Protocol

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Protocol for interfering peptide injection into adult mouse hippocampus and spatial memory testing

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SUMMARY
Metabotropic glutamate receptor-dependent long-term depression (mGluR-LTD) occurs in diverse brain regions and contributes to the plasticity of behavior, learning, and memory. mGluR-LTD relies on rapid (in minutes) local protein synthesis. Here, we describe a detailed protocol for delivering an interfering peptide into the adult mouse hippocampus. The delivered peptide disrupts the interaction between polyglutamine binding protein 1 and eukaryotic elongation factor 2, resulting in impaired hippocampal mGluR-LTD and mGluR-LTD-associated behaviors.

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

BEFORE YOU BEGIN
Here we describe materials and methods for a cannula insertion surgery in the adult mouse hippocampus, combined with an intracerebral infusion of interfering peptide and spatial memory testing (Shen et al., 2021). The stereotaxic coordinates for injection sites are obtained from the mouse brain atlas (Paxinos and Franklin, 2004).

Preparation of cannula

© Timing: 2 weeks

1. Customize the cannula with a specific length. The cannula consists of a protective cap, a catheter, and an injection tube (Figures 1A–1C). The length of the catheter under the plastic base (L1) is determined by the depth of a specific injected brain region (e.g., here hippocampus, 1.6 mm) to bregma referring to the mouse brain atlas (Paxinos and Franklin, 2004). The lengths of the injection tube (L2) and the protective cap (L3) protruding out of the catheter are both 0 mm. The values of L1 to L3 are variable based on the experimental requirements. Moreover, the outer diameter of the protective cap is 4 mm (D1). The outer diameters of the plastic base (D2) and the catheter (D3) are 3 mm and 0.5 mm, respectively. The outer diameter of the injection tube (D4) is 0.26 mm, and the outer diameter of the metal rod under the protective cap (D5) is 0.25 mm (Figures 1A–1E). The values of D1 to D5 are fixed. The protective cap is sheathed on the catheter after cannula insertion (Figure 1D). When the drug is administered, the injection tube and catheter are installed together (Figure 1E). The cannulas are sterilized by ultraviolet light before cannula insertion. See Troubleshooting 1.
Peptide preparation

2. Peptide design. Shen et al. found polyglutamine binding protein 1 (PQBP1) bound directly to the non-phosphorylated linear peptide around Thr56 of eukaryotic elongation factor 2 through its N-terminal WW domain (residues 45–81). The conserved amino acid of the WW domain is 52–68. Therefore, two membrane-permeable interference (TAT)-peptides corresponding to residues 52–68 (TAT-52-68aa) and 60–78 (TAT-60-78aa) of PQBP1 were synthesized (Shen et al., 2021). The peptide of TAT-52-68aa was used in this study. A TAT-scramble peptide was generated with the same amino acids but in random order. The sequences are shown in the key resource table.

3. The peptides with 98% purity were synthesized by Genscript and purified by high-performance liquid chromatography (HPLC).

4. The peptides were dissolved in 0.9% NaCl (normal saline) at a final concentration of 800 nM and stored at −20°C. The dissolved peptides can be stored at −20°C for up to 6 months.

Pipette preparation

© Timing: 5 min

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**Figure 1. Preparation of cannula**
The cannula contains three components, a protective cap (A), a catheter (B), and an injection tube (C).
(D) The protective cap is sheathed on the catheter.
(E) When delivering peptide, the injection tube and the catheter are assembled together.
(F) Parameters of each cannula component. The values of D1-D5 are fixed values. The values of L1-L3 can be customized according to experimental requirements.

| Parameters         | Value (mm) | Remarks     |
|--------------------|------------|-------------|
| D1                 | 4          | Fixed value |
| D2                 | 3          |             |
| D3                 | 0.5        |             |
| D4                 | 0.26       |             |
| D5                 | 0.25       |             |
| L1                 | 1.6        | Changeable  |
| L2                 | 0          |             |
| L3                 | 0          |             |

D1: Outer diameter of protective cap
D2: Outer diameter of plastic base of catheter
D3: Outer diameter of catheter
D4: Outer diameter of injection tube
D5: Diameter of the steel rod of protective cap
L1: Length of catheter under the plastic base
L2: Length of injection tube protruding catheter
L3: Length of protective cap protruding catheter
5. Prepare pipettes using a Sutter P-1000 puller. The glass capillary is 100 mm long, and the diameter is 0.5 mm. The parameters are shown below: one-step pulling; heat: 600°C; pull: 30 N; Vel: 31 V; time: 120 ms; pressure: 500 pa. The capillary tip deforms when touching the skull, so it is more accurate to use the pipette to adjust the horizontal plane of the brain. An unbroken pipette after pulling will be used for skull level adjustment.

### Key Resources Table

| REAGENT or RESOURCE                        | SOURCE               | IDENTIFIER       |
|--------------------------------------------|----------------------|------------------|
| Chemicals, peptides, and recombinant proteins |                      |                  |
| Chloral hydrate                            | Sangon Biotech       | Cat# 320-17-0    |
| Isoflurane                                 | RWD                  | Cat# R510-22     |
| Saline                                     | RHAWN                | Cat# R052040     |
| Mineral oil                                | Sigma-Aldrich        | Cat# M8410       |
| Tissue adhesive(n-butylicyanoacrylate)     | 3M Vetbond           | Cat#1469SB       |
| Dental acrylic                             | Feiyi                | N/A              |
| Ophthalmic ointment                        | Jieqi                | N/A              |
| DAPI                                       | Sangon Biotech       | Cat# E607303     |
| Optimal cutting temperature (OCT) compound | Sakura               | Cat# 4583        |
| Phosphate buffer saline (PBS)              | Sangon Biotech       | Cat# B548117-0500|
| Paraformaldehyde (PFA)                     | Sigma-Aldrich        | Cat# 158127      |
| VECTASHIELD Antifade Mounting Medium       | Vector Labs          | Cat# H-1000      |
| VECTASHIELD Hardset Mounting Medium with DAPI | Vector Labs          | Cat# H-1500      |
| Peptide TAT-scramble:                      | Shen et al.,2021     | N/A              |
| YGRKRRQRRSVFYKDWLYGCPNVW                    |                     |                  |
| Peptide TAT-52-68:                         | Shen et al.,2021     | N/A              |
| YGRKRRQRRWKFDPSCGLPLLWYWNV                  |                     |                  |
| Experimental model: strains                | Jackson Laboratory   | Cat# JAX-000664, RRID:IMSR_JAX:000664 |
| - C57BL/6J                                 |                      |                  |
| Software and algorithms                    |                      |                  |
| EthoVision XT 7 video tracking software    | Noldus               | https://www.noldus.com/download/trial-download-ethovision-xt-us |
| GraphPad Prism                             | GraphPad Software    | https://www.graphpad.com/scientific-software/prism/          |
| Other                                      |                      |                  |
| Sutter P-1000 puller                       | Sutter               | Cat# P - 1000    |
| Cannula                                    | Kedoubc              | N/A              |
| Glass capillary                            | WPI                  | Cat# 1B120F-4    |
| Surgical scissors                           | JZ surgical instruments | Cat# JC2101     |
| Tweezers                                   | JZ surgical instruments | Cat# J3C030    |
| Razor blade                                | Feiyi                | Cat# 74-C        |
| Stereotaxic instrument                     | RWD surgical instruments | Cat# 68045   |
| Animal platform                            | RWD surgical instruments | Cat# 68030   |
| Syringe holder                             | RWD surgical instruments | Cat# 68207   |
| Animal surgical microscope                 | JNOEC                | Cat# JS25        |
| Drill                                      | RWD surgical instruments | Cat# 78001   |
| A drill bit                                | RWD surgical instruments | Cat# 78043   |
| Microsyringe                               | Harvard Apparatus surgical instruments | Cat# Pump 11 Elite |
| Microsyringe needle                        | Googe                | Cat # LPM-S-10   |
| Isoflurane vaporizer                       | Friends honesty      | Cat # F700       |
| Freezing microtome                         | Leica                | Cat # CM1950     |
| Fluorescence microscope                    | Leica                | Cat # DM50008    |
| Cover slide                                | Citotest             | Cat # 80340-3610 |
| Microscope slide                           | Citotest             | Cat # 80302-2104 |

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MATERIALS AND EQUIPMENT
All materials, equipment, and sources are listed in the key resources table.

Alternatives: For alternative reagents, materials, equipment, and sources, please consult local medical, pharmaceutical, and laboratory equipment suppliers.

STEP-BY-STEP METHOD DETAILS
Surgical preparation

△ Timing: 15 min

△ CRITICAL: All animal experiments must be performed under the guidelines approved by the Institutional Animal Care and Use Committee (IACUC). The procedure and drugs must be adapted according to the IACUC approved protocol.

1. Two-month-old male C57BL/6J mice are weighed. The weights of mice are around 20–23 g.

Note: This technique can be used with mice of different weights over two months of age.

2. Animal anesthesia. Mice are induced to deep anesthesia with 7% (v/v) chloral hydrate (320-17-0, Sangon Biotech) dissolved in normal saline at room temperature (R.T.) by intraperitoneal injection ($150\,\mu\text{L}$/20 g body weight).

△ CRITICAL: Mice will be anesthetized within 5 min and returned to their cage until deep anesthesia is induced. Check the anesthetic state by a hard pinch to the tail and a puff of air to the eye. If neither causes any responses, the mouse is considered to have achieved deep anesthesia.

Alternatives: Isoflurane is a good alternative for animal anesthesia.

3. Transfer the anesthetized mouse to a stereotaxic instrument (68045, RWD), fit the front teeth into the incisor bar’s hole, and secure the incisor bar. Adjust ear bars in front of ears to tightly hold the head (Figure 2).

△ CRITICAL: The height of the incisor bar should not be too low. Otherwise, it will affect the breath of the mouse. The ear bars should be the same height. Fix ear bars on both sides at the same length, which can be calibrated according to the scale on the ear bars. These are done basically to ensure left-right symmetry.

4. Apply ophthalmic ointment (Jieqi) to the eyes for physical protection and drying prevention.

5. Remove the scalp hair of the mouse with a razor blade (74-C, Feiying). Carefully shave the hair to expose an area of about 1.5 cm² on the top of the head.

6. Incision and skull preparation.
   a. A 1.5–2 cm cut is made sagittally in the center of the scalp. Remove the whole scalp skin using small sharp scissors. Remove the overlying membranes on the skull with sterile swabs.
   b. The edges of the scalp skin are glued to the skull with tissue adhesive (1469SB, 3M Vetbond) (Figure 3A).

Note: Ensure the edges of the scalp skin stick tightly to the skull.

Surgical procedure

△ Timing: 1 h
7. Skull level adjustment.
   a. A pipette is installed on a microsyringe needle, fixed on the microsyringe (Pump 11 Elite, Harvard apparatus). The pipette is used to level the skull along the midline axis at the suture intersection points of bregma and lambda.
   b. Lower the pipette onto bregma to zero the coordinates (Figure 3A).
   c. Adjust the height of the incisor bar to make sure the readings of Z-axis coordinates (2.3 mm anterior to bregma, 2.3 mm posterior to bregma) is less than 0.03 mm. Next, adjust the height of the incisor bar to make sure the readings of Z-axis coordinates (2.3 mm anterior to bregma, 2.3 mm posterior to bregma) is less than 0.03 mm.

   Note: When there is a big difference in the readings of the Z-axis between two sites, let the pipette go back to bregma and repeat zeroing the coordinates at bregma.

8. Coordinates determination. Coordinates for drilling are measured using bregma as a reference point. The coordinates are marked using a sterile drill bit and re-confirmed for accuracy before drilling starts. Coordinates for specific brain regions of interest (e.g., here hippocampus) are determined, referring to the mouse brain atlas (Paxinos and Franklin, 2004). The coordinates of the drill sites we used for hippocampal microdialysis are 2.15 mm lateral to the midline, 2.45 mm posterior to bregma (Figure 3B).

9. Drilling. Drill four holes through the skull. Be careful to apply a minimal force so that the trauma to the dura and underlying membranes is minimal or absent. Two holes are at the measured sites of cannula installation, and the other two are away from the injection sites and used for the insertion of skull screws. Clear up the holes with a cotton-tipped swab after drilling.
10. Cannula placement.

a. Insert skull screws of the specific size (e.g., 2.0 mm self-tapping screws; RWD) into the holes without impacting the brain beneath, which are used to anchor the dental cement (Figure 3C).

b. Coordinates are re-confirmed to be the sites of the hole drilled and lowered to the target depth. The coordinates we used are ± 2.15 mm lateral to the midline (X-axis). Four holes are drilled. Two are at the injection sites; the other two are away from the injection sites.

c. Insert two cranial nails at the holes away from the injection sites.

(d) Place the first cannula and secure the nail and cannula with dental cement.

(c) Loosen the protective cap and make sure the protective cap can be removed.

(f) Insert another cannula and secure it with dental cement.

(g) Loosen the protective cap.

(h) Recouple the protective cap.

The orientation: a, anterior; p, posterior; l, lateral; m, medial. Scale bar, 5 mm.

Note: Ensure the dental cement is completely set before proceeding to the next step. See Troubleshooting 2 and 3.
Post-surgical care

**Timing:** 15 min (immediate care) and 3 days (follow-up care)

11. Post-surgical care and recovery.
   a. Acute post-surgical care. Mice are placed in an empty cage with a heating pad until full recovery from the anesthesia. Then, return the mice to their cage and mark the date of surgery.
   b. Follow-up care in the recovery days following surgery. It takes about 7–10 days for recovery. Animals are examined at least once a day for the first three days. The overall health status of mice is checked according to institutional animal care guidelines.

    ⚪️ **Pause point:** peptide injection and behavior testing can be performed 7–10 days after cannula insertion.

Peptide injection

**Timing:** 20 min

12. Animal anesthesia.
   a. Sufficient amount of isoflurane (R510-22, RWD) is added into the isoflurane vaporizer (F700, Friends honesty) (Figure 4A). The switch of the isoflurane vaporizer is turned on to the anesthesia chamber.
   b. Mice are anesthetized with airflow at 1 L/min and isoflurane at 4% in the anesthesia chamber.
c. The anesthetized mouse is transferred to a stereotaxic instrument. Fit the front teeth into the incisor bar, and insert the nose into an anesthetic mask. Adjust ear bars to hold the head tightly. Switch the isoflurane vaporizer to the mask, with airflow at 1 L/min and isoflurane at 1.5% (Figures 4B and 4C).

**Note:** The most suitable anesthetic mix of isoflurane is at 1.5%. It must be monitored discreetly to avoid a potentially lethal isoflurane overdose.

13. Miniature syringe connection.
   a. The microinjection needle filled with mineral oil (M8410, Sigma-Aldrich) is installed on the microsyringe (Pump 11 Elite, Harvard apparatus) and connected to the hose, filled with mineral oil in advance.
   b. Adjust the microsyringe to the withdrawal mode and suck up 5 mL liquid (e.g., here peptides) into the hose.
   c. Connect the hose to the drug injection tube. Adjust the microsyringe to the injection mode and push the syringe forward until liquid comes out from the front of the injection tube.
   d. Connect the injection tube to the catheter.

14. Peptides injection. Inject 1.5 mL of the peptides solution into each side of the hippocampus at a rate of 0.3 mL/min. When the injection is complete, keep the mouse in position for 2 min to ensure a complete infusion. Recouple the protective caps. See Troubleshooting 4 and 5.

15. Recovery. Return the injected mice to the cage, and the mice will recover from anesthesia in 5 min.

**Behavioral testing**

© Timing: 3 days

Animals are respectively infused with saline, scramble peptide, and peptide TAT-52-68aa for three consecutive days. One hour after peptide delivery, the injected mice will perform the spatial recognition tasks.

16. Habituation. Clean the field arena with 70% (v/v) alcohol before each experiment and wait until it is completely dry. An hour after the peptide infusion, gently introduce the mouse into the arena for 10 min free movement and return to its original cage.

17. Training. Place two identical objects (i.e., A and B) at one side of the arena, re-introduce the testing mouse into the arena and allow it to explore freely in the arena for 10 min. Record its movements using the EthoVision XT7 video tracking software (Noldus). Finally, return the mouse to its cage. Clean the arena with 70% (v/v) alcohol between each mouse.

18. Re-exposure. Keep object A in the original position, and move object B to the opposite side. Introduce the testing mouse into the area and allow it to explore freely in the arena for 10 min. Record its movements by the camera. Finally, return the mouse to its cage. Clean the arena with 70% (v/v) alcohol between each mouse.

19. Result analysis. Analyze the record movements and calculate the exploration time for objects. Sniffing on the objects (with nose contact or head directed toward the object) within a 2-cm radius of the objects is regarded as an exploration of the objects. Sitting or standing on the objects is not considered an exploration.

20. Data presentation. Data are expressed as a percentage of moved-object exploration time relative to the total exploration time (familiar and moved-object exploration during ‘test’).

***Pause point:*** injection sites and spread region analysis can be performed 1–15 days after behavioral testing.

**Injection sites and spread region analysis**

© Timing: 2 days
21. Injection sites analysis.
   a. Perfuse the mouse with 1 × phosphate buffer saline (PBS) (B548117-0500, Sangon Biotech), followed by 4% paraformaldehyde (PFA) (158127, Sigma Aldrich) in PBS (Figure 5A).
   b. Keep the brain in 4% PFA overnight at 4°C. On the second day, wash the brain with 1× PBS 3 times, 5 min each time.
   c. Embed the brain in optimal cutting temperature (OCT) compound (4583, SAKURA).
   d. Produce 14-μm-thick frozen brain sections on a freezing microtome (CM1950, Leica), then the brain tissue sections are placed on microscope slides (80302-2104, CITOTEST).
   e. Dry the brain tissue slide at R.T., then wash the slide with 1× PBS 3 times, 5 min each time.
   f. Administer small drops of VECTASHIELD HardSet Mounting Medium with DAPI (H-1500, Vector Labs), then place cover slide (80340-3610, CITOTEST) on top of the microscope slide.
   g. Analyze the brain slide by using a fluorescence microscope (DM5000B, Leica). The staining of DAPI shows that the trauma caused by the cannula is located above the hippocampus (Figure 5B).

22. The spread region analysis.
   a. Produce the mixed solution by adding DAPI (dilution, 1:200) into the peptide solution.
   b. Inject 1.5 μL of the mixed solution at 0.3 μL/min into each side of the hippocampus.
   c. Keep the infusion cannula in position for another 5 min after the delivery of the mixed solution.
   d. Remove the step-design cannula from the skull and kill the mouse immediately.
   e. The brain is dissected and embedded in OCT compound.
   f. Produce 14-μm-thick frozen brain sections on a freezing microtome, then the brain tissue sections are placed on microscope slides.
   g. Dry the brain tissue slide at R.T., then wash the slide with 1× PBS 3 times, 5 min each time.
   h. Administer small drops of VECTASHIELD Antifade Mounting Medium (H-1000, Vector Labs), then place the cover slide on top of the microscope slide.
i. Analyze the brain slide by using a fluorescence microscope. The positive signal of DAPI is mainly in the dorsal hippocampus (Figure 5C). The results indicate that the peptide mainly spread in the dorsal hippocampus.

**EXPECTED OUTCOMES**

By following the protocol described here, we have respectively delivered saline, scramble peptide, and peptide TAT-52–68aa into the hippocampus (Figure 6A). On the first day of behavior testing, the open field test was performed to analyze the movements and anxiety levels of peptide-treated mice (Figure 6B). The results show that peptide TAT-52–68aa treated mice show normal moving distances in the open field test compared with saline and scramble peptide treated mice (Figure 6C). Moreover, the peptide TAT-52–68aa treated mice spent comparable time in the center and showed the same number of times into the center as saline and scramble peptide treated mice (Figure 6D). The results indicate that peptide TAT-52–68aa treated mice show normal anxiety levels. Next, the spatial recognition task was performed (Figure 6B). One hour after the peptide delivery, perform the behavioral testing to analyze the peptide’s effect on mGluR-LTD related memory. We characterized the peptide TAT-52–68aa disrupts the interaction of PQBP1 and eEF2, and results in a poor performance in the spatial recognition task (Figure 6E).
QUANTIFICATION AND STATISTICAL ANALYSIS
Statistical analysis was performed using Prism 7.0 (GraphPad Software). All the quantitative data are presented as the mean ± SD. A two-tailed unpaired Student’s t test was used to compare the different conditions. Statistical significance was set as: * p < 0.05; ** p < 0.01; *** p < 0.001; ns, no significance, p > 0.05.

LIMITATIONS
This protocol is not suitable for cannula insertion in small nuclei, deep nuclei, and embryonic mice. The catheter’s outer diameter is 0.5 mm, which is large enough to destroy many small nuclei. Moreover, it takes a large space to place two cannulas at the same time. The distance between the two injection sites should be greater than the diameter of the cannula. Therefore, not all brain regions are suitable for cannula insertion.

Injection volume, especially for new-found nuclei, is uncertain. Considerable effort is required to determine the optimal injection volume.

Two- to three-month-old mice are most suitable for cannula insertion surgery, as their skulls are fully developed but not very thick. The skulls are not fully developed and calcified in younger mice. However, the trauma caused by the operation to the elder mice is greater, and the recovery time for them is longer.

TROUBLESHOOTING
Problem 1
Unable to insert two cannulas at the injection sites (preparation of cannulas, step 1 in "before you begin").

Potential solution
The distance between the two injection sites should not be too small. Otherwise, the cannula cannot be installed. It must be greater than the diameter of the cannula. Identify the coordinates of the injection sites according to the mouse brain atlas. Alternatively, a small diameter cannula can be customized by a different manufacturer.

Problem 2
The protective cap cannot be uncapped (surgical procedure, step 10).

Potential solution
The protective cap may be fixed to the catheter by dental cement. Be careful not to apply it to the protective cap when using dental cement.

Problem 3
Detachment of cannula from mouse head (surgical procedure, step 10).

Potential solution
The cannula may be detached from the mouse head under the effect of external force or mice interaction. Two or more cranial nails should be installed and taken as an anchor for the dental cement. The prepared dental cement should not be too sticky and should be set until it is fully solidified.

Problem 4
The mouse was in poor condition after drug injection (peptide injection, step 14).
Potential solution
Slower the injection speed or reduce the injection volume. Besides, the duration of drug delivery should be reduced appropriately. Especially, the injection volume should not be too large, and the optional injection volume should be determined in advance.

Problem 5
Inappropriate injection volume (peptide injection, step 14).

Potential solution
Optimize the injection volume according to the published researches and assess the spread region using dye additive, such as DAPI.

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and fulfilled by the lead contact, Junhai Han (junhaihan@seu.edu.cn).

Materials availability
This study did not generate new reagents.

Data and code availability
This study did not generate any data, sophisticated custom computer code, or algorithm.

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AUTHOR CONTRIBUTIONS
S.C. and Y.S. exerted the protocol. S.C. wrote the detailed procedure, and Z.C.Z. and J.H. conceived this project and contributed to the protocol’s writing.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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