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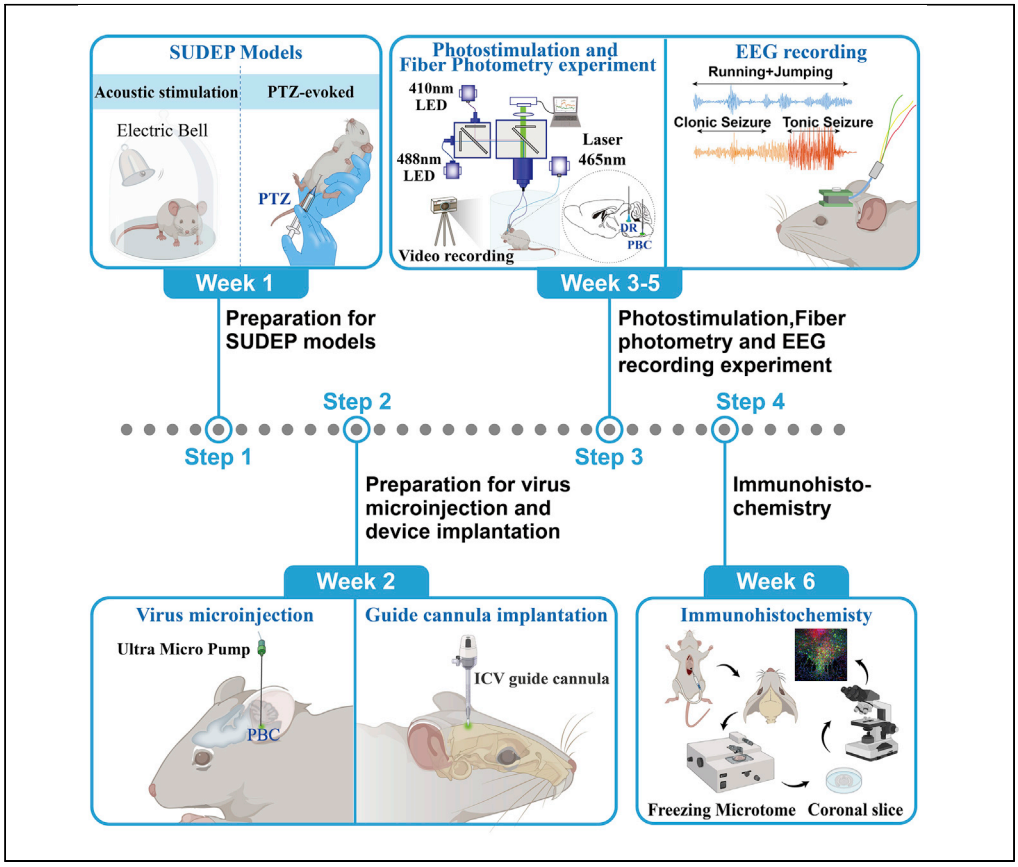
Protocol for modulation of the serotonergic DR-PBC neural circuit to prevent SUDEP in the acoustic and PTZ-induced DBA/1 mouse models of SUDEP



The dorsal raphe nucleus (DR) and the pre-Bötzinger complex (PBC) may play an important role in regulating seizure-induced respiratory arrest (S-IRA), the main contributor to sudden unexpected death in epilepsy. Here, we describe pharmacological, optogenetic, and retrograde labeling approaches to specifically modulate the DR to PBC serotonergic pathway. We detail steps for implanting optical fibers and viral infusion into DR and PBC regions and optogenetic techniques for exploring the role of 5-hydroxytryptophan (5-HT) neural circuit of DR-PBC in S-IRA.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

YuLing Wang, Qing Xu, Qian Yu, ..., JiaXuan Gu, Lu Liu, HongHai Zhang

zhanghonghai_0902@163.com

Highlights

A protocol for implantation of optical fibers and viral infusion into the DR and PBC

Optogenetic techniques explore the role of 5-HT neural circuit of DR-PBC in S-IRA

Optimized coordinates and volume for viral infusion into male DBA/1 mice

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Protocol for modulation of the serotonergic DR-PBC neural circuit to prevent SUDEP in the acoustic and PTZ-induced DBA/1 mouse models of SUDEP

YuLing Wang,[1,4] Qing Xu,[2,4] Qian Yu,[1,4] LeYuan Gu,[1,4] HaiXiang Ma,[1] Yue Shen,[1] XiTing Lian,[2] WeiHui Shao,[1] JiaXuan Gu,[1] Lu Liu,[2] and HongHai Zhang[1,2,3,5,6,*]

[1]Department of Anesthesiology, the Fourth Clinical School of Medicine, Zhejiang Chinese Medical University, Hangzhou 310006, China

[2]Department of Anesthesiology, Affiliated Hangzhou First People's Hospital, Zhejiang University School of Medicine, Hangzhou 310006, China

[3]Westlake Laboratory of Life Sciences and Biomedicine, Hangzhou 310006, China

[4]These authors contributed equally

[5]Technical contact

[6]Lead contact

*Correspondence: zhanghonghai_0902@163.com
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SUMMARY

The dorsal raphe nucleus (DR) and the pre-Bötzinger complex (PBC) may play an important role in regulating seizure-induced respiratory arrest (S-IRA), the main contributor to sudden unexpected death in epilepsy. Here, we describe pharmacological, optogenetic, and retrograde labeling approaches to specifically modulate the DR to PBC serotonergic pathway. We detail steps for implanting optical fibers and viral infusion into DR and PBC regions and optogenetic techniques for exploring the role of 5-hydroxytryptophan (5-HT) neural circuit of DR-PBC in S-IRA.

For complete details on the use and execution of this protocol, please refer to Ma et al. (2022).[1]

BEFORE YOU BEGIN

S-IRA is the main factor in SUDEP and that enhancement of 5-HT function in the DR can significantly reduce the incidence of S-IRA in the DBA/1 mouse model of SUDEP. In addition, physiological studies reported that electrical and chemical stimulation of raphe nuclei, changed respiratory patterns and serotonergic neurons in raphe nuclei projected throughout the VRC (ventral respiratory column), especially PBC.[2] Thus, we assume that there is a neural circuit between the DR and the PBC, which might contribute to preventing SUDEP. The approach is mainly based on the stereotactic injection to deliver drugs or viruses to the target brain regions in rodents.[3]

Institutional permissions

1. All experimental procedures are in line with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, approved by the Animal Advisory Committee of Zhejiang University, and followed proper animal use protocols and institutional guidelines. The users of the protocol must obtain similar permissions from corresponding institutions.

Breeding animals

Timing: 4–5 months prior to the experiment

This section describes the process of DBA/1 mice breeding. Since DBA/1 mice are sensitive to sound, it is recommended to keep them in a soundproof environment.

2. House and breed DBA/1 mice in the Animal Center of Zhejiang University School of Medicine and give them rodent food and water ad libitum in the soundproof room to protect the sensitivity to sound. DBA/1 mice are the model for chronic susceptibility to audiogenic seizures followed by sudden death associated with respiratory arrest.[4]

   Note: During the rearing of the mice, keep the room quiet and avoid excessive decibels to ensure the sensitivity of the mice to sound.

3. Use DBA/1 mice of either gender in the experiments.

   Note: Gender is not a variable affecting S-IRA in DBA/1 mice.[4] Therefore, use both male and female DBA/1 mice in the experiment according to the reproduction.

4. To minimize the effect of temperature on mice, use the heat preservation and sound insulation boxes to transport the mice from the outside to inside.

   Note: Because the temperature affects the sound stimulation of the DBA/1 mice, and lower temperature reduces the incidence of S-IRA, we use heat preservation and sound insulation boxes during the transport of the mice.

Preparation for acoustic and PTZ-induced models

This section describes how to establish SUDEP models in two ways.

5. For the acoustic stimulation murine model, "prime" DBA/1 mice starting from postnatal days 26–28 by subjecting to acoustic stimulation daily for 3–4 days to establish consistent susceptibility to audiogenic seizures and S-IRA.

   Note: Use primed DBA/1 mice at approximately 8 weeks of age (10–15 g) in experiments with acoustic stimulation.

6. For the pentylenetetrazole (PTZ)-evoked seizure model, intraperitoneal inject PTZ (75 mg/kg) into non-primed DBA/1 mice at approximately 8 weeks of age.[5]
7. Put the mice in a soundproof room to prevent other mice from being affected by the sound stimulus and then ring the bell.

Seizure induction and resuscitation

This section describes the ways of inducing seizure in the two models and the rescue measures after the occurrence of S-IRA.

8. For the acoustic stimulation model, each mouse in 26–28 days is placed in a cylindrical plexiglass chamber in a sound-isolated room, and generalized audiogenic seizures (AGSz) are evoked by an electric bell (96 dB SPL, Zhejiang People's Electronics, China).[6]

   Note: Acoustic stimulation is given for a maximum duration of 60 s or until the onset of tonic seizures and S-IRA. Resuscitate DBA/1 mice with S-IRA within 5 s after the final respiratory arrest using a rodent respirator. The respiratory arrest is defined visually by the appearance of a

deep respiratory gasp and relaxation of the pinnae, which are invariant indicators that respiratory arrest and death are imminent. When the DBA/1 mouse develop respiratory arrest after seizures, we record the state as S-IRA. The rodent respirator operates at 180 strokes/minute, 1:1.5 of I/E and a volume of 1 cc with room air.[7] Formal experiments are performed in mice at 8 weeks of age.

9. For the PTZ-evoked seizure model, evoke S-IRA in non-primed DBA/1 mice by IP administration of a single dose of PTZ at a dose of 75 mg/kg.[6,8] Formal experiments are performed in mice at 8 weeks of age.

Note: Formal experiments are performed in mice at 8 weeks of age.

Preparation of surgical area

Timing: 3 h

This section describes the environment and equipment preparation of mice before stereotactic surgery.

10. Disinfect Surgical tools with a laboratory autoclave pot. Clean the surgical area with 75% ethanol (Figure 1A).
11. Connect the gauge needle (10μL, virus injection) (Figure 1C) to the microinfusion pump (Figure 1A), and assemble the stereotaxic instrument and dental drill (Figure 1A).

Note: Before virus loading, fill the gauge needle with paraffin solution. The connection between the gauge needle and the glass electrode is sealed with glue to ensure that the syringe was airtight (Figure 1C). The appearance of small paraffin droplets at the tip of the needle and the formation of bubbles in the syringe are observed to test the air impermeability.

⚠ CRITICAL: Adjust the needle to be perfectly straight before use. When connecting the gauge needle to the microsyringe pump, note that the needle tip is vertical and no air bubbles in the needle.

12. Turn on a heating pad throughout the entire surgical process to keep the body temperature of anesthetized mice constant at 37°C.
13. In order to avoid impaired sound sensitivity of DBA/1 mice during surgery, put a cotton ball in the ear of DBA/1 mice to protect the tympanic membrane, and remove the cotton ball before weighing and after surgery.

Reagents preparation

Timing: 20 min

This section describes the preparation and storage methods of reagents required for stereotactic surgery and behavioral experiment.

14. Dissolve 3.5 g chloral hydrate in 100 mL sterilized 0.9% saline to make 3.5% chloral hydrate solution, for anesthetization.

Note: The chloral hydrate (3.5%) can be stored at room temperature (RT, 15°C–25°C) for up to 1 month. Sterilized 0.9% saline can be stored at 4°C for up to 3 months. It's better to use it in time. Weigh the mice and calculate the dosage of chloral hydrate solution used for anesthetization.

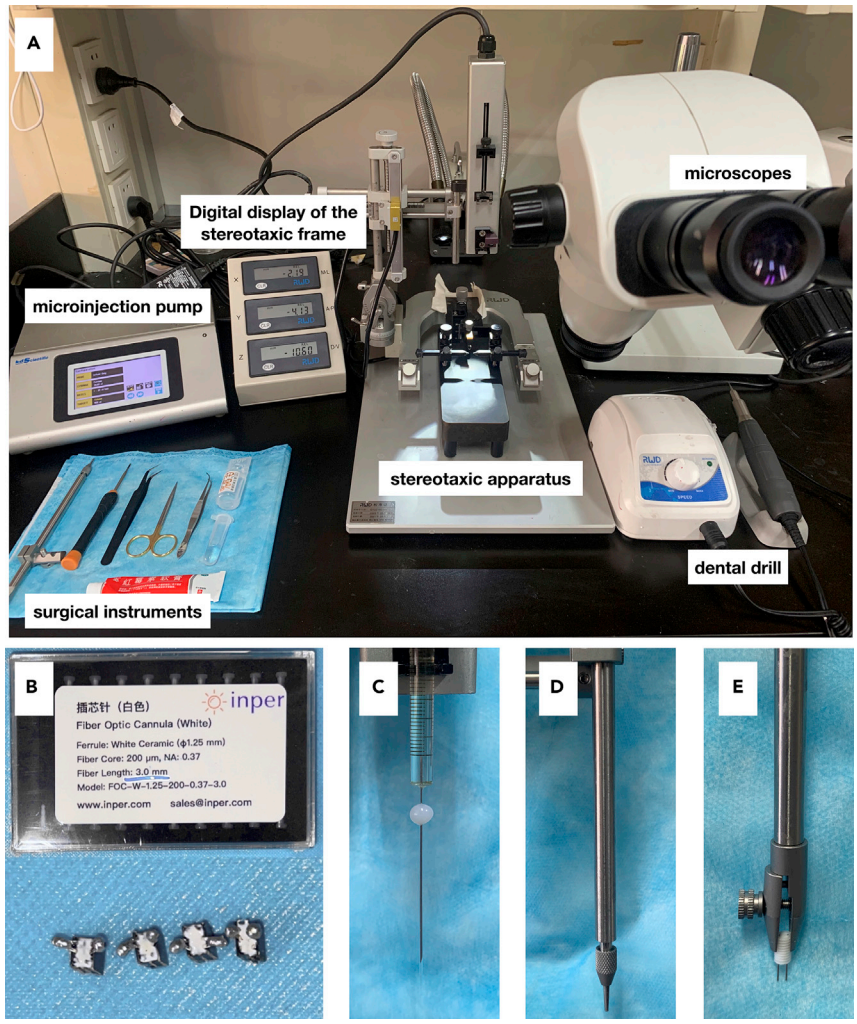

Figure 1. Preparation for stereotactic surgical and device implantation

(A) Main components of the stereotactic system.

(B–E) Some core instruments: optical fiber with proper length and cortical EEG electrodes (B), a gauge needle for the specification of 10 μL glass with electrodes inserted in the front end (C), ceramic pin holder (D), cannula holder (E). EEG, electroencephalogram.

15. Dissolve ketanserin (KET) in 100% dimethyl sulfoxide (DMSO). After the KET is completely dissolved, add double distilled water to dilute 100% DMSO to 25%.

Note: Compound KET when it is used, avoid storage for more than three hours and do not leave it at 21°C–26°C for too long.

16. Take out an adeno-associated virus (pAAV-TPH2 PRO-ChETA-EYFP-WPRES-PAS; AAV2/9-mCaMKIIa-GCaMP6f-WPRE-pA), and put it on the ice right before viral loading.

Note: Store viruses in a freezer at − 80°C. Avoid multiple freeze-thaw processes. For storage, it is recommended that the virus be stored at 4°C for no more than one week. Virus exposure to light should be avoided before and during the injection.

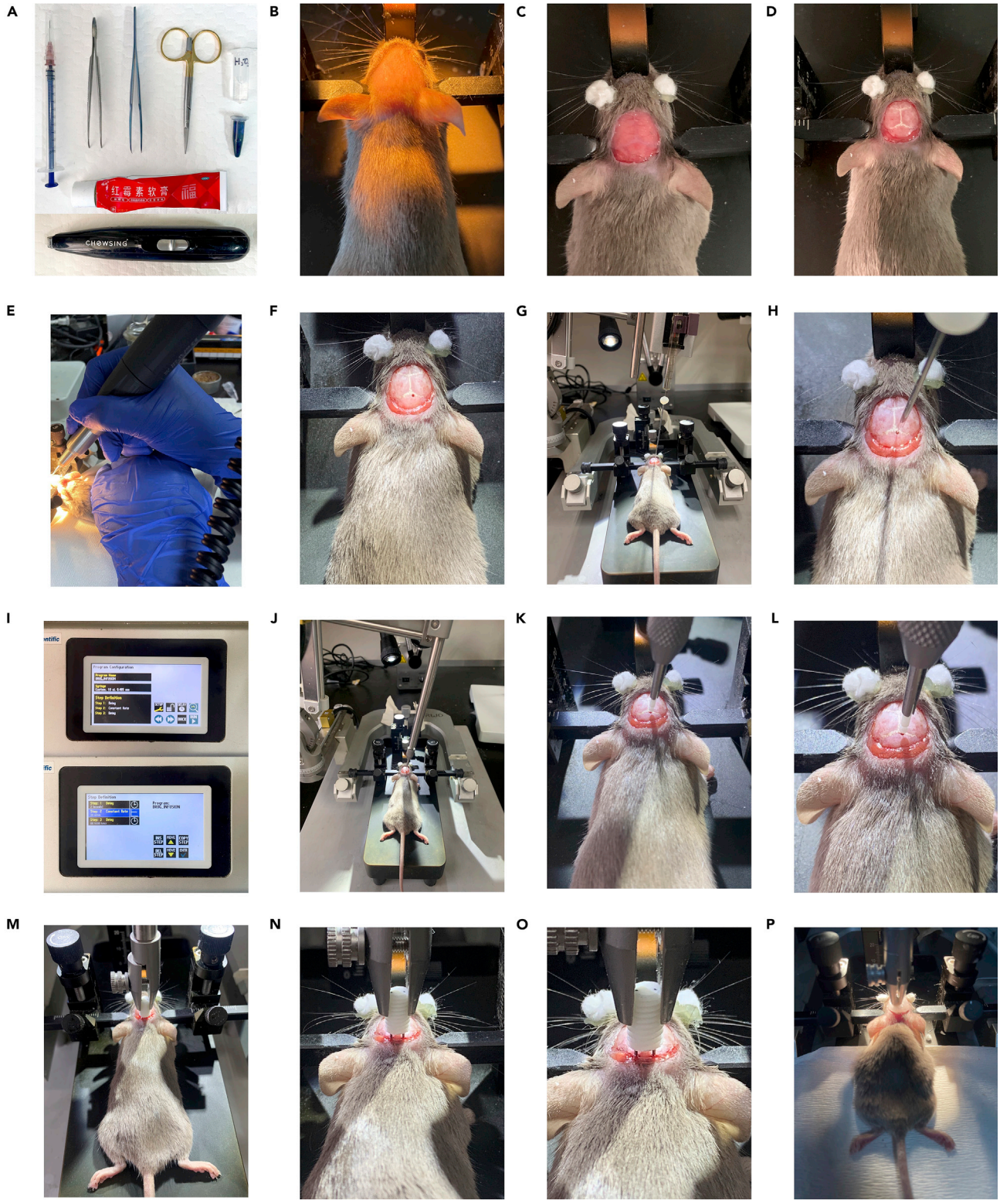

Figure 2. Stereotactic surgical materials and key procedures

(A) Surgical tool for virus injection. From left to right, from top to bottom: 1 mL syringe (for intraperitoneal injection of 3.5% chloral hydrate), anatomical forceps, sharp forceps, sharp surgical scissor (small), hydrogen peroxide, colored dye, erythromycin ointment for external use, pet electric shaver.

Figure 2. *Continued*
(B) Fix the mouse on the stereotactic instrument with the ear bar. Then hang the front tooth on the tooth bar, and move the tooth bar far away from the mouse until we feel obvious resistance. Once we fixed the tooth bar, we can tighten the nose clip.
(C) In order to protect the eyesight of mice, we smear erythromycin eye ointment on the surface of mouse eyeballs and then cover them with sterile cotton balls. Remove the skin of the mouse head, and wipe the connective tissue on the skull surface with a cotton ball soaked in alcohol.
(D) Wipe the bone seam with a cotton ball dipped in hydrogen peroxide to expose bregma and lambda.
(E) The drilling must be well controlled, otherwise, it is easy to injure the brain tissue accidentally after drilling through the skull.
(F) Open an appropriate bone window, which should be as small as possible.
(G) Diagram of DR virus injection (AP − 4.55 mm, ML − 0.44 mm, DV − 2.80 mm). Adjust the needle entry angle to avoid the blood vessels above DR when injecting.
(H) Amplification of (G).
(I) The specific parameter settings of the virus injection program. (Program name: drug infusion; step 1: delay 2 s, step 2: constant rate: 20 nl/min, step 3: delay 10 min) The length of stay of the syringe before and after the virus injection can be adjusted according to the amount of virus injection and the injection speed.
(J) Implant the optical fiber above DR (AP − 4.55 mm, ML − 0.44 m, DV − 2.75 mm, 10°right).
(K and L) Amplification of (J).
(M) Implant the bilateral PBC guide cannulas (AP − 6.80 mm, ML − 1.25 mm, DV − 4.90 mm).
(N and O) Amplification of (M).
(P) Use dental cement to fix the PBC cannula after the operation. DR, dorsal raphe nucleus; PBC, pre-Bötzinger complex; ICV, intracerebroventricular injection.

17. To ensure uniform distribution of the drug when injected into the lateral ventricle, we vibrate evenly when dispensing the drug to ensure no drug precipitation and adherence to the wall, and rinse the syringe with prepared drug before injection to ensure no air bubbles inside the syringe and cannula core.

Affixing the mouse to the stereotaxic device

Timing: 30 min

This section describes the process of anesthesia, shaving, head fixation and vision protection of mice.

18. Intraperitoneal injection of anesthetic into the mouse, and put it back into the empty cage, wait for 5–10 min, check the mouse's responsiveness to noxious stimuli (for example, pinch tail reflex), to ensure that it is in a state of deep anesthesia.

Note: In this protocol, for 20 g male DBA/1 mice, 8 weeks old, 0.30 mL of 3.5% chloral hydrate for adult DBA/1 mice, 15 mL/kg body weight is recommended. If the DBA/1 mice show pain in response to a paw pinch during the maintenance period, an additional 10% of the initial dosage of chloral hydrate is given to guarantee a painless state.

19. Shave the mouse head according to the position of the target nucleus. Referring to the AP value of PBC and DR, our shaving range extends back to the level of two ears (Figure 2B).

Note: The shaved area is slightly larger than the surgical wound to avoid adhesion between the hair and the wound.

20. Fix the anesthetized and shaved mice on the stereotaxic device.

Note: When fixing, hold the mouse head with the left hand, insert the left ear bar into the mouse ear canal, adjust the left and right ear bars to keep the animal in the center of the U-shaped opening, lock and fix one ear bar first, and then tighten the other ear bar, so that the animal head cannot shake, and at the same time tighten, the door teeth clamp screws.

21. Check whether the fixation is successful. The standard for successful fixation is no medial-lateral or front-back movements allowed while free up-down rotation.

Note: Be careful of the status of the mice. The prone position and the clamping of the brain stereotaxic device can cause asphyxia and death in mice. Therefore, it is necessary to confirm that the airway of the mice is unobstructed at any time.

22. In order to avoid irreversible damage to the vision of mice caused by strong light, smear erythromycin eye ointment on the surface of mouse eyeballs and then cover them with sterile cotton balls to achieve the protective effect.

Note: Unmedicated ophthalmic ointment is an acceptable alternative.

Preparation before virus injection

Timing: 1 h

This section describes the procedures for exposing skull marks, including bregma and lambda, leveling of the stereotactic apparatus, dye pre-injection and coordinate adjustment.

23. Cut the skin of the mouse head with a scalpel, and wipe the connective tissue on the skull surface with an alcohol-soaked cotton ball (Figure 2C).
24. To better expose bregma and lambda, gently wipe the bone seam with a cotton ball dipped in hydrogen peroxide (Figure 2D).

Note: If the surgical incision is too large or the scope of alcohol wiping is too large, it is easy to cause blood leakage or bleeding in the tissue. Use sterile cotton balls dipped in normal saline to press the bleeding point.

⚠ CRITICAL: The exposed part of the operation should be wiped with a cotton ball dipped in normal saline to prevent the wound from being too dry, which may lead to tissue necrosis.

25. Gently touch bregma and lambda with a glass electrode, level them back and forth according to the DV value, and then return to the level of bregma for horizontal leveling. After the left and right leveling, check bregma and lambda leveling again.

Note: Before precise leveling under the microscope, the placement of the skull can be visually inspected and roughly adjusted.

26. According to the coordinates of DR (AP − 4.55 mm, ML − 0.44 mm, DV − 2.80 mm) and PBC (AP − 6.80 mm, ML − 1.25 mm, DV − 4.95 mm), carefully drill through the skull above the nucleus with a dental drill (Figure 2E).
27. Pre-injection experiment of colored dye.
    a. Microinject colored dye (40 μL) into DR or PBC.
    b. Sacrifice DBA/1 mice and perfuse with phosphate-buffered saline (PBS) containing 4% paraformaldehyde (PFA).
    c. Each mouse brain is sectioned into 30-μm-thick coronal slices with a freezing microtome (CM30503, Leica Biosystems, Buffalo Grove, IL, USA).
    d. Confirm that the injected nuclear sites are correct according to the atlas.

Note: When sectioning the brain, it should be noted that the sections should be cut from the outside of the target nucleus (not yet at the level of the nucleus). At the same time, the experimenter should pay attention to adjusting the angle of the freezing table to make the section angle consistent with the atlas.

28. Compare the obtained sections with some references in the atlas to determine whether the infection site is accurate.

Note: The references can be large and clear hippocampus, optic chiasma, special structures near the ventricles and nuclei, such as fiber cords, etc.

⚠ CRITICAL: When the animal is too light, there may be a certain distance from the actual location of the nucleus according to the reference coordinates of the atlas.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
pAAV-TPH2 PRO-ChETA-EYFP-WPRES-PAS	Sheng BO, Co., Ltd.	N/A
AAV2/9-mCaMKIIa-GCaMP6f-WPRE-pA	Taitool Bioscience Co., Ltd.	N/A
Chemicals, peptides, and recombinant proteins		
PTZ	Sigma-Aldrich, St. Louis, MO	Cat #P6500
KET	Sigma-Aldrich, St. Louis, MO	Cat #8006
5-HTP	Sigma-Aldrich, St. Louis, MO	Cat #107751
CTB-555	BrainVTA Technology Co. Ltd	N/A
Chloral hydrate	Sangon Biotech (Shanghai) Co., Ltd. Shanghai, China	I418BA0004
Dimethyl sulfoxide (DMSO)	Sigma, St. Louis, MO	SHBK2703
Saline	China Otsuka Pharmaceutical Co., Ltd. Tianjin, China	DB01-1-0701
Experimental models: Organisms/strains		
DBA/1 mice	The Animal Center of Zhejiang University School of Medicine, Harlan Laboratories	Male, 8 weeks old
Software and algorithms		
SPSS	Chicago, IL, USA	N/A
Other		
Rodent respirator	ShangHai, China.	Yu yan
Glass electrode	Sutter Instrument, Novato, USA	B100-58-10
Microinfusion pump	Kd Scientific	N/A
Microliter syringes	Shanghai Gaoge Industry and trade Co., Ltd, China	G019105
Stereotaxic device	RWD Life Science Inc., Shenzhen, China	68,018
Dental drill	RWD Life Science Inc., Shenzhen, China	78001
Denture base meterials	Shanghai new century dental material Co., Ltd, China	N/A
Artificial teeth resin	Shanghai new century dental material Co., Ltd, China	N/A
Glue	Komax, Zhejiang, China	N/A
The mouse atlas of Paxinos and Franklin	4th Edition, 2013	N/A
Syringes	JIANGXI HONGDA MEDICAL EQUIPMENT GROUP LTD.	N/A
Medical cotton balls	JIANGXI HONGDA MEDICAL EQUIPMENT GROUP LTD.	N/A
Photometry system and laser	Inper, Hangzhou, China	N/A
Optical fiber	Inper, Hangzhou, China	FOC-W-1.25-200-0.37-3.0
ICV guide cannula	RWD Life Science Inc., China	O.D.I.41 × I.D.O.0.25 mm/M3.5, 62004
PBC guide cannulas	RWD Life Science Inc., China	O.D.0.48 × I.D.0.34 13 mm/M3.5,62033

STEP-BY-STEP METHOD DETAILS

Virus injection

Timing: 1 h

This section includes the procedure of drilling, screw implantation, DR and PBC virus microinjection and the parameter setting of the injection process.

1. According to the determined position parameters of the injected brain area, Bregma of the anterior fontanel is 0 point.
2. Move the dental drill.
   a. Mark the position range of the nucleus cluster with a pen.
   b. Open the appropriate bone window according to the size of the range.

   Note: The window should be as small as possible without interfering with the experiment.

3. Carefully grind the skull at the injection site with a dental drill to thin the skull slowly.
   a. When the skull cracks, use the needle of a medical syringe to carefully prick it to prevent damage.

   Note: If there is bleeding during the process, use a very small medical cotton ball to pull a long strip to suck the blood away.

   ⚠ CRITICAL: The drilling must be well controlled, otherwise it is easy to accidentally cause the brain tissue damage after drilling through the skull (Figures 2E and 2F).

4. Implant four miniature screws along the lateral portions of the front and back of the skull, forming roughly a square (Figure 3B).
5. Slowly insert the glass electrode until it reaches the target nucleus.

   Note: In our experiment, there are blood vessels flowing above the target nucleus DR, so we avoid the blood vessels by adjusting the needle entry angle to achieve better injection effect (Figure 2G).

   ⚠ CRITICAL: There may be blood vessels flowing above some nuclei. Vertical needle insertion will inevitably damage the blood vessels and cause bleeding at the injection site. Improper treatment may easily cause blockage of the glass electrode tip (Figure 2H).

6. Based on the mouse atlas of Paxinos and Franklin (4th Edition, 2013), microinject (100 nl, at a rate of 20 nl/min) pAAV-TPH2-PRO-ChETA-EYFP-WPRES-PAS according to the following stereotaxic coordinates of DR (AP − 4.55 mm, ML − 0.44 mm, DV − 2.80 mm, 10°right) (Figure 2I).

   ⚠ CRITICAL: It is recommended that the injection speed should be set at 10–40 nl/min and the injection time should be controlled at 10 min.

   Note: This step is to observe the effect of activating 5-HT neurons in DR on the incidence of S-IRA.

7. Deliver the viruses via a gauge needle for the specification of 10 μL (cat# 60700010, Gao Ge, Co., Ltd, ShangHai, China) by an Ultra Micro Pump (160494 F10E, WPI) over a period of 10 min (including the residence time of the needle tip in the brain area before and after injection).

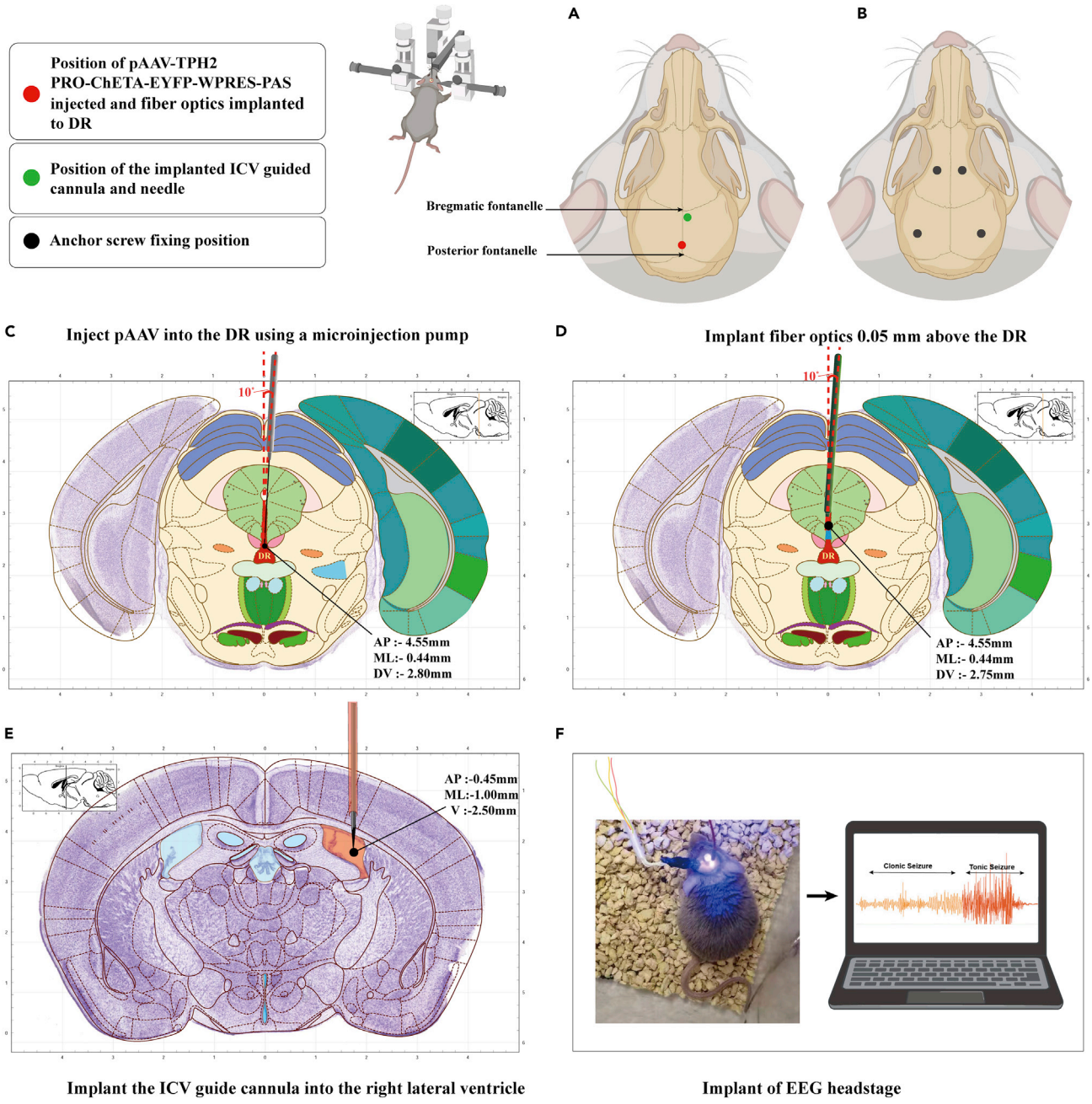

Figure 3. The implantation of optical fiber in DR and ICV guide cannula

(A) The drilling point in the mouse skull indicates the virus injection site(red) in DR and the implantation site of the ICV cannula (green).

(B) The four gray dots correspond to the recommended location of the anchor screws.

(C and D) Schematic illustration of pAAV-TPH2-PRO-ChETA-EYFP-WPRES-PAS delivery into the DR of DBA/1 mice and implantation of the optic fiber.

(E) We implanted an ICV guide cannula on the right side of the skull to microinject KET, which indicates the lower incidence of S-IRA after photostimulation DR was remarkably reversed by ICV injection of KET at a dose of 18.3 nmol.

(F) Representative images of implanted EEG, ICV and optic fiber devices in a DBA/1 mouse. ICV, intracerebroventricular injection; PTZ, pentylenetetrazole; DR, dorsal raphe nucleus; KET, ketanserin; EEG, electroencephalogram; ICV, intracerebroventricular injection.

Note: During use, place the virus on ice or in a 4°C refrigerator to minimize the time at room temperature.

Treatment for blocked needle tip of virus injection: firstly, wipe the needle tip with normal saline cotton ball. If the needle tip is still blocked after the injection pump is quickly pushed, cut off some electrode tips.

⚠ CRITICAL: During microinjection, observe whether the liquid level stratification of the virus and liquid paraffin in the glass electrode drops through the microscope.

8. After the end of infusion, do not remove the syringe until 15–20 min to allow the diffusion of the viruses.

Note: Determine the injection amount according to the target nucleus, virus titer (in this protocol the titer of photogenetic virus is greater than 5×10^{12} vg/mL) and virus type to achieve ideal experimental results.

⚠ CRITICAL: To avoid viruses leaking to the nucleus above the target brain area, the needle stopping time after injection shall not be less than 10 min. On the one hand, it allows the virus to spread, and on the other hand, it prevents the virus from leaking out of the nucleus as the needle is pulled out.

9. For retrograde labeling of projection neurons, microinject CTB-555 (100 nl, 1 μg/μL, BrainVTA Technology Co. Ltd, Wuhan, China) in the DR (AP − 4.55 mm, ML − 0.44 mm, DV − 2.80 mm, 10°right) or the right side of the PBC (AP − 6.80 mm, ML − 1.25 mm, DV − 4.95 mm), and we waited approximately 1 week (Figure 4).

Note: For virus expression, we wait approximately 1 week. This step is to directly verify the bidirectional projection between DR-PBC.

10. For the photostimulation of the bilateral PBC, deliver pAAV-TPH2 PRO-ChETA-EYFP-WPRES-PAS in the bilateral PBC (AP − 6.80 mm, ML − 1.25 mm, DV − 4.95 mm).

Note: This step is to test the effect of activating 5-HT neurons in PBC on the incidence of S-IRA.

Device implantation

Timing: 1 h

To avoid the damage caused by the second operation and the difficulty in locating the nucleus, we completed virus injection and device implantation at the same time in one operation. This section describes the scheme of implanting optical fiber in DR and PBC, implanting guide cannula and embedding cortical EEG electrodes in mice.

11. Implant the optical fiber (FOC-W-1.25-200-0.37-3.0, Inper, Hangzhou, China) above DR for 0.05 mm (AP − 4.55 mm, ML − 0.44 m, DV − 2.75 mm, 10°right) (Figures 2J–2L).

Note: Implanted the optical fiber at a right tilt of 10° to avoid damaging the blood vessels above the DR, and reducing the postoperative mortality of the mice.

12. Use the dental cement fix the optical fiber.

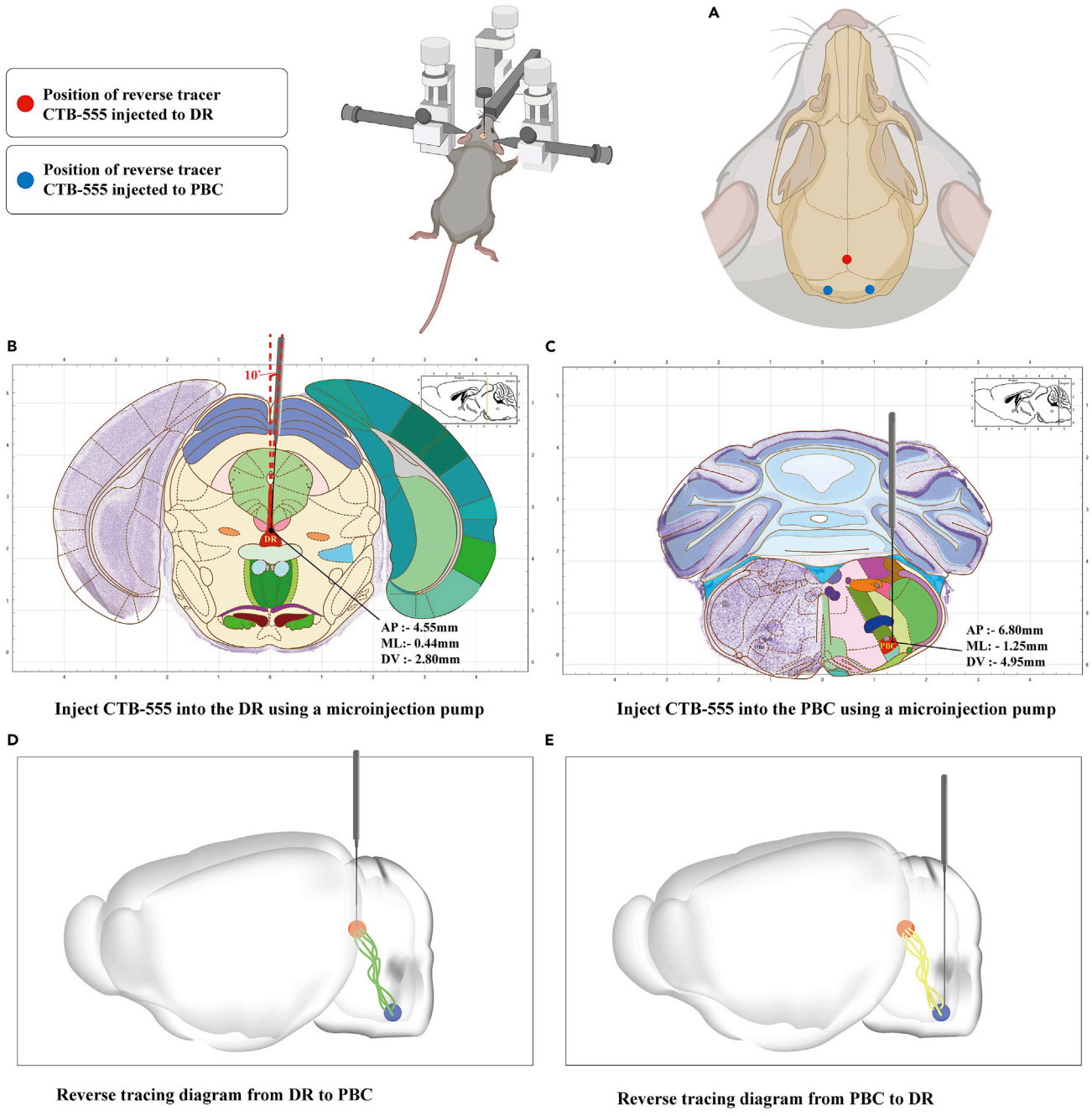

Figure 4. The location of CTB-555 injection in DR and PBC

(A) The drilling point in the mouse skull indicates the CTB-555 injection site(red) in DR and in bilateral PBC(blue).

(B and C) Microinjection of CTB-555 in DR and the right side of PBC.

(D and E) Neural projection from the DR to the PBC established by the application of the nerve tracer CTB-555. DR, dorsal raphe nucleus; PBC, pre-Bötzinger complex.

a. For intracerebroventricular injection (ICV) surgery, ICV guide cannula implantation (AP - 0.45 mm; ML - 1.0 mm; V - 2.50 mm) and fixation were completed using the protocol as described above and implant a headstage for electroencephalogram (EEG) in the same mice[9] (Figure 3).

Note: This step is to investigate the role of 5-HT2A receptor in the brain in the regulation of S-IRA. We observed whether the suppression effect of S-IRA mediated by activating 5-HT neurons in DR or intraperitoneal injection of 5-HTP could be reversed by the ICV delivery of KET.

b. For microinjection of KET in the bilateral PBC, implant guide cannulas (O.D.0.48 × I.D.0.34 mm/M3.5,62033, RWD Life Science Inc.) on both sides of PBC (AP − 6.80 mm, ML − 1.25 mm, DV − 4.90 mm) (Figures 2M–2O, Figure 5).

Note: This step is to further explore the role of 5-HT2A receptor located in the PBC in the inhibition of S-IRA by activating 5-HT neurons in DR.

c. For the photometry recordings, deliver AAV2/9-mCaMKIIa-GCaMP6f-WPRE-pA via microinjection using the same protocol for the bilateral PBC (AP − 6.80 mm, ML − 1.25 mm, DV − 4.95 mm), and implant an optical fiber (FOC-W-1.25-200-0.37-3.0, Inper) over 0.05 mm (AP − 6.80 mm, ML − 1.25 mm, DV − 4.90 mm) (Figure 6).

Note: This step is to record the change of calcium signal of $PBC^{5\text{-}HT}$ neurons during seizures and the effect of photostimulating DR on the calcium signal of $PBC^{5\text{-}HT}$ neurons.

⚠ CRITICAL: Before the bone cement effectively fixes the cannula, do not place and tighten the tube core to avoid the displacement of the casing position caused by external forces (Figure 2P).

Note: When using dental cement to fix the cannula, the anchor screws must be completely covered with cement, and only the upper part of the guide sleeve is visible outside the implant structure. It is not appropriate to apply too much dental cement to avoid adverse effects on the recovery and physiological activities of mice.

13. For the photostimulation of the bilateral PBC, deliver pAAV-TPH2 PRO-ChETA-EYFP-WPRES-PAS in the bilateral PBC (AP − 6.80 mm, ML − 1.25 mm, DV − 4.95 mm), and implant an optical fiber within a guide cannula (O.D.0.48 × I.D.0.34 mm/M3.5, 62033, RWD Life and FOC-W-1.25-200-0.37-3.0, Inper, Hangzhou, China) over 0.05 mm (AP − 6.80 mm, ML − 1.25 mm, DV − 4.90 mm) (Figure 7).

Note: After implantation, allow the virus to express for 3 weeks. It is worth noting that CTB-555 only needs 1week.

This step aims to explore the effect of activation of $PBC^{5\text{-}HT}$ neurons on the incidence of S-IRA and the effect of inhibiting PBC 5-HT2A receptors during PBC photostimulation on the incidence of S-IRA.

14. At the end of the experiment, the expression of viruses and the location of an optic fiber cannula tip and guide cannula tip in the nucleus of each mouse were verified by histology.

Photostimulation and fiber photometry experiment

Timing: 1 h

This section describes how to conduct photogenetics and fiber photometry experiment as well as parameter setting for specific experiments.

Since calcium signal recording is susceptible to the influence of external light, we recommend that it be carried out in a dark environment.

15. Animal handle and habituation.

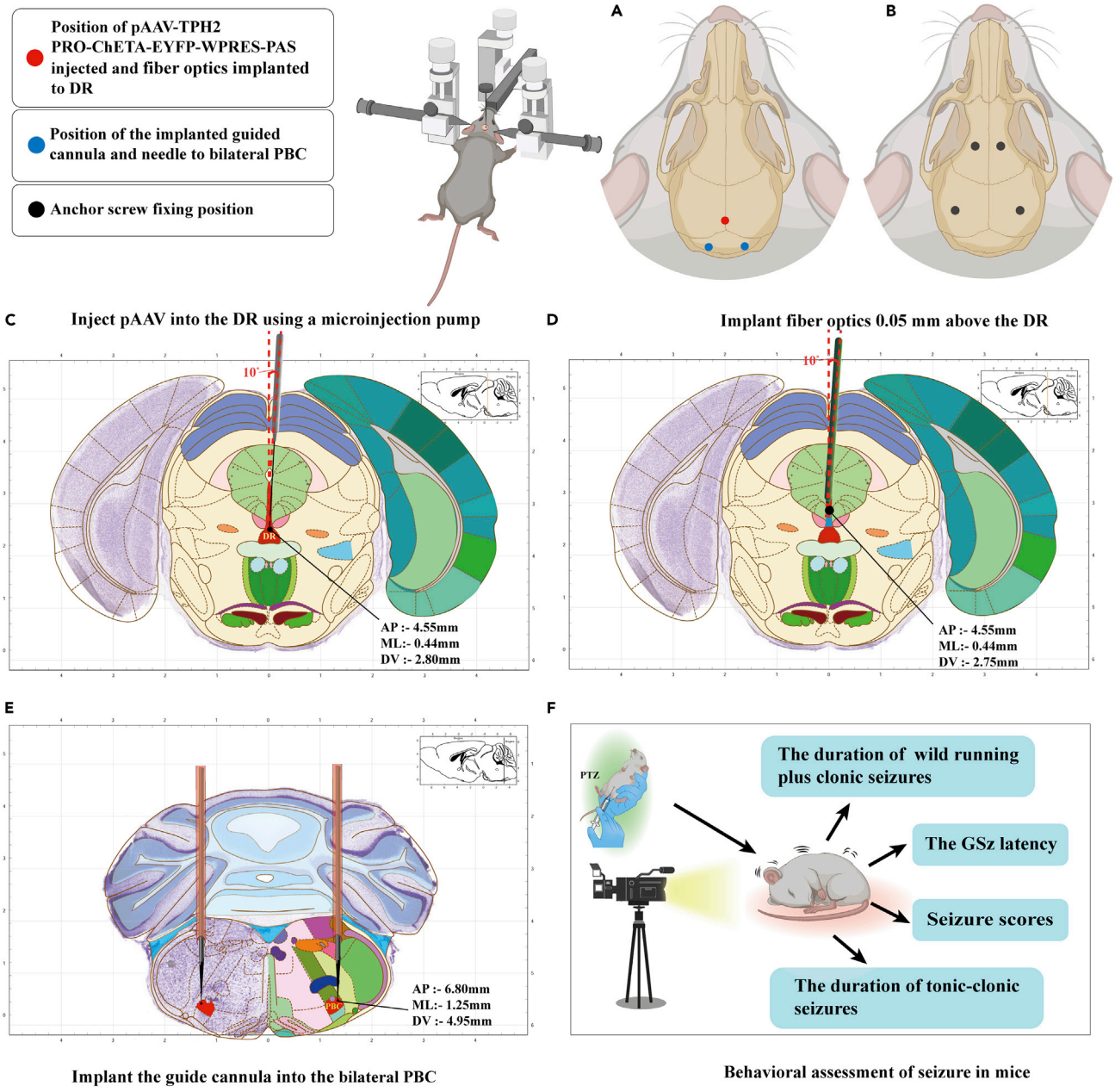

Figure 5. The implantation of optical fiber in DR and bilateral guide cannula in PBC

(A) The drilling point in the mouse skull indicates the virus injection site(red) in DR and the implantation site of bilateral guide cannula in PBC(blue).

(B) The four gray dots correspond to the recommended location of the anchor screws.

(C and D) Schematic illustration of pAAV-TPH2-PRO-ChETA-EYFP-WPRES-PAS delivery into the DR of DBA/1 mice and implantation of the optic fiber.

(E) We implanted bilateral guide cannula in PBC to microinject KET, which indicates optogenetic activation of TPH2-ChETA neurons in DR-mediated reduction of PTZ-induced S-IRA was significantly reversed by injection of KET into the PBC.

(F) Schematic diagram of intraperitoneal injection PTZ and behavioral assessment of seizure by recording. PBC pre-Bötzinger complex; PTZ, pentylenetetrazole; DR, dorsal raphe nucleus; KET, ketanserin.

a. Handle the mice injected with the virus and implanted with optical fiber two days in advance, mainly touching the back of the mice to reduce the stress reaction of the mice during the experiment.

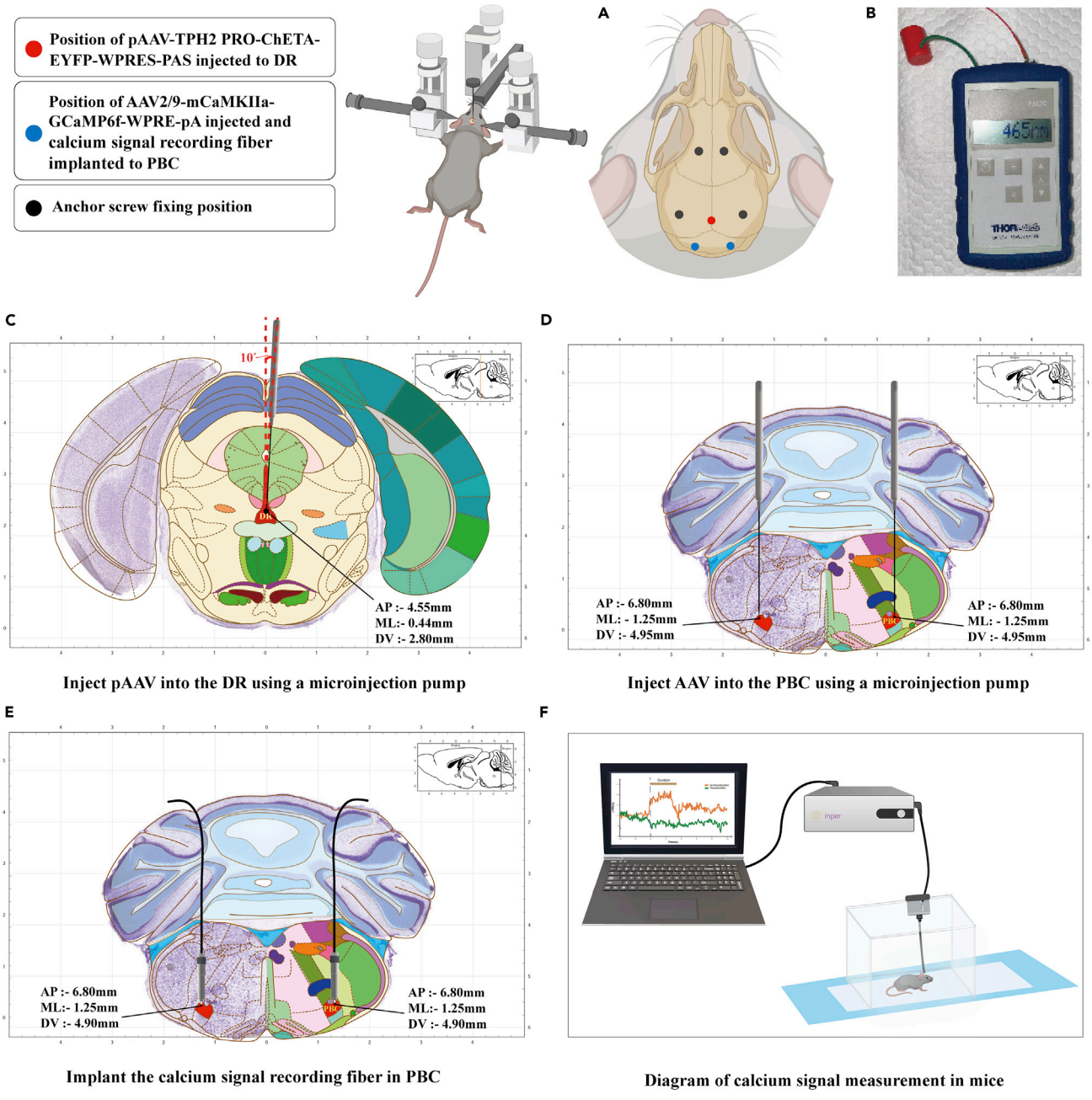

Figure 6. The implantation of optical fiber in DR and bilateral calcium recording fiber in PBC

(A) The drilling point in the mouse skull indicates the virus injection site(red) in DR and the implantation site of bilateral guide cannula in PBC(blue).

(B) Portable laser power meter. Before the optogenetic experiments and photometry recordings, we use portable laser power meter to test whether the light intensity of the front end of the optical fiber reaches the expected value.

(C) Schematic illustration of pAAV-TPH2-PRO-ChETA-EYFP-WPRES-PAS delivery into the DR of DBA/1 mice.

(D and E) Bilateral microinjection of AAV2/9-mCaMKIIa-GCaMP6f-WPRE-pA and the implantation of calcium signal recording fiber in PBC.

(F) Photometric recordings from DBA/1 mice infected with ChETA and GCaMP6f in the DR and the bilateral PBC and of neural activity based on calcium signaling in the bilateral PBC. DR, dorsal raphe nucleus: PBC, pre-Bötzinger complex.

b. Each mouse stimulation lasts about 5–7 min.

c. On the third day, place the mice implanted with optical fiber in the test device to adapt to the environment of the box, and each mouse takes about 10 min.

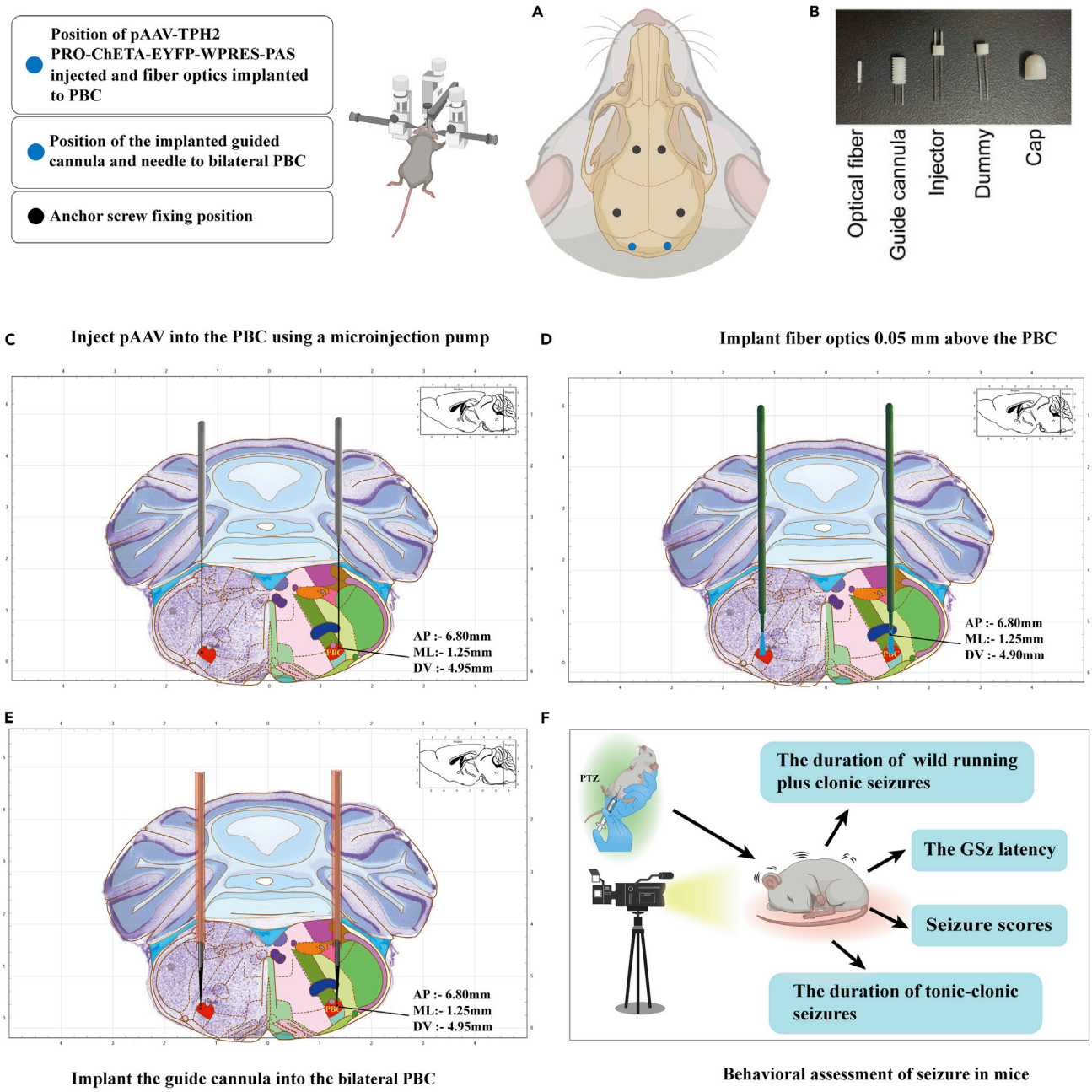

Figure 7. The implantation of bilateral optical fiber and guide cannula in PBC

(A) The drilling point in the mouse skull indicates the virus injection site and the implantation site of bilateral optical fiber and guide cannula in PBC (blue).

(B) All implantation devices in a DBA/1 mouse.

(C and D) Schematic illustration of pAAV-TPH2-PRO-ChETA-EYFP-WPRES-PAS delivery into the PBC of DBA/1 mice and implantation of the optic fiber.

(E) We implanted bilateral guide cannula in PBC to microinject KET.

(F) Schematic diagram of intraperitoneal injection PTZ and behavioral assessment of seizure by recording. PBC, pre-Bötzinger complex; KET, ketanserin; PTZ, pentylenetetrazole.

Note: Scrub the device with 75% alcohol before and after placement to eliminate external interference.

16. Connect the optic genetic system and the photometry recording system with the experimental animal through the ceramic sleeve, optical fiber, rotary joints and fiber patch cables.

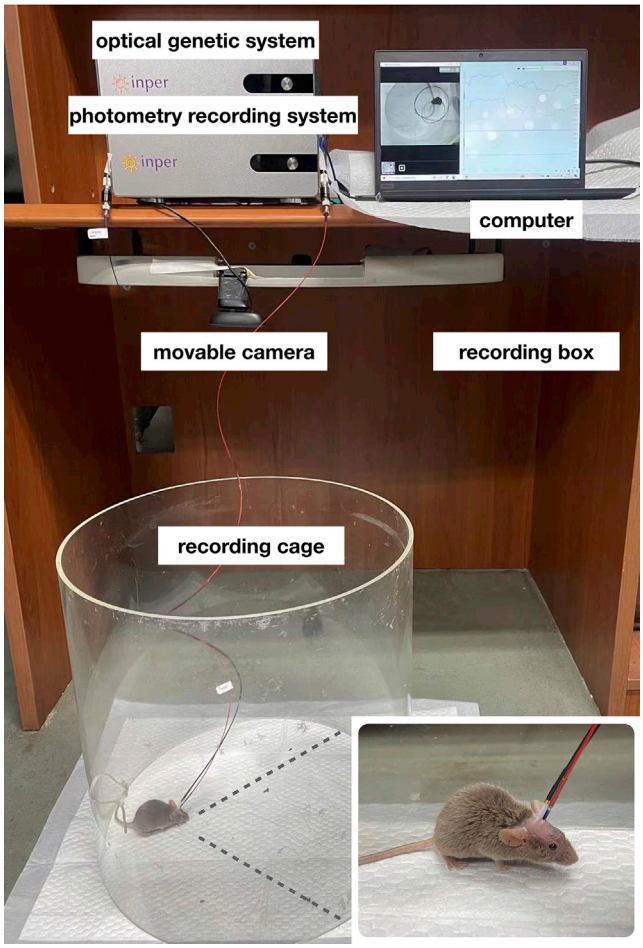

Figure 8. Photostimulation and Fiber photometry experiment

The main components of photostimulation and fiber photometry experiment: a computer with movable camera, a recording box, a recording cage, an optic genetic system and a photometry recording system with some related accessories, such as fiber patch cables and rotary joints.

Note: To ensure the accuracy of calcium signal recording, it is recommended to carry out the fiber photometry experiment in a laboratory with stable and dark light.

17. In the recording box, we place a camera to facilitate synchronous analysis of calcium signal data and animal behavior data (Figure 8).
18. Set the required experimental parameters, such as pulse width, frequency and time, on the optical genetic equipment.

Note: In our experiment, the fiber photometry system (Inper, Hangzhou, China, C11946) used a 488-nm diode laser. The parameter for photostimulation of the DR: blue-light, 465 nm, 20 Hz, 20-ms pulse width, 15 mW, and 20 min) delivered by the laser (B12124, Inper) through a 200-μm optic fiber.

⚠ CRITICAL: Before inserting the optical fiber, use the light intensity tester to test whether the light intensity at the front end of the ceramic sleeve reaches the set value (Figure 6B).

19. In the fiber photometry experiment, it is necessary to record 410 nm signal simultaneously.

Note: 410 nm laser will not affect the signal of fluorescent protein, but it can reflect the signal changes of the above interference factors to reflect the background noise signal and can remove the background noise signal to directly obtain the real calcium signal data.

20. After the animals adapt to the environment, start recording.

Note: In the selection of consumables, black ceramic inserts and black ceramic sleeves can well avoid the interference of ambient light on the changes of fluorescence in the brain of experimental animals.

EXPECTED OUTCOMES

The peripheral injection of 5-hydroxytryptophan (5-HTP) can significantly reduce the incidence of S-IRA (n = 7), and this effect can be reversed by IP injection of a certain concentration of KET. The incidence of S-IRA in the group treated with 5-HTP and KET (20 mg/kg) was significantly increased in the model of acoustic stimulation (n = 6). Injecting KET (18.30 nmol) into the lateral ventricle significantly reverses the suppressive effects of 5-HTP in the model of PTZ (n = 7) and the dosage of the reversal effect of KET produced no effects on the mortality rate of DBA/1 mice. Refer to our recently published paper (Ma et al. 2022) for more detailed data and analysis. The reduction in the incidence of PTZ-induced S-IRA via optogenetic activation of TPH2-ChETA neurons in the DR was remarkably reversed by ICV injection of KET. Subsequently, the reduction in the incidence of PTZ-induced S-IRA by photostimulation of the DR was significantly reversed by microinjection of KET into the bilateral PBC, in turn, suggesting that photostimulation of the DR remarkably reduced the incidence of S-IRA by activating 5-HT2AR in the PBC in our model.

Virus expression in nerve cells requires at least 3 weeks. The epifluorescence of endogenous EYFP is strong enough to observe a green signal without immunohistochemical staining. After the cryostat section, TPH2 and c-fos strongly apparent fluorescence and could be observed in the nerve cells of DR and PBC. The placement of the optical fiber and cannula tip within the DR and bilateral PBC in each mouse was verified by histology. The PBC region was identified by neurokinin-1 receptor (NK1R) staining. For the figure of histology, please refer to Ma et al. (2022).

To further examine the bridge between the DR and PBC and its role in modulating S-IRA in our models, we used CTB-555, a nerve retrograde tracer, to confirm and establish the neural circuit between the DR and PBC. CTB555 fluorescence was seen in the PBC after injection of CTB-555 into DR.

QUANTIFICATION AND STATISTICAL ANALYSIS

All data are presented as the mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed using SPSS 23 (SPSS Software Inc., Chicago, IL, USA). The incidence rates of S-IRA in different groups were compared using Wilcoxon signed rank test. Seizure scores, the latency to AGSz, the duration of wild running and clonic seizures, and duration of tonic-clonic seizures were evaluated using one-way analysis of variance (ANOVA), unpaired t-test. The standard for seizure scores: A score of zero was assigned if there was no seizure. A wild running is scored as one point. Two or more wild running are given two points. The presence of tonic-clonic or S-IRA was scored as 3 points.[10–12] One-way ANOVA test was used to compare the clonic seizures and tonic seizures peak $\Delta F/F$. Two-way ANOVA test was used for EEG analysis. Statistical significance was inferred if $P < 0.05$.

LIMITATIONS

We used CTB-555, a nerve retrograde tracer, to confirm and establish the neural circuit between the DR and PBC. However, there is insufficient evidence for the specificity of DR 5-HT neuronal manipulations and retrograde prediction between DR and PBC. When CTB-555 was injected into DR, it showed up in the PBC and vice versa, but these manifestations emerged one week after the CTB-555

injection, suggesting that this approach may not be the most effective for proving the existence of direct synaptic connections between the DR and PBC. Furthermore, activation of c-fos in PBC with DR optogenetic stimulation may not be a direct way to validate the projection between these two nuclei. It needs to employ more specific methods, using tracers other than CTB-555, to trace and investigate the neuronal circuit between DR and PBC as well as the direct synaptic connections from DR 5-HT neurons to PBC.

We used optical fiber photometry in our experiments to record calcium signals, but it can only record the activity of population cells, not single neuronal cells. In the future, we will try to use two-photon calcium imaging to record the activity of neurons within nuclei, which has good resolution and can show calcium activity not only of individual neurons, but also of subcellular structure axon terminals.

TROUBLESHOOTING

Problem 1

When the optical fiber is implanted in DR, the region has many blood vessels around, which are very vulnerable to damage, easily leading to massive hemorrhage and death of mice (steps 6, 9 in "virus injection").

Potential solution

During the experiment, we found that there were abundant blood vessels around DR, so if the optical fiber was implanted vertically into DR, it would be more likely to damage the blood vessels around the brain area, leading to massive hemorrhage and death of mice. Therefore, we implanted the optical fiber at a right tilt of 10° to avoid damaging the blood vessels directly above the DR, thereby reducing the postoperative mortality of the mice. The optical fiber (FOC-W-1.25-200-0.37-3.0, Inper, Hangzhou, China) was implanted above the area (AP − 4.55 mm, ML − 0.44 mm, DV − 2.80 mm, 10°right) for 0.05 mm (AP − 4.55 mm, ML − 0.44 m, DV − 2.75 mm, 10°right). If bleeding persists, dip a few hydrogen peroxide with a small cotton ball and place it over the bleeding site.

Problem 2

The injected drug distribution may be uneven, when administered through the lateral ventricle (step 12 in "device implantation").

Potential solution

When configuring the drug, we uniformly vibrate to ensure that there is no particle precipitation and adherence to the tube wall to test if the drug distribution has been homogeneous. To be sure that the drug was homogeneously distributed when injected into the lateral ventricle, we rinsed the syringe with the prepared drug before injection and ensured that there were no bubbles in the syringe and cannula cores. Although a part of the drug is wasted, it ensures that the drug injection is evenly distributed in the lateral ventricle.

Problem 3

Due to the sound sensitivity of DBA/1 mice, ambient sound may have an impact on the acoustic stimulation seizure of mice during the rearing and experimental processes (step 2 in "breeding animals", step 13 in "preparation of surgical area").

Potential solution

During the process of rearing the mice, ensure that the room is quiet and avoid excessive decibel sounds. To protect the sound sensitivity of DBA/1 mice at the time of surgery, cotton balls were placed into the ears of DBA/1 mice to protect the tympanic membrane. Before weighing and after the operation, the cotton ball was removed.

Problem 4

When the sound stimulates mice, other mice are likely to be affected by the sound (step 7 in "Preparation for acoustic and PZT induced models").

Potential solution

When acoustic stimulation, we put the mice in a soundproof room to prevent other mice from being affected by the sound stimulus and then ring the bell. The walls of the room are covered with soundproof sponges. Measure the decibels of the raising room before and during the experiment, especially when acoustically stimulating the mice, to ensure that there is no difference in the decibels between soundproof room and raising room.

Problem 5

DBA/1 mice with acoustic stimulation were affected by temperature. Lower temperature reduced the incidence of S-IRA in acoustically stimulated mice (step 4 in "breeding animals").

Potential solution

In order to reduce the influence of temperature on mice, we made a box with heat preservation and sound insulation to transport mice outdoors. During feeding and experiment, the room temperature was kept at 25°–26°.

RESOURCE AVAILABILITY

Lead contact

Further information and requests should be directed to and will be fulfilled by the lead contact, Honghai Zhang (zhanghonghai_0902@163.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate new unique data or code.

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AUTHOR CONTRIBUTIONS

Conceptualization and methodology, H.H.Z.; writing – original draft, methodology and investigation, H.X.M., Y.S., X.T.L., W.H.S., J.X.G., and L.L.; writing – review & editing and investigation, Y.L.W., Q.X., Q.Y., and L.Y.G.; funding acquisition, supervision and resources, H.H.Z. We are grateful for YuDong Zhou and Yi Shen for the experimental design.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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