arms, t (14) = 4.194, p = 0.0009. Total distance, t (14) = 0.4946, p = 0.6285. C Representative images and the percentage of c-Fos+ neurons in the eLPB ChAT, ICeA PKCδ and ovBNST PKCδ neurons. Two-tailed unpaired t test. n = 6 mice per group. eLPB (c-Fos+/ChAT+ / ChAT%) t (10) = 3.539, p = 0.0054; eLPB (c-Fos+/ChAT+ / c-Fos+%), t (10) = 2.413, p = 0.0365; ICeA, t (10) = 3.636, p = 0.0046; ovBNST, t (10) = 0.02581, p = 0.9799.
Figure 4. Inhibiting eLPB ChAT–ICeA PKCδ pathway alleviates the anxiety-like behaviors in METH-withdrawn male mice. A. Experimental design and timeline of Gi virus in the eLPB. B. Schematics and representative images of rAAV2/9-ChAT-hM4D(Gi)-mCherry injection in the eLPB, cannula implantation in the ICeA, and mCherry+ axon terminals in the ICeA of WT mouse. C. Representative images and the percentage of c-Fos+ neurons in the ICeA PKCδ+ and PKCδ− neurons. Two-way ANOVA with Sidak’s multiple comparisons test. Upper, n = 6 mice per group. F (1, 20) = 0.6286, p = 0.4372; S-CNO group, t = 3.024, p = 0.0395 vs S-Vhl group; M-CNO group, t = 4.145, p = 0.0030 vs M-Vhl group; M-Vhl group, t = 3.405, p = 0.0167 vs S-Vhl group. Lower, n = 6 mice per group. F (1, 20) = 0.2019, p = 0.6580; S-CNO group, t = 0.2862, p = 0.9999 vs S-Vhl group; M-CNO group, t = 0.3492, p = 0.9996 vs M-Vhl group; M-Vhl group, t = 0.1561, p = 0.9999 vs S-Vhl group. D. EPM test. Two-way ANOVA with Sidak’s multiple comparisons test. S-Vhl group, n = 6 mice; S-CNO group, n = 6 mice; M-Vhl group, n = 16 mice; M-CNO group, n = 12 mice. Time in open arms, F (1, 36) = 7.915, p = 0.0079; S-CNO group, t = 0.2862, p = 0.9999 vs S-Vhl group; M-CNO group, t = 4.269, p = 0.0006 vs M-Vhl group; M-Vhl group, t = 3.054, p = 0.0021 vs S-Vhl group. Entries into open arms, F (1, 36) = 2.688, p = 0.1098; S-CNO group, t = 0.0004 vs S-Vhl group. E. Experimental design and timeline of Gq virus in the eLPB. F. Schematics and representative images of rAAV2/9-ChAT-hM3D(Gq)-mCherry injection in the eLPB, cannula implantation in the ICeA, and mCherry+ axon terminals in the ICeA of WT mouse. G. Representative images and the percentage of c-Fos+ neurons in and ICeA PKCδ+ and PKCδ− neurons. Two-tailed unpaired t test. n = 6 mice per group. c-Fos+ PKCδ+/PKCδ−, t (10) = 5.514, p = 0.0003; c-Fos− PKCδ+/PKCδ−, t (10) = 1.596, p = 0.1417. H. EPM test. Two-way ANOVA with Sidak’s multiple comparisons test. n = 6 mice per group. Time in open arms, F (1, 20) = 0.1872, p = 0.6699; S-CNO group, t = 0.1417, p = 0.4599 vs S-Vhl group; M-CNO group, t = 3.129, p = 0.0313 vs M-Vhl group; M-Vhl group, t = 5.041, p = 0.0004 vs S-Vhl group. Entries into open arms, F (1, 20) = 1.180, p = 0.2844 vs M-Vhl group; M-Vhl group, t = 1.063, p = 0.8827 vs S-Vhl group. Similarly, we conducted inhibition of eLPB ChAT terminals in the ovBNST PKCδ in METH-exposed mice (Figures 7A, 7B and 7C). Consistent with the findings of eLPB ChAT−ICeA PKCδ, inhibiting the eLPB ChAT−ovBNST PKCδ pathway also had no influence on METH-primed reinstatement of CPP in mice (Figure 7D). Subsequently, we locally activated eLPB ChAT terminals in the ovBNST PKCδ in METH-exposed mice (Figures 7E and 7F), and found that activating eLPB ChAT−ovBNST PKCδ pathway effectively blocked...
METH-primed reinstatement of METH CPP in mice (Figure 7G). We thought that the activation of eLPBChAT neurons by METH priming may represent a compensatory response of the brain, potentially attenuating sensitivity to drug priming by activating ovBNSTPKC<sup>δ</sup> neurons.

Taken together, these findings demonstrate that the eLPBChAT-ovBNSTPKC<sup>δ</sup> pathway, rather than eLPBChAT-CeAPKC<sup>δ</sup> pathway, is encoding the METH-primed reinstatement of CPP.

Figure 5. METH-primed reinstatement of CPP triggers excitability of the eLPBChAT, iCeA PKC<sup>δ</sup> and ovBNSTPKC<sup>δ</sup> neurons in male mice. A Experimental design and timeline. B Heatmap of spent duration by mice in CPP apparatus and ΔCPP score. Two-way ANOVA with Sidak’s multiple comparisons test. S group, n = 6 mice per group; M group, n = 14 mice per group; M-M group, t = 1.267, p = 0.7630 vs S-S group; M-S group, t = 5.846, p < 0.0001 vs M-S group. C Representative images and the percentage of c-Fos<sup>+</sup> neurons in the eLPBChAT, iCeA PKC<sup>δ</sup> and ovBNST PKC<sup>δ</sup> neurons. Two-way ANOVA with Sidak’s multiple comparisons test. n = 6 mice per group. eLPB, F(1, 20) = 0.04656, p = 0.8313; S-M group, t = 4.967, p = 0.0004 vs S-S group; M-M group, t = 5.272, p = 0.0002 vs M-S group; M-M group, t = 3.183, p = 0.0277 vs S-S group; M-M group, t = 5.047, p = 0.0004 vs M-S group; M-M group, t = 2.149, p = 0.2367 vs S-M group; ovBNST, F(1, 20) = 24.78, p < 0.0001; S-M group, t = 1.587, p = 0.5608 vs S-S group; M-M group, t = 8.627, p < 0.0001 vs M-S group; M-M group, t = 6.764, p < 0.0001 vs S-M group. Scale bar, 100 μm. N.S., p > 0.05, *, p < 0.05, **, p < 0.01 vs S-S or M-S or Vhl or CNO.
Figure 6. Manipulation of eLPB\textsuperscript{C}AT-\textit{iceCA}\textsuperscript{PKC}-pathway had no influence on METH-primed reinstatement of CPP in mice. A Experimental design timeline. B Schematics and representative images of rAAV2/9\textsuperscript{C}AT-miMD4(Gq)-mCherry injection in the eLPB, cannula implantation into the \textit{iceCA}, and mCherry\textsuperscript{+} axon terminals in the \textit{iceCA} of WT mouse. C Representative images and the percentage of c-Fos\textsuperscript{+} neurons in PKC\textsuperscript{δ} and PKC\textsuperscript{ε} neurons of \textit{iceCA}. Two-way ANOVA with Sidak’s multiple comparisons test. CPP scores, M-S group, t = 3.141, p = 0.0459 vs M-S-Vhl group; M-M group, t = 3.940, p = 0.0048 vs M-S-Vhl group; M-M-Vhl group, t = 3.380, p = 0.0097 vs extinction; M-M group priming, t = 4.884, p = 0.0001 vs extinction. ∆CPP score, n = 6 mice per group. F (1, 20) = 1.169, p = 0.2924; M-S-CNO group, t = 0.4610, p = 0.6514 vs M-S-Vhl group; M-M group, t = 1.629, p = 0.1249 vs M-S-CNO group; M-M-CNO group, t = 0.4007, p = 0.6999 vs M-M-Vhl group. Scale bar, 100 µm. D CPP scores during the pre-test, CPP test, extinction and priming in M-S and M-M group. ∆CPP score (priming CPP score minus extinction CPP score). Two-way ANOVA with Sidak’s multiple comparisons test. CPP scores, M-S group, n = 6 mice per group. F (3, 40) = 0.2925, p = 0.8035; M-S-Vhl group priming, t = 0.3979, p = 0.7203 vs extinction; M-S-CNO group priming, t = 0.0363, p = 0.9999 vs extinction; M-M group, n = 6 mice per group. F (3, 40) = 0.1466, p = 0.9552 vs M-M-Vhl group, M-M-CNO group, t = 0.3362, p = 0.7203; M-M-CNO group, t = 0.3304, p = 0.9997 vs M-S-Vhl group; M-M-CNO group, t = 1.629, p = 0.0524 vs M-Vhl-Vhl group; M-M-Vhl group, t = 3.224, p = 0.0253 vs M-S-Vhl group. Heatmap of spent duration by mice in CPP apparatus and total distance traveled in CPP apparatus during priming test. Two-way ANOVA with Sidak’s multiple comparisons test. n = 6 mice per group. F (1, 20) = 1.919, p = 0.1812; M-S-CNO group, t = 0.5205, p = 0.5324 vs M-Vhl-Vhl group; M-M group, t = 0.5528, p = 0.4450; M-M-CNO group, t = 4.855, p = 0.0006 vs M-M-Vhl group; M-M-CNO group, t = 1.990, p = 0.0524 vs M-M-Vhl group; M-M-Vhl group, t = 3.404, p = 0.0098 vs M-S-Vhl group; M-M-Vhl group, t = 0.9710 vs M-S-Vhl group; M-M group, t = 0.4610, p = 0.6514 vs M-S-Vhl group; M-M group, t = 0.3362, p = 0.7203; M-M-CNO group, t = 0.8018, p = 0.4872; M-M group, t = 0.8305, p = 0.3853; M-S-CNO group, t = 4.855, p = 0.0006 vs M-S-Vhl group; M-S-Vhl group, t = 1.068, p = 0.8085 vs M-S-Vhl group; M-M group, t = 0.3304, p = 0.7778; M-M-CNO group, t = 0.3362, p = 0.7203 vs M-M-Vhl group; M-M-CNO group, t = 0.4450 vs M-M-Vhl group. Scale bar, 100 µm. G CPP scores during the pre-test, CPP test, extinction and priming in M-S and M-M group and ∆CPP score. Two-way ANOVA with Sidak’s multiple comparisons test. CPP scores, M-S, n = 6 mice; M-S-CNO, n = 6 mice; M-M group, n = 6 mice; M-M-CNO, n = 7 mice; M-M-Vhl group, n = 6 mice; M-M-CNO group, n = 6 mice; M-S group, F (3, 40) = 3.6040, p = 0.0011 vs extinction; M-M group priming, t = 3.380, p = 0.0097 vs extinction; M-M-Vhl group priming, t = 3.830, p = 0.0048 vs extinction; M-M-CNO group priming, t = 0.6514, p = 0.5324 vs extinction. ∆CPP score, n = 6 mice per group. F (3, 44) = 0.8805, p = 0.4450; M-M-Vhl group, t = 3.0510, p = 0.0048 vs M-S-Vhl group; M-M-CNO group, t = 0.9710 vs M-S-Vhl group. E Schematics and representative images of rAAV2/9\textsuperscript{C}AT-miMD4(Gq)-mCherry injection in the eLPB, cannula implantation into the \textit{iceCA}, and mCherry\textsuperscript{+} axon terminals in the \textit{iceCA} of WT mouse. F Representative images and the percentage of c-Fos\textsuperscript{+} neurons in PKC\textsuperscript{δ} neurons of lCeA. Two-way ANOVA with Sidak’s multiple comparisons test. Upper, n = 6 mice per group. F (3, 40) = 3.1303, p = 0.8242 vs M-S-Vhl group; M-M-CNO group, t = 0.2366, p = 0.5205 vs M-S-Vhl group; M-M-Vhl group, t = 0.3224, p = 0.7778; M-S-CNO group, t = 0.9314, p = 0.3853; M-S-Vhl group, t = 0.9999 vs M-S-Vhl group; M-M group, F(3, 44) = 0.1465, p = 0.5010 vs M-S-Vhl group; M-M-Vhl group, t = 0.3304, p = 0.7778; M-M-CNO group, t = 0.9710 vs M-M-Vhl group; M-M group, t = 0.4610 vs M-S-Vhl group, M-S-CNO group, t = 0.5205, p = 0.9999 vs M-M-Vhl group; M-M-CNO group, t = 0.1249, p = 0.9552 vs M-M-Vhl group; M-M-Vhl group, t = 1.811, p = 0.0805 vs M-S-Vhl group; M-M-CNO group, t = 0.8252, p = 0.9552 vs M-M-Vhl group; M-M-Vhl group, t = 1.629, p = 0.0524 vs M-Vhl-Vhl group; M-M-CNO group, t = 0.8242 vs M-Vhl-Vhl group. Scale bar, 100 µm. Experimental design

**Vhl**, vehicle; **CNO**, clozapine-N-oxide; **M-S**, saline-primed reinstatement test following METH CPP extinction training; **M-M**, METH-primed reinstatement test following METH CPP extinction training; **N.S.**, p > 0.05, ***, p < 0.005, ****, p < 0.0001.

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Figure 7. Activating eLPB-ChAT-ovBNSTδ-CRE pathway suppresses METH-primed reinstatement of CPP in male METH-exposed mice. A: Experimental design and timeline of rAAV2/9-ChAT-hM3D(Gq)-mCherry injection in the eLPB, cannula implantation into the ovBNST, and mCherry+ axon terminals in the ovBNST of WT mouse. B: Representative images and the percentage of c-Fos+ neurons in PKCδ neurons in the ovBNST PKCδ neurons of WT mouse. C: Schematics and representative images of experimental design and timeline. D: Viral injection in the eLPB, cannula implantation in the ovBNST, and mCherry+ axon terminals in the ovBNST of WT mouse. Scale bar, 100 μm. D: Schematics and representative images of rAAV2/9-ChAT-hM4D(Gi)-mCherry injection in the eLPB, cannula implantation in the ovBNST, and mCherry+ axon terminals in the ovBNST of WT mouse. Scale bar, 100 μm. E: Representative images and the percentage of c-Fos+ neurons in PKCδ neurons of WT mouse. Scale bar, 100 μm.

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Discussion

In summary, our study has made a novel discovery by identifying two distinct anatomically and functionally pathways of eLPB\textsuperscript{ChAT} neurons, namely eLPB\textsuperscript{ChAT–lCeA\textsuperscript{PKC\textgreek{d}}} pathway and eLPB\textsuperscript{ChAT–ovBNST\textsuperscript{PKC\textgreek{d}}} pathway. We further elucidate the involvement of synaptic elements of presynaptic Ach release and postsynaptic nAChRs in the positive innervation of the two cholinergic pathways. Importantly, our findings demonstrate that METH withdrawal anxiety and METH-primed reinstatement of CPP respectively recruit eLPB\textsuperscript{ChAT–lCeA\textsuperscript{PKC\textgreek{d}}} and eLPB\textsuperscript{ChAT–ovBNST\textsuperscript{PKC\textgreek{d}}} pathway in METH-exposed male mice (Figure 8).

In our previous study [14], chemogenetic activation of either the eLPB\textsuperscript{ChAT} neurons or the CeA-projecting eLPB\textsuperscript{ChAT} neurons significantly suppressed METH primed-reinstatement of CPP in mice. Here, our chemogenetic experiments specifically targeted the eLPB\textsuperscript{ChAT} projection to the ICeA using DREADD virus in the eLPB, followed by a site-specific infusion of the CNO into the ICeA to evaluate the effects of eLPB\textsuperscript{ChAT–ICeA} pathway in METH primed-reinstatement. We found that chemogenetic modulation of local presynaptic activity at eLPB\textsuperscript{ChAT} axon terminals within the eLPB\textsuperscript{ChAT–ICeA} pathway had no effect on METH primed-reinstatement of CPP. These seemingly contradictory findings raise significant concerns regarding whether eLPB\textsuperscript{ChAT} neurons projecting to other brain regions encode METH primed-reinstatement of CPP.

Figure 8. Schematic diagram of the present study. The ChAT\textsuperscript{+} neurons in the eLPB send projections to PKC\textgreek{d}\textsuperscript{+} neurons in the ICeA and ovBNST, forming eLPB\textsuperscript{ChAT–ICeA\textsuperscript{PKC\textgreek{d}}} and eLPB\textsuperscript{ChAT–ovBNST\textsuperscript{PKC\textgreek{d}}} pathways. At least in part, the eLPB\textsuperscript{DAT} neurons positively excite the ICeA\textsuperscript{PKC\textgreek{d}} neurons and ovBNST\textsuperscript{PKC\textgreek{d}} neurons through synaptic elements of presynaptic Ach release and postsynaptic nAChRs. Chemogenetic inhibiting the eLPB\textsuperscript{DAT} terminals within the ICeA alleviates the anxiety-like behaviors in METH-withdrawn mice, and chemogenetic activating the eLPB\textsuperscript{DAT} terminals within the ovBNST blocks METH-primed reinstatement of CPP in METH-exposed mice. These results indicate that METH withdrawal anxiety and METH-primed relapse recruit distinct eLPB\textsuperscript{DAT} projections, as identified by eLPB\textsuperscript{DAT–ICeA\textsuperscript{PKC\textgreek{d}}} pathway and eLPB\textsuperscript{DAT–ovBNST\textsuperscript{PKC\textgreek{d}}} pathway, respectively.
In addition to fMOST results [14], anterograde and retrograde tracing results in the present study also revealed an additional region of substantial innervation, namely ovBNST, which is in line with previous immunohistochemical studies [13, 31]. Convincing empirical support has substantiated the pivotal role of the BNST in facilitating stress-induced reinstatement of drug seeking [32-35]. For example, mimic α2a-adrenergic receptor [33] or activating pituitary adenylate cyclase-activating peptide (PACAP) systems [32] in the BNST was sufficient to induce reinstatement of cocaine. Notably, injections of the corticotropin releasing factor (CRF) receptor antagonist into the BNST attenuated stress-induced reinstatement of cocaine [36] and morphine [37] seeking, whereas no such effect was observed in the amygdala. However, the role of BNST and CeA in reinstatement of drug seeking is controversial, as conflicting findings have been reported in various studies. For example, injections of the CRF receptor antagonist into the amygdala, but not the BNST, attenuated morphine primed-reinstatement of CPP [37]. Furthermore, the blockade of noradrenergic receptors in both the BNST and the CeA effectively prevented stress-induced reinstatement of cocaine seeking, but did not influence cocaine primed-reinstatement [38]. Reversible tetrodotoxin (TTX) lesions of either the BNST or the CeA blocked stress-induced reinstatement of heroin seeking [35]. To data, the investigation of BNST and CeA, particularly those neurons receiving eLPBChAT projections, has been sparsely documented in METH seeking. Here, our study presents the first evidence of the eLPBChAT–lCeAPKC5 pathway and eLPBChAT–ovBNSTPKC5 pathway. Results showed that activating eLPBChAT–ovBNSTPKC5 pathway, but not the eLPBChAT–lCeA PKC5 pathway, suppressed METH-primed reinstatement of CPP in male METH-exposed mice. In light of these findings, we wondered whether distinct outputs from the eLPBChAT mediate different components of the METH-related behavior.

Studies indicated the CeA as a target for anxiolytic agents [39, 40]. The lCeA is often referred to as the “nociceptive amygdala” due to its high concentration of neurons that respond to noxious stimuli which receive nociceptive-specific information through the PBN [41, 42]. The lCeA predominantly consists of PKCδ neurons [43, 44], and optogenetic activation of lCeA PKC5 was anxiolytic [45]. Additionally, LPB neurons projected to the lCeA, forming a functionally significant circuit involved in appetite suppression [31] and the formation of aversive memories [6]. A recent study demonstrated that chemogenetic stimulation of LPB–CeA pathway heightened anxiety-like behavior [13]. In this study, we found that inhibiting eLPBChAT–lCeA PKC5 pathway alleviated anxiety-like behaviors in METH-withdrawn male mice, indicating the eLPBChAT may serve as the primary source of lCeA PKC5 relevant to METH withdrawal-induced anxiety-like behavior.

In the current study, we found that both eLPBChAT and ovBNSTPKC5 neurons were activated following METH-primed reinstatement of CPP, while it was to activating rather than suppressing eLPBChAT–ovBNST pathway blocked METH-primed reinstatement of CPP in mice. We predict that the activation of this pathway may be a compensatory effect for the METH-primed reinstatement of METH CPP. Notably, eLPBChAT neurons were activated as early as METH withdrawal period, while ovBNSTPKC5 neurons were activated after METH priming rather than the METH withdrawal period. Further, we found that activating eLPBChAT neurons enhanced the ACh release within the ovBNST, as well as triggered ovBNSTPKC5 neurons in mice. As such, we suspect that the activation of eLPBChAT neurons is a direct result of the concomitant effect with METH exposure, while the activation of ovBNSTPKC5 neurons might be secondary occurrence only after METH priming during the reinstatement of METH CPP. The BNST mainly consists of GABAergic neurons, which send inhibitory projections to some addiction-related brain regions, such as ventral tegmental area [46] and nucleus accumbens [47]. Therefore, activating eLPBChAT–ovBNSTPKC5 pathway has potential to enhance the inhibitory outputs from ovBNST, which might inhibit these addiction-related regions and subsequently suppress the METH-primed reinstatement of CPP.

There are several limitations in the current study. First, there is lack of female mice models to explore the potential sex differences in the impact of eLPBChAT projections on METH toxicity. Second, there is the absence of PKCδ-promoter-tagged viral tools or animal models, which hindered the precise manipulation of CeA PKC5 or BNSTPKC5 neurons in vivo.

Conclusions

Collectively, our data show that the eLPBChAT is a critical node in the neural networks governing METH withdrawal anxiety and METH primed-reinstatement of CPP through its projections to the lCeA PKC5 and ovBNSTPKC5, respectively.

Abbreviations

Ach: acetylcholine; AUC: area under the curve; CeA: central nucleus of the amygdala; BNST: bed nucleus of the stria terminalis; ChAT: choline acetyltransferase; CNO: clozapine-N-oxide; CPP: conditioned place preference; CRF: corticotropin releasing factor; DREADDs: Designer receptors
exclusively activated by designer drugs; eLPB: external lateral portion of parabrachial nucleus; EPM: elevated plus maze; IACUC: Institutional Animal Care and Use Committee; IceA: lateral portion of central nucleus of amygdala; MEC: Mecamylamine; METH: methamphetamine; MUD: METH use disorders; nAChRs: nicotinic acetylcholine receptors; ovBNST: oval portion of bed nucleus of the stria terminalis; PACAP: pituitary adenylate cyclase-activating polypeptide; PBN: parabrachial nucleus; PFA: paraformaldehyde; PKCδ: protein kinase C delta; sEPSC: spontaneous excitatory postsynaptic currents; TTX: tetrodotoxin; WT: wild type.

Supplementary Material
Supplementary figures. https://www.thno.org/v14p2881s1.pdf

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Author contributions
Chen W, Guo H, Zhou N and Xu X performed the experiments and collected the data. Hu T and Mai Y performed the experiments and data analysis during the revision of the manuscript. Fan Y., Mai Y, and He T performed the data curation and analysis. Wen J, Qin S, Liu C and Wu W assist data analysis and some experiments. Fan Y, Ge F and Guan X prepared figures and wrote the draft. Kim HY edited the manuscript. Guan X, Liu C and Wu W raised the funding. Guan X conceptualized and supervised the project.

Data availability statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing Interests
The authors have declared that no competing interest exists.

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