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Protocol for induction of heterosynaptic long-term potentiation in the mouse hippocampus via dual-opsin stimulation technique

Cholecystokinin (CCK) is the most abundant neuropeptide that broadly regulates the physiological status of animals. Here, we present a two-color laser theta burst stimulation (L-TBS) protocol for simultaneous activation of Schaffer collateral and perforant pathway in the hippocampus of CCK Cre mice. We describe steps for heterosynaptic long-term potentiation induction by L-TBS. This technique allows for the examination of the neurotransmitter roles in synaptic modulation and facilitates the exploration of pathological mechanisms in genetic models of brain disorders in mice.

Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
Protocol for induction of heterosynaptic long-term potentiation in the mouse hippocampus via dual-opsin stimulation technique

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SUMMARY
Cholecystokinin (CCK) is the most abundant neuropeptide that broadly regulates the physiological status of animals. Here, we present a two-color laser theta burst stimulation (L-TBS) protocol for simultaneous activation of Schaffer collateral and perforant pathway in the hippocampus of CCK Cre mice. We describe steps for heterosynaptic long-term potentiation induction by L-TBS. This technique allows for the examination of the neurotransmitter roles in synaptic modulation and facilitates the exploration of pathological mechanisms in genetic models of brain disorders in mice.
For complete details on the use and execution of this protocol, please refer to Su et al.1

BEFORE YOU BEGIN
This protocol describes a novel technique that can be performed in two kinds of neuronal circuits expressing red and blue light sensitive opsins. This technique is optimized for heterosynaptic long-term potentiation (hetero-LTP) induction in dorsal hippocampal (DHP) slices of CCK Cre mice. Here, we observe that simultaneous and specific light theta burst stimulation (L-TBS) of ChrimsonR-expressing Schaffer collateral (CA3-CA1) and Chronos-expressing CCKergic perforant pathway (medial entorhinal cortex-CA1) successfully elicited robust and persistent hetero-LTP.

Note: The medial entorhinal cortex (MEC) provides most of the CCK-positive neurons projecting to the DHP region (Figure 1). Thus, we investigate the effect of CCKergic perforant pathway projection on the CA3-CA1 pathway in the DHP of CCK Cre mice.

Training for AAV injection and electrophysiology recording (optogenetic stimulation)
To use mouse brain slices for electrophysiology recording, the experimenter must be well trained in animal experiments and, if necessary, anatomy experiments. Additionally, the experimenter should have a deep understanding of how to set up the electrophysiology recording system and conduct experiments using the system. Thus, it is important to have a comprehensive training from technician for standalone operation.
Acquisition of the stereotaxic coordinates of target brain regions

**Timing:** 2 days

We show the steps needed to acquire the coordinate of two study areas CA3 and MEC in adult mice (2–3 months).

1. Subject adult male mice to anesthesia with 1.4% isoflurane during the surgery.
2. Head-fix the anesthetized mouse on a stereotaxic device (RWD Life Science, Shenzhen, China),
   a. Perform a skin incision above the mice skull.
   b. Balance the position of bregma and lambda by manual calibration.
3. Prepare AAV8-hSyn-mCherry in a tough quartz glass micropipette with a fine tip mounted on a nanoliter 2000/Micro4 system (World Precision Instruments [WPI], Sarasota County, FL, USA).
4. For the positional parameters of unilateral CA3 and MEC in adult mice brain, refer to Allen Brain Atlas of the adult mouse (https://atlas.brain-map.org).
   a. Make craniotomies (diameters 0.5 mm) over the target location of the mice brain.
   b. Inject AAV8-hSyn-mCherry in MEC (AP: - 4.90 mm, ML: +/- 3.10 mm, DV: -3.0/-3.5/-4.0 mm with an 8-degree angle tilt).
   c. For the CA3, injection parameters are AP: -1.75 mm, ML: - 2.35 mm, DV: -1.85 mm.

   **CRITICAL:** It is important to ensure that the injection locations are precise and limited to the target regions.

5. Microinject 30 nL of AAV8-hSyn-mCherry at a rate of 15 nL/min in CA3, and in the three DV coordinates of MEC. Allow 4 weeks for AAV expression.
6. After AAV expression, transcardially perfuse mice with 0.1 M PBS, followed by fixation with 4% PFA.
7. Carefully detach and submerge mouse brain into prepared PFA solution for subsequent fixation.

   **Pause point:** Keep the brain at 4°C for ~12 h.

8. Preparation of brain sections.
Section brains into 60 μm-thick slices by using a vibratome (Leica VT1000 S).

Brain slice staining.

i. Stain slices with DAPI (1:5000, Santa Cruz Biotechnology) for 5 min.

ii. Mount onto glass slides containing 70% glycerol in PBS (Santa Cruz Biotechnology).

iii. Observe the AAV expression and verify the injection site.

Use the coverslips to cover the brain slices and seal with adhesive glue.

9. Scan fluorescent images of brain slices using a Nikon Eclipse fluorescence microscope (Figure 2).

Verification of AAV expression and neuronal response to laser stimulation

Timing: 4 weeks

10. Verify the AAV expression after injection into mouse brain prior to optogenetic experiment (4 weeks for AAV expression).

11. Ensure the AAV expression is efficient for in vivo electrophysiology recordings (Figure 3).

12. Dilute the AAV (AAV9-hSyn-ChrimsonR-tdTomato, AAV9-Flex-Chronos-GFP) to an appropriate titer (~ 5.0 × 10^{12} gc/ml in our case).

Note: Non-specific expression in the target region might be caused by high-titer AAV.

Assembling of electrophysiology recording system and laser generator

13. Combine the microelectrode array (MEA) system (MED64-Basic; Alpha Med Scientific, Japan) with two-color laser generator (473 nm and 635 nm; Inper, China) for in vitro extracellular electrophysiology.

14. Use laser monitor (Sper Scientific 840011, USA) to measure the power of red and blue laser.

15. Fix 200 μm optical fiber for laser stimulation by an adjustable holder on the operation platform.

△ CRITICAL: It is important to maintain the distance between the optical fiber and brain slice during the whole process of recording.

16. Use opsin-expressing slice to test the recording system to see if normal light-evoked field post-synaptic potentials (L-fEPSPs) can be elicited by laser stimulation (Figure 4).
17. During the pre-test phase, use ~3.5 mw 635 nm red-light and ~3.0 mw 437 nm blue light to activate the ChrimsonR and Chronos, respectively.

△ CRITICAL: Intensity of surrounding light needs to be maintained at the same level, since the low power of light from the bulb or outside also affects the response of brain slice.

Institutional permissions
All male mice were housed in a 12-h light/dark cycle and were provided food and water ad libitum. All experimental procedures were approved by the Animal Subjects Ethics Sub-Committee of the City University of Hong Kong.
## KEY RESOURCES TABLE

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<th>REAGENT or RESOURCE</th>
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## MATERIALS AND EQUIPMENT

**Buffers/solutions/media**

### aCSF solution

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<tr>
<td>Glucose</td>
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5 L (pH ~7.2)
Note: ACSF can be stored at 4°C for up to one week.

### 10× PBS solution

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<td>KH₂PO₄</td>
<td>2.4</td>
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</table>

1 L (pH ~7.2)

Note: 10× PBS can be stored at 4°C during the experiment period.

### 4% PFA solution

For 4% paraformaldehyde solution, add 4 g paraformaldehyde to 50 mL of ddH₂O. Add 1 mL NaOH (1 M) and stir slowly on digital hotplate magnetic stirrer at 50°C until the paraformaldehyde is completely dissolved. Then, add 10 mL 10× PBS and allow the mixed solution to cool to 25°C.

Pause point: Store the prepared PFA in 4°C refrigerator.

### STEP-BY-STEP METHOD DETAILS

#### Preoperative preparation of mouse

**Timing:** 1.5 h (per mouse)

In this section, we need to prepare all the experimental materials and surgical tools before conducting the experiment.

1. Inject CCK-Cre mice with dexamethasone (2 mg/kg, i.p., Sigma-Aldrich, Darmstadt, Germany) and carprofen (5 mg/kg, i.p., Sigma-Aldrich, Darmstadt, Germany) at least 1.5 h before anesthesia to prevent brain swelling and inflammation.

   CRITICAL: The age and weight of all mice should be similar for the AAV injection. Also, the sex of the mice. This step can promote the accuracy of AAV injection.

2. Anesthetized mice with pentobarbital sodium and head-fix on a stereotaxic device.

3. Dilute the titer of AAV virus to ~5.0 × 10¹² gc/mL, to avoid the non-specific expression in target regions.

4. Prepare AAV virus in a tough quartz glass micropipette with a fine tip mounted on a nanoliter 2000/Micro4 injector (details can be seen in above preparation work).

5. Balance the bregma and lambda.
   a. Place the mouse into the stereotaxic device by fixing steel ear bars and keep the mouse’s upper incisors into the mouth holder (Figure 5A).

   Note: Bregma is the intersection of the coronal and sagittal sutures (Figure 5B), and lambda is the intersection of the lambdoid and sagittal sutures (Figure 5C).

   b. Observe the location of bregma and lambda.
      i. Use the microscope to observe the location of the glass micropipette, until it just touches bregma on the mouse’s skull and note the DV value.
      ii. Move the glass micropipette and lower it to lambda and note the DV value.
Note: The difference of DV value between bregma and lambda must be less than 0.05 mm. If not, adjust the height of the mouth holder until the skull is levelled.

c. Return the glass micropipette to bregma, move it 2 mm to the left of bregma and lower it to the skull surface and note the DV value.
d. Move the glass micropipette 2 mm to the right of bregma and lower it to the skull surface and note the DV value.

Note: The DV value between the left and right should be the same. If not, adjust the height of the ear bars and repeat until bilateral DV value is equal.

**CRITICAL:** Since different mice have different profile of bregma and lambda, we also used the superior sagittal sinus and confluence of sinuses as reference site for calibrating the leveling (Figure 5D). This step enables us to precisely inject the AAV virus into the target brain regions.

### AAV preparation and injection

- **Timing:** 1 h (per mouse)

In this section, we describe the details on AAV injection of our protocol, which are given below:

6. Set the coordinate value of indicator (AP, ML, DV) to zero.
7. Place glass micropipette with AAV virus on the surface of mouse brain, reset the DV value to zero.
8. Lower micropipette to the area Cornu ammonis 3 (CA3), and fix coordinate values at AP: -1.70 mm, ML: -2.35 mm, DV: 0.00 mm.
9. Inject the AAV into brain.
   a. Set AAV (AAV9-hSyn-ChrimsonR-tdTomato) volume to 250 nL for area CA3. The glass micro-
      pipette is held in the target region for ~ 5 min before injection.
   b. Start the injection procedure with a speed of 50 nL/min.

   △ CRITICAL: Observe whether there is a bubble in the glass micropipette during the AAV
   preparation. The bubble needs to be injected out from the glass micropipette, or this
   can result in an incorrect volume of AAV injection.

10. Slowly withdraw the injector from the brain and reset coordinates to zero upon the reference site.
11. Adjust the angle of injector to 8°, then place the injector on the MEC region (AP: -4.90 mm, ML:
   3.10, DV: -4.0 mm/-3.5 mm/-3.0 mm).
12. Set the AAV (AAV9- Flex-Chronos-GFP) volume for area MEC to 300 nL for each site. After a
    while (5 min), begin the injection procedure with a speed of 50 nL/min.

    Note: We can draw a black line at the interphase between the AAV and silicon oil in the glass
    micropipette. This step helps to verify that the AAV is successfully delivered into mouse brain.

Postoperative care of the mice

   ⊗ Timing: 1 h (per mouse)

In this section, we emphasize the importance of the postoperative care of animals, this step pro-
   motes awareness of recovery of the mice from the anesthesia, and also prevents or reduces the
death rate of experimental mice.

   13. Seal the wounds with sterile sutures after completing the injection procedure, and apply eryth-
       romycin ointments to prevent infection during the recovery period.
   14. Monitor all the animals in a heating device before fully regaining consciousness.
   15. Return mice to the Laboratory Animal Research Unit for regular holding.

Slice preparation

   ⊗ Timing: 2 h for brain slicing

In this section, we give an outline on the preparation of dual-opsin expressing hippocampal slices,
this step allows us to obtain the target slice as soon as possible and to distinguish the available brain
slice for further processing.

   16. Anesthetize mice with gaseous isoflurane (Wellona Pharma, Surat, India) and decapitate in the
       ventilation hood.
   17. Rapidly extract the brain and immerse in a cold bath of oxygenated (95% O2-5% CO2) ACSF.
   18. Keep the mouse brain cool for 30 s, remove appropriate portions of the brain and fix the
       remaining part onto the base in a vibrating slicer (Leica VT1000S; Leica Microsystems, Tokyo,
       Japan).
   19. Obtain coronal slices containing the dorsal hippocampus region (300 μm thick) and transfer
       them to the incubation chamber.

   △ CRITICAL: Additionally, the ice-cold ACSF should be prepared well before the brain
       slicing, which can better maintain the neuronal activity of the slice during the process
       of brain slicing.

   20. Allow brain slices to recover for 2 h before optogenetic and electrophysiological recording.
21. Lastly use the fluorescent microscope to check the viral expression in the target regions before the next step.

**Note:** Because the gaseous isoflurane is harmful to the health, the mouse anesthesia should be conducted in the ventilation hood.

**Slice recording**

** Timing: 2–3 h**

In this section, we provide details on light stimulation of brain slice and L-fEPSP recording.

22. Place a freshly prepared hippocampal slice on the MED probe and cover the CA1 region with the recording electrodes.

23. Fix the brain slice and gently place a fine-mesh anchor (Warner Instruments, Harvard) to ensure slice stabilization during the L-fEPSP recording.

24. After 30 min of recovery, apply 2 ms pulse of red light (3.5 mW) via an optical fiber (200 μm diameter) positioned upon the CA1 region by visual observation through optical microscope.

25. Identify the typical L-fEPSP, and determine the input/output curve by measuring the slopes of L-fEPSP at different light intensities (Figure 6A).

**CRITICAL:** Optical fiber for light stimulation should be placed upon the region containing the SC and PP pathway.

26. Apply the two-color laser theta-burst stimulation (L-TBS) to the stimulation optical fiber at the intensity that induces 75% of maximum response of L-fEPSP (Figure 6B).

27. For the two-channel-rhodopsin experiment, 635 nm red light activates ChrimsonR-expressing SC projections, while 473 nm light-activates Chronos in MECDHP pathway.

28. Examine the cross-excitation of ChrimsonR and Chronos expressing axons by red vs. blue light. The power of 473 nm wavelength for activation of Chronos exceeds 3.50 mW, which has a significant effect on the ChrimsonR.

29. Control the maximum power of the 473 nm laser under 3.5 mW during the laser stimulation protocols. The maximum power (16.25 mW) of 635 nm wavelength for activation of ChrimsonR showed no effect on Chronos in our electrophysiology system.

30. After establishing a stable optically evoked fEPSP baseline for 30–40 min, apply stimulation protocols to the target region. Then, continuously acquire fEPSP (60 min) in the recording system.

31. Extract and analyze the fEPSP data by the MED Mobius software. Normalize and express the slope of fEPSP as a percent change from the baseline level, and use the mean value of the last 10 min of recordings for statistical analysis (Figures 6C–6E).

**Note:** For the L-fEPSPs recording, the bad quality (poor response for the light stimulation or unusual AAV expression in the slice) and excitatory slices (small amplitude with population spikes) during the input-output test should be excluded. Moreover, the baseline of L-fEPSPs should be stable for at least 30 min, since the recovery responses might contribute to the increase of L-fEPSPs thereby complicating the conclusion (Figure 6F).

**EXPECTED OUTCOMES**

The protocols described here provide a reliable method for producing the heterosynaptic LTP in the mice hippocampus. To mimic the classic electrical theta burst stimulation (TBS) protocol, we developed the light-TBS, one burst with five pulses set at 50 Hz with 5 Hz interburst interval (Figure 6B), which is used for simultaneously activating the ChrimsonR-expressing SC inputs and Chronos-expressing CCK-ergic PP inputs in the DHP of CCK Cre mice. The L-fEPSPs of SC projections show significant increase when two-wavelengths of L-TBS are applied to SC and CCK+/PP inputs (Figure 7). Additionally,
we also applied the two-color-LTB for stimulating the SC inputs without CCK+ PP inputs (Data is presented in the paper1). However, insignificant change of L-fEPSPs was observed. This indicates that specific co-activation of SC inputs and CCK+ PP inputs triggers the hetero-LTP in the DHP.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

1. For the analysis of L-fEPSPs, calculate the slope for comparing the L-fEPSPs change before and after the L-TBS.
2. Normalize and express the slope of fEPSP as a percent change from the baseline level, and use the mean value of the last 10 min of recordings for statistical analysis.
3. Use two-way mixed ANOVA for the statistical analysis.

**LIMITATIONS**

We noticed that optically evoked fEPSP and population spike (PS) show an attenuation trend throughout the induction train (L-TBS), which may prevent reliable action potential generation and cause insignificant depolarization. Previous reports have shown that light-evoked synaptic responses often exhibit artificial synaptic depression in the hippocampus and amygdala.3,4 However, the mechanism underlying this phenomenon remains unclear, and further investigation is required. Additionally, laser stimulation of ChrimsonR-expressing SC inputs without GABA receptors antagonist treatment might affect the LTP induction if the GABA neurons are significantly suppressed or activated by L-TBS.

**Figure 6. Steps for calculation of L-fEPSP before and after the L-TBS**

(A) Representative result of input-output curve presenting L-fEPSP slope in response to increasing light stimulus.
(B) The protocol of two-color light TBS for activating the SC pathway (ChrimsonR) and PP projection (Chronos).
(C) L-fEPSP was quantified by measuring the slope.
(D) Example of calculation of the L-fEPSP change before and after L-TBS of the SC pathway.
(E) Representative result of statistical analysis.
(F) Figure shows the recovery of L-fEPSP before and after L-TBS of the SC pathway.
TROUBLESHOOTING

Problem 1
AAV expression in non-specific regions (step 5).

Potential solution
The expression of AAV in non-specific regions can occur partly because the leveling of bregma and lambda are done by manual calibration. Additionally, different mice have different profiles of the skull. These factors contribute to AAV expression outside the target region, such as area CA2, DG or CA1, which can complicate experimental analyses and interpretations. To ensure reliable and accurate outcomes for this experiment, consider implementing the following potential strategies. Firstly, we observe that the superior sagittal sinus or confluence of sinuses can serve as a good reference site and is more stable during brain development. Additionally, it is essential to use mice of the same sex, and similar age and weight. This can minimize variations during the AAV injection, which will help reduce non-specific region expression and enhance the reproducibility of results.

Problem 2
Recording electrodes are contaminated by the brain tissues after many trials, which can affect the stability of the L-EPSPs recording (step 22).

Potential solution
The decaying tissues tightly cover the electrodes and adhere to it. According to our experience, it is a good practice to immerse the electrode in the trypsin solution at 40°C for 1 h, and wash it with double distilled water, which can remove tissue debris effectively.

Problem 3
Multiple L-fEPSPs or population spikes are produced by light stimulation (step 24).

Potential solution
The amplitude of L-fEPSPs evoked by light stimulus can be affected by the distance between the optical fiber and brain slice, the stimulation site in the SC fibers, as well as the light intensity. Therefore, we should adjust these variations to elicit a typical L-fEPSPs (Figure 3) for the LTP induction.

Problem 4
Poor neuronal activity of brain slices (step 25).

Potential solution
Some of slices show excitatory or poor response when laser is given. Thus, these brain slices need to be excluded. A better way to test the quality of brain slice is by conducting the IO curve; the slope of
L-fEPSPs should have a good positive correlation with laser intensity which can suggest that the slices are suitable for electrophysiology recording.

**Problem 5**
Although most ChrimsonR-expressing SC projections can follow the L-TBS (50 Hz), in some cases these projections exhibit severe attenuation, which prevents reliable depolarization (step 26).

**Potential solution**
We observed that the response of L-fEPSPs evoked by 40 Hz L-TBS is more stable compared to that of 50 Hz. Based on previous studies, LTP can be induced when the electrical high frequency stimulation is higher than 20 Hz.

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Prof. He Jufang (jufanghe@cityu.edu.hk).

**Technical contact**
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**Materials availability**
This study did not produce new unique reagents.

**Data and code availability**
This study did not generate or analyze new datasets or code.

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**AUTHOR CONTRIBUTIONS**

**DECLARATION OF INTERESTS**
The authors declare no competing interests.

**REFERENCES**


