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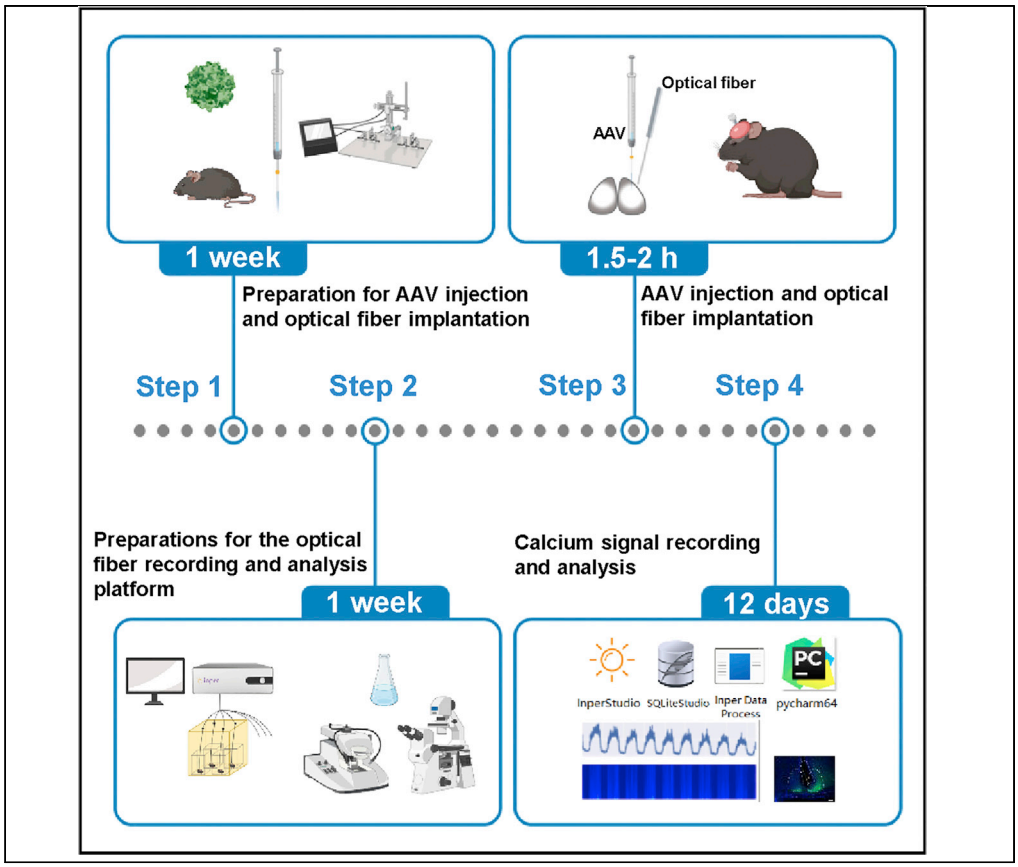
Long-term SCN calcium signal recording in freely moving mice



The suprachiasmatic nucleus (SCN) is the master circadian pacemaker of the mammalian biological clock. Here, we provide a detailed protocol for long-term recording of calcium signals in SCN neurons of freely moving mice through a multichannel optical fiber recording system. This system can simultaneously collect calcium signals from up to seven animals. The calcium signals can be visualized by the appropriate software and code. This protocol can be used to explore the long-term response of SCN to external environmental stimulation.

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

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Highlights

Simultaneous recording of calcium signals from multiple freely moving mice

Screening of mice with rhythmic SCN calcium signal

Long-term rhythmic calcium signal processing and visual analysis

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Long-term SCN calcium signal recording in freely moving mice

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SUMMARY

The suprachiasmatic nucleus (SCN) is the master circadian pacemaker of the mammalian biological clock. Here, we provide a detailed protocol for long-term recording of calcium signals in SCN neurons of freely moving mice through a multichannel optical fiber recording system. This system can simultaneously collect calcium signals from up to seven animals. The calcium signals can be visualized by the appropriate software and code. This protocol can be used to explore the long-term response of SCN to external environmental stimulation.

For complete details on the use and execution of this protocol, please refer to Zhai et al. (2022).

BEFORE YOU BEGIN

At the electrophysiological level, mouse SCN neurons are active during the day and rest at night, exhibiting robust rhythmicity (Houben et al., 2009). Recording the neuron calcium activity through optical fibers has become an effective research method to study whether neuronal activity regulates the occurrence of the behavior in recent years (Miyamoto and Murayama, 2016). At present, some progress has been made in the research of SCN function through optical fiber recording systems. The researchers successfully recorded long-term rhythmic calcium activity in VIP neurons of freely moving mice (Jones et al., 2018; Mei et al., 2018) and the response of VIP neurons to light stimulation (Jones et al., 2018; Todd et al., 2020) using the sixth-generation GCaMP. This protocol uses a 200 μm diameter, 0.5 NA optical fiber, the calcium indicator GCaMP7s (Dana et al., 2019), and a higher sampling resolution (record 35 s every 10 min) to obtain more abundant SCN calcium activity information than previous studies mentioned above.

Institutional permissions (if applicable)

The animal procedures were approved by the Animal Care and Use Committee of Soochow University.

Preparation for AAV injection and optical fiber implantation

Timing: 1 week

1. Preparation of AAV.
   a. AAV-VGAT-Cre and AAV-flox-GCaMP7s (titer $\geq 1 \times 10^{13}$ vg/mL) can be bought from Shumi Technology Co., Ltd (Brain VTA) and shipped on dry ice.

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b. Thaw the virus stock solution on ice and store it at −80°C after aliquoting.

2. Preparation of experimental animals.
   a. The SPF C57BL/6J mice aged 6–8 weeks can be bought from GemPharmatech Co., Ltd.
   b. In our case, the mice used in the experiment were all raised in the SPF facility of the Soochow University. The temperature of the animal housing environment was 22 ± 2°C, the humidity was 60%–70%, and the light/dark cycle consisted of 12 h of light and 12 h of darkness with free access to water and food.

Note: All mice used in the experiment should be at least two months old and their skull size is staying steady at this stage.

3. Preparation of glass micropipette.
   a. Glass micropipette with an outer diameter of 1.2 mm and a length of 10 cm can be bought from Nanjing Liuhe Springshui Teaching Experiment Equipment Co., Ltd.
   b. Use the P-1000 micropipette horizontal puller to pull the glass micropipette for virus injection according to the following conditions.

Heat	Pull	Vel	Time	Pressure
560	90	95	30	440

Note: The pulling conditions should be decided according to the material of the glass micropipette.

4. Set up the brain stereotaxic system.
   a. The AAV injection and optical fiber implantation system consists of six main parts: stereo microscopes, gas anesthesia system, digital stereotaxic instrument, microinjection pump, homeothermic system, and skull drill (Figure 1A).
   b. Add the proper amount of isoflurane to the gas anesthesia machine before the experiment.
   c. Prepare the glass micropipette holder (Figure 1B), syringe pump holder (Figure 1C) and optical fiber holder (Figure 1D) in the stereotaxic apparatus for skull leveling, virus injection and optical fiber implantation respectively.
5. Preparation of AAV syringe.
   a. Trim the tip of the glass micropipette to a diameter of 15–20 μm.
   b. Turn on the hot melt glue gun to preheat.
   c. Fill the 10 μL syringe with liquid paraffin oil (Figure 2A), and insert the needle (700 μm diameter) of the syringe into the glass micropipette. Pinch the junction site between the syringe and the glass micropipette, and push the plunger until the glass micropipette is filled with the paraffin oil (Figure 2B).
   d. Wipe off the paraffin oil spilled at the junction site, apply the hot melt glue at the junction site, and leave it at room temperature for 5–10 min to completely turn into a solid (Figure 2C).
   e. Continue to push the plunger of the 10 μL syringe until the paraffin oil overflows from the glass micropipette tip, and check that there is no excess paraffin overflowing from the junction site between the glass micropipette and the syringe.
   f. Place the prepared AAV syringe at room temperature for use.

⚠ CRITICAL: Before applying the hot melt glue, make sure that there is no paraffin oil spilling out of the junction site, otherwise it may cause problems with the airtightness of the AAV syringe, and cause paraffin oil to flow out of the junction site during AAV injection, while the AAV itself cannot enter the brain tissue.

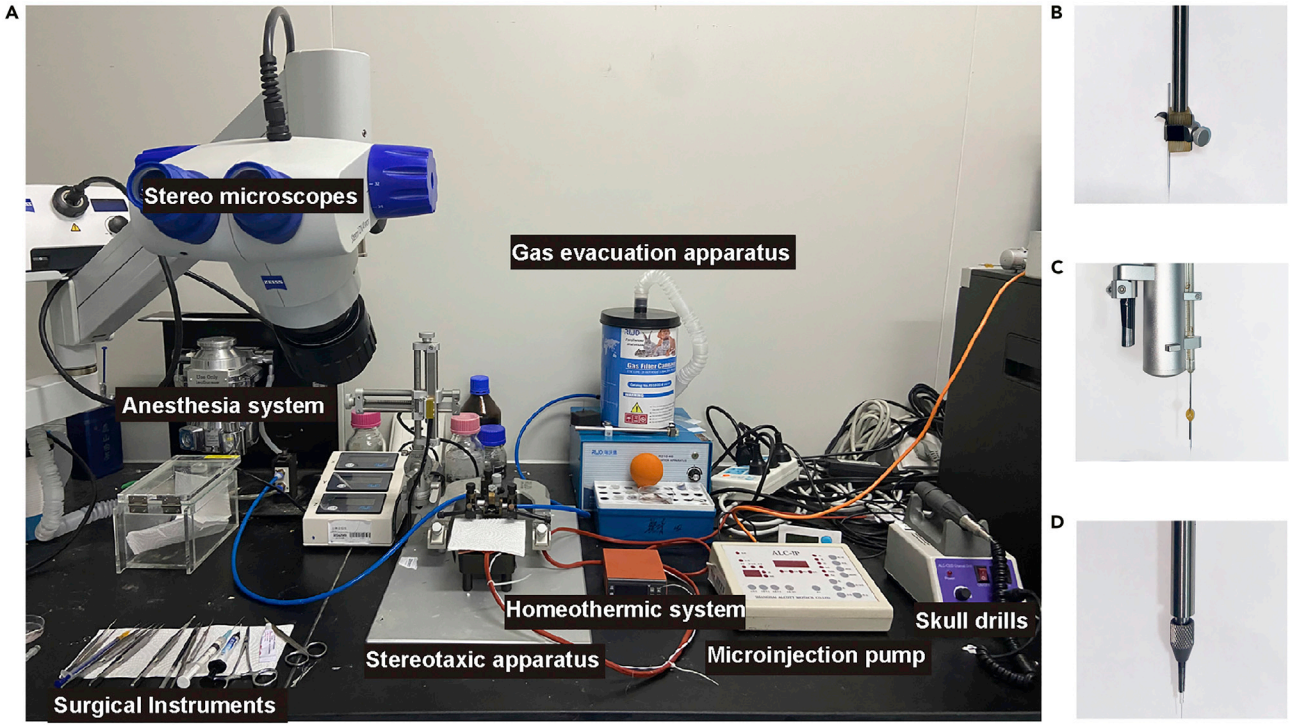

Figure 1. Brain stereotaxic system for AAV injection and optical fiber implantation

(A) Main components of a brain stereotaxic system, including stereo microscopes, gas anesthesia system, digital stereotaxic instrument, microinjection pump, homeothermic system, and skull drill.

(B) Micropipette holder with glass micropipette for skull leveling.

(C) Syringe pump holder fitted with a homemade syringe for AAV injection.

(D) Optical fiber holder fitted with a fiber tip for implantation.

6. Preparation of other surgical-related instruments and reagents, including:
   a. 75% alcohol.
   b. 3% H_2O_2.
   c. Fine scissors.
   d. Ophthalmic forceps.
   e. Glass micropipette (for leveling).
   f. Skull drill and drill bit (0.8 mm diameter).
   g. Rubber suction bulb.
   h. Optical fiber tips (200 μm diameter, 0.5 NA, 6 mm length).
   i. Dental cement.
   j. Vetbond tissue adhesive.

Preparations for the optical fiber recording and analysis platform

Timing: 1 week

7. Set up the optical fiber recording platform, which consists of a multichannel optical fiber recording instrument, a computer, and a recording box with cages (Figure 3).
   a. A multichannel optical fiber recording instrument can be bought from Inper Co., Ltd. The recording and analysis software supporting the instrument should be installed on the computer in advance of the experiment.

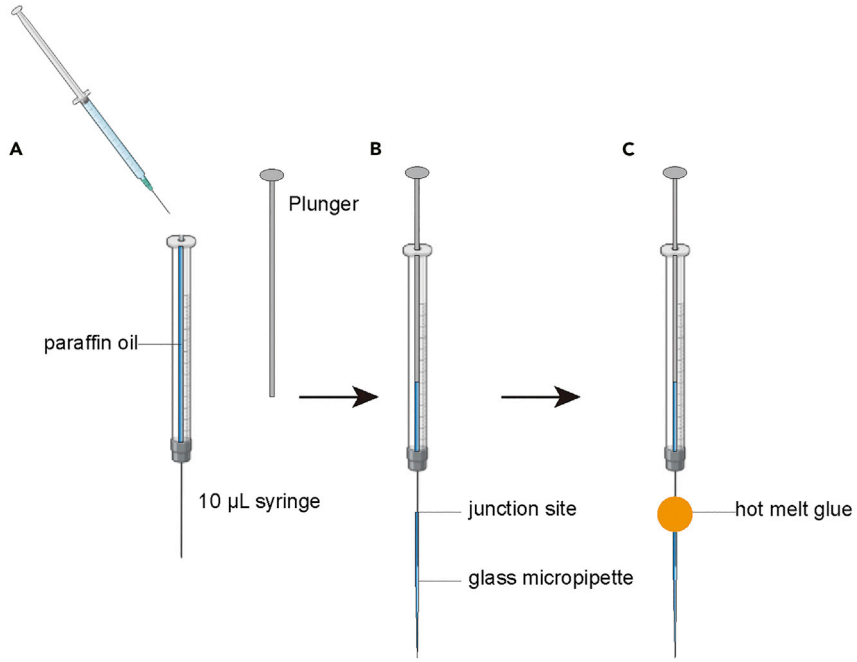

Figure 2. Schematic diagram of making an AAV syringe

(A) Remove the plunger and fill the 10 μL syringe with paraffin oil.

(B) Connect the glass micropipette with a syringe and push the plunger until the glass micropipette was filled with paraffin oil.

(C) Apply the hot melt glue to the junction site until it is completely solidified.

b. Prepare a 55 × 55 × 55 cm wooden box, drill four circular holes with a diameter of 1.5 cm on the top of the box, fix part of the fiber rotary joints on the foam board with hot melt glue, and partially suspend the joints in the holes, allowing them to rotate freely.

c. Connect the rotary joints and the recording instrument with optical fibers. Another lightweight monofiber patch cord with a length of 55 cm should also be connected to the rotary joint, and the optical fiber tip should be exposed with a mated sleeve for the later connection to the optical fiber interface of the mouse head.

d. Make holes on the cages and in the corresponding positions on the wooden box for the placement of drinking bottles.

8. Install the optical fiber recording and analysis software, which is given by Inper company. Open the recording software, turn on the LED through the software, adjust the light power of each channel to be about the same, and make sure of that the light power of each channel is about 15 μw during recording.

9. Install PyCharm software on a personal computer for later wavelet analysis and visualization of calcium signals.

10. Preparation of reagents for SCN section.

a. Preparation of 2% sodium pentobarbital.

Weigh 1 g of sodium pentobarbital, add it to 50 mL of distilled water, mix until dissolved, and store at 4°C in the dark.

b. Prepare for 10× PBS and 4% PFA.

11. Corresponding experimental equipment and materials needed for SCN section preparation, including Leika Vibratome, blades, glass slides, coverslips, glycerol, nail polish, 24-well plate, and fluorescence microscope.

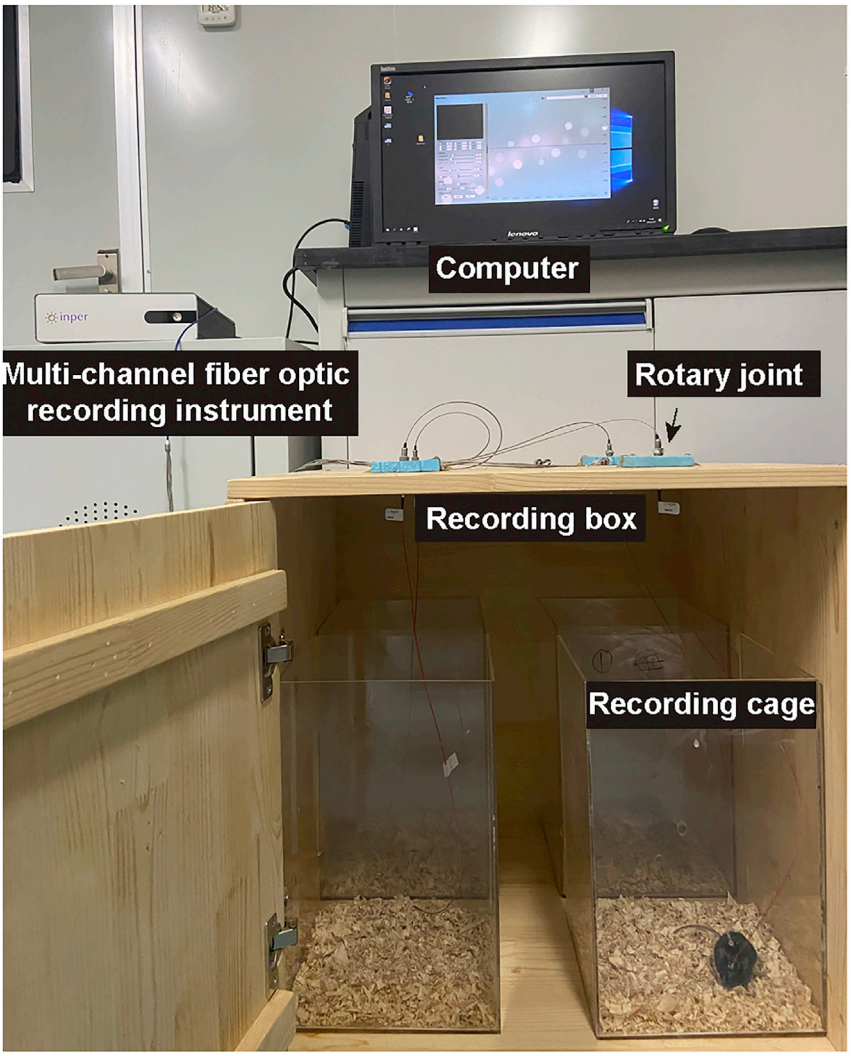

Figure 3. Multichannel optical fiber recording platform

The main components of the optical fiber recording platform include a computer, a homemade recording box, a set of recording cages and a fiber optic recording instrument with some related accessories, such as rotary joints and lightweight monofiber patch cords.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
rAAV-VGAT1-Cre-mCherry-WPRE-pA	BrainVTA	Cat#PT-0533
rAAV-CAG-FLEX-jGCaMP7s-WPRE-SV40 pA	BrainVTA	Cat#PT-1421
Chemicals, peptides, and recombinant proteins		
NaCl	Sangon Biotech	Cat#A100241
KCl	Sangon Biotech	Cat#A100395
$Na_2HPO_4 \cdot 12H_2O$	Sangon Biotech	Cat#A501725

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
KH_2PO_4	Sangon Biotech	Cat#A100571
NaOH	Sangon Biotech	Cat#AF29483100
HCl	Sinopharm	Cat# 10011018
Isoflurane	RWD	Cat#D8418
Mineral oil	Beyotime	Cat# ST1524
C_2H_5OH	Sinopharm	Cat# 10009218
30% hydrogen peroxide	Rockland	Cat#KHJ001
DAPI	Sangon Biotech	Cat# E607303
Paraformaldehyde	Sangon Biotech	Cat# A500684
Erythromycin eye ointment	Baiyunshan	Cat#62686817844
Pentobarbital sodium	Sigma	Cat# P3761
Vetbond tissue adhesive	3M	Cat# 1469SB
Dental cement	New Century Dental	Cat# 20173170702
Experimental models: Organisms/strains		
Mouse: C57BL/6J, 2 months, male	GemPharmatech	Cat#N000013
Software and algorithms		
Inper Studio	Inper	N/A
Inper Data Process	Inper	N/A
SQLiteStudio	SQLiteStudio	https://www.sqlitestudio.pl/
PyCharm 2020.1.2	JetBrains	https://www.jetbrains.com/pycharm/
Code for Calcium signal wavelet analysis	This paper https://doi.org/10.5281/zenodo.6635701	https://github.com/zhaiqiaocheng/Calcium-wave-analysis.git
Other		
−80°C refrigerator	Haier	Cat#DW-86L959W
Glass capillaries	Rantai	Cat#020091
Micropipette puller	Sutter	Model#P-1000
Ophthalmic tweezers	RWD	Cat#F11027-13
Fine scissors	RWD	Cat#S18004-10
10 mL syringe	Gaoge	Cat#GG-032003
Stereotaxic apparatus	RWD	Cat#68025
Isoflurane vaporizer and anesthesia system	Renyi	Model#MSS-3
Gas evacuation apparatus	RWD	Model#R546
Stereo microscopes	ZEISS	Model#CL-6000
Microinjection pump system	ALC Bio	Model#IP600
Homeothermic system	Cinontech	Cat#HP-10
Optical fiber holder	RWD	Cat#68217
Hot melt glue gun	Deli	Cat#DL5044
Skull drills	ALC Bio	Model#CED8
Multichannel fiber optic recording instrument	Inper	https://www.inper.com/shop/product/15#attr=461
Computer	Lenovo	Model#M8500t-N000
Optical power meter	Sanwa	Model#LP10
Vibratome	Leica	Model#VT 1000S
Blade	Gillette	Cat#YC-XH99002
Adhesive slides and cover slides	CITOTEST	Cat#80312-7141
Fluorescence microscope	Olympus	Model#IX53
Dissecting microscope	Motic	Model#K-500L
Autoclave	TOMY	Model#SX-500E
Pipette	Eppendorf	Cat#3120000224
24-well plate	Corning	Cat#3527

MATERIALS AND EQUIPMENT

10× phosphate buffer solution (PBS)		
Reagent	Final concentration	Amount
NaCl	1370 mM	80 g
KCl	27 mM	2 g
$Na_2HPO_4 \cdot 12H_2O$	100 mM	35.8 g
KH_2PO_4	17.6 mM	2.4 g
ddH_2O	n/a	up to 1 L
Total	n/a	1 L

Note: The 10× PBS can be stored at room temperature (20°C–22°C) for 24 months.

4% PFA for perfusion and fixation		
Reagent	Final concentration	Amount
Paraformaldehyde powder	4%	8 g
10 M NaOH	n/a	10 μL
10× PBS	n/a	20 mL
hydrochloric acid (36%–38%)	n/a	10 μL
ddH2O	n/a	up to 200 mL
Total	n/a	200 mL

Note: 4% PFA solution should be prepared by the following steps. Add 10 μL of 10 M NaOH solution to 180 mL of ddH_2O, heat to 55°C, add 8 g of PFA powder, stir until dissolved, add 20 mL of 10× PBS solution, and finally add 10 μL of concentrated hydrochloric acid to neutralize. Store 4% PFA at 4°C and use in one week.

Alternatives: The multichannel fiber optic recording instrument can be replaced by other brands, such as RWD (R810, R820), Plexon, etc. The Inper Data Process software can also be replaced by other algorithms, as long as data extraction and splicing can be achieved.

STEP-BY-STEP METHOD DETAILS

AAV injection and optical fiber implantation

Timing: 1.5–2 h

This step describes how to inject AAV expressing GCaMP7s at the site of the mouse SCN and implant an optical fiber for recording calcium signaling. By completing AAV injection and optical fiber implantation at the same time in one operation, we avoided the damage to mice caused by the second operation and the difficulty of positioning the SCN nucleus.

1. AAV loading.
   a. Mix AAV-VGAT-Cre and AAV-FLEX-GCaMP7s at a ratio of 1:2 (final virus titer) by pipetting.
   b. Cut a small piece of parafilm and stick it on the adhesive slide, and pipette 3 μL of virus mixture onto the parafilm.
   c. Fix the AAV injection pump on the stereotaxic apparatus, pipette the AAV into the glass micropipette, and mark the level of the virus suspension with an oil pen to observe the situation of AAV injection in real-time (Figure 4A).
2. Anesthesia and skull leveling.

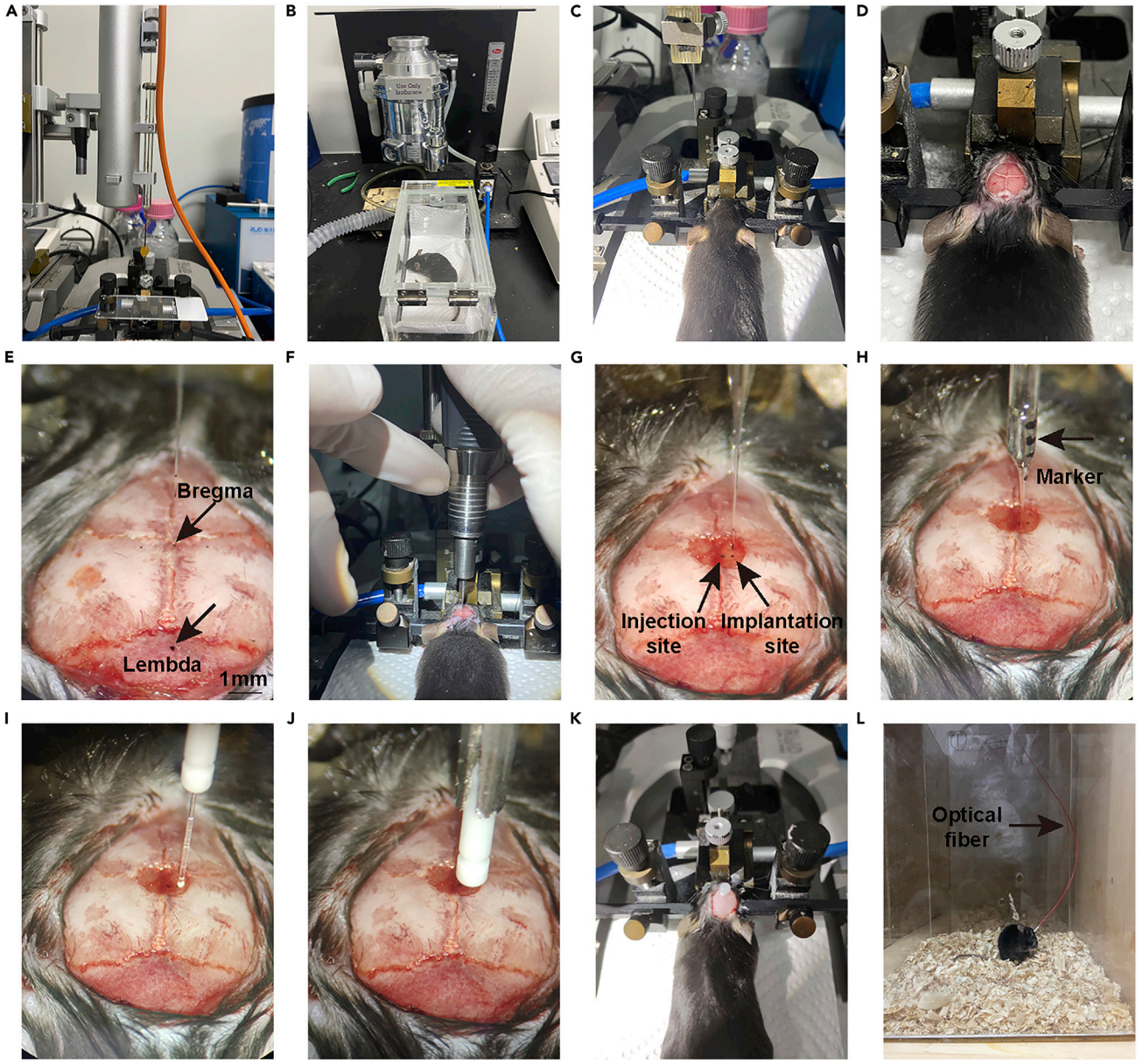

Figure 4. The main experimental steps of AAV injection and fiber implantation

(A) Load the AAV in the homemade syringe.
(B) Anesthetize the mouse with isoflurane.
(C) Fix the head of the mouse in the stereotaxic apparatus.
(D) Clean the surface of the skull with hydrogen peroxide.
(E) Level the skull (Scale bar: 1mm).
(F) Drill a hole in the skull at the bregma.
(G) Mark the AAV injection and fiber implantation sites.
(H) Inject AAV.
(I and J) Implant the fiber tip.
(K) Fix the optical fiber in place using dental cement.
(L) Dock the optical fiber between the head of the mouse and the fiber patch cord connected by rotary joints.

a. Anesthetize a 2-month-old C57 wild-type mouse with 2.5% isoflurane, then fix its head on a stereotaxic apparatus; the concentration of isoflurane was maintained at 1.5% during surgery (Figures 4B and 4C).

b. Turn on the animal homeothermic system and adjust the temperature to 35°C。
c. Apply the proper amount of erythromycin ophthalmic ointment to the eyes of the mouse.
d. Cut the hair on the mouse head with scissors, and disinfect the scalp with 75% alcohol. Make an incision to expose the skull. Wash the surface of the skull with PBS and then wipe it with 3% hydrogen peroxide to show the sutures of the skull.
e. Secure the skin of the head to the skull with 3 M tissue adhesive (Figure 4D).

3. Skull leveling (Figure 4E).
   a. Place the glass micropipette on the holder, dip the tip of the micropipette with the ink of the oil pen, move the micropipette to the bregma point of the mouse skull using the stereotaxic apparatus, lower the glass micropipette until it touches the skull surface, and set coordinates (X, Y, Z) to (0, 0, 0) mm.
   b. Move the micropipette up and 1 mm (a longer distance will make the leveling more precise) to the left of the bregma point and then lower the micropipette to the surface of the skull to read the Z-axis value.
   c. Move the micropipette to the right of the bregma point by 1 mm in the same way, and read the Z-axis value. If the Z-axis difference is $\pm$0.01 mm, the left and right skull surfaces are considered to be at the same level, otherwise, continue to adjust the left and right heights.
   d. After the left and right horizontal adjustment is completed, return the micropipette to the bregma point and set the coordinates to zero, then move the micropipette upward and move it 4.2 mm to the lambda point. If the Z-axis reading is $\pm$0.03 mm, the anteroposterior axis of the skull is considered level; otherwise, continue to adjust the front and rear heights. After confirming that the anterior and posterior skull surfaces are level, continue to check whether the left and right are still level. If so, the leveling step is completed.
4. AAV injection.
   a. Use an 0.8 mm diameter skull drill to drill a hole with a diameter of about 1.5 mm at the bregma point of the mouse skull (Figure 4F). Carefully remove the dura with ophthalmic forceps to expose the central blood vessel.
   b. Label the AAV injection site. Move the glass micropipette to the position of coordinates (AP -0.05, ML +0.15, DV 0) mm, lower it to the cortical surface, record the distance X (about 250 μm) between the skull surface and the cortex, and mark it with ink (Figure 4G).
   c. Label the fiber implantation site. Move the glass micropipette further to the right by 0.15 mm, lower the micropipette to the surface of the cortex, and mark it with ink. Add PBS to the puncture hole dropwise to keep it moist.
   d. Mount the syringe pump that contains the AAV on the stereotaxic apparatus. Move the syringe micropipette to the AAV injection site, zero the Z-axis coordinate and lower the micropipette to a depth of 5.85-X mm slowly.
   e. Inject 150 nL of the mixed virus at a rate of 50 nL per minute, leave the glass micropipette in place for 10 min after injection, and then slowly withdraw it (Figure 4H).
5. Optical fiber implantation.
   a. Prepare an optical fiber tip with a diameter of 200 μm, a length of 6 mm, and a numerical aperture of 0.5. Clean the exposed part of the optical fiber with 75% alcohol and then remove the residual alcohol with sterile PBS (Figure 4I).
   b. Install the holder with the optical fiber tip on the stereotaxic instrument. Rotate the holder to the left at an angle of 5 degrees, then inhale the PBS on the cortical surface, move the optical fiber to the fiber implantation site, and set the coordinates to zero.
   c. Puncture the cortex of the fiber implantation site with a syringe needle, then drop a small amount of PBS again, and slowly lower the fiber tip to a depth of 5.65-X mm (Figure 4J).

   Note: If the cortex is not pierced with a syringe needle, it will be hard for the fiber to enter the tissue and cause considerable damage to the brain.

6. Optical fiber fixation (Figure 4K).

a. Aspirate the PBS on the surface of the skull and cortex, and then drop a small amount of 3 M tissue adhesive to fill the drilled hole, and wait for the adhesive to completely turn into a solid.
b. First fix the fiber at the junction site of the fiber tip and the skull with a small amount of dental cement (core fixation), and then fix the entire fiber tip to the skull surface with dental cement. Be careful to minimize contact between the dental cement and the scalp.
c. Withdraw the optical fiber holder and put a dust cap on the fiber tip.

⚠ CRITICAL: If the tissue adhesive is not completely solidified, the optical fiber will not be fixed firmly, which will damage the mouse brain tissue during long-term recording, reduce the signal quality or record no calcium signal.

7. House mice in individual cages and wait 2–3 weeks for virus expression.

Calcium signal recording and analysis

Timing: 12 days

This step describes the software settings, screening of positive mice, long-term calcium signal recording, analysis, and SCN slice verification. Through the method of screening first and recording later, we can reduce the overall time of the experiment and ensure that a batch of experiments can get enough positive results.

8. Wait 3 weeks for mice to recover after surgery. After the mice are anesthetized with isoflurane, the dust cap on the mouse head should be removed and docked with the fiber patch cord (Figure 4L). The mice should be placed in the recording cage, and food and water should then be added.
9. Recording software settings.
   a. Turn on the multi-channel fiber optic recording instrument and the software. After selecting the recording channel, set the sampling rate to 30 Hz, the exposure time to 30 ms, the gain to 10, and adjust the 470 nm LED light power to 15% and the 410 nm LED light power to 10%. (The light power is about 10–15 μW.).
   b. Select the interval recording mode, with a recording time of 35 s and an interval time of 565 s; that is, 35 s will be recorded every 10 min.
   c. Select the storage location for the record file, close the recording box, turn off the lights in the room, and click record to start. Minimize human activity around the instrument during recording.
10. Mice screening (Figure 5).
    a. One day after recording, import the raw data with the Inper Data Process software.
    b. Remove the invalid 565 s of data with De-Inter.
    c. Extract the 35 s of valid date with Extract Trend.
    d. Select deinter_MCorrected on the upper panel to display the normalized calcium signal with blue color.
    e. Select deinter_conrol_signals to display the baseline reference signal (environmental noise) of the 410 nm channel in purple color in the lower panel.
    f. Screen the mice with rhythmic calcium signaling by the above method.
11. After recording for 9.5 days in screened mice, use the same method described in step 10 and get the result of normalized raw SCN calcium signals.
12. Use the SQLiteStudio software to extract the deinter_MCorrected data from normalized raw data, and store it in CSV format.

Note: To avoid a baseline difference caused by the inconsistent virus expression of each mouse, it is acceptable to normalize the data from 0 to 100. Normalization will have little effect

Figure 5. Protocols for screening of positive mice using Inper Data Process software

By importing raw data (step 1, Data Source), removing invalid data (step 2, De-Inter), extracting valid data (step 3, Extract Trend), displaying normalized calcium signal data (step 4, deinter_MCorrected) and displaying baseline reference signal data (step 5, deinter_control_signals), mice with obvious rhythmic calcium signals within one day can be selected as positive mice (the recorded signal is from the SCN). This graph shows data from two positive mice.

on the results when exploring changes in SCN circadian phase. While external stimuli can affect SCN amplitude, we do not recommend data normalization.

13. Visualize calcium signal data by PyCharm software and related codes (4-channels data, from c-1 to c-4)。
    a. Select the path of the CSV file in the 135 lines of code (Figure 6A).
    b. Select the output file format and the frequency resolution of the wavelet analysis in lines 157–167 (Figure 6B).
    c. Run the code to output calcium signal visualization results and energy value files of calcium signals at different frequencies. The frequency parameters can be adjusted manually.
14. Slice verification of the recording site.
    a. Anesthetize mice by intraperitoneal injection of sodium pentobarbital.
    b. Lay the mouse on its back with its limbs fixed on the foam board. Open the mouse chest to expose the heart. The needle for perfusion should be inserted into the left ventricle, and the needle should be fixed; then, the right atrium should be cut, and the mouse should be perfused with 10 mL of 1× PBS and 10 mL of 4% PFA solution.
    c. Carefully pull out the optical fiber and dental cement on the surface of the mouse skull, collect the mouse brain tissue, and place it in 4% PFA for 48 h at 4°C.
    d. Cut 40–50 μm coronal sections with a Leica vibratome and collect them in 24-well plates containing 1× PBS.

A

```
def main_process():
    files = [r'D:\data\after3day.csv']
    chns = ['c-1', 'c-2', 'c-3', 'c-4']
```

Modification for file path

B

```
if __name__ == '__main__':
    ShowFigure = False

    SaveSVG = True
    SavePNG = True
    ExportResult = True

    Wavelet = 'cgau8'    # 'cgau8' 'morl' etc.
    FrequencyResolution = 14
    ZLimits = np.arange(-0.02, 1.0, 0.01)

    main_process()
```

Modification for image format

Modification for frequency parameter

Figure 6. Visualization of long recorded calcium signals using code

(A) Change the storage path of the CSV file to be analyzed.

(B) Change the format of the output image, including SVG and PNG formats. (True means output, False means no output.) Change the frequency resolution of the calcium signal wavelet analysis. The larger the value is, the more frequency bands will be analyzed.

e. Under the dissecting microscope, observe whether there is a tissue defect at the position of the optical fiber implantation.

f. Pick up the brain slice where the optical fiber implantation site is located, put it into 1× PBS solution containing 10000× DAPI and incubate for 5 min, then wash the brain slice with 1× PBS for 5 min.

g. Add about 50 μL of 1× PBS solution dropwise to the adhesive slide. After it forms droplets, spread the brain slices in the droplets, remove the droplets and air dry at room temperature. Then, seal the slides with 50% glycerol and nail polish.

h. Take pictures using a fluorescence microscope.

Optional: For clearer seeing of the damaged area caused by optical fiber, it is necessary to get brain slice by cryostat to check the fiber position. Alternatively, it is more convenient and quicker to use a vibratome in processing multiple brain samples.

EXPECTED OUTCOMES

Through the above experimental methods, we first did AAV injection and optical fiber implantation, and after three weeks of complete virus expression, we performed preliminary screening on all operated mice and got the positive mice with robust rhythmic calcium signals. The success rate of the surgery is about 30%–40%. Subsequently, long-term (9.5 days) calcium signal recordings were performed on positive mice in complete darkness to reveal the autonomous calcium rhythm in the SCN. The light conditions do not affect the operation of the optical fiber recording system, so there is no problem if you want to record SCN calcium signals under light-dark cycles. Through data processing, we obtained rhythmic calcium signal maps and spectrograms of wavelet analysis at different frequencies (Figure 7A). The calcium signals in a 10 s timescale showed a more active calcium signaling during the subjective day (bedtime) than the subjective night (wakeup time) (Figure 7B). We further verified the site of optical fiber recording and GCaMP7s expression by sectioning (Figure 7C).

LIMITATIONS

Although most of the neurons in the SCN are GABAergic neurons (Moore and Speh, 1993), there are still a certain number of GABAergic neurons in the hypothalamus around the SCN, which leads to

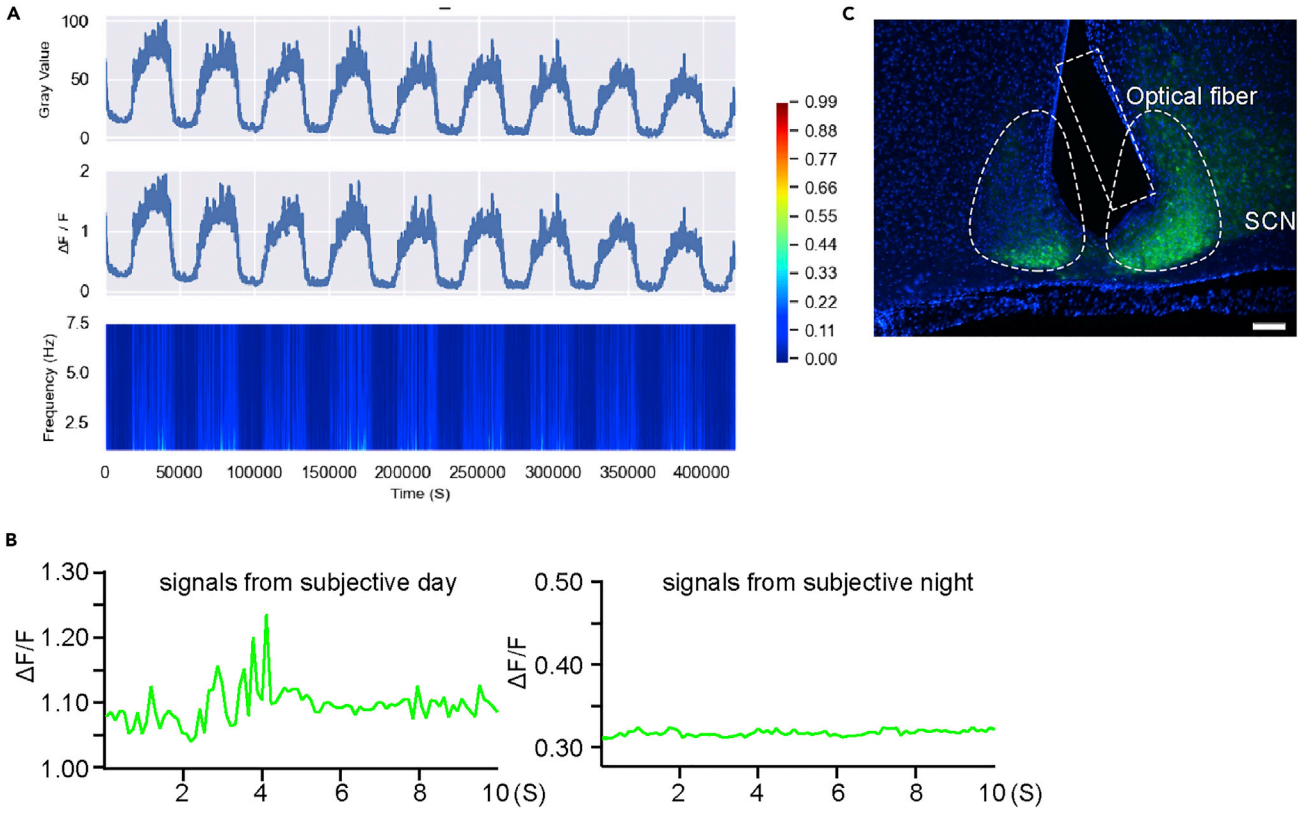

Figure 7. Calcium signal visualization and slice validation of long-term recordings

(A) A screenshot of the output file of the SCN calcium signal analyzed by the code.

(B) The calcium signals in a 10 s timescale during the subjective day and night. The data was extracted from the same mice as (A).

(C) The expression of GCaMP7s (green) and DAPI (blue) and the location of optical fibers in a 50 μm SCN slice. (Scale bar: 200 μm).

nonspecific expression of GCaMP outside the SCN area through a mixed injection of VGAT-Cre and Cre-dependent GCaMP AAV. However, the location of the optical fiber and the rhythmic calcium signal can confirm whether the recorded signal comes from the SCN. To overcome this limitation, using Cre mice with SCN neuron-specific promoters may be a good choice, but it should be noted that if the distribution of specific neurons is relatively scattered or the promoter is too weak to drive the expression of calcium indicator, it may affect the sensitivity of GCaMP and long-term recording quality of the calcium signal.

Also, the current wavelet analysis code for calcium signals has not been integrated into the analysis software, which may not be very friendly to beginners. Later, we will optimize the analysis software to make the analysis steps easier.

TROUBLESHOOTING

Problem 1

Abnormal skull surface morphology (step 2).

Potential solution

Abnormal skull surface morphology will lead to difficulty in positioning the bregma and lambda point, which in turn affects the leveling of the skull and the positioning of the SCN. It is recommended to replace the mouse with a normal skull.

Problem 2
Bleeding during skull drilling and dura removal (step 4).

Potential solution
It is recommended to use a skull drill with a smaller diameter (0.8 mm). First, slowly grind a hole at the bregma site, and then appropriately widen it into the surrounding area to increase the diameter of the hole.

When removing the dura, use ophthalmic tweezers to slowly peel off the dura from the edge. During this process, watch the condition of the central blood vessel and do not break it. If bleeding happens, the wound needs to be continuously irrigated with PBS until the bleeding stops.

Problem 3
Tissue adhesive does not solidify for a long time (step 5).

Potential solution
Try to clean up the liquid in the drilled hole until there is no tissue fluid leakage or PBS remaining.

Problem 4
The fluid level of AAV does not change during virus injection (step 4).

Potential solution
The tip of the glass micropipette is blocked by the tissue. The glass micropipette can be withdrawn, then suck a small amount of PBS with a piece of absorbent paper and wipe the tip to remove the tissue block. The micropipette tip can also be trimmed properly, but note that the Z-axis needs to be re-zeroed after trimming.

There is a problem with the tightness of the connection between the syringe and the glass micropipette, and the virus injection needle needs to be remade.

Problem 5
There is no signal in the recording or there is a signal at the beginning, and the signal disappears after a few days of recording (steps 9–11).

Potential solution
The absence of signal after three weeks of virus expression may be more due to problems with AAV injection or that the fiber is not positioned above the GCaMP-expressing neurons, and it is recommended to sacrifice the mice.

The signal was strong at first and disappeared after a few days of recording. The possible reason is that there was a problem with the fixation of the dental cement. Loosening of the fiber tips will lead to inflammation or necrosis of the brain tissue. It is recommended to wait patiently for the tissue adhesive and the dental cement to fully solidify during the operation before ending the experiment.

Problem 6
The calcium signal amplitudes recorded over a long period continuously diminished (step 13).

Potential solution
This problem can be solved by reducing the light power of the LED and the sampling time.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ying Xu (yingxu@suda.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The code generated in this paper can be accessed at https://github.com/zhaiqiaocheng/Calcium-wave-analysis.git.

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AUTHOR CONTRIBUTIONS

All technical platforms were constructed by Q.Z., who wrote the manuscript. Y.Z. and Z.L. provided photographs of the animal surgery. Youjia Xu and Ying Xu revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Dana, H., Sun, Y., Mohar, B., Hulse, B.K., Kerlin, A.M., Hasseman, J.P., Tsegaye, G., Tsang, A., Wong, A., Patel, R., et al. (2019). High-performance calcium sensors for imaging activity in neuronal populations and microcompartments. Nat. Methods *16*, 649–657. https://doi.org/10.1038/s41592-019-0435-6.

Houben, T., Deboer, T., van Oosterhout, F., and Meijer, J.H. (2009). Correlation with behavioral activity and rest implies circadian regulation by SCN neuronal activity levels. J. Biol. Rhythm. *24*, 477–487. https://doi.org/10.1177/0748730409349895.

Jones, J.R., Simon, T., Lones, L., and Herzog, E.D. (2018). SCN VIP neurons are essential for normal light-mediated resetting of the circadian system. J. Neurosci. *38*, 7986–7995. https://doi.org/10.1523/jneurosci.1322-18.2018.

Mei, L., Fan, Y., Lv, X., Welsh, D.K., Zhan, C., and Zhang, E.E. (2018). Long-term in vivo recording of circadian rhythms in brains of freely moving mice. Proc. Natl. Acad. Sci. USA *115*, 4276–4281. https://doi.org/10.1073/pnas.1717735115.

Miyamoto, D., and Murayama, M. (2016). The fiber-optic imaging and manipulation of neural activity during animal behavior. Neurosci. Res. *103*, 1–9. https://doi.org/10.1016/j.neures.2015.09.004.

Moore, R.Y., and Speh, J.C. (1993). GABA is the principal neurotransmitter of the circadian system. Neurosci. Lett. *150*, 112–116. https://doi.org/10.1016/0304-3940(93)90120-a.

Todd, W.D., Venner, A., Anaclet, C., Broadhurst, R.Y., De Luca, R., Bandaru, S.S., Issokson, L., Hablitz, L.M., Cravetchi, O., Arrigoni, E., et al. (2020). Suprachiasmatic VIP neurons are required for normal circadian rhythmicity and comprised of molecularly distinct subpopulations. Nat. Commun. *11*, 4410. https://doi.org/10.1038/s41467-020-17197-2.

Zhai, Q., Zeng, Y., Gu, Y., Li, Z., Zhang, T., Yuan, B., Wang, T., Yan, J., Qin, H., Yang, L., et al. (2022). Time-restricted feeding entrains long-term behavioral changes through the IGF2-KCC2 pathway. iScience *25*, 104267. https://doi.org/10.1016/j.isci.2022.104267.