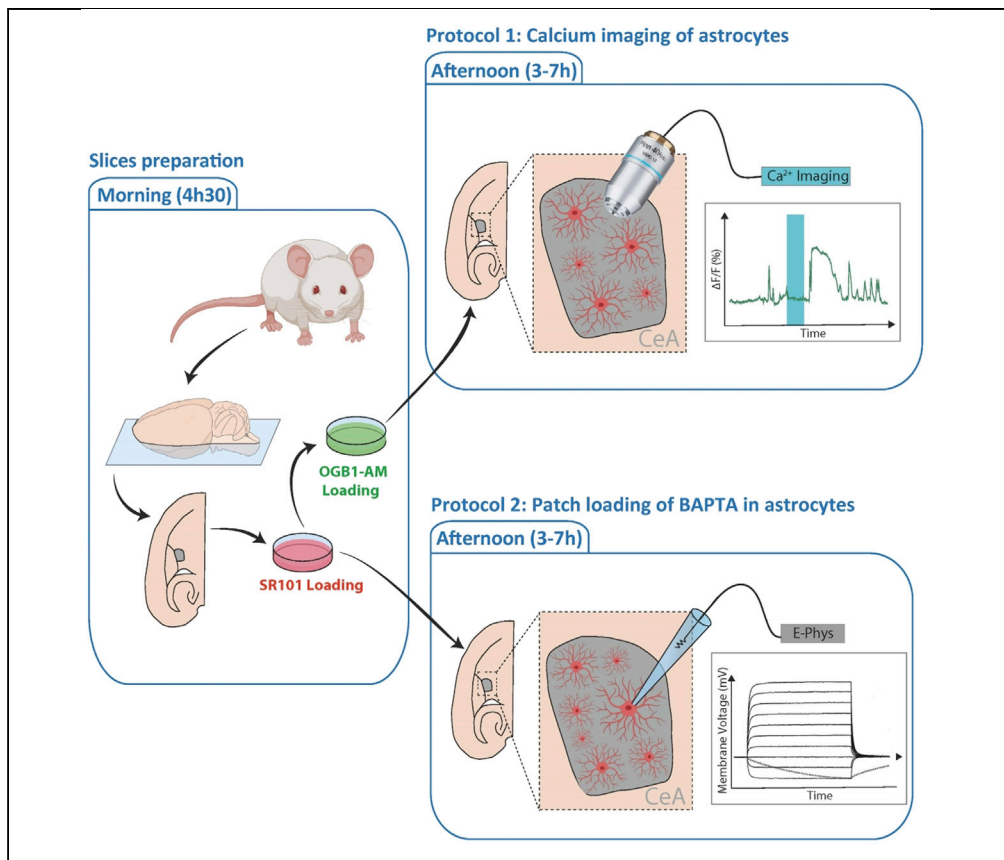


Protocol

Calcium imaging and BAPTA loading of amygdala astrocytes in mouse brain slices



Astrocytes are glial cells that exhibit calcium signaling-mediated activity. Here, we present a protocol to monitor and manipulate astrocyte calcium activity from mouse amygdala slices. In the first part of this protocol, we describe the procedure of astrocyte calcium imaging. In the second part, we detail how to disrupt astrocyte calcium activity by patch-clamp-mediated loading of BAPTA. These two approaches are presented separately but they can also be used simultaneously to monitor the effects of disruption on an astrocyte network.

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Highlights
Protocol describes calcium imaging and BAPTA loading on astrocytes in mouse brain slices

Calcium imaging is a simple yet reliable tool to study astrocyte activity

Patch-clamp-mediated loading of BAPTA allows the disruption of astrocytic activity

Calcium imaging can assess BAPTA diffusion within the astrocytic network

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Protocol

Calcium imaging and BAPTA loading of amygdala astrocytes in mouse brain slices

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SUMMARY

Astrocytes are glial cells that exhibit calcium signaling-mediated activity. Here, we present a protocol to monitor and manipulate astrocyte calcium activity from mouse amygdala slices. In the first part of this protocol, we describe the procedure of astrocyte calcium imaging. In the second part, we detail how to disrupt astrocyte calcium activity by patch-clamp-mediated loading of BAPTA. These two approaches are presented separately but they can also be used simultaneously to monitor the effects of disruption on an astrocyte network. For complete details on the use and execution of this protocol, please refer to Wahis et al. (2021).

BEFORE YOU BEGIN

Calcium imaging requires a confocal fluorescence microscope and an illumination system capable of achieving a high temporal resolution (sampling frequency > 1 Hz). For the calcium indicator, we use Oregon Green BAPTA 1 coupled to an AcetoxyMethyl ester group (OGB1-AM) because it preferentially stains astrocytes, but this protocol can be used with other AM-coupled calcium indicators as well. For example, Rhod2-AM can be used as a red wavelength alternative. However, in our hands, we observe more cell death with Rhod2-AM than with OGB1-AM. One can also use ratiometric calcium indicators such as Fura 2, Fluo-4 or Indo-1. However, while this approach allows for an accurate estimation of the intracellular calcium concentration, it also has the drawback of requiring three wavelengths of light, thereby restricting the use of other fluorescent labeling approaches. The K_d value should also be considered when choosing a calcium indicator. For example, if the goal of the experiment is to monitor small events, then an indicator with a high K_d (e.g., Fluo-4FF) would be ideal, whereas a dye with a lower K_d (e.g., OGB1) would be preferable for monitoring bigger calcium events due to a lower degree of saturation (Delvendahl et al., 2015). Yet another approach that may be used in conjunction with this protocol would be the use of genetically encoded calcium indicators such as GCaMP, or the newly developed Salsa6f ratiometric analogs (for use in astrocytes, see Wakida et al., 2020).

For required tools, please see [key resources table](#) and [materials and equipment](#) for references.

In the present paper, the two protocols were performed in mouse horizontal slices, containing the central nucleus of the amygdala.



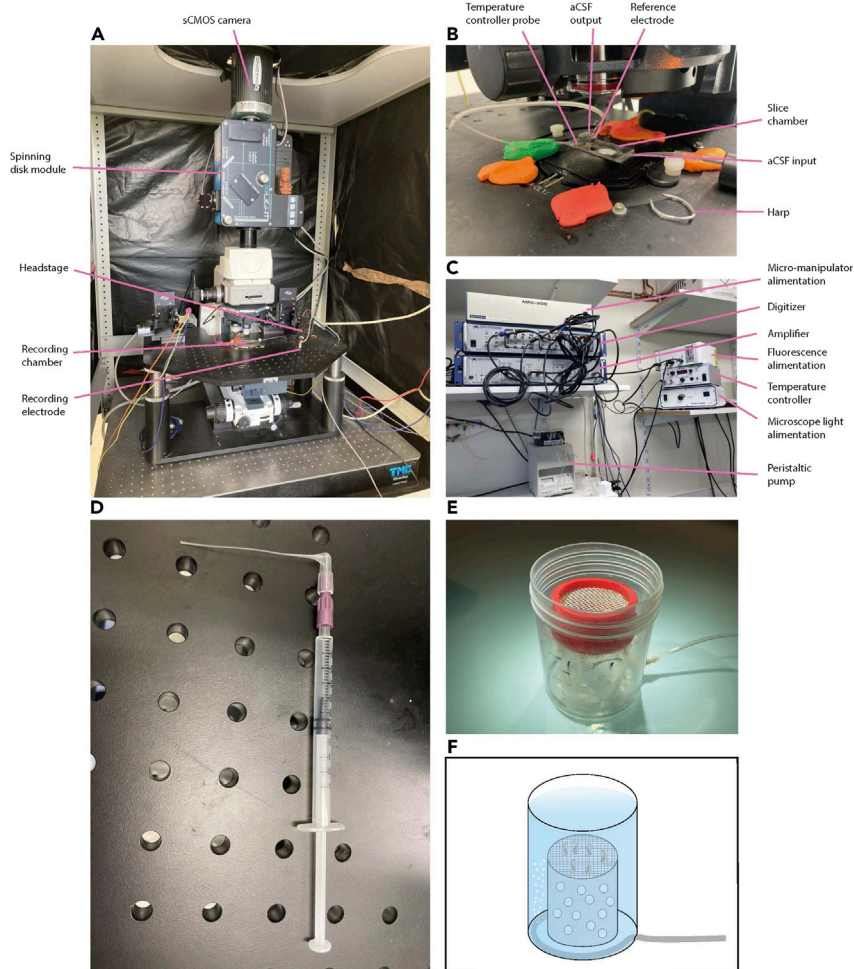


Figure 1. Material for calcium imaging and patching of astrocytes

(A) Microscope equipped with a spinning disk module and a sCMOS camera for calcium imaging. This microscope is placed on a Gibraltar table along with the recording chamber, the headstage and the recording electrode. The assembled instruments are placed on an anti-vibration table.

(B) Magnified photo of a recording chamber divided into 3 parts: one space for the slice and aCSF input, one for the aCSF output and one for the reference electrode. The harp is placed on the slice to avoid any movement during recordings.

(C) All the equipment needed for patch clamp (for more details, see [key resources table](#)).

(D) Example of a manually pulled tip used to fill the pipette in patch-clamp. The tip is mounted on a filter placed on a 1 mL syringe to avoid clogging the tip.

(E) Photo of a recovery chamber.

(F) Scheme of the recovery chamber, a pierced tube is fixed at the bottom of the chamber to bring carbogen in the aCSF of the chamber.

The calcium imaging setup is composed of a Zeiss Axio examiner microscope equipped with a 40× water immersion objective and a mounted X-Light Confocal unit – CRESTOPT spinning disk (Figure 1A). We use a LED light source for excitation and emission data are acquired at 2 Hz with an optiMOS sCMOS camera (Figure 1A). All of these hardware elements are interfaced with a computer running MetaFluor (ver. 7.8.8.0) software.

The patch-clamp setup is composed of an Axon MultiClamp 700B amplifier coupled to an ADC Digidata 1440A Digitizer (Figure 1C). Data are acquired at a sampling rate of 20 kHz and stored on a computer using the pClamp (ver. 10) software suite.

If needed, prepare aCSF, the perfusion solution and the internal solution in advance (see [materials and equipment](#)).

Experiments have to be performed in accordance with ethical rules and approbation from your local institutions.

Slice preparation

⌚ Timing: 2 h

1. Anesthetize the mouse with an *i.p.* injection of ketamine and paxman at 400 mg/kg and 80 mg/kg respectively.

Note: one can also use isoflurane inhalation followed by quick decapitation.

2. Access the heart by performing a laparotomy and a thoracotomy, being careful not to damage the internal organs ([Figures 2A and 2B](#)). Use a 50 mL syringe filled with cold (4°C) perfusion solution (see [materials and equipment](#)), insert the needle into the left ventricle and cut the right atrium. Exsanguinate the animal until there is no more blood flowing from the heart (typically, 10 mL is enough).

Note: Heart perfusion is not essential for young animals (<P30-40) to obtain proper slices.

Note: To perform the exsanguination, you can also use a peristaltic pump to have better control over the flow of the perfusion solution.

Note: Observe the liver during the perfusion. If the perfusion procedure is done properly, its color should change from red to almost white.

3. Once perfusion has finished, decapitate the animal using scissors. Place the head in a Petri dish containing cold oxygenated perfusion solution. Open the scalp using the scalpel and remove the part of the skull covering the brain using scissors and forceps ([Figure 2C](#)). Extract the brain from the skull and place it in another Petri dish filled with clean cold oxygenated perfusion solution.

Note: If you are fast enough (< 3 min), this step can be done outside the perfusion solution to save both time and solution. For the sake of reproducibility, we highly recommend that the first few attempts be done as described here until you get used to it.

⚠ CRITICAL: It is important to transfer the brain into a clean Petri dish immediately after extraction as the first Petri dish will contain blood and parts of the skull that might damage the brain tissue.

4. Using a scalpel, remove the cerebellum and the olfactory bulbs. Separate the two hemispheres by cutting along the sagittal axis ([Figure 2D](#)). Then, slice off a portion of the cortex to flatten the dorsal part of the brain ([Figure 2E](#)).

⚠ CRITICAL: It is important to cut the dorsal part of the brain as flat as possible to fix the brain correctly and have identical slices throughout the days.

5. Put some glue on the vibratome sample holder. Reassemble the brain ([Figure 2F](#)), fix it on its dorsal surface by sliding it from the spoon onto the sample holder, and slice it using a vibratome to obtain 350 μm thick slices. This will allow the slicing of both hemispheres together with the same accuracy ([Figure 2G](#)).

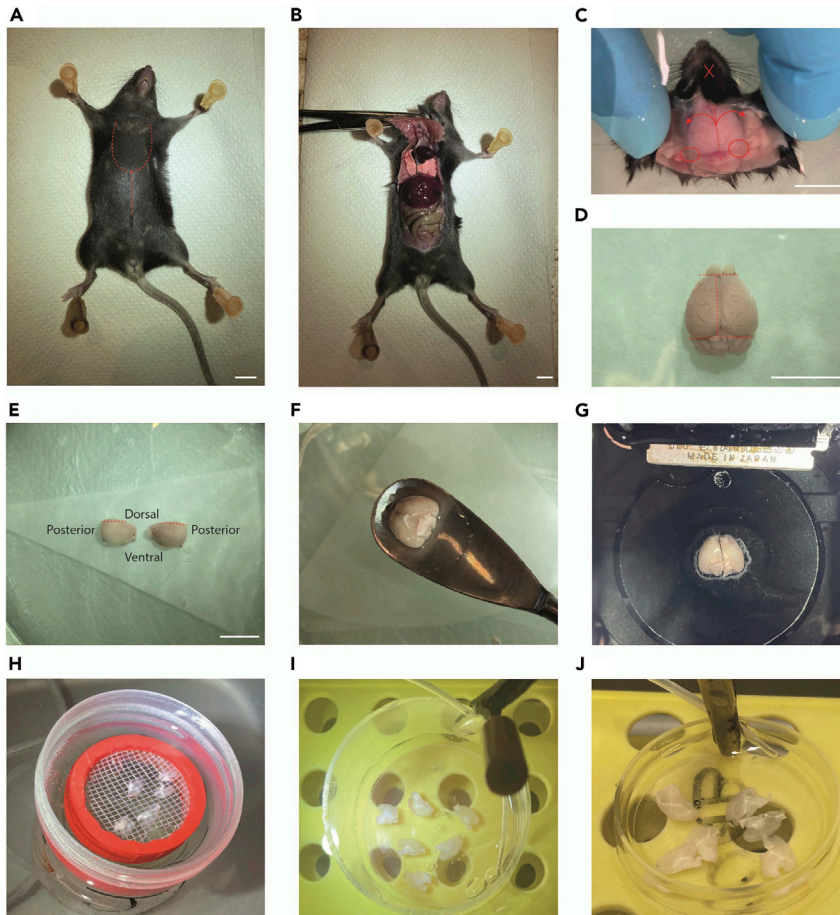


Figure 2. Slice preparation and dye loading

(A) The mouse is fixed by the paws using syringe needles. The red dotted lines show where to cut for clear access to the heart.

(B) Photo of the mouse after performing the laparotomy and thoracotomy.

(C) After opening the scalp, cut the skull following the dotted red line. Then remove the two parts of the skull as indicated by the red arrows. The two red circles show important bones to remove to avoid damaging the brain during the extraction. Stick the tips of closed scissors in the red cross and open them to remove the lateral part of the skull. This last step is optional but facilitates brain extraction.

(D) Brain after extraction. The red dotted lines show where to cut to remove the cerebellum, olfactory bulbs and hemisect the brain.

(E) After hemisecting the brain, cut the two parts following the red dotted lines to flatten the dorsal part of the brain. Scale bar A–E = 1 cm.

(F) Use a spoon-shaped tool to retrieve the brain after re-assembling it. Dry the remaining liquid and fix it on its dorsal surface.

(G) Fixed brain before slicing.

(H) Photo of the slices placed in the recovery chamber.

(I) Photo of the slices placed in the culture well for SR101 staining.

(J) Photo of the slices placed in the culture well for OGB1-AM loading.

Note: Use a spoon-shaped tool to put the brain on the vibratome sample holder. The glue we use sets when in contact with a liquid. Thus, dry the spoon and the dorsal surface of the brain as much as possible.

Note: We use a Leica VT1000S vibratome with the following parameters: (1) 0° blade angle; (2) 7.5 (75 Hz) vibration frequency; (3) 1 (0.05 mm/s) progression speed.

△ **CRITICAL:** during the slicing, it is essential to keep the solution containing the brain as cold as possible by adding ice around the slicing tank (you can also pour ethanol into the ice to keep it cooler). Failing to keep the solution cold enough will cause the brain to soften, which may cause it to be crushed by the razor blade. Furthermore, keeping the brain cold will reduce both the activity of degradation enzymes and the metabolic activity within the cells.

6. Transfer the slices into a recovery chamber containing aCSF (see [materials and equipment](#)), oxygenated with carbogen for 1 h at 25°C ([Figure 2H](#), for recovery chamber details, see [Figures 1E](#) and [1F](#)).

△ **CRITICAL:** Starting from this point, all manipulations have to be performed in the dark.

Slice incubations for SR101 dye loading

⌚ **Timing:** 30 min

7. While waiting for the slices to recover in the chamber, pour 10 mL of aCSF into a culture well and add 10 µL of SR101 (1 mM) so the final concentration of SR101 will be 1 µM. Connect a carbogen line to the well to make it bubble at a modest rate (~2–3 bubbles/second). Carefully position the bubbling culture well into the heating bath at 37°C ([Figure 2I](#)).

Note: You can make a platform with a test tube rack so the well will heat properly and not sink.

8. After 1-h recovery (step 6), transfer the slices from the recovery chamber into the culture well containing SR101 using either a brush or a Pasteur pipette and let them incubate for 20–30 min ([Figure 2I](#)).

Note: Make sure that the slices do not overlap with each other and that the carbogen bubbling does not move them around.

Slice incubations for calcium dye loading

⌚ **Timing:** 1 h

9. The following step is only applicable for calcium imaging.
 - a. During the incubation period of step 8, prepare the OGB1-AM solution. Add 2 µL of PF-127 (20% in DMSO) and 8 µL of Cremophor EL (0.5% in DMSO) in the OGB1-AM vial. Vortex 1 min. Wrap the vial in aluminum foil to avoid photodamage and ultrasonicate it for 10–20 min.

Note: If you do not have a sonication bath, you can use a thermomixer at max speed, 40°C for 10 min.

- b. Centrifuge the aliquot for 5 s to get all the droplets to the bottom of the vial. Carefully add 20 µL of aCSF to the vial and homogenize the solution by gently pipetting up and down without making bubbles.
 - c. At the end of the SR101 incubation time, transfer the slices into a small culture well containing 3 mL aCSF. Put a carbogen line in the well and adjust the carbogen flow to produce 1 bubble every 2 s. Carefully position the bubbling culture well into the heating bath at 37°C ([Figure 2J](#)).

Note: Maintain the orientation of the slices such that the same side is loaded with both SR101 and OGB1-AM.

Note: Surfactants tend to produce foam that will desiccate the slices. Try to adjust the carbogen flow in such a way as to keep the bubbles along the well wall and not floating above the slices.

Note: You can also put the carbogen line just above the level of the aCSF such that it oxygenates the solution without producing foam. To do so, pipet 3 mL into the well and make a hand-made hole just above the level of the aCSF.

d. Slowly and carefully, deposit 6–10 μ L of OGB1-AM solubilized into Cremophor and PF-127 directly onto your region of interest to ensure that it will receive exposure to a high concentration of dye. Repeat the operation for all other slices. Incubate for 45 min to 1 h in the dark.

△ CRITICAL: Cover the incubation well with a lid to avoid evaporation and dehydration of the slices. Leave a tiny space between the lid and the well to allow carbogen to escape from the well.

Recovery

⌚ Timing: 1 h

10. After the incubation period, transfer the slices to a recovery chamber filled with aCSF oxygenated with carbogen. Incubate at 25°C for at least 1 h before starting experiments (Figure 2H).

Note: At this point, you can take a break to eat.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Oregon Green 488 BAPTA-1, AM, cell permeant – Special Packaging	Thermo Fisher Scientific	Cat#O6807
Sulforhodamine 101 (SR101)	Merck	Cat#S7635
NaCl	Sigma-Aldrich	793566
KCl	Carlo Erba	471177
NaH ₂ PO ₄	Sigma-Aldrich	S9638
NaHCO ₃	Sigma-Aldrich	S5761
D-Glucose	Sigma-Aldrich	G8270
MgSO ₄	Merck	63139
CaCl ₂	Carlo Erba	433381
NMDG	Sigma-Aldrich	M2004
HEPES	Sigma-Aldrich	H4034
L-Ascorbic Acid	Sigma-Aldrich	95209
Thiourea	Sigma-Aldrich	T8656
Sodium Pyruvate	Sigma-Aldrich	P2256
N-Acetyl-L-Cysteine	Sigma-Aldrich	A7250
Kynurenic acid	Alomone labs	K-110
BAPTA	Thermo Fisher Scientific	B1204
Na ₂ ATP	Sigma-Aldrich	A2383
Na ₂ GTP	Sigma-Aldrich	51120
MgCl ₂	Sigma-Aldrich	208337
PF-127	Sigma-Aldrich	P2443

(Continued on next page)

<i>Continued</i>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Cremophor EL	Sigma-Aldrich	238470-1 SET
DMSO	Sigma-Aldrich	D8418
Experimental models: Organisms/strains		
1–2 months old C57BL6/J male or female mice	Chronobiotron UMS3415	n/a
Software and algorithms		
MetaFluor 7.8.8.0	Molecular Devices	n/a
pClamp 10	Molecular Devices	n/a
uEye Cockpit	iDS software suite	n/a
MultiClamp 700b commander	Molecular Devices	n/a
Fiji	Schindelin et al. (2012)	https://imagej.net/software/fiji/downloads
Deposited data		
Example code	Alexandre Charlet's lab	https://github.com/AngelBaudon/Canal.git
Other		
Vibratome	Leica Biosystems	VT1000S
pH meter	Hanna instruments	HI 2210
Micro-osmometer	Löser	Type 6
Ultrasonic cleaner	Branson	5510-MT
Amplifier	Axon Instruments	Digitizer
Digitizer	Axon Instruments	MultiClamp 700B
Microscope	Zeiss	Examiner A1
Manipulators	Sutter Instrument	MPC-325
Light source (patch)	Lumen Dynamics	XT640-W
LED light source	Lumencor	7-CLX-NA
Peristaltic pump	Gilson	Minipuls 3
Anti-vibration table	Photon Lines	63-7590M
Recording chamber	Multichannel systems	RC-26G
Spinning disk	CrEST	X-LIGHT
sCMOS camera	QImaging	optiMOS
uEye camera	iDS	UI-1240LE-NIR-GL
Electrode holder + Ag wire	Warner instruments	QSW-T15P
Flaming/Brown micropipette puller	Sutter Instrument	P-97
Borosilicate glass	Sutter instrument	BF150-86-7.5HP
Nalgene 4-mm syringe filters purple	Thermo Fisher Scientific	176-0020
26G needle	Agani	SAN2613R1
Syringe 1 mL	Sigma-Aldrich	Z683531
Syringe 20 mL	Sigma-Aldrich	Z116882
Syringe 50 mL	Sigma-Aldrich	Z118400
Ketamine	Virbac France	211101
Paxman	Virbac France	221631
Qualitative filter paper 413	VWR	516-0817
Scalpel	Fine Science Tools	10004-13
Scalpel blades	Fine Science Tools	10021-00
Dumont forceps	Fine Science Tools	11254-20
Bonn scissors	Fine Science Tools	14184-09
Fine scissors – Sharp-blunt	Fine Science Tools	14028-10
Paintbrush	Princeton	Round – natural red sable
Loctite Superglue-3	Dutscher	999227

Note: The resources listed here are what we use at the time of writing this protocol. There are currently many different alternatives and new versions of some products may become available in the future.

MATERIALS AND EQUIPMENT

- aCSF

Reagent	Final concentration (mM)	Amount
H ₂ O	n/a	1 L
NaCl	124	7.2416 g
KCl	2.5	0.1864 g
NaH ₂ PO ₄	1.25	0.1725 g
NaHCO ₃	26	2.1843 g
D-Glucose	15	2.7030 g
MgSO ₄	2	0.493 g
CaCl ₂	2	0.294 g

Should be stored at 4°C. Maximum storage time ~2 weeks.

- Perfusion solution

Reagent	Final concentration (mM)	Amount
H ₂ O	n/a	5 L
NMDG	93	90.7773 g
KCl	2.5	0.9319 g
NaH ₂ PO ₄	1.25	0.8625 g
NaHCO ₃	30	12.6015 g
HEPES	20	23.8310 g
D-Glucose	25	22.5250 g
L-Ascorbic Acid	5	4.4030 g
Thiourea	2	0.7612 g
Sodium Pyruvate	3	1.6506 g
N-Acetyl-L-Cysteine	10	8.1595 g
Kynurenic acid	2.5	2.3646 g
MgSO ₄	10	12.324 g
CaCl ₂	0.5	0.3675 g

Should be stored and used at 4°C. Maximum storage time ~2 weeks. If you prepare large volumes, you can freeze it at -20°C and keep it for up to 1 year.

Note: You will have to use a lot of HCl while adjusting the pH.

Alternatives: You can also use sucrose instead of NMDG. We use it at a concentration of 180 mM and adjust the N-acetyl-L-Cysteine to 5 mM instead of 10 mM.

- Internal solution for astrocyte patch-clamp

Reagent	Final concentration (mM)	Amount
H ₂ O	n/a	50 mL
KMeSO ₄	125	838.75 mg
HEPES	10	119.15 mg
BAPTA	10	314.4 mg
Na ₂ ATP	4	110.228 mg
Na ₂ GTP	0.4	10.4636 mg
MgCl ₂	4	19.042 mg

Should be stored at -20°C and used at 4°C. Maximum storage time ~1 year.

Note: This last solution should be prepared and used on ice as ATP/GTP is unstable at room temperature. We recommend weighing and adding these two components last. As soon as ATP/GTP is added, keep the solution at 4°C.

Note: For the aCSF and perfusion solutions- after adding all powders to the water, bubble the solution with carbogen for about 15–20 minutes to activate the bicarbonate pH buffer. For all solutions, titrate the pH to 7.3–7.4 at room temperature (RT; 20°C–25°C) (except the internal solution at 4°C to avoid ATP/GTP degradation), as needed, using HCl or NaOH to acidify or alkalize the solution, respectively. Because CaCl₂ equilibrates slowly, we add it after pH titration.

If needed, adjust the osmolality to 300 ± 10 mOsm/kg by adding water or NMDG/glucose/sucrose to decrease or increase the osmolality, respectively.

Note: It is not recommended to adjust the osmolality as it will change ions concentrations. It is preferable to optimize the protocol beforehand to have the correct osmolality every time.

- Required tools

Equipment	Surgical instruments	Consumable
<ul style="list-style-type: none"> • Carbogen gas supplier (95% O₂, 5% CO₂) • Vibratome Leica VT1000S • Heating bath • Fluorescence microscope • Recording chamber (Figure 1B) • Peristaltic pump • For calcium imaging: spinning disk (Figures 1A–1C) • For BAPTA loading: Patch-clamp set-up (Figures 1A–1C) • Recovery chamber (Figures 1E and 1F) • pH meter • Osmometer 	<ul style="list-style-type: none"> • Scalpel • Forceps • Scissors • Paintbrush • Superglue 	<ul style="list-style-type: none"> • Ketamine (400 mg/kg) • Paxman (80 mg/kg) • 1 mL/20 mL/50 mL syringe • 26G needle • Blotting paper • aCSF • Perfusion solution • OGB1-AM • SR101 • PF-127 (20% in DMSO) • Cremophor EL (0.5% in DMSO)

STEP-BY-STEP METHOD DETAILS

Calcium imaging of astrocytes

⌚ Timing: 3–7 h

Astrocytes, as with any glial cell, do not fire action potentials. However, they do exhibit calcium-based excitability in certain contexts (Charles et al., 1991). For instance, the cytosolic calcium concentration of astrocytes increases in response to many types of stimuli such as neurotransmitter signaling, mechanical stimulation and variations in pH (Khakh and Benjamin, 2019). The following protocol describes a method to monitor these calcium fluctuations in astrocytes.

Follow the initial steps (1–10) for slice preparation, as described in the preparation section above.

1. Transfer the slices into an aCSF filled recording chamber (see [key resources table](#)) connected to a peristaltic pump that provides a continuous flow of oxygenated aCSF (~2 mL/min) at room temperature (or any temperature as needed by the experiment).
2. Place a holding harp on top of the slice to prevent movement.

Note: make sure that the strings of the harp are not too close to the recording site. Otherwise, it will bend your slice and thereby prevent consistent focus with the microscope.

3. Use an Axio examiner Zeiss microscope (or Olympus BX51WI Japan for example) with a 4× objective to identify your region of interest. Set the microscope to infrared differential interference contrast (IR-DIC, $\lambda = 750\text{--}790\text{nm}$) and visualize the image with the uEye cockpit software (or Andor iQ 3.2 for example).

Note: Use an appropriate atlas to identify landmarks that will help you locate your structure of interest.

4. Switch to a 40× water-immersion objective and find the surface of the slice.
5. Open the MetaFluor software and click the “Configure” menu to set the following parameters:
 - a. Spinning disk pinhole diameter: 70 μm .
 - b. Spinning disk rotation speed: 15,000 rpm.
 - c. Wavelength: 575 nm for SR101, 475 nm for OGB.
 - d. Exposure time: 20 ms for SR101, 80 ms for OGB.
 - e. Illumination intensity: 220 μW for SR101, 580 μW for OGB.
 - f. Exposure time: 20 ms for SR101, 80 ms for OGB.
 - g. Sampling frequency: 2 Hz (500 ms interval between acquisitions).

△ CRITICAL: Before switching on the fluorescent light, do not forget to put your microscope in confocal mode. The high intensity of the wide-field mode will bleach the calcium indicator.

△ CRITICAL: Finely tune your illumination time and intensity to avoid fluorescence bleaching. To do so, we recommend starting at an illumination intensity of 0 μW and an illumination time of 20 ms then progressively increase both values. In our hands, we typically set the illumination intensity between 580 μW and 1040 μW and find that good OGB1 loading can be seen at an illumination time of 20 ms. We never illuminate more than 100 ms.

6. Cells labeled with SR101 are astrocytes.
 - a. Find a focal plane that includes several of them ([Figure 3A](#) & [Method video S1](#)).
 - b. Check the shape of the cells: a healthy cell must have a ramified morphology with observable proximal processes ([Figures 3A](#) and [3B](#)). If the cells exhibit a round, swelled shape, it may indicate that these cells are dead or dying. If so, refer to [Troubleshooting](#).
 - c. You can observe 3 kinds of cells: (1) the ones that are labeled with both markers (SR101 and OGB, yellow merge in [Figure 3A](#)), indicating that astrocytes are well loaded with both indicators; (2) the cells SR101 negative, that are probably neurons; and (3) cells labeled with SR101 but that are OGB1 negative.

Note: In this last configuration, try to run the experiment anyway, the labeling is maybe too weak to be seen at resting state but can be enough to detect calcium transients. If you did not see any variation of the OGB1 fluorescence, refer to [Troubleshooting](#).

Note: To guarantee a good signal/noise ratio, you must adjust the focal plane in such a way as to both see the cell and distinguish the proximal processes.

7. Click on “Save pictures” and choose an appropriate folder. Click on “Zero clock” and then “Start”. Your data must be saved as independent pictures in the selected folder. At the end of a 10-min recording, you should have 2400 pictures (1200 for the SR101 channel and 1200 for the OGB1 channel).

Note: Because the files produced are very large, be sure that you correctly set the sampling frequency regarding the event you want to observe. The sampling frequency should be at

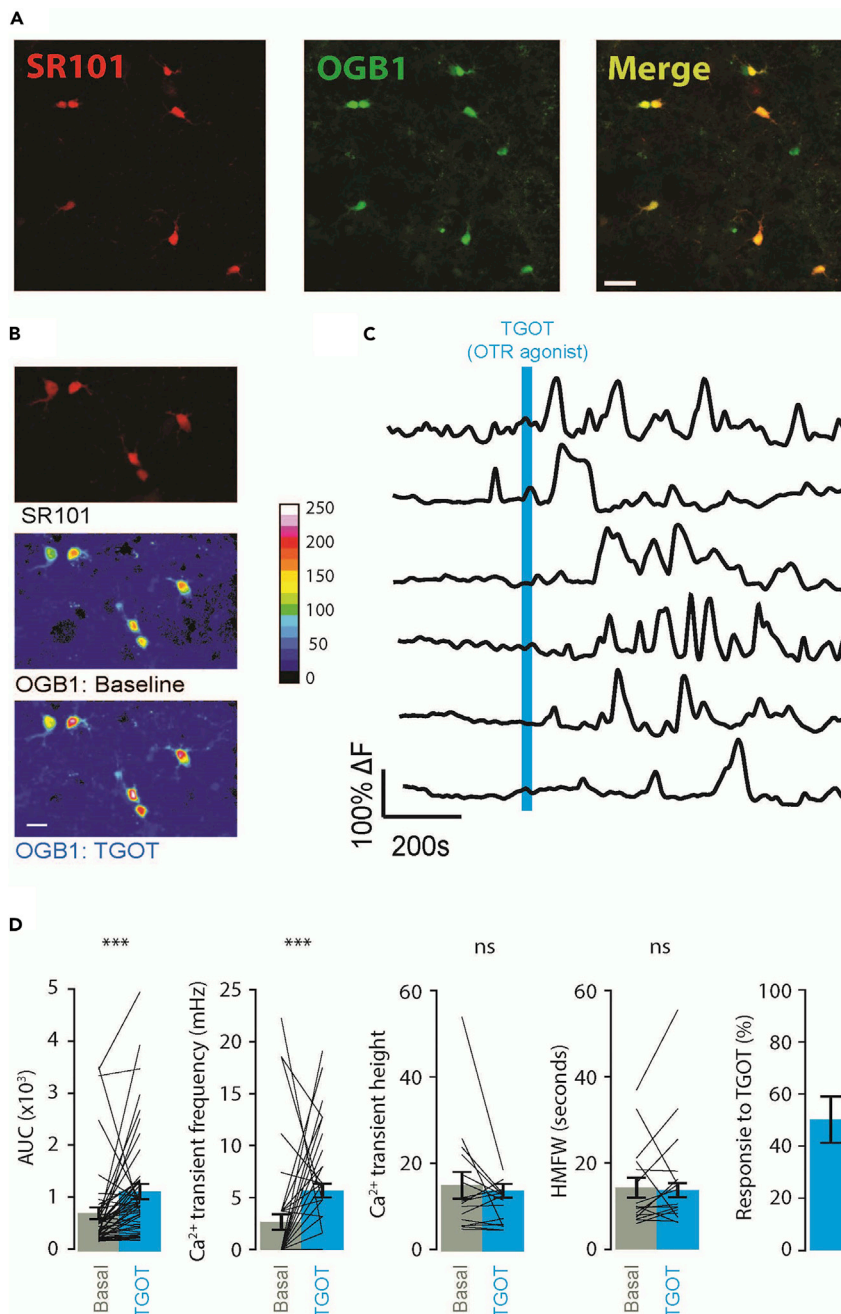


Figure 3. Expected results from a calcium imaging experiment

(A) Stacked picture of SR101 staining (left), OGB1 staining (middle) and the merge of both labels (right). Scale bar = 50 μ m.

(B) Pseudo-colored images showing the increase of fluorescence after application of an OTR agonist (TGOT 1 nM 10 s duration) on OGB1-loaded astrocytes. Upper panel: SR101 staining; middle panel: basal OGB1 fluorescence; lower panel: OGB1 intensity after application of an OTR agonist (TGOT 1 nM, 10-s duration). Scale bar = 50 μ m.

(C) Traces of OGB1 fluorescence over time. The blue bar represents the application of TGOT 1 nM 10-s duration.

(D) Quantification of the effect of TGOT 1 nM on astrocytic activity. From left to right: variation of AUC, frequency, calcium transient height, half-max full width (FWHM), and proportion of responsive cells after TGOT application. The gray histograms represent baseline values and the blue histograms represent the quantification of the TGOT effect. Data are represented as mean \pm SEM. *** $p < 0.001$, Paired t test.

least twice as high as the maximal frequency of the events recorded (see Nyquist-Shannon theorem). For somatic global calcium transients that last for more than 2 seconds, a 2 Hz sampling frequency is sufficient. In our hands, a 10-minute recording at 2 Hz results in an individual picture size of ~1 MB (~2.4 GB of pictures in total).

8. At the desired time, apply a drug or any kind of stimulation to see if it changes astrocyte calcium activity. We apply an oxytocin receptor agonist, the TGOT at [1 nM] for 10 s through the perfusion system (Figures 3B–3D).

Note: If your perfusion aCSF is in a close loop, be sure to discard the solution for ~5 minutes to avoid contamination of your clean aCSF stock.

Patch-clamp-mediated BAPTA loading of astrocytes

⌚ Timing: 3–7 h

Astrocyte communication is largely based on intracellular calcium signaling (Khakh and Benjamin, 2019). Thus, blocking these transients using calcium chelators will inhibit astrocytic activity. As astrocytes create a large network interconnected via gap junctions, loading a chelator in just a few astrocytes is sufficient to block calcium transients across a local network. Thus, this approach can be used to observe the effects of blocking calcium-dependent astrocyte communication on the activity of a nearby neuronal network. In this section, we describe the detailed steps needed to perform patch clamp on an astrocyte and the protocol used to load BAPTA into the astrocytic network. This experiment can also be performed with other calcium chelators such as EGTA, but we choose to use BAPTA because its binding kinetics is ~40 times faster than EGTA, allowing for the blockade of local and rapid calcium transients (Naraghi and Neher, 1997).

Optional: this protocol can be used in combination with the calcium imaging of astrocytes. If you intend to do so, please follow the ‘optional’ steps such as this one that indicates what steps are required from both protocols.

Follow the initial steps (1–10, except step 9) for slice preparation, as described in the preparation section above.

Optional: If you intend to perform BAPTA loading together with calcium imaging, follow the initial steps (1–10, including step 9) for slice preparation, as described in the preparation section above.

9. Transfer the slices into an aCSF filled recording chamber connected to a peristaltic pump that provides a continuous flow of oxygenated aCSF (~2 mL/min) at room temperature (or any temperature as needed by the experiment).
10. Use an Axio examiner Zeiss microscope (or Olympus BX51WI Japan for example) with a 4× objective to identify your region of interest. Set the microscope to infrared differential interference contrast (IR-DIC, $\lambda = 750\text{--}790\text{ nm}$) and visualize the image with the uEye cockpit software (or Andor iQ 3.2 for example).
11. Switch to a 40× water-immersion objective and find the surface of the slice.

Note: At this point, you should visualize the cells with infrared illumination to verify that the slice is ‘healthy’. Check that there are no or only a few dead cells, which can be discriminated by their round shape and absence of clear edges.

12. Visualize SR101 labeled astrocytes by illuminating the slice with yellow ~575 nm light. Once you identify your shining star (i.e., the cell you want to patch), do not adjust the microscopic stage position (in neither the x nor y-axes) or you may lose track of it (Figure 4A).

Note: One should keep several considerations in mind when choosing a cell to patch: (1) Deep cells are harder to visualize and are more easily disrupted by the pipette-induced movement of the tissue above. (2) A healthy cell is not round. (3) Bigger cells are easier to patch.

Optional: If you intend to perform BAPTA loading together with calcium imaging, also refer to step 5 and 6 and try to find several healthy astrocytes in the same stage position.

Note: In uEye Cockpit, press 'Ctrl + right click' to place a marker on the screen. This will help you return to the cell more easily. Alternatively, place a small chunk of putty (e.g. Play Dough) on the screen for each target cell.

13. To get a pipette above the slice:
 - a. Raise the objective far away from the slice, while still keeping the lens submerged in the solution.
 - b. Take a pulled pipette (borosilicate glass; outer \varnothing : 1.5 mm; inner \varnothing : 0.86 mm; length: 7.5 cm, see [key resources table](#)) previously filled with the internal solution.

Note: To create a pipette filler: place a pipetman tip on a 1 mL syringe. Use a flame to heat the tip and pull it until it is thin enough to enter the pipette, blow air into it with the syringe while doing this to avoid clogging. Use a razor blade to cut the end of the pulled tip and then verify that it is not blocked by pushing water through it. If the water does not flow through the tip, replace it and start over. It may take several attempts to obtain a working tip (see [Figure 1D](#) for an example of how it should look).

- c. Insert the pipette onto the AgCl electrode.
- d. Put positive pressure (about 1 mL of air with a syringe). This will help avoid clogging your pipette while moving through the tissue. Position the pipette in the bath using the manipulator.
- e. If you are using pClamp, use the membrane test to determine the resistance of the pipette (if the signal is drifting, see [troubleshooting](#)). Otherwise, in voltage-clamp, apply a voltage step of 5–10mV and measure the current transients to calculate the pipette's resistance using Ohm's law. It must be $\sim 6 \pm 2 \text{ M}\Omega$ (see [troubleshooting](#) for more details). A lower value means the tip is too large (or broken) and a higher value means the tip is too thin. In either case, it will be difficult to patch a cell.

Note: The instruction manual of your pipette puller contains helpful instructions on how to pull the pipette to achieve the desired resistance.

- f. Under infrared light, bring the pipette tip under the objective using the micromanipulator. Move the pipette along the x and y axes until you see a shadow moving on the screen of the computer and then adjust the objective to focus on it. Then, move the pipette until the tip is in focus.

Note: While positioning the pipette tip, it is important to continuously monitor both the computer screen and the actual pipette to ensure the tip does not hit any obstacles (e.g. the objective).

△ CRITICAL: Once the pipette is in the bath, extreme caution must be used when moving it along the z-axis. Uncontrolled movement of the pipette can result in damage to both the tissue and the recording chamber.

- g. The next goal is to safely lower the pipette just above the slice.
 - i. First, lower the objective.

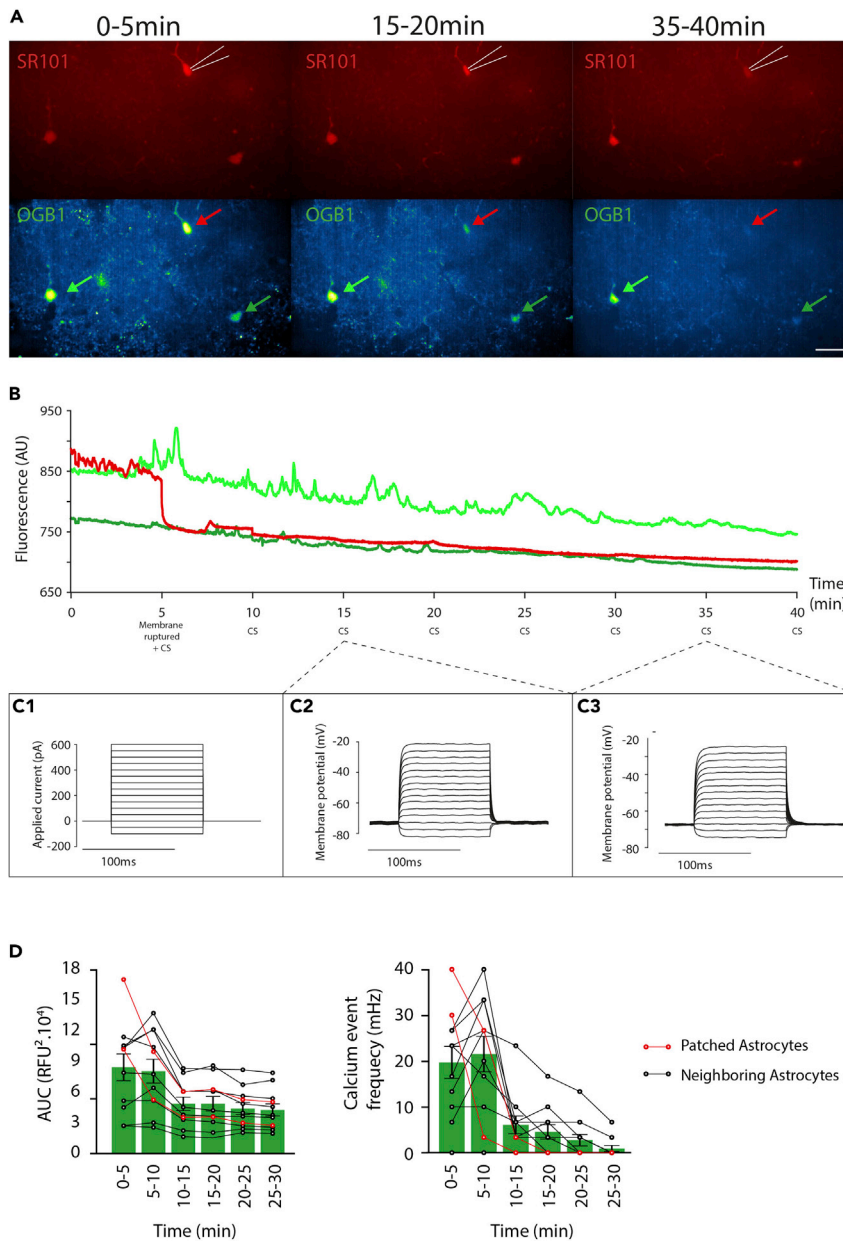


Figure 4. Patch-clamp mediated BAPTA loading in astrocytes

(A) Pseudo-colored images showing SR101 (top) and OGB1 (bottom) at the beginning of the recording (left), after 15 min (middle) and 35 min (right) of recording. The white lines represent the pipette. The three arrows indicate the signals of the cells shown in (B). Scale bar = 50 μm .

(B) Raw signals showing the evolution of OGB1 fluorescence from the same three cells shown in A that were recorded for 40 min. At 5 min, the patched cell membrane is ruptured (orange).

(C) Example of current step (CS) realized during the recording in (B). Left: command step of the CS: 15 steps of 100 ms starting at -100 pA and incremented by 50 pA. Right: membrane potential of the astrocyte recorded in response to the CS.

(D) Quantification of the effect of BAPTA loading in astrocytes. Left: Quantification of the area under the curve (AUC) of calcium measurement in astrocytes. This value is expressed in relative fluorescence unit² (RFU²). Right: Quantification of calcium transients in astrocytes. The astrocyte membrane is ruptured at 5 min. The red points indicate the values for patched astrocytes and the black ones for neighboring cells. Data are represented as mean \pm SEM.

- ii. Then lower the pipette until you can see it again.
- iii. Repeat this sequence until you are just above the slice.

Note: you cannot go all the way down with the objective because you will bend/break the pipette and you cannot go down only with the pipette because you will not know when you will be near the tissue.

14. Once you are just above the tissue.
 - a. Position the pipette tip above the targeted cell, slightly to its side.
 - b. Switch the microscope channel to see the fluorescent labeling of astrocytes.
 - c. Lower the pipette down into the tissue until you are on the same level as the top of the target cell.
 - d. Move the pipette toward the cell (see [Method video S1](#)).

Note: To make positioning of the pipette easier, you can move the objective up and down to help identify your current location within the tissue.

Note: Usually, it is easier to approach the cell diagonally.

15. Two indicators can be used to confirm you are on the target cell.
 - a. The first is visual: the positive pressure within the pipette creates a visible deflection (dimple) on the membrane.
 - b. The second indicator is the pipette resistance: when you get close enough to the cell, the tip of the pipette becomes enclosed by a pocket of membrane and thus increases the resistance. You can expect an increase of about 0.2 M Ω –1 M Ω (see [Method video S1](#)).
16. Release the positive pressure (this increases the resistance as more membrane enters the pipette).

Note: When releasing the positive pressure, the resistance should increase up to at least 15–20 M Ω . The better the pipette is positioned on the cell, the higher the resistance will go (sometimes even directly up to gigaseal).

17. Gently apply negative pressure by mouth until you achieve a gigaseal (i.e., a pipette resistance of several G Ω).

Note: After reaching 100 M Ω , start holding the potential at the desired membrane potential value (–80 mV), this will help reach the gigaseal.

Note: Astrocytic membranes are much more fragile than neuronal membranes. If you are accustomed to patching neurons, then be extra gentle during seal formation and membrane opening.

Optional: To confirm that calcium has been correctly chelated by the BAPTA in the patched cell, start the calcium imaging (see step 7) once the giga seal is reached. Wait 5 min before opening the cell.

18. When the gigaseal is reached, apply negative pressure in the pipette by repetitive suction until the membrane opens.
19. If the astrocyte opens correctly, you will see capacitive transients and the resistance will drop by several tens of M Ω . You can also look at the calculated membrane resistance by clicking the “Cell” button on pClamp. This resistance must be ~20 M Ω for astrocytes and ~200 M Ω for neurons in the central amygdala.

Optional: If you are performing calcium imaging at the same time, you should see a drop in the fluorescence of the patched cell (Figure 4B & Method video S1).

20. Switch to current-clamp mode to check the resting membrane potential, which should be around $-80/-90$ mV.
 - a. Apply current steps of 15 steps starting at -100 pA incremented by 50 pA and lasting 100 ms each. This is to verify that the patched cell is truly an astrocyte.
 - b. Thus, the membrane potential should have increased to at least -30 mV and you should not have seen any action potentials (Figure 4C).

Note: If the patched cell is a neuron or even if you just patched a presynaptic terminal, you should see action potentials (Bischofberger et al., 2006).

21. Wait 20 min (Wallraff et al., 2006) to let the BAPTA diffuse in the astrocyte network. Monitor the access resistance regularly to make sure the membrane of the astrocyte is still open and that the astrocyte is still alive.

Optional: If you are performing calcium imaging and patch-mediated BAPTA loading simultaneously, you will observe an immediate fluorescence quenching in the patched cell and a progressive loss of fluorescence in the neighboring astrocytes after 10–20 min. For more details, see [expected outcomes](#) and Figure 4.

22. Remove the pipette very slowly and carefully along the x-axis, as this is the best direction for avoiding damage to the cell. It is extremely important to avoid any damage to the cell during this step, as the goal is to reseal the membrane.

Note: When the membrane is resealed, you should see the pipette resistance reach the $G\Omega$ range again as you will inevitably tear off a part of the membrane during removal.

23. Repeat all the steps from 13 to 21 on another astrocyte located approximately one screen ($200 \mu\text{m}$) apart to ensure the efficient diffusion of BAPTA within the local network.

EXPECTED OUTCOMES

Here, we share a protocol to investigate the involvement of astrocytes and their networks in brain function. We present *ex vivo* approaches to both monitor astrocytic calcium activity (Figure 3) and interfere with their activity at the microcircuit scale (Figure 4).

While one can also investigate astrocytic membrane potential changes using patch clamp in a current clamp mode, tracking of fluorescence variations using a calcium sensor, such as OGB1, in the cytosol further allows one to test whether astrocytes respond to a given stimulus using calcium-associated mechanisms. To distinguish astrocytes from other cells, we use SR101 because it allows a relatively simple way to label astrocytes in wild-type mice. Thus, by selecting cells labeled with both SR101 and OGB1 (Figure 3A), we can restrict our measurements of calcium-mediated OGB1 fluorescence variations to putative astrocytes only (Figures 3B and 3C). Alternatively, one can use genetically labeled astrocytes (e.g., GFAP-GFP mice, Zhuo et al., 1997).

The protocol described here can achieve a variety of experimental goals. One classical experiment is the application of a given drug to quantify its effects on astrocytic calcium signaling. For example, we applied the oxytocin receptor agonist, TGOT [1 nM], for 10 s via the peristaltic perfusion system used to supply aCSF to the recording chamber. One of the outputs of this experiment was a video recording that allowed us to assess visually the effects of an applied pharmacological agent to astrocytic calcium activity. The output data can further be used to quantify several effects of interest

through calculations such as the global area under the curve (AUC), the frequency of calcium transients, the duration of calcium transients, etc. (Figure 3D). You can find an example of these calculations in the following section, [quantification and statistical analysis](#).

A fine analysis of the calcium signals may answer numerous critical questions regarding the upstream mechanisms that trigger calcium transients in astrocytes. However, this technique alone does not provide information about the downstream effects of calcium signaling in astrocytes. Therefore, we also present here a protocol to load the calcium chelator, BAPTA, specifically into a local astrocyte network to disrupt calcium-dependent cellular mechanisms (Figure 4). This approach does not alone produce results *per se* but it may be coupled to other recording techniques. For example, one method to validate BAPTA diffusion in the astrocytic network is to incorporate the use of calcium imaging. To do so, we load astrocytes with SR101 and OGB1 (See Calcium imaging of astrocytes) and then patch SR101 positive cells (See [patch-clamp-mediated BAPTA loading of astrocytes](#)). The output recording allows us to observe a rapid reduction of OGB1 fluorescence in the patched cell followed by a slower reduction of fluorescence in other cells within the same field of view (Figures 4A, 4B, and 4D; [Method video S1](#)). Another application of this method is to load BAPTA into astrocytes and then record the neuronal activity from a nearby network. For example, our group has previously used this method to uncover the critical role of astrocyte-mediated neuromodulation on oxytocin signaling mechanisms in amygdala neuronal networks ([Wahis et al., 2021](#)).

QUANTIFICATION AND STATISTICAL ANALYSIS

In this section, we provide some examples of how to analyze the calcium imaging data. Note that the following examples are by no means an exhaustive list of possible analyses, which should always be chosen within the context of the scientific question being addressed.

1. Launch the open-source FIJI software ([Schindelin et al., 2012](#), available here) and import your recordings.
2. Adjust the brightness and contrast to visualize the cells (Image → Adjust → Brightness/Contrast) and check the quality of your recording. If you see motion artifacts, correct them with a motion correction macro (see Nicholas Schneider's macro available here, instructions here). In our case, we perform recordings in a single plane, therefore all recordings that drift along the z-axis cannot be corrected and must be removed.
3. Run the Bleach correction module (Image → Adjust → Bleach Correction, documentation here). We use the "Simple ratio" method as we have found that it produces adequate correction with minimal distortion of the data.
4. Select the "Oval" tool on the FIJI toolbar and manually draw the Region of Interest (ROI) on the SR101 video, then add it to the ROI manager by pressing the [t] shortcut. Click "Show all" to see which ROIs you have already drawn.
5. Extract the fluorescence signal from each ROI by clicking "More → Multi Measure" in the ROI manager panel. A window containing a table of the results will pop up when it is complete. You can save the table directly or copy the results into a spreadsheet.
6. Open the saved results in the analysis software of your choice. Below, we describe an analysis pipeline that uses a python-based routine (available in the [resource availability](#) section), but the analysis can be applied to any programming language.
7. Preprocessing:
 - a. Read-in the ROI data extracted from Fiji and plot the SR101 data to check if the curve is stable. The intensity of the dye should be constant over time, so if the SR101 curve is not straight then consider rechecking the raw recording for motion or drift in the z-axis. If a motion correction routine is not enough to correct SR101 intensity changes, then exclude the recording from further analysis.
 - b. Correct potential micromovements by subtracting the OGB1 signal from the SR101 signal. Note that a simple subtraction method would introduce noise from the SR101 signal into

the OGB1 signal. For that reason, we perform a polynomial regression on the SR101 curve using the Savitzky-Golay filter of the `scipy.signal` library (window = 51, order = 1) to estimate the trend of the SR101 curve. Doing so, we can subtract the trend of the SR101 trace without introducing noise into the OGB1 signal.

- c. Smoothen the resulting curve without modifying the calcium transient dynamics by applying another, less-stringent polynomial filter (`savgol_filter`, windows = 11, order = 3).
- d. Define two analysis windows: one for the quantification of the baseline activity and another for the quantification of the effect of stimulation. In our recordings, we stimulate astrocytes by applying the oxytocinergic agonist TGOT [1nM] for 10 s, 5 min after the recording begins. Therefore, the windows we select are: (1) 0–5 min for baseline, and (2) 5–10 min for the TGOT effect.
- e. If the windows you choose have different sizes, calculate a ratio to normalize the data according to the duration of each window. For example, if the first window (baseline) is 5 min and the second is 10 min, you must normalize the analyzed parameters (number of calcium transients, area under the curve...) according to the relative duration of each window. To do so, calculate a ratio based on the duration of each window with the following formula:

$$r = \frac{\text{duration of window 1}}{\text{duration of window 2}}$$

8. Processing:

- a. Find the area under the curve (AUC) for each cell using a trapezoid method. Ensure that the function you choose to apply does not evaluate the negative AUC, otherwise it will bias your results by summing the upward and downward deflections. In our case, we remove all negative values before using the “`trapz`” function of the “`NumPy`” package. Split the resulting AUC values according to your analysis windows then sum the data and normalize it with the previously calculated ratio.
- b. Find calcium transients in your curve using the “`find_peak`” function of the “`scipy.signal`” library. The parameter of the detection depends on the events you want to identify. An important consideration here is the detection threshold, which must be proportional to the standard deviation (SD) of the curve. One issue with this method is that highly active cells have higher SDs, which results in fewer detectable peaks. To avoid this issue, we perform a non-stringent polynomial regression (`savgol_filter`(windows = 31, order = 3)) and subtract it from the curve to obtain the residuals (i.e., the noise without the peaks). We then calculate the SD of these residuals and use it as the threshold for peak detection. In our case, we use a minimal height of $8 \times \text{SD}$, a prominence of $5 \times \text{SD}$ and a minimal inter-peak distance of 10 points (corresponding to 5 s in our recordings with a 2 Hz sampling frequency). The height is the minimum height in absolute value and the prominence is the relative height compared to neighboring peaks. This last parameter avoids multiple detections of the same peak that satisfy the “height” parameter. Once you have the index of each peak, compute their frequency within each of the chosen analysis windows.
- c. For each calcium transient index, find the amplitude of each peak. Then, calculate the half-maximum amplitude to measure the Full Width at Half Maximum (FWHM) to evaluate the duration of each peak. We choose to compute the FWHM because it is more reliable than the total duration, wherein you have to find the precise foot peak, which can be especially difficult in calcium imaging due to the potential for slow rise/decay.
- d. Evaluate the responsiveness of each cell to the stimulation. To do so, compare the calcium transient frequency and the AUC during the baseline and drug exposure. We base the response criteria on both calcium transient frequency and AUC to cover the diverse variety of responses observed (e.g., numerous calcium transients and/or single important transient). The baseline activity of astrocytes is heterogeneous due to the stochastic occurrence of

calcium transients. Therefore, we do not evaluate the response based on the SD of the baseline, but rather as a percentage of calcium transient frequency and/or the AUC increase before versus after drug exposure. We classify an astrocyte as responsive if the frequency of calcium spike or AUC increases by 20% after drug exposure.

9. Run paired statistical tests to evaluate if the applied stimulus modified astrocyte activity. If you have two quantification windows, evaluate the normality of your data using a Shapiro-Wilk test and, depending on the result, run either a parametric paired t test or a non-parametric Wilcoxon test. If you have three or more quantification windows, check the normality of the data and their homoscedasticity with a Levene's test. If data follow a normal distribution and their variances are equal, run a One-Way repeated measure ANOVA followed by a pairwise Tukey HSD post-hoc. Otherwise, run a Friedmann test followed by a Conover post-hoc test and a Bonferroni correction of the p value.

LIMITATIONS

The present protocol provides a relatively easy method to selectively label, monitor and disrupt the activity of astrocytes. However, there are several technical limitations to consider.

First, the loading of the AM coupled calcium indicator involves the use of a detergent, which affects the viability of the cells. Indeed, if the slices spend too much time in the AM dye mix, you will likely see several dead cells under the microscope (i.e., swollen and circular).

Another potential problem of this dye loading method is the intracellular distribution of the indicator. While AM loading of calcium indicators is an easy way to infuse the dye in the cytosol of cells, it only provides calcium signals from the soma and the proximal processes. This is of particular importance for astrocytes because they have a huge surface/volume ratio, where the processes represent ~95% of the cell surface (Khakh and Benjamin, 2019) and therefore the inability of the dyes to reach the distal processes may have a significant impact on the resulting observations. To overcome this limitation, Khakh's laboratory has developed membrane-tethered variants of the GCaMP3 and GCaMP6f genetically encoded calcium indicators (reviewed in Shigetomi et al., 2016). Another potential approach is to patch astrocytes with a version of OGB1 that cannot cross the membrane. In this way, high concentrations of OGB1 can be achieved in the cytosol, which would cause the dye to diffuse further into the processes and thereby allow for better visualization. However, this latter approach involves a disruption of the astrocytic membrane and a dilution of the cytosolic content by the intra-pipette solution, which can severely affect astrocytic integrity.

Another limitation to consider is that calcium indicators tend to bleach easily. While this is not a problem for short recordings (<30 min), the change in gain can be substantial across longer recordings. Additionally, in this protocol, we load a non-ratiometric calcium indicator (Maravall et al., 2000). This can be a limitation if you want to compare the AUC across different astrocytes because it is impossible to load each cell identically and thus the gain will vary from cell to cell. However, this issue can be overcome by normalizing the data for each cell with its baseline values.

Since calcium indicators are derived from chelators (e.g., OGB comes from BAPTA), indicator loading in the cell perturbs the endogenous calcium toolbox. Indeed, it has been shown that the loading of such indicators changes the diffusion dynamics of calcium into the cell, shortening its reach and decreasing the concentration of free calcium ions in the cytosol (Semyanov et al., 2020). For these reasons, it is not recommended to couple that type of loading with the measurement of the downstream effects of calcium signaling.

We base our identification of astrocytes on SR101 staining. This method is widely used because it is quite simple to implement either *in vivo* or *ex vivo*. SR101 is taken up into the cytosol of astrocytes through the thyroid hormone transporter OATP1C1 (Schnell et al., 2015). However, the specificity of such labeling is controversial and quite heterogeneous among brain regions (Hülsmann et al., 2017).

In our case, all SR101-positive patched cells did not fire action potentials and had a low membrane resistance, strongly suggesting an astrocytic identity. Moreover, it has been shown that SR101 can be cytotoxic at high concentrations and produce seizure-like local field potential activity *in vivo* (Rasmussen and Nedergaard, 2016). In our hands, we did not observe excessive cell death during the experiments.

The size of the astrocytic network within the structure being studied introduces another limitation concerning the loading of BAPTA. Simply put, the more astrocytes within a given network, the harder it will be to disrupt the activity of the whole astrocytic network. This limitation could technically be overcome by patching more astrocytes; however, this would result in very long protocols. An alternative approach could be to disrupt astrocytic network function using genetic models. For instance, the exogenous expression of Calcium Exchanger (CalEx) in astrocytes can decrease calcium signaling in these cells by extruding cytosolic calcium (Nagai et al., 2021).

Finally, the present protocol describes how to monitor/disrupt astrocyte activity in brain slices. However, this approach introduces bias in that the slicing procedure considerably changes cell activity and connectivity. To overcome this issue, one can perform *in vivo* calcium imaging using genetically encoded calcium indicators, such as GCaMP (for a complete protocol, see Tang et al., 2021).

TROUBLESHOOTING

Problem 1

The slices roll when the razor blade cuts the brain (step 5 of “Slice preparation”).

Potential solution

Try to reduce the speed of the Vibratome or increase the vibration frequency of the blade. If this is not enough to produce good slices, be sure that your slicing milieu is cold enough (4°C) by adding some ice and/or some ethanol around the tank.

Problem 2

There are many dead cells in the slice (step 6 of “calcium imaging of astrocytes”).

Potential solution

A high occurrence of cell death can be explained by several factors:

The brain extraction was too slow and/or it was damaged during the process.

The perfusion solution is not cold enough during the slicing procedure. To prevent this, you can put a thermometer in the slicing tank. If the temperature is above 4°C, add some ice until it reaches the very top of the slicing tank. You also can add some ethanol on the ice.

The incubation time of AM dye is too long. Consider reducing it. For some potentially cytotoxic indicators (Rhod2-AM), loading at room temperature instead of 37°C can increase cell viability.

The slices were agitated too much. Be careful that the bubbles present during the recovery period do not agitate your slices. Also, take extra care when transferring them to different locations. One alternative to consider is using the back of a Pasteur pipette instead of a brush.

Avoid using old solutions (aCSF, perfusion solution) and always ensure that there is no bacterial growth before using them. It is ideal to use fresh solutions for each recording day.

Always verify the pH and osmolality when preparing the solutions.

Regularly check that your slices are properly oxygenated.

Problem 3

It is hard to focus on the entire field of view when performing calcium imaging (step 6 of “[calcium imaging of astrocytes](#)”).

Potential solution

This problem can occur when the strings of the harp that hold the slice are too close to each other. In this case, the slice surface becomes bent and is no longer parallel to the optic plane of the microscope. To solve this, you can cut out a string from the middle of the harp and then position the harp on the slice such that your recording area lies in the gap you just created. If this does not solve the issue, then double-check that your recording chamber and microscope are perfectly horizontal and not tilted in any direction.

Problem 4

The slice moves during the calcium imaging (step 6 of “[calcium imaging of astrocytes](#)”).

Potential solution

The solution depends on the axis of the movement:

If the slice moves in the x/y axes, verify that your harp is correctly positioned at the bottom of the recording chamber. Use harps that fit perfectly in the chamber (as is the case with those provided by Harvard Apparatus). Alternatively, your harp may not be heavy enough to counterbalance the flow produced by the peristaltic pump. To correct this you can:

Build a heavier harp.

Build/buy an arena where the harp fits the chamber better.

Check that there is no air entering the tubing, as this can introduce air bubbles into the circuit, which then make the recording milieu vibrate as they pass through the recording chamber.

The flow of the peristaltic pump may be too high and should be reduced as needed.

If the slice moves in the z-axis, it is probably because the strings of your harp are spaced too far apart. Either move the slice to a narrower gap of the harp or try using a different harp.

Problem 5

The calcium indicators bleach too much (step 6 of “[Calcium Imaging of astrocytes](#)”).

Potential solution

If the calcium indicator bleaching is so strong that it will change the interpretation of your data, you have to adjust the duration and intensity parameters of the illumination. In our case, we never illuminate for more than 100 ms. For the illumination intensity, we find the best values are between 580 μW and 1040 μW . However, since all setups are different, we strongly recommend you try several intensity values within your setup.

Problem 6

OGB1 signal is absent/weak (step 6 of “[calcium imaging of astrocytes](#)”).

Potential solution

If you cannot see any signal in the green channel (OGB1), there is probably an issue with OGB1-AM solubilization in the Cremophor EL + PF-127 mix. This can be due to the degradation of the PF-127, which must be kept in an air-proof vial. We usually keep aliquots of PF-127 in 1 mL tubes, covered with Parafilm. Issues in dye solubilisation can also be due to the sonication step. Consider increasing

the time/intensity of the sonication. You can also try to incubate the mix in a heating bath at 40°C for 20 min; however, in our hands, this produces less solubilisation of the mix.

Problem 7

Opening the cell is difficult (step 17 of “[patch-clamp-mediated BAPTA loading of astrocytes](#)”).

Potential solution

Patching small cells is more challenging than bigger ones. Consider some of the following steps to make it easier:

Try using a small pipette tip (around 6–8 MΩ) for smaller cells, but do not exceed 9 MΩ as this would make it even harder.

If after several trials you still struggle to achieve a gigaseal and/or cannot open the cell, it may be because you are not actually on the cell but rather only nearby. Try to get on the cell as you would normally and then move either slightly closer or away from it to understand how to get on a cell correctly. Practice makes perfect in this case.

Problem 8

During the membrane test, the signal is constantly drifting, even after doing the pipette offset (step 13 of “[patch-clamp-mediated BAPTA loading of astrocytes](#)”).

Potential solution

The recording electrode is probably no longer adequately chlorinated. To chlorinate it, run an electrolysis reaction in 3 M KCl. Connect the electrode to the positive pole of a battery and a cable to the negative one. Dip both in the KCl solution and let them sit for 15s. You should see the solution bubble.

Problem 9

The pipette is clogged (step 13 of “[patch-clamp-mediated BAPTA loading of astrocytes](#)”).

Potential solution

Try to remove the clog by applying a strong pressure (with the syringe for example). If the clog is still there, change the pipette.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Alexandre CHARLET acharlet@unistra.fr.

Materials availability

This study did not generate new unique reagents.

Data and code availability

This protocol did not include a particular code. However, to see an example of the code that can be used, feel free to check our public repository where our routines are available at <https://github.com/AngelBaudon/Canal.git>.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101159>.

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AUTHOR CONTRIBUTIONS

Patch loading of astrocytes, E.C.C.; Calcium imaging, A.B.; Supervision, A.C. All authors wrote and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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