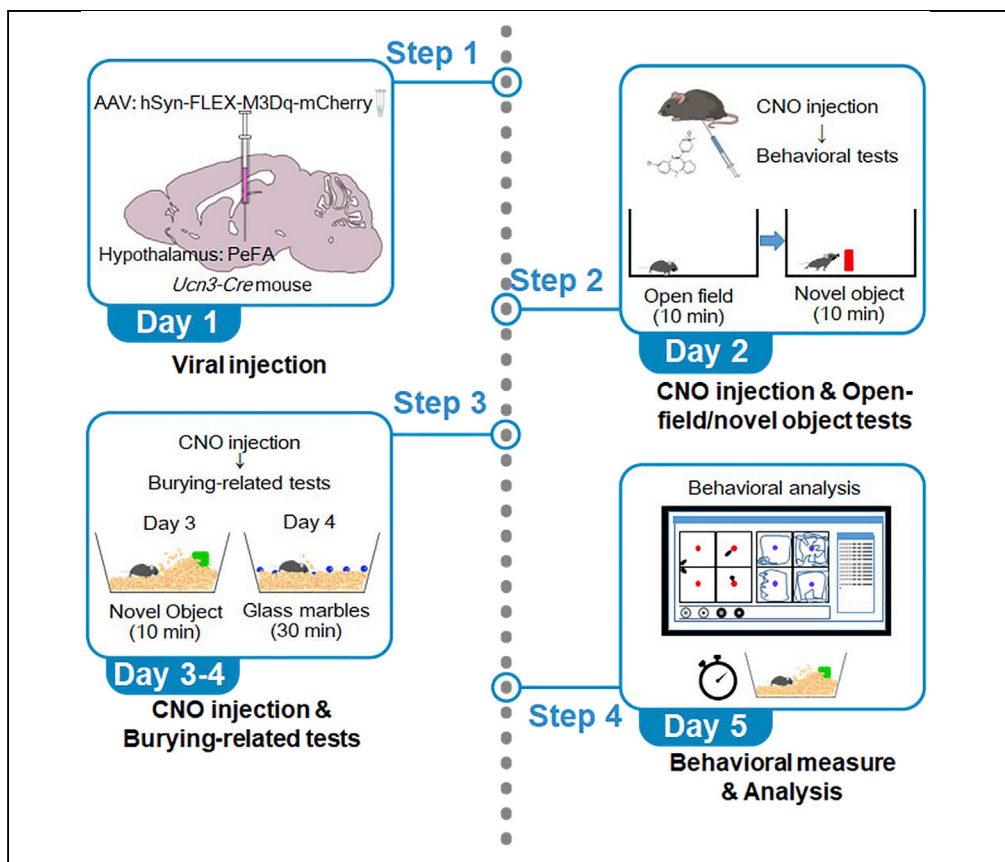


Protocol

Protocol for behavioral tests using chemogenetically manipulated mice



Behavioral analyses using mice chemogenetically manipulated by designer receptors exclusively activated by designer drugs are powerful tools to elucidate neural functions. Here, we describe the detailed protocols for stereotaxic surgery, AAV-mediated introduction to Gq-DREADDs in mice, and for behavioral testing and analyses related to anxiety, risk assessment, and burying behaviors. A series of these tests are useful in evaluating animal anxiety and their defensive response patterns to potential threats.

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Highlights
Detailed protocols for stereotaxic viral injection and behavioral testing in mice

Chemogenetic activation of targeted neurons and behavioral testing

Behavioral tests are related to anxiety, risk assessment, and burying

Behavioral results are useful in evaluating animal anxiety and defensive responses

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Protocol

Protocol for behavioral tests using chemogenetically manipulated mice

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SUMMARY

Behavioral analyses using mice chemogenetically manipulated by designer receptors exclusively activated by designer drugs (DREADDs) are powerful tools to elucidate neural functions. Here, we describe the detailed protocols for stereotaxic surgery, adeno-associated virus (AAV)-mediated introduction to Gq-DREADDs in mice, and for behavioral testing and analyses related to anxiety, risk assessment, and burying behaviors. A series of these tests are useful in evaluating animal anxiety and their defensive response patterns to potential threats. For complete details on the use and execution of this protocol, please refer to Horii-Hayashi et al. (2021).

BEFORE YOU BEGIN

Designer receptors exclusively activated by designer drugs (DREADDs) are powerful tools to manipulate neuronal activity through the Gi-, Gq- or Gs-protein-coupled signaling pathway (Khambhati and Bassett, 2016; Roth, 2016). DREADDs have been used to elucidate neural functions, including those related to behavior, perception, emotion, motor functions, and innate drives (Roth, 2016). The most widely applied DREADDs are based on human muscarinic receptors engineered to lose their affinity for acetylcholine, but to gain responsiveness to the artificial ligand clozapine-N-oxide (CNO), a metabolite of the atypical antipsychotic clozapine (CLZ) (Khambhati and Bassett, 2016).

While DREADDs become popular among neuroscientists, a study reported that systemically applied CNO does not cross the blood-brain barrier, CNO can be reversely metabolized to CLZ, and the CLZ acts as the DREADD activator in rats (Gomez et al., 2017). Another study demonstrated that interoceptive sensation induced by CLZ is recaptured after injection of 10 mg/kg CNO, but not after that of 5 mg/kg or lower doses. Contrary to rats, a mouse study reported that CNO can penetrate the blood-brain barrier and reach brain tissue and cerebrospinal fluid at the concentrations that can activate the receptors at least 15 min after intraperitoneal injection (Jendryka et al., 2019). Based on these issues, this protocol recommends CNO doses lower than 5 mg/kg in mice and performing control experiments using mice injected with control viral vector lacking the receptors for DREADDs. A recently introduced DREADD ligand, compound-21, is also applicable to activate both hM3Dq and hM4Di *in vivo* to avoid unspecific effects of reverse-metabolized CLZ from CNO (Thompson et al., 2018).

This protocol describes behavioral testing methods designed to research anxiety, risk assessment, and burying behaviors in mice manipulated with Gq-based DREADD technology. This protocol describes the combined use of *Ucn3*-Cre mice (*Ucn3* promoter controlling Cre recombinase



expression), and delivery of adeno-associated virus (AAV) in the hypothalamus, which allows for Cre-dependent expression of hM3Dq and mCherry. As expected, activation of hypothalamic Ucn3 neurons by CNO increases risk assessment and burying behaviors towards a novel object without affecting anxiety levels. However, this protocol is also applicable to different combinations of Cre-mouse lines and other types of DREADDs.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacteria and virus strains		
AAV(DJ)-hSyn-FLEX-hM3Dq-mCherry	(Inutsuka, 2014)	N/A
AAV(DJ)-hSyn-FLEX-mCherry	Provided by Dr. Yamanaka	N/A
Chemicals, peptides, and recombinant proteins		
Isoflurane inhalation solution [Pfizer] ^a	Mylan Seiyaku Ltd.	1119701G1092
Xylocaine jelly 2%	Aspen Japan	886-002815
Isodine solution (povidone-iodine)	Mundipharma	N/A
Clozapine N-oxide	Abcam	ab141704
Experimental models: organisms/strains		
Mouse: STOCK Tg(Ucn3-Cre) KF43Gsat/Mmucd	MMRRC	032078-UCD
Software and algorithms		
TopScan LITE	CleverSys Inc.	http://cleversysinc.com/CleverSysInc/csi_products/topscan-lite/
Other		
Mouse cage	CLEA Japan, Inc.	CL-0104-2
Clean chip (bedding material)	CLEA Japan, Inc.	CL-4161
Small animal anesthetizer	Muromachi Kikai Co., Ltd.	MK-AT210D
Stereotaxic frame	Narishige	SR-5M-HT
Mouse anesthesia mask	Narishige	GM-4
Micromanipulator	Narishige	SM-15L
Electric microinjector	Narishige	IMS-20
7001 Neuros syringe	Hamilton	4015-63002
Surgical operation stereomicroscope	Shodensha	AFN-405W
Dental drill	URAWA CORPORATION	Minitor Jet II
Razor	Amazon	ASIN : B071WL6JF5
Disposable scalpel	Feather	No. 11
Tweezers	Natsume	A-1
Needle holder	Natsume	Olsen Hegar 8-15TC
Surgical sutures	Bear Medic Corporation	SO13A04N-45
Open field box (40×40×40 cm)	Hazaiya (custom made)	https://www.hazaiya.co.jp/
Open-field recording apparatus (camera and LED dimming panel light)	OHARA & CO., LTD.	https://ohara-time.co.jp/
Mouse cage recording apparatus (camera and cage-dedicated recording box)	OHARA & CO., LTD.	https://ohara-time.co.jp/
Glass marbles	Amazon	ASIN B01GLKVM50
Objects (Plastic ball and ceramic toy etc.)	Various shops	N/A
Double-sided tape	Various shops	N/A
Illumination photometer	Amazon	ASIN : B011220FYI
Hypochlorite solution	Amazon	ASIN: B00A6LXTUG
Alcohol swab	Amazon	ASIN : B000FQUZEU
Paper towel	Nippon Paper Cnesia	61000
Mouse brain atlas	Academic Press	ISBN-13 : 978-0123910578

^aFor use in surgery

MATERIALS AND EQUIPMENT

Alternatives:

- AAV vector for GqDREADD and its control are commercially available from Addgene (hSyn-DIO-hM3Dq-mCherry: #44361 and hSyn-DIO-mCherry: #50459).
- Other anesthetics, such as ketamine/xylazine mix, can be used in place of isoflurane. However, isoflurane anesthesia has some advantages, such as faster induction and recovery and relative sparing effect on cardiovascular function and cerebral blood flow autoregulation (Ludders, 1992).
- Other manufacturers' stereotaxic instruments, including automated ones, can be substituted for the Narishige stereotaxic apparatus, David Kopf, Stoelting, and RWD Life Science.
- EthoVision and ANY-maze software can be used for behavioral tracking.
- In the absence of a dedicated apparatus in the mouse cage, a transparent acrylic lid on the cage enables video recordings (Figure 1C).
- Compound-21 (HelloBio, #HB6124) can be used instead of CNO.

Animal breeding

Although this protocol uses adult male *Ucn3-Cre* mice, it can be applied to females and/or younger animals. However, expected outcomes indicated below are observed in adult male *Ucn3-Cre* mice (Horii-Hayashi et al., 2021). Females and/or younger animals have not been investigated.

- Breed heterozygous *Ucn3-Cre* mice.
- Prepare 8–24-week-old adult male mice

CNO stock solution

The solid power of CNO is not sensitive to either light or air, and CNO in solution is also chemically stable for at least 4 weeks at room temperature (<https://www.hellobio.com/clozapine-n-oxide-stability-solubility-review/>). The manufacturer instructions recommend preparing and using the solution on the same day (<https://www.abcam.com/clozapine-n-oxide-metabolite-of-clozapine-ab141704.html>). However, when you need to make up the stock solution in advance, the same instructions recommend storing the solution as aliquots in tightly sealed vials at -20°C .

This protocol store the stock solution at the concentration of 2 mg/mL at -20°C . One milliliter of the solution serves 80 times of intraperitoneal injection for mice weighing 25 g or 66 times for 30 g ones when administrating 1 mg/kg of CNO.

Reagent	Final concentration	Amount
CNO	2 mg/mL	5 mg
Saline	n/a	2.5 mL
Total		2.5 mL

After dispensing, store at -20°C for a maximum of 1 year.

Diluted hypochlorite solution

Reagent	Final concentration	Amount
6% Hypochlorite Solution	0.02%	1 mL
Water	n/a	300 mL
Total		301 mL

Store the solution in a spray bottle at 23°C – 25°C for a maximum of 1 month.

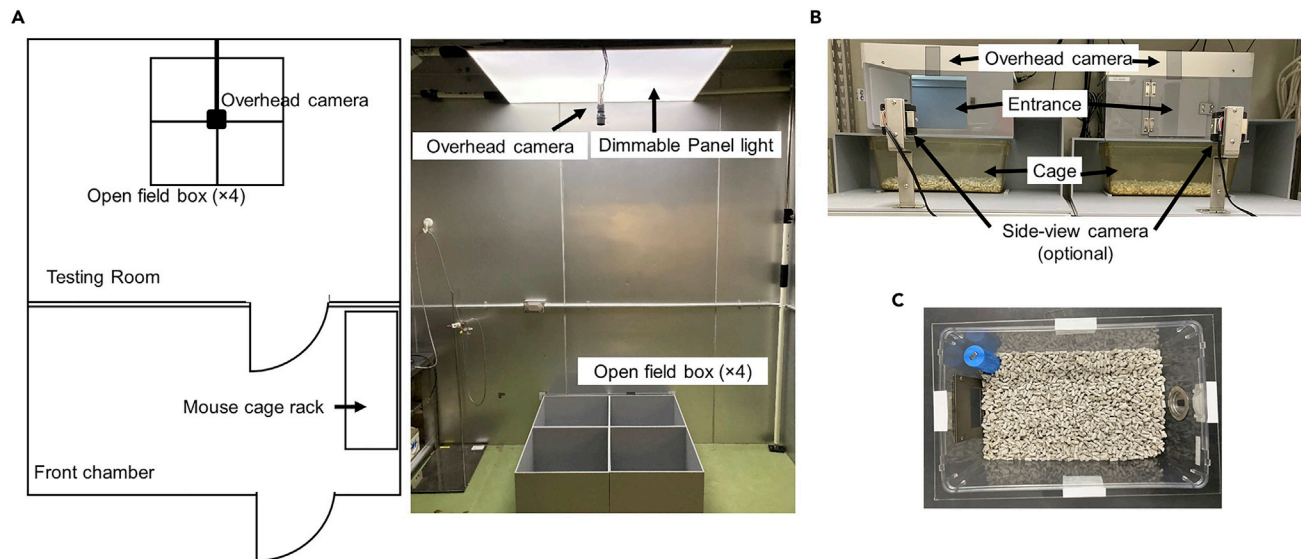


Figure 1. Apparatus for behavioral testing

(A) The layout of a behavioral testing room.

(B) A mouse cage recording apparatus.

(C) A mouse cage covered with a transparent acrylic lid can be substituted for the mouse cage recording apparatus.

Sterilization of surgical instruments

- Put surgical instruments into an autoclave box.
- Autoclave the instruments at 121°C for 30 min.

Preparation of critical equipment

- **Behavioral testing room:** Prepare a behavioral testing room and set up the instruments, including a dimmable panel light, open field boxes, an overhead video camera (Figure 1A), and a video apparatus near the mouse cages (Figure 1B). A transparent acrylic lid on the cage enables video recording without the dedicated apparatus in the mouse cage (Figure 1C). It is better to have a front chamber adjacent to the behavioral testing room where the animals can be kept before commencing the tests. The brightness of the waiting area must be adjusted to approximately 5–10 lx, similar to conditions in the breeding room.

△ **CRITICAL:** The brightness during the testing conditions affects the behavioral test results.

- **Stereotaxic apparatus:** The stereotaxic apparatus must be set up beforehand (Figure 2A), including a micromanipulator connected to a stereotaxic frame, an electric microinjector connected to the arm of the micromanipulator, a surgical stereomicroscope, an arm light, dental drill, alcohol swabs, cotton swabs, and surgical tools (shaver, scalpel, tweezers, needle holder, and surgical sutures). During surgery, an anesthesia mask is connecting to the isoflurane anesthetizer via two tubes for supply gas and exhaust one, respectively (Figure 2B).

STEP-BY-STEP METHOD DETAILS

Figure 3 indicates an experimental workflow.

Determine the stereotaxic coordinates

⌚ Timing: 30 min

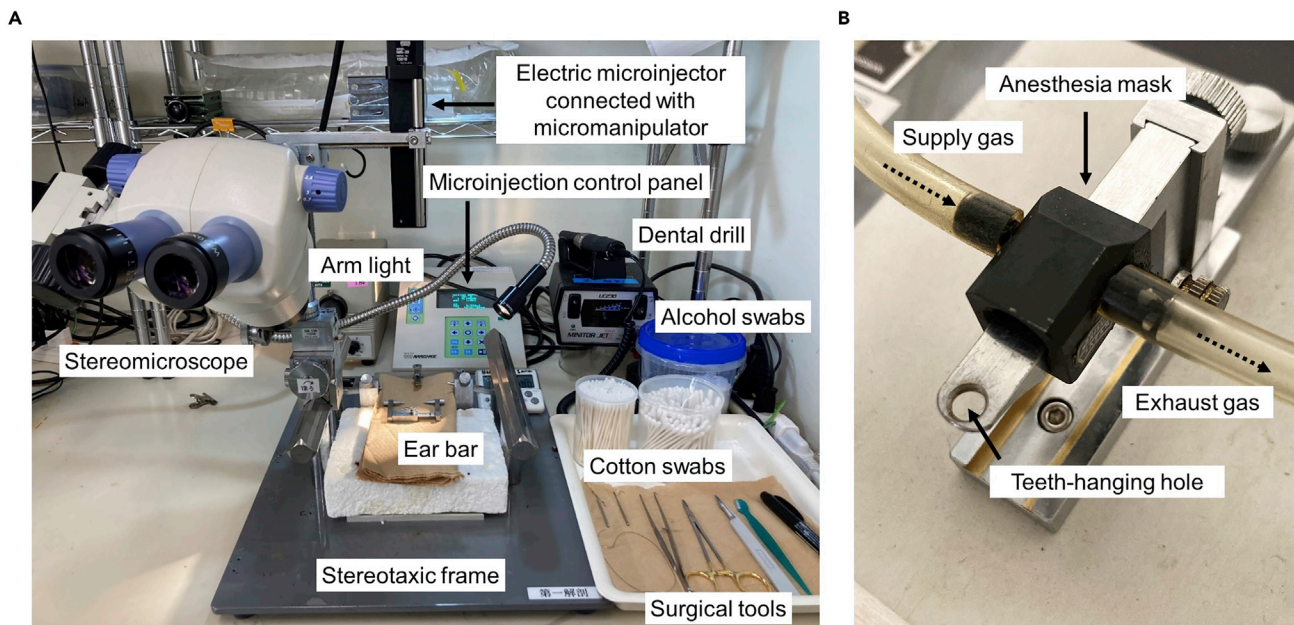


Figure 2. Setup for stereotaxic surgery

(A) An example for the arrangement of stereotaxic instruments.

(B) An anesthesia mask is connected to an anesthetizer via two tubes for supply gas and exhaust one.

The skull sutures are used to determine the two landmarks, the bregma and lambda (Figure 4A). The bregma point is formed by the intersection of the coronal and sagittal sutures, whereas the lambda point is formed by the intersection of the sagittal and lambdoid sutures. Stereotaxic coordinates for specific brain regions can be easily determined using a brain atlas (for example, *The mouse brain in stereotaxic coordinates*, by Paxinos and Franklin) (Franklin and Paxinos, 2007). This atlas provides the medial-lateral (X), rostral-caudal (Y), and dorsal-ventral (Z) distances from the bregma when its position is assigned the coordinates $(X, Y, Z) = (0, 0, 0)$.

1. Determine the stereotaxic coordinates of a targeted brain region using a mouse brain atlas (Figure 4B). The coordinates for the hypothalamic perifornical area are $(0.47, -0.7, 4.75)$.

Note: There are at least two methods to determine the z coordinate. One utilizes the distance from the bregma on the skull surface, while the other uses the distance of the targeted region from the pia surface. In the former case, we use the scale provided in the brain atlas. For the

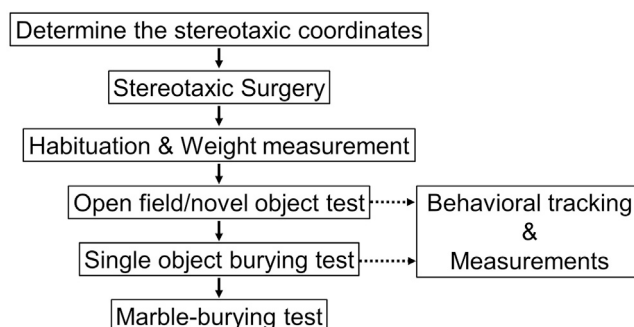


Figure 3. Experimental workflow

12. Lower the microsyringe, mark the drilled position using a marker if necessary, and drill the skull surface to make a hole of approximately 1-mm diameter.

Note: When accidental bleeding occurs, apply pressure on the hole with a cotton swab to stop bleeding.

13. Fill the microsyringe with the viral solution.

Note: Injection needles can be easily clogged. Please confirm that the viral solution is released from the needle when pushing the plunger. After that, wipe off the solution with a cotton swab.

14. Lower the microsyringe to the targeted z-coordinate.
15. Inject 0.25 μL of viral solution (1×10^{13} copies/mL) at a flow rate 0.1 $\mu\text{L}/\text{min}$ and leave the needle in place for 3 min after injection.
16. Slowly pull out the microsyringe to prevent the backflow and suture up the incision.
17. Apply isodine solution and then xylocaine gel on the wound.

Note: Please keep this order because xylocaine gel makes a barrier to antiseptics when administered prior to isodine solution (Doshi et al., 2011).

18. Leave the mouse on a heated pad until it awakens.
19. House the mouse alone in a home cage for 4 weeks until behavioral testing.

⚠ **CRITICAL:** Please refer to institutional guidelines for specific instructions on aseptic and analgesic managements.

⚠ **CRITICAL:** The fixation of the head is a very important step for accurate stereotaxic injection.

Habituation and weight measurement

⌚ **Timing:** 2–3 days

This procedure is performed in order for animals to habituate to the testing environments.

20. Transport animals in their home cages to a behavioral testing room or to its front chamber a few days prior to beginning the behavioral testing.
21. Place the cages on a rack and leave for 2–3 h.
22. Return the animals to their breeding room.
23. Repeat the above steps for at least 2 days.
24. On the last day of habituation, weigh the animals and determine the volume of CNO that will need to be injected.

⚠ **CRITICAL:** Behavioral results are influenced by stress and novel experiences. It is important to carry out this procedure in the same way as in the day of testing.

⚠ **CRITICAL:** Behavioral tests should be performed within a fixed time period of the day. It is better to perform the tests during the dark phase to avoid disturbing the animals' sleep. However, this might not be possible in animal experimental facilities. In this case, we are going to perform the tests during the initial few hours of the light phase, from 9:00 to 13:00 (12 h light-dark cycle, lights on at 8:00).

Open field/novel object test

⌚ Timing: 2–4 h

Both open field and novel object tests can be used for evaluating animal anxiety (Belzung and Le Pape, 1994; Calhoun and Tye, 2015). These tests can be sequentially performed on the same day (Anthony et al., 2014; Dietrich et al., 2015; Horii-Hayashi et al., 2021), which can save time and evaluate behavioral responses to novel environment and novel objects.

Anxious animals prefer to stay along the edges and the corners of the enclosure and tend to avoid the center of the field, which is frequently accompanied with decreased locomotion (Calhoun and Tye, 2015). Similar to the open field test, high anxiety decreases object investigation time represented by sniffing and stretch-attend posture behaviors toward the object (Ren et al., 1981).

The following procedure describes how to perform the open field test followed by the novel object test using chemogenetically activated mice. However, this protocol can also be applied to animals subjected to other manipulations. Table 1 shows behavioral categories, parameters, and analysis methods in the open field and novel object tests.

Table 1. Behavioral categories, parameters, and analysis methods in open field and novel object tests

Test	Behavioral category	Parameter	Method/tracking point
Open field	Anxiety	No. of entries into the center area	TopScan LITE/Body center
	Anxiety	Time spent in the center area (s)	TopScan LITE/Body center
	Anxiety	Time spent in corners (s)	TopScan LITE/Body center
	Anxiety	Distance moved in the center area (mm)	TopScan LITE/Body center
	Locomotor activity	Distance moved (mm)	TopScan LITE/Body center
Novel object	Investigation	No. of sniffing events	TopScan LITE/Nose
	Investigation	Duration of sniffing events	TopScan LITE/Nose
	Risk assessment	No. of stretch-attend posture movements	Visually observed
	Locomotor activity	Distance moved (mm)	TopScan LITE/Body center

25. Prepare the required volume of diluted CNO solution immediately before the test:
 - a. Calculate the volume of the diluted CNO solution required. When administering 1 mg/kg of CNO to mice, the stock solution (2 mg/mL) is diluted 20 times to obtain a final concentration of 0.1 mg/mL, of which 100 μ L is injected per 10 g of a mouse's body weight (e.g., 250 μ L of the diluted solution should be injected into a mouse weighing 25 g).
 - b. Dilute the stock solution (2 mg/mL) with saline. Saline is used for control animals.

Note: The CNO concentration of 1 mg/kg is recommended for the first trial.

26. Transport animals to the experimental room in the same way as during the habituation period (step 20).
27. Place the cages on a rack and leave them for at least 30 min before commencing testing.
28. Using a photometer, adjust the brightness in the open field box to 100 lx. Intraperitoneally inject the CNO solution (1 mg/kg) or saline 15 min prior to the test and return the mouse to their home cage.

Note: Intraperitoneally injected CNO (3.5 mg/kg) reaches cerebrospinal fluid and brain tissue at 15–30 min after the injection (Jendryka et al., 2019).

Note: Duration between CNO injection and behavioral testing should be the same for all of the mice.

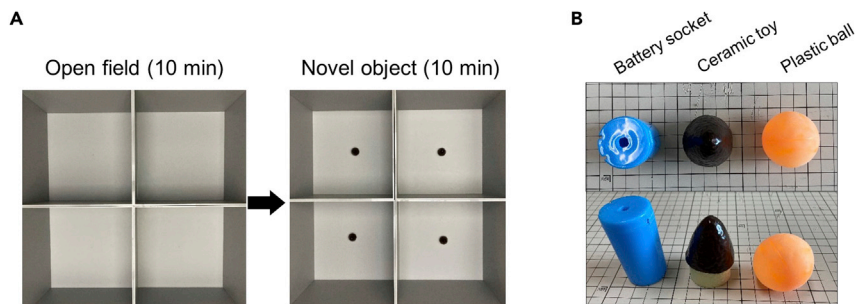


Figure 5. Open field/novel object test

(A) Open field test followed by a novel object test using the same box.

(B) Examples of novel objects.

29. Start behavioral testing

- a. **Open field test:** Start the video recording, put the mouse in the corner of the open-field box, and leave for 10 min.
- b. Return the mouse to its home cage.
Clean the field with the diluted hypochlorite solution and fix a novel object at the center of the field using double-sided tape (Figure 5A).
- c. **Novel object test:** Start video recording, put the mouse in a corner of the field, and leave for 10 min.
- d. Return the mouse to the home cage.

Note: It is recommended to use the following shaped objects, column, conical, or spheroidal (Figure 5B). Because these shapes are uniform, one can easily surround and determine the object area with a circle on the behavioral software, while one would have difficulty in the accurate surrounding of ununiformed-shape objects with a mouse pointer. Besides, anxious mice sometimes stay at a particular corner in the box. In that case, they would see a limited aspect of the objects from one angle, which might generate redundant differences in individual animals. A uniformed shape can resolve this problem because mice can see the same scene wherever they stay in the box.

Note: [Methods video S1](#) shows examples of stretch-attend posture during single object burying test. A similar behavior could be observed in the novel object test. Please refer to the movie.

△ **CRITICAL:** Do not put the mouse in the center of the box in both tests.

△ **CRITICAL:** It is recommended to avoid using shiny materials as novel objects, as this could cause bias depending on the way the light hits them.

△ **CRITICAL:** Use the same object consistently, so that the measured values can be compared.

△ **CRITICAL:** Limit to one behavioral test per day, except for open field/novel object tests.

△ **CRITICAL:** Do not repeat open field/novel object tests using the same animals.

Single object burying test

⌚ **Timing:** 2–4 h

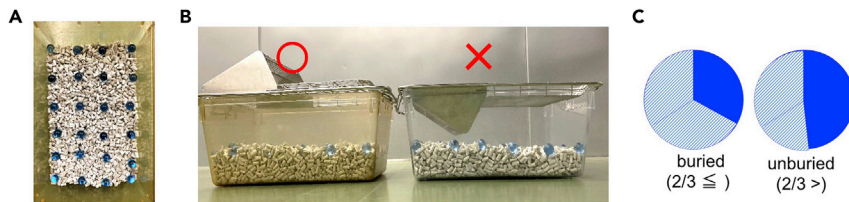


Figure 6. Marble-burying test

- (A) An example of a cage for the marble-burying test.
 (B) An example of a marble-burying cage covered with an upside-down stainless steel lid.
 (C) Illustrations indicating the criteria for buried marbles.

The following procedure describes how to perform the single object burying test. One should keep at least a 1-day interval between the former and the present tests to relieve animals' stress. Ethologically, burying and risk assessment in rodents are categorized into defensive behavior (Blanchard et al., 1991; De Boer and Koolhaas, 2003; Horii-Hayashi et al., 2021; Koolhaas et al., 1999). Activation of hypothalamic Ucn3 neurons increases both behaviors (Horii-Hayashi et al., 2021). This test directly measures burying action and concurrently enables the evaluation of investigatory behaviors, including risk assessment and sniffing. This point is different from the marble-burying test, in which burying activity is evaluated by counting the number of buried marbles. Table 2 shows behavioral categories, parameters, and analysis methods in the single object burying test.

Table 2. Behavioral categories, parameters, and analysis methods in single object burying test

Test	Behavioral category	Parameter	Method/tracking point
Single object burying test	Active defense	Duration of burying (s)	Visually observed
	Investigation	No. of sniffing events	TopScan LITE/Nose
	Investigation	Duration of sniffing events	TopScan LITE/Nose
	Locomotor activity	Distance moved in the whole field	TopScan LITE/Body center

Optional: The number of stretch-attend postures is also countable. However, the discrimination of the end of burying action and the withdrawal following the posture is difficult, especially in CNO-treated animals (Methods video S1).

30. Dilute the CNO stock solution in the same manner as described above (step 25) and transport animals in the same way as in the habituation period (step 20).
31. Place the cages on a rack and leave them for at least 30 min before commencing testing.
32. Adjust the brightness to 5 lx using a photometer for mouse cage recording (approximately the same brightness as in breeding conditions).
33. Prepare new cages containing bedding material at a height of up to 4 cm from the floor.
34. Intraperitoneally inject CNO solution (1 mg/kg) or saline 15 min prior to the test and return them to the home cage.

Note: Duration between CNO injection and behavioral testing should be the same for all of the mice.

35. **Field exploration session:** Start video recording, put the mouse in the new cage, and leave for 10 min.
36. Return the mouse to the home cage.
37. Fix a novel object in a corner of the cage.
38. **Novel object session:** The mouse is placed in the cage and left for 15 min.
39. Return the mouse to the home cage.

△ **CRITICAL:** When using the same animals as in the open field/novel object tests, use an object that is different from that used in the novel object test.

△ **CRITICAL:** When the field exploration session (step 35) is skipped, mice tend to utilize the time for field exploration during the novel object session (step 38).

Marble-burying test

⌚ **Timing:** 1–2 h

The marble-burying test is simple, easy to perform, and widely used for evaluating repetitive/stereotypic behaviors and the effects of anxiolytic drugs (Fodor et al., 2016; Thomas et al., 2009). One should keep at least a 1-day interval between the former and the present tests to relieve animals' stress. The procedures given below describe how to perform this test. However, it is also notable that burying is a natural and innate behavior in rodents. Thus, result interpretation does not always provide a single conclusion; rather, they depend on the animal models and types of treatments used (De Boer and Koolhaas, 2003; de Brouwer et al., 2019). Table 3 shows a behavioral category, parameter, and analysis method in the marble-burying test.

Table 3. Behavioral category, parameter, and analysis method in marble-burying test

Test	Behavioral category	Parameter	Method/tracking point
Marble-burying test	Repetitive/stereotypic	No. of marbles buried	Manually counted

40. Dilute the CNO stock solution in the same manner as described above (step 25) and transport animals in the same way as in the habituation period (step 20).
41. Place the cages on a rack and leave them for at least 30 min before commencing testing.
42. Adjust the brightness in the testing room to 5 lx using a photometer (approximately the same brightness as in the breeding conditions).
43. Prepare new cages containing bedding material placed up to a height of 4 cm from the floor, and align 24 glass marbles (6 × 4) at specific intervals (Figure 6A).
44. Inject the CNO (1 mg/kg) or saline solution intraperitoneally 15 min prior to the test and return the mouse to its home cage.
45. Place the mouse in a cage containing glass marbles, cover the cage with an upside-down stainless steel lid, and leave for 30 min (Figure 6B).
46. Carefully return the mouse to its home cage so that the bedding material is not scattered.
47. Count the number of marbles that remain unburied and thus calculate the number of marbles buried.

Note: The criterion for considering a marble to be buried is that more than two-third of it is covered with bedding material (Figure 6C).

Behavioral analysis

⌚ **Timing:** 1–2 weeks

48. Open field test
 - a. Assign 30% of the open field as the center area using TopScan LITE software.
 - b. Measure the behavioral parameters listed in Table 1 with the same software.
49. Novel object test
 - a. Assign a 1-cm-diameter area around the novel object as the object area with the TopScan LITE software.

- b. Measure the behavioral parameters listed in [Table 1](#), except for the number of stretch-attend posture movements, using the same software.
- c. Experimenters blinded to the animal groups calculate the number of stretched approach posture movements.

Note: Each time a mouse places its nose less than or equal to 1 cm from the novel object, it is defined as a sniffing event.

Note: A stretch-attend posture is defined as when the mouse lowers its back and stretches its neck towards an object while either standing still or moving forward without moving its hind paws.

50. Single object-burying test

- a. Assign a 1-cm-diameter area around the novel object as the object area using TopScan LITE.
- b. Measure the behavioral parameters listed in [Table 2](#), except for the duration of burying, using the same software.
- c. The duration for which a mouse is engaged in burying behavior is measured by experimenters who are blinded to the animal groups.

Note: Each time a mouse places its nose less than or equal to 1 cm from the novel object, it is defined as a sniffing event.

Note: Burying is defined as the action in which a mouse replaces or push bedding material toward a novel object, regardless of whether the object is covered ([Methods video S1](#)). Although mice frequently dig at the corner of the cage with their back to the object, it is not counted as burying behavior.

51. Marble-burying test: Data are expressed according to the number of marbles buried.

△ CRITICAL: After behavioral testing, it is critical to histologically confirm the accuracy of the viral injection and reporter gene expression under a fluorescent microscope.

EXPECTED OUTCOMES

When the M3-DREADD vector is injected into the hypothalamic perifornical area in *Ucn3-Cre* mice, mCherry expression is observed in the perifornical area between the fornix and the paraventricular nucleus ([Figure 7A](#)). Our previous immunohistochemical results indicate that $87.0 \pm 1.68\%$ of mCherry⁺ cells are *Ucn3*⁺ ($n = 16$ mice) and $90.4\% \pm 2.0\%$ of mCherry⁺ cells are c-Fos⁺ (activated neuron marker, $n = 5$) 2 h after intraperitoneal CNO injection (5 mg/kg) ([Horii-Hayashi et al., 2021](#)). Although intraperitoneal injection of CNO (0.7 mg/kg) does not induce any behavioral differences in the open field test, it increases the number of sniffing and stretch-attend posture events in the novel object test ([Figure 7B](#)). It also increases the incidence of burying behavior in the single object burying and marble-burying tests ([Figures 7C and 7D](#), respectively). In contrast, when the control vector is injected into the same region ([Figure 7E](#)), there are no behavioral differences in the open field and novel object tests between CNO (5 mg/kg) and saline groups ([Figures 7F and 7G](#), respectively).

LIMITATIONS

In this protocol, computer-based automated software is not utilized for the measurements in the stretch-attend posture and burying events. These behaviors are instead measured by experimenters blinded to the animal groups. However, this requires proficiency and considerable time. A MATLAB-based software, MATSAP, would potentially be useful for evaluating stretch-attend posture events. MATSAP is an open-source program that provides an objective and automated analysis of stretch-attend posture events in rodent behavioral experiments ([Holly et al., 2016](#)). HomeCageScan (CleverSys) might be able to analyze burying behavior as foraging or digging.

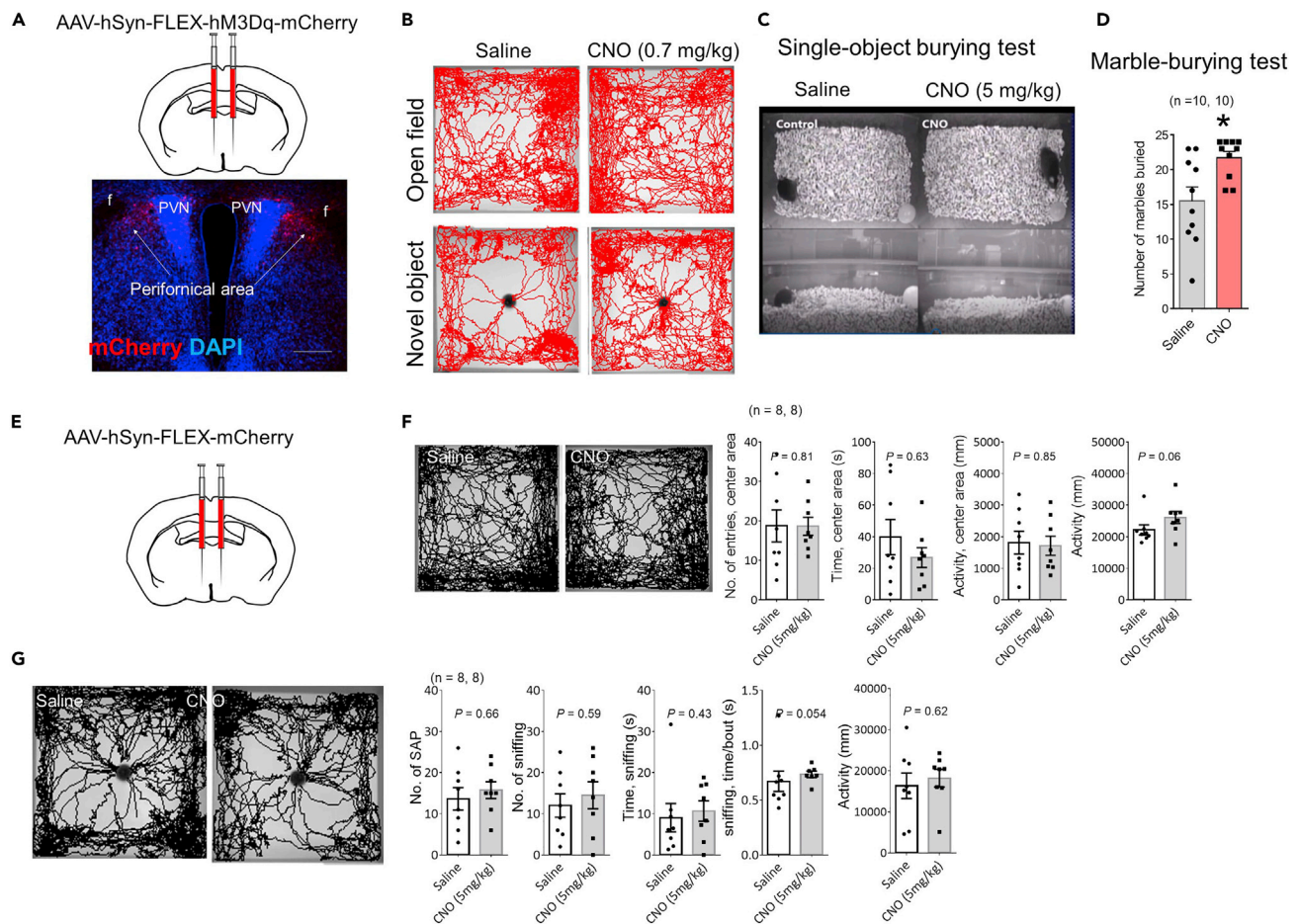


Figure 7. Expected results

(A) A schema indicating the injection of AAV-hSyn-FLEX-hM3Dq-mCherry to the hypothalamus. A fluorescent micrograph showing mCherry (red) and dapi (blue) signals. mCherry⁺ cells are observed between the fornix and paraventricular nucleus. Scale bar = 250 μm.

(B) Representative behavioral tracings using saline- or CNO-injected mice in the open field (left) and novel object (right) tests.

(C) Representative images of the single object burying test.

(D) Results of the marble-burying test. Data are represented as the mean ± SEM. *P<0.05 (n=10, Mann-Whitney U test).

(E) A schema indicating the injection of the control vector AAV-hSyn-FLEX-mCherry to the hypothalamus.

(F and G) There are no behavioral differences between saline and CNO groups in either open field (F) or novel object (G) test using the control vector-injected animals.

When target neurons project to multiple brain regions, intraperitoneal CNO injections activate all of the pathways between the neurons and their respective projection areas. Thus, the present protocol is not suitable for specific manipulation of a single neural pathway. To activate a single pathway using the DREADD technique, it is recommended to use a retrograde AAV vector (Addgene # 44361-AAVrg).

There is a limitation in cell-type specificity: although the majority of mCherry⁺ neurons are Ucn3⁺, a small population of neurons (approx. 13% of mCherry⁺ neurons) ectopically express hM3Dq even though they do not express Ucn3 (Horii-Hayashi et al., 2021).

The expected outcomes of increases in risk assessment, sniffing, and burying behaviors are observed in Ucn3-Cre male mice. It is unknown whether the same results will be obtained in female Ucn3-Cre mice. The expected outcomes indicated are the results of behavioral testing performed in the light phase from 9:00 to 13:00 (12 h light-dark cycle, lights on at 8:00).

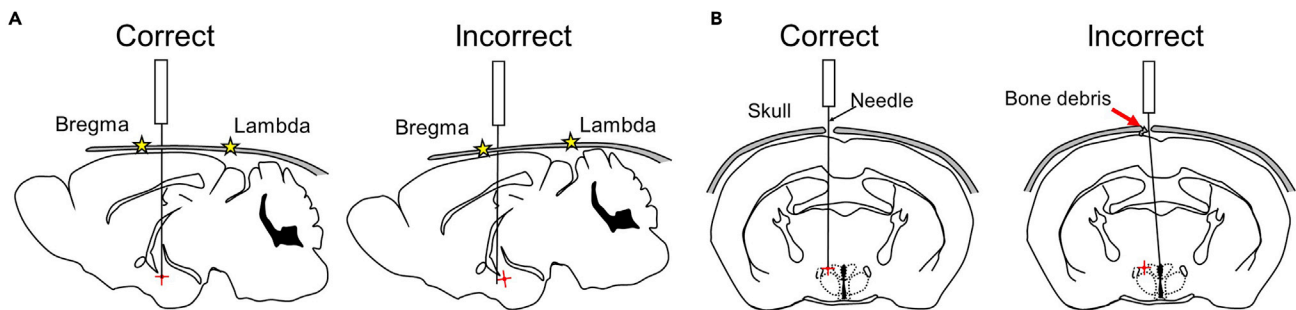


Figure 8. Possible causes of failure during stereotaxic surgery

(A) It is necessary for the success of stereotaxic surgery to adjust the bregma and lambda positions to the same height. (B) Bone debris after drilling disturbs the vertical lowering of the injection needle towards the target region.

TROUBLESHOOTING

Problem 1

Failure of the viral injection (step 5–10)

To achieve an accurate viral injection, fixation of the head, horizontal adjustment of the bregma and lambda, and smooth vertical lowering of the injection needle are necessary.

Potential solutions

Solution 1: The placement of an ear bar is important to firmly fix the head to the stereotaxic frame. When this step is not performed, the head remains mobile during surgery.

Solution 2: Ensure that the bregma and lambda levels are adjusted equally (Figure 8A).

Solution 3: After drilling, bone debris occasionally remains around the drilled hole, which can disturb the smooth vertical lowering of the needle (Figure 8B). In particular, the hypothalamus is located deep in the brain, and even a small amount of debris can cause a large positional deviation from the target region. To prevent this, carefully remove the bone debris under stereomicroscopic guidance.

Problem 2

mCherry reporter gene is not expressed in cells (step 13–16).

Potential solutions

Solution 1: Virus solution may not be released from the microsyringe needle. As describe as a note in step 13, confirm the release of virus solution before each injection.

Solution 2: The injection site is out of the target neurons expressing Cre recombinase.

Solution 3: The mouse is not a Cre-mice. Re-genotype the animal.

Problem 3

No behavioral differences are observed between CNO and saline groups (steps 25–47).

Activation of hypothalamic perifornical Ucn3 neurons increases sniffing, risk assessment, and burying behaviors towards a novel object. However, these outcomes are affected by the viral injection site and are likely influenced by stress.

Potential solutions

Solution 1: Confirm that the mCherry reporter gene is expressed in the hypothalamic perifornical Ucn-3 neurons in the region -0.58 to -1.06 mm from the bregma by immunohistochemical staining with anti-Ucn3 antibody (Dilution, 1:500; Yanaihara Institute, #Y364).

Solution 2: Confirm the activation of mCherry⁺ cells by immunohistochemical staining for activated neurons' markers (e.g., c-Fos) about 1.5–2 h after CNO injection (Horii-Hayashi et al., 2021).

Solution 3: There are individual differences in behaviors. It is recommended to use at least 8–10 animals in each group.

Solution 4: Try higher CNO doses. Although 0.7 mg/kg of CNO increases sniffing, risk assessment, and marble burying behaviors, a higher dose (e.g., 5 mg/kg) further accelerates these behaviors (Horii-Hayashi et al., 2021).

Problem 4

Mice does not bury a novel object (steps 30–39).

Potential solutions

Solution 1: Burying behavior is substantially different in individual animals. It is recommended to use at least 8–10 animals in each group.

Solution 2: When habituation to the field without the object is not enough, mice tend to consume the time for field exploration, which can disturb the expression of burying behavior during the novel object session. Longer time for the field exploration session may resolve this problem.

Solution 3: As described as a note in step 50, burying is defined as the action in which a mouse replaces or push bedding material toward a novel object, regardless of whether the object is covered. If one only counts the time that bedding material covers the object, measured values will be quite low.

Problem 5

Judging stretch-attend posture and burying is difficult (steps 49 and 50).

Potential solutions

Solution 1: Please refer to [Methods video S1](#).

Solution 2: The stretch-attend posture is frequently followed by quick withdrawal and retreat from the object in the first half of the novel object test, in particular. In the latter half, mice tend to repeat this posture without fleeing.

Problem 6

Mice push objects during the tests (steps 29 and 38).

Potential solutions

Firmly fix the objects using double-sided tape.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Noriko Horii-Hayashi (hayashi@naramed-u.ac.jp).

Materials availability

The study did not generate any unique reagents.

Data and code availability

The study did not generate/analyze any datasets or codes.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2021.100418>.

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AUTHOR CONTRIBUTIONS

N.H. performed the experiments and wrote the manuscript. M.N. read and approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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