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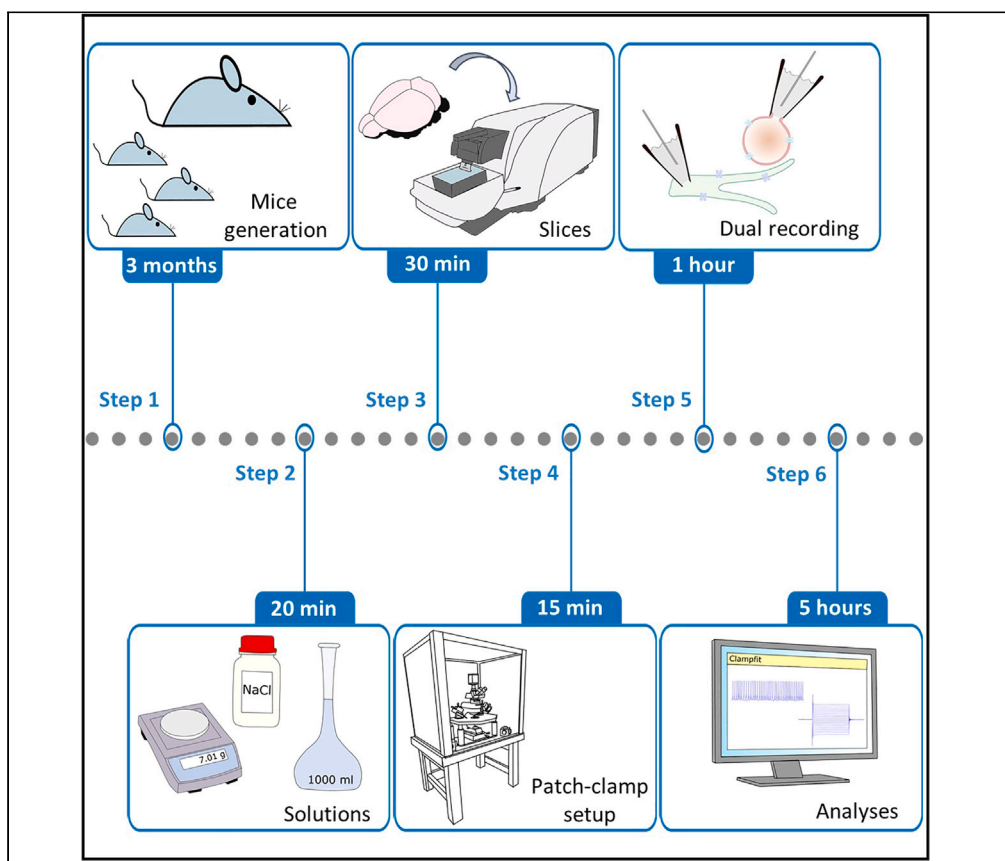
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Protocol

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Here, we present a protocol for tanycyte-neuron paired whole-cell patch-clamp recording in living mouse brain slices. We describe steps for mice generation, solution preparation, and dissection. We then detail realization of slices and patch-clamp recordings. While we use, as an example, tanycytes of the arcuate nucleus of the hypothalamus and pro-opiomelanocortin neurons, this protocol can be adapted to study metabolic coupling between tanycytes and any neuronal population.

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

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Highlights

Key steps for mouse brain extraction and viable living brain slices preparation

Procedure for setting up a patch-clamp experiment in the hypothalamus

Detailed approach for paired whole-cell patch-clamp recording of tanycytes and neurons

A protocol describing the analysis of electrophysiological data

Lhomme & Prévot, STAR
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Protocol

Protocol for simultaneous patch-clamp recording from tanycytes and neurons in living mouse brain slices

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SUMMARY

Here, we present a protocol for tanycyte-neuron paired whole-cell patch-clamp recording in living mouse brain slices. We describe steps for mice generation, solution preparation, and dissection. We then detail realization of slices and patch-clamp recordings. While we use, as an example, tanycytes of the arcuate nucleus of the hypothalamus and pro-opiomelanocortin neurons, this protocol can be adapted to study metabolic coupling between tanycytes and any neuronal population.

For complete details on the use and execution of this protocol, please refer to Lhomme et al. (2021).¹

BEFORE YOU BEGIN

Whole-cell recording in mouse brain slices is a well-known electrophysiological technique to study the electrical properties of single neurons.

The advantage of performing recordings in brain slices, as compared to other types of preparations such as cells in culture or isolated neurons, is that it allows the neuronal and glial environment of the targeted neuron to be preserved. Thus, using these preparations, it is possible to investigate the influence of glial cells such as astrocytes or tanycytes on the electrical activity of a neuron.

In a recent publication, a method based on optogenetics, allowing tanycytes to be activated or inhibited, was used to test the influence of these glial cells on the activity of neurons regulating food intake.²

Here, we present a technique to more precisely modulate a tanycytic cellular pathway using the patch-clamp technique in whole-cell configuration in order to investigate its consequences to the electrical activity of POMC neurons.¹ The rupture of the plasma membrane in this configuration allows the intrapipette medium to diffuse freely into the cytoplasm, and can be used to introduce pharmacological agents to manipulate very specific targets without altering the function of neighboring cells.

Institutional permissions

The presented study was performed on animals and was carried out with the approval of the Institutional Ethics Committee for the Care and Use of Experimental Animals of the University of Lille and the Ministry of Higher Education and Research (APAFIS#2617-2015110517317420 v5 and 13387-2017122712209790 v9), and under the guidelines defined by the European Council Directive of 22 September 2010 (2010/63/EU).



Generation of POMC-tdTomato mice

⌚ Timing: 90 days

Tanycytes are easily differentiated from neurons under infrared differential interference contrast (IR-DIC) microscopy (Figure 1). They are localized along the surface of the third ventricle. The cell bodies of arcuate tanycytes have a ladder-bar-like appearance in the wall of the third ventricle in coronal brain slices containing the tuberal region of the hypothalamus (Figure 1A). In contrast, to record specifically from POMC neurons, transgenic mice in which these neurons spontaneously express a fluorescent marker are needed. The breeding strategy is described in Figure 1B.

1. Purchase 2-month-old male *Pomc-cre* and female *Rosa26-loxP-stop-loxP tdTomato* mice.
2. After an acclimatization period of at least one week, house one male *Pomc-cre* with two female *Rosa26-loxP-stop-loxP-tdTomato* mice in a temperature-controlled room (21°C–22°C) on a 12-h day/night cycle, with free access to water and standard food.
3. Check for vaginal plugs on the following morning (between 7.30 a.m. and 9.30 a.m.) to identify potentially pregnant females and pinpoint the beginning of gestation.
4. Transfer these females to individual cages.
5. Monitor mice daily for good health and check for births between 19.5 and 21.5 days of gestation.
6. Control litter size. The average is 6–8 pups per litter. This can influence metabolism, reproductive function and behavioral development.
7. Wean the pups on postnatal day 21 (P21).
8. Genotype mice according to the protocol provided by the supplier and select *Pomc-tdTomato* mice (Figure 1B).^{3,4}
9. Maintain the lineage on the C57BL/6J genetic background. To do this, backcross male *Pomc-tdTomato* mice with female *Rosa26-loxP-stop-loxP-tdTomato* mice.

Preparation of solutions

⌚ Timing: 2.5 h

⌚ Timing: 30 min (for step 10)

⌚ Timing: 2 h (for step 11)

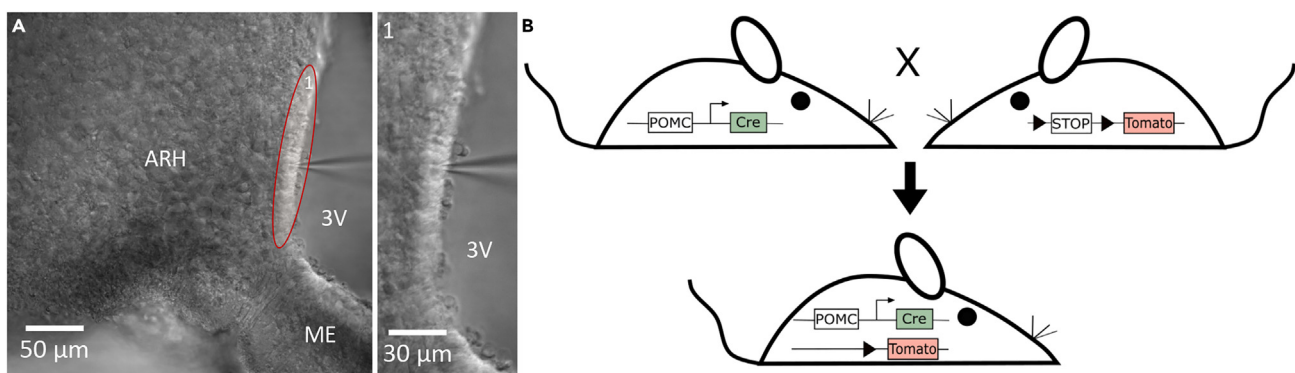


Figure 1. Visualization of tanycytes in infrared coupled differential interference contrast (IR-DIC) and generation of *Pomc-tdTomato* mouse model
(A) Anatomical distribution and shape of tanycytes on coronal hypothalamic slice delineated by the oval shape. ARH: arcuate nucleus of the hypothalamus. ME: median eminence. 3V: third ventricle.
(B) Crossbreeding *Pomc-cre* and *Rosa26-loxP-stop-loxP-tdTomato* mice to obtain *Pomc-tdTomato* mice.

10. Prepare two 1 L batches of fresh artificial cerebrospinal fluid (aCSF) the day of experimentation. Refer to the materials and equipment for a list of recipe table (Figure 2A).
 - a. For each batch of 1 L, in about 2/3 of the total volume of distilled water, dissolve 7 g of sodium chloride (NaCl), 0.24 g of potassium chloride (KCl), 0.12 g of sodium dihydrogen phosphate (NaH_2PO_4), 2.18 g of sodium bicarbonate (NaHCO_3) and 0.45 g of D-glucose.
 - b. Then add 1 mL of 1 M magnesium chloride (MgCl_2) and 2 mL of 1 M calcium chloride (CaCl_2) while stirring the solution to avoid precipitation.
 - c. Make up the volume to 1 L with distilled water.
 - d. Check the osmolarity of the solutions, which should be about 295 mOsm.
 - e. Use one batch for dissection and slices preparation and the other for recordings (Figure 2A).
 - i. Gas the dissection solution with carbogen to saturate it with oxygen and obtain a pH of 7.4.
 - ii. Keep the dissection solution on ice to reach a temperature of 4°C.
 - iii. Allow the recording solution to reach room temperature (about 20°C) and oxygenate it just before recordings.
11. Prepare the intrapipette medium for tanyctes and POMC neurons. Refer to the materials and equipment for a list of recipe tables.
 - a. First, prepare a solution of potassium hydroxide (KOH) 2.5 mM by dissolving 1.4 mg KOH in 10 mL distilled water.
 - b. Then, prepare a 50 mL solution of 10 mM HEPES by dissolving 119.15 g of HEPES in 50 mL of distilled water. Adjust the pH to 7.3 with KOH 2.5 mM.



Figure 2. Representative images of tools required for brain extraction and slices preparation

(A) aCSF solution. 1. Dissection solution. 2. Recording solution.

(B) Overview of the disposition of all equipment for brain dissection and slices preparation.

(C) Dissection area. 1. Absorbent paper. 2. Dissection tank. 3. Micro-scissor. 4. Fine scissor. 5. Forceps. 6. Scissor. 7. Spatula. 8. Bent tip forceps. 9. Petri dish. 10. Whatman filter paper disk. 11. Vibratome sample holder. 12. Razor blade. 13. Glue. 14. Lamp.

(D) 1. Petri dish. 2. Base of Petri dish removed. 3. Nylon mesh glued instead of the base of Petri dish. 4. Basket for slices finalized. 5. 150 mL beaker. 6. Bubbling compartment. 7. Side view of incubation chamber assembled. 8. Top view of incubation chamber assembled. 9. Carbogen bubbler. 10. Water bath with incubation chamber.

(E) Vibratome. 1. Ice tank. 2. Buffer tank. 3. Razor blade on blade holder. 4. Syringe with inclined needle. 5. Dropper for transferring slices.

- c. To 15 mL of HEPES, add while stirring 576.13 mg of potassium gluconate (K-gluconate), 11.93 mg of KCl, 1.52 mg of EGTA, 44.09 mg of Na₂-ATP, 3.13 mg of Na-GTP and 40 μL MgCl₂ 1 M.
- d. For the intrapipette medium for tanyocytes, add the chemical or drug to be tested. It must be added, at the desired concentration.
- e. Adjust the volume to 20 mL with 10 mM HEPES solution.
- f. Filter the solution and check the osmolarity (275 mOsm) and the pH (7.3).
- g. Make aliquots of 500 μL in Eppendorf tubes and store at −20°C.
 - i. Mark the Eppendorf tubes with neuron and tanyocyte intrapipette solutions so that they can be differentiated.
 - ii. Store during about 6 months.

Preparation of materials for making mouse brain slices

⌚ Timing: 1.25 h

⌚ Timing: 15 min (for step 12)

⌚ Timing: 45 min (for step 13)

⌚ Timing: 15 min (for step 14)

(Figures 2B–2E)

All the materials and equipment described below must be available nearby (Figure 2B).

12. Prepare the dissection area (Figure 2C).
 - a. On absorbent paper, place the dissection tank in which brain extraction will be done, a spatula, a 6 cm diameter Petri dish, a Whatman filter paper disk, the vibratome sample holder, a razor blade (Gem Scientific), the glue and the lamp.
 - b. Add dissection instruments (micro-scissor, fine scissor, scissor, forceps, bent tip forceps). The layout of the materials and the different dissection tools can be found in Figure 2C.
13. Preparation of the slice incubation chamber (Figure 2D)
 - a. Make an incubation chamber containing two compartments, the basket for slices and the bubbling compartment. The basket for slices holds slices and the bubbling compartment allows the installation of the carbogen bubbler.
 - i. For the basket for slices, replace the base of a 3.5 cm diameter Petri dish by a nylon mesh Figure 2D.1–4.
 - ii. For the bubbling compartment, take a 10 mL syringe, remove the plunger and cut at the 3 mL mark.
 - iii. Place these two compartments in a 150 mL beaker as shown in Figure 2D.5–8.
 - b. Add approximately 100 mL of aCSF in slices compartment.
 - c. Remove any bubbles under the basket for slices with a dropper, sucking up and ejecting the liquid.
 - d. Place the incubation chamber in a water bath at 34°C and oxygenate the aCSF continuously with a carbogen bubbler.
14. Preparation of the vibratome (Figure 2E).
 - a. After powering up the apparatus, set the following parameters:
 - Cutting speed: 0.12 mm per second.
 - Amplitude: 2 mm.
 - Thickness of the slices: 250 μm.
 - b. Place the buffer tank in the ice tank.

- c. Put ice in the ice tank around the buffer tank.
- d. Install the whole on the vibratome.
- e. On the blade holder, place a razor blade (Feather) that has been cleaned beforehand to remove all residual glue, then tilt it to the cutting position.
- f. Next to the vibratome, place a syringe with a bent 26G needle that will be used to manipulate slices, as well as a plastic dropper for transferring slices.

Preparation of patch-clamp pipettes for tuncytes and neurons

⌚ Timing: 45 min

15. Configure the stretching program to have tuncyte and neuron pipettes with respectively a resistance of 7 M Ω and 6 M Ω (Figure 3A).

Note: Different parameters (Heat, Pull, Velocity, Time and Pressure) need to be added. These parameters depend on the filament of the puller, the glass capillaries and pipettes characteristics required. The Heat setting for the filament is determined by running a Ramp Test. It is required to adjust others parameters in function of characteristics pipettes; refer to the notice of the puller. Here is detailed an example of a stretching program to pull pipettes for neurons and tuncytes:

Heat: 478 units.

Pull: 0 unit.

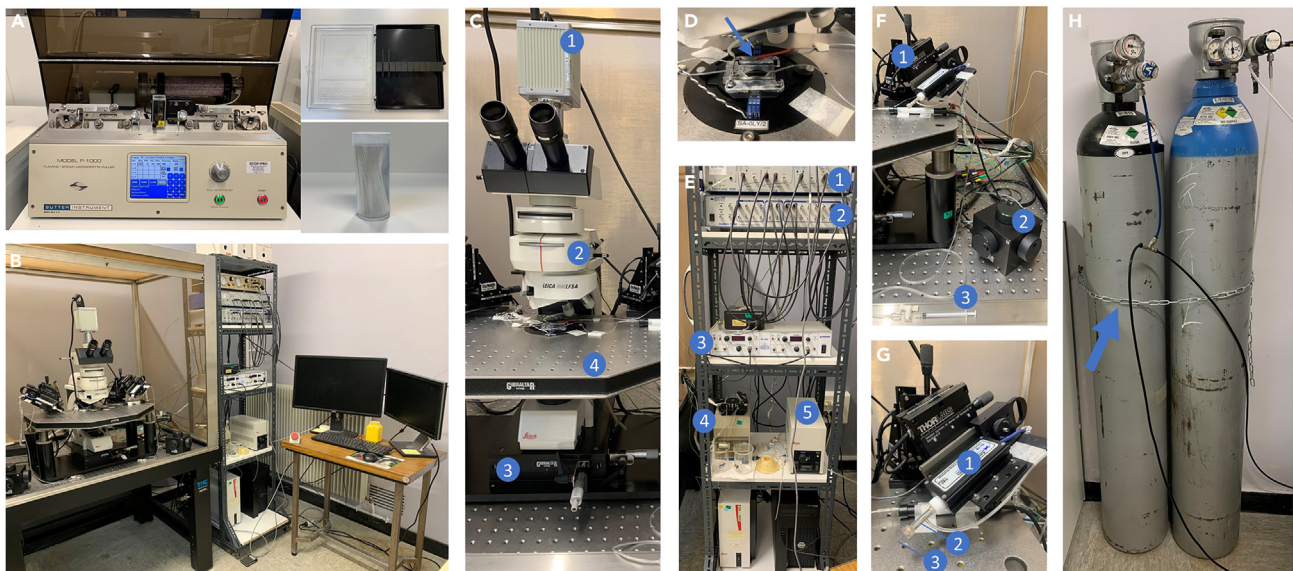


Figure 3. Representative images of patch-clamp setup

- (A) Pipette puller.
- (B) Overview of all equipment to recording.
- (C) Zoom on the camera (1), microscope (2) and the two parts of manual XY translation platform (3 and 4).
- (D) Zoom on the recording chamber with the reference electrode placed in the bath (arrow).
- (E) Zoom on the rack containing the amplifier (1), digidata (2), heater controller (3), peristaltic pump (4), fluorescent lamp (5).
- (F) Zoom on the two parts of micromanipulator (1 and 2) and the system (syringe + tubing) to regulate the positive pressure (3).
- (G) Zoom on the headstage (1) with its holder electrode (2) and chlorinated silver wire (3).
- (H) Image showing nitrogen and carbogen bottles secured by a wall-mounted chain (arrow).

Velocity: 79 units for neurons and 81 units for tanyocytes (increasing the velocity parameter for the tanyocyte pipette allows for an increase in the pipette resistance and enables to reduce the diameter of its tip).

Time: 250 units.

Pressure: 500 units.

16. Center the glass capillary under the heating filament of the stretcher with care, these filaments are very fragile and expensive.
17. Start the "pull cycle".
18. Collect the pipettes and store them in a closed pipette storage box, protected from dust, until recording.

Setting up an electrophysiology setup

⌚ Timing: 3 days

Electrophysiology setup (Figures 3B–3H)

Step	Details	Function
19	Install the Faraday cage and an anti-vibration table . Connect the antivibration table to a nitrogen bottle . Regulate the level of the table by using pneumatic regulators and adjusting the height of the legs.	Isolates the patch clamp set-up from noise from external interference or floor vibrations that could disturb the signal.
20	Place a carbogen bottle near the Faraday cage and anti-vibration table. It is important to secure these bottles with a wall-mounted chain; they can be dangerous when knocked over.	Is used to oxygenate the aCSF that perfuses the slices.
21	Place the manual XY translation platform in the Faraday cage, on the anti-vibration table.	The platform allows to screw different tools such as micromanipulators and recording chamber. The manual control allows the stage to be moved along the X and Y axes to precisely position the region of interest of the slice during the experiment.
22	Install the recording chamber on the manual XY translation platform.	Compartment into which the slices are transferred to be observed.
23	Place the microscope , equipped with fluorescence and infrared (IR) sources and capable of differential interference contrast (DIC), on the manual XY translation platform in such a way that the recording chamber is below the objective. Connect the fluorescent lamp to the microscope.	Allows slices to be observed in IR-DIC and fluorescence modes.
24	Install the camera on the microscope and connect it to a computer with image analysis and processing software.	Allows images of the slices to be observed on a monitor and uploaded.
25	Install the perfusion system as follows: connect the recording chamber to a peristaltic pump with PVC tubing, which are connected to the chamber via polyethylene tubing with the help of coupler. Adjust the flux rate to about 2 mL/min.	Continuously perfuse the slices with oxygenated aCSF.
26	Connect the temperature controller to the recording chamber.	Allows recordings to be done at a constant temperature.
27	Place a silver wire chlorinated with bleach (i.e., AgCl-coating) in the electrode holder to form the recording electrode.	It is on the electrode holder that the patch pipette containing the intrapipette medium is placed to record from a cell.
28	Connect the electrode holder to a headstage . Then, place the reference electrode in the bath and connect it to the headstage.	This configuration allows ionic variations between the bath and the intrapipette medium to be detected during recording, and converts these variations into an electrical signal.
29	Attach a micromanipulator on the manual XY translation platform. Then, attach the headstage to the micromanipulator.	Allows fine movements of the recording electrode over small distances along the X, Y and Z axes and thus the accurate and stable positioning of the pipette on the cell membrane.
30	Connect a 1 mL syringe to the electrode holder via Tygon tubing.	Allows the positive pressure in the pipette to be regulated during the patch-clamp approach.
31	Connect the headstage to an amplifier which must itself be connected to a Digidata .	The amplifier amplifies the electrical signal and the digitizer converts this signal into a digital signal.
32	Repeat steps 27–31 for the second headstage.	Allows a dual patch recording to be carried out.
33	Connect the amplifier and the digitizer to a computer equipped with signal acquisition and analysis software.	Allows the digital signal to be collected.
34	Make the necessary connections of the different devices to the ground with ground connectors.	Allows noise reduction and the generation of a clean and analyzable signal. For whole-cell recordings, the peak-to-peak noise amplitude must be less than 10 pA to be acceptable. Troubleshooting 1 .

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Sodium chloride (NaCl)	Fisher Chemical	Cat#7647-14-5
Potassium chloride (KCl)	VWR	Cat#7447-40-7
Sodium dihydrogen phosphate (NaH ₂ PO ₄)	Sigma	Cat#7558-80-7
Sodium bicarbonate (NaHCO ₃)	Sigma	Cat#144-55-8
Magnesium chloride 1.0 M (MgCl ₂)	Honeywell	Cat#7786-30-3
Calcium chloride 1.0 M (CaCl ₂)	Honeywell	Cat#10043-52-4
D-glucose	Sigma	Cat#50-99-7
Potassium hydroxide (KOH)	Sigma	Cat#1310-58-3
Potassium gluconate (K-gluconate)	Sigma	Cat#299-27-4
Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)	Sigma	Cat#67-42-5
Adenosine 5'-triphosphate disodium salt hydrate (Na ₂ -ATP)	Sigma	Cat#34369-07-8
Guanosine 5'-triphosphate sodium salt hydrate (Na-GTP)	Sigma	Cat#36051-31-7
2-[4-(2-hydroxyethyl)piperazine-1-yl]ethanesulfonic acid (HEPES)	Sigma	Cat#7365-45-9
Carboxymethylcellulose sodium salt	Sigma	Cat#7421-40
Experimental models: Organisms/strains		
Mouse: <i>Pomc-cre</i> , male, aged 2 months (genotyping information: https://www.jax.org/Protocol?stockNumber=010714&protocolID=22392)	The Jackson Laboratory	RRID: IMSR_JAX: 010714
Mouse: <i>Rosa26-loxP-stop-loxP-tdTomato</i> , female, aged 2 months (genotyping information: https://www.jax.org/Protocol?stockNumber=007914&protocolID=29436)	The Jackson Laboratory	RRID: ISMR_JAX: 007914
Software and algorithms		
MetaMorph	Molecular Devices	https://support.moleculardevices.com/
pClamp/Clampfit	Molecular Devices	https://support.moleculardevices.com/
Prism	GraphPad, LLC	https://www.graphpad.com
Other		
Osmometer	Vapro	Model 5600
pH meter	Eutech Instruments	PH 510
Magnetic stirrer	Labovolt	9000001406
Vibratome	Leica Biosystems	VT1200S
Lamp	Photonics	MSECLLED12F
Water bath heater controller	Fisherbrand	Julabo PC
Pipette puller	Sutter Instrument	P1000 Flaming/Brown
Antivibration table	TMC CleanBench	81-302-12
Faraday cage	Sutter Instrument	AT-3648
Microscope (IR-DIC)	Leica Microsystems	DM LFS
Fluorescent lamp	Leica Microsystems	EL6000
Manual XY translation platform	Burleigh	GHB-BX
Recording chamber	Warner Instruments	RC 26G
Camera	Hamamatsu	C11440
Micromanipulator (×2)	Thorlabs	PCS-5400N
Headstage (×2)	Molecular Devices	CV-7B
Electrode holder and silver wire	Molecular Devices	HL-U
Reference electrode	Luigs & Neumann	200-100 500 0192
Multiclamp	Molecular Devices	Model 700B
Digidata	Molecular Devices	Model 1440A
Temperature controller	Warner Instruments	TC-344C
Peristaltic pump	Gilson	Minipuls 3
Computer	Dell	Dell precision T1700
Gas anesthesia table for rodents	TEMSEGA	MiniTag
Carbogen (95% O ₂ -5% CO ₂)	Linde	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Nitrogen	Linde	N/A
Graduated flask 1 L (x2)	Schott	Cat#2820393
Beaker 150 mL	Scientific Laboratory Supplies	Cat#SLS5072
Magnetic bar	Fisherbrand	Cat#001.51.3
Microdissection tools	Fine Science Tools	N/A
Spatula 150 mm	Bochem Instrumente	Cat#3400
Cyanoacrylate glue	Loctite	N/A
Plastic pipette 3 mL	Labbox	Cat#PIPP-003-500
Petri dish 6 cm diameter	Falcon	Cat#353004
Petri dish 3.5 cm diameter	Falcon	Cat#353001
Nylon mesh	Genesee Scientific	Cat#57-105
Filter paper 90 mm diameter	Whatman	Cat#1001-090
Razor blade	Feather	Cat#99077
Razor blade	Gem Scientific	Cat#71950
Tube 1.5 mL	Eppendorf	Cat#0030121872
Borosilicate glass capillary	World Precision Instruments	Cat#TW150F-3
Syringe 1 mL	B. Braun Omnifix	Cat#9161406V
Syringe 10 mL	B. Braun Omnifix	Cat#4616103V
Syringe filter 0.22 μm	Merck Millipore	Cat#SLGV004SL
Needle 22G×1 1/2"	Henke-ject	Cat#HK4710007040
Needle 26 G	Terumo	Cat#AN*2613R
Slice anchor	Warner Instruments	Cat#SHD-26GH/15
Tygon tubing ID 1.6 mm × OD 3.20 mm	Warner Instruments Saint-Gobain Performance Plastics Verneret	Cat#VACF00017
PVC tubing 1.29 mm peristaltic pump	Gilson	Cat#F117940
Polyethylene tubing 0.97 mm OD × 0.58 mm ID	Warner Instruments	Cat#64-0753
Luer-to-Tubing Coupler Assortment Kit	World Precision Instruments	Cat#504954
Ground connectors	RS Pro	Cat#361-816
Pipette storage box	Sutter Instrument	Cat#BX10
Bleach	La Croix	N/A
Soldering iron	Antex	Cat#CS18

MATERIALS AND EQUIPMENT

The following is a list of solutions and reagents

aCSF solution

aCSF solution

Reagent	Final concentration (mM)	Amount (for 1 L)
NaCl	120	7 g
KCl	3.2	0.24 g
NaH ₂ PO ₄	1	0.12 g
NaHCO ₃	26	2.18 g
D-Glucose	2.5	0.45 g
MgCl ₂	1 (Molarity 1 M)	1 mL
CaCl ₂	2 (Molarity 1 M)	2 mL
Distilled water	-	Fill up to 1 L

Storage at 20°C during 1 day.

Note: Prepare two 1 L batches of aCSF. Check the osmolarity (295 mOsm). Keep one on ice for at least 30 min before use and gas it with carbogen.

Alternatives: The NaCl in the dissection solution can be replaced by an equimolar concentration of sucrose to increase the survival of the slices.

Alternatives: Instead of always freshly preparing the aCSF solution, 1 L of stock solution containing all the powders (NaCl, KCl, NaH₂PO₄, NaHCO₃, D-Glucose) and concentrated at 10× can be prepared. This solution should be kept at 4°C for one week. On the day of the experiment, and for a 1 L solution of aCSF at the concentrations mentioned in the table above, simply add while stirring, 1 mL of 1 M MgCl₂ and 2 mL of 1 M CaCl₂ to 100 mL of 10× stock solution, then adjust volume to 1 L with distilled water.

Intrapipette medium

KOH solution		
Reagent	Final concentration (mM)	Amount (for 10 mL)
KOH	2.5 mM	1.4 mg
Distilled water	-	Fill up to 10 mL

Storage at 4°C during 6 months.

HEPES solution		
Reagent	Final concentration (mM)	Amount (for 50 mL)
HEPES	10	119.15 mg
Distilled water	-	Fill up to 50 mL
KOH 2.5 mM	-	To adjust the pH

Storage at 4°C during 1 day.

Neuronal intrapipette medium		
Reagent	Final concentration (mM)	Amount (for 20 mL)
K-gluconate	123	576.13 mg
KCl	2	11.93 mg
EGTA	8	1.52 mg
Na ₂ -ATP	0.2	44.09 mg
Na-GTP	4	3.13 mg
MgCl ₂	0.3	40 μL
HEPES	10	Fill up to 20 mL

Storage at -20°C during 6 months.

Note: Prepare 20 mL of intrapipette medium in 10 mM HEPES solution and adjust the pH to 7.3 with 2.5 mM KOH solution. Filter the solution and make aliquots of 500 μL and store at -20°C.

Reagents and electrophysiology setup

For all reagents, equipment and software, there are many company options and they are not limited to those listed in the [key resources table](#). E.g.

- Chemical products are available from Sigma, Fisher Chemical, Santa Cruz Biotechnology, Tocris, VWR.
- Laboratory products are available from Scientific Laboratory Supplies, Falcon, Labbox.
- Microscope is available from Leica, Olympus, Zeiss.
- Electrophysiological equipment are available from Axon Instruments, Scientifica, Sutter Instruments, HEKA, Luigs & Neumann, Thorlabs, Warner Instruments.
- Software are available from Molecular Devices, MathWorks, Scientifica.

STEP-BY-STEP METHOD DETAILS

Brain extraction and slices preparation

⌚ Timing: 30 min

⌚ Timing: 10 min (for step 1)

⌚ Timing: 20 min (for step 2)

Paired whole-cell patch-clamp recordings are performed on living brain slices. This procedure details how to extract the brain and prepare slices (Figures 4.1–3 and 5).

1. Dissection.

- a. Fill the dissection tank and the buffer tank of the vibratome with aCSF that has been saturated with oxygen and kept at 4°C.
- b. Anesthetize the mouse with Isoflurane.
 - i. Place the mouse in the induction box of a gas anesthesia table for rodents.

Note: The induction is realized with a concentration of isoflurane of 3%.

- ii. Check mouse reflexes (i.e., by pinching its paw) to check if the anesthesia has worked.

△ CRITICAL: Isoflurane is dangerous, it is mandatory to use specific equipment to protect the experimenter of Isoflurane vapor.

- c. Euthanize the mouse by decapitation and quickly immerse its head in ice-cold aCSF.
- d. Extract the brain into the ice-cold aCSF using microdissection tools.
 - i. Cut the skin along the midline of the brain from the neck to the nose with fine scissor and stretch it on each side (Figure 5.1).
 - ii. Hold the head with one hand by the skin flaps, cut around the cranial cavity with fine scissor and remove the top of the skull with forceps (Figure 5.2).
 - iii. Cut the olfactory bulbs perpendicularly and slide micro-scissors at the ventral part of the skull to cut the optic nerves (Figure 5.3).

△ CRITICAL: If the optic nerves are not cut, the median eminence will be torn during brain harvesting.

- iv. Carefully turn the brain over and drop it into the aCSF by cutting the trigeminal nerves with micro-scissor (Figure 5.4).

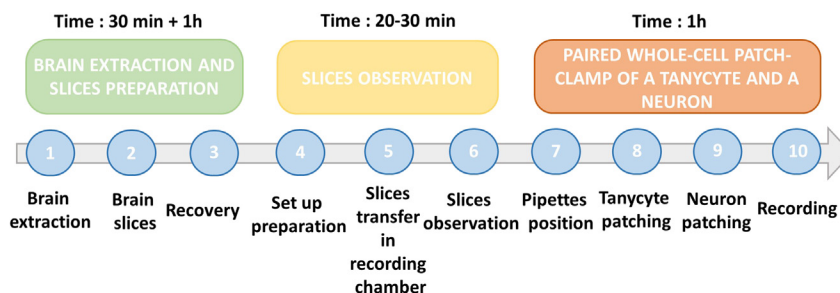


Figure 4. Workflow from brain extraction to recordings

Step 1–3: Brain extraction and slices preparation. Step 4–6: Slices observation. Step 7–10: Paired whole-cell patch-clamp of a tancyte and a neuron.

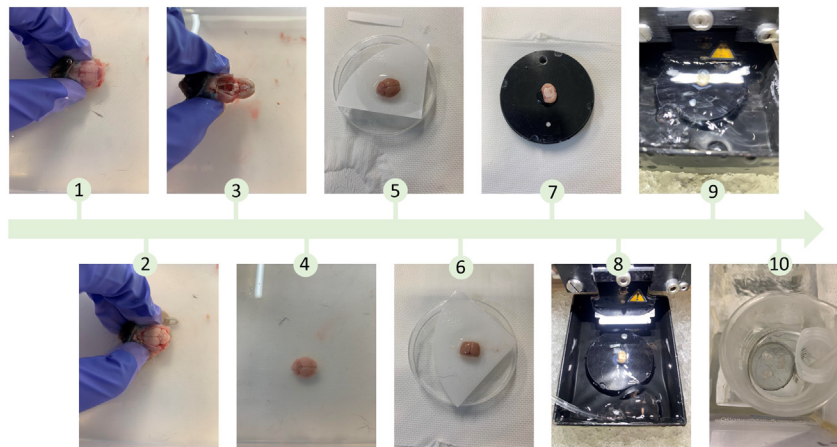


Figure 5. Representative image of the different steps of brain extraction and slices preparation

1. Cutting the skin along midline of the brain using fine scissors. 2. Upper skull cut off and removed using scissors. 3. The olfactory bulbs and optic nerves are sectioned using micro-scissors and the brain is gently flipped over. 4. The trigeminal nerves are sectioned using micro-scissors and the brain is harvested in aCSF solution. 5. The brain is placed on a damp disk of Whatman paper. 6. The cerebellum and rostral end of the brain are cut out and removed with a razor blade. 7. The rostral surface of the delimited brain block is glued on the vibratome sample holder. 8. The vibratome sample holder with the glued brain is placed in the tank of the vibratome containing ice-cold aCSF. 9. Brain slices are obtained using the vibratome. 10. The collected slices are placed in the incubation chamber.

- e. Moisten the disk of Whatman paper with aCSF, retrieve the brain with a spatula and place it on top [Figure 5.5](#).
- f. Using a razor blade, cut off the cerebellum and the rostral end of the brain to make a block ([Figure 5.6](#)).

Optional: For more precision, this step can be done using a brain cutting matrix.

- g. Glue the rostral surface of the blocked brain on to the vibratome sample holder. To do this, use the bent tip forceps ([Figure 5.7](#)).

Note: These steps should be done as quickly as possible to optimize slice survival.

2. Preparation of coronal slices of the hypothalamus.

- a. Place the vibratome sample holder with the attached brain in the tank containing ice-cold aCSF ([Figure 5.8](#)).

Note: To optimize median eminence slices, rotate the brain so that cutting begins with the left hemisphere and ends with the right hemisphere.

- b. Define the cutting window, carry out rough cutting and then cut the slices to a thickness of 250 μm ([Figure 5.9](#)).
- c. With the dropper, collect slices containing the median eminence and arcuate nucleus and place them in the incubation chamber.

△ CRITICAL: Do not stack slices on top of each other and check that they do not move to optimize slice survival ([Figure 5.10](#)).

▮▮ Pause point: Allow at the slices to recover at 34°C for 1 h, then leave them at room temperature (about 20°C) until use.

Observation of slices by video microscopy

⌚ Timing: 20–30 min

After a stabilization period, slices have recovered their physiological properties. The procedure details how to observe slices and identify neurons of interest (Figure 4.4–6 and 6).

3. Turn on all equipment needed for slices observation and patch-clamp recordings.
 - a. Perfusion system to bring newly oxygenated aCSF into the recording chamber,
 - b. Computer with pClamp acquisition software and MetaMorph imaging software,
 - c. Microscope,
 - d. Camera,
 - e. Fluorescent lamp,
 - f. Digidata,
 - g. Amplifier,
 - h. Temperature controller at 32.8°C,
 - i. Micromanipulators.
4. Using a cut dropper, transfer a slice into the recording chamber and immobilize it at the bottom of the chamber with a nylon grid to prevent movement.
5. Observation of slices.

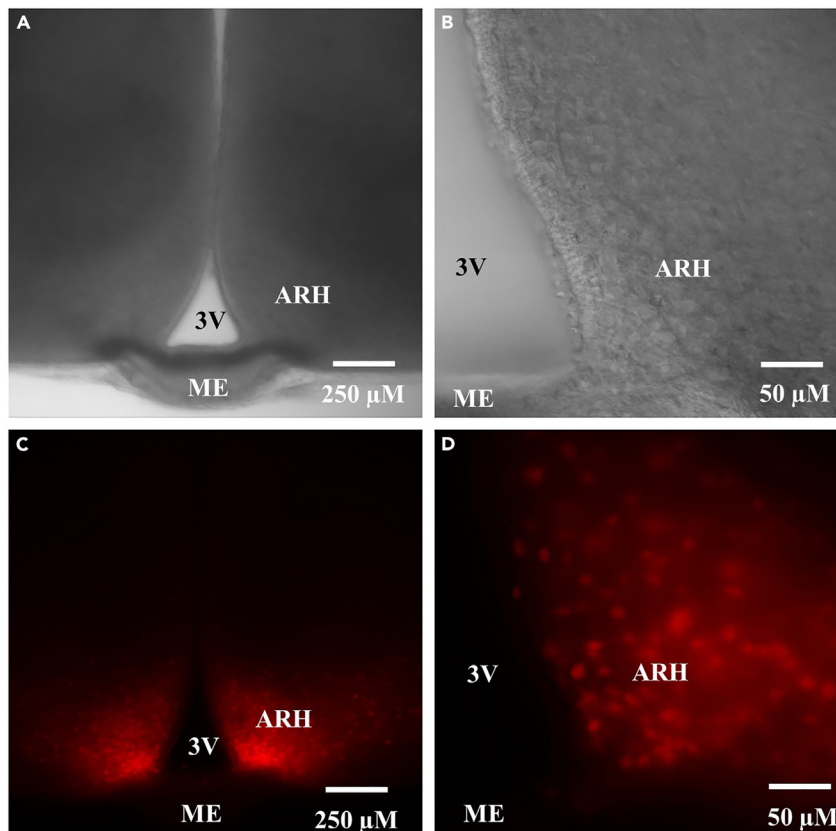


Figure 6. Microscopic observation of a *Pomc-tdTomato* mouse hypothalamus slice

(A and B) Observation in infrared coupled differential interference contrast (IR-CID) mode at 10× (A) and 40× (B) magnification.

(C and D) Observation under fluorescence at 10× (C) and 40× (D). ARH: arcuate nucleus of the hypothalamus. ME: median eminence. 3V: third ventricle.

- a. Place the region of interest under the microscope's field of observation.

△ **CRITICAL:** Check that the cells are healthy. They should have a three-dimensional appearance with a smooth appearing cell membrane and no visible nucleus (Figure 6B). [Troubleshooting 2](#).

- b. Observe slices under fluorescence with a 40× objective to locate spontaneously fluorescent POMC neurons expressing the tdTomato protein.
- c. Circle them with the "drawing" tool of the imaging software.
- d. Locate the same neurons under IR-DIC.

Note: At this step, do not move the field of observation.

Paired whole-cell patch-clamp of a tanycyte and a POMC neuron

⌚ **Timing:** 1 h

The following steps detail how to patch a tanycyte to fill it with a specific compound, then how to patch a neuron to record its electrical activity (Figures 4.7–10 and 7).

6. Position the electrode pipettes under the microscope.
 - a. Thaw an aliquot of the intrapipette medium for tanycytes.
 - b. Put the intrapipette medium in 1 mL syringe with a 22Gx1¹/₂" needle.

Note: It is a good idea to add a sterile syringe filter between the syringe and the needle.

- c. Place the syringe filled with intrapipette medium on ice.
- d. Fill the tanycyte pipette with its intrapipette medium.

Note: Do not overfill the pipette. Fill about 1/3 of its length.

- e. Remove any air bubbles that are visible to the naked eye with a few gentle taps.
- f. Mount the patch pipette on the electrode holder.

Note: When inserting the patch pipette into the amplifier headstage, avoid scratching the AgCl-coating of the electrode, as a freshly scratched surface will cause an unstable offset potential.

- g. Locate the pipette under the 40× objective immersed in aCSF and center the tip in the field of view.

Note: The pipette can be placed directly under the 40× objective or, for greater ease, be placed under a lower magnification first.

- h. Check that the pipette tip is not broken.
 - i. Apply airflow to verify that the pipette is not clogged and to push any remaining air bubbles out of the pipette.
 - j. Apply a small amount of constant positive pressure (between 0.3–0.5 mL) to prevent debris from sticking to the pipette tip.

Note: The positive pressure depends on the cell type and age of the mice.

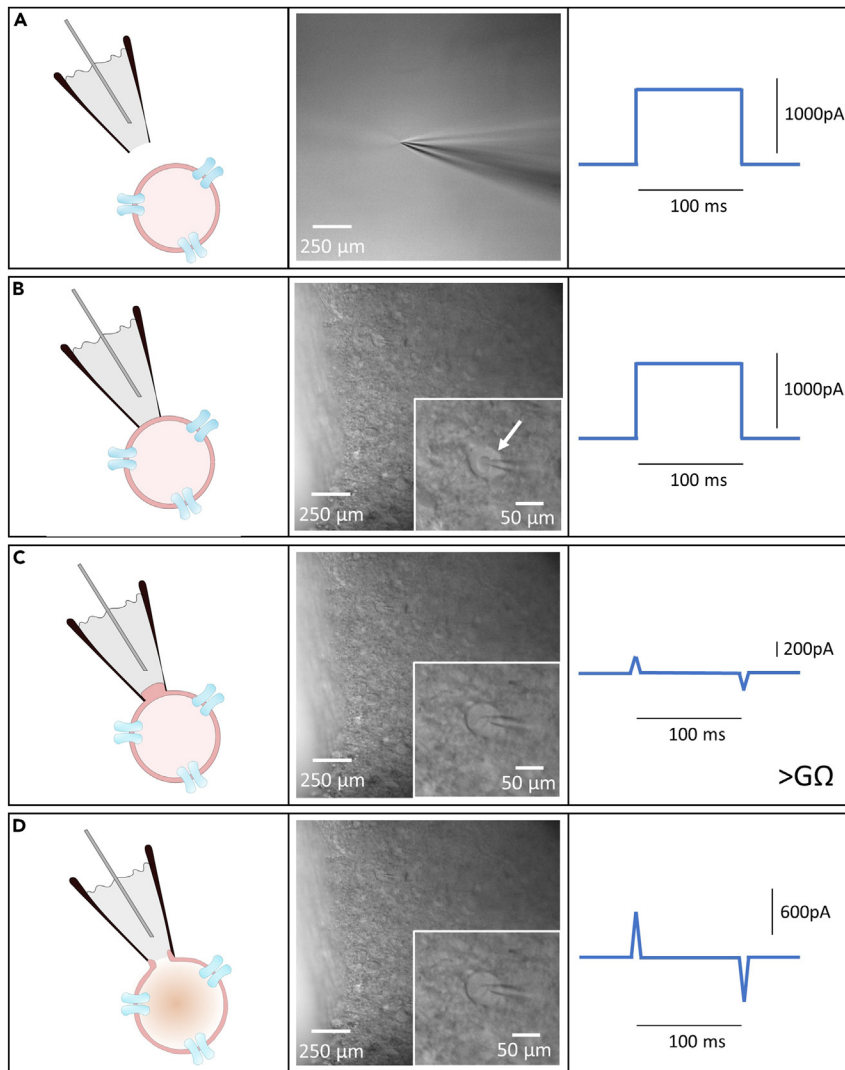


Figure 7. Cell observation under IR-DIC and membrane test recording during pipette approach to obtain a whole-cell configuration

(A) A pipette filled with intrapipette medium is placed in the bath and a positive pressure is applied to its tip. Visualization of the membrane test of a current in response to the application of a voltage pulse.
 (B) Pipette stuck to the membrane is represented by the dimple (arrow) formed around the pipette tip. At this stage, there is no change in current.
 (C) Positive pressure removal results in a decrease of the current, which corresponds to an increase in resistance between the pipette and the cell up to a gigaohm.
 (D) The rupture of the membrane under the pipette results in a brief suction and an increase of the capacitive transients.

- k. Approach the pipette to just above the surface of the slice without touching it, using the "coarse" setting of the micromanipulator.

Note: To approach the pipette, first lower the objective a little and then follow with the pipette until its tip is focus again. Repeat this operation and stop just above the slice.

- l. Repeat steps 6a to 6k for the neuron pipette, mounting it on the second headstage of the amplifier. At this step, you need to use the intrapipette solution for neurons.

7. Whole cell patch clamp of a tanyocyte.
 - a. Run the "membrane test" protocol of pClamp software in "bath" mode to visualize a current in response to the application of a 10 mV voltage pulse during 100 ms (Figure 7A).
 - b. Depending on the configuration of the amplifier and digidata, choose the analog output that corresponds to the headstage chosen to patch the tanyocyte.
 - c. Adjust the offset potential to 0 and check that it remains stable.
 - d. Check the resistance of the pipette. It should be between 4 and 6 M Ω .
 - e. Using the "fine" setting of the micromanipulator, lower the pipette onto the center of the cell membrane surface of a tanyocyte until a "dimple" is created around the pipette tip by the positive pressure (Figure 7B).

Note: Too much positive air pressure or physical pressure by pushing the cell with the pipette tip might cause the cell to move within the slice. The integrity of such a cell may be compromised, and it should not be recorded.

- f. Quickly relieve the positive pressure and apply gentle suction to draw a part of the membrane into the pipette.

Note: This suction should be maintained until a tight contact between the pipette wall and the cell membrane, called a gigaseal, is achieved. The quality of the gigaseal formed can be evaluated by the increase in resistance, up to a gigaohm, which corresponds to a decrease in the membrane test current response (Figure 7C). [Troubleshooting 3](#).

- g. Switch the holding potential to -80 mV on the amplifier.
- h. Switch to "patch" mode.

Optional: At this step, the capacitance of the pipette can be cancelled. However, since the pipette capacitance depends mainly on the length of the pipette in contact with the extracellular solution, which depends on the liquid level in the recording chamber, the liquid level must remain very stable after pipette capacitance compensation.

- i. Rupture the membrane by applying a brief but strong suction to obtain whole-cell configuration and to deliver the chemical or pharmacological substance of interest.

Note: The rupture is successful if large capacitive transients in response to a voltage pulse are seen on the scope obtained with "membrane test" protocol (Figure 7D). [Troubleshooting 4](#).

- j. Switch to cell mode and check the stability of the whole-cell configuration over 2–3 min.
- k. Note the access resistance (Ra) value given directly by the cell mode of the "membrane test".

Optional: Perform an episodic stimulation protocol, pulse test, corresponding to the application of a voltage pulse of -10 mV for 100 ms. This allows you to calculate the value of the Ra to confirm the value given by the "membrane test".

Note: The access resistance value must be taken at the beginning and end of the recording. If the access resistance changes by more than 20% during the recording, the recording must be excluded. The access resistance provides information on the membrane opening. If it changes by more than 20% during recording, this can lead to changes in activity independent of the substance tested. [Troubleshooting 5](#).

- l. Perform the recordings necessary for measurement of the electrical properties of the membrane and allowing functional confirmation that the patched cell is indeed a tanyocyte.

- i. Plot a current-voltage (I-V) curve in voltage-clamp mode by applying a series of 300 ms voltage pulses ranging from -100 mV to $+100$ mV at 10 mV increments.
- ii. Perform a "gap-free" protocol of pClamp Software to record spontaneous tanyocyte activity in current-clamp mode.

Note: Tanyocytes exhibit membrane properties typical of glial cells, including a highly polarized membrane potential (-80 mV), a linear I-V relationship and low membrane resistance (30 M Ω).

Note: It is necessary to consider that unlike other cells, tanyocytes of the arcuate nucleus of the hypothalamus form a network of cells interconnected by gap junctions made up of connexin 43.^{1,5} These gap junctions allow the intercellular diffusion of small molecules with a molecular weight of less than 1.5 kDa. Thus, the use of drugs below this molecular weight will result in the diffusion of the substance from one tanyocyte to another and the modulation of the targeted cellular pathway in all cells of the network. If the goal of the experiment is to investigate the effect of a substance on a single tanyocyte, it is possible to pre-incubate the slices for 20 min with a gap junction inhibitor such as carbenoxolone (50 μ M).^{6,7}

8. Whole-cell patch clamp of a POMC neuron.
 - a. In the "membrane test", choose the analog output that corresponds to the headstage chosen to patch the neuron.
 - b. Carry out the steps from 7c to 7k previously described. For step 7g, clamp the cell at -60 mV instead of -80 mV.
9. Using a gap-free protocol, co-record the spontaneous activity of the tanyocyte and the neuron in current-clamp mode.
 - a. Do not inject any current.
 - b. After a period of stabilization and baseline recording, apply the different conditions foreseen in the experimental protocol to answer the scientific question. [Troubleshooting 6](#).

Note: As tanyocytes, like all other glial cells, are non-excitabile cells that do not generate action potentials, the current-clamp plot helps to ensure that the tanyocyte is not lost during recording from the neuron.

EXPECTED OUTCOMES

The protocol described here allows the effects of a modification of the cytoplasmic content of a cell on the electrical activity of a neighboring cell to be evaluated. For example, this technique has been recently used to test the possibility that the lactate that fuels electrical activity in POMC neurons is provided by tanyocytes.¹ In this paired patch-clamp experiment, we dialyzed a tanyocyte with lactate via a patch-clamp pipette and simultaneously recorded the electrical activity of a nearby POMC neuron to investigate whether tanyocytic lactate could maintain the electrical activity of POMC neurons during exogenous glucose deprivation. While exogenous glucose deprivation led to a decrease in the electrical activity of POMC neurons, this decrease was not induced when a neighboring tanyocyte was filled with lactate. In contrast, this preventive effect was abolished by bath application of 4-CIN, a blocker of lactate transporters (MCTs) ([Figures 8A–8C](#)). Interestingly, the loss of the tanyocyte during the course of the experiment induced a reduction in action potential firing that could be reversed by a bath application of 5 mM lactate ([Figure 8D](#)). We also investigated in greater detail whether the lactate fueling POMC neurons was produced by the tanyocytes or whether the tanyocytes were just intermediate cells. For this, we specifically inhibited lactate dehydrogenase in a tanyocyte by filling it with oxamate. The results showed that POMC neurons lost their activity when lactate dehydrogenase was inhibited in tanyocytes. This inhibition was reversed by the application of lactate in the bath ([Figures 8E–8G](#)). These data demonstrated that the neuronal activity of POMC neurons was dependent on lactate released from tanyocytes by metabolizing glucose. Taken together, these data

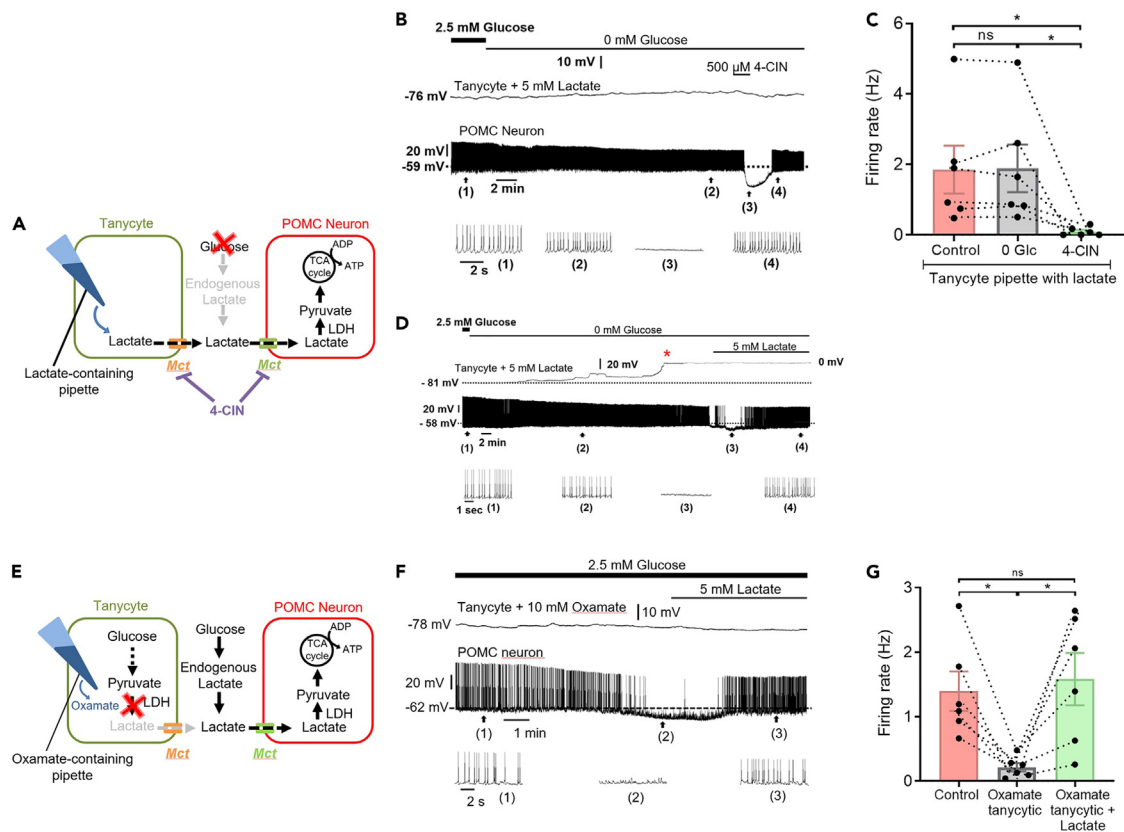


Figure 8. Recording of a paired arcuate nucleus tanyocyte and POMC neuron in whole-cell patch-clamp mode

(A) Schematic models of experiments in B and D.

(B) Recording showing the effect of exogenous glucose deprivation on the spontaneous activity of a POMC neuron when a tanyocyte is filled with lactate.

(C) Quantitative analyses of the mean of the spike frequency of the experiment in B. Data are represented as mean \pm SEM. ns: non-significant, * $p < 0.05$.

(D) Recording showing that the loss of the patched tanyocyte, represented by the red asterisk, leads to a decrease in the firing rate of the POMC neuron.

(E) Schematic models of the experiment in F.

(F) Recording showing effect of a lactate dehydrogenase blockade induced by oxamate infusion in a tanyocyte on the spontaneous activity of a POMC neuron.

(G) Graph illustrating the frequency discharge of action potentials in experiment F. Data are represented as mean \pm SEM. Adapted with permission from Lhomme et al. (2021).¹ ns: non-significant, * $p < 0.05$.

demonstrate the utility of this method in identifying new cellular mechanisms that may involve both glial and neuronal cells.

QUANTIFICATION AND STATISTICAL ANALYSIS

Analysis of electrophysiological data

Electrophysiological data analysis is performed with Clampfit.

1. Measurement of access resistance (Ra).

Here we describe how to measure Ra from the episodic stimulation protocol obtained in the "step-by-step method details" section, step 7.k.

- Open the recording in Clampfit.
- Place cursors 1 and 2 as shown in Figures 9A and 9B.
- Measure the difference (Δ).
- Using Ohm's law, calculate Ra as follows: $Ra = -10 \text{ mV} / \Delta$.

2. Establishment of the linear current-voltage relationship

Here we describe how to establish the linear current-voltage relationship from the IV curve obtained in the "step-by-step method details" section, step 7.L.i.0.

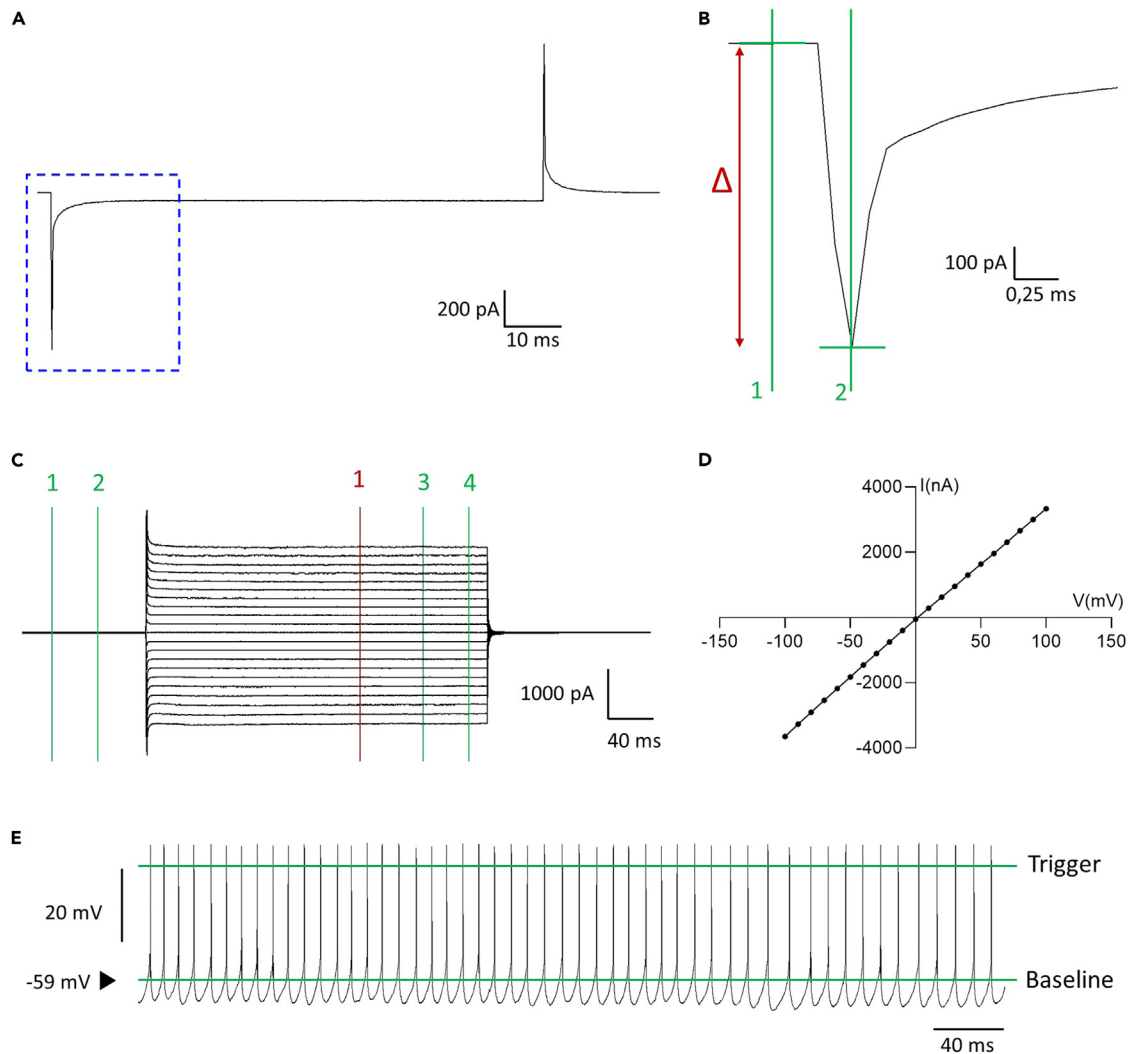


Figure 9. Analysis of access resistance (R_a), membrane resistance (R_m) and resting membrane potential (RMP)

(A) Example of a pulse test recording of a neuron.

(B) Expansion of the area delineated by the blue square in A, showing how to place cursors 1 and 2 to measure the R_a .

(C) Example of a current-voltage curve. Cursors 1, 2, 3 and 4 in green and 1 in red correspond to the cursors for measuring R_m and to plot the current-voltage relationship, respectively.

(D) Graphic showing the current-voltage relationship from the tanyocyte recorded in C.

(E) Example of the spontaneous activity of a neuron, showing how to place the « baseline » and « trigger » markers to measure the firing rate.

- a. Open the IV curve in Clampfit.
 - b. Place cursor 1 (red) as shown in [Figure 9C](#).
 - c. Use the "IV plot" function and go to "Results" to get current values as a function of the applied voltage pulses.
 - d. Make a graph as shown in [Figure 9D](#).
3. Measuring the membrane resistance.
- Here we describe how to measure the membrane resistance from the IV curve obtained in the "step-by-step method details" section, step 7.1.i.
- a. Open the IV curve in Clampfit.
 - b. Place cursors 1 (green), 2, 3 and 4 as shown in [Figure 9C](#).
 - c. Use the "Resistance" function and go to "Results" to get the membrane resistance value.

4. Measuring the resting membrane potential

Here we describe how to measure the membrane potential from the gap-free recording obtained in the "[step-by-step method details](#)" section, step 9.

- Open the recording in Clampfit.
- Use the "Statistics" function.
- In "Baseline Region" check "Fixed at 0 units" and in "measurements" check "mean (units)".
- Read the value in "Results".

Note: The difference in composition between the extracellular medium (aCSF) and the intracellular medium (intrapipette medium) of the cell, creates a potential difference at the interface of these two liquids, called the liquid junction potential. If this liquid junction potential is not directly compensated during the recordings, do not forget to subtract it from the value obtained.

5. Frequency.

Here we describe how to measure the frequency from the gap-free recording obtained in the "[step-by-step method details](#)" section, step 9.

- Open the recording in Clampfit.
- Use the "Threshold Search" function.
- Place markers "baseline" and "triggers" as described in [Figure 9E](#).
- Start the automatic calculation of the number of action potentials.
- Report the number of potentials to the duration of the recording analyzed to obtain the Frequency (in Hz) (= number of action potentials / time in seconds).

Note: From the gap-free recordings of the spontaneous electrical activity of the neurons, other parameters of the cell can be determined (threshold for triggering action potentials, amplitude of the action potentials, interval between action potentials, characteristics of the after-hyperpolarization or -depolarization, ...).

Statistical analysis

Data are presented as means and standard error of the mean (SEM). All statistical analyses are performed using GraphPad Prism software, version 7. P values < 0.05 are considered statistically significant.

- To calculate normality, the Shapiro-Wilks test was used.
- To compare two groups, an unpaired or paired Student's t-test is used for normal distributions and a Wilcoxon or Mann-Whitney U test for non-Gaussian distributions.
- To compare multiple groups, a one-factor analysis of variance (ANOVA) is used, followed by Tukey's post hoc multiple comparison test. Repeated-measures ANOVAs are used when multiple measurements are made over time in the same group.

LIMITATIONS

This protocol has been optimized to perform paired recordings of a tanycyte and a POMC neuron in order to reveal metabolic coupling between these two cell types. This protocol is perfectly adaptable to NPY neurons. However, it is not adaptable to neuronal populations that might lie further away from tanycytes. Indeed, the two cell types of interest must lie in the same slice plane and, more precisely, in the same field of observation to be able to patch them simultaneously. This is a first limitation.

In our project, tanycytes were one of the cells of interest. A peculiarity of these specialized glial cells is that they form a network of interconnected cells through gap junctions made of Cx43. Thanks to this network, the substance of interest is transmitted in a volumetric manner,

increasing the probability that the target cell receives the signal. The use of a different cell type that does not have this characteristic could reduce this probability. This makes it a second limitation.

TROUBLESHOOTING

Problem 1

Too much noise to have a clean and analyzable signal (step 34 – [before you begin](#)).

Potential solution

The system or rig should be well grounded.

- Look for the specific equipment that is producing the noise. Turn off the devices one at a time and see if you have noise reduction. If there is a significant drop in noise when a device is turned off, connect that device to ground.
- Test the Patch 1U Model cell. Sometimes the grounding cable and the electrode need to be soldered. Bad soldering might cause extra noise.
- Move inverters and power strips as far away as possible from the amplifier.
- Set Sampling rate at 5 kHz and the Bessel filter at 2 kHz. 5 or 10 kHz are frequently used for the sample rate. The filter frequency is often 2 or 5 kHz. The trace is noisier when the sample rate is high.

Problem 2

The slice is unhealthy (step 5 – [step-by-step method details](#)).

Potential solution

- Increase dissection speed.
- Check the composition, osmolarity and pH of the solutions.
- Check that the cells have been constantly oxygenated.
- Use a cutting solution in which the NaCl is replaced by an equimolar concentration of sucrose and in which the Mg^{2+}/Ca^{2+} ratio is increased. This allows neuronal death to be reduced during brain extraction and slicing.

Problem 3

Difficulty in making a Gigaseal (step 7f – [step-by-step method details](#)).

Potential solution

Not enough negative pressure applied.

- Apply some more negative pressure, close the three-way valve and wait a few minutes.
- Setting the voltage at -60 to -70 mV can help.

Cells are unhealthy.

- See [problem 2](#).

The pipette resistance is under $4\text{ M}\Omega$ or over $8\text{ M}\Omega$.

- Change the resistance of your pipette. To do this, you need to modify stretching program of the puller. The increase or the decrease of the velocity, pull, time and pressure values change the resistance of the pipette. To know exactly what you need to modify and have a good pipette resistance, refer to notice of the puller.

Problem 4

Difficulty in breaking through the membrane (step 7i – [step-by-step method details](#)).

Potential solution

The pipette resistance is over 8 MΩ.

- Lower the resistance of the pipette.

The pressure system is not working.

- Check it.

Mouth suction is not enough.

- Use the zap function in the amplifier to provide short electric pulses through the pipette to create small holes in the membrane that will help break through.

Problem 5

The access resistance increases over time (step 7k – [step-by-step method details](#)).

Potential solution

The patch is resealing.

- Try to break through again very smoothly.

Problem 6

Difficulty maintaining the patch and leading to loss of spontaneous activity during recording (step 9 – [step-by-step method details](#)).

Potential solution

The pipette is moving.

- Check the antivibration table and the stability of the micromanipulator and the microscope stage.

The slice is moving.

- Check the aCSF flow (around 2 mL/min).
- Make sure that the inflow equals the outflow. The in or outflow may be bottlenecked at some point.
- Make sure there are no bubbles in the aCSF introduced into the bath.
- Make sure of the quality of the nylon grid used to immobilize slices in the recording chamber.

The slice is unhealthy.

- See [problem 2](#).

The intrapipette medium is not suitable.

- Optimize the intrapipette medium. Different types of neurons may do better with different types of media.

RESOURCE AVAILABILITY

Lead contact

Further information and request for resources and reagents should be directed to the lead contact, Vincent Prévot (vincent.prevot@inserm.fr).

Materials availability

This study did not generate unique new reagents.

Data and code availability

No data sets or code were generated during this study.

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AUTHOR CONTRIBUTIONS

Conceptualization, T.L. and V.P.; methodology, T.L.; investigation, T.L. and V.P.; writing, T.L. and V.P.; funding acquisition, V.P.; resources, V.P.; supervision, V.P.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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