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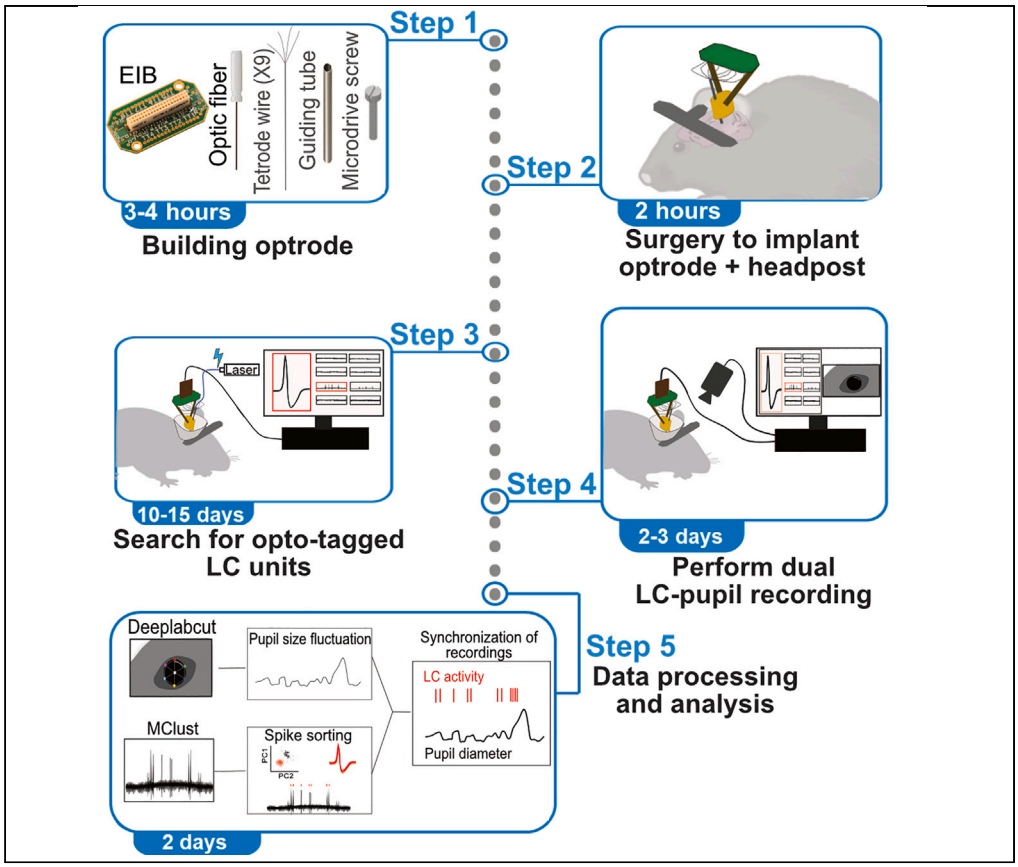
Protocol

Simultaneous recordings of pupil size variation and locus coeruleus activity in mice



An extensive literature describes how pupil size reflects neuromodulatory activity, including the noradrenergic system. Here, we present a protocol for the simultaneous recording of optogenetically identified locus coeruleus (LC) units and pupil diameter in mice under different conditions. We describe steps for building an optrode, performing surgery to implant the optrode and headpost, searching for opto-tagged LC units, and performing dual LC-pupil recording. We then detail procedures for data processing and analysis.

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

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Highlights

Protocol for simultaneous recording of pupil size and locus coeruleus activity in mice

Adaptable to targeting different brain regions for a variety of behavioral experiments

Enables correlation between pupil size change and locus coeruleus activity

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Simultaneous recordings of pupil size variation and locus coeruleus activity in mice

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SUMMARY

An extensive literature describes how pupil size reflects neuromodulatory activity, including the noradrenergic system. Here, we present a protocol for the simultaneous recording of optogenetically identified locus coeruleus (LC) units and pupil diameter in mice under different conditions. We describe steps for building an optrode, performing surgery to implant the optrode and headpost, searching for opto-tagged LC units, and performing dual LC-pupil recording. We then detail procedures for data processing and analysis.

For complete details on the use and execution of this protocol, please refer to Megemont et al.[1]

BEFORE YOU BEGIN

Institutional permissions

We used both male and female mice, between 8-20 weeks old. To be able to identify the noradrenergic (NA) neurons in the locus coeruleus (LC) we express channelrhodopsin 2 (ChR2) in NA neurons. Mice were either crossed DBH-Cre (B6.FVB(Cg)-Tg(Dbh-cre) KH212Gsat/Mmucd, 036778-UCD, MMRRC); Ai32 (RCL-ChR2(H134R)/EYFP, 012569, JAX), or DBH-Cre injected with AAV5-EF1α-DIO-hChR2(H134R)-EYFP (UNC Vector Core). A recent study comparing different strategies to target the LC supports the choice of using the DBH-cre line over the TH-cre line.[2] Mice were housed in a vivarium with reverse light–dark cycle (9 a.m.–9 p.m. dark). To perform the protocol, obtaining permissions from the relevant institutional committees is required.

Setting up the recording rig

Timing: 1 day

The recording setup, designed for simultaneous LC electrophysiological and pupil videographic recordings, requires a light-proof, sound-attenuated Faraday cage, crucial for accurately capturing isoluminant pupil size variations. Considering the extended recording sessions (≥1 h) and the substantial amount of data generated, we utilize two computers: one records LC neuronal activity, and the other records pupil. Synchronization of these systems is ensured through a TTL pulse from the camera. In the following sections, we detail the components required to assemble this recording apparatus.

1. Install all the software needed to perform the recording:
   a. MATLAB (https://www.mathworks.com/products/matlab.html , with WaveSurfer https://wavesurfer.janelia.org/, to control the laser stimulation).
   b. Pylon Viewer (for the camera recording, https://www.baslerweb.com/en/downloads/software-downloads/).
   c. Intan Technologies RHD2000 interface (https://intantech.com/downloads).
2. Set up the apparatus for head-fixation (apparatus extensively described by previous authors[3]).
   a. Assemble a head bar holder and clamps to firmly hold the headpost fixed on the skull of the mouse.
   b. Cut a plexiglass tube to restrain body movement and allow the mice to rest their front paws on the front edge.
3. Set up the camera for pupil recording.
   a. Use an infrared light (850 nm, Vishay, Cat#VSMB2943GX01) to illuminate the face of the animal for pupil recording.
   b. Properly position and focus the camera on the pupil of the mouse so that the pupil is clearly visible, illuminated and in focus.

Caution: The video quality greatly impacts the accuracy of DeepLabCut pupil tracking. High contrast and absence of reflections on the pupil are important factors for optimal results.

Note: We typically record the pupil ipsilateral to the recorded/stimulated LC. Unilateral LC stimulation evokes bilateral pupil responses, but the ipsilateral side exhibits faster and more prominent dilation.[4]

4. Set up all the connections needed to perform the dual recording:
   a. The LC neural activity will be recorded using the RHD recording system (Intan Technologies, https://intantech.com/RHD_system.html).
      i. Connect the RHD2132 amplifier board (#C3314) to the RHD2000 interface board (#C3100).
      ii. Ground the board using the dedicated port.
      iii. Connect the RHD2000 interface board to the recording computer with the USB port.
   b. Connect the camera (Basler, acA1300-200 μm) to the computer (USB 3.0).

Note: The camera has an opto-coupled I/O output line that will be used to synchronize the video with neuronal activity. This output is connected to the ADC Input of the RHD2000 interface board and to a data acquisition (DAQ) system (DAQ card: PCIe-6323, National Instrument; breakout box: BNC-2090A, National Instruments).

   c. Set the parameters in the Basler software to have the camera send an output TTL pulse train. Go to > Digital I/O Control > Line 2 > Line mode "Output". Set the Line source to "Exposure active".
   d. When running the recording in the RHD2000 Interface software, you should see the TTL pulse train appear in the Board ADC Inputs.
   e. Open MATLAB and run the application WaveSurfer. Go to Tools > Device & Channels. Select the DAQ board and set up the input for the TTL pulse from the camera.
5. Set up an ambient LED light matching the laser's wavelength (in our case blue light for 450 nm) inside the recording chamber to provide ambient illumination.

Note: A properly constricted pupil is needed to observe pupil size changes. In addition, the ambient light helps to mask any potential light leakage from optogenetic stimulation. It is crucial to avoid any light leakage from optogenetic stimulations for several reasons: (1) to avoid luminance-mediated pupil constriction; (2) the potential to startle the animal; (3) the leaking light may serve as an unintended cue during behavior. To prevent light leakage, the connection of the optic fiber will be covered with black tape.

6. Set up the laser for optogenetic stimulation, controlled by WaveSurfer.

   Note: For more detailed information on how to use optogenetic stimulation in WaveSurfer, refer to the manual available on their website (https://wavesurfer.janelia.org/manual-0.945/index.html).

   a. Connect the DAQ to a blue light source (in our studies we used the 450 nm blue diode laser, UltraLasers, MDL-III-450–200 mW).
   b. In Tools > Device & Channels, set the output for the laser stimulation. In the main window, click the Stimulation > Enabled checkbox to turn on the stimulation.
   c. In the "Stimulus library" window, you can set the parameters for the different pulses you want to use to activate the laser in your protocol.
7. Connect the light source to the optogenetic fiber patch cables (Ø200 μm Core, 0.39 NA, Thorlabs, M72L02, M89L01).
8. Measure the laser output at the tip of the ferrule using a Laser power Meter (Coherent, SKU 1098293).
   a. Align centrally the laser beam on the sensor and read the value.
   b. Adjust the power to be 5–10 mW to stimulate LC neurons.

Create and train a neural network for pupil tracking

Timing: 2 days

In this section of the protocol, we detail the creation and training of a neural network for pupil tracking, using DeepLabCut.[5] We choose to use this tool for its user-friendliness, high degree of customizability and its accuracy and efficiency. Moreover, this tool is open-sourced and now a well-established tool with a robust support system.

Note: We have published a premade and trained network on the DeepLabCut website (Jim McBurney-Lin, http://www.mackenziemathislab.org/dlc-modelzoo). However, to maximize the efficiency of the pupil tracking, we highly recommend creating your own neural network trained on a subset of video from your own experiment to tailor the tracking for your needs.

9. Install DeepLabCut following the instructions available on the website[5] (http://www.mackenziemathislab.org/deeplabcut).
10. Either download a pre-made neural network for pupil tracking on their website or create your own.
    a. We build the skeleton for tracking the pupil on DeepLabCut based on the guidelines available on their website (https://deeplabcut.github.io/DeepLabCut/README.html) (Figure 1):
       i. Use 6 points to contour the edges of pupil.
       ii. Use 1 point in the center.
       iii. Link all neighboring points.
11. Load a set of videos to train the network, extract frames and label them manually.

    Note: This step is determinant for the tracking to work, so be sure to select representative examples and to be consistent in the manual labeling.

12. Train the artificial neural network. We recommend following the directions on the DeepLabCut website to choose the best parameters to create and train the neural network.

    Note: For our experiment, we used the resnet_50 network and the default training parameters.

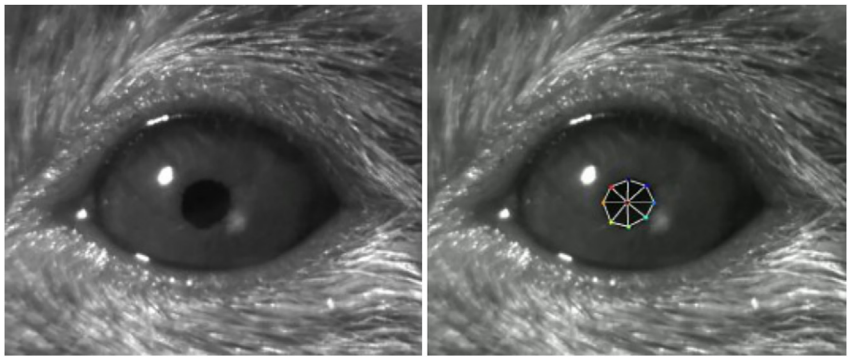

Figure 1. Side-by-side representative examples from a pupil recording and the same video frame analyzed by DeepLabCut

See how the pupil is clearly visible, illuminated and in focus. DeepLabCut accurately recognizes the contour of the pupil.

13. Run a test to see the performance of the network.
14. If the performance is acceptable, analyze one video and extract the result as a .csv file.

Note: This is the file to feed to MATLAB to extrapolate pupil size variation and then synchronize with other recordings.

15. Generate a labeled video (Figure 1) to do a visual quality check of the performance of your network in tracking pupil size.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Tyrosine hydroxylase polyclonal antibody	Thermo Fisher Scientific	OPA1-04050
Goat anti-rabbit IgG (H + L) secondary antibody, Alexa Fluor 488	Thermo Fisher Scientific	A11008
Chemicals, peptides, and recombinant proteins		
Bupivacaine	Hospira	00409-1162-01
Buprenorphine	Par Pharmaceutical	42023-0179-05
Enrofloxacin	Norbrook	ANADA 200-513
Isoflurane	Covetrus	029404
Ketoprofen	Zoetis	NADA 140-269
Paraformaldehyde (PFA)	Sigma-Aldrich	P6148
Puralube vet ointment	Dechra	17033-211-38
Software and algorithms		
DeepLabCut	Mathis et al.,[5] Nature Neuroscience 2018	http://www.mackenziemathislab.org/deeplabcut
Intan Technologies RHD2000 interface	Intan Technologies	Version 1.5.2
MATLAB	MathWorks	Version R2020.a
MClust	Redish Lab	http://redishlab.neuroscience.umn.edu/MClust/MC
pylon Viewer	Basler	Version 5.2.0.13457 64-bit
WaveSurfer	HHMI Janelia	http://wavesurfer.janelia.org/
Other		
450 nM blue diode lasers	UltraLasers	MDL-III-450–200 mW
850 nm infrared lights	Vishay	Cat#VSMB2943GX01
Camera	Basler	acA1300-200 µm
Ceramic ferrule	Thorlabs	CFLC230-10

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Data acquisition system card	National Instruments	PCIe-6323
Data acquisition system breakout box	National Instruments	BCN-2090A
Dental cement	LangJet	Denture repair powder #1220
EIB pins small	Neuralynx	EIB small pins 500 pack
Electronic interface board (EIB)	Neuralynx	EIB-36-PTB
Electric heat gun	Wagner	HT1000
Electrode impedance tester	Bak Electronics	IMP-2A
Epoxy glue	Devcon	GLU-720.90
Fiber optic mating sleeve connectors	Thorlabs	ADAFCB3
Gold plating solution	Neuralynx	Gold plating solution 10 mL
Laser power meter	Coherent	SKU 1098293
Miniature stainless steel tubing	McMaster-Carr	0.008″ OD, 0.004″ ID, 33 tubing needle gauge #8988k84
Miniature stainless steel tubing	McMaster-Carr	0.0032″ OD, 0.0025″ ID, 21 tubing needle gauge, #5560K56
Nichrome wires	Sandvik	PX000004
Optic fiber (19 mm long, 0.39 NA, Ø200 μm core)	RWD Life Science	R-FOC-L200-39NA
Optogenetic fiber patch cables (Ø200 μm core, 0.39 NA)	Thorlabs	M72L02, M89L01
Polyimide tubing	HPC Medical Products	35-gauge, Item # 72113900001-012
RHD2132 amplifier board (32-channel recording headstage)	Intan Technologies	#C3314
RHD USB interface board (RHD2000)	Intan Technologies	Version 1.0 #C3100
Seamless round tubes	McMaster-Carr	89495K775, 89495K55
Stainless steel wire	A-M Systems	792800
Stimulus isolator	WPI	A365
Super glue (all purpose)	Krazy glue	KG585
Telecentric lens	Edmund Optics	55–349
Tetrode spinner	Neuralynx	Version 2.0

STEP-BY-STEP METHOD DETAILS

Building optrode

Timing: 3–4 h across 3 days

In the following paragraph, we outline the process of constructing an optrode from scratch. Our step-by-step protocol is based on previous work,[6] but modified for LC recording/stimulation. The individual parts for the holder are 3D printed by the user (technical sketch available at https://doi.org/10.5281/zenodo.10070203).

1. Assemble the microdrive.
   a. Print the different pieces (custom 3D printed parts).
   b. Assemble the holder with the microdrive screw, the microdrive nut and the 3 metallic tubes (Figure 2).
   c. Assemble the holder and the Electrical Interface Board (EIB, Neuralynx, Bozeman, MT).

   Note: We used the wood handle from cotton swabs, see Figures 2 and 3. 3D-printed parts may work better.

      i. Secure it to the EIB using Krazy glue.
      ii. Reinforce it with Epoxy.
      Caution: Be careful not to put glue on the EIB pinholes.

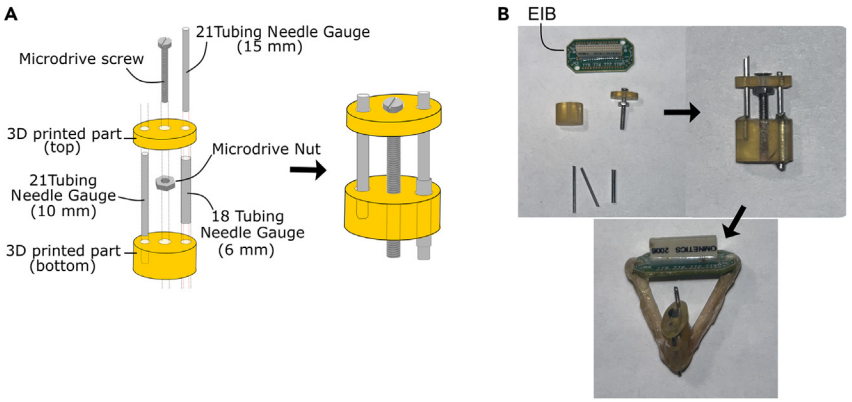

Figure 2. Assembling the tetrode holder

(A) Schematic of the microdrive.

(B) Example of a microdrive with the EIB fixed on top (see also figure below).

d. Add 9 pieces of polyimide tubing (about 3 inches long each, HPC Medical, 35-gauge, I#72113900001-012) and the optic fiber (RWD Life Science, R-FOC-L200-39NA 19 mm long, 0.39 NA, 200 μm core) inside the guiding metallic tube (Figure 3A).

Note: Try to place the optic fiber in the middle of the polyimide tubes.

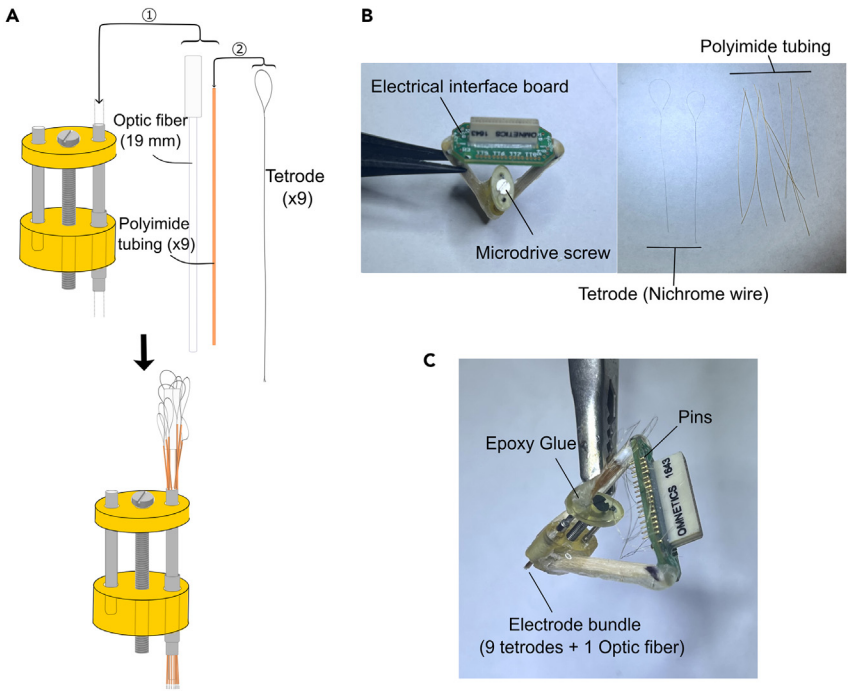

Figure 3. Details to build an optrode

(A) Diagram illustrating the assembly of the optical fiber and polyimide tubing. Individual wire bundles are inserted into individual polyimide tubes. The finalized assembly is shown in the lower illustration. Following this, the tetrode loop will be cut open and connected to the EIB.

(B) Example of an assembled microdrive (top view) and tetrode after spinning, ready to be wired, and the polyimide tubing used to guide each tetrode.

(C) Optrode ready to be implanted.

e. Using Krazy glue, secure polyimide tubing, guiding metallic tube, optic fiber, and the top part of the holder together.
f. Let it dry completely and then cut the polyimide tubes to the desired length.

Note: We recommend ~2.5 mm out of the guiding metallic tube to target the LC.

Caution: Be careful to leave enough space on the optic fiber adapter to plug in the laser. Make sure that after each gluing step the microdrive screw can be advanced and retracted.

2. Prepare the tetrode wires.
   a. Cut pieces of about 15 inch (40 cm) of insulated nichrome wire (Sandvik, #PX000004).

Note: Our EIB board has 32 channels, thus our construct contains 9 tetrodes (8 for recording, 1 for reference).

   b. Fold twice and twist the bundled four wires using a tetrode spinner (Neuralynx, 70 forward, 10 backward).
   c. Use an electric heat gun (Wagner, HT1000) to melt the coating of the nichrome wires so they are all joined together.
   Caution: Do not overheat, this could damage the insulation of individual nichrome wires.
   Tips: To make the wiring easier try to make the tetrode wire as straight as possible (Figure 3B).
3. Tetrode loading.
   a. Insert the tetrode wire bundle into the polyimide tubing (1 bundle per tube, Figure 3A).
   b. Cut the loop of the tetrode to release the 4 wires.
   c. Connect each of the four wires to a group of four pinholes in the EIB and secure by pushing in a pin.
   d. Repeat the operation for the 8 recording tetrodes + the reference tetrode.
   e. Once they are all wired, glue the tetrode on both sides of the polyimide tubing using "No run gel" Krazy glue to create a single implantable bundle with fixed wires.

Caution: Avoid glue on the tip of the optic fiber.

4. Let it dry completely and cut the wire bundle about 0.5 mm extending out of the polyimide tubes.

Note: Make sure to prevent bending the wires to achieve a clean cut.

5. Wire a grounding screw, to help eliminate noise and artifacts in the recordings.
   a. Cut a piece (about 3 inches) of stainless-steel wire (A-M Systems, 792800).
   b. Create 2 loops around the grounding screw on one end and connect the other end to the reference pin (R1 or R4 channel) on the EIB.
6. Adjust the impedance of the wire using Gold Plating solution (Neuralynx) and a stimulus isolator (WPI, #A365).
   a. Dip the electrode bundle in the gold plating solution.
   b. Pass DC current (about 0.3 μA) on the pin of each channel.
   c. Measure the impedance with an Electrode impedance tester (Bak Electronics, IMP-2A) until reaching the right value.

Note: In our studies, the target impedance before recording wires is around 300 kΩ; for the reference wire, the impedance should be as low as possible, but typically not higher than 10 kΩ.

⚠ CRITICAL: Make sure to never put glue on the microdrive screw. After gluing, never bend the wires (during cutting or gold plating process).

Surgery to implant optrode and headpost

Timing: 2 h

Our headpost and the surgery procedures are based on a previous study,[3] but modified for recording from the LC. It is essential that the surgery is carried out in an aseptic environment, especially since the implant will remain in place for at least 2–3 weeks. Surgical instruments must be autoclaved prior to surgery and sterilized between surgeries (if more than one surgery is occurring per surgery day). Disinfecting with 70% alcohol may be used in addition to bead sterilization.

7. Prepare the skull for implant.
   a. Anesthetize the animal (induction 2.5%–3% isoflurane) and administer the necessary drugs for pain control according to your institutionally approved protocol.
   b. Move the animal to the stereotaxic frame and place the ear bars.

   Note: Ensure ear bars and nose cone are tight enough to avoid movement of the head.

   c. Use a heating blanket to maintain body temperature.
   d. Shave, clean (70% ethanol and iodine) and cut the scalp using scissors.
   e. Once the scalp is cut, make sure to remove the periosteum and clean the skull again (ethanol / iodine 3 times).
   f. Glue the skin around the exposed skull with Krazy glue and let it dry.
   g. Using the stereotaxic frame, align bregma and lambda.
   h. Calculate the coordinates for the targeted recording site and make a mark using a dental drill. The hole will be drilled later in the process.

   Note: To target LC in adult mice, we use the coordinates of A/P: -5.30 mm. M/L: 0.9 mm relative to the bregma. Depth: 2.6 mm relative to the surface of the brain. The LC is located deeper (3.1–3.3 mm).The microdrive will be advanced slowly after the recovery of the mouse to reach the target brain region.

   i. Using the dental drill, scratch the skull to ensure a strong hold of the dental cement.
   j. Clean the skull again to remove any dust.
8. Place the headpost (Figure 4A).
   a. Place a sterilized titanium headpost on top of the skull and expose one hemisphere (left hemisphere in our case).
   b. Use Krazy glue to secure the placement.

   Note: The headpost will be sealed with dental cement after the optrode is implanted.

9. Place the grounding screw (Figure 4C).
   a. Using an alligator clip to hold the optrode over the skull, visualize the position for the grounding screw.
   b. Make a mark on the skull with the drill.
   c. Use a screwdriver to screw in the skull a grounding screw.

Caution: Do not implant it too deep, or there is a risk to damage the brain.

10. Implant the optrode (Figure 4D).
    a. Drill a craniotomy (about 2 mm diameter) for the optrode implant.

    ⚠ CRITICAL: As the coordinates for targeting the LC are located over big blood vessels, there is a risk of considerable bleeding during the procedure. To stop the bleeding, use

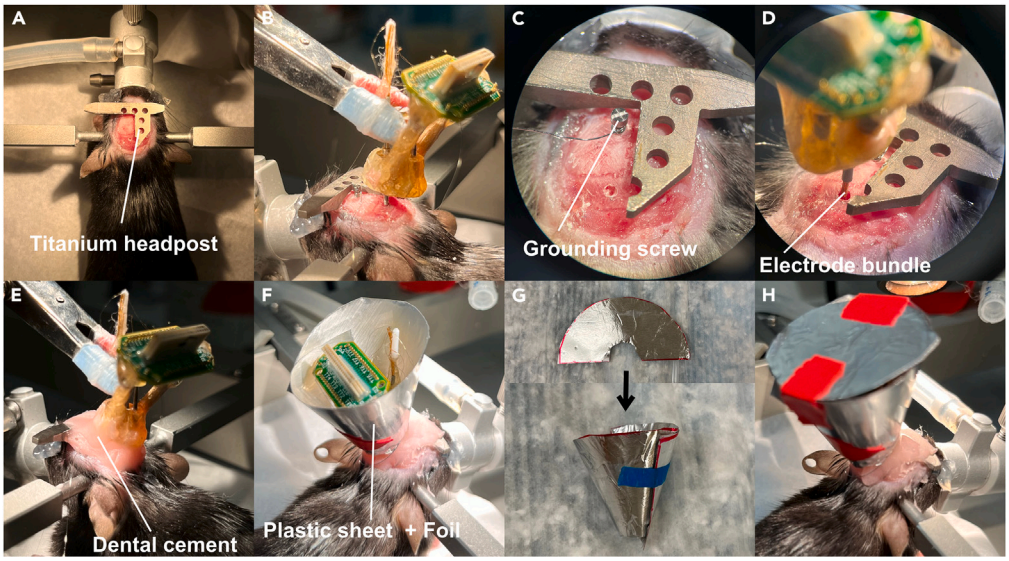

Figure 4. The surgical procedures for optrode implant

(A) Adult mice anesthetized, with the head fixed in a stereotaxic frame. After shaving, the scalp is removed, and the headpost is placed.

(B) Overview of the optrode during implantation in the LC.

(C) Close-up of the grounding screw placed away from the implantation site. On this picture the craniotomy where the optrode will be implanted is visible.

(D) Close-up of the electrode bundle slowly inserted in the brain.

(E) The implant is fixed on the skull with dental cement.

(F) Last step, a protective cone is fixed around the optrode.

(G) The protective cone is crafted by combining a plastic sheet with foil glued on one side, and then folded to form the cone.

(H) The surgery concludes with the addition of a lid on top of the protective cone. The total weight of the implant is approximately 5 g.

sterile cotton swabs or a hemostatic sponge. Ensure bleeding has stopped before proceeding.

b. Place the optrode above the craniotomy.
c. Verify the vertical alignment of the probe.
d. Apply sterile optical ointment (Dechra, Puralube, vet ointment, NDC 17033-211-38) on the guiding tube of the optrode.

Note: The ointment will prevent the dental cement from slipping into the tubes and blocking the movement of the wires.

e. Slowly advance the tetrode to the brain surface, making sure the tetrode gets in the brain without bending, which could be due to unremoved skull fragments or dura.
f. Slowly advance to the target depth (2.6 mm).

Note: Clean any bleeding during optrode advancement. If there is significant bleeding, pause and use a hemostatic sponge.

⚠ CRITICAL: A dry, clean surface is necessary for the dental cement to bond properly with the skull. This will ensure that the implant stays securely attached.

11. Secure the implant (Figure 4E).
    a. Use dental cement to fill any space around the implant and under the headpost.

b. Use thicker dental cement to build up around the implant.
c. Wait until the cement is completely cured (~30 min).
d. Carefully detach the implant from the holder.
e. Make sure the microdrive screw can advance using a screwdriver.

12. Build a cone to protect the implant.
    a. Attach a piece of aluminum foil to a sheet of plastic film.
    b. Wrap it around the implant (Figure 4G)
    c. Glue the bottom of the cone to the dental cement.
    d. Cover the top of the cone.
13. Remove the animal from the stereotaxic frame. Allow the mouse to recover in a cage over a heating pad.

Note: Once recovered, singly house the mouse in the cage. Within several days, the mouse should resume normal activity. The total weight of the implant is less than 5 g, allowing the mouse to resume normal locomotor activity. The implant must show no sign of inflammation or edema around the dental cement.

Look for pupil response and opto-tagged LC units

Timing: across several days

After the mouse has recovered, the signal from the optrode is monitored daily for tagged LC units. The microdrive is advanced in steps of ~100 μm at the end of the recording until reaching LC (1 full turn of the microdrive screw is about 300 μm). Usually, LC neurons can be recorded between 3.1 and 3.8 mm deep. The identification of LC neurons has traditionally been based on their characteristic's wide waveform and response to tail pinch.[7,8] Studies using pharmacology have shown both wide and narrow units within the LC.[9] As a result, a more specific approach using pharmacology (clonidine) or opto-tagging can better identify genetically and neurochemically defined LC neurons. To opto-tag noradrenergic neurons, we combine extracellular recording with an optrode and the expression of Cre-dependent ChR2 in neurons expressing dopamine beta hydroxylase. Putative LC units are identified by apparent spiking responses to optogenetic stimulation. We also examine whether these putative opto-tagged units respond to tail pinch. These units are further analyzed offline to confirm their identity based on response latency and waveform.

14. Put the animal under anesthesia (2% isoflurane) and head fixation in the recording rig.

Note: We do opto-tagging under anesthesia to reduce spontaneous activity, increase the signal-to-noise ratio for opto-tagged units, and to avoid the mouse reacting to the stress associated with tail pinch.

15. Start the recording.
    a. Plug in the RHD2132 Amplifier Board (Figure 5) and connect the optogenetic fiber patch cable to the optic fiber using a mating sleeve.
    b. To avoid potential light leak, cover the connection with black tape.
    c. Start Intan RHD2000 Interface software. We set the recording parameters as follows: Sampling rate 20 or 30 kHz/s, filter the signal online 0.10 Hz–7.50 kHz.
    d. Run the impedance measurement to make sure the wires are connected and in a good range of impedance (100–500 kΩ).
    e. Turn on online streaming to look for spiking activity in any of the 32 channels.
16. Look for spiking response to tail pinch and/or jaw opening.

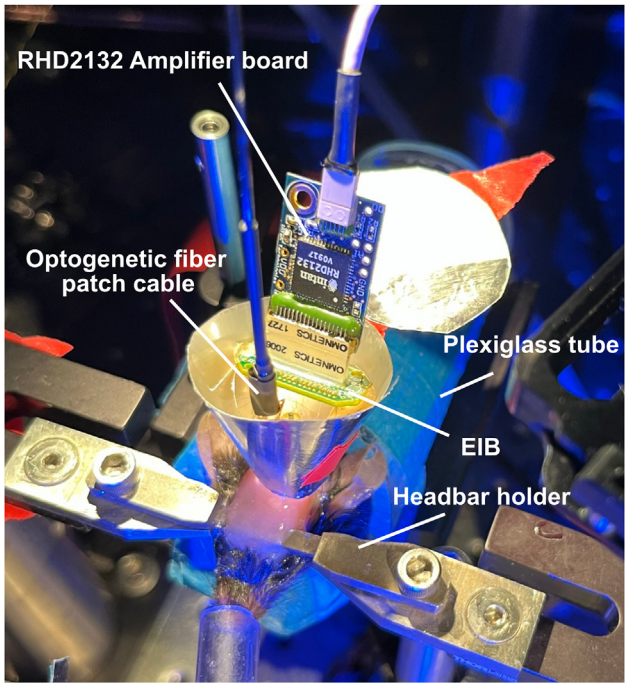

Figure 5. Recording setup

The mouse is placed in a plexiglass tube and then head-fixed. The protective hat is open, and the amplifier board is plugged in the electrical interface board. For the optogenetic, the ferrule of the fiber patch cable is connected to the optic fiber with a mating sleeve. This connection is covered with black tape to prevent light leakage.

Note: The trigeminal mesencephalic nucleus (Me5) is located right next to the LC, on the lateral side. Me5 neurons fire when the muscles of the jaw are stretched; it can be used as a landmark to know if the optrode gets closer to the LC.

17. Start optogenetic stimulation.
    a. Use a train of 200–300 ms pulses at 0.3–0.5 Hz.

Note: We choose a longer pulse duration than what has been typically used in the literature (5–10 ms).[10] We found that using a longer pulse evokes a train of action potential rather than a single action potential that is more reliable and easier to identify. Typically, tagged units show first spike latency < 10 ms with respect to pulse onset.[11] We also have found some units that have a latency of response to optical stimulation >10 ms as reported by others.[12,13] The unit's consistent and short-latency responsiveness to the optogenetic stimulation serves as a hallmark to identify opto-tagged LC units.

    b. Look for units responding to the laser stimulation (Figure 6A).
    c. Using the Spike Scope, check the shape of the action potential.
18. Check the pupil response to verify the targeting of LC with the optrode.
    a. While doing the opto-tagging, turn on the camera and monitor the change in pupil diameter.

Note: A 300 ms laser pulse should activate LC neurons (even if they can't be recorded by the optrode) and evoke a noticeable pupil dilation (Figure 6B). Pupil responses to optogenetic stimulation serve as an important proxy to ensure that the tetrode implant is correctly targeting the LC.[14] Indeed, observable pupil dilation in response to opto-tagging can occur even when no unit is recorded by the tetrode. This serves as a useful indicator to slow down or pause the lowering of the optrode to monitor for nearby unit activity. We typically begin to

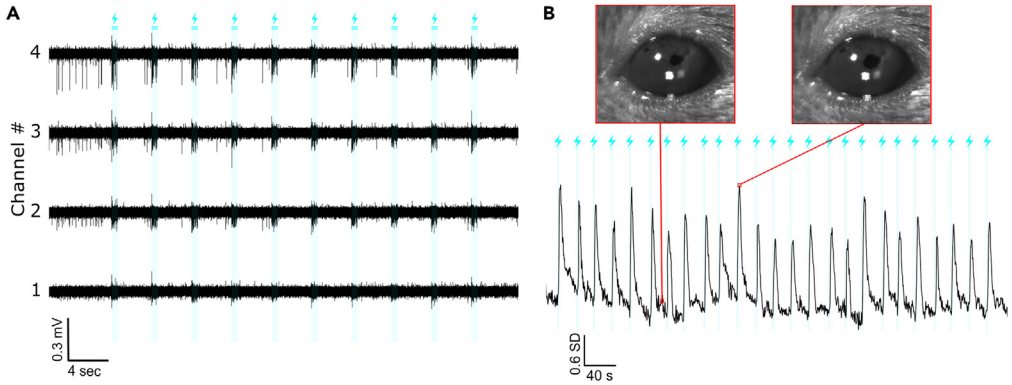

Figure 6. Opto-tagged LC unit and pupil tagging

(A) Example of the response of a LC neuron to 300 ms laser pulses (10 mW). The unit can be recorded clearly on the channel 4 of the tetrode. The same unit is also recorded in channel 2 and 3, but not clearly in channel 1.

(B) Variation of the pupil size in response to the opto-stimulation of LC in an anesthetized mouse. The two example pictures show the visible difference in size of the pupil before and after the laser stimulation.

look for LC units after confirming a pupil response. If the pupil is too dilated, it might be difficult to see the pupil dilation. Decrease the level of isoflurane (1.5%) or increase ambient light level inside the recording rig to constrict pupil. Once the pupil is smaller (like the example in Figure 1), test the pupil response to optical stimulation again.

19. Once an opto-tagged LC unit is identified, note the channel number.
20. If no opto-tagged unit can be identified:
    a. Turn off the recording and unplug the RHD2132 Amplifier Board.
    b. Advance the microdrive of about ~100 μm.
21. Turn off the isoflurane and let the animal completely recover before proceeding.
22. If an opto-tagged unit has been identified, you can proceed to the recording. If not, start again from step 15 on the next day.

Dual recording pupil/locus coeruleus activity

Timing: 2 h

Once an opto-tagged LC unit is acquired, the dual recording can be performed. In this section we detail the process to conduct the recording.

Note: Although we typically perform simultaneous LC-pupil recordings in awake behaving animals, this protocol can be applied to animals under different arousal/behaving conditions (e.g., under anesthesia, quiet awake, or awake behaving)

23. At least 1 day before the day of the recording, put the mouse in the recording rig and head-fix for habituation.
24. Put the animal in the plexiglass tube, place the tube on the recording rig and secure the head bar.
25. Remove the cover of the protective cone, plug in the amplifier board and the optic fiber to the tetrode.
26. Turn on the camera and the laser.
27. Check the signal from the optrode to confirm the presence of opto-tagged units.
    a. Start the different software used to perform the recording.
        i. Intan for recording LC neurons.
        ii. MATLAB with WaveSurfer to control laser.
        iii. Pylon Viewer for the camera.

Note: The optical zoom and focus of the camera are adjusted for individual mice and do not change across the days of recording to allow for comparing pupil size between experiments.

Tips: Recording spiking activity evoked by optical stimulation in the same file as the spontaneous activity will allow for the spontaneous and opto-evoked spikes to be sorted together, thus facilitating the identification of LC neurons.

28. Remove fiber optic cables and unplug the amplifier board before releasing the mouse from the head-restraint clamps.
29. Put the mouse back in the housing cage.

Note: To facilitate the synchronization of all the recording offline, the Intan recording should be started first and stopped last so that it contains the onset and offset of the TTL pulse from the pupil camera.

Spike signal sorting

Timing: 1 day

After recording the neural activity, spikes are sorted offline to isolate single units for classification and analysis. We have performed LC spike sorting using MClust (Redish Lab, https://redishlab.umn.edu/mclust). It is a MATLAB-based spike sorting toolbox for the separation of putative cells from multi-site neurophysiological recordings.

30. Export Intan data on MATLAB using the m-file provided by Intan Technologies. (https://www.intantech.com/downloads.html?tabSelect = Software&yPos = 0_).
31. Export the data for spike sorting using MClust.
32. MClust spike sorting.
    a. Use different Principal Component analysis to separate and isolate the potentials units.

Note: We find using Energy and Peak usually yields better clustering (Figure 6), Valley would also be useful in some cases.

    b. For each cluster of neurons, we require clustering quality measures as L-ratio < 0.05 and ID > 20. Those parameters can be found in the window "Check cluster".
    c. Export the clustering by writing the *t-files*. Those files contain time stamps of sorted spike events.

Note: See MClust manual for more detailed instructions on sorting (https://redishlab.umn.edu/sites/redishlab.neuroscience.umn.edu/files/2021-04/MClust-4-4%20documentation.pdf).

Analyze pupil size variation

Timing: 2–3 h

In this section, we describe the steps to analyze pupil size variations using the artificial neuronal network in DeepLabCut as described in "before you begin".

33. Load the neural network and load the video to analyze.
34. Run DeepLabCut analyze with the parameter "Yes" for "Want to save result as csv.?".

Note: The .csv file will contain the coordinate of each tracking point for every frame of the video.

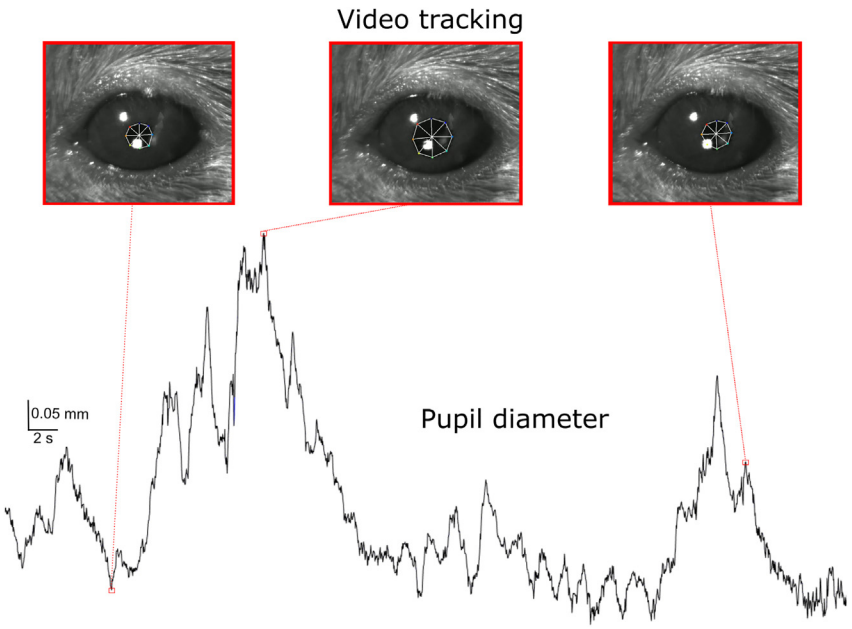

Figure 7. Representative example of the variation of pupil size in an awake mouse
For 3 selected time points you can see the corresponding frame of the video.

Optional: We recommend creating the labeled video after the tracking of each video. Doing this visual control quality will ensure the accuracy of pupil tracking.

35. Extract the size of the pupil (Figure 7).
    a. Extract the diameter of the pupil by measuring the distance between 2 opposite points.

Note: As each point has a likelihood score (between 0 and 1, 1 meaning the tracking was accurate), you can select the most reliable pair of tracking points to have the best estimate of pupil diameter.

Optional: The measured distance will be in pixels. As we z-score the pupil variation in our analysis we haven't converted the pupil size to mm. To convert the unit to mm, a calibration is needed.

36. Detect pupil events. For this step we used the zero-crossing of pupil derivatives method described in detail by Joshi et al.[15]
    a. The raw pupil diameter data was first smoothed using a median filter.

Note: We used a window width of 500 ms for the filter, but this parameter can change. This will reduce the noise of the data and remove potential artifacts due to blinking.

    b. Calculate the derivative of the smoothed data to obtain the rate of change of the pupil diameter over time.
    c. Find all the zero-crossings in the derivative data, which indicate a change in the slope direction.
    d. Identify the pupil events as local maxima (positive slope, dilation) and minima (negative slope, constriction) between each pair of zero-crossings.

Synchronizing pupil and electrophysiology recordings

Timing: 1 h

Once the spike sorting process is done and the pupil size variation extracted you can synchronize the 2 recordings. The pupil video and the LC activity are recorded on different computers. The small difference between the internal clocks will cause a slow drift over time. When the recording is ~ 1 h, the shift could be on the order of hundreds of milliseconds, which renders correlating two time series inaccurate.

37. Check if the number of frames matches.
    a. In the Intan file, count the number of TTL pulses in the pupil video based on the recorded TTL pulse train.
    b. The number of pulses in the Intan file should match (± 1 frame) the number of frames in the .csv file from DeepLabCut and the number of frames in the pupil video.

Warning: If there is a mismatch in the number of frames, synchronization with LC recording would be challenging, as we need to locate where in the recording the frames were dropped.

38. Synchronize the 2 recordings (Figure 8).
    a. Using the onset of the TTL pulse, align the recordings from the 2 computers (pupil diameter and spiking activity of the LC unit).
    b. Check the end of the TTL pulse train for both recordings. You will have a small drift that needs to be readjusted.
    c. There are various ways to correct the drift. We take the simplest one by scaling the duration of one recording to match the other.
39. Once the 2 recordings are synchronized, the relationship between neural activity and variation in pupil size can be analyzed.

Note: See our study about pupil size change and LC spiking[1] for example, as well as Joshi et al.[15] and Cazettes et al.[16] for other examples of correlation between change on pupil size and neural activity from other brain area.

Histology

Timing: 2 days

At the end of the experiment, an electrolytic lesion is made before perfusing the animal to be able to visualize the location of the tetrode (Figure 9).

40. Deeply anesthetize the animal with 5% isoflurane.
41. Make the lesion using a 50 μA current for 50 s targeting the tetrode where the LC units have been recorded.
42. Euthanize the animal with an overdose of Ketamine/Xylazine.
43. Perfuse the animal with PBS followed by 4% paraformaldehyde, postfix the brain overnight.
44. Cut the brain into 100-μm coronal sections.
45. Stain the slices with anti-tyrosine hydroxylase antibody (Primary antibody: 1/1000, Tyrosine Hydroxylase Polyclonal Antibody, Thermo Fisher, OPA1-04050, secondary antibody: 1/1000, Goat anti-Rabbit IgG (H + L) Secondary Antibody, Alexa Fluor 488, Thermo Fisher, A11008) to allow visualization.

EXPECTED OUTCOMES

The 32-channel optrode recording technique is used to find and identify opto-tagged LC units that can be combined with tracking of pupil size variation. The present protocol has been used to show that pupil diameter has an overall positive and monotonic relationship with LC spiking activity. Only the infrequent and large pupil dilation events (>1.5–2 SD amplitude, <10% occurrence, Figure 10) can accurately

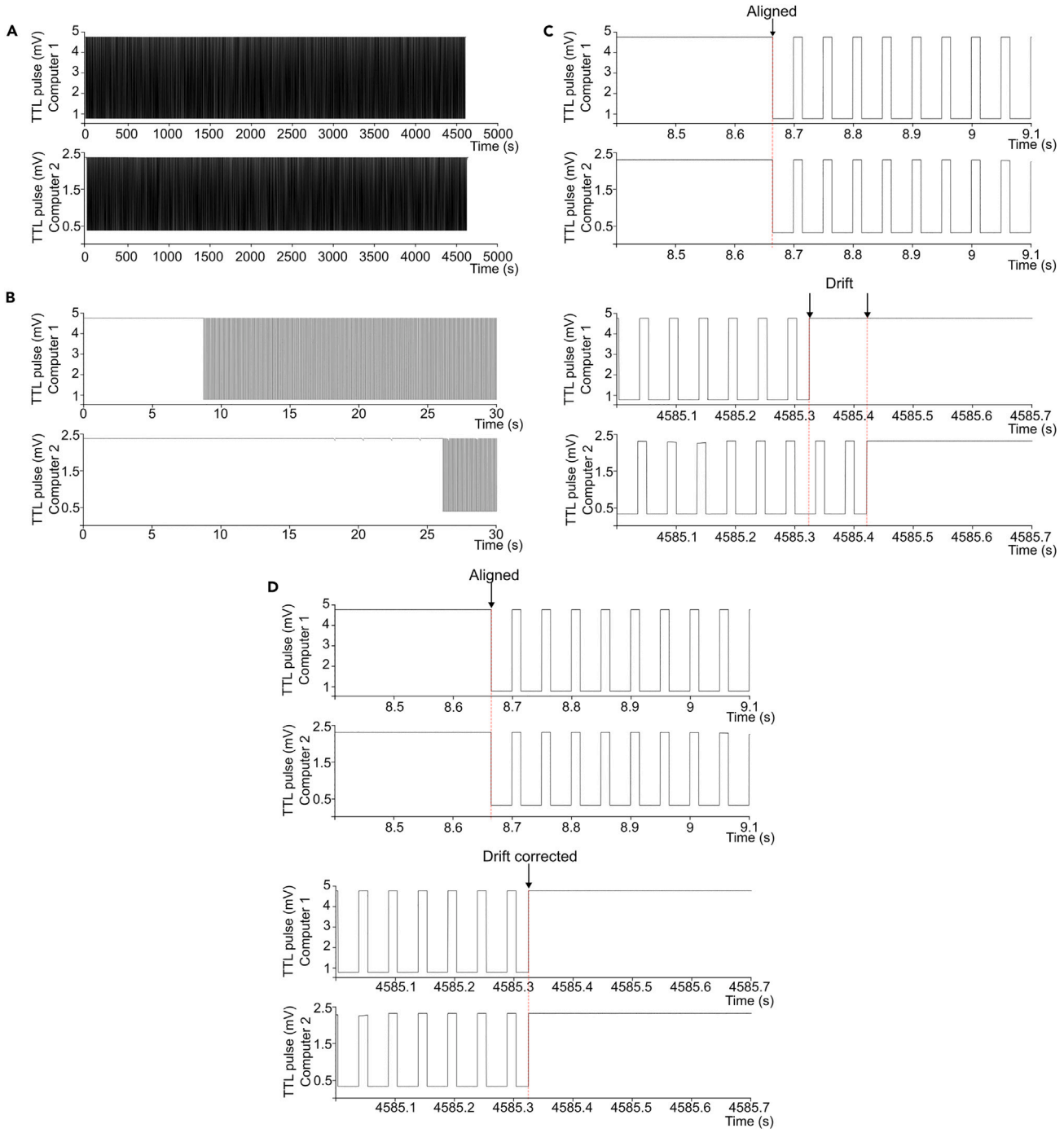

Figure 8. Aligning different recordings

(A) Example simultaneous recordings of the TTL pulse from 2 computers.

(B) Close up of the beginning of pulse train. As the 2 recordings are started separately, the onset of the TTL pulse is different.

(C) The 2 recordings are aligned based on the onset of the TTL pulse (upper panel). The small drift between the 2 computers introduces a mismatch in the offset of the TTL pulse (lower panel).

(D) After correcting the drift, the onset and offset of the TTL pulse are perfectly aligned.

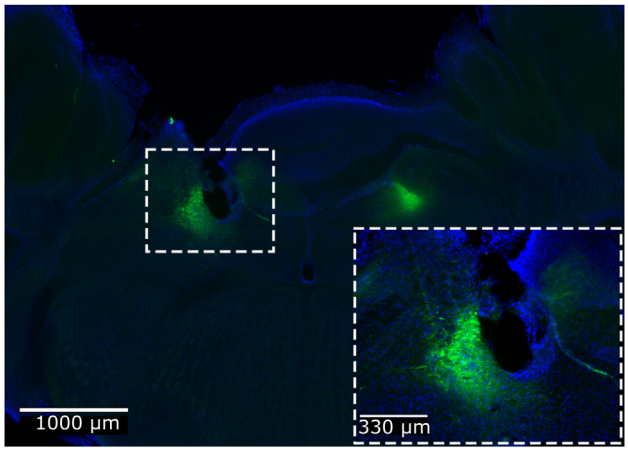

Figure 9. Representative example of an electrolytic lesion in LC after recording with an optrode

Blue: DAPI, Green: LC neurons visualized with the Tyrosine Hydroxylase antibody. The lesion indicates that the optrode was correctly aiming at the LC.

predict LC spiking on a moment-by-moment basis. Monitoring pupil to check optrode placement can inform when to speed up or slow down the advancement of the implant.

The current protocol is to link pupil size variation with LC activity. The protocol integrates two primary methods: 1) pupil size tracking and 2) LC tetrode recording. These two recordings can be

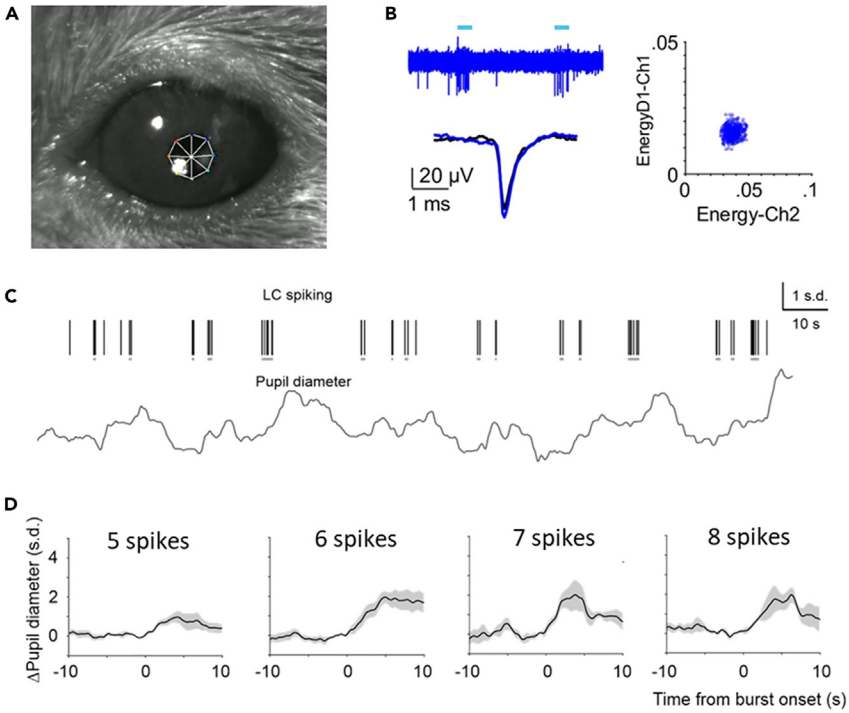

Figure 10. Example of pupil tracking (Z-scored) and the LC spiking

Data from Megemont et al.[1]

(A) Example of pupil tracking.

(B) LC unit spike sorting. Blue bars indicate laser stimulation, evoking action potentials in the recorded neuron.

(C) Synchronized LC spiking and pupil variation (Z-scored).

(D) Mean LC cluster-triggered pupil responses (± standard error of the mean [SEM]) for cluster sizes. The time 0 corresponds to the onset of the first AP of the cluster. Adapted from Megemont et al.[1]

used either in combination (with proper synchronization)—or independently. Beyond its primary focus on LC neurons, this protocol offers the flexibility to be adapted for neural recordings in other brain regions as well. One of the advantages of using the tetrode in this setting is its robustness. In our own experiments, we have observed that the implant remains functional in mice for as long as three months after the surgical procedure. However, it should be emphasized that recording a unit across several days is challenging. Therefore, if the protocol is used in a behavioral study, we recommend lowering the implant at the targeted recording site only right before the intended recording session. Another advantage is the implant's lightweight, weighing approximately 5 g, which minimizes interference with normal behavior. Post-surgical recovery for the mice is quick, with animals resuming normal locomotion within a few days. This allows to conduct studies involving either head-fixed[17] or freely moving behavioral paradigms. However, a critical limit to consider is that pupil tracking is more challenging when employing freely moving experimental conditions, but has been achieved in a recent study[18] using a miniature camera mounted of the head of the mice). Moreover, video recording and DeepLabCut tracking can be used to track other parts of the body, not only the change of pupil size. Indeed, neural activity related to movement is increasingly recognized to play important role in the response of non-motor related brain areas.[19,20] Tracking facial movement for example (eyelid, nose, whisker pad, lower lips) can be easily achieved by creating a dedicated neural network on DeepLabCut.

LIMITATIONS

A home-made tetrode system can be time-consuming to build compared to commercially available systems (like silicon probes), but it's cost-efficient. The size of the implant needs also to be considered when combined with other recording/perturbation methods, such as optogenetic stimulation of another brain structure.

TROUBLESHOOTING

Problem 1

Optrode not functional (step 6 – step-by-step method details).

Some tetrode channels are disconnected.

Potential solution

If the pin is not correctly inserted in the EIB, it can result in a poor connection between the wires and the board. Try to push the pin back inside the EIB to ensure proper contact. Additionally, the wire can also be damaged during the surgery to implant the optrode. To prevent this, it is important to make a craniotomy large enough and that the electrode bundle does not touch the skull during the implantation process. This can help to minimize the risk of damaging the wire and ensure a stable recording.

Problem 2

Signal is noisy (step 15 – step-by-step method details).

Signal-to-noise ratio is not good due to background noise.

Potential solution

The good quality signal in our protocol relies on both reference tetrode and the grounding. For the reference tetrode, we recommend to gold-plate to adjust the impedance to 10 kΩ, and make sure it's well connected on the EIB. For the grounding, the placement of the grounding screw during the surgery is important. It should be positioned at a distance from the recording tetrodes but also in a location where it won't meet the headpost (as shown in Figure 4). This is to prevent any electrical interference that could affect the quality of the recorded signals.

Problem 3

Not being able to record LC (step 23 – step-by-step method details).

Unable to find an opto-tagged unit in the locus coeruleus, even though tetrode is advanced beyond the ventral boundary of LC.

Potential solution

Targeting LC is challenging. Indeed, this nucleus is small and deep in the brain. After each implant, do the electrolytic lesion, perfuse the animal and perform histology to adjust the coordinate if needed. Pupil response to laser stimulation is a very good proxy to know if the problem comes from a misplacement of the implant. It is important to keep in mind as LC is a small nucleus, it might be hard to record from it even with precise targeting. When the pupil dilation is observed, we recommend slowing down the advancing of the optrode to enhance the chances to record an opto-tagged unit.

Problem 4

Infection/implant is ripped off (step 13 – step-by-step method details).

The surgery to implant the optrode is time consuming and sophisticated, making the animal at risk of not recovering properly or ripping off the implant when head-fixed in the rig.

Potential solution

When performing surgery to implant an optrode in a mouse, it is important to be careful about the conditions of the mouse during the surgery. This includes maintaining the body temperature, using an appropriate level of isoflurane, and maintaining aseptic conditions, including autoclaving the surgery tools. Make sure not to skip the step of scratching the skull surface and cleaning again after to remove any dust. This greatly increases the durability of the implant. Additionally, it's important to use the appropriate amount of antibiotics to prevent infections. When habituating the mouse to head fixation, monitor the mouse, and shorten the time of fixation if the animal is trying to escape too vigorously. Some animals need prolonged habituation time to be able to stay quietly head fixed.

Problem 5

Pupil tracking isn't accurate (step 34 – step-by-step method details).

Your pupil tracking doesn't accurately measure the pupil dilation, or you have a lot of glitches during the tracking.

Potential solution

First, verify that the infrared light conditions are adequate for clearly distinguish the pupil and ensure that the pupil is properly focused during video recording. If despite good quality video, tracking issues persist, consider work on your DeepLabCut neural network. Increase the number of labeled video and train again the network until reaching a satisfactory performance. You can also visit the DeepLabCut help page (https://deeplabcut.github.io/DeepLabCut/README.html#) for any problem related to video tracking.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Marine Megemont (marinem@ucr.edu).

Technical contact

Technical questions should be directed to and will be fulfilled by the technical contacts, Marine Megemont (marinem@ucr.edu) and Hongdian Yang (hongdian@ucr.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Original data for Figure 10 in the paper is available on https://doi.org/10.7554/eLife.70510.

Technical sketch for the tetrode holder is available on https://doi.org/10.5281/zenodo.10070203.

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AUTHOR CONTRIBUTIONS

M.M., J.M.-L., J.Y.C., D.H.O.C., and H.Y. conceptualized the methods. M.M., L.S.T., and J.M.-L. performed the experiments. M.M., L.S.T., J.M.-L., and H.Y. performed the analysis. M.M., L.S.T., and H.Y. drafted the manuscript with contributions from all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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