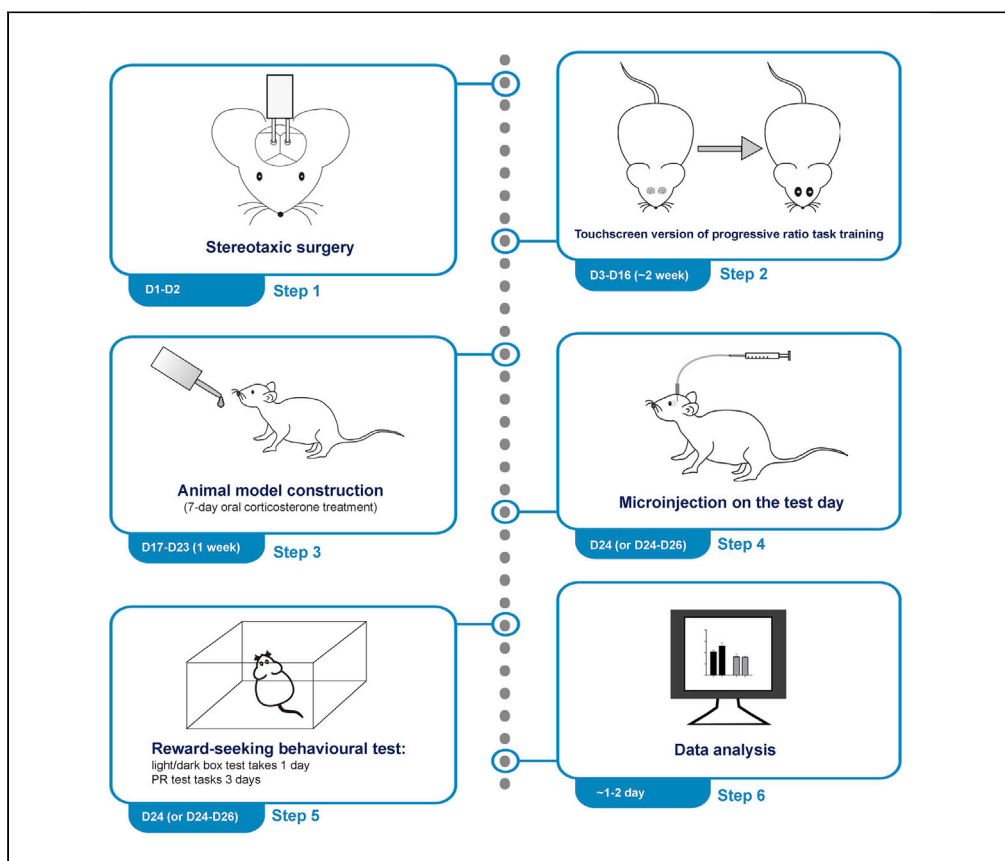## Protocol

Using intra-brain drug infusion to investigate neural mechanisms underlying reward-seeking behavior in mice

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Highlights

Intra-brain drug infusion for studying neural mechanisms underlying behavioral deficit

Behavioral paradigms for testing reward-seeking behavior

Python-based analysis tool for behavioral data processing

Brain-region-specific drug infusion is a key way to investigate neural mechanisms underlying behavior and neurological diseases. Here, we present a detailed protocol for cannula implantation, intra-brain drug infusion, and two reward-seeking-related behavioral paradigms in mice: the light/dark box test and touchscreen version of progressive ratio test. In addition, we provide a user-friendly Python-based tool for behavioral data analysis. This protocol can be easily adapted to address various research questions related to behavioral pharmacology.

STAR Protocols

Protocol

Using intra-brain drug infusion to investigate neural mechanisms underlying reward-seeking behavior in mice

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https://doi.org/10.1016/j.xpro.2022.101221

SUMMARY

Brain-region-specific drug infusion is a key way to investigate neural mechanisms underlying behavior and neurological diseases. Here, we present a detailed protocol for cannula implantation, intra-brain drug infusion, and two reward-seeking-related behavioral paradigms in mice: the light/dark box test and touchscreen version of progressive ratio test. In addition, we provide a user-friendly Python-based tool for behavioral data analysis. This protocol can be easily adapted to address various research questions related to behavioral pharmacology.

For complete details on the use and execution of this protocol, please refer to Peng et al. (2021).

BEFORE YOU BEGIN

The protocol below describes using microinjection to investigate neural mechanisms underlying corticosterone (CORT)-induced reward-seeking deficits. The same microinjection protocol has been used to explore how the satiety signal, insulin, influences feeding behaviors (Labouèbe et al., 2013; Liu et al., 2016).

Animal use and procedures in this protocol are in accordance with the ethical guidelines established by the East China Normal University Animal Care Committee.

Animal model: 7-day oral CORT treatment to mice

© Timing: 1 week

Chronic CORT treatment induces a variety of behavioral deficits, from anxiety-related phenotype (David et al., 2009) to impaired positive valence behaviors (Dieterich et al., 2019; Gourley et al., 2008; Moda-Sava et al., 2019). Consistently, we showed that 7-day CORT treatment decreased food-seeking behavior in a mildly risky environment, which is possibly be explained by elevated anxiety (Peng et al., 2021). However, we did not observe any depressive-like phenotypes, which is reported in mice with 21-day CORT treatment (Moda-Sava et al., 2019). Hence, we suggest that
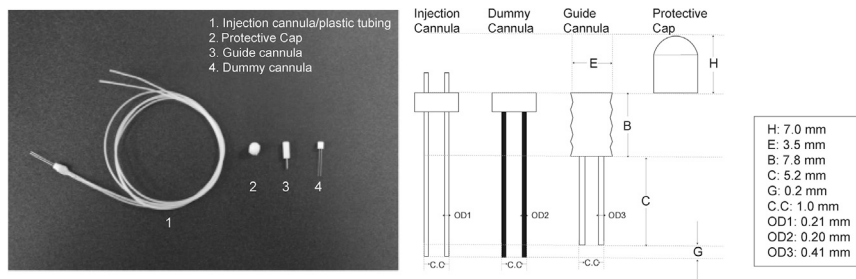
Figure 1. Photograph (left) and cording diagram (right) of the double cannula
The parameters listed are specific for bilateral intra-VTA drug infusion to C57BL/6J mice. Guide cannula (RWD 62064): outer diameter (OD) 0.41 mm × inner diameter (ID) 0.25 mm, center to center distance (C.C) 1 mm, length C 5.2 mm. Dummy cannula (RWD 62164): OD 0.20 mm, C.C 1 mm, length 5.4 mm. Injector (RWD 62264): OD 0.21 mm × ID 0.11 mm, length 5.4 mm. Protective cap (RWD 62523): Height H 7.0 mm, OD 5.5 mm.
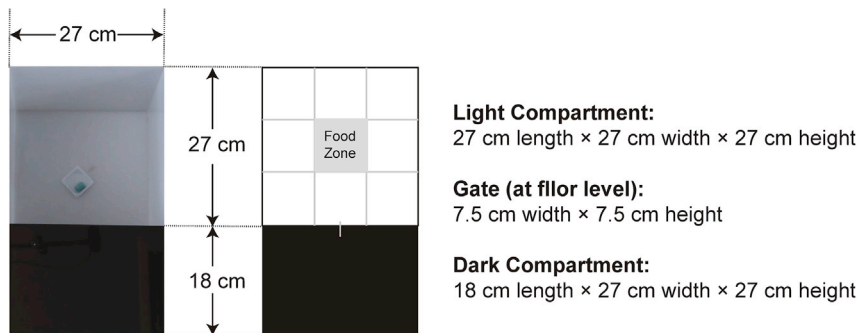
one should check the CORT treatment protocol carefully while reviewing relevant publications and adapt it according to the experimenter's research questions/objectives.

1. Animals:
   a. P21 male C57BL/6J mice are from The Jackson Laboratory. The age and sex specified are for studying the effects of chronic CORT treatment in periadolescent male mice. Other age, sex and strain can be used according to the experimenter's own research questions.
   b. Animals are housed in a humidity (40%–60%)- and temperature (18°C–23°C)-controlled environment in a 12 h light/12 h dark cycle with free access to food and water.
   c. Animals are acclimated to the environment and habituated to handling for 3–5 days prior to the behavioral tests.
2. Drug preparation:
   a. Due to its poor solubility in the aqueous solution, CORT (Cayman) is dissolved in ethanol (1 mg/mL). Dissolve CORT completely using a vortex mixer (VWR) before storing aliquots at −20°C or below up to 1 month.
   b. Before usage, dilute CORT/ethanol solution with water to a final concentration of 100 μg/mL.
3. Drug treatment: after surgery recovery or behavioral training, mice are exposed to drinking water containing 100 μg/mL CORT or vehicle (1% ethanol) for 7 days. It is recommended to check physiological conditions daily after surgery. Mice in post-surgery poor condition should be removed. Please refer to the surgical procedures part 9.d. for signs of pain and distress.

Cannula preparation

⏱ Timing: few hours

4. Cannulas. A set of cannulas includes a guide cannula (GC), an injection cannula (IC), a dummy cannula (DC) and a projective cap (Figure 1).
   a. Guide cannula: as its name indicated, GC guides the IC to the target brain region. It comprises a stainless-steel tube and a threaded cylindrical plastic pedestal.
   b. Injection cannula: IC is inserted into the GC to deliver drug or sample.
   c. Dummy cannula: DC remains in the GC to prevent cerebral-spinal fluid and tissues from entering the bottom of the GC. Screw the protective cap to prevent DC from dropping out.
   d. For bilateral intra-VTA (ventral tegmental area) drug infusion, we order cannulas from a commercial company (RWD Life Science) with customized parameters.
      i. Guide cannula (RWD 62064): 26-gauge, OD (outer diameter) 0.41 mm, ID (inner diameter) 0.25 mm, C.C (center to center distance) 1 mm, length 5.2 mm.

Figure 2. The acrylic light/dark box

Light Compartment:
27 cm length × 27 cm width × 27 cm height

Gate (at flor level):
7.5 cm width × 7.5 cm height

Dark Compartment:
18 cm length × 27 cm width × 27 cm height

  ii. Injection cannula (RWD 62264): 32-gauge, OD 0.21 mm, ID 0.11 mm, length 5.4 mm, C.C 1 mm.
  iii. Dummy cannula (RWD 62164): OD 0.20 mm, length 5.4 mm, C.C 1 mm.
  iv. Protective cap (RWD 62523): OD 5.5 mm, height 7.0 mm.
5. Injector preparing.
   a. Prepare two 50 cm long polyethylene tubes (0.85 mm OD*0.42 mm ID, RWD 62321).
   b. Tightly connect one end of the plastic tubing with the injection cannula.
   c. Seal the connection with hot-melt adhesive.
   d. Check the injection cannula/plastic tubing assembly: either leakage or clog can cause micro-injection failure. Always check the assembly before the experiment.
      i. Fill a 1 mL syringe with sterile water. Do not use saline as it could clog the plastic tubing.
      ii. Slide the plastic tubing onto the needle (26 gauge) attached to the syringe.
      iii. Flush the plastic tubing. There should be no resistance while filling the plastic tubing. Water should spray from the injection cannula tip and a straight stream should be observed.
      iv. Fill the plastic tubing and keep it upright. Water should drop fluidly. Otherwise, flush the assembly and check it again.

Preparing for behavior tests

⏱ Timing: n/a

6. Light/dark box ordering/customization (Figure 2).
   a. The light/dark box is an acrylic box (45 cm length × 27 cm width × 27 cm height) divided into dark (black acrylic, 18 cm length × 27 cm width × 27 cm height) and light (white acrylic, 27 cm length × 27 cm width × 27 cm height) compartments. A gate (7.5 cm width × 7.5 cm height) at floor level allows free shuttle between chambers.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Pentobarbital sodium	Sigma-Aldrich	P3761
Sulpiride	Tocris Bioscience	Cat# 0894
Corticosterone	Cayman Chemical	Cat# 16063
Vaseline	Sangon Biotech	Cat# A510146
Super-Bond C&B Kit	Sun medical	N/A
Ketoprofen	Cayman chemical	CAT# 10006661
High-fat food pellet	Research diets	Cat# d12492
Strawberry milkshake	Yazoo	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Sodium chloride	Sangon Biotech	Cat# A610476-0001
Potassium chloride	Sangon Biotech	Cat# A610440-0500
Sodium dihydrogen phosphate	Sangon Biotech	Cat# A610879-0500
Magnesium chloride	Sangon Biotech	Cat# A610328-0500
Calcium chloride	Sangon Biotech	Cat# A610050-0500
Sodium hydrogen carbonate	Sangon Biotech	Cat# A100865-0500
Glucose	Sangon Biotech	Cat# A600219-0500
4% paraformaldehyde solution	Sangon Biotech	Cat# E672002-0500
Sucrose	Sangon Biotech	Cat# A502792-0500
Phosphate buffer solution	Sangon Biotech	Cat# E607008-0500
Experimental models: Organisms/strains		
C57BL/6J (p21)	The Jackson Laboratory	N/A
Software and algorithms		
ABET II (ABETII_TOUCH2.19.5834.17763)	Lafayette Instrument Company	https://lafayetteneuroscience.com/products/abetii-touch-screen-software
Python (Spyder)	Anaconda	https://docs.anaconda.com/anaconda/install/index.html
Prism 8	GraphPad	https://www.graphpad.com/scientific-software/prism/
Any-Maze	Any-Maze	https://www.any-maze.com
Deposited data		
The code generated for PR task performance analysis ('PR_parameters_ABETII_TOUCH2. 19.5834.17763.py', 'PR_parametersSessionMean_ ABETII_TOUCH2.19.5834.17763.py')	This study	https://github.com/xcccc402/Touchscreen-version-of-PR-task-data-analysis.git (https://doi.org/10.5281/zenodo.5987836)
Other		
Polyethylene plastic tubing	RWD	Cat# 62321
Dry sterilizer	VWR	Cat# 102095-946
Stereotaxic instrument	RWD	Cat# 68030
Digital microscope	RWD	Cat# 77001D
Surgical scissors	RWD	Cat# S12003-09
Surgical tweezers	RWD	Cat# F12006-10
Surgical spatula	RWD	Cat# R51003-11
Drill	RWD	Cat# 78001
Drill holder	RWD	Cat# 68605
Standard probe holder	RWD	Cat# 68201
Cannula holder	RWD	Cat# 68217
Protective cap	RWD	Cat# 62523
Dummy cannula	RWD	Cat# 62164
Injection cannula	RWD	Cat# 62264
Guide cannula	RWD	Cat# 62064
Microsyringes (5 μL)	Hamilton	Cat# 87900
Syringe pump	RWD	Cat# R404
Light/dark box / acrylic box	N/A	N/A
Bussey-Saksida mouse touch screen chamber	Campden Instruments	Cat# 80614
Vapor pressure osmometer	wescor	Cat# 5520
Digital lux meter	Shenzhen Master Industrial	MS 6612
Vortex mixer	VWR	Cat# 97043-562
Hot-melt adhesive	Suoli	Cat# SL-RJ1120
Camera	Logitech	Cat# C270 HD
Cryotome	Thermo Fisher Scientific	Cat# A78910101
Light microscope	LW scientific	Cat# R3M-TN4A-DAL3

MATERIALS AND EQUIPMENT

Artificial cerebrospinal fluid (aCSF)		
Reagent	Final concentration	Amount
NaCl	126 mM	7.36 g
KCl	1.6 mM	0.12 g
$NaH_2PO_4.2H_2O$	1.1 mM	0.17 g
$MgCl_2.6H_2O$	1.4 mM	0.29 g
$CaCl_2.2H_2O$	2.4 mM	0.35 g
$NaHCO_3$	26 mM	2.18 g
Glucose	11 mM	1.98 g
ddH_2O	N/A	Adjust to 1 L
Total		1L

Note: Measure osmolarity of prepared aCSF with the osmometer (Wescor). The acceptable value is 300 ± 5 mOsm/L. Store aCSF at 4°C for up to 2 days.

STEP-BY-STEP METHOD DETAILS
Surgical procedures

⏲ Timing: 0.5 h/cannula implantation

Main procedures include pre-surgery preparation, anesthesia, incision/craniotomy, cannula implantation, and post-operative care. Figure 3 illustrates all instruments and reagents. A checklist is also provided (Table 1). Cannula implantation is an invasive surgery that may lead to a 10% mortality rate in preadolescent mice.

1. Surgical instruments sterilization. Turn on the sterilizer Germinator 500™. Insert the tips of surgical instruments in the stainless steel glass beads for 15 s.

   ⚠ CRITICAL: Surgery must be carried out in a sterilized environment. All surgical instruments must be sterilized between procedures. Decontamination with 75% alcohol may be used if a more effective sterilizer such as Germinator 500™ is not available in the lab. However, 75% alcohol is not recommended for sterilization purpose due to its poor sporicidal action (Rutala and Weber, 2008).

2. Anesthesia. Anesthetize C57BL/6J mice with pentobarbital sodium solution (80 mg/kg, dissolved in saline 0.6% w/v, intraperitoneal). After 5 min, assess anesthesia level by toe-pinch to ensure the anesthesia is adequate for surgery. If necessary, give a supplementary dose less than 25% of the first dose.

   ⚠ CRITICAL: Animal use and procedures must be in accordance with the guidelines approved by Institutional Animal Care and Use Committee (IACUC). Alternative anesthesia protocol approved by IACUC can be used.

3. Placement in the stereotaxic apparatus (Figure 4.1). After checking anesthesia, shave mouse head, coat its eyes with Vaseline (Sangon Biotech), and headfix the mouse in a stereotaxic apparatus (RWD 38313). To mount mice on the head stage, insert ear bars into the ear canal and secure the mouth with the incisor bar. Inject 0.3 mL warm sterile saline to prevent dehydration during surgery.

4. Scalp incision (Figure 4.2). Swab scalp with 75% alcohol and then with iodine. Pick up the scalp with a sterilized tweezer and make a sagittal incision with a sterilized scissor. The incision should
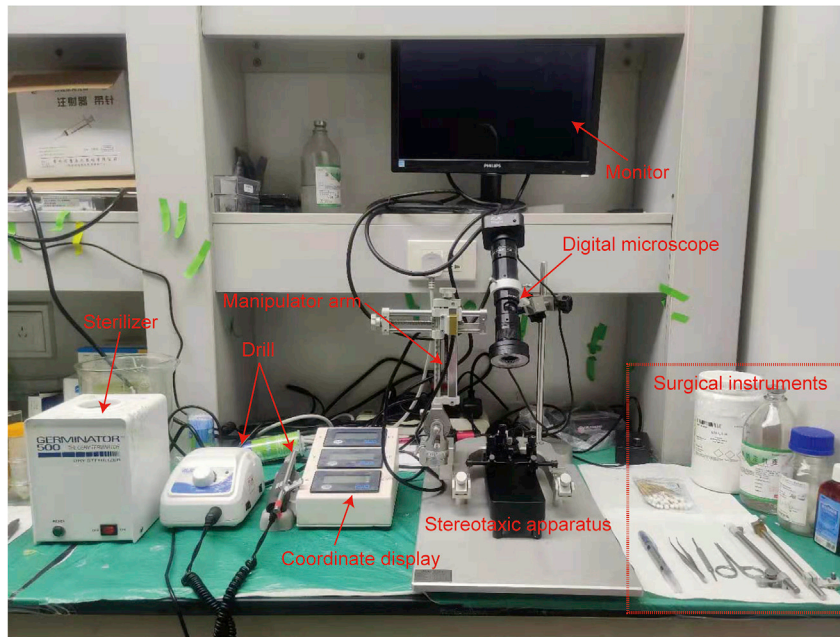
Figure 3. Surgery equipment

be long enough to expose bregma and lambda (Franklin and Paxinos, 2008). Scrape away tissues on the top of the skull with a surgical spatula and clean the skull with a cotton swab.

> △ CRITICAL: Make sure you can see sutures and blood vessels clearly under an operating microscope. If not, swab the skull again and wait a few minutes to let the skull dry completely.

5. Leveling.
   a. Use a needle mounted on a probe holder in the manipulator arm of the stereotaxic instrument to mark bregma (Figure 4.3).
   b. Set bregma X (mediolateral, ML)-, Y (anteroposterior, AP)-, Z (dorsoventral, DV)-axis coordinates to zero (or record coordinates if coordinate display is not available).
   c. Move the needle tip to lambda and read Z-axis coordinate (Figure 4.3).
   d. Adjust the incisor bar if the difference between bregma and lambda DV coordinates is greater than ± 0.02 mm.
   e. Repeat steps b-d until the criterion is met.
   f. Move the holder to Bregma-post and set X-, Y-, and Z-axis coordinates to zero.
   g. Posit the holder to AP -3.2 mm, ML +1.5 mm. Lower the needle tip to the skull and read Z-axis coordinate (DV1, Figure 4.3).
   h. Posit the hold to AP -3.2 mm, ML -1.5 mm. Lower the needle tip to the skull and read Z-axis coordinate (DV2, Figure 4.3).
   i. Adjust the ear bar if the difference between DV1 and DV2 is greater than ± 0.02 mm.
   j. Repeat steps b-i until the criterion is met.
   k. Posit the needle tip to Bregma-final and set the AP, DA, ML coordinates to zero.

> △ CRITICAL: The purpose of steps b-e is to make sure the mouse head is flat. This is the most crucial step for accurate cannula implantation. AP and ML coordinates mentioned in steps f-h can be adjusted according to reader's own brain region of interest. Steps f-h are for bilateral symmetry check. Please follow manufacturer's instruction, because these steps may vary among different stereotaxic instruments.

Table 1. A surgical checklist for cannula implantation

Item	Necessity
Cannulas	Yes
(Digital) Operating Microscope (with a monitor)	Yes
(Digital) Stereotaxic Instruments, including a left-hand manipulator arm, (digital display module), (adaptor), ear bars and a standard probe holder.	Yes
Drill	Yes
Sterilizer	No but recommended
Surgical tweezers, tweezers, and spatulas	Yes
Cannula holder	Yes
Marker	Yes
75% alcohol, iodine	Yes
Dental cement	Yes
Skull screws	No
Vaseline (or ophthalmic ointments)	Yes
Cotton/swab	Yes

> △ CRITICAL: Lower the needle holder slowly because a sharp needle can pierce the skull.

6. Craniotomy.
   a. Mark the position to be drilled (ML $\pm$0.50 mm; AP -3.20 mm for VTA, Figure 4.3).
   b. Mount the drill on a drill holder and place the holder in the manipulator arm.
   c. Drill two holes carefully to prevent dura and brain tissues from damaging (Figure 4.4). One may apply warm sterile saline to the skull before drilling to prevent brain tissues from heating.
   d. Clean the skull and/or stop bleeding using a cotton swab.

> △ CRITICAL: Drill tips 0.6 mm in diameter are used in the protocol. The diameter of the drill tip should be bigger than the outer diameter of the guide cannula. Sterilize the drill tip between procedures to avoid cross infection.

7. Cannula placement.
   a. Mount guide cannula on a cannula holder. The line between tips of the guide cannula should be perpendicular to the sagittal plane (Figure 4.5).
   b. Mount the holder in the manipulator arm, posit the cannula tip above the two holes (Figure 4.6).
   c. When the cannula tip touches dura (Figure 4.7), set the Z-axis coordinate to zero.
   d. Lower the cannula slowly until the cannula is posited to the predetermined DV coordinate (DV -4.60 mm for VTA). When bleeding happens, stanch before continuing.

> △ CRITICAL: Do not lower the cannula when there is excessive bleeding.

> △ CRITICAL: Cannula tip is blunt. One may use a sterilized needle tip to pierce dura before cannula insertion. This helps to reduce bleeding.

8. Fixing.
   a. Get super-bond C&B kit (Sun medical) from the refrigerator. Mix up $^3/_4$ cup of polymer, four drops of monomer and one drop of catalyst V.
   b. Secure the cannula with dental cement (Figure 4.8).
   c. Let dental cement dry completely before removing the cannula holder.
   d. Remove the mouse from the stereotaxic apparatus.
   e. Screw the protective cap on the guide cannula. Hold the cannula with a tweezer to prevent head rotation.
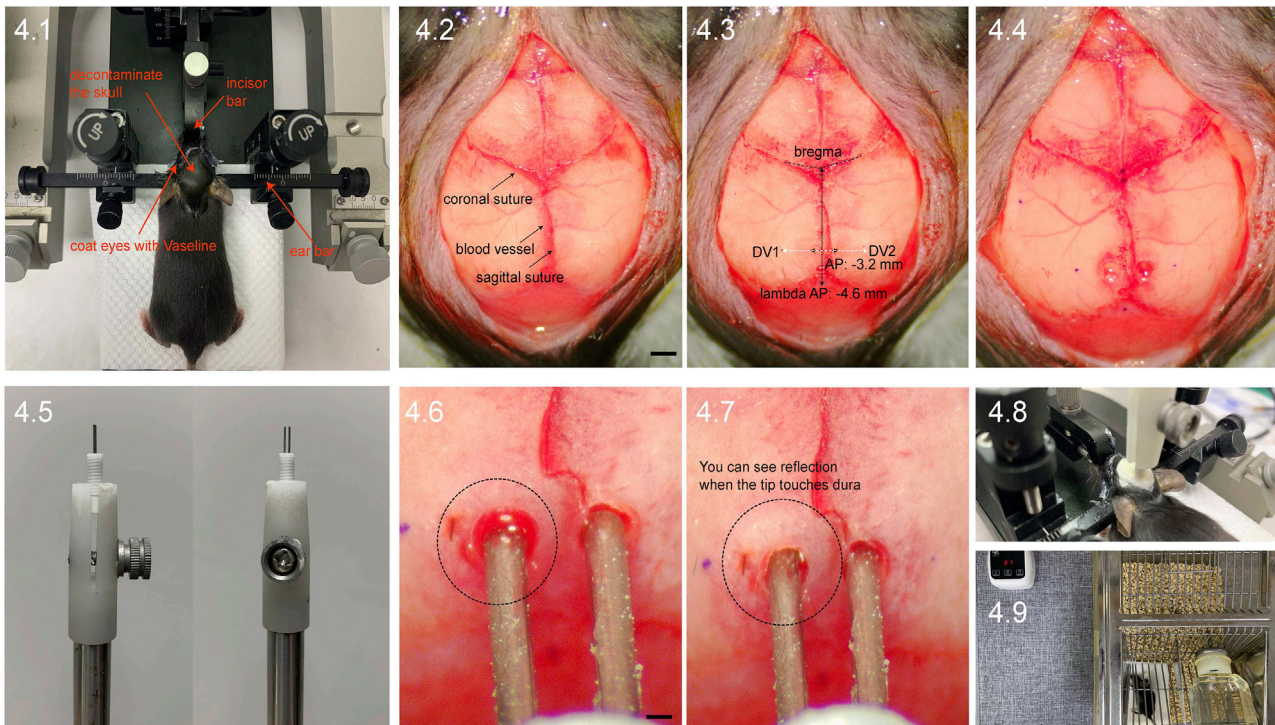
Figure 4. The main steps of a stereotaxic surgery for cannula implantation
4.1 Mounting a mouse on the stereotaxic apparatus;
4.2–4.4 Incision and craniotomy, scale bar in 4.2: 1 mm;
4.5–4.8 Cannula placement, scale bar in 4.6: 0.25 mm;
4.9 Post-operative care.

> ⚠ CRITICAL: Avoid applying the dental cement to the skin or eyes of the mouse, or to the cannula-holder junction. Occasionally, protective cap and dummy cannula drop out. Daily check is necessary to ensure the two accessories remain in place.

9. Post-operative care.
   a. Inject ketoprofen (5 mg/kg, Cayman chemical) subcutaneously.
   b. Place the mouse in a clean cage and let it recover on the heating pad (Figure 4.9).
   c. After full recovery from the anesthesia, transfer the mouse to its home cage.
   d. Monitor the animal daily until its full recovery from surgery. Signs of infection, pain and distress include (not exclusively):
      i. Self-isolation (if group-housed).
      ii. Low spontaneous locomotion.
      iii. Little water/food intake.
      iv. Hunched posture.
      v. Lack of grooming.
      vi. Excess weight loss.

Consult animal care staff when necessary.

> ⚠ CRITICAL: Ketoprofen belongs to non-steroidal anti-inflammatory drugs (NSAIDs), which are frequently used post-surgery to relieve pain and reduce inflammation. However, ketoprofen non-specifically blocks the induction of both cyclooxygenase-1 (Cox1) and Cox2 (Lamon et al., 2008). Losing protective effects of Cox1 can result in severe gastrointestinal

and renal problems. Hence, avoid using ketoprofen for more than 3 days. Other IACUC approved analgesia protocols can be used.

Mouse light/dark box test

⏱ Timing: 2 days

The risk/reward decision making is dynamic and sensitive to multiple factors including metabolic and emotional state. For example, hunger state increases salience of the risk of starvation, prompting foraging for food despite the risk of predation (Lockie et al., 2017). On the other hand, negative emotions such as anxiety make animals reluctant to seek food rewards in a battery of conflicts, such as the conflict between food consumption and neophobia (Heinz et al., 2021).

Food seeking in a natural environment frequently involves evaluating the risk of foraging against reward value (Lockie et al., 2017). Based on the innate aversion of rodents to open, brightly illuminated arenas, a modified light/dark box is designed to assess risk/reward decision making in laboratory (Bourin and Hascoët, 2003; Liu et al., 2016; Lockie et al., 2017). A plateable high-fat food pellet is placed in the center of the light box. Mice must resolve the conflict between staying in a comfort zone and foraging for food in an anxiogenic zone.

With this behavior paradigm, we show that long-term CORT treatment attenuates food-seeking in the open light compartment. To test if D2 receptor antagonist sulpiride can restore food-seeking, intra-VTA sulpiride or control aCSF (artificial cerebrospinal fluid) infusion is performed via injection cannula using a syringe pump. Here, we describe the major steps of the modified light/dark box test. Please refer to the intra-VTA drug infusion part for microinjection guidance.

10. Set up. It is necessary to minimize exposure of the test mice to unnecessary stressful events such as noise and over-bright light source.
    a. Measure brightness with a lux meter (Master). Adjust the light source to ensure that the luminous flux in the light chamber is approximately 100 lux.
    b. Mount the camera (Logitech) on a tripod and place the camera above the light/dark box. Make sure it captures the entire field.
11. Habituation. This step is required to prevent food neophobia and to familiarize mice with the apparatus.
    a. Transfer mice to the test room at least 30 min before experiments.
    b. Clean the light/dark box with 75% alcohol before use.
    c. Put a palatable high-fat food pellet (Research diets, d12492) in the food zone (9 cm × 9 cm arena) of the light chamber.
    d. Place a mouse at the corner of the light chamber and let it freely explore the box for 5 min.
    e. Return the mouse to its home cage.
    f. Repeat steps a-d for another mouse until finishing.
12. Food-seeking behavior test. During the test day, mice first receive sulpiride or vehicle infusion into the VTA and wait in their home cage for a quarter hour before the light/dark box test. Make sure the camera is on to record mouse's behavior, which is analyzed *post hoc* with Any-Maze software or other software available in the lab. Same as the habituation session, place high-fat food in the food zone and introduce mouse into the box. Each trial lasts 10 min.

Intra-VTA drug infusion

⏱ Timing: 3 days

Infuse D2R antagonist sulpiride into the VTA before the light/dark box test to investigate its effect on reward-seeking behavior. Pump a bolus of drug or vehicle through an injection cannula with a
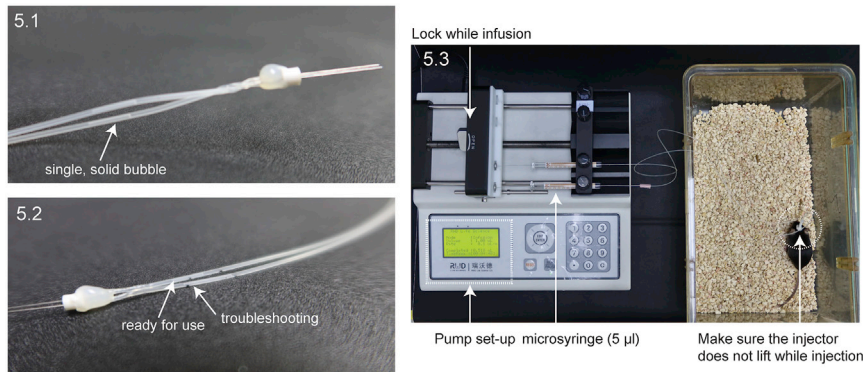
Figure 5. Demonstration of the infusion system
5.1 and 5.2 Injection process can be inferred from movement of the bubble.
5.3 The syringe pump (RWD R404) pushes the microsyringes (Hamilton 87900) forward to deliver a bolus of drug or vehicle through the injector.

syringe pump (RWD R404). It is necessary to test the infusion system before each microinjection and check for injection process *post hoc*. Details are described below.

13. Preparing reagents for injection.
    a. aCSF contains the following (in mM): 126 NaCl, 1.6 KCl, 1.1 NaH_2PO_4, 1.4 $MgCl_2$, 2.4 $CaCl_2$, 26 $NaHCO_3$, and 11 glucose. Measure osmolarity of prepared aCSF with the osmometer (Wescor). The acceptable value is 300 $\pm$ 5 mOsm/L.
    b. Dissolve 1 mg sulpiride (Tocris) in aCSF with a final concentration of 2.2 mM.
    c. Filter prepared solution by a syringe filter and pipette 200 μL filtered solution into a 0.2 mL centrifuge tube. Mark the centrifuge tube.
14. Checking the injection cannula/plastic tubing assembly. The assembly may be re-used for the same drug but must be checked for leakage and patency. Please refer to Cannula preparation section for details.
15. Infusion system set up.
    a. Mount 4 microsyringes (Hamilton 87900) on the syringe pump (Infuse vehicle (aCSF) and drug (sulpiride) separately).
    b. Slide the water-filled plastic tubing onto the microsyringe tip. Make sure the connection is tight.
    c. Pull the syringe plunger back 1 μL to create an air space.
    d. Place the injector tip into sulpiride or vehicle solution and then pull the plunger back 4 μL. You should see a single, solid bubble isolating the reagent to be injected from sterile water (Figure 5.1).
16. Habituation. To minimize stress, acclimate mice to the system by receiving two mock injections.
    a. Handle the mouse and gently place it on a cotton cloth.
    b. Gently restrain the mouse, hold the guide cannula with a tweezer, unscrew the protective cap, unplug the dummy cannula, and insert the injection cannula into the guide cannula.
    c. Place the mouse back to its cage and let it move freely in the cage for five minutes. Pay attention to the mouse. Press the injection cannula when it lifts and stop the mouse from biting the plastic tubing.
    d. After habituation, pull out the injection cannula, place the dummy cannula into the guide cannula, and screw the dust cap.
    e. Return the mouse to its home cage.
17. Microinjection. On the test day, deliver sulpiride or vehicle (aCSF) into the VTA.
    a. Power on the syringe pump.
    b. After drug loading (step 15d), lock the microsyringe into the rack of the syringe pump.

c. Check the pump setting: diameter of microinjection needle is _0.343 mm_; volume is _1 μL_; rate of infusion is _0.1 μL/min_; and mode is _infusion_.

d. Mark the position of the bubble to allow injection process monitoring.

e. Press [RUN]. A droplet should appear at the tip of the injection cannula and the bubble should move forward after running for a while (Figure 5.2). Do not advance to the next step until drips are observed. Potential causes of failure include clog and leak. Please refer to Cannula preparation section for troubleshooting. Loose connection between the micro-syringe and plastic tubing also leads to failure.

f. Press [RESET].

g. Mark position of the bubble-post.

h. Mildly handle and restrain the mouse for injector insertion as described in step 16.b

i. Place the mouse back to its cage and press [RUN] (Figure 5.3). Again, keep an eye on the mouse.

j. Press [PAUSE] when the injection volume reaches 0.2 μL.

k. Leave the injection cannula in place for another 3 min to allow drug diffusion. After injection, pull out the injection cannula, fill the dummy cannula into the guide cannula, and screw the dust cap.

l. Leave the mouse in its home cage for 15 min before the behavioral test.

m. Check the injection process. The bubble should move towards the cannula end of the plastic tubing. Write down any exceptions and exclude them for analysis.

△ CRITICAL: Adjust the pump setting and injection volume according to the experimenter's own needs.

△ CRITICAL: Reload reagents between animals because air bubbles may exist in the injector after each microinjection.

△ CRITICAL: Perform post-hoc histology check to ensure cannula implantation in the target brain area. After behavioral sessions, dissect brain out and directly transfer it to 4% para-formaldehyde (PFA) solution (Sangon Biotech) for post-fixation at 4°C. After 24 h, transfer the brain to 30% (w/v) sucrose solution (Sangon Biotech) dissolved in phosphate buffer so-lution (Sangon Biotech) for 48 h at 4°C. Thereafter, prepare coronal slices containing the VTA (60 μm thickness) following the frozen section procedure using cryotome (Thermo Fisher Scientific). Check cannula placement under the light microscope (LW Scientific).

18. Clean up.
    a. Remove the plastic tubing from the microsyringe.
    b. Flush the tubing with sterile water several times and then with air. Keep the cannula/tubing in a plastic bag. Or discard it.
    c. Remove microsyringes from the rack and push the plunger fully back before placing them into packaging.
    d. Clean up the table and make sure electronic equipment is power off before leaving.

Touchscreen version of progressive ratio test

⊙ Timing: 2 weeks

PR test was first introduced to overcome shortcomings of using the obstruction technique to mea-sure reward strength (Hodos, 1961). Nowadays, PR paradigm has been widely used to assess moti-vation by measuring the ability of a rodent to keep responding for a reward in the face of sequentially incremented response requirement (Heath et al., 2015). Different forms of response have been em-ployed in a PR schedule, including lever press, nose poke and screen touch. In this protocol, we describe a touchscreen version of PR test established by Time Bussey and Lisa Saksida (Figure 6,
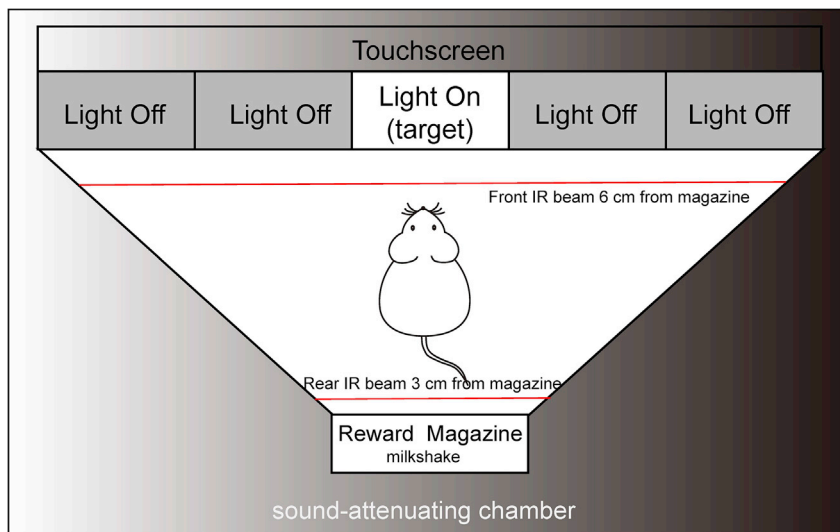
Figure 6. Demonstration of the Bussey-Saksida mouse touch screen chamber
The operant arena resides in a sound-attenuating chamber. A perforated stainless steel floor is enclosed by trapezoidal walls, aiming to focus the animal's attention. A standard '5-choice' black Perspex mask is placed in front of the touchscreen to guide responding. The central response aperture is illuminated, while the rest four apertures are never illuminated. Infra-red (IR) beam arrays positioned in front of the touchscreen surface (<5 mm from the surface) record screen touches. Only target touch results in tone delivery, magazine illumination and reward delivery. Reward magazine entry is recorded by magazine IR beam. Front and rear IR beams run across the floor allow on-line locomotor activity monitoring. IR-sensitive camera placed above the arena allows online monitoring.

'Bussey-Saksida chamber', Campden Instruments) to test if intra-VTA sulpiride infusion can normalize motivated behavior impaired by 7-day CORT treatment. Major steps include food restriction, apparatus set, fixed and progressive ratio task training, and motivational assessment.

19. Food restriction. To encourage engagement, maintain mice at approximately 85%–90% of their ad-libitum feeding weights (Toth and Gardiner, 2000). Frequently, provide mice with limited amounts of standard laboratory chow pellets if food rewards such as high-fat food pellets and milkshakes are given (Heath et al., 2015). In this protocol, we use strawberry milkshake (SM, Ya-zoo). Alternatively, water restriction can be introduced, and 10% sucrose is given as a reward.
    a. Weigh mice to establish stable free-feeding weights. It is recommended to weigh animals twice during a day and take the average.
    b. Weigh food intake daily for 3 consecutive days to establish daily intake.
    c. Provide animals with 85%–90% of ad-libitum daily intake.
    d. Monitor weights daily to prevent excessive weight loss or insufficient food restriction.

One may need to adjust daily food provision according to task performance.

> △ CRITICAL: Food restriction in adolescent and young adult mice (< 14 weeks) requires special concern as they are more vulnerable during starvation (Rowland, 2007). Apart from comparing with free-feeding weights, reference to weights of age/sex/strain-matched control with free access to food and water or established growth charts is also recommended.

20. Apparatus setting. Detailed instructions are provided by the manufacturer. In the protocol, we assume that hardware has been set up properly and is ready for use. All schedules loaded to the software (ABET II) are brought from the supplier. One may also modify the schedules according to the objective of the experiment.

21. Habituation. To minimize neophobia, habituate mice to the touchscreen system for two consecutive 20-min sessions. During each session, record infrared (IR) beam breaks, screen touches and magazine entries, with no programmed consequences of these responses.
    a. Transfer animals to the test room at least 30 min in advance.
    b. Pipette 200 μL milkshake into the reward magazine.
    c. Load the schedule [PR Mouse Habituation 1], introduce a mouse into the touchscreen behavioral chamber, and run the schedule.
    d. Return the mouse to its home cage when the schedule is completed.
    e. Clean up the chamber with 75% alcohol.
    f. Repeat steps b-e for each mouse.
22. Fixed ratio (FR) task training.

    Note: During a 60-min training session, 20 μl SM will be delivered when the response requirement is met. In FR1, for example, a single screen touch results in reward delivery. Similarly, two touchscreen responses are required to earn a single reward in FR2. Each screen touch is accompanied by a 500ms removal of the screen stimulus and a 10ms, 3kHz 'chirp' tone. Reward delivery is coincident with reward magazine illumination and tone delivery (1 s, 3 kHz). FR training starts from FR1 and advances to 2, 3 and 5, sequentially. A completion of 30 trials within 60 min is required for advancing to the next FR schedule.

    Note: Record off-target screen touches during each session. This allows experimenters to calculate the *target/blank touch ratio*, which indicates the specificity for the central, illuminated target screen location over the other four unlighted blanks. With sufficient FR5 training, mice show a significant preference for the target screen location as indicated by a high target/blank touch ratio (10.2±2.1:1, Heath et al., 2015). A *target/blank touch ratio* of at least 3:1 and 30 trials in 60 mints are both required for advancing to the PR training (Heath et al., 2016).

    Note: In our experiments, it takes approximately 10 sessions for the most mice to achieve stable FR5 performance. Keep mice on FR5 training until all meet the criteria for advancing. Once all mice acquire the FR5 rule, they need to do three PR sessions before CORT treatment to ensure stable PR performance after drug exposure.

    a. Start daily training at approximately the same time. Transfer animals to the test room at least 30 min in advance.
    b. Keep the pump running until SM is dripping from the tube (priming).
    c. Load and run the calibration schedule. The system will then pump SM for [N] * [T] ms bursts, defined by the operator. Typically, ~800 ms pulse pumps 20 μL SM through the tube. Yet, the amount depends on the viscosity of the liquid reward used and the patency/diameter of the plastic tubing. Weigh the liquid and adjust the Pulse Time [T] to calibrate.
    d. Load the FR training schedule. Set FIXED RATIO to the required value, starting from 1.
    e. Transfer one animal into the behavioral chamber and run the schedule.
    f. After 60 min or 30 trials completed, remove the mouse, and return it to its home cage.
    g. Clean the chamber with 75% alcohol.
    h. Repeat steps d-g for each mouse.

    △ CRITICAL: Milkshakes are sticky. Rinse the plastic tubing after use and change the plastic tubing regularly.

    △ CRITICAL: Establish training timeline according to the experiment's objective.

23. PR schedule. All task parameters are the same as the FR procedure, except the response requirement is incremented on a linear +4 basis (1, 5, 9, 13 etc.). Response ratio can also be determined

by other equations, for example, $Ratio = 5e^{injection\ number*0.2} - 5$ used by Labouèbe et al. (2013). The PR session ends if the animal neither touches the illuminated target screen nor collects a reward for 5 min or the session is forced to stop after 60 min. Repeat PR sessions daily until performance stabilizes. Breakpoint (the number of target screen touches for the last successful trial of a session) performance usually becomes stable after the first three sessions (Heath et al., 2015).

   a. Same as 22.a

   b. Same as 22.b

   c. Same as 22.c

   d. Load the PR schedule.

      i. Transfer one animal into the behavioral chamber and run the schedule.

   e. Return the mouse to its home cage once session ends

   f. Clean the chamber with 75% alcohol.

   g. Repeat steps d-f until all animals being trained.

24. Motivational assessment. Once performance stabilizes, treat animals with CORT or vehicle for 7 days. Before PR sessions, mice receive intra-VTA drug infusion (steps 13–18). Calculate breakpoint average from three consecutive session

### PR task data analysis

  ⏲ Timing: <1 h

PR task performance is frequently assessed by breakpoint, which is automatically displayed by the end of each session. Significantly, the touchscreen testing chamber is equipped with IR beams at multiple locations to keep track of animal's general activity. Detailed analysis may bring unexpected bonuses (Heath et al., 2015). The ABET II software is equipped with Data Viewer Tab to perform PR analysis, giving the following performance parameters: 1) Session length; 2) Number of trials completed; 3) Breakpoint; 4) Number of target screen touches; 5) Number of blank touches; 6) Number of front beam breaks; 7) Number of rear beam breaks; 8) Number of magazine entries; 9) Reward collection latency (RCL): the interval between reward delivery and magazine entry; 10) Post reinforcement pause (PRP): the interval between magazine exit and next target touch to start a new trial; 11) Total response time: time between first target touch and final touch that reaches the ratio; 12) Inter response interval: time between each target touch of a given trial.

Here, we provide a Python-based analysis tool (Figure 7), which we consider as a more powerful and flexible tool. There is no need to gain access to the ABET II software. Hence, data can be analyzed anywhere. Besides, data from all sessions can be analyzed within few seconds in one go.

25. Export raw data with session information. Save all files that need to be analyzed in a single folder.

26. Open Jupyter Notebook or Spyder. Here we use Spyder for demonstration.

27. Load the 'PR_parameters_ABETII_TOUCH2.19.5834.17763.py' file, input folder path and run the file. You will get performance parameters, including **breakpoint**, mean post reinforcement pause across trials (**PRP_mean**), mean reward collection latency across trials (**RCL_mean**), blank screen touch rate (**BlankTouch_Rate**), rate of front IR beam breaks (**FIR_rate**), rate of rear IR beam breaks (**BIR_rate**), food magazine entry rate (**MagazineEntry_Rate**), total response time for each required ratio and interval between each target touch of a given trial. Rate is calculated considering various session lengths. If running multiple sessions, average across sessions is calculated, using the 'PR_parametersSessionMean_ABETII_TOUCH2.19.5834.17763.py' file.

28. Proceed with statistical analysis, using Python or other statistical softwares such as Prism.

  ⚠ CRITICAL: We notice that variable names listed in raw data vary between ABET software versions. Our code is specific for ABETII_TOUCH2.19.5834.17763. One may
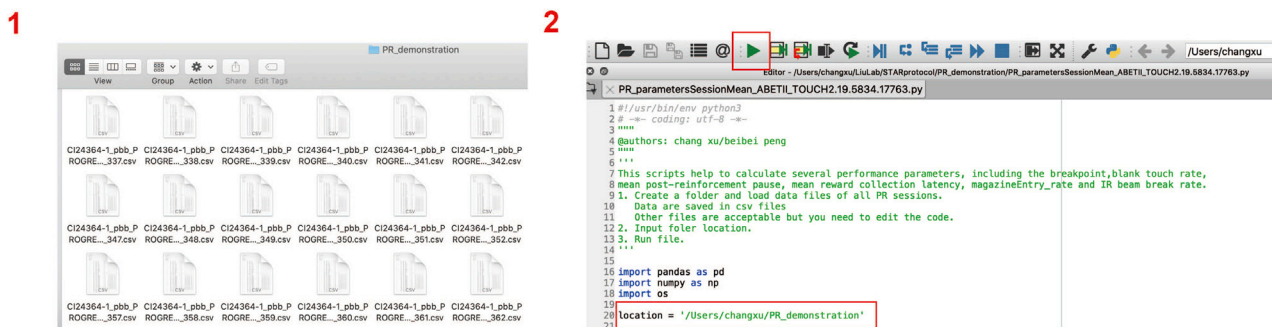
Figure 7. Demonstration of PR analysis with customized code in Spyder

Save all files that need to be analyzed in a single folder (step 1). Open the script with Spyder, input folder path and run the file (step 2). All performance parameters are saved in excel files.

need to equip with basic knowledge about the Python language to troubleshoot. Nevertheless, we find that our colleagues with limited Python experience can understand the error messages and perform troubleshooting. Basically, one only needs to change the variable names.

EXPECTED OUTCOMES

7-day oral CORT treatment reduced foraging for food in a mildly aversive environment (Figure 8A), as well as general motivation for food reward (Figure 9A). Intra-VTA sulpiride restored food-seeking in the light compartment of a light/dark box without affecting food zone time and general activity (Figures 8A–8C). The restoration could not be explained by normalized motivation for food because drug infusion did not normalize breakpoint in CORT mice (Figure 9A). Detailed PR performance analysis revealed a trend increase in reward collection latency in the CORT group (Figure 9C), which was also suggestive of reduced motivation to collect any rewards earned. No significant effect of CORT on other parameters was observed (Figures 9D–9H). Although sulpiride reduced post reinforcement pause (Figure 9B), our analysis did not show that sulpiride normalized motivation in CORT mice
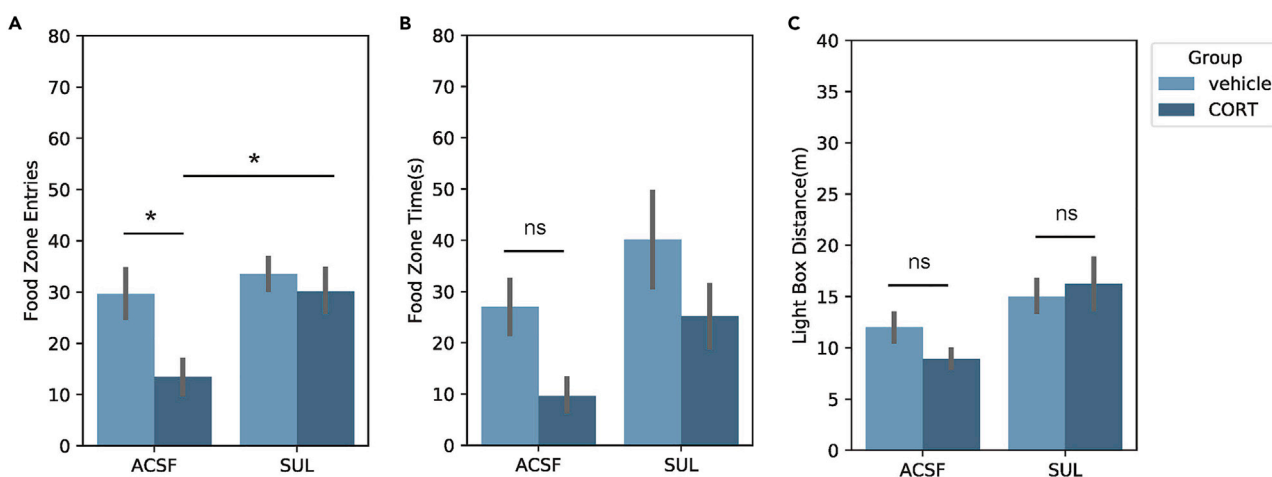

Figure 8. Reward-seeking deficit was restored by intra-VTA sulpiride infusion

(A) Number of food zone entries was significantly reduced in CORT mice (p=0.025), which was reversed by sulpiride treatment (p=0.04). Two-way ANOVA revealed a significant treatment (F $(1,41)$ =5.692, p=0.02) and drug infusion (F $(1,41)$ =6.282, p=0.016) effect, but no interaction between the two factors (F $(1,41)$ =2.406, p=0.1286).

(B) Time in the food zone was not significantly reduced in CORT treatment (p=0.2042).

(C) Sulpiride treatment did not affect general locomotor activity. All data were analyzed using 2-way ANOVA with post-hoc Tukey multiple comparisons. Vehicle + aCSF: n=13; CORT + aCSF: n=12; vehicle + sulpiride: n = 11; CORT + sulpiride: n = 9. Error bars draw 68% confidence interval for the mean.
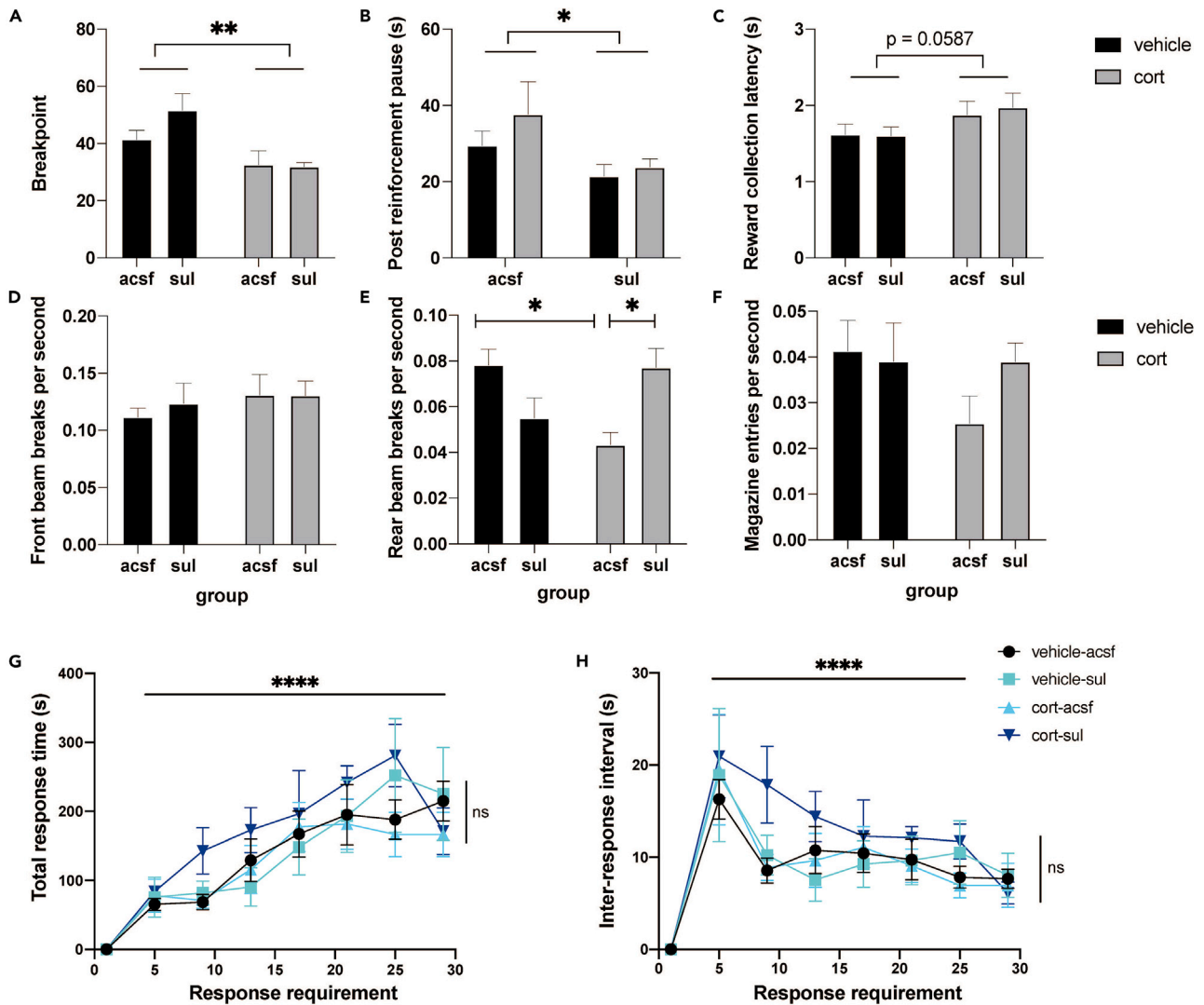
Figure 9. 7-day CORT treatment suppressed PR performance

(A) Breakpoint values of control and CORT mice receiving either aCSF or sulpiride. Two-way revealed a significant group (vehicle or CORT) effect (F (1,20) = 11.74, p=0.0027).

(B) There was a significant treatment (aCSF or sulpiride) effect on the post reinforcement pause (two-way ANOVA, F (1,20) = 4.7, p=0.0419).

(C) CORT treatment increased reward collection latency, although the increase was not statistically significant (F (1,20) = 4.0, p=0.0587).

(D) Neither CORT nor sulpiride influenced front IR beam break rate.

(E) There was a significant group × treatment effect on the rear IR beam break rate (F (1,20) = 14.48, p=0.0011). Post-hoc Tukey's multiple comparisons test revealed that the rear IR beam break rate was significantly reduced in CORT mice (p=0.0177), which was restored by intra-VTA sulpiride infusion (p=0.022).

(F) Magazine entry rate was not affected by CORT or sulpiride.

(G and H) Total response time and inter-response interval increased as response requirement incremented (three-way ANOVA, F (7,140) = 27.98, p<0.0001 (G); F (7,140) = 20, p<0.0001 (H)). N = 6 for each group. All p values are adjusted using post-hoc Tukey's multiple comparisons test. Data are represented as mean ± SEM.

(Figures 9A and 9C), except that it restored the reduced rear beam break rate (Figure 9E), which was constructive in interaction with the reward zone. Instead, Peng et al. (2021) found that intra-VTA sulpiride alleviated anxiety-like behavior using the elevated plus maze (data not shown here). Overall, these results indicate that CORT treatment impairs reward-seeking behavior, possibly due to its pro-anxiogenic effect. Sulpiride infusion in the VTA restores foraging for food in the light box, implying that CORT induced reward-seeking deficits via D2R signaling in the VTA.

LIMITATIONS

In this protocol, we describe micro drug infusion into the ventral tegmental. We cannot rule out the possibility that drugs diffuse to adjacent brain areas. Infusing dyes such as Chicago sky blue 6B or fluorescently tagged drugs (Moreines et al., 2017) can be used to estimate drug diffusion. Nevertheless, diffusion properties of these chemicals are different from drugs themselves. Another limitation of microinjection is that the size of guide cannula excludes its use for delivering drugs to small brain areas. Besides, implantation of guide cannula is invasive.

TROUBLESHOOTING

Problem 1

High mortality rate. (Surgical procedures steps 1–9)

Potential solution

Underweight mice (<10 g) should not be subjected to cannula implantation surgery. Follow the protocol strictly, especially the sterilization protocol.

Problem 2

Inconsistent distance of bubble movement between animals. (Intra-VTA drug infusion steps 13–18)

Potential solution

- Check for patency and leakage as detailed in Cannula preparation step 5 and VTA drug infusion steps 13–18.
- Use spare injection cannula/plastic tubing assembly.
- Use spare microsyringes.

Problem 3

Mice rotate in one direction after microinjection. (Intra-VTA drug infusion steps 13–18)

Potential solution

Try a lower drug dose for intra brain injection.

Problem 4

Poor engagement in the light/dark box test. (Light/dark box test steps 10–12)

Potential solution

Make sure animals are not exposed to unnecessary stressful events such as noise, over-bright light sources or food/water deprivation. Check temperature/humidity settings in the test and housing rooms. All mice should be able to perform the light/dark box test in an appropriately set experimental environment.

Problem 5

Poor performance in the touchscreen chamber or breakpoint performance does not stabilize. (Progressive ratio test steps 19–24)

Potential solution

- Monitor body weight daily, ensuring that weights are within acceptable percentage of ad-libitum weights.
- Check hardware, ensuring that touchscreen and pulse feeder work properly.
- Follow priming and calibration procedures, ensuring that approximately 20 μL milkshake is delivered for each successful trial.

- The cannula may prevent animals from entering the reward magazine. Enlarge the entrance if necessary.
- Experimenters should keep an eye on behavioral training, because occasionally cannula may get stuck in the hole on the chamber floor

⚠ CRITICAL: all mice should be able to pass the FR training and perform the PR schedule within 2 weeks. This is in accordance with other labs (Heath et al., 2015). Otherwise, perform troubleshooting as we suggested.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Shuai Liu (sliu@psy.ecnu.edu.cn).

Materials availability
This study did not generate any reagents.

Data and code availability
The code generated for PR task performance analysis is available at

https://github.com/xcccc402/Touchscreen-version-of-PR-task-data-analyasis.git

(https://doi.org/10.5281/zenodo.5987836)

AUTHOR CONTRIBUTIONS

Conceptualization, B.P., C.X., and S.L.; Methodology, Data Analysis, and Original Draft Writing, C.X. and B.P.; Python scripts, C.X. and B.P.; Review and Editing, S.L., C.X., and B.P.; Supervision and Funding Acquisition, S.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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