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

Protocol to downregulate GABAergic-astrocyte signaling via astrocyte-selective ablation of GABA_B receptor in adult mice

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SUMMARY

Here, we present a protocol to selectively downregulate GABA_B receptor (GABA_BR) expression in astrocytes of mouse medial prefrontal cortex (mPFC). We first describe the procedure of surgeries and viral injections. We then detail genetic, histological, and functional characterizations of astrocytic GABA_BR ablation using RT-PCR, imaging, and behavioral assays. The use of GABA_B flox mice can be easily adapted to generate astrocyte-selective GABA_BR ablation in different brain areas and postnatal stages, leading to local downregulation of GABAergic-astrocyte signaling without developmental issues.

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

BEFORE YOU BEGIN

The protocol below describes the specific steps for deleting Gabbr1 gene expression specifically in astrocytes using viral approaches in a GABA_B flox transgenic mouse. The strategy described in the following sections allows to evaluate the correct ablation of GABAergic signaling in astrocytes in the mPFC of adult mice, without interfering with brain developmental processes. Before you begin, review the key steps of the protocol.

Institutional permissions

All the procedures relating to the handling and sacrificing of the animals have been approved and supervised by the local Bioethics Committee (2013/53/RD) in accordance with the European Commission (2010/63/EU) and FELASA guidelines for the welfare of experimental animals.

Be aware that to use animals and perform the following procedures you should have the corresponding permissions from the relevant institutions.

Set up operating table

© Timing: 30 min

1. Materials needed
   a. Stereotaxic frame to fix the skull and take the appropriate coordinates.
   b. Stereotaxic injector to infuse the viral particles.
   c. Drill to make a craniotomy in the skull.
d. Gas anesthesia system to anesthetize the mice with isoflurane.
e. Heating pads to avoid hypothermia during surgery and recovery.
f. Surgical instruments (scissors, forceps, sutures).
g. Surgical microscope.
h. Skin antiseptic.
i. Local anesthetic.
j. Analgesics.
k. Eye gel.
l. Cotton buds.

2. Viral vectors
   a. 5 μL aliquots of virus are stored at –80°C. Take one and keep it at –20°C or in ice until use.
   b. To place the virus into the glass pipette:
      i. First, place the pipette in the stereotaxic injector and make sure it is fixed.
      ii. Fill the glass pipette with mineral oil to avoid air bubbles mixed with the viral particles and
          injection flow obstruction (using the withdrawal mode of the microinjector).
      iii. Eject the mineral oil from the glass pipette until reaching the first quarter of the pipette.
      iv. Place the total volume of the virus on a flat and sterile surface and fill the pipette using the
          withdrawal mode.

   Note: Glass pipettes (Drummond Wiretrol, 10 μL) can be used to drive the virus into the
   selected brain area. Sharp tips are generated using a puller in order to reduce brain damage.
   Make sure that the pulled end is long enough for the targeted brain area. For example, if the
dorso-ventral coordinate is 2.5 mm, the thinner portion must be longer than 2.5 mm and
   tapered 50 μm tip diameter. Alternatively, use a syringe instead of a glass pulled pipette,
   i.e Hamilton syringe for dispensing volumes in the range of nanoliters, with successful results.

As a reference example of pulling pipette in P-1000 puller (1 cycle):

| Heat (°C) | Pull (force) | Velocity | Delay | Pressure |
|----------|--------------|----------|-------|----------|
| 550      | 60           | 60       | 225   | 500      |

   Note: Heat is determined from the Ramp test.

Brain slicing solutions: NMDG-HEPES and aCSF solution preparation

© Timing: 45 min each solution

3. Prepare 1l of N-Methyl-D-glucamine (NMDG)-HEPES Recovery Solution (Ting et al., 2014).
4. Prepare 1l of aCSF (Artificial Cerebrospinal Fluid Solution).

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Guinea pig polyclonal Anti-GABA B Receptor R1 (1:1000 dilution) | Millipore | Cat#AB2256; RRID: AB_11210385 |
| Mouse monoclonal Anti-NeuN (1:500 dilution) | Millipore | Cat#MAB377; RRID: AB_2298772 |
| Rabbit polyclonal Anti-S100 (1:200 dilution) | Abcam | Cat#ab868; RRID: AB_306716 |
| Mouse monoclonal anti-GAD67 (1:500 dilution) | Merck (Sigma-Aldrich) | Cat#MAB5406; RRID: AB_2278725 |
| Goat polyclonal Anti-Mouse IgG (H+L) Pacific Blue (1:500 dilution) | Thermo Fisher Scientific | Cat#P31582; RRID: AB_10374586 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Goat polyclonal Anti-Rabbit IgG (H+L) Alexa Fluor 488 (1:500 dilution) | Thermo Fisher Scientific | Cat#A11008; RRID: AB_143165 |
| Goat polyclonal Anti-Rabbit IgG (H+L) Pacific Blue (1:500 dilution) | Thermo Fisher Scientific | Cat#P10994; RRID: AB_2539814 |
| Goat polyclonal Anti-Guinea Pig IgG (H+L) Alexa Fluor 647 (1:500 dilution) | Thermo Fisher Scientific | Cat#A21450; RRID: AB_2735091 |
| **Bacterial and virus strains** | | |
| AAV2.5-GFAP-Cre-mCherry | UNC Gene Therapy Center Vector Core | N/A |
| AAV2.5-hGFAP-cyto-GCaMP6f | Penn Vector Core | N/A |
| **Chemicals, peptides, and recombinant proteins** | | |
| D-AP5 | Tocris | Cat#0106; CAS: 79055-68-8 |
| CNQX | Tocris | Cat#0190; CAS: 115066-14-3 |
| AM251 | Tocris | Cat#1117; CAS: 183232-66-8 |
| MPEP hydrochloride | Tocris | Cat#1212; CAS: 219911-35-0 |
| LY367385 | Tocris | Cat#1237; CAS: 198419-91-9 |
| Tetrodotoxin (TTX) | Alomone Labs | Cat#T-550; CAS: 18660-81-6 |
| (RS)-Baclofen | Tocris | Cat#0417; CAS: 1134-47-0 |
| ATP | Sigma-Aldrich | Cat#A7699; CAS: 34369-07-8 |
| N-Methyl-D-glucamine | Sigma-Aldrich | Cat#66930; CAS: 6284-40-8 |
| L(+)-Ascorbic acid | VWR | Cat#20150; CAS: 50-81-7 |
| Glycerol 3% (C3H8O3) | PanReac | Cat#141339 |
| Sodium pyruvate | Sigma-Aldrich | Cat#P2256; CAS: 113-24-6 |
| HEPES | Sigma-Aldrich | Cat#H3375; CAS: 7365-45-9 |
| Glucose | Biotechnology Grade | Cat#0188; CAS: 50-99-7 |
| Thiourea | Sigma-Aldrich | Cat#T8656; CAS: 62-56-6 |
| Dithiothreitol (DTT) | Sigma-Aldrich | Cat#D0632; CAS: 3483-12-3 |
| Tris hydrochloride | Merck-Roche | Cat# 10812846001; CAS: 1185-53-1 |
| Sodium dodecyl sulfate (SDS) | Sigma-Aldrich | Cat#L3771; CAS: 151-21-3 |
| Proteinase K (20 mg/mL) | Life Technologies | Cat#25530049 |
| Ethylene glycol 3% in PB (CH2OHCH2OH) | PanReac | Cat#141316 |
| **Critical commercial assays** | | |
| Extracta DNA prep for PCR—tissue kit | Quantabio | Cat#95091-002 |
| Applied Biosystems™ Power SYBR™ Green | Thermo Fisher Scientific (Applied Biosystems) | Cat#4368577 |
| DireCtQuant 100ST | DireCtQuant | Cat#DCQ100ST |
| PureLink™ PCR Purification Kit | Thermo Fisher Scientific (Invitrogen) | Cat# K3100-01 |
| SsoFast™ EvaGreen® Supermix | Bio-Rad | Cat#1725204 |
| **Experimental models: Organisms/strains** | | |
| Mouse: Gabbr1-floxed: Gabbr1floxed | Haller et al. (2004) | MGI:3512742 |
| Sex: Male / Female | | |
| Age: 3–4 months for behavior; 1.5 months for ex-vivo recordings | | |
| **Oligonucleotides** | | |
| Gabbr1 forward | Nagai et al. (2019) | (ACAGACAAATCTACCGGGC) |
| Gabbr1 reverse | Nagai et al. (2019) | (GTGCGTGCTAGTAGCGGAT) |
| Gabbr2 forward | Nagai et al. (2019) | (AAGCTCAAGGGGAACGACG) |
| Gabbr2 reverse | Nagai et al. (2019) | (ACTTGCTGCAAACATGCCT) |
| **Software and algorithms** | | |
| GenEx | MultiD Analyses | v.5.4.4 |
| LAS X | Leica Microsystems | N/A |
| Fiji-ImageJ | Schindelin, et al., 2012 | https://imagej.net/software/fiji/ |
| NIS-Elements | Nikon | N/A |
| MATLAB | MathWorks | 2020 |
| EthoVisionXT | Noldus | 11.5 |

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**MATERIALS AND EQUIPMENT**

**T-maze**

Build your own maze or buy it according with these guidelines. Start arm (46 cm long x 11 cm wide x 10 cm high), choice arms (80 cm long x 11 cm wide x 10 cm high). The arms are arranged to form a letter “T” with doors that separate the start and choices arms for test trials (Figure 4) (Korotkova et al., 2010).

**Solutions and drug preparation**

For calcium imaging experiments, make the following drug aliquots prior to use:

| Reagent                  | Stock recommended (mM) | Final (mM) | Action                                      |
|--------------------------|------------------------|------------|---------------------------------------------|
| D-AP5                    | 50                     | 0.05       | NMDA antagonist                             |
| CNQX                     | 10                     | 0.01       | AMPA receptor antagonist                    |
| AM251                    | 5                      | 0.002      | CB1 receptor antagonists                    |
| MPEP hydrochloride       | 10                     | 0.05       | mGlu5 receptor non-competitive antagonist    |
| LY367385                 | 100                    | 0.1        | mGlu1a receptor antagonist                  |
| Tetrodotoxin             | 1                      | 0.001      | Sodium channel blocker                      |
| Baclofen                 | 20                     | 2          | GABAb receptor agonist                      |
| ATP                      | 10                     | 1          | Purinergic receptor agonist                 |

**Note:** Dilute Baclofen and ATP stock aliquots in artificial cerebrospinal fluid (aCSF) to reach final concentration at the day of the experiment.

**Note:** Stock solution aliquots can be stored at –20°C or –80°C if they are used during the following 3–5 months.
CRITICAL: Tetrodotoxin (TTX) is a potent, selective and reversible, use-dependent inhibitor of voltage-dependent Na+ channels involved in action potentials generation. Wear respiratory protection and protective gloves/protective clothing/eye protection/face protection.

NMDG-HEPES solution:

| Reagent                               | Final concentration (mM) | Amount    |
|---------------------------------------|--------------------------|-----------|
| N-Methyl-D-glucamine (NMDG)           | 93                       | 18.16 g   |
| KCl                                   | 2.5                      | 0.19 g    |
| NaH2PO4                               | 1.2                      | 0.17 g    |
| NaHCO3                                | 30                       | 2.52 g    |
| HEPES                                 | 20                       | 4.77 g    |
| Glucose                               | 25                       | 4.51 g    |
| Sodium ascorbate                      | 5                        | 0.99 g    |
| Thiourea                              | 2                        | 0.15 g    |
| Sodium pyruvate                       | 3                        | 0.33 g    |
| MgSO4                                 | 10                       | 1.20 g    |
| CaCl2                                 | 0.5                      | 0.073 g   |
| miliQ H2O                             | N/A                      | 1 l       |
| Total                                 | N/A                      | 1 l       |

Note: Store at 4°C for maintenance (no more than 48 h). This volume should be enough to perfuse and brain slicing of 2–3 mice.

In case the final pH is deviated from 7.3-7.4, it will be adjusted by using HCl (3M) or NaOH (1M), accordingly. pH will be then maintained stable by the gas mixture.

CRITICAL: HCl is a corrosive agent and inhalation/exposure may cause eye, skin and respiratory tract irritation. It should be manipulated with gloves and under a fume hood. If manipulated out of a fume hood, use a 3M Respirator Cartridge and ventilate the room.

aCSF solution:

| Reagent                               | Final concentration (mM) | Amount    |
|---------------------------------------|--------------------------|-----------|
| NaCl                                  | 124                      | 7.247 g   |
| KCl                                   | 2.7                      | 0.201 g   |
| KH2PO4                                | 1.25                     | 0.17 g    |
| MgSO4                                 | 2                        | 0.241 g   |
| NaHCO3                                | 26                       | 2.184 g   |
| Glucose                               | 10                       | 1.802 g   |
| CaCl2                                 | 2                        | 0.222 g   |
| L(+)-Ascorbic acid                    | 0.4                      | 0.071 g   |
| miliQ H2O                             | N/A                      | 1 l       |
| Total                                 | N/A                      | 1 l       |

Note: Bubble with 95% O2, 5% CO2 for 30 min after adding the glucose and CaCl2 in order to maintain aCSF stability and prevent calcium carbonate precipitation. Keep bubbling since then.

Note: Prepare fresh aCSF for every day of experiment. Do not store.

Note: In case the final pH of aCSF is deviated from 7.3-7.4, it will be adjusted by using HCl (3M) or NaOH (1M), accordingly. pH will be then maintained stable by the gas mixture.
CRITICAL: Proteinase K digests itself; therefore, keep the lysis buffer as shortly as possible at room temperature (RT, 21–23°C). Keep lysis buffer stock at -20°C for 1 month and only defrost the required volume to perform the experiments (keep it on ice).

STEP-BY-STEP METHOD DETAILS

Surgeries and viral injections

© Timing: 1 h per mouse

In order to generate transgenic mice with selective downregulation of GABA
 subcellular receptor signaling in astrocytes from mPFC (GFAP/PFC
 mice), use 3–4 months old Gabbr1 (GABA
) floxed mice (Haller et al., 2004) and inject them with adenoviral particles to express Cre recombinase in mPFC astrocytes (AVV2.5-GFAP-Cre-mCherry, viral titer 4.3 × 10¹² viral genomes (vg) per mL). See Figure 1.

Note: This study did not quantify the number of infected astrocytes based on the mouse age. Be aware of potential differences in transfection levels between juvenile and adult mice.

1. Weigh mice prior to anesthesia (see Figure 2).
2. Inject 0.1 mg per kg of buprenorphine subcutaneously.
3. Anesthetize mice with isoflurane (1.5–2% in O₂).
4. Once anesthetized, place the animal in the stereotaxic frame.
   a. Put a heating pad below to maintain body temperature at approximately 36°C.
   b. Keep the mouse anesthetized with a gas anesthesia mask, which will also help you position the animal’s head.
   c. Fix the mouse’s head placing the ear bars of the stereotaxic frame in front of the ears. Make sure the skull is well fixed.

Lysis buffer 1x:

| Reagent                        | Final concentration | Volume (mL) |
|--------------------------------|---------------------|-------------|
| Dithiothreitol (DTT) 1 M       | 20 mM               | 0.2 mL      |
| Tris HCl (pH 7.4) 1 M          | 10 mM               | 0.1 mL      |
| Sodium dodecyl sulfate (SDS) 10% | 0.5%               | 0.5 mL      |
| Proteinase K (20 mg/mL)        | 0.5 μg/μL           | 0.25 mL     |
| ddH₂O                          | N/A                 | 8.950 mL    |
| Total                          | N/A                 | 10 mL       |

Figure 1. Generating GFAP/PFC
conditional knockout mice

(A) Schematic drawing showing the viral approach to downregulate gene expression using conditional mice. Gabbr1-floxed mice injected with a AAV2.5-GFAP-Cre-mCherry virus in mPFC such that the target gene becomes inactivated in vivo within the expression domain of Cre, generating a knockout mouse GFAP/PFC
. (B) Left, general workflow of the genome editing in Gabbr1 gene floxed by loxp sites, where excision is done. Right, scheme of AAV2.5-GFAP-Cre-mCherry virus.
5. Cover the eyes with eye gel to avoid dryness.
6. Expose the skull.
   a. Shave the animal’s head with a razor blade.
   b. Apply ethanol with a cotton bud and an antiseptic (Betadine or similar).
   c. Make an incision from back to front with the scissors.
   d. Remove the tissue above the skull using cotton buds and forceps.

7. Take stereotaxic coordinates.
   a. Using the stereotaxic and surgical microscope, place the tip of the pipette at bregma and set this point at X=0, Y=0.
   b. Raise the pipette and place it on the X and Y coordinates of the targeted area. The coordinates used for the mPFC are: anterior-posterior (AP) or Y = +1.68 and + 1.8; lateral (L) or X = ±0.35; and ventral (V) or Z = −2.5 to −1.3 mm (Franklin and Paxinos, 2008).

   Note: For this experiment, bilateral injections with two injections per hemisphere are made (a total of 4 injections).

   △ CRITICAL: For calcium imaging experiments, a mix of both viral constructions (AAV2.5-gfaABC1D-cyto-GCaMP6f (viral titer range 2–6 × 10^{13} vg/ml) and AVV2.5-GFAP-Cre-mCherry (4.3 × 10^{12} vg/ml; 1:1 ratio) are injected.

   c. Place the tip of the needle in the first position and make a mark scratching softly on the surface of the skull with forceps. Do this for the positions described.
8. Drill the skull.
   a. Make a craniotomy at the marked positions with a drill.
i. Carefully, apply pressure to the skull with the drill until you feel a drop-in resistance. At that point, the brain should be accessible.

**Note:** If bleeding, use cotton buds and saline solution to stop it.

ii. Repeat the process with the 4 areas.

9. Viral injections.
   a. Place the tip of the needle at the top of the hole and move down in the Z axis until the surface of the brain is reached.
   b. At that point, set the Z axis to 0 and take the Z coordinate in which you want to start the injection. For mPFC experiments, use Z = −2.5.
   c. Set the injection speed to 70 nl min$^{-1}$ and start the injection.
   d. 70 nl steps are made along the Z axis in the coordinates: −2.5, −2.3, −2.1, −1.9, −1.7, −1.5, −1.3 mm to cover the entire mPFC. Inject a total volume of 500 nl of viral particles.
   e. Wait for 10 min before you remove the pipette to avoid reflux and slowly withdraw the pipette.
   f. Repeat these steps for the 4 injection sites.

△ **CRITICAL:** Before you insert the pipette into the brain, make sure it is not clogged by injecting and checking liquid is coming out of the tip under microscope.

10. Suture
   a. Moisten the skin with a cotton bud and saline solution to help suture the skin.
   b. Use 6.0 suture or skin glue.
   c. Apply antiseptic (betadine or similar), local anesthetics (lidocaine), and liquid glue to avoid the animal removing the stitches.

11. Recovering
   a. Turn off anesthesia.
   b. Remove the ear bars and the gas anesthesia mask.
   c. Check the animal’s breathing and consciousness while keeping it in the heating pad.
   d. Place the animal in the cage with a heating pad below and monitor until it shows regular breathing and locomotion.
   e. Buprenorphine (0.05 mL, 0.1 mg/mL) is given once daily for 48 h post-surgery.

△ **CRITICAL:** Check the mouse’s breathing during the surgical process to adjust anesthesia levels. Breathing must be regular and without gasping.

**Note:** For control experiments, viral injections are also made in GABA$_B$ flox$^{-/-}$ mice.

### Genetic characterization of astrocytic GABA$_B$ receptors ablation

** Timing: 3 days

△ **CRITICAL:** Timing for infection is constrained from 3 weeks, when expression of viral particles is enough to reach desire number of astrocytes being targeted, to 5 weeks to prevent possible sides effects. After AAV2.5-GFAP-Cre-mCherry expression, analyze the presence of the knockout allele following Cre-mediated deletion of exon 7 and exon 8 by quantification of Gabbr1 and Gabbr2 gene expression in astrocytes (see Figure 3).

** Alternatives:** Perform quantitative western blots to analyze the protein levels in the transfected astrocytes using fluorescence-activated cell sorting (FACS) from mPFC tissue (bulk collection).
Note: Although FACS and quantitative western blots are commonly used, we have experienced inaccuracies in quantitation using immunodetection (cf. Walker, 2009), making these approaches not reliable in our hands. If it is also your case, analyze the mRNA levels of \( \text{Gabbr1} \) and \( \text{Gabbr2} \) by RT-qPCR, and the protein levels by looking at the GABAB puncta located at the astrocytic membrane by immunohistochemical labeling (Figure 4, co-localization analysis).

12. Collecting cells from cortical slices for Magnetic Bead Purification of RNA and RT-qPCR
   a. Anesthetize control mice (GABA\(_B\) flox\(^{-/-}\)) and GFAP/PFC\(^{\Delta \text{Gb}}\) animals with sodium pentobarbital 50 mg/kg, administered intraperitoneally.
   b. Check for the absence of reflex and transcardially perfuse animals with NMDG-HEPES solution at 4°C using a 10 mL syringe.
   c. Decapitate the mouse using scissors or a guillotine.
   d. Remove the brain from the skull:
      i. Cut the skin to expose the skull using fine scissors
      ii. Make two horizontal cuts through the jaw bones
      iii. Cut the skull along the midline very carefully not to damage the brain
      iv. Make a cut at the olfactory bulbs
      v. From the caudal midline, grab one half of the skull with forceps and open it to expose the brain. Then do it with the other half.
      vi. Extract the brain with a spatula from the ventral zone of the olfactory bulbs.

Figure 3. Workflow of collection and testing of GABA\(_B\) receptors knockout in astrocytes from mPFC
(1) Collect cells with a patch-clamp pipette ensuring the cell content is suctioned, under a fluorescence microscope to guide the selection of transfected astrocytes by selective expression of the mCherry reporter protein. (2) RNA extraction by magnetic beads from the astrocytic pull collected in the lysis buffer. (3) Library preparation of cDNA out of the extracted RNA. (4) Preparation of the reaction mix for PCR and running the qRT-PCR assay. Cycles for gene detection: \( \text{Gabbr1}\ #21, \text{Gabbr2}\ #22\) for wildtype astrocytes; \( \text{Gabbr1}\ #32, \text{Gabbr2}\ #30\) for GFAP/PFC\(^{\Delta \text{Gb}}\) astrocytes. (5) Acquisition of the amplification results.
Figure 4. Characterization and validation of GABA<sub>B</sub> receptors downregulation in astrocytes

(1) **Histological characterization.** Left, Scheme of serial coronal sections of the brain acquired using a vibratome. Cre-expression levels in neurons (determined by the NeuN marker), and in astrocytes (reported by the S100 antibody labeling). No labeling should be detected in neurons. Confocal fluorescence images of coronal brain sections with successful AAV injection. Right, Co-localization of puncta for Gababr1 and S100 in GFAP/PFC<sup>Gb</sup> generated mice and control animals.

(2) **Ex-vivo functional characterization.** Left, Examples of GCaMP6f successful expression in astrocytes: the calcium sensor is expressed throughout the mPFC in astrocytes as it co-localized with S100 (marker for astrocytes specific cell-type). Co-localizing astrocytes are shown in yellow. Scheme of local application of baclofen to the brain slices, specific agonist of GABA<sub>B</sub>Rs. Right, Time-lapse analysis of calcium transients recorded in GCaMP6f expressing astrocytes in response to different agonist applied. Representative calcium traces for control and GFAP/PFC<sup>Gb</sup> astrocytes after baclofen stimulation. Note control astrocytes will show calcium events triggered by baclofen, sensitive to the specific

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**Functional characterization in-vivo**

**T-maze behavioral test**

- Sample run (forced)
- Test run (choice)
- Accuracy achievement (90%)

**Behavioral outcome**

- Control
- GFAP/PFC<sup>Gb</sup>

- Correct trials
- Days
- # Trials
- 1-20
- 21-40
- 41-60

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**Protocol**

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During the entire process of brain extraction and slicing, NMDG-HEPES solution should be continuously bubbled with the gas mix.

e. Slice the brain at 350 μm with NMDG-HEPES solution at 4°C. Use a vibratome (Leica Biosystems, VT1200 S or similar). Place the mPFC slices in a chamber with NMDG-HEPES at 37°C for 10 min, then place them into a chamber with aCSF at RT (21°C–23°C) for the rest of the experiment.

Note: The slices are held in nylon nets placed on 250 ml of aCSF.

f. After 45–60 min transfer the slices to the recording chamber of the microscope (Nikon Eclipse FN1) in aCSF. Use a 40X/60X objective and CCD camera (Hamamatsu C4742-95), illumination system (CoolLED), and appropriated software (NIS-Elements Software, Nikon) to identify mCherry-positive astrocytes based on fluorescence signal. Excitation wavelength 535 nm.

g. Fill out the borosilicate glass pipette with 2–3 μL of Lysis buffer 1x. To prevent tip plugged, no negative pressure is applied to the pipette while approaching the cells. In contrast, be aware no to apply excessive positive pressure avoiding leak out of the lysis buffer.

h. Using the patch-clamp technique to collect the intracellular content (Figure 3, step 1):
   i. Identify a mCherry-positive astrocyte.
   ii. Approach with the borosilicate glass pipette filled with lysis buffer and place it touching the astrocyte membrane and apply negative pressure to follow a cell-attach configuration.
   iii. Two possible actions can be followed:
      First, in cell-attached configuration, move slowly up the tip of the pipette making sure that the cell follows the tip and it gets out of tissue. Once the tip and the cell are out the tissue, apply gentle negative pressure by mouth pipetting or using an external syringe to incorporate the intracellular content into the glass pipette. Then, carefully remove the pipette from the headstage and break the tip of the pipette into vials containing a 45 μL lysis buffer.

   Note: There is no need to accomplish a whole cell access.

Second, in cell-attached configuration, apply negative pressure as described above to get access to the cell (whole-cell configuration). Next, gentle negative pressure will be applied to incorporate the intracellular content into the recording pipette, and then remove the pipette slowly out from the tissue. Pull off the pipette from the headstage and break the tip of the pipette into vials containing a 45 μL lysis buffer.

Note: Visualize the entire process under the microscope to follow the tip and the cell continuously. In case of mCherry-labeled astrocytes, the fluorescence tagging can be used. For control samples, non-labeled neurons are isolated by using the same approach with differential interference contrast optics to visualize the cells.

Note: If the process to reach the cell and collect its intracellular content is done in less than 2 min, the rate of success of cell-attached sealing did not differ from regular intracellular solutions.
iv. Directly after collecting cell, close the vial containing lysis buffer and lysed cell, and keep in dry ice. Successively, repeat the process and collect 20–25 cells into the same vial. Once all cells have been collected perform a quick spin down and incubate for 15 min at 65°C in a water bath (Figure 3, step 2).

**Note:** As cells will be pulled together in the same Eppendorf with lysis buffer, keep the vial in dry ice until all the astrocytes are collected.

△ **CRITICAL:** The total volume of cellular content into the lysis buffer vial may not exceed 5 μL (total volume 50 μL).

△ **CRITICAL:** Water bath right close to the collecting set up to avoid RNA degradation.

i. After 65°C incubation, spin down the vial to recover possible drops formed.

**Note:** At this point, store samples at −20°C or −80°C until processing.

j. Isolate RNA with magnetic beads using the DireCtQuant 100ST protocol (http://directquant.eu/wp-content/uploads/manuals/DireCtQuant%20100ST%20USER%20GUIDE%2020171016.pdf) (Figure 3, step 3).

i. Perform a complementary DNA synthesis, library preparation and amplification following Pico Profiling workflow, which uses Whole Transcriptome Amplification (WTA) to generate sufficient cDNA for microarray expression analysis.

ii. Add SYBR Green to the amplification reaction to monitor the efficiency in real-time.

**Note:** Stop the amplification reaction once the SYBR Green signal reaches a plateau (Gonzalez-Roca et al., 2010).

k. Pre-amplify cDNA obtained from the reverse transcription: prepare cDNA mix for PCR amplification and divide it in three equivalent parts in order to have technical replicates (Figure 3, step 4).

**Note:** Include a sample without RNA as a control.

| PCR cycling conditions |
|------------------------|
| **Steps** | **Temperature** | **Time** | **Cycles** |
| Initial Denaturation  |  |  |  |
| Denaturation          | 94°C | 15 s | 23 cycles |
| Annealing            | 60°C | 15 s |  |
| Extension            | 72°C | 1 min |  |
| Hold                 | 4°C | Forever |  |

l. Purify cDNA using a PureLink Quick PCR Purification kit (Invitrogen) and elute it in 40 μL of milliQ water.

m. Obtain cDNA concentration with a Nanodrop 1000 spectrophotometer.

n. qPCR of Gabbr1 and Gabbr2:

i. Use the following primer sets to analyze Gabbr1 and Gabbr2 expression: Gabbr1 (152 bp), forward (ACAGACCCAAATCTACCGGC), reverse (GTGCTGTGCGTGAGTAGCCGAT); Gabbr2 (106 bp), forward (AAGCTACGGTCACGACG), reverse (ACTTGCTGCACAGACGCTC).
ii. Fill the wells of a hard-shell 384-well white well clear shell PCR plate (Bio-Rad, CN HSP-3805) with 5 μL of Sso Fast EvaGreen Supermix (Bio-Rad, CN 172-5204), 1 μL of primer mix (5 μM of each primer) and 5 ng of pre-amplified cDNA to reach a total volume of 10 μL per well.

**Note:** Use housekeeping genes, i.e., ARBP (ribosomal protein 36B4 mRNA) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) for data normalization of RT-qPCR.

**Note:** To prevent potential contamination of reagents and/or primer–dimer artifacts, a no-template control reaction must be carried out using all the reagents except the sample.

**Note:** Perform technical triplicates to correct pipetting errors in plate loading.

iii. Perform qPCR in a CFX 384 real-time system (Bio-Rad).

iv. Include a melting curve from 60°C to 95°C (0.5°C s⁻¹) at the end of the program to verify the specificity of the PCR.

v. Use the GenEx v.5.4.4 software (MultiD Analyses) for data processing using the $2^{-ΔCq}$ method.

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### PCR cycling conditions

| Steps                  | Temperature | Time  | Cycles |
|------------------------|-------------|-------|--------|
| Initial Denaturation   | 95°C        | 30 s  |        |
| Denaturation           | 95°C        | 5 s   | 40 cycles |
| Annealing and extension| 60°C        | 5 s   |        |
| Hold                   | 4°C         | Forever |        |

**Note:** Gabbr1 and Gabbr2 are detected at 21 and 22 cycles, respectively, in wildtype astrocytes, and at 32 and 30 in GFAP/PFC^Gb^ astrocytes.

**Histological characterization of GABA$_b$ receptors in astrocytes**

**Timing:** 2 days for step 13

**Timing:** 2 days for step 14

Similar to genetic identification, 3–5 weeks after AAV2.5-GFAP-Cre-mCherry injection into PFC of GABA$_b$ flox$^{+/−}$, the so-called GFAP/PFC$^{Gb}$ mice, characterize the absence of GABA$_b$ receptor expression in astrocytes by analyzing the puncta density immunofluorescence using confocal microscopy (Figure 4).

13. Immunohistochemistry

a. Anesthetize the animal with sodium pentobarbital 50 mg/kg, administered intraperitoneally.

b. Transcardially perfuse the animal with 10 mL of phosphate-buffered saline (PBS) followed by 40 mL PFA 4% using a perfusion pump.

c. Keep the brain in PFA 4% overnight.

**Note:** If brain is not analyzed right now, saturate the brain in sucrose gradient solutions (7%, 32%, shaking at 4°C), and keep it in cryoprotected solution (glycerol 3%) and ethylene glycol 3% in PBS.

d. Once ready, use a vibratome (Leica Biosystems, VT1000 S) to cut the brain in 50 μm coronal slices and collect them in PBS in a multiwell plate.
e. Wash the slices 3 times (20 min each) with PBS.
f. Wash the slices 3 times (20 min each) with 0.2% Triton/PBS.
g. Block non-specific binding with PBS containing 1% goat serum and 0.3% Triton-X 100 for 1 h (blocking solution).
h. Incubate the slices with primary antibodies in blocking solution overnight at 4°C. Primary antibodies used: guinea pig anti-GABA<sub>B</sub>R1 (1:1,000), mouse anti-NeuN (1:500), rabbit anti-S100 (1:200) and mouse anti-GAD67 (1:500).
i. Wash the slices 3 times (20 min each) with blocking solution.
j. Incubate the slices with secondary antibodies in blocking solution for 1 h at RT. The secondary antibodies are labeled with Pacific Blue, AlexaFluor-488 or AlexaFluor-647 and diluted 1:500 in blocking solution.
k. Wash the slices with 0.1% Triton/PBS 3 times (20 min each).
l. Wash the slices 3 times (20 min each) with PBS.
m. Mount the slices using Vectashield antifade mounting medium.

14. Confocal imaging analysis
Co-localization analysis of the encoded constructs by viral vectors (mCherry-Cre, Cyto-GCaMP6f) with astrocytic (S100) and neuronal (NeuN) markers.
a. Acquire 1 μm single optical sections using a x63 objective (1.40 NA, oil-inmersion) at 2,048 x 2,048 resolution (physical size: 246.03 x 246.03 μm) with a confocal microscope.

Note: For this article, Leica SP-5 confocal microscope (Leica Biosystems) is used, but particular settings will be adapted based on the user’s confocal microscope.

b. Take Z-stack images of 7 μm in thickness containing mCherry-Cre/Cyto-GCaMP6f positive astrocytes in PFC using LAS X (Leica Application Suite X) software.
c. Open the z-stack images with ImageJ to analyze colocalization of mCherry-Cre/Cyto-GCaMP6f with S100 and NeuN labeling (astrocytic and neuronal markers, respectively) in the Z-stack images to quantify the number of double-positive cells using the Fiji software.
i. Open the file which contains the desired image and select the Z stack maximum projection image generated previously.
ii. Select the channel of the infection (mCherry-Cre or Cyto-GCaMP6f).
iii. Use the ROI manager tool in Fiji to select manually the fluorescent somatic regions (Analyze → Tools → ROI manager).
iv. Once all the ROIs are selected, press More in the ROI manager window, and select OR (combine).
v. Create a mask that will be superimposed to the other channels to analyze co-localization by clicking on Edit → Selection → Create mask.
vi. Click on Edit → Selection → Create Selection and press Shift+E. To paste the mask selection in the other channels (S100 and NeuN), press Shift+E after having selected the channel window where we want to apply the mask (just by clicking in the window).
vii. With the ROI manager still open, add 3 more ROIs drawn in these channels (3 for S100 and 3 for NeuN) to measure the background fluorescence intensity.
viii. With the ROI manager of each channel completed, click on Measure in the ROI manager window to obtain the fluorescence intensity of each ROI.
ix. To determine double-positive cells, the intensity of S100 or NeuN in the corresponding ROIs has to be higher than the value obtained, as per the following formula: mean value (background ROI intensity) + 4 times standard deviation (background ROI intensity).
x. Tag the positive ROIs with 1 and negative ROIs with 0 and calculate the percentage of positive ROIs for each channel.
d. Analyze GABA<sub>B</sub>R1 puncta density using Fiji.
i. From the Z-stacks obtained, demarcate astrocyte somata and projections in the S100 channel using the ROI manager tool only in mCherry-positive astrocytes.
Create a mask and paste it in the GABA_R channel as in 14c (v-x) to measure the GABA_R intensity in these ROIs.

Analyze the puncta density at ROIs: Binary images are obtained from these astrocytes (threshold of 25–255), and the number of puncta is acquired using Analyze Particles in ImageJ. The puncta density is calculated as the number of puncta versus ROI area.

**Note:** Using this approach it is possible to establish a linear correlation between the total area of viral expression of GFAP-Cre-mCherry (mCherry fluorescence) in the mPFC and the behavioral performance in the T-maze test. Check also [https://biop.github.io/ijp-imagetoatlas/registration_with_deepslice.html](https://biop.github.io/ijp-imagetoatlas/registration_with_deepslice.html), [https://docs.brainglobe.info/brainreg-segment/user-guide/segmenting-3d-structures](https://docs.brainglobe.info/brainreg-segment/user-guide/segmenting-3d-structures), for correlation between labeling areas and alignment to brain atlas.

**Functional characterization of the GABA_R receptors ablation in astrocytes**

**Timing:** 1 day per mouse for step 15

**Timing:** 3 h for step 16

**Timing:** 9 days for step 16

To determine the functional impact of downregulating GABA_R receptors signaling in mPFC astrocytes, two different strategies can be followed: **calcium imaging** to test the astrocytic calcium response to baclofen (GABA_R agonist), and **behavioral tests** (T-maze alternation task) to monitor the impact of astrocytic GABA_R downregulation.

15. **Calcium imaging.** Figure 4
   a. Sacrifice control and GFAP/PFC^Gb^ mice 3–5 weeks after AAV2.5-GFAP-Cre-mCherry and AAV2.5-hGFAP-cyto-GCaMP6f injections to get the mPFC slices as previously described in point 12.
   b. After 1 h in aCSF at RT, transfer the slices to the chamber of the microscope (Nikon Eclipse FN1).
   c. Isolate calcium-driven GABAergic responses by using tetrodotoxin (TTX, 1 μM) and a cocktail of antagonists, which include D-(-)-2-amino-5-phosphonopentanoic acid (D-AP5; 50 μM) and 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX; 10 μM), N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251; 2 μM), 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP; 50 μM) and LY367385 (100 μM). Apply these drugs in the aCSF (see solutions and drug preparation) (Araque et al., 2002; Perea et al., 2014).
   d. Identify GCaMP6f-positive astrocytes using a 40X-water immersion objective using 488 nm as excitation wavelength.
   e. Apply baclofen (2 mM) by a pressure micro-injector (Picospritzer; 1 bar) for 5 s using a glass micropipette placed into the slice close to the recorded astrocytes.
   f. Use NIS-Elements Software or similar to record 2–3 min video of calcium dynamics images.

**Note:** Considering the kinetics of calcium responses, the acquisition rate would be at least 1 frame per second.

**Note:** Control astrocytes show fluorescence increases after baclofen stimulation, while GFAP/PFC^Gb^ astrocytes do not show changes in their calcium levels.

**Note:** Baclofen pipette placed in the field of view to ensure correct cell stimulation.
g. Use ATP (1 mM) local air puff as positive control to test viability of the astrocytes for GFAP/
PFC\textsuperscript{AGb}, when no baclofen response is observed. Robust fluorescence changes are ob-
tained (Guthrie et al., 1999).

16. Imaging data analysis
Perform time-lapse image-series analysis using ImageJ of the Fiji software.
   a. Open video with ImageJ in AVI format
   b. Correct the x-y drift if needed with Turboreg plugin in ImageJ.
   c. Define ROIs automatically by using MATLAB based software Caltracer3, although visual
       confirmation should be made (Mederos et al., 2019; Poskanzer and Yuste, 2016).
   d. Extract time traces of fluorescence intensity of each ROI and convert it to $\Delta F/F$ values, where
       $\Delta F/F_0 = (F(t) - F_0)/(F_0)$, being $F_0$ the baseline fluorescence defined as the average minimum
       fluorescence across pre-stimulus frames. This process can be made by custom-written soft-
       ware in MATLAB.
   e. Consider a calcium event when the fluorescence signal shows maximum values above three
       times the standard deviation of mean values of pre-stimulus frames.
   f. For superimposed calcium events, consider more than one calcium event when maximum
       values are above two times the standard deviation of the previous steady signal.
   g. Discard ROIs with no calcium signal changes during the entire recording (signals above five
       times the standard deviation of the baseline).
   h. Discard events with duration fewer than two frames.
   i. Analyze calcium event frequency, amplitude and area per ROI per min.

17. Behavioral assay: T-maze alternation task. Figure 4
   a. Keep the GFAP/PFC\textsuperscript{AGb} mice and control mice (GABA\textsubscript{B} flox\textsuperscript{-/-}) under food restriction
      maintained above 80–85% of baseline body weights, which motivates mice to perform
      the behavioral task, and under water deprivation for 12 h before sessions (Toth and
      Gardiner, 2000).
   b. Keep the temperature and humidity of the experimental rooms at 23 ± 2°C and 55 ± 5%,
      respectively.
   c. Transfer mice to the testing room at least 30 min before performing behavioral task to reduce
      stress.
   d. Habituate mice to the T-maze by placing them in the maze for 15 min for a 3-day period
      before the test.
   e. Perform behavioral trials, each trial consists of one sample run and one test run.
   f. Sample run: block one side of the T arm with a wooden block and place a 0.1 mL reward (wa-
      ter or condensed milk) in the food well at the end of the opened arm. Place the animal in the
      start arm and let the animal enter into the opened arm with the reward.

Note: To choose which arm to block, use a pseudorandom sequence with equal numbers
of left and right turns per session and not more than two consecutive turns in the same
direction.

   g. After the sample run, leave the mouse for 15 s at the beginning of the start arm, in a compart-
      ment separated by a door.
   h. Test run: remove the block and place the reward at the previously unvisited arm. Open the
      door for the mouse to start the test.
   i. Take note of the decision made by the animal (alternation or not alternation).

Note: Consider an entry when the mouse has placed all four paws into the arm.

   j. Repeat the trial 10 times per day for 6 days (60 trials in total) with each animal with an inter-
      trial-interval of 5 min.
EXPECTED OUTCOMES

Refined targeting of the brain regions is possible by limiting the number of injections. A single injection resulting in local expression, limited to an area of one hemisphere. As the aim is to cover as much PFC as possible to have a consistent effect in behavior, broad expression of viral particles is achieved by several injections at multiple depths through the two hemispheres. Fluorescence reporting proteins will confirm the achievement of this coverage after three weeks after viral injections.

Amplification of the RNA content is expected from initial values of 5 ng. Patch-clamp pipettes are used to isolate the intracellular content of cells expressing the Cre-recombinase, visualized under the fluorescence microscope. Adding pre-amplification of RNA reagents in the pipettes allows to obtain a consistent amount of RNA sample content per cells, with the later possibility to focus on how efficient is the deletion of the GABA\textsubscript{B} mRNA in the transfected animals. Furthermore, this approach also allows to study other RNAs in the same samples in order to test the specificity and stability of the genetic content of targeted cells.

Cellular responses to different agonists should be selective enough to test functionality. Based on such responses, it will help to determine the functional presence/absence of a particular pathway.

Immunofluorescence studies will show that Cre-recombinase is specifically express in astrocytes, and double staining for other cell types will determine that there is no residual expression and only GABA\textsubscript{B} is being deleted from astrocytes. Finally, by immunofluorescence studies is possible to test the presence/activation of inflammatory response following AAV expression using specific markers (Iba1, CD68, among others) indicating if the tissue shows signs of neuroinflammation. If procedure has been done correctly, the later are not expected.

For the T-maze task, the process of training and experiment requires up to 4 sessions with 10 trials per day for wild-type animals to achieve a performance over 80% of correct trials. Tracking over days and computing the results of correct and incorrect trials over sessions will allow to monitor if indeed wild-type control animals are correctly learning. These details can then be used to examine if the protocol is correctly being performed and if control animals are reaching the expected levels of success rate in the task. For this reason, it is highly recommended to carry out in the same day and sessions wild-type animals with the same background as targeted mice in order to correct for other plausible deviations on learning. The sequence of correct vs. incorrect choices can be used to follow the learning process. Additionally, other animal behaviors such as sitting positions, grooming and movement into the arms can also be examined. Depending on the analysis software, detailed tracking of other explorations or behaviors during the task can also be assessed.

LIMITATIONS

Not enough number of astrocytes express the virus. Small volume of astrocytic infections would be inefficient to study the electrophysiology and behavioral effects of GABA\textsubscript{B} receptors downregulation in astrocytes.

Cre-driver gene manipulation strategy requires a carefully analysis of the expression pattern of the Cre-recombinase in brain tissue. The particular control of gene expression by the Cre-lox method in non-targeted cells can significantly impact the results of all subsequent steps being accomplish and could imply non-specific results.

Brain slices for collecting cells or functionality testing are not healthy. If the viability of the cells is not in safety margins, data may be unreliable and obtaining good samples for RNA content will also be an issue to preserve stability of the RNA material.
Calcium signals measurements not stable. Calcium indicators tend to bleach easily. This should not be a problem in the calcium recordings made in this study (<30 min), the change in fluorescence gain can be substantial across longer recordings.

Amplification of the GABA<sub>B</sub> collected material from cells of cortical slices not yields to sufficient numbers. This might be a problem in order to perform further amplification steps and followed subsequent analysis of gene expression. It can be implemented by increasing the number of collected cells, as the posterior amplification processes it is done from a pull of cells.

Mice control group do not learn the test around the 4 days established through literature. In our experience, this limitation could be influenced by the experimenter. The potential solution would be the automation of the maze apparatus.

**TROUBLESHOOTING**

**Problem 1**

No efficient viral expression by immunobiological analysis (step 14).

**Potential solution**

There are several factors that can account for the transduction efficiency by AAV in a given tissue and/or cell populations, including: AAV preparation, AAV tropism (promoter, size and nature of the transgene), and specific factors related to the procedure, specifically time. For optimal results, revise the Surgery and viral injections protocol to obtain high purity, high titering AAV particles in small aliquots, avoid repetitive freeze-thaw cycles and keep them on ice once out the freeze (for thawing until final use).

Furthermore, to avoid clogging at the tip of the injection pipette, before entering the brain always check under microscope that the nanoinject is indeed delivering volume through the tip. Specially, after removing the pipette from the first injection site when bleeding or tissue debris might block the tip of the injection pipette.

**Problem 2**

There is viral expression but limited with scarce number of transfected astrocytes, indicating that brain area under study is not being satisfactorily covered and affected to observe behavioral deficits (step 14).

**Potential solution**

A positive correlation has been found between area of infection in PFC and behavioral performance (Figure 5). Therefore, limited area of infection can be circumvented by optimizing the Surgery and viral injection protocol: i) using AAV constructs in which the transgene expression is driven by more-specific AVV constructs (e.g., in case the area being targeted is not the cortex, some other AAV serotypes could potentially be more effective). ii) To increase the number of injection sites and increasing the amount of virus delivered at each injection site. As in problem 1, it is also important to check for the stability of the virus, titering, number of cycles of freezing (if reused), and total time of the injection procedures. Pilot experiments for determining the efficacy of other AAV constructs for specific experimental readouts may be needed.

**Problem 3**

High variability of RT-qPCR data from the amplification experiments using single cells collected by the patch -pipette under florescence labeling (step 12).

**Potential solution**

Low or absent of RNA material to amplify may suggest that not enough or none viable cells are collected. Revise the protocol of collecting cells from cortical slices: one possibility would be increasing
the number of cells being collected for each case. Also see solutions to problem 6, as a concern could be the viability of the slice used to collect the samples. If no RNA material is detected, it is possible that although cells are being patch through the pipette they are being lost before entering the Eppendorf containing the lysis buffer solution. Ensure you apply enough negative pressure to the pipette so the cell is being completely attached to the tip of the pipette enabling to isolate it from the tissue. Experimentalist can ensure that the pipette is carrying the cell under the microscope (Figure 6).

Problem 4
Contamination/Degradation of collected RNA (step 12).

Potential solution
To prevent contaminations and degradations that might yield to RNA loss during experiment revise the section of Genetic characterization of astrocytic GABA<sub>B</sub> receptors ablation, with particular attention to: i) always wear gloves during the entire process, from the performance of cell patching and in subsequent steps. ii) Previous to the experiment clean possible RNase-contaminated surfaces of the set-up. iii) Use RNase-free solutions. iv) Correct storage of RNA is also critical to avoid RNA degradation. In the short-term, RNA may be stored in the lysis buffer as soon as cells are isolated from tissue and freeze under −80°C.

Problem 5
Antibody staining not properly labeling cells (step 13).

Potential solution
While we never have experienced complete absence of the IHC signal, we noticed that it could be case of weaker IHC signal. Pay attention to the Immunohistochemistry protocol, and consider incubating with the primary antibody for a longer time (2 instead of 1 day) and increase the antibody concentration. If possible, can also increase the levels of Triton to enhance antibody penetration. It is

Figure 5. Viral expression in mPFC
(A) Histological verification of bilateral transfected astrocytes in mPFC. Scale bar, 200 μm.
(B) Correlation between transfected area and T-maze performance. Dots represented the number of incorrect trials at day 4 for individual GFAP/PFC<sup>G458</sup> mice (linear fitting curve, black line). Figure modified from Mederos et al. Nat Neuroci. 2021.

Figure 6. Visual reference of the single cell collecting procedure under the microscope
Left: Touch the cell membrane to get cell-attached configuration.
Center: Confirmation of pulling out the cell from the tissue.
Right: Same as b with DIC optics. Scale bar, 25 μm. Note: as example, the image corresponds to cortical slice from ALDH1L1 eGFP reporter mouse.
important to use fresh collected brains, so do not store them more than 2 days prior to the antibody staining after the PFA perfusion. Do not keep brains under PFA solution for longer 1 overnight period.

**Problem 6**
Viability of brain slices (step 15).

**Potential solution**
Brain extraction procedure can impact the viability of the cells, if the entire process is too slow and/or brain is damaged during the extraction. Revise the calcium imaging protocol (point 15) and try to reduce the speed of the vibratome or increase the vibration frequency of the blade. If this is not enough to improve the viability of the slices, check whether the slicing milieu is cold enough (below 4°C) during cutting by adding some ice and/or some ethanol around the tank. Avoid using non-fresh solutions (aCSF or perfusion solution prepared days before the procedure), and prepare freshly every day before acquiring brain slices. Always verify the pH and osmolality when preparing the solutions. Regularly check that your slices are properly oxygenated with CO₂ (5%) and O₂ (95%).

**Problem 7**
Non-significant learning in behavioral task (step 17).

**Potential solution**
Revise the Behavioral assay protocol (point 17) to make the required changes. Not all animals will succeed in learning a particular task. In these cases, the group animal must be reported as not learning, and if necessary, additional animals must be trained. In any case, it is important to keep a control group that is consistent over several sessions to make sure it is comparable to the animals accounting for the down-regulation of GABA₉ receptor in astrocytes. If learning is not consistent for the control group, it is required to analyze possible interferences that might be affecting the learning curves, such as periods of stress in the animal facility or non-well-trained experimenters. If possible, the same experienced experimenters should conduct the entire process, from animal selection to experiment, to ensure comparable results. Perform all sessions at the same time-frame of the day.

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Gertrudis Perea (gperea@cajal.csic.es).

**Materials availability**
This study did not generate new unique reagents.

**Data and code availability**
The published article includes all datasets generated and analyzed during this study. Analyses used in this study are largely standard approaches for this type of data.

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**AUTHOR CONTRIBUTIONS**
S.M., A.S.-R., and G.P. wrote the manuscript. S.M. designed the figures.
DECLARATION OF INTERESTS
The authors declare no competing interests.

REFERENCES
Araque, A., Martin, E.D., Perea, G., Arellano, J.I., and Buño, W. (2002). Synaptically released acetylcholine evokes Ca2+ elevations in astrocytes in hippocampal slices. J. Neurosci. 22, 2443–2450.
Franklin, K., and Paxinos, G. (2008). The Mouse Brain in Stereotaxic Coordinates, Compact - 3rd Edition (Academic Press).
Gonzalez-Roca, E., García-Albéniz, X., Rodríguez-Mulero, S., Gomis, R.R., Kornacker, K., and Auer, H. (2010). Accurate expression profiling of very small cell populations. PLoS One 5, e14418.
Guthrie, P.B., Knappenberger, J., Segal, M., Bennett, M.V., Charles, A.C., and Kater, S.B. (1999). ATP released from astrocytes mediates glial calcium waves. J. Neurosci. 19, 520–528.
Haller, C., Casanova, E., Müller, M., Vacher, C.M., Vigot, R., Doll, T., Barbieri, S., Gassmann, M., and Bettler, B. (2004). Floxed allele for conditional inactivation of the GABAB(1) gene. Genesis 40, 125–130.
Korotkova, T., Fuchs, E.C., Ponomarenko, A., von Engelhardt, J., and Monyer, H. (2010). NMDA receptor ablation on parvalbumin-positive interneurons impairs hippocampal synchrony, spatial representations, and working memory. Neuron 68, 557–569.
Mederos, S., Hernández-Vivanco, A., Ramírez-Franco, J., Martín-Fernández, M., Navarrete, M., Yang, A., Boydén, E.S., and Perea, G. (2019). Melanopsin for precise optogenetic activation of astrocyte-neuron networks. Glia 67, 915–934.
Mederos, S., Sánchez-Puelles, C., Esparza, J., Valero, M., Ponomarenko, A., and Perea, G. (2021). GABAergic signaling to astrocytes in the prefrontal cortex sustains goal-directed behaviors. Nat. Neurosci. 24, 82–92.
Nagai, J., Rajbhandari, A.K., Gangwani, M.R., Hachisuka, A., Coppola, G., Masmanidis, S.C., Fanselow, M.S., and Khakh, B.S. (2019). Hyperactivity with Disrupted Attention by Activation of an Astrocyte Synaptogenic Cue. Cell 177, 1280–1292.
Perea, G., Yang, A., Boydén, E.S., and Sur, M. (2014). Optogenetic astrocyte activation modulates response selectivity of visual cortex neurons in vivo. Nat. Commun. 5, 3262.
Poskanzer, K.E., and Yuste, R. (2016). Astrocytes regulate cortical state switching in vivo. Proc. Natl. Acad. Sci. USA 113, E2675–E2684.
Schindelin, J., Arganda-Carreras, I., Frise, E.,Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682.
Ting, J.T., Daigle, T.L., Chen, Q., and Feng, G. (2014). Acute brain slice methods for adult and aging animals: application of targeted patch clamp analysis and optogenetics. Methods Mol. Biol. 1183, 221–242.
Toth, L.A., and Gardiner, T.W. (2000). Food and water restriction protocols: physiological and behavioral considerations. Contemp. Top. Lab. Anim. Sci. 39, 9–17.
Walker, J.M. (2009). The Protein Protocols Handbook (Humana Press).