Dopamine receptor 1 on CaMKII-positive neurons within claustrum mediates adolescent cocaine exposure-induced anxiety and electro-acupuncture therapy

Liying Chen[a,b,1], Zhaoyu Liu[a,1], Ziheng Zhao[a,1], Demin Du[a], Weichao Pan[a], Xiaoyan Wei[a], Jiaxun Nie[a], Feifei Ge[a], Jianhua Ding[c], Yu Fan[a,*], Hee Young Kim[d,*], Xiaowei Guan[a,*]

[a]Department of Human Anatomy and Histoembryology, Nanjing University of Chinese Medicine, Nanjing, China

[b]Department of Critical Care Medicine, Jiangxi Province Hospital of Integrated Chinese and Western Medicine, Nanchang, China

[c]Department of Pharmacology, Nanjing Medical University, Nanjing, China

[d]Department of Physiology, Yonsei University College of Medicine, Seoul, South Korea

[1]These authors are equally contribute to this work

* Correspondence authors: guanxw918@njucm.edu.cn (XG), yfan@njucm.edu.cn (YF), vet202001@yuhs.ac (HYK)

Phone number: +86-25-85811387

Running title: Claustrum D1R^{CaMKII} in cocaine anxiety and EA

Abstract

Adolescent cocaine exposure (ACE) increases risk of developing psychiatric problems such as anxiety, which may drive relapse in later life, however, its underlying molecular mechanism remains poorly understood.

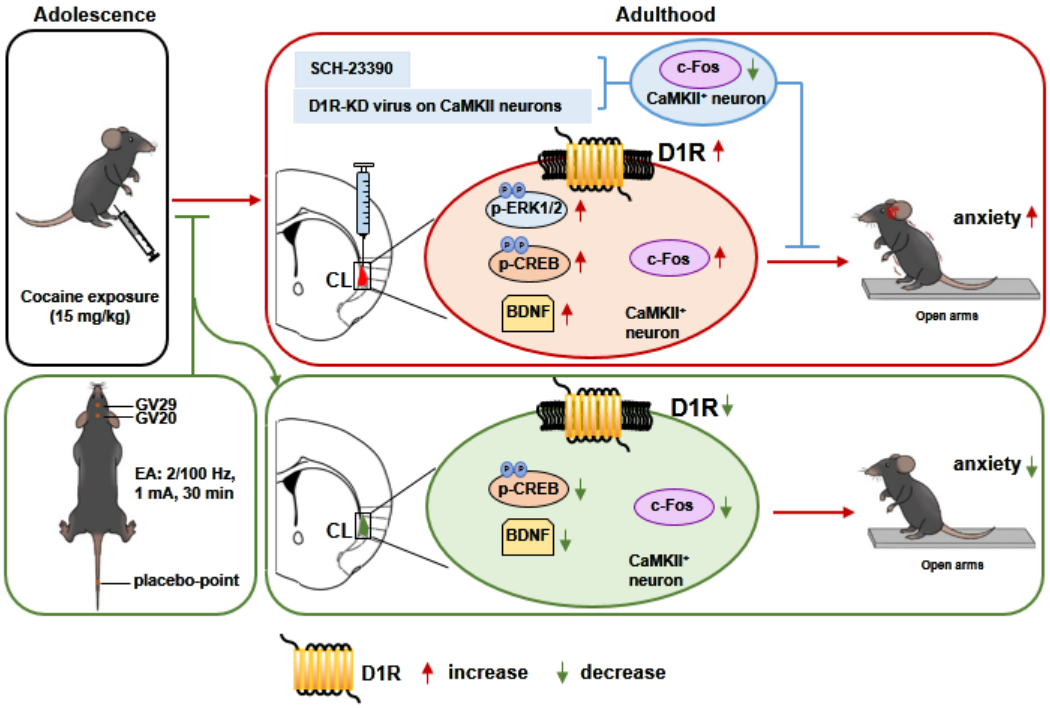
Methods: ACE male mice model were established by exposing to cocaine during adolescent period. Elevated plus maze (EPM) were used to assess anxiety-like behaviors in mice. Within claustrum, local injection of SCH-23390, a specific antagonist for dopamine receptor 1 (D1R), or D1R knocking-down virus were used to regulate D1R function or expression on CaMKII-positive neurons ($D1R^{CaMKII}$) *in vivo*. Electro-acupuncture (EA) treatment was performed at acupoints of Baihui and Yintang during withdrawal period.

Results: We found that ACE mice exhibited anxiety-like behaviors, along with more activated CaMKII-positive neurons and increased $D1R^{CaMKII}$ levels in claustrum during adulthood. Locally inhibiting D1R function or knocking-down $D1R^{CaMKII}$ levels in claustrum efficiently reduced claustrum activation, and ultimately suppressed anxiety in ACE mice during adulthood. EA treatment alleviated ACE-evoked claustrum activation and anxiety by suppressing claustral $D1R^{CaMKII}$.

Conclusion: Our findings identified a novel role of claustrum in ACE-induced anxiety, and put new insight into the claustral $D1R^{CaMKII}$. The claustral $D1R^{CaMKII}$ might be a promising pharmacological target, such as EA treatment, to treat drugs-related psychiatric disorders.

Keywords: Adolescent cocaine exposure; Anxiety; Claustrum; $D1R^{CaMKII}$; Electro-acupuncture

Graphic abstract:



1. Introduction

Adolescents present higher vulnerability to substance abuse [1,2]. Adolescent experience of drugs increase the risk of developing psychiatric disorders that even persists into adulthood [3,4]. Anxiety is a prominent psychiatric symptom of adolescent drug abuse, which is generally believed as critical factor for driving relapse to drugs in later life [5,6]. Previously, we showed that adolescent cocaine-exposed (ACE) mice exhibit more anxiety in their adulthood [7,8], however, the involved brain region remains much to be clarified.

The claustrum is a thin stripped nucleus located between insular cortex and putamen, being architected in anterior-posterior and dorsal-ventral organization. Above 90% neurons in claustrum are glutamatergic (CaMKII-positive) neurons [9]. Anatomically, the claustrum has extensive reciprocal connectivity with most neocortical regions [10-13] and communicates densely with limbic structure and thalamus [14,15], implying that claustrum may be a hub for integrating and relaying “executive process” of cortex. At present, the functions of claustrum remain poorly understood. Emerging studies demonstrate that claustrum may be key nucleus involved in consciousness [16], attention [17,18] and cognition [19-21]. The claustrum has been implicated in numerous brain disorders, such as seizures [22], autism [23] and Parkinson Disease [24] , but it has not been evidently as the core structure of any specific disease. In marijuana users [25,26] or internet game addicts [27], the claustrum shows greater activity. Recently, Terem et al. [28] found that the dopamine receptor 1 (D1R)-positive neurons in claustrum is required for acquisition of cocaine-preferred behaviors, and stimulating these neurons could drive context-reinstatement of cocaine preference. In addition, the other recent study shows that the claustrum

mediates stress-induced anxiety in mice [29]. These studies demonstrate that claustrum is involved in addiction and anxiety, but its role in drugs-related anxiety is unknown.

Currently, there is few pharmacotherapy options for adolescent substance use-induced anxiety, partially due to serious side-effects caused by anxiolytic medicine. As a non-invasive therapeutic approach, acupuncture has been considered as an efficient intervention on substance use, especially for treatment of the protract abstinence syndrome (such as anxiety) [30,31], but its pharmacological targets remain largely unclear. The electro-acupuncture (EA), a kind of peripheral acupuncture with electric stimulation, has been shown to improve anxiety without adverse effects [32,33]. Previously, we found that EA at acupoints of Baihui (GV 20) and Yintang (GV 29) effectively reduced the anxiety in ACE mice during adulthood [34]. Early in 2006, Shapo et al. [35] reported that acupuncture at Weizhong points (BL40) could significantly evoke claustrum in humans. Whether claustrum is one of key targets for EA to treat ACE-related anxiety, and its potential pharmacological mechanism, has not been explored yet.

In the present study, male mice were exposed to cocaine during adolescent period from postnatal day 28 (P28) to P42 [36]. EA treatment at acupoints of Baihui (GV 20) and Yintang (GV 29) were performed in male mice during withdrawal period. When growing up to adult ages, the mice were subjected to anxiety behavioral tests to explore the role of claustral D1R, especially expressed on CaMKII-positive neurons ($D1R^{CaMKII}$), in ACE-induced anxiety and EA therapy.

2. Materials and methods

2.1 Animals

Male C57BL/6 wild type (WT) mice weighing 12-16 g were used. All animals were housed at constant humidity (40~60%) and temperature (24 ± 2ºC) with a 12-hour light/dark cycle (lights on at 8 a.m.) and allowed free access to food and water. Handling of the animals was carried out for three days before onset of experiments. 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.

2.2 Drug treatment

On P28, male C57BL/6 WT mice were assigned to receive a single daily injection of cocaine hydrochloride (15 mg/kg, dissolved in saline, i.p., Qinghai Pharmaceutical, China) or saline (0.2 mL, i.p.) once daily at 10 a.m. for 14 consecutive days. From P42-P70, these mice were kept at their home cage (four mice per cage). On P71, elevated plus maze (EPM) tests were performed in mice. On P77, the mice were sacrificed within 90 min after stimulus of EPM procedure, and the brain tissue was collected for qPCR, western blot and immunofluorescence.

2.3 Electro-acupuncture treatment

During P42 to P70, two acupuncture needles (0.16 × 7 mm, Hwato, China) were inserted gently in a depth of 2–3 mm at the acupoints of Baihui (GV20, the midpoint between the auricular apices) and Yintang (GV29, the midpoint between the medial ends of the two eyebrows) in each mouse, the acupoints

were electric mixed stimuli of 2/100 Hz at an intensity of 1 mA (HANS Electronic Apparatus, China) for 30 min once a day at acupoints of GV20 and GV29 in EA mice, or at "placebo-point" (at 1 cm from base of the tail) in Sham EA (SEA) mice.

2.4 Behavioral tests

On P71, EPM was performed to assess anxiety-like behaviors. All behavioral tests were conducted during light cycle. The EPM apparatus (TopScan, Kansas City, MO, USA) consists of 4 elevated arms (52 cm above the floor) arranged in a cross pattern with two 1-cm walled arms (open arms) and two 40-cm walled arms (closed arms). Each mouse was placed in the center portion of the EPM with its head facing an open arm and allowed freely to explore the maze for 5 min. The time spent, the entries and the distance traveled in the open arms were recorded.

2.5 Western blot

On P77, total protein was extracted from claustrum in mice using RIPA lysis buffer (Beijing ComWin Biotech Co., China) according to the manufacturer's instructions. Protein samples (15 μg) was separated by 10% SDS–PAGE, 12% SDS–PAGE and electrophoretically transferred onto PVDF membranes. The transferred membranes were blocked with 5% non-fat dry milk or 5% BCA and 0.1% Tween 20 in 10 mM Tris–HCl (TBST buffer, pH 7.5) for 1.5 h at room temperature, then subsequently incubated with the following primary antibodies: D1R (1:1000, Rabbit, RRID:AB_10598308, Proteintech, USA), D2R (1:1000, Rabbit, ER1907-70, Hua An Biotechnology Company, China), D3R (1:1000, Mouse, ab155098, Abcam, UK), $ERK_{1/2}$ (1:1000, Rabbit, BM4326, Boster,

China), p-ERK$_{1/2}$ (1:1000, Rabbit, BM4326, Boster, China), CREB (1:1000, Rabbit, RRID:AB_10699020, Cell Signaling Technology, USA), p-CREB (1:1000, Rabbit, RRID:AB_2561044, Cell Signaling Technology, USA), c-Fos (1:2000, Rabbit, RRID:AB_2247211, Cell Signaling Technology, USA), BDNF (1:1000, Mouse, RRID:AB_2881675, Proteintech, USA). The next day, the membranes were washed three times in Tris-buffered saline with Tween 20 (TBS-T) and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody goat anti-rabbit or goat anti mouse (1:5000, Beijing ComWin Biotech Co., China) at room temperature for 1 h. The blots were visualized by the ECL kit (Beyotime, China) and the signal was visualized by imaging system (Tanon-5200, Shanghai, China). The blots were washed with stripping buffer (Beyotime Institute of Biotechnology) to allow reprobing with other antibodies. In this study, GAPDH or β-actin was used as the loading control. Values for target protein levels were calculated using Image J software (NIH, USA). The relative level of each protein expression (blot volume) was normalized to GAPDH or β-actin.

2.6 RNA extraction and quantitative real-time PCR

On P77, mice were decapitated within 90 min after stimulus of EPM procedure. Claustrum were collected immediately. Total RNA was extracted from the middle of claustrum using FastPure Cell/Tissue Total RNA Isolation Kit (Vazyme, China), according to the manufacturer's instructions. All samples were tested for concentration and purified using a 4200 TapeStation (Agilent Technologies, Santa Clara, USA). After RNA extraction, the amount of RNA was normalized across samples, and cDNA was created using iScript (1708891, Bio-Rad, Hercules, USA). Actin was used as the internal control. The primers of *D1R,*

D2R, *D3R* and Actin were as follows: *D1R* (forward, ATGGCTCCTAACACTTCTACCA; reverse, GGGTATTCCCTAAGAGAGTGGAC); *D2R* (forward, ACCTGTCCTGGTACGATGATG; reverse, GCATGGCATAGTAGTTGTAGTGG); *D3R* (forward, AGACAACATGGAGAGCTGAAACGCT; reverse, TTCAGGGCTGTGGATAACCTGCC); Actin (forward, GGCTGTATTCCCCTCCATCG; reverse, CCAGTTGGTAACAATGCCATGT). *D1R*, *D2R* and *D3R* mRNA levels were normalized to Actin mRNA levels. The relative mRNA level was calculated by the comparative CT method ($2^{-\Delta\Delta Ct}$).

2.7 Immunofluorescence

On P77, mice brains were collected within 90 min after stimulus of EPM procedure and perfused with 4% paraformaldehyde (PFA). The coronal brain sections (30 μm) were cut on a cryostat (Leica, Germany). 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: rabbit anti-c-Fos (1:2000, RRID: AB_2247211, Cell Signalling Technology, USA), mouse anti-CaMKIIα (1:100, RRID: AB_626789, Santa Cruz, USA) and rabbit anti-DRD1 (1:100, RRID: AB_10598308, Proteintech, USA), followed by the corresponding fluorophore-conjugated secondary antibodies for 1 h at room temperature. The following secondary antibodies were used here: Alexa Fluor 555-labeled donkey anti-rabbit secondary antibody (1:500, RRID: AB_162543, Invitrogen, USA), Alexa Fluor 488-labeled donkey anti-mouse secondary antibody (1:500, RRID: AB_141607, Invitrogen, USA). Fluorescence signals were visualized using a Leica DM6B

upright digital research microscope (Leica, Germany) or Leica TCS SP8 (Leica, Germany). The mean gray value of D1R on CaMKII-positive neurons of claustrum was analyzed by Image J software (NIH, USA).

2.8 Local injection of SCH-23390 in claustrum

On P64, the cannula (O.D. 0.41 mm, C.C 3.0 mm, Cat 62004, RWD, China) were planted into claustrum (AP + 0.62 mm, ML ± 3.05 mm, DV – 2.50 mm) of ACE mice. After surgery, mice were maintained at home cage about 1 week. On P71, mice were subjected to EPM. The D1 receptor antagonist R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride (SCH-23390, Selleck chemicals, China) was dissolved in 0.9% saline and stored -80°C. A volume of 70 nL of SCH-23390 were bilaterally injected into claustrum at a rate of 10 nL/min at 20 min prior to behavioral tests.

2.9 Local knockdown of D1R on CaMKII-positive neurons of claustrum

On P50, mice were anesthetized with 2% isoflurane in oxygen, and were fixed in a stereotactic frame (RWD, Shenzhen, China). A heating pad was used to maintain the core body temperature of the mice at 36°C. The coordinates of claustrum were defined as AP + 0.62 mm, ML ± 3.05 mm, DV –3.50 mm. A volume of 70 nL virus of *CaMKIIap-EGFP-MIR155(shDrd1)-SV40 PolyA* (KD group, Titer: 8.89E+13v.g/ml, GeneChem, Shanghai, China) and *CaMKIIap-EGFP-MIR155(MCS)-SV40 PolyA* (Ctrl group, Titer: 1E+13v.g/ml, GeneChem, Shanghai, China) were injected bilaterally into claustrum at a rate of 10 nL/min. After surgery, mice were kept at home-cage about 3 weeks.

2.10 Statistical analysis

Statistical analysis was carried out using GraphPad Prism software (Version 8.0.2). Data are presented as the Mean ± SEM. All data was analyzed by unpaired t-tests. Statistical significance was set as $p < 0.05$.

3. Results

3.1 ACE evokes claustral CaMKII-positive neurons and D1R^{CaMKII} levels, and anxiety during adulthood

To examine anxiety-like behaviors in mice, mice were subjected to behavioral tests of EPM during their adulthood (Figure 1A). As shown in Figure 1B, ACE mice (n = 16 mice) exhibited less time spent (t = 3.813, p = 0.0006) and less entries (t = 2.823, p = 0.0084) into the open arms, while similar total distances traveled in EPM apparatus (t = 0.6731, p = 0.5061) than those of adolescent saline exposure (ASE) mice (n = 16 mice) during adulthood. These results indicate that ACE cause anxiety in mice during adulthood.

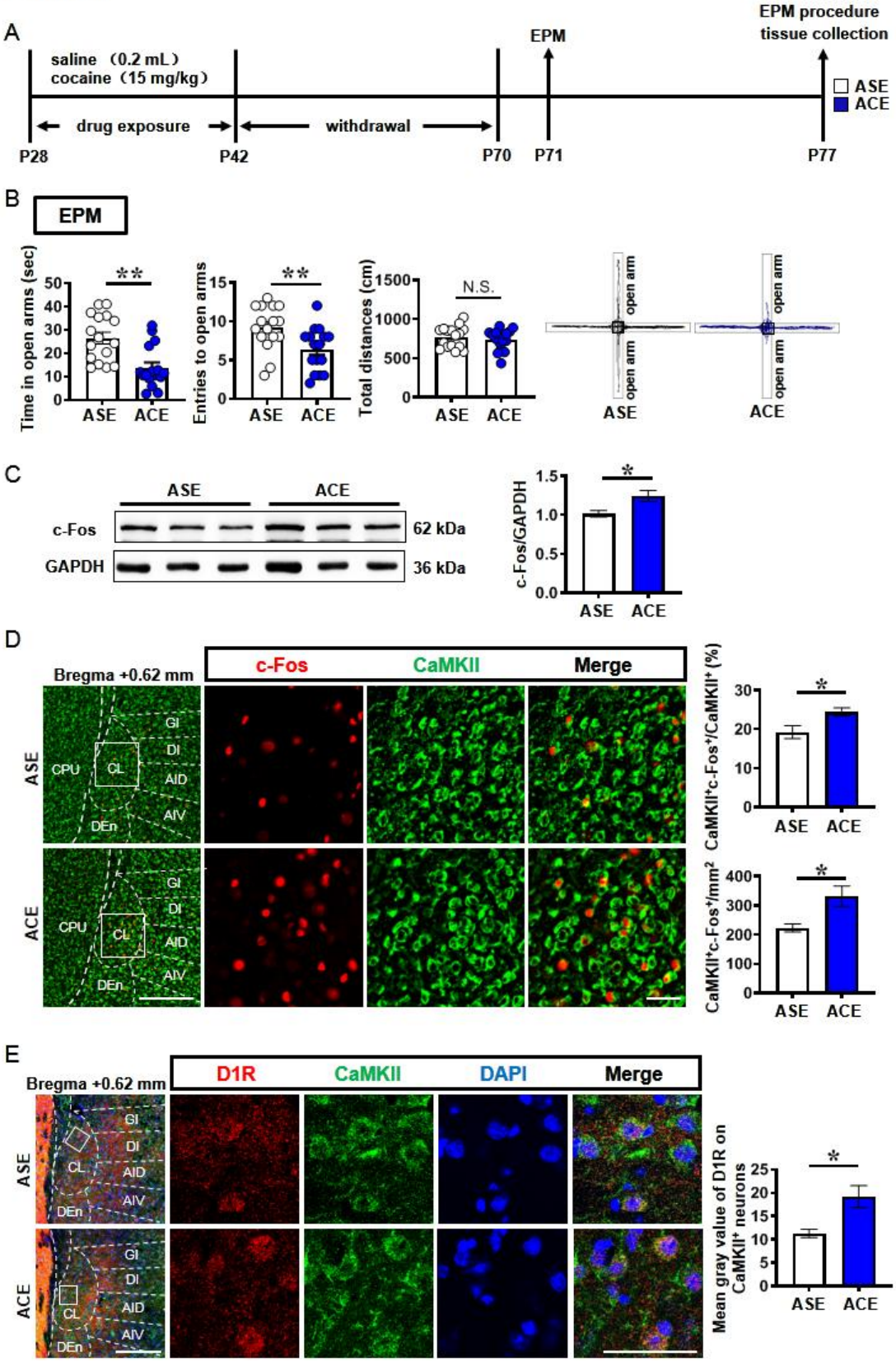
The c-Fos and CaMKII were used as markers to detect the neuronal activity and glutamatergic neurons, respectively. The total protein level of c-Fos (t = 2.811, p = 0.0483) was higher in ACE (n = 3 mice) than in ASE mice (n = 3 mice) during adulthood (Figure 1C). Both the percentage (t = 2.466, p = 0.0358) and number (t = 3.106, p = 0.0126) of c-Fos-positive & CaMKII-positive neurons were higher in ACE mice (n = 6 mice) than those in ASE mice (n = 5 mice) during adulthood (Figure 1D). These results indicate that ACE increase claustrum activity, especially the CaMKII-positive neurons, in male mice during adulthood.

On CaMKII-positive neurons, D1R level was much higher (t = 3.177, p = 0.0336) in ACE (n = 3 mice) than in ASE mice (n = 3 mice) during adulthood

(Figure 1E). The total levels of mRNA (t = 3.502, p = 0.0249, Figure 1F) and protein (t = 7.389, p = 0.0018, Figure 1G) of claustral D1R was higher in ACE (mRNA, n = 3 mice; protein, n = 3 mice) than ASE mice (mRNA, n = 3 mice; protein, n = 3 mice) during adulthood. There were no significant differences in claustral dopamine receptor 2 (D2R) mRNA (t = 0.5756, p = 0.5957, Figure 1F) and protein (t = 0.1621, p = 0.8791, Figure 1G), and claustral dopamine receptor 3 (D3R) mRNA (t = 0.1051, p = 0.9214, Figure 1F) and protein (t = 1.159, p = 0.2035, Figure 1G) between ACE mice (mRNA, n = 3 mice; protein, n = 3 mice) and ASE mice (mRNA, n = 3 mice; protein, n = 3 mice) during adulthood. These results indicate that ACE obviously increase D1R level, particularly those expressed on CaMKII-positive neurons in claustrum during adulthood.

In parallel, the protein levels of p-$ERK_{1/2}$ (t = 4.905, p = 0.0080), ratio of p-$ERK_{1/2}$ to $ERK_{1/2}$ (t = 3.239, p = 0.0317), p-CREB t = 5.871, p = 0.0042), p-CREB/ CREB (t = 3.502, p = 0.0248) and BDNF (t = 2.940, p = 0.0424) were higher in ACE mice (n = 3 mice) than ASE mice (n = 3 mice), but that of $ERK_{1/2}$ (t = 0.9475, p = 0.3970) and CREB (t = 0.4336, p = 0.6869) had no significant difference between ACE (n = 3 mice) and ASE mice (n = 3 mice) during adulthood (Figure 1H, 1I). These results indicate that ACE increase $D1R^{CaMKII}$ level and its potential subsequent signals in claustrum, which may be associated with ACE-induced claustral activation and anxiety during adulthood.

Figure 1


A
saline (0.2 mL)
cocaine (15 mg/kg)
drug exposure
withdrawal
EPM
EPM procedure
tissue collection
ASE
ACE
P28
P42
P70
P71
P77
B
EPM
Time in open arms (sec)
Entries to open arms
Total distances (cm)
N.S.
open arm
ASE
ACE
C
c-Fos
GAPDH
62 kDa
36 kDa
c-Fos/GAPDH
D
Bregma +0.62 mm
c-Fos
CaMKII
Merge
CPU
CL
GI
DI
AID
AIV
DEn
CaMKII+c-Fos+/CaMKII+ (%)
CaMKII+c-Fos+/mm2
E
D1R
CaMKII
DAPI
Merge
Mean gray value of D1R on CaMKII+ neurons

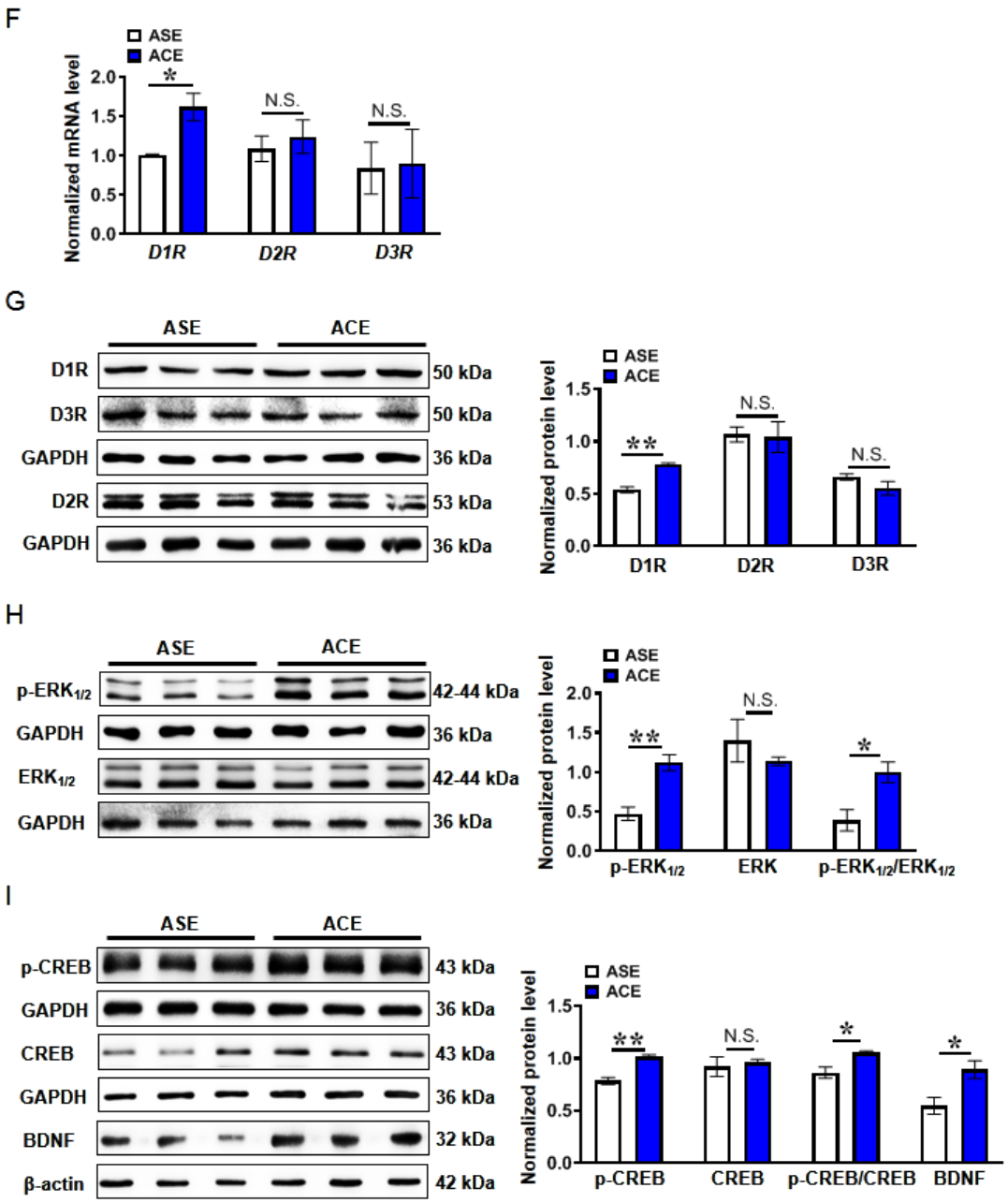


Figure 1. ACE enhances anxiety along with increased CaMKII-positive neuron activity and D1R^{CaMKII} level in claustrum during adulthood. A, Experimental timeline. **B**, EPM test. **C**, Total protein levels of c-Fos. **D**, The percentage and number of c-Fos-positive & CaMKII-positive neurons. Scale bar, 300 μm/50 μm. **E,** The expression of D1R^{CaMKII}. Scale bar, 200 μm/50 μm. **F**, The mRNA levels of *D1R, D2R and D3R*. **G,** The protein levels of D1R, D2R

and D3R. **H,** Levels of p-ERK$_{1/2}$ and ERK$_{1/2}$. **I,** Levels of p-CREB, CREB and BDNF. ASE, adolescent saline exposure; ACE, adolescent cocaine exposure; CL, claustrum. N.S., $p > 0.05$, *, $p < 0.05$, **, $p < 0.01$ vs ASE.

3.2 Locally inhibiting claustral D1R suppresses ACE-induced anxiety and claustrum activation during adulthood

To evaluate the role of claustral D1R in ACE-induced anxiety, the selective D1R antagonist SCH-23390 was bilaterally injected into claustrum 20 min prior to the tests of EPM (Figure 2A). Compared with vehicle-treated mice (n = 10 mice), SCH-23390 treatment (n = 8 mice) significantly increased the time spent in open arms ($t = 2.689$, $p = 0.0161$), and did not affect the entries into the open arms ($t = 0.7529$, $p = 0.4624$) and the total distances traveled in EPM apparatus ($t = 0.8365$, $p = 0.4152$) in ACE mice during adulthood (Figure 2B). These results imply that inhibiting claustral D1R could ameliorate ACE-induced anxiety during adulthood.

As shown in Figure 2C, local SCH-23390 treatment (n = 6 mice) significantly attenuated the number of c-Fos-positive neurons ($t = 3.097$, $p = 0.0113$), when compared with vehicle-treated mice (n = 6 mice). These results indicate that inhibiting claustral D1R attenuate claustral activity during adulthood.

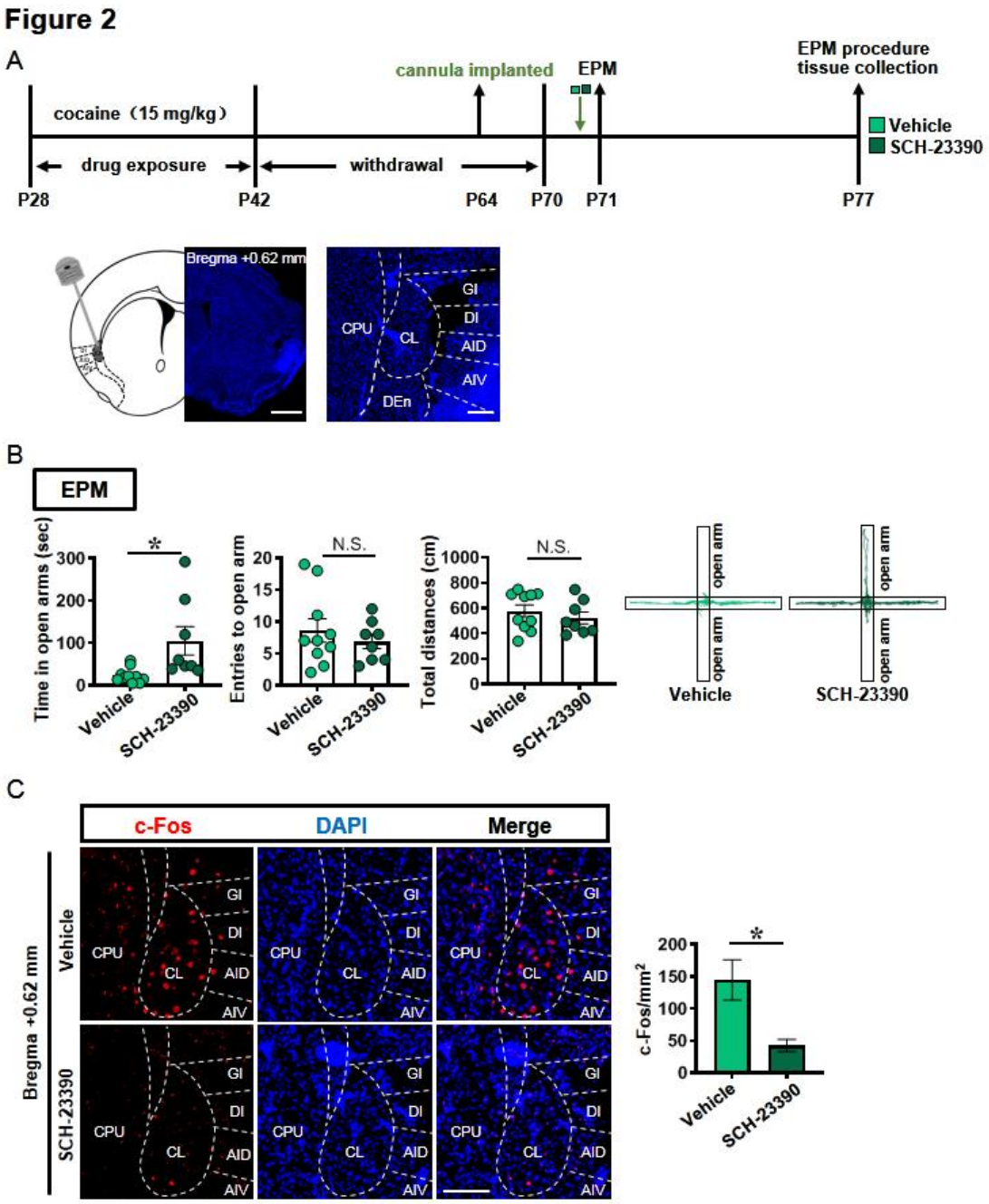


Figure 2. Inhibiting claustral D1R suppresses ACE-induced anxiety and claustrum activation in mice during adulthood. A, Experimental timeline and local injections of SCH-23390 into claustrum. Scale bar, 400/100 μm. **B**, EPM test. **C**, The number of c-Fos-positive neurons. Scale bar, 100 μm. Vehicle, vehicle-treated ACE mice; SCH-23390, SCH-23390-treated ACE mice; CL,

claustrum. N.S., $p > 0.05$, *, $p < 0.05$, **, $p < 0.01$ vs Vehicle.

3.3 Knocking-down claustral $D1R^{CaMKII}$ level suppresses ACE-induced anxiety and claustrum activation during adulthood

Specific knockdown of claustral $D1R^{CaMKII}$ was performed in ACE mice (Figure 3A). Adeno-associated virus of either *CaMKIIap-EGFP-MIR155(shDrd1)-SV40 PolyA* (ACE_{KD}) or *CaMKIIap-EGFP-MIR155(MCS)-SV40 PolyA* (ACE_{Ctrl}) was bilaterally infused into claustrum, and CaMKII-positive neurons within claustrum were transfected with virus (Figure 3B). Compared with ACE_{Ctrl} mice (n = 3 mice), the levels of D1R protein (t = 3.465, p = 0.0257, Figure 3C) was decreased in claustrum of ACE_{KD} mice (n = 3 mice). These results confirmed the efficient effects of knocking-down virus on claustral $D1R^{CaMKII}$ used in claustrum.

As shown in Figure 3D, ACE_{KD} mice (n = 9 mice) exhibited more time spent (t = 3.073, p = 0.0083) and more entries into the open arms (t = 2.695, p = 0.0174), while did not affect the total distances traveled (t = 1.148, p = 0.2701) in EPM apparatus, when compared with ACE_{Ctrl} mice (n = 7 mice). These results indicate that knocking-down claustral $D1R^{CaMKII}$ could suppress ACE-induced anxiety during adulthood.

In parallel (Figure 3E), the number of c-Fos-positive neurons in EGFP-transfected neurons (t = 3.207, p = 0.0327) and the mean gray value of c-Fos in EGFP-transfected neurons (t = 3.006, p = 0.0397) were much lower in ACE_{KD} mice (n = 3 mice) than that in ACE_{Ctrl} mice (n = 3 mice).

Figure 3

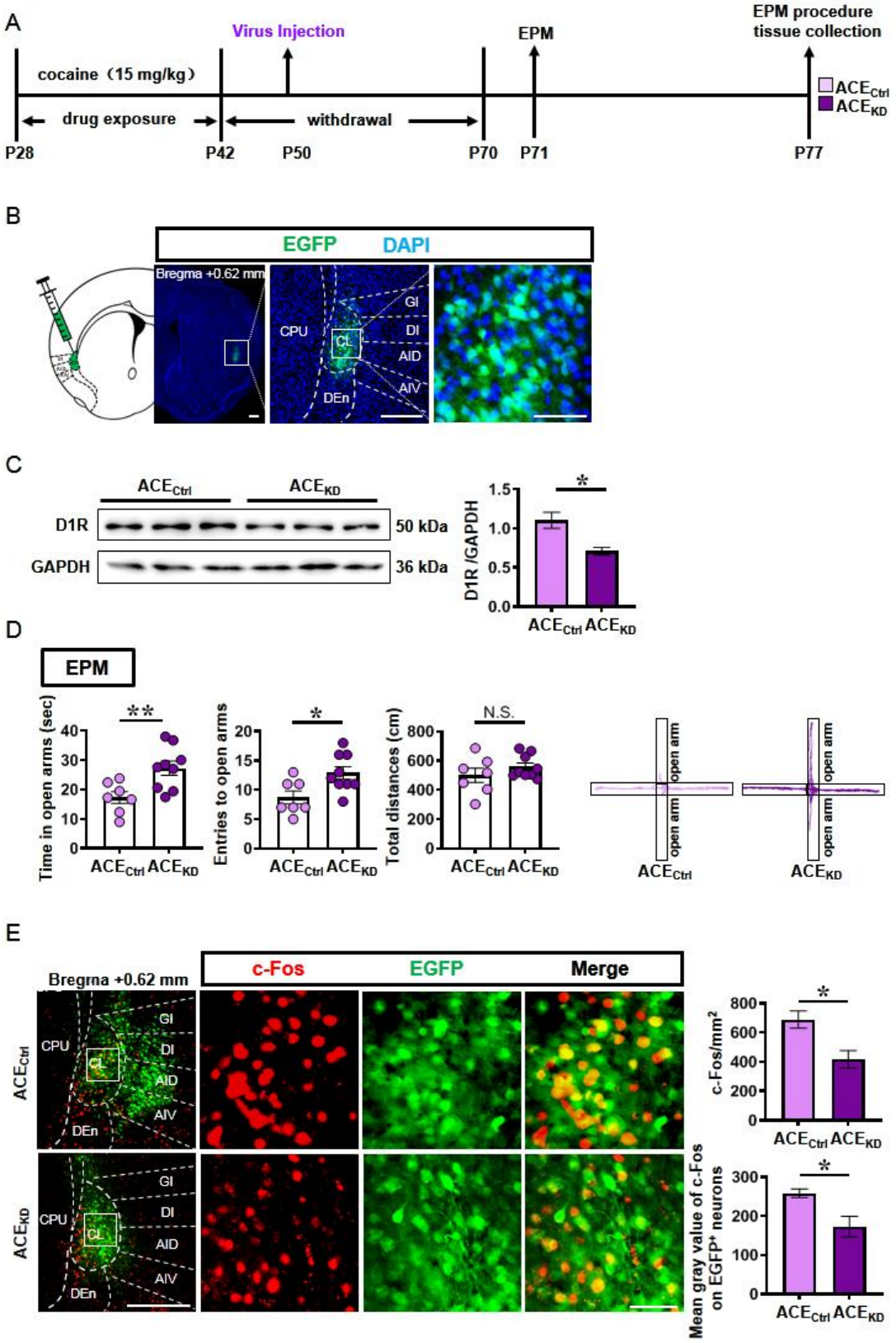


Figure 3. Knocking-down claustral D1R^{CaMKII} level suppresses ACE-

induced anxiety and claustrum activation during adulthood. A, Experimental design and timeline. **B**, Local injections of virus into claustrum. Scale bar, 300 μm. **C**, The D1R protein level. **D**, EPM test. **E**, The percentage of c-Fos-positive neurons in EGFP-transfected neurons and the mean gray value of c-Fos in EGFP-transfected neurons. Scale bar, 400 μm/50 μm. ACE_{Ctrl}, ACE mice injected with control virus; ACE_{KD}, ACE mice injected with knockdown virus; CL, claustrum. N.S., $p > 0.05$, *, $p < 0.05$, **, $p < 0.01$ vs ACE_{Ctrl}.

3.4 EA treatment rescues anxiety, the activated claustrum activation and the increased $D1R^{CaMKII}$ in ACE mice during adulthood

EA treatments at acupoints of GV20 and GV29 during P42-P70 were carried out in ACE mice (ACE_{EA}, n = 10 mice), and placebo acupoints were used as sham EA treatment (ACE_{SEA}, n = 13 mice) (Figure 4A, 4B). Compared with ACE_{SEA} mice, ACE_{EA} mice spent more time ($t = 2.084$, $p = 0.0496$), similar entries into the open arms ($t = 1.536$, $p = 0.1394$) and similar total distances traveled in EPM apparatus ($t = 0.7660$, $p = 0.4522$, Figure 4C). These results indicate that EA at GV20 and GV29 efficiently alleviate ACE-induced anxiety during adulthood.

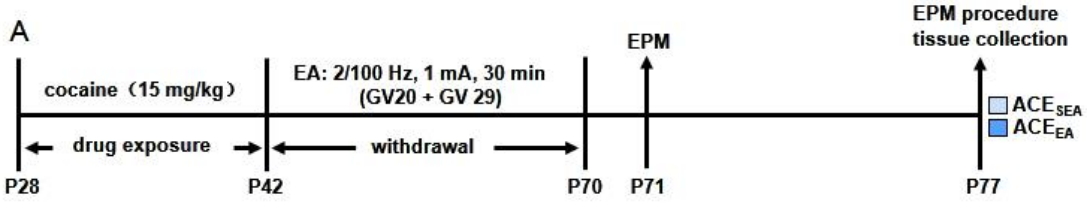
Compared with ACE_{SEA} mice (n = 6 mice, Figure 4D), both the percentage ($t = 4.246$, $p = 0.0017$) and the number ($t = 3.076$, $p = 0.0117$) of c-Fos-positive & CaMKII-positive neurons were much lower in ACE_{EA} mice (n = 6 mice). In parallel, both levels of total D1R protein in claustrum ($t = 4.396$, $p = 0.0117$, Figure 4E) and $D1R^{CaMKII}$ staining ($t = 4.874$, $p = 0.0082$, Figure 4F) were lower in ACE_{EA} mice (n = 3 mice) than that in ACE_{SEA} mice (n = 3 mice). These results

indicate that EA at GV20 and GV29 could suppress CaMKII-positive neuron activity and $D1R^{CaMKII}$ level in claustrum.

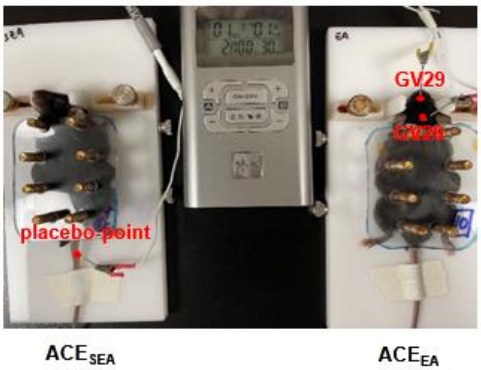
As shown in Figure 4G-H, there were no differences on protein levels p-$ERK_{1/2}$ (t = 0.2242, p = 0.8336), $ERK_{1/2}$ (t = 0.7388, p = 0.5010), CREB (t = 0.9656 p = 0.3889) and ratio of p-$ERK_{1/2}$ to $ERK_{1/2}$ (t = 0.3008, p = 0.7786) in claustrum between ACE_{EA} mice (n = 3 mice) and ACE_{SEA} mice (n = 3 mice). The levels of p-CREB (t = 4.464, p = 0.0111), BDNF (t = 2.957, p = 0.0417) and radio of p-CREB to CREB (t = 3.191, p = 0.0332) were lower in ACE_{EA} mice (n = 3 mice), when compared with ACE_{SEA} mice (n = 3 mice). These results imply that p-CREB and BDNF might be downstream signals of D1R targeted by EA treatment.

Figure 4

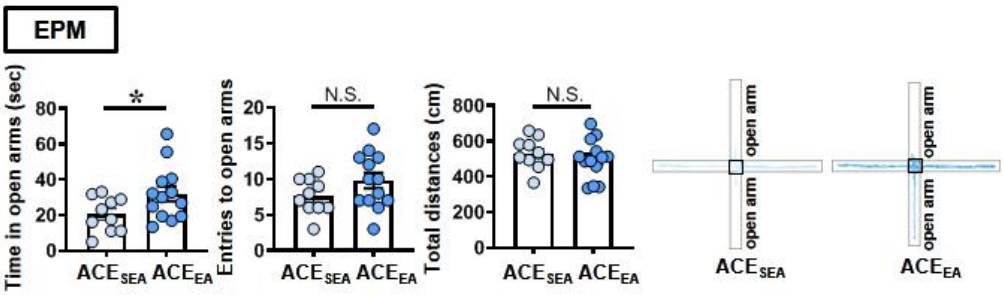
A



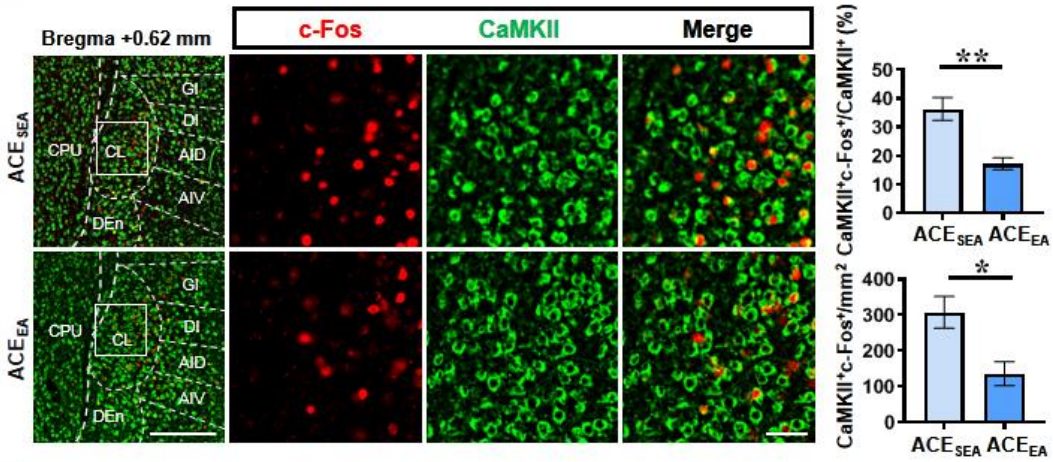
B



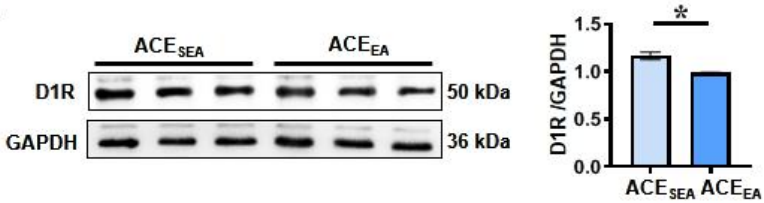
C



D



E



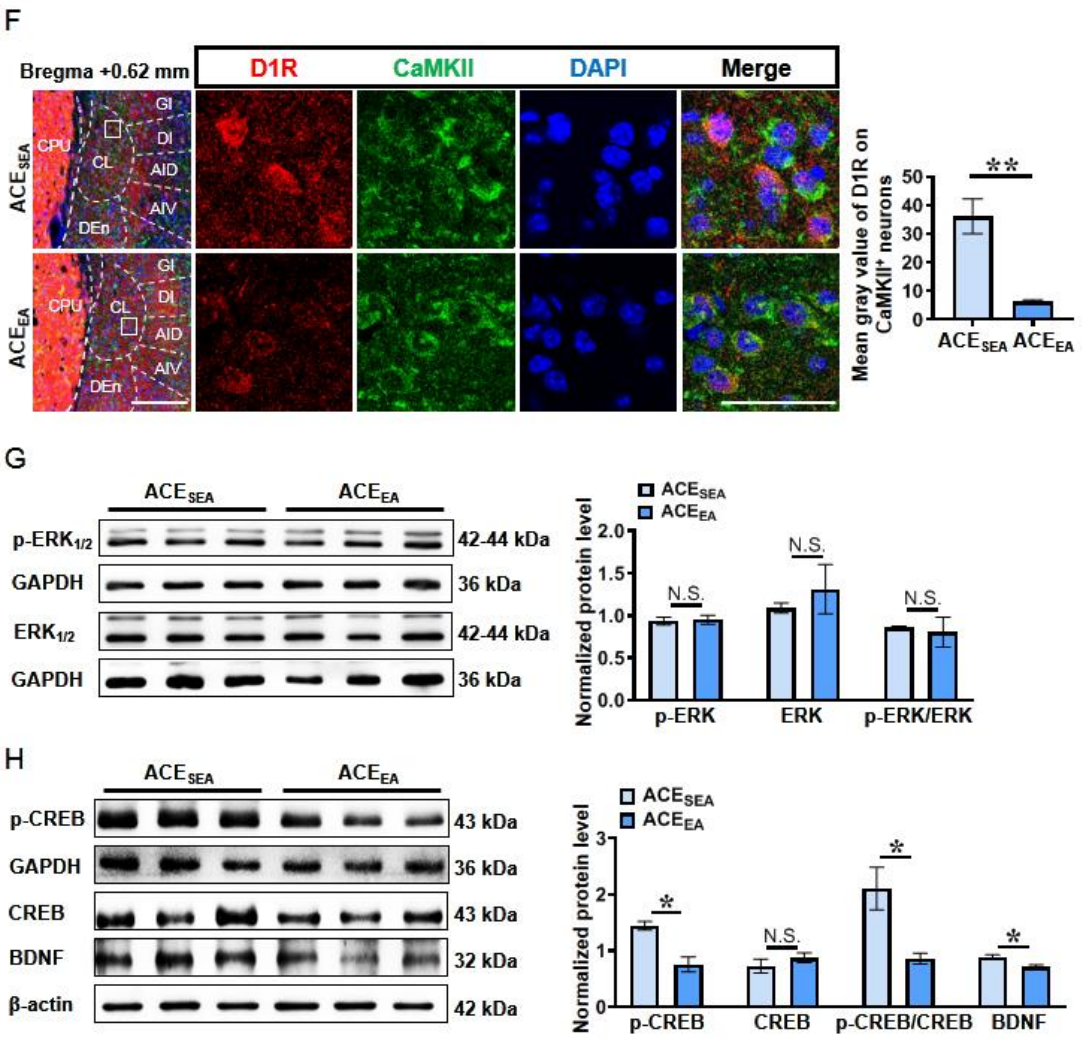


Figure 4. EA treatment rescues ACE-increased anxiety, claustrum activation and D1R^{CaMKII} level. A, Experimental design and timeline. **B**, Schematic diagram of EA and SEA treatments in mice. **C**, EPM test. **D**, The percentage and number of c-Fos-positive & CaMKII-positive neurons. Scale bar, 300 μm/50 μm. **E**, The D1R protein level. **F**, The expression of D1R^{CaMKII}. Scale bar, 200 μm/50 μm. **G**, Levels of p-ERK$_{1/2}$ and ERK$_{1/2}$. **H**, Levels of p-CREB, CREB and BDNF. ACE$_{SEA}$, sham electro-acupuncture-treated ACE mice; ACE$_{EA}$: electro-acupuncture (GV 20 and GV 29)-treated ACE mice; CL, claustrum. N.S., $p > 0.05$, *, $p < 0.05$, **, $p < 0.01$ vs ACE$_{SEA}$.

4. Discussion

The current study explored the role of claustrum in ACE-induced anxiety during adulthood. We found that ACE produces obvious anxiety in male mice during adulthood, along with being evoked CaMKII-positive neurons and increased $D1R^{CaMKII}$ in claustrum. Locally inhibiting D1R function or knocking-down $D1R^{CaMKII}$ levels within claustrum efficiently suppresses ACE-increased claustrum activation, and ultimately rescues ACE-induced anxiety in mice during adulthood. EA at acupoints of GV 20 and GV 29 alleviates ACE-induce anxiety, potentially by targeting claustral $D1R^{CaMKII}$ (Figure 5).

Figure 5

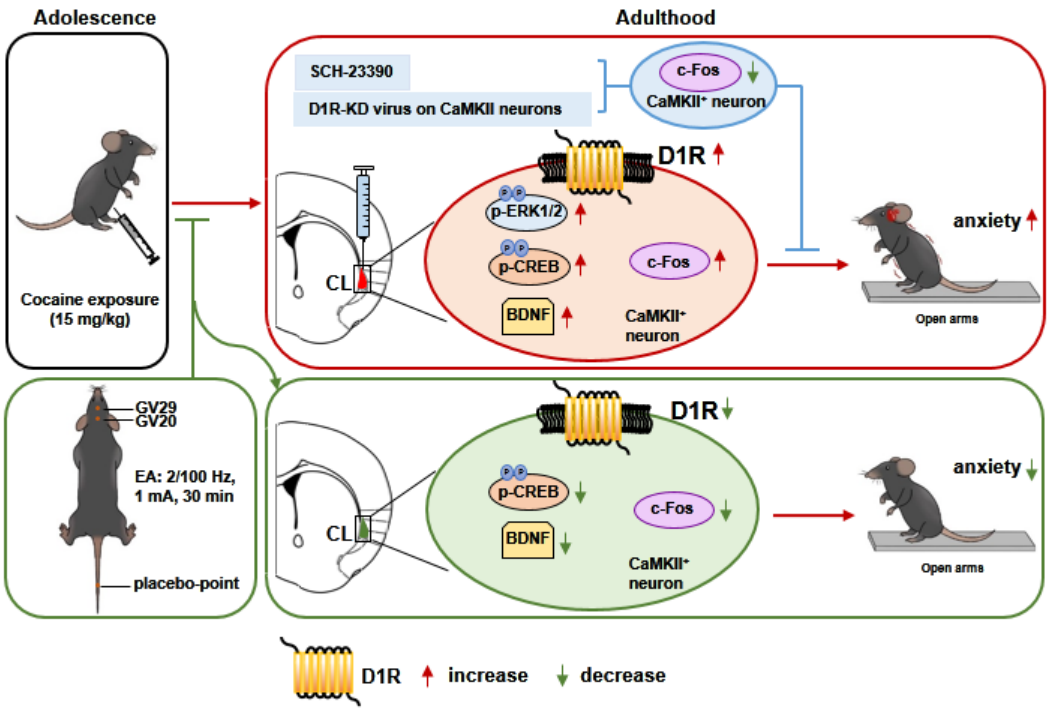


Figure 5. Schematic summary of present study. Adolescent cocaine exposure (ACE) enhances anxiety-like behaviors in male mice during adulthood, accompanied with triggered activation of CaMKII-positive neurons and more dopamine receptor 1 (D1R) on CaMKII-positive neurons ($D1R^{CaMKII}$)

in claustrum. Either blocking D1R with SCH23390 or knocking-down $D1R^{CaMKII}$ with virus in the claustrum efficiently reduces claustrum activation, and ultimately suppresses ACE-induced anxiety in mice. By targeting claustral $D1R^{CaMKII}$, electro-acupuncture (EA) at acupoints of GV20 and GV29 rescues ACE-increased anxiety in mice during adulthood. In the process, the p-CREB and BDNF may be the potential subsequent signals to $D1R^{CaMKII}$.

Consistent with human and animal studies on ACE [7,34,37,38], including ours, here we found that ACE mice exhibited more anxiety in their adulthood. Further, we found that CaMKII-positive neurons in claustrum seem to be involved in the process. Recently, Niu et.al [29] report that claustrum is crucial encoding stress-induced anxiety, as indicated by attenuation of stress-induced anxiety with silencing CaMKII-positive neurons in claustrum. Claustrum receives inputs and sends back axonal outputs to the prefrontal cortex (PFC), a critical region coding cocaine-related anxiety [39,40]. In our previous study, we found that ACE altered the function of local circuit from GABAergic interneuron to pyramidal neurons in mPFC, which mediates ACE-increased anxiety in adult mice [7]. Due to the robust communications between mPFC and claustrum, ACE-triggered claustrum should contribute to the anxiety in mice of adulthood. Firstly, we explored the potential molecules that underlie the evoked claustrum by ACE. A recent study by Terem et al. [28] found that D1R-positive ($D1R^{+}$) neurons in claustrum, especially those connected with PFC are required for acquisition and reinstatement of cocaine preference. They focus the cell subgroups of $D1R^{+}$ and $D1R^{-}$ in cocaine-preferred behaviors. Here, our findings show that it is $D1R^{CAMKII}$ that were elevated by ACE in claustrum of adult mice, and we focus on the role of D1R in activation of CaMKII-positive neurons and

ACE-related anxiety. Moreover, locally blocking D1R or knocking-down $D1R^{CaMKII}$ in claustrum reduced ACE-evoked claustrum and subsequent anxiety. These findings indicate that activity of claustrum CaMKII-positive neurons encodes ACE-increased anxiety, which is mediated at least partially by $D1R^{CaMKII}$. In other D1R-rich brain regions, such as PFC and hippocampus, cocaine abuse could alter p-ERK [41], p-CREB [42] and BDNF [43,44]. Here, we found that protein levels of p-ERK, p-CREB and BDNF were significantly increased in claustrum of ACE mice, implying that they are potential downstream signals for claustral $D1R^{CaMKII}$ in the process of ACE-induced anxiety.

At present, neither D1R antagonists nor specific virus-regulated D1R could be clinically used to treat emotional disorders such as ACE-induced anxiety in humans. EA has been widely applied in management of substance abuse, especially for drugs-caused withdrawal symptoms, including anxiety [33,34,45]. Shao et al [46] reported that EA at Zusanli (ST36) and Kunlun (BL60) could reduce anxiety-like behaviors in animals by suppressing neuropeptide Y in anterior cingulate cortex. Wu et al. [47] found that EA at ST36 and Sanyinjiao (SP6) alleviated anxiety by activating D1R or antagonizing D2R in the basolateral amygdala. Previously, we found that EA with a stimulus of a mixed frequency of 2/100 Hz could alleviate ACE-induced anxiety in mice [34]. With the findings of claustrum here, we speculated that claustral $D1R^{CaMKII}$ might be one pharmacological targets of EA treatment. As expected, we found that EA at acupoints of GV20 and GV29 reversed ACE-enhanced claustrum activation by suppressing claustral $D1R^{CaMKII}$, which ultimately ameliorated ACE-induced anxiety during adulthood.

5. Conclusions

Our findings identified a novel role of claustrum in ACE-induced anxiety, and put new insight into the function of claustral $D1R^{CaMKII}$. EA treatments rescue ACE-induced anxiety potentially by targeting claustral $D1R^{CaMKII}$. The claustral $D1R^{CaMKII}$ might be a promising pharmacological target to treat drugs-related psychiatric disorders.

Abbreviations

ACE: Adolescent cocaine exposure; ASE: adolescent saline exposure; Ctrl, control; D1R: dopamine receptor 1; $D1R^{CaMKII}$, dopamine receptor 1 on CaMKII-positive neuron; D2R: dopamine receptor 2; D3R: dopamine receptor 3; EA: Electro-acupuncture; EPM: Elevated plus maze; KD, knocking-down; SEA: Sham electro-acupuncture; N.S., no significance.

Competing interests

The authors have no conflict of interests to declare.

Fundings

This work is supported by National Natural Science Foundation of China (82211540400, 82271531 and 82071495), Natural Science Foundation of Jiangsu Province, China (BK20201398) and Natural Science Foundation of the Higher Education Institutions of Jiangsu Province, China (21KJB360007).

Author contributions

Chen L and Liu Z have equal contribution to the manuscript. **Chen L, Fan Y, Liu Z and Zhao Z:** Methodology, Investigation. **Du D, Pan W, Wei X and Nie J:** Data Curation. **Fan Y, Ge F and Ding J:** Data Curation. **Guan X and Fan Y:** Formal analysis, Writing - Original Draft, Writing - Review & Editing. **Kim HY:**

Writing - Review & Editing. **Guan X:** Supervision, Conceptualization, Project administration.

Data availability statement

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

References

1 Laviola G, Adriani W, Terranova ML, Gerra G. Psychobiological risk factors for vulnerability to psychostimulants in human adolescents and animal models. Neuroscience and biobehavioral reviews. 1999;23(7):993-1010.

2 Chambers RA, Taylor JR, Potenza MN. Developmental neurocircuitry of motivation in adolescence: a critical period of addiction vulnerability. The American journal of psychiatry. 2003;160(6):1041-52.

3 Moss HB, Chen CM, Yi HY. Early adolescent patterns of alcohol, cigarettes, and marijuana polysubstance use and young adult substance use outcomes in a nationally representative sample. Drug and alcohol dependence. 2014;136:51-62.

4 Rutherford HJ, Mayes LC, Potenza MN. Neurobiology of adolescent substance use disorders: implications for prevention and treatment. Child and adolescent psychiatric clinics of North America. 2010;19(3):479-92.

5 Stopponi S, Soverchia L, Ubaldi M, Cippitelli A, Serpelloni G, Ciccocioppo R. Chronic THC during adolescence increases the vulnerability to stress-induced relapse to heroin seeking in adult rats. European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology. 2014;24(7):1037-45.

6 Erb S. Evaluation of the relationship between anxiety during withdrawal and stress-induced reinstatement of cocaine seeking. Progress in neuro-psychopharmacology & biological psychiatry. 2010;34(5):798-807.

7 Shi P, Nie J, Liu H, Li Y, Lu X, Shen X, et al. Adolescent cocaine exposure enhances the GABAergic transmission in the prelimbic cortex of adult mice. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2019;33(7):8614-22.

8 Zhu W, Ge X, Gao P, Li M, Guan Y, Guan X. Adolescent cocaine exposure induces prolonged synaptic modifications in medial prefrontal cortex of adult rats. Brain structure & function. 2018;223(4):1829-38.

9 Hinova-Palova DV, Edelstein L, Landzhov B, Minkov M, Malinova L, Hristov S, et al. Topographical distribution and morphology of NADPH-diaphorase-stained neurons in the human claustrum. Frontiers in systems neuroscience. 2014;8:96.

10 Wang Q, Ng L, Harris JA, Feng D, Li Y, Royall JJ, et al. Organization of the connections between claustrum and cortex in the mouse. The Journal of comparative neurology. 2017;525(6):1317-46.

11 Tanné-Gariépy J, Boussaoud D, Rouiller EM. Projections of the claustrum to the primary motor, premotor, and prefrontal cortices in the macaque monkey. The Journal of comparative neurology. 2002;454(2):140-57.

12 Atlan G, Terem A, Peretz-Rivlin N, Groysman M, Citri A. Mapping synaptic cortico-claustral connectivity in the mouse. The Journal of comparative neurology. 2017;525(6):1381-402.

13 Jackson J, Smith JB, Lee AK. The Anatomy and Physiology of Claustrum-Cortex Interactions. Annual review of neuroscience. 2020;43:231-47.

14 Markowitsch HJ, Irle E, Bang-Olsen R, Flindt-Egebak P. Claustral efferents to the cat's

limbic cortex studied with retrograde and anterograde tracing techniques. Neuroscience. 1984;12(2):409-25.

15 Brennan EKW, Jedrasiak-Cape I, Kailasa S, Rice SP, Sudhakar SK, Ahmed OJ. Thalamus and claustrum control parallel layer 1 circuits in retrosplenial cortex. eLife. 2021;10:e62207.

16 Stiefel KM, Merrifield A, Holcombe AO. The claustrum's proposed role in consciousness is supported by the effect and target localization of Salvia divinorum. Frontiers in integrative neuroscience. 2014;8:20.

17 Bayat A, Joshi S, Jahan S, Connell P, Tsuchiya K, Chau D, et al. A pilot study of the role of the claustrum in attention and seizures in rats. Epilepsy research. 2018;140:97-104.

18 Atlan G, Terem A, Peretz-Rivlin N, Sehrawat K, Gonzales BJ, Pozner G, et al. The Claustrum Supports Resilience to Distraction. Current biology : CB. 2018;28(17):2752-62.e7.

19 Madden MB, Stewart BW, White MG, Krimmel SR, Qadir H, Barrett FS, et al. A role for the claustrum in cognitive control. Trends in cognitive sciences. 2022;26(12):1133-52.

20 White MG, Mu C, Qadir H, Madden MB, Zeng H, Mathur BN. The Mouse Claustrum Is Required for Optimal Behavioral Performance Under High Cognitive Demand. Biological psychiatry. 2020;88(9):719-26.

21 White MG, Panicker M, Mu C, Carter AM, Roberts BM, Dharmasri PA, et al. Anterior Cingulate Cortex Input to the Claustrum Is Required for Top-Down Action Control. Cell reports. 2018;22(1):84-95.

22 Kurada L, Bayat A, Joshi S, Koubeissi MZ. The Claustrum in Relation to Seizures and Electrical Stimulation. Frontiers in neuroanatomy. 2019;13:8.

23 Wegiel J, Flory M, Kuchna I, Nowicki K, Ma SY, Imaki H, et al. Brain-region-specific alterations of the trajectories of neuronal volume growth throughout the lifespan in autism. Acta neuropathologica communications. 2014;2:28.

24 Ayyildiz S, Velioglu HA, Ayyildiz B, Sutcubasi B, Hanoglu L, Bayraktaroglu Z, et al. Differentiation of claustrum resting-state functional connectivity in healthy aging, Alzheimer's disease, and Parkinson's disease. Human brain mapping. 2022;n/a(n/a).

25 Yanes JA, Riedel MC, Ray KL, Kirkland AE, Bird RT, Boeving ER, et al. Neuroimaging meta-analysis of cannabis use studies reveals convergent functional alterations in brain regions supporting cognitive control and reward processing. Journal of Psychopharmacology. 2018;32(3):283-95.

26 Acheson A, Ray KL, Hines CS, Li K, Dawes MA, Mathias CW, et al. Functional activation and effective connectivity differences in adolescent marijuana users performing a simulated gambling task. Journal of addiction. 2015;2015:783106.

27 Zarrindast MR, Khakpai F. The Modulatory Role of Dopamine in Anxiety-like Behavior. Archives of Iranian medicine. 2015;18(9):591-603.

28 Terem A, Gonzales BJ, Peretz-Rivlin N, Ashwal-Fluss R, Bleistein N, Del Mar Reus-Garcia M, et al. Claustral Neurons Projecting to Frontal Cortex Mediate Contextual Association of Reward. Current biology : CB. 2020;30(18):3522-32.e6.

29 Niu M, Kasai A, Tanuma M, Seiriki K, Igarashi H, Kuwaki T, et al. Claustrum mediates bidirectional and reversible control of stress-induced anxiety responses. Science

advances. 2022;8(11):eabi6375.
30 Kim DH, Kim NJ, Zhao RJ, Kim DH, Yang CH, Kim HY, et al. Effects of acupuncture on the anxiety-like behavior induced by withdrawal from chronic morphine use. Neuroscience letters. 2018;664:38-42.
31 Chae Y, Yeom M, Han J-H, Park H-J, Hahm D-H, Shim I, et al. Effect of acupuncture on anxiety-like behavior during nicotine withdrawal and relevant mechanisms. Neuroscience letters. 2008;430(2):98-102.
32 Amorim D, Amado J, Brito I, Fiuza SM, Amorim N, Costeira C, et al. Acupuncture and electroacupuncture for anxiety disorders: A systematic review of the clinical research. Complementary therapies in clinical practice. 2018;31:31-37.
33 Zeng L, Tao Y, Hou W, Zong L, Yu L. Electro-acupuncture improves psychiatric symptoms, anxiety and depression in methamphetamine addicts during abstinence: A randomized controlled trial. Medicine. 2018;97(34):e11905.
34 Nie J, Wei X, Xu X, Li N, Li Y, Zhao Y, et al. Electro-acupuncture alleviates adolescent cocaine exposure-enhanced anxiety-like behaviors in adult mice by attenuating the activities of PV interneurons in PrL. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2020;34(9):11913-24.
35 Shapo GR, Ma L, Liu C. [Effects of acupuncture at Weizhong (BL40) on brain function with PET/CT]. Zhongguo Zhong xi yi jie he za zhi Zhongguo Zhongxiyi jiehe zazhi = Chinese journal of integrated traditional and Western medicine. 2006;26(11):969-72.
36 Spear LP. The adolescent brain and age-related behavioral manifestations. Neuroscience and biobehavioral reviews. 2000;24(4):417-63.
37 Estelles J, Lluch J, Rodríguez-Arias M, Aguilar MA, Miñarro J. Cocaine exposure during adolescence affects anxiety in adult mice. Brain research bulletin. 2007;71(4):393-403.
38 Lopez B, Turner RJ, Saavedra LM. Anxiety and risk for substance dependence among late adolescents/young adults. Journal of anxiety disorders. 2005;19(3):275-94.
39 Jackson J, Karnani MM, Zemelman BV, Burdakov D, Lee AK. Inhibitory Control of Prefrontal Cortex by the Claustrum. Neuron. 2018;99(5):1029-39.e4.
40 Zingg B, Dong HW, Tao HW, Zhang LI. Input-output organization of the mouse claustrum. The Journal of comparative neurology. 2018;526(15):2428-43.
41 Siemsen BM, Lombroso PJ, McGinty JF. Intra-prelimbic cortical inhibition of striatal-enriched tyrosine phosphatase suppresses cocaine seeking in rats. Addiction biology. 2018;23(1):219-29.
42 Valzachi MC, Teodorov E, Marcourakis T, Bailey A, Camarini R. Enhancement of Behavioral Sensitization, Anxiety-Like Behavior, and Hippocampal and Frontal Cortical CREB Levels Following Cocaine Abstinence in Mice Exposed to Cocaine during Adolescence. PloS one. 2013;8(10):e78317.
43 Caffino L, Giannotti G, Messa G, Mottarlini F, Fumagalli F. Repeated cocaine exposure dysregulates BDNF expression and signaling in the mesocorticolimbic pathway of the adolescent rat. The world journal of biological psychiatry : the official journal of the World Federation of Societies of Biological Psychiatry. 2019;20(7):531-44.
44 Caffino L, Mottarlini F, Mingardi J, Zita G, Barbon A, Fumagalli F. Anhedonic-like behavior and BDNF dysregulation following a single injection of cocaine during adolescence. Neuropharmacology. 2020;175:108161.

45 Kim DH, Kim NJ, Zhao RJ, Kim DH, Yang CH, Kim HY, et al. Effects of acupuncture on the anxiety-like behavior induced by withdrawal from chronic morphine use. Neuroscience letters. 2018;664:38-42.

46 Shao F, Du J, Wang S, Cerne R, Fang J, Shao X, et al. Electroacupuncture Alleviates Anxiety-like Behavior in Pain Aversion Rats by Attenuating the Expression of Neuropeptide Y in Anterior Cingulate Cortex. Clinical Complementary Medicine and Pharmacology. 2022;2(2):100019.

47 Wu M, Chen Y, Shen Z, Zhu Y, Xiao S, Zhu X, et al. Electroacupuncture Alleviates Anxiety-Like Behaviors Induced by Chronic Neuropathic Pain via Regulating Different Dopamine Receptors of the Basolateral Amygdala. Molecular neurobiology. 2022.

Figure legends

Figure 1. ACE enhances anxiety along with increased CaMKII-positive neuron activity and D1R^{CaMKII} level in claustrum during adulthood. A, Experimental timeline. **B**, EPM test. **C**, Total protein levels of c-Fos. **D**, The percentage and number of c-Fos-positive & CaMKII-positive neurons. Scale bar, 300 μm/50 μm. **E,** The expression of D1R^{CaMKII}. Scale bar, 200 μm/50 μm. **F**, The mRNA levels of *D1R, D2R and D3R*. **G,** The protein levels of D1R, D2R and D3R. **H,** Levels of p-ERK$_{1/2}$ and ERK$_{1/2}$. **I,** Levels of p-CREB, CREB and BDNF. ASE, adolescent saline exposure; ACE, adolescent cocaine exposure; CL, claustrum. N.S., $p > 0.05$, *, $p < 0.05$, **, $p < 0.01$ vs ASE.

Figure 2. Inhibiting claustral D1R suppresses ACE-induced anxiety and claustrum activation in mice during adulthood. A, Experimental timeline and local injections of SCH-23390 into claustrum. Scale bar, 400/100 μm. **B**, EPM test. **C**, The number of c-Fos-positive neurons. Scale bar, 100 μm. Vehicle, vehicle-treated ACE mice; SCH-23390, SCH-23390-treated ACE mice; CL, claustrum. N.S., $p > 0.05$, *, $p < 0.05$, **, $p < 0.01$ vs Vehicle.

Figure 3. Knocking-down claustral D1R^{CaMKII} level suppresses ACE-induced anxiety and claustrum activation during adulthood. A, Experimental design and timeline. **B**, Local injections of virus into claustrum. Scale bar, 300 μm. **C**, The D1R protein level. **D**, EPM test. **E**, The percentage of c-Fos-positive neurons in EGFP-transfected neurons and the mean gray value of c-Fos in EGFP-transfected neurons. Scale bar, 400 μm/50 μm. ACE$_{Ctrl}$, ACE mice injected with control virus; ACE$_{KD}$, ACE mice injected with knockdown virus; CL, claustrum. N.S., $p > 0.05$, *, $p < 0.05$, **, $p < 0.01$ vs

ACE_{Ctrl}.

Figure 4. EA treatment rescues ACE-increased anxiety, claustrum activation and $D1R^{CaMKII}$ level. A, Experimental design and timeline. **B**, Schematic diagram of EA and SEA treatments in mice. **C**, EPM test. **D**, The percentage and number of c-Fos-positive & CaMKII-positive neurons. Scale bar, 300 μm/50 μm. **E**, The D1R protein level. **F**, The expression of $D1R^{CaMKII}$. Scale bar, 200 μm/50 μm. **G**, Levels of p-$ERK_{1/2}$ and $ERK_{1/2}$. **H**, Levels of p-CREB, CREB and BDNF. ACE_{SEA}, sham electro-acupuncture-treated ACE mice; ACE_{EA}: electro-acupuncture (GV 20 and GV 29)-treated ACE mice; CL, claustrum. N.S., $p > 0.05$, *, $p < 0.05$, **, $p < 0.01$ vs ACE_{SEA}.

Figure 5. Schematic summary of present study. Adolescent cocaine exposure (ACE) enhances anxiety-like behaviors in male mice during adulthood, accompanied with triggered activation of CaMKII-positive neurons and more dopamine receptor 1 (D1R) on CaMKII-positive neurons ($D1R^{CaMKII}$) in claustrum. Either blocking D1R with SCH23390 or knocking-down $D1R^{CaMKII}$ with virus in the claustrum efficiently reduces claustrum activation, and ultimately suppresses ACE-induced anxiety in mice. By targeting claustral $D1R^{CaMKII}$, electro-acupuncture (EA) at acupoints of GV20 and GV29 rescues ACE-increased anxiety in mice during adulthood. In the process, the p-CREB and BDNF may be the potential subsequent signals to $D1R^{CaMKII}$.