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

Tetrode recording of rat CA1 place cells in an observational spatial working memory task

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SUMMARY

Social observation facilitates spatial learning by activation of hippocampal place cell patterns. Here, we describe an observational spatial working memory task to investigate the neural circuits underlying observational learning. This approach trains observer rats to learn to run a T-maze by observing a demonstrator’s spatial trajectory while recording their hippocampal CA1 place cell activities in a course of several hours. The protocol provides a tool to study neural activities at population level in a social setting. For complete details on the use and execution of this protocol, please refer to Mou et al. (2021).

BEFORE YOU BEGIN

The following materials need to be prepared before a researcher begins the protocol.

1. Sufficient dedicated space (> 20 m² recommended) for a T-maze, an observation box, and necessary computer equipment to control programs. It is suggested an experimental room be devoted to this protocol and for movement of equipment to be minimal.
2. Materials for hyperdrive assembly.
3. A procedure suite for rat survival surgery.
4. Histological suite to verify tetrode recording sites in the brain.

Institutional permissions

All behavioral and recording procedures followed the guidelines from the US National Institutes of Health and were approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine.

Hyperdrive assembly, tetrode fabrication, and loading

© Timing: 2 days

Hyperdrives with tetrodes are used to record hippocampal CA1 cells in our experiment. We suggest gathering materials for hyperdrive construction before or during behavioral training. Tetrode fabrication and loading, hyperdrive assembly are detailed in other work (Mou and Ji, 2018) and are described below.
5. Design hyperdrive body for 24 tetrodes using a computer-aided design software (e.g., SOLIDWORKS, Figure 1A), then 3-D print the product with strong and rigid material (e.g., Accura 55).

6. Individual drive fabrication (Figure 1B). For each drive, glue a ~15 mm small screw and 23-gauge guide tube (~15 mm, Component Supply company) with a piece of dental acrylic (Co-oral-ite Dental Mfg, CA).

7. Assemble drives and electrical interface board (EIB, Neuralynx, Bozeman, MT) to the hyperdrive body obtained in step 5.
   a. In this step, each drive is guided by a 60 mm 30-gauge guide tubing.
   b. Bundle all 30-gauge tubings within a guide cannula and glue to hyperdrive body using dental acrylic.
   c. Fix the EIB into pre-designed posts in the hyperdrive body using two screws (Figure 1C).

8. Make tetrodes.
   a. Cut a strand of ~40 cm insulated nichrome wire, 13 μm in diameter (Sandvik, Fair Lawn, NJ).
   b. Fold twice, then cut one end open to make four strands.
   c. Hang and rotate the bundled four wires above a wire twister (Neualynx, Bozeman, MT), ~60 forward rotations followed by ~20 backward rotations.
   d. Use an electronic heat gun (Steinel, Bloomington, MN) to blow the tetrode up and down from ~2 cm away on three sides for about 10 s for each side. The purpose is to melt the coating on the nichrome wire so that the four wires are joined together.

   **Note:** Do not heat too much as you may short the wires. Spare the open end of the tetrode so the four wires can be separated and connected to the EIB individually.

9. Tetrode loading.
   a. First, insert a polyimide tube (HPC Medical Products) into the 30-gauge guide tube of each tetrode drive under a dissection microscope, glue at its top.

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**Figure 1. Hyperdrive fabrication**

(A) A sketch of a drive body designed in SOLIDWORKS (left) and its 3D–printed product (right).
(B) A tetrode drive with a screw and a guide tube bound with a top piece.
(C) An assembled 24-tetrode hyperdrive with tetrodes loaded to guide tubes and connected to an EIB.
(D) Wires at the open end of a tetrode secured to pre-designated EIB holes by small pins.
(E) A guide cannula at the bottom of the hyperdrive loaded with tetrodes.
b. Load a tetrode and glue to the polyimide.

c. Connect each of the four wires at the open end to the pre-designated holes on the EIB and secure by pushing an EIB pin down from the top with a serrated jaw plier (Figure 1D).

10. Tetrode plating.

a. Turn drive screw to lower each tetrode out of the guide cannula (Figure 1E).

b. Plate each channel of a tetrode in a 1%–5% gold chloride solution.

c. Pass DC current (0.1–0.5 mA) to each channel for 1–5 s using a current isolator (Model A365, World Precision Instruments, Sarasota, FL).

d. Wash the tetrode in distilled water before measuring each channel’s impedance. Repeat this step a few times until a stable 200–300 kΩ impedance is reached.

Note: Check connections between any two channels of a tetrode to ensure there are no shorts among channels.

Construction of behavioral apparatus

© Timing: ~2 days

Construction of this apparatus (Figures 2A and 2B) is necessary for the behavioral procedures that will be outlined. Once setup, the apparatus can be used repeatedly with little adjustment.
11. Construct a steady continuous T-maze.
   a. Attach horizontal arm (120 cm long), a central arm (110 cm long), and two side arms (each 125 cm long) together (all segments 10 cm wide). Use square edge whitewood common board as material for arms. Connect pieces together with glue as adhesive. Screw down flat corner braces at the joints between horizontal arm and central arm, and horizontal arm and side arms to reinforce their connections.
   b. Use plexiglass (120 cm long × 5 cm high) as a barrier on the outside of the horizontal arm (facing away from central arm, see Figure 2A), and 5 cm wood barrier for rest of maze. This barrier will both guide the rat and prevent him from falling off the maze.
   c. Attach foam board cutout (50 cm wide × 50 cm high) as guides on both sides of the central arm, aligned to the end farthest from the horizontal arm.
   d. Attach rest box (20 cm wide × 40 cm long × 50 cm high) to bottom of T-maze (farthest end from horizontal arm). Suggested material is dark foam board.
   e. Use dark foam board or similar material to create sliding door to control entry to central arm of T-maze from rest box. Place this sliding door between the rest box and the bottom of the T-maze. The experimenter should be able to manually slide this door to control rat’s entry to and exit from the rest box.
   f. Elevate maze structure and rest box ~50 cm above the floor using materials such as traffic cones with metal braces to support the wood maze.

12. Construct a steady trapezoidal observation box.
   a. Connect three, opaque walls together. Use material such as black foam board for opaque walls and glue as adhesive. Tilt the walls ~30 degrees such that the (open) top is wider than the base, as it will facilitate easier placement of the rat into and out of the box (Figure 2A). Wall facing away from maze should be 10 cm wide × 50 cm high. Angled walls should be approximately 16 cm wide × 50 cm high.
   b. Attach clear plexiglass wall, 20 cm wide at top of trapezoid × 50 cm high, using glue as adhesive and facing the maze. Ensure plexiglass is cut and fits such that an observer rat inside the box can have a clear view of the T-maze.
   c. Attach a firm and steady base made of black form board under structure with glue. The entire observation box should be placed on a chair or stool.
   d. Ensure observation box is at same or slightly higher elevation relative to T-maze such that an observer rat in the observation box would be able to see turns made by a demonstrator rat in the maze.

13. Mount water ports on T-maze and observation box.
   a. Mount one water port (Lafayette Instrument, IN) on each side of observation box, cutting rectangles (10 cm wide × 15 cm high) through foam side walls such that the conical opening (2.54 cm in diameter) of the water port can be accessed by an observer rat inside the box. Each water port has a photo beam detector placed 6.35 mm behind the front of the opening.
   b. Mount one water port on each side of the horizontal arm of the T-maze using glue as adhesive.
   c. Connect all water ports to peristaltic water pumps bundled with water reservoir. Place water pumps at the center under the T-maze horizontal arm.

14. Animal’s poking into the water port will be detected by the infrared beam in the water ports, thus triggering water delivery. Set up the workflow in ABET II (Lafayette Instrument, IN). Make sure each device (water ports and pumps) in the setup can be activated independently and activities (beam break on water ports, water delivery on pumps) on different devices do not conflict. The core logic is that a certain reward volume of water should be delivered upon a nosepoke (poke at water port) by a rat, with specific limitations described in relevant behavioral procedure section below.

Note: It is best practice to test the workflow manually before use in animal training.
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Lidocaine hydrochloride | Sigma-Aldrich | 1366013 |
| Bupivacaine hydrochloride | Sigma-Aldrich | 1078507 |
| Buprenorphine SR-LAB | ZooPharm | #239173 |
| Isoflurane | Covetrus | NDC 11695-6777-2 |
| Oxygen | Airgas | UN1072 |
| 70% Ethanol | McKesson | NDC 68599-2302-6 |
| Povidone-iodine | Purdue Products L.P. | NDC 67618-154-16 |
| Euthasol | Virbac Animal Health | ANADA #200-071 |
| Formaldehyde | Fisher Scientific | Item # F79P-4 |

**Experimental models: Organisms/strains**

| Experimental models: Organisms/strains | SOURCE | IDENTIFIER |
|---------------------------------------|--------|------------|
| Male, wild type Long-Evans rats, 4–10 months | Charles River Laboratories | Substrain: Crl:LE, RRID: RGD_2308852 |

**Software and algorithms**

| Software and algorithms | SOURCE | IDENTIFIER |
|-------------------------|--------|------------|
| Solidworks Desktop 3D CAD | SOLIDWORKS | https://www.solidworks.com/product/solidworks-3d-cad |
| EthoVision XT14 | Noldus | https://www.noldus.com/ethovision-xt |
| Digital Lynx | Neuralynx | https://neuralynx.com/hardware/digital-lynx-sx |
| ABET II | Lafayette Instrument | https://lafayetteneuroscience.com/products/abetii-operant-ctrl-software |

**Other**

| Other | SOURCE | IDENTIFIER |
|-------|--------|------------|
| Square edge whitewood common board | The Home Depot | https://www.homedepot.com/p/1-in-x-4-in-x-8-ft-Premium-Kiln-Dried-Square-Edge-Whitewood-Common-Board-914681/100023465 |
| Wood glue | Gorilla | https://www.homedepot.com/p/Gorilla-8-oz-Wood-Glue-62000/100672167 |
| Flat corner brace, 8 in | Everbilt | https://www.homedepot.com/p/Everbilt-8-in-Steel-Zinc-Plated-Flat-Corner-Brace-13617/203170059 |
| Clear Plexiglass, 0.125 in. thickness | OPTIX | https://www.homedepot.com/p/OPTIX-18-in-x-24-in-x-0-093-in-Clear-Acrylic-Sheet-Glass-Replacement-MC-05/202038047 |
| Water resistant black foam board, 0.18 in. thickness | Office Depot | #460851 |
| PVC non-reflective traffic cone, 18 in. height | The Home Depot | https://www.homedepot.com/p/Unbranded-18-in-Orange-PVC-Flow-Molded-Cone-HDFM18/308848264 |
| Nosepoke | Lafayette Instrument | https://lafayetteneuroscience.com/products/nosepoke-tricolor-light-conical-entry-liquid |
| Precision liquid pump with tubing | Lafayette Instrument | https://lafayetteneuroscience.com/products/precision-liquid-feed-pump |
| 3-D printer | Formlabs | Forlab 2 |
| Resin | Formlabs | Tough resin 2000 |
| Custom “half moon” drive screw head | Neuralynx | N/A |
| Stainless steel tubing, 23 gauge, 30 gauge | Component Supply | Item #: HTX-23T, HTX-30T |
| Polymide tubing | HPC Medical Products | Item #: HPCM000001-012 |
| Dental acrylic | Co-oral-ite Dental Mfg | Item #: 110-8102 |
| Teets cold curing liquid | Co-oral-ite Dental Mfg | Item #: 110-8503 |
| Insulated nichrome wire, 13 μm diameter | Sandvik | Item #: PF000591 |
| Straight iris scissors | Word Precision Instruments | Item #: 500216 |
| Tetrode assembly station | Neurolyx | https://neuralynx.com/hardware/tetrode-assembly-station |
| Electronic heat gun | Steinel | Type 3483 |
| Super glue gel | Gorilla | https://www.homedepot.com/p/Gorilla-0-71-oz-Super-Glue-Gel-7700103/205485334 |
| Small EIB pin | Neurolyx | https://neuralynx.com/hardware/small-eib-pins |

(Continued on next page)
### MATERIALS AND EQUIPMENT

**Materials and equipment for behavioral apparatus**

Square edge whitewood common board (1 in. D × 4 in. W × 8 ft. L, The Home Depot).

Wood glue (Gorilla).

Flat corner braces (8 in. Zinc-Plated, Everbilt).

Clear Plexiglass (0.125 in. thickness, The Home Depot).

Water resistant black foam board (0.18 inch thickness, Office Depot).

Orange PVC non-reflective traffic Cone (18 in. Height, The Home Depot).

Nosepoke / water port (Model 80117RM, Lafayette Instrument, IN).

Precision liquid feed pump with tubing (Model 80204, Lafayette Instrument, IN).

### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Serrated jaw pliers | Neuralyx | [https://neuralynx.com/hardware/eib-pins-tooling](https://neuralynx.com/hardware/eib-pins-tooling) |
| Gold chloride solution | Neuralyx | [https://neuralynx.com/hardware/gold-plating-solution](https://neuralynx.com/hardware/gold-plating-solution) |
| Current isolator | World Precision Instruments | Model A365 |
| Electrode impedance tester | Bak Electronics | Model IMP-2 |
| Isoflurane anesthesia system | Veterinary Anesthesia Systems | Matrix VIP 3000 |
| Stereotaxis instrument | David Kopf | Model 962 |
| Fiber optic illuminator | AmScope | HL 150-B |
| Homeothermic blanket system | Harvard Apparatus | Model 507220F |
| High speed drill | Foredom Electric Company | Model 1474 |
| Drill bits | Stoelting | Item # 514551, 514554 |
| Glass bead sterilizer | Fine Scientific Tools | Model 250 |
| Animal fur trimmer | Andis | Item # 69176 |
| Hair remover lotion | Veet | Item # 8235654 |
| Surgical tools: fine scissors, scalp, fine forceps, coarse forceps | Fine Science Tools | [https://www.finescience.com/en-US/](https://www.finescience.com/en-US/) |
| Nylon surgical suture | ETHILON | Item # 667 |
| Artificial tears ointment | AKORN | NDC-59399-162-35 |
| Sterile saline | Hospira | NDC 0409-4888-02 |
| Syringe, 3 mL | BD Syringe | Ref # 309586 |
| Syringe, 1 mL | Covidien | Ref # 8881501400 |
| Syringe needle, 27 gauge, 30 gauge | BD PrecisionGlide | Ref # 305109, 305110 |
| Anchoring screws | Stoelting | Item # 51457 |
| Silicone grease | Fine Science Tools | Item # 29051-45 |
| Vibratome | Leica | VT1000S |
| Cresyl violet | MilliporeSigma | Item # 190-12 |
| Tissue-Tek O.C.T. compound | Sakura Finetek USA, Inc. | Item # 4583 |
| VWR micro slides | VWR International, LLC. | Item # 16004-422 |
| VWR micro cover glass | VWR International, LLC. | Item # 48382-136 |
| Elevated flowerpot in a sleep box | Walmart | [https://www.walmart.com/ip/](https://www.walmart.com/ip/) Pennington-Red-Terra-Cotta-Clay-Planter-2-inch-Pot/16795425 |
| Guide cannula | Component Supply | Item #: HTX 16T |
| Dissection microscope | A-M Systems | EM2-STR |
Materials and equipment for hyperdrive assembly and tetrode fabrication

3-D printer (Formlab 2, Formlabs).

Resin (Tough resin 2000, Formlabs).

Custom “half moon” drive screw head (Neuralynx).

Stainless steel tubing, 23 Gauge (Item #: HTX 23T, Component Supply company), 30 Gauge (Item #: HTX-30T), Polyimide tubing (35-gauge, Item # 72113900001-012, HPC Medical Products).

Dental acrylic (Item # 110-8102, Co-oral-ite Dental Mfg, Diamond Springs, CA).

Teets cold curing liquid (Item # 110-8503, Co-oral-ite Dental Mfg, Diamond Springs, CA).

Insulated nichrome wire (13 µm diameter, Sandvik, Fair Lawn, NJ).

Straight iris scissors (Item # 500216, Word Precision Instruments, Sarasota, FL).

Tetrode assembly station (Neuralyx, Bozeman, MT).

Electronic heat gun (Type 3483, Steinel, Bloomington, MN).

Super glue gel (Gorilla, The Home Depot).

Small EIB pin (Neuralynx, Bozeman, MT).

Serrated jaw pliers (Neuralynx, Bozeman, MT).

Gold chloride solution (Neuralynx, Bozeman, MT).

Current isolator (Model A365, World Precision Instruments, Sarasota, FL).

Electrode impedance tester (Model IMP-2, Bak Electronics, Umatilla, FL).

Dissection microscope (EMZ-5TR, A-M Systems).

Equipment and supplies for surgery

Local analgesics: 50/50 mix of 2% lidocaine & 0.5% bupivacaine diluted 1:20 in sterile saline (stored at room temperature for maximum 1 year). Injected subcutaneously around the site of incision.

| Reagent        | Final concentration | Amount |
|----------------|---------------------|--------|
| 2% Lidocaine   | 0.048%              | 0.5 mL |
| 0.5% Bupivacaine | 0.012%           | 0.5 mL |
| Sterile saline |                     | 20 mL  |

General analgesics: Buprenorphine 1 mL/kg body weight, injected subcutaneously.

Isoflurane anesthesia system (Matrix VIP 3000, Veterinary Anesthesia Systems, Inc.).

Oxygen supply, medical grade (UN1072, Airgas).
Small animal stereotaxic instrument (Model 962, David Kopf).

Stereo microscope (Stemi, 2000-C, ZEISS).

Fiber optic illuminator (Model 725910, A-M Systems).

Homeothermic blanket system (Model 507220F, Harvard Apparatus).

High speed drill (Model 1474, Foredom Electric Company).

Glass bead sterilizer (Model 250, Fine Scientific Tools).

Animal fur trimmer (Item # 69176, Andis).

Hair remover lotion (Item # 8235654, Veet).

Surgical tools: fine scissors, scalpel, fine forceps, coarse forceps (Fine Science Tools).

Isoflurane (NDC 11695-6777-2, Covetrus).

Nylon surgical suture (Item # 667, ETHILON).

Drill bits (0.7 mm tip, 0.45 mm, Stoelting).

Artificial tears ointment (NDC-17478-062-35, AKORN).

Sterile saline (NDC 0409-1966-02, Hospira, Inc.).

70% Ethanol.

Povidone-iodine.

1 mL and 3 mL syringe, 27- and 30-gauge needle.

Anchoring screws (Item # 51457, Stoelting, Wood Dale, IL).

Silicone grease (Item # 29051-45, Fine Science Tools).

**Equipment and setup for tetrode recording**
Continuous T-maze with a rest box attached at the bottom.

Observation box.

ABET II software.

EthoVision XT14.

Digital Lynx acquisition system (Neuralynx, Bozeman, MT).

Elevated flowerpot in a sleep box.

**Materials for histology**
Vibratome (VT1000S, Leica).
Cresyl violet (MilliporeSigma).

Tissue-Tek O.C.T. compound (Sakura Finetek USA, Inc.).

VWR micro slides (VWR International, LLC.).

VWR micro cover glass (VWR International, LLC.).

**Alternatives:** The specific products listed above are examples that we have used successfully, but they could be replaced by similar products.

**STEP-BY-STEP METHOD DETAILS**

**Behavioral procedures**

© Timing: 6–10 weeks

This behavioral protocol trains rats to engage in an observational working memory task. Behavioral procedures are split into three phases, followed by additional conditions. The demonstrator and observer rat should be cage mates (two male rats living in same cage). The purpose of the first phase is to familiarize all rats (including both demonstrator rats and observer rats) to the maze and reward acquisition, whereas the second and third phases focus on observational learning by an observer rat. During the second phase, the observer rat should learn to observe the demonstrator rat and poke the same side in the observation box during the defined time frame for a reward. During the third phase, the observer rat should learn to observe the demonstrator rat and poke the same side in the maze during the defined time frame for a reward. Training days need not be every weekday, but should not be spaced more than two days apart. The default position of the experimenter should be behind the rest box whenever not performing any experimental procedure.

1. Observational working memory task (Figure 3).
   a. Restrict all rats’ water consumption for three days (rats receive 5% of the weight of the rat in water per day). Maintain body weight at > 85% of the ad libitum level. Keep food available ad libitum at all times. Resume free water consumption if animal’s body weight drops below 85%.
   b. Phase 1: Habituation.
      i. Each trial starts with releasing the demonstrator rat from rest box (~10 s in rest box between trials) onto T-maze such that it can run an outbound trajectory (move along central arm, make a free choice at choice point, and reach a water port in the maze).
      ii. Demonstrator rat should poke at the water port in the maze to trigger water delivery (100 µL) as reward.
      iii. After water consumption, the demonstrator rat should return from the corresponding side arm (inbound trajectory). Retain the demonstrator rat in the rest box for 10 s before starting next trial. Manage entry and exit from rest box through experimenter manually controlling sliding door. A successful trial will have the demonstrator rat run along the central arm, turn at choice point, poke water port in the maze for reward, and return along the same side into the rest box.

   **Note:** Ensure the demonstrator rat returns to rest box through side arms rather than turn around (see troubleshooting problem 1).

   iv. Each session lasts 20 trials or 20 min, whichever occurs first. Total phase one should be one session per day for 6 training days. Animals are considered familiarized when they can make > 10 successful runs each session.
CRITICAL: To avoid bias, if the demonstrator rat chooses same side for three trials in a row, halt water reward on that side until the demonstrator rat chooses the alternative side. This is achieved by setting the maximum number of consecutive water deliveries on the same side to be three in ABET II software.

c. Phase 2: Pre-training.
   i. In ABET II, change settings of water ports in the maze such that a nosepoke results in 7 pulses of water, each pulse lasting 0.7 s with a 3 s interval (total reward 250 μL).
   ii. In each trial, the demonstrator rat should run along the central arm, make a free choice, and obtain reward from one of the water port reward sites in the T-maze, and return to the rest box as described in Phase 1.
   iii. Define settings of water ports in observation box such that a nosepoke in the T-maze starts a 10 s window for poking in the observation box. A poke on the same side in the box as the demonstrator rat running in the T-maze during the 10 s window should start 4 pulses of water in a 7 s interval, each pulse 0.5 s, for total reward of 100 μL (Data S1).
   iv. Take two cage mate rats, place one in the observation box, and the other in the T-maze apparatus. Both rats should have gone through previous training phase.
   v. Each session should last 20 trials. Total Phase 2 should be one session per day for 2–3 weeks. Expected outcome is that observer rat poke times should move closer to

Figure 3. Task structure
(A) Workflow of the task training procedure. Note that only observer rats after training are subject to test on other conditions.
(B) Habituation. Rats learn to properly run in T-maze and obtain water reward.
(C) Pre-training. Observer rat in observation box learns association between demonstrator rat’s direction choice and reward.
(D) Training. Observer rat must make same decision as demonstrator rat in maze to obtain water reward.
(E) Tetrode advancing. Tetrodes are advanced to CA1 pyramidal layer.
(F) Recording. Tetrodes monitor LFP and spiking activity.
demonstrator rat poke time (Figure 7A). If an observer rat is rewarded in > 15 trials, it is ready for Phase 3.

d. Phase 3: Training.
i. In this phase, the procedure for the demonstrator rat is identical to Phase 2. However, after the demonstrator rat returns to the rest box, the observer rat should be transported from the observation box to the T-maze central arm (facing away from choice point). Experimenter should return to the default position of behind the rest box.

Note: If the observer rat fails to poke the water port in the maze within the 10 s window, the current trial terminates. Transport the observer rat back to the box. Pause for ~30 s before next trial begins.

ii. The observer rat should get rewarded with 300 µL for poking the same water port in the maze the demonstrator rat poked.

iii. After obtaining reward, the observer rat should return towards the rest box along the same side arm he poked and be transported back to observation box on a plate.

iv. Each session should last > 30 trials (each ~2 min), one session per day. Such sessions should continue until the percentage of correct trials is > 70% for at least 2 consecutive sessions.

△ CRITICAL: The experimenter’s movement and picking side from the observation box could give the observer visual cues and bias the observer’s choice of direction on the T-maze (e.g., the observer rat could choose the side from which it was picked up by the experimenter). To avoid potential bias by human cues, the experimenter should transport the observer rat out of and return to the observation box on the same side of the T-maze.

Note: See troubleshooting section for potential issues and solutions.

2. Additional behavioral conditions: for observer rats that reached the desired performance level from Phase 3, test one of the following conditions (Figure 3). Test only one condition per day.

a. Object - This condition tests whether social cues are required for correct observer rat performance.
   i. Replace the demonstrator rat with a moving object (10 cm × 20 cm black rectangular plastic block attached to the end of a 1.5 m wood pole).
   ii. Control pole to mimic Phase 3 demonstrator movements in the T-maze, including triggering water port in the maze and remaining at poking location for a comparable duration.
   iii. Procedure for the observer rat remains identical to Phase 3.

b. Empty - This condition tests whether the observer rat’s performance requires the presence of a moving subject / object.
   i. Leave the T-maze empty during the demonstrator rat portion of trials. Rather, trigger water rewards every 2 min in the maze with a wood pole controlled by the experimenter. Ensure that the pole remains at the reward location for a comparable duration as the rats in Phase 3.

c. No lick - This condition tests whether the observer rat simply follows acoustic cues from licking.
   i. Keeping all else same as Phase 3, do not deliver water to the demonstrator rat following the nosepoke.

d. Mixed bedding - This condition tests whether the observer rat follows olfactory cues.
   i. Keeping all else same as Phase 3, lay regular cage bedding along the top half of the central arm as well as half of the left and right horizontal arms (Figure 4A).
   ii. After the demonstrator rat returns to the rest box, scramble the bedding thoroughly across both sides of the choice point prior to the observer rat’s run.

e. Blocked-view - This condition tests whether vision plays a role in the observer rat’s performance.
Keeping all else same as Phase 3, cover the front panel of the observation box with a black cloth such that the observer rat can no longer see the T-maze (Figure 4B).

**Note:** If multiple conditions were used for the same observer rat and the performance in the maze was below the criterion (>70% for at least 2 consecutive sessions) under one condition, re-train the observer rat back to criterion (Phase 3) before the next condition.

**Surgery and hyperdrive implantation**

© Timing: 4–6 h

Well-trained observer rats are subject to survival surgery to implant hyperdrive. Stop water restrictions two days before surgery. Surgical procedures should be conducted under aseptic conditions as described in other work (Haggerty and Ji, 2015; Mou and Ji, 2016; Wu et al., 2017).

3. Implant observer rats with a hyperdrive. Target hippocampal CA1 region at coordinates antero-posterior (AP) -3.8 mm and mediolateral (ML) 2.4 mm relative to the Bregma and the right dorsal anterior cingulate cortex at the coordinates AP 1.9–1.3 mm and ML 1.0 mm (Paxinos and Watson, 2006).

4. Prepare instruments and supplies for surgery.

5. Surgical implantation of the hyperdrive.
   a. Inject Buprenorphine (1.0 mg/kg, S.C.) 30 min prior to anesthesia.
   b. Anesthetize the animal with 2.5% isoflurane in oxygen (1.0 L/min flow rate) in an induction chamber. Ensure adequate depth of anesthesia by making sure there are no tail- or foot-pincho reflexes.
   c. Shave the surgical region with a fur trimmer.
   d. Fix the animal in a stereotaxic frame under anesthesia by locking the incisor and inserting ear bar into the ear canals (Figure 5A). Cover animal’s eyes with ophthalmic ointment to protect them from dryness and bright light.
   e. Disinfect the skin of the surgical region with 70% ethanol and povidone-iodine three times.
   f. Inject a 50/50 mix of 2% lidocaine and 0.5% bupivacaine diluted 1:20 in sterile saline (0.04 mL/10 g body weight) subcutaneously along the incision line.
   g. Make an incision with the scalpel to the scalp along the midline to expose the skull. Use blunt-end forceps to pull the skin to the sides and clean the skull with cotton swabs.
   h. Mark the recording sites for dorsal hippocampal CA1 (anteroposterior – 3.8 mm, mediolateral 2.4 mm relative to the Bregma) and dorsal anterior cingulate cortex (AP 1.9–1.3 mm, ML 1.0 mm).
   i. Drill holes for ~10 anchoring screws without touching the brain around the skull edge, typically 2 screws/bone piece (Figure 5B). Tightly turn the anchoring screws into the holes.
The screws need to be further away from the recording site and should be as close to the skull edge as possible to cover a sufficiently large area of the skull.

j. Drill circular holes slightly larger than the recording cannula while carefully removing bone residues with an air tube attached to the drill (Figure 5C).
k. Use a fine needle (27 gauge) to gently penetrate and remove the dura.
l. Attach the hyperdrive to a drive holder mounted on the stereotaxic frame. Lower the hyperdrive slowly right above the brain. Apply silicone grease around the guide cannula to completely cover the hole.
m. Secure the hyperdrive to the anchoring screws and the skull by applying dental acrylic.
n. Pull the skin closely around the hyperdrive and suture the opening, while leaving the hyperdrive in place. Add a protective cap to the top of the hyperdrive.

Tetrode placement and recording

© Timing: 3–6 weeks

6. In the 2–3 weeks following the surgery, advance tetrodes daily to CA1 pyramidal layer and monitor hippocampal local field potential (LFP) and cells’ spiking activity using a Digital Lynx acquisition system (Neuralynx, Bozeman, MT) as described in other articles (Haggerty and Ji, 2015; Mou and Ji, 2016; Wu et al., 2017).

Note: No restrictions are necessary immediately after surgery. Resume water restriction 1 week after surgery (as in step 1a) for implanted observer rats.

7. In the second to third week after surgery, re-train the implanted animal to Phase 3 procedure for 2–3 sessions, one session per day, to get rats accustomed to hyperdrive and overhead tethers.

8. After characteristic sharp-wave ripple (100–250 Hz oscillation, Figure 6B) on LFPs are observed and stable single unit spike clusters have formed (Figure 6A) on half of the tetrodes (> 12 out of all 24 tetrodes), start recording the observer rat when it performs the observational working memory task under either the standard conditions or under the Object or Empty conditions described in step 2. Track animal’s head and body center positions with EthoVision XT system at 30 Hz.

Note: Start recording sessions after tetrodes have not been moved for at least 24 h.

Note: Before and after each session, let the observer rat rest on an elevated flowerpot outside of the sleep box for 30 min to collect neural data in resting condition.
9. Record each observer rat for several weeks, one session per day. Before and after each training session, rest the observer rat in a sleep box with an elevated flowerpot (Figure 2C) to rest for 30 min.

**Histology**

© Timing: 1–2 days

This portion of the protocol verifies tetrode locations.

10. After experiments, euthanize observer rats with Euthasol (200 mg/kg). Then, pass a 30 μA current for 10 s on each tetrode to generate a small lesion at each recording site.
11. Dissect the observer rat brain and store brain tissues in 4% formaldehyde solution at least 12 h before sectioning.
12. Section the brain at 90 μm thickness with vibratome (VT1000S, Leica) and mount the sections on the glass slides.
13. Stain brain sections using 0.2% Cresyl violet and use coverslip for storage.
14. Identify tetrode locations by matching lesion sites with tetrode depths and their relative positions.

**EXPECTED OUTCOMES**

Our experiments with rats show that this protocol trains the observer rat to follow a demonstrator rat’s trajectories in a T-maze by observing the demonstrator rat’s actions from a separated box nearby. The entire training course takes 40–60 days. In our experience, > 90% of observer rats can be trained to synchronize their poking activity in the box to their corresponding demonstrator rat’s pokes in the maze (Figures 7A and 7B). Furthermore, the observer rats followed the demonstrator rat’s trajectories during later self-running (Figure 7C). Under control conditions without the demonstrator rat, both poking synchrony in the box and the behavior of following the demonstrator rat’s trajectories in the maze were reduced (Figure 7D and Mou et al., 2021). These results show that rats can be trained to perform a spatial working memory task through observational learning.

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**Figure 6. Representative spiking and LFP in the hippocampal CA1 at resting state**

(A) Spiking clusters on a tetrode in the CA1. Each window represents the combination of any two of the four channels of a tetrode. Each white dot represents the amplitude of an action potential sampled by the same two channels. (B) Top: spike raster of the active place cells replayed in example ripple events. Cells are ordered by their place field position on the Outbound (blue) or Inbound (red) trajectory. Bottom: ripple LFP for corresponding replay events on the top.
In our setup, we typically record 50–100 cells (about 2–5 cells per tetrode). Our tetrode correctly located in the anterior cingulate cortex and dorsal hippocampal CA1 region (Figure 8). During tetrode recording, the observer rat’s position and neural data were acquired. Our data show that while consuming water reward in the observation box, simultaneously recorded hippocampal CA1 place cells replayed their activity patterns in the T-maze during ripples (Figure 6B). Of particular interest is that the trajectories leading to the reward sites along the side arms were consistently re-played in reverse order, even though the observer rats never traversed in this direction (Mou et al., 2021).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

To assess the degree of synchronization between the demonstrator rat’s pokes in the maze and the observer rat’s in the box, quantify nosepokes rate at a 0.25 s time bin and examine observers’ poke rate around the demonstrator’s first pokes (reference time) (Figure 7A). For the control (Object, Empty) conditions without a demonstrator, the reference time is the time when the moving object makes the nose poke after reaching the reward site in the maze under the Object condition, or when the poke is manually triggered under the Empty condition. We further defined a percentage of correct first pokes in the box. For each trial, evaluate the observer’s first poke in the box within the

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**Figure 7.** Observer rats were trained to follow their demonstrator’s choices in the observation box and in the maze

(A) Poke rate of an example observer rat in example sessions before (Pre-) and after (Post-) training (Post-training: 600 s shown for comparison with Pre-). Red line: demonstrator rat’s first poke time in each trial. Note the clustering of the observer rat’s nosepokes following the demonstrator’s first pokes during the post-training session. Time bin = 0.25 s. Positive and negative values represent left and right pokes, respectively.

(B) Percentage of correct first pokes during Pre-training. Gray thin lines: individual rats. Black thick lines: average performances.

(C) Maze performances (percentage of trials observer rats followed the demonstrator rat’s choices) in all training sessions of observer rats (adopted from Figure 2B in Mou et al., 2021 with permission). Gray thin lines: individual rats. Black thick lines: average performances.

(D) Maze performances in the first three test sessions of observer rats under various control conditions.
A 1 mm B 1 mm

Figure 8. Verification of tetrode placement
(A) Crystal violet staining of coronal brain sections. Arrow, tetrode locations in the right dorsal anterior cingulate cortex (AP: 1.9–1.3 mm, ML: 1.0 mm).
(B) Dense staining area shows hippocampal cell bodies. Arrow, tetrode locations in dorsal CA1 (AP: -3.8 mm, ML: 2.4 mm).

[1–2] s window around the demonstrator’s first poke in the maze. The first poke is deemed correct if it is on the same side of the demonstrator’s in the maze (Figure 7B).

We quantify animal’s performance in the maze by a percentage of correct trials. A trial is defined as correct if the observer makes the same choice and pokes the same water port in the maze as the demonstrator rat.

LIMITATIONS
In this protocol, behavioral training takes majority of the time. Our experience suggests that the behavioral procedure could take up to 10 weeks before the observer rats reach the criterion (correct trials > 70% for at least 2 consecutive sessions). However, once trained, animals’ performance can be maintained given that they are retrained once a week afterwards.

With two animals in the arena, researchers need to pay attention to activities of both, and sometimes coordinate their activities while operating the neural recording and video tracking systems. Thus, tetrode recording in behaving observer rats in our observational learning task can be challenging for unexperienced researchers at first. However, one will develop skills with practice. Experimenters can even try trial runs with untrained rats as practice before trying with rats that have gone through the behavioral training.

TROUBLESHOOTING
Problem 1
Animals linger at the reward sites or turn back to the opposite direction after water consumption in the T-maze (step behavioral procedures, 1, 2). This happens more often in early training.

Potential solution
If this happens, researcher can use a long wood pole to prevent the animal from turning back or to gently guide the animal down to the side arm. After animals are acquainted with the training procedure, they tend to return from the side arms more often.

Problem 2
Difficulty in training observer rats to follow the demonstrator’s trajectories in the maze (step behavioral procedures, 1d).

Potential solution
We recommend handling all animals before the Behavioral procedures start. Using cage mates as demonstrators is more likely to draw the observer rats’ attention and facilitate learning. However,
if an observer rat does not show any sign of improvement in performance in the maze after 4 weeks in step 1d, training can stop. Our experiences show that > 90% of observer rats can be trained to surpass our criterion.

Problem 3
The observer rats could use experimenter’s position, rather than the demonstrator rat’s activities, to guide their choices in the maze (step behavioral procedures, 1d, 2; step tetrode placement and recording 7, 8, 9).

Potential solution
To prevent experimenter’s position from becoming a confounding factor and biasing observers’ choices in the maze, experimenter should stand quietly behind the rest box at the bottom of the T-maze after placing the observer rat in the maze. A curtain separating the arena and the experimenter is recommended.

Problem 4
While moving in the observation box, the hyperdrive mounted on the observer rat’s head can hit the walls and cause mechanical damage. In addition, tethers connecting the hyperdrive and Digital Lynx interface can fall out of hyperdrive when animals exert tension on them during the task (step tetrode placement and recording 7, 8, 9).

Potential solution
We recommend stabilizing the tether connectors and receivers with hot gun glue on the EIB board of the hyperdrive (Mou and Ji, 2018) and adding protection cone to cover all electronics on the hyperdrive during recording.

Problem 5
Environmental noise during tetrode recordings (step tetrode placement and recording, 8, 9).

Potential solution
Shield the recording room if possible and keep all noisy power sources outside the room. Ground the apparatus and other equipment properly. Electrical noise during recording can be reduced by noise cancellation via referencing. Therefore, we recommend placing a reference electrode in a local “quiet” area (e.g., white matter in hippocampus).

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xiang Mou (xmou@bcm.edu).

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate code.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101501.

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Conceptualization, X.M. and D.J.; Methodology, X.M. and D.J.; Investigation, X.M. and P.S.; Writing, X.M., P.S., and D.J.; Supervision, X.M. and D.J.; Funding acquisition, D.J.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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