STAR Protocols

Protocol

Combining low-density cell culture, single-cell tracking, and patch-clamp to monitor the behavior of postnatal murine cerebellar neural stem cells

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Highlights

Protocol to culture
postnatal mouse
cerebellar neural
stem cells (NSCs) in
low density

The behavior of
isolated postnatal
cerebellar NSC can
be monitored at
single-cell level

Protocol for
simultaneous
monitoring of the three
postnatal cerebellar
neurogenic niches

Procedures of live
imaging and single
cell tracking for
lineage tracing

Low-density cell culture of the postnatal cerebellum, combined with live imaging and single-cell tracking, allows the behavior of postnatal cerebellar neural stem cells (NSCs) and their progeny to be monitored. Cultured cerebellar NSCs maintain their neurogenic nature giving rise, in the same relative proportions that exist *in vivo*, to the neuronal progeny generated by the three postnatal cerebellar neurogenic niches. This protocol describes the identification of the nature of the progeny through both post-imaging immunocytochemistry and patch-clamp recordings.

Protocol

Combining low-density cell culture, single-cell tracking, and patch-clamp to monitor the behavior of postnatal murine cerebellar neural stem cells

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SUMMARY

Low-density cell culture of the postnatal cerebellum, combined with live imaging and single-cell tracking, allows the behavior of postnatal cerebellar neural stem cells (NSCs) and their progeny to be monitored. Cultured cerebellar NSCs maintain their neurogenic nature giving rise, in the same relative proportions that exist *in vivo,* to the neuronal progeny generated by the three postnatal cerebellar neurogenic niches. This protocol describes the identification of the nature of the progeny through both post-imaging immunocytochemistry and patch-clamp recordings.

For complete details on the use and execution of this protocol, please refer to Paniagua-Herranz et al. (2020b).

BEFORE YOU BEGIN

Prepare the tools required

1. Prepare the following tools
   a. A beaker containing 70% ethanol
   b. A Petri dish (60 mm × 15 mm) for the brain extraction
   c. The lid of a Petri dish for dissection
   d. Tools required for dissection: 30 G syringe needles, microdissection scissors (8.5 cm surgical scissors: Fine Science Tools), curved forceps (Dumont no. 5SF Forceps: Fine Science Tools)
   e. Siliconized and normal glass Pasteur pipettes (see troubleshooting 5).
   f. Petri dish (35 mm × 10 mm) with EBSS medium (supplied in the Papain Dissociation System) into which the cerebellums can be placed and for the papain digestion
   g. P1000 and P200 Eppendorf pipette tips
   h. 15 mL conical tubes

Note: All the tools must be sterilized in an autoclave or washed for 20 min in 70% ethanol on the day of dissection.

Prepare the dissociation media

⏱ Timing: 20 min

2. Reconstitute the albumin-ovomucoid inhibitor (vial 4) with 32 mL of Earle's Balanced Salt Solution (EBSS, **vial 1**).

   Note: Aliquots of this vial (4 mL) can be prepared and refrigerated between experiments. Maintain them at 4°C until the moment of use (to be used within the day of preparation) or at −20°C for longer storage. Aliquots can be stored at −20°C for up to 6 months.

3. Add 500 μL of EBSS and 5 μL APV (5 mM, Amino phosphonopentanoic acid) to one DNase vial (**vial 3**).
4. Reconstitute the papain solution (**vial 2**) by adding 5 mL of EBSS and 50 μL of APV (5 mM) and incubate for 15 min in a 5% CO_2 37°C incubator or in a 37°C water bath until the papain fully dissolves. After incubation, add 250 μL of the reconstituted DNase to the papain solution prior to dissection. The final concentration of this preparation is 0.005% DNase, 20 units/mL papain and 47.61 μM APV, and it has to be freshly prepared.

Prepare the cell culture media

5. Prepare cell culture medium (to be used in step 15 in "step-by-step method details")

Chemical	Volume (mL/100 mL Neurobasal medium)	Final concentration (mM)
Antibiotic/Antimycotic Solution (100×)	1 mL	100 U/mL penicilin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin
L-glutamine	1 mL	0.5
B27 (50×)	2 mL	2%

Storage the cell culture medium at 4°C for a week maximum.

Prepare the external and internal solutions for patch-clamp recording

⏱ Timing: 2 h

These solutions can be made days or weeks in advance, as the amounts prepared are sufficient for several days or weeks of daily patch-clamp experimentation.

6. Prepare a 100 mL stock solution of 1 M NaOH by adding 4 g of NaOH to double-distilled water (ddH_2O).

   ⚠ CRITICAL: NaOH is corrosive. Handle with care and wear gloves.

7. Prepare a 100 mL stock solution of 1 M KOH by adding 5.61 g of KOH to ddH_2O.

   ⚠ CRITICAL: KOH is corrosive. Handle with care and wear gloves.

The solutions prepared in steps 6 and 7 are used to adjust the pH of the internal and external solutions and they can be stored at room temperature (RT, 19°C–23°C). Solutions should be discarded if any precipitate is detected.

8. Prepare the external solution

Chemical	Amount (g/ 1 L solution)	Final concentration (mM)
NaCl	8.4738	145
KCl	0.2087	2.8
$CaCl_2$	0.2219	2
$MgCl_2$	0.0951	1
D-(+)-Glucose	1.8016	10
HEPES	2.3830	10
ddH_2O	Complete volume up to 1 L	

    a. The external (bathing) solution aims to mimic the extracellular medium. To prepare the external solution add the amounts of chemicals specified to 0.95 L of stirred ddH_2O.

    b. Carefully titrate to pH 7.3–7.4 with the 1 M NaOH solution.

    c. Adjust to the final volume with ddH_2O.

    d. Measure the osmolarity to ensure it is ≈300 mOsm.

Note: If the osmolarity is not between 295–305 mOsm, the solution should be discarded and a new solution prepared.

Note: The external recording solution can be kept for up to 1 week at 4°C, although it is usually recommended that the glucose is added just before use.

9. Prepare the internal solution

Chemical	Amount (g/100 mL solution)	Final concentration (mM)
KCl	1.0810	145
$MgCl_2$	0.0190	2
EGTA	0.0114	0.3
$ATP.Na_2$	0.1138	2
$GTP.Li_3$	0.0163	0.3
HEPES	0.2383	10
ddH_2O	Complete volume up to 100 mL	

    a. The internal (*pipette*) solution aims to mimic the composition of the intracellular milieu. To prepare the internal solution, add the amounts of chemicals specified to 90 mL of stirred ddH_2O. It is usually recommended that the ATP and GTP salts are added just before use on the day of recording to minimize their autohydrolysis.

    b. Carefully titrate the pH to 7.1–7.2 with the 1 M KOH solution and add ddH_2O to adjust to the final volume.

    c. Measure the osmolarity to ensure it is ≈280 mOsm.

⚠ CRITICAL: The osmolarity of the internal solution should be 10 mOsm lower than that of the external solution to prevent cell swelling during recording.

Note: Aliquot 100 mL of the internal solution into small volumes (0.5–1.0 mL) to be used for individual experiments on the day of recording and store these at −20°C for up to 1 year. Once thawed, the internal solution should be kept on ice and protected from light, and it should be discarded at the end of the day.

Note: Both the internal and external solutions can be used in the voltage-clamp and current-clamp modes of the patch-clamp technique.

10. Prepare drug stock-solutions for patch-clamp recordings

Stock solutions of the drugs to be employed in patch-clamp experiments should be prepared in advance and they can be kept for several months.

a. Prepare a 1 mL stock solution of 10 mM GABA by dissolving 1.0312 mg in ddH$_2$O. The stock GABA solution is diluted 1:100 in the extracellular solution to a final concentration of 100 μM to elicit GABA$_A$ receptor mediated currents. Divide into 0.1 mL aliquots and store at −20°C for up to 1 year. Avoid freeze-thawing cycles.

b. Prepare a 1 mL stock of 10 mM serotonin (5-HT) hydrochloride by dissolving 2.1268 mg in ddH$_2$O. 5-HT is diluted 1:100 in the extracellular solution to a final concentration of 100 μM to elicit 5-HT$_3$ receptor mediated currents. Divide into 0.1 mL aliquots and store at −20°C for up to 1 year. Avoid freeze-thawing cycles.

c. Prepare a 1 mL stock of 100 mM monosodium glutamate by dissolving 18.813 mg in ddH$_2$O. Glutamate is diluted 1:100 in the extracellular solution to a final concentration of 1 mM to elicit ionotropic glutamate receptor mediated currents. Divide into 0.1 mL aliquots and store at −20°C for up to 1 year. Avoid freeze-thawing cycles.

d. Prepare a 1 mL stock of 10 mM MK-801 by dissolving 2.2130 mg in ddH$_2$O. MK-801 is diluted 1:1000 in the extracellular solution to a final concentration of 1 μM to block NMDA receptor mediated currents, as it is an NMDA antagonist. Divide into 0.1 mL aliquots and store at −20°C for up to 1 year. Avoid freeze-thawing cycles.

Chlorinate a silver wire electrode and check the ground electrode

© Timing: 1 day before recording

The recording electrode is made of a silver wire which should be chlorinated regularly, especially if the chloride coating is wearing off. This is evident when the grey coat is not uniformly distributed, and shiny silver spots appear on the electrode surface.

11. Connect one of the poles of a 9 V battery to the bare end of the recording electrode (the one soldered to a gold pin that plugs into the head stage of the amplifier), while the other pole is left in contact with a silver wire spring of about 20 turns that extends over 4–5 cm. Both silver wires are immersed in a 0.1 M HCl solution for about 1 min, the time required for AgCl to be deposited on the surface of the recording electrode. Hydrogen air bubbles are produced simultaneously due to the reduction of H$^+$ in the solution.

12. Check the appearance and clean the surface of the AgCl pellet of the ground electrode. Immerse the recording electrode and the ground electrode together into the external solution to equilibrate their surface potentials.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-SOX2 (1:100)	Abcam	Cat# ab137385
Mouse anti-GFAP (1:200)	Sigma-Aldrich	Cat# G3893
Mouse anti-βIII Tubulin (1:800)	Sigma-Aldrich	Cat# T8660
Rabbit anti-VGLUT1 (1:500)	Synaptic Systems	Cat# 135302
Mouse anti-VGAT (1:100)	Synaptic Systems	Cat# 131011
Alexa Fluor 594 goat anti-Rabbit IgG (H+L) (1:500)	Thermo Fisher Scientific	Cat# A-11012
Alexa Fluor 647 goat anti-Mouse IgG1 (1:500)	Thermo Fisher Scientific	Cat# A-21240

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Alexa Fluor 488 goat anti-Mouse IgG2b (1:1000)	Thermo Fisher Scientific	Cat# A-21141
Alexa Fluor 488 goat anti-Rabbit IgG (H+L) (1:500)	Thermo Fisher Scientific	Cat# A-11008
Biotin goat anti-Mouse IgG (H+L) (1:500)	Thermo Fisher Scientific	Cat# 62-6540
Streptavidin, Alexa Fluor™ 594 Conjugate (1:500)	Thermo Fisher Scientific	Cat# S32356
Chemicals, peptides, and recombinant proteins		
DL-2-Amino-5-phosphonopentanoic acid lithium salt (APV)	Sigma-Aldrich	Cat# A6553
Poly-L-lysine	Biochrom AG	Cat#L7240
Gibco™ B-27 Supplement (50×), serum-free	Thermo Fisher Scientific	Cat# 17504044
Gibco™ Neurobasal Medium	Thermo Fisher Scientific	Cat# 21103049
L-glutamine	Sigma-Aldrich	Cat# G8540
Antibiotic (penicillin, streptomycin)/Antimycotic (amphotericin) Solution (100×)	Sigma-Aldrich	Cat# A5955
(+)-MK-801 hydrogen maleate	Sigma-Aldrich	Cat# 475878
Sodium L-glutamate	Sigma-Aldrich	Cat# 1446600
Gamma Amino Butyric Acid (GABA)	Sigma-Aldrich	Cat# 03835
Serotonin (5-HT) hydrochloride	Sigma-Aldrich	Cat# H9523
NaCl	Sigma-Aldrich	Cat# S9888
KCl	Sigma-Aldrich	Cat# P3911
$CaCl_2$ (anhydrous)	Sigma-Aldrich	Cat# C1016
$MgCl_2 \cdot 6H_2O$	Sigma-Aldrich	Cat# M9272
$ATP.Na_2$	Sigma-Aldrich	Cat# 1191
$GTP.Li_3$	Sigma-Aldrich	Cat# G5884
HEPES	Sigma-Aldrich	Cat# H3375
EGTA	Sigma-Aldrich	Cat# E4378-
D-(+)-Glucose	Sigma-Aldrich	Cat# G8270
HCl	Sigma-Aldrich	Cat# 320331
KOH	Sigma-Aldrich	Cat# 417661
NaOH	Sigma-Aldrich	Cat# S8045
Ethanol	Sigma-Aldrich	Cat# 32221-M
Phosphate Buffered Saline (PBS)	Biosolve	Cat#162323
Triton X-100	Sigma-Aldrich	Cat#T8787
Paraformaldehyde (PFA)	Sigma-Aldrich	Cat#P6148
Bovine serum albumin (BSA)	Sigma-Aldrich	Cat#A9418
Trypan Blue	Sigma-Aldrich	Cat#T6146
Critical commercial assays		
Papain dissociation system	Worthington Biochemical Corporation	Cat#LK003150
Papain vials	Worthington Biochemical Corporation	Cat#LK003176
DNase vials	Worthington Biochemical Corporation	Cat#LK003170
Albumin-ovomucoid inhibitor vials	Worthington Biochemical Corporation	Cat#LK003182
Earle's Buffer vial	Worthington Biochemical Corporation	Cat#LK003188
Experimental models: Organisms/strains		
Mice C57BL/6J (male and female, P0)	Animal Facility, School of Medicine from Universidad Complutense de Madrid, and Universidad Autónoma de Madrid	Ref No. ES-28079-0000086 and ES-28079-0000097

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
PATCHMASTER	HEKA Elektronik	https://www.heka.com/
GraphPad Prism	GraphPad Software Inc.	https://www.graphpad.com/scientific-software/prism/
IGOR Pro	WaveMetrics, Inc.	http://www.wavemetrics.com
TTT (The tracking tool)	ETH Zurich (Prof Timm Schoreder)	https://bsse.ethz.ch/csd/software/ttt-and-qtfy.html
Other		
Brightfield/Phase contrast/fluorescence microscope	Nikon	Model TE-2000-E
CFI PLAN FLUOR DLLL 10× objetives	Nikon	Ref 280MRH10101
CFI SUPER PLAN FLUOR ELWD AMD 20× objetives	Nikon	Ref 280MRH48230
Software: NIS-Elements AR4.5	Nikon	Model NIS-Elements AR4.5 -Hasp ID: 13CE819E
pE-300 LED fluorescence	Cool LED	Serial number 1981
310M-201 Incubation system (temperature)	OKO-Lab	Serial Nº VOF007307
OKO touch Incubation system (CO2)	OKO-lab	Serial Nº 1716
Pro-ScanII Motorized stage system	Prior	Serial Nº 60018
High precision microscope camera version 4.2 Andor zyla USB3	ANDOR Zyla	VSC-03650
Microdissection scissors (8.5 cm surgical scissors)	Fine Science Tools	Cat#15003-08
Curved forceps (Dumont no. 5SF Forceps)	Fine Science Tools	Cat#11252-00
Siliconized and normal glass Pasteur pipettes	Brand	Cat#747720
Hemocytometer	Brand	Cat#BR717810
Petri dish (60 mm × 15 mm)	Corning	Cat# 353004
Petri dish (35 mm × 10 mm)	Corning	Cat# 353001
15 mL Conical Centrifuge Tube	Corning	Cat# 430791
24 well plate	Jet Biofil	Cat# TCP011024
Aqua-Poly/Mount	Polysciences	Cat# 18606-20
BD microlance needles 30G×0.5" 0.3 × 13 mm	BD Biosciences	Cat# 304000
Silver wire	World Precision Instruments	Cat# AGT0510
Gold pin	HEKA Elektronik	Cat# 895226
Ag/AgCl ground pellet	World Precision Instruments	n/a
Borosilicate glass capillaries for electrophysiology	Kimble Chase	1.5 mm ID and 1.8 mm OD
Vertical two-step puller	NARISHIGE GROUP	Cat# PP-830
Microforge	NARISHIGE GROUP	Cat# MF-830
Pipette holder	HEKA Elektronik	Cat# 895230
EPC10 patch-clamp amplifier	HEKA Elektronik	Cat# 89-5001
Upright fluorescence microscope equipped with 10× and 63× water immersion objectives for patch-clamp recording	Olympus Corporation	Model BX51WI
I-R CCD camera	Andor	Model DL604M-OEM
Micromanipulator	Burleigh	Model PCS-P560
Computer	Hewlett Packard	Model HP xw4300
Anti-vibration air table	Newport	Model LW303036B-OPT
Faraday case	In house	n/a
Recording chamber	Warner Instruments	Model DH-40iL
Gravity-fed perfusion system (includes a vacuum pump)	In house	n/a
Pneumatic drug ejection system	NPI electronic	Model PDES-02DX
Silicone tubing	United States Plastic Corp.	n/a

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Polyethylene tubing	United States Plastic Corp.	n/a
1.5 mL Eppendorf tube Silicone tubing	Eppendorf AG	n/a
5 mL syringes	B. Braun	Cat# 4617053V-02
10 mL syringes	B. Braun	Cat# 4617100V-02
Non-sterile syringe filters	Merck Millipore	Cat# SLGP05010
Erlenmeyer flasks	Fisher Scientific SL	Cat# 15409103
Forceps (curved and straight)	B. Braun	n/a
10 mm diameter glass coverslips Menzel Glässer	Avantor	Cat# 630-2115
Freezing-point osmometer	Wescor	Model VAPRO 5520
CO_2 cylinder	Air liquide	n/a

Note: This protocol involves an EPC-10 patch-clamp amplifier and PATCHMASTER (HEKA Elektronik) software to record voltage- and ligand-gated currents as well as the changes in membrane potential, and an Olympus BX51WI microscope (Olympus). Other amplifiers and programs can also be used for the recordings and analyses, such as the MultiClamp 700B, pCLAMP and Clampex (Molecular Devices, CA, USA). Other microscopes are also suitable (Nikon, Leica, Zeiss, etc.).

STEP-BY-STEP METHOD DETAILS
Poly-L-lysine cell attachment

⏱ Timing: 3–4 h

1. Poly-L-lysine coating of the cell culture surface.

   Note: To perform time-lapse experiments, the cells must be plated on Poly-L-lysine coated 24-multiwell, while the electrophysiology experiments are done in Poly-L-lysine coated coverslips.

   a. Dilute the poly-L-lysine in sterile water from the stock solution (0.1 mg/mL) to a final concentration of 10 μg/mL.
   b. Add the appropriate amount of diluted poly-L-lysine to the culture surface; typically 1 mL per 25 cm^2 is required in sterile conditions (see troubleshooting 4).
   c. Cover and incubate at 37°C for 1–2 h.
   d. Remove the solution by aspiration and thoroughly rinse the surface with sterile water.
   e. Cover and allow to dry at RT for at least 1 h.

Dissection and papain digestion

⏱ Timing: 2 h (see troubleshooting 5)

2. Sacrifice the P0 mice in accordance with current regulatory guidelines and using protocols approved by the institution. Euthanize the mouse pup by decapitation, separate the head from the body and wipe the head of the pup with 70% ethanol.
3. Place the embryo's head in a 60 mm Petri dish with cold Neurobasal (NB) medium and hold the head with 13 cm stainless forceps to clearly see the back of the skull. Insert the microdissection scissors into the foramen magnum and cut straight toward the eyes. Using curved forceps, gently peel away the skin of the head and lift the skull to expose the brain.

   △ CRITICAL: Take care to not cut through the brain tissue when removing the skull bone.
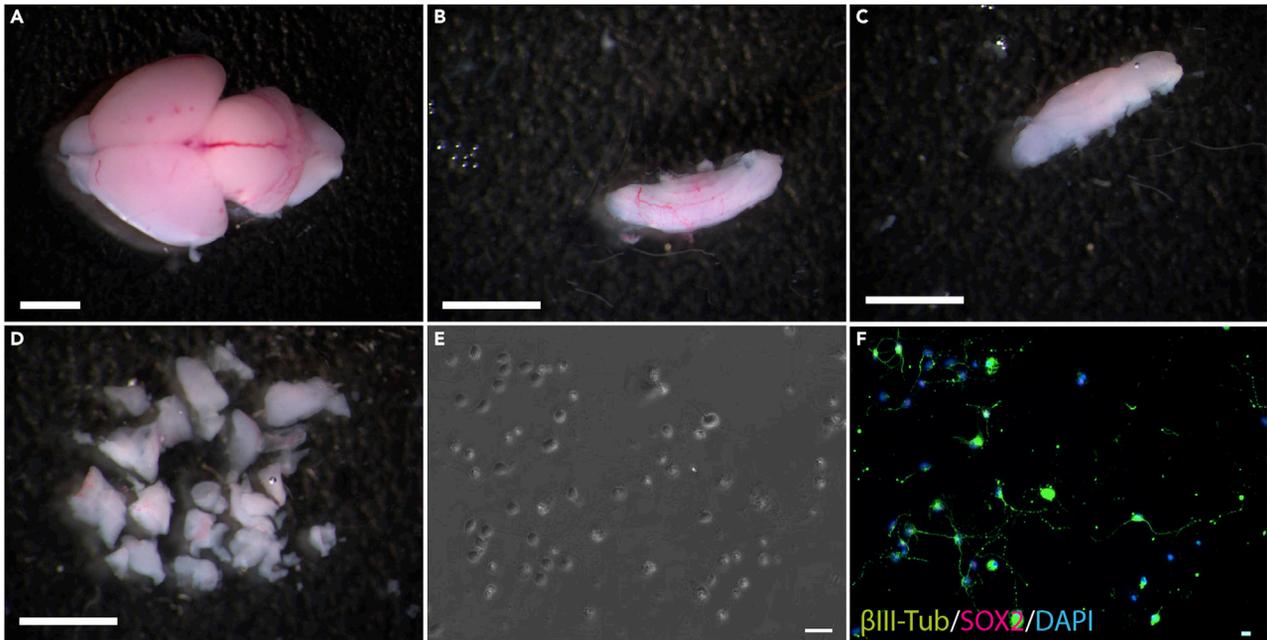
Figure 1. Preparation of the P0 cerebellum

(A) P0 mouse brain after extraction.

(B) View of the isolated P0 cerebellum.

(C) P0 cerebellum after removal of the meninges.

(D) Cerebellum after mechanical dissociation into pieces of approximately 1 mm.

(E) Bright field image showing the general aspect of the cell culture.

(F) Identification of the neural progeny by immunocytochemistry. Scale bar (A–D: 5 mm) (E and F: 30 μm)

4. Pinch off the cerebellum and transfer it to the 35 mm Petri dish with EBSS medium. Under the dissecting microscope, gently remove the meninges from the cerebellum with one 30G syringe needle while using the other needle to fix the cerebellum to the dish (Figure 1).

   Note: The meninges can be easily identified by observing the blood vessels on the surface of the cerebellum. Remove the meninges until the cerebellum appears to be completely white.

5. Once the dissection has been completed, carefully remove all the EBSS medium with a Pasteur pipette and leave only the tissue.

6. Add the papain solution (vial 2, prepared previously) to the dish and chop up the tissue with a surgical blade (see troubleshooting 1).

7. Incubate for 45 min at 37°C in 5% CO_2 in sterile conditions.

8. Transfer the pieces of cerebellum to a 15 mL conical tube with a siliconized Pasteur pipette. Mechanically dissociate the tissue by pipetting up and down until the lysate is homogeneous. About 15 up and down strokes with the pipette should be sufficient to dissociate the tissue, producing a cloudy solution (see troubleshooting 3).

   ⚠ CRITICAL: Avoid making bubbles and the formation of any froth.

9. Centrifuge at 200 ×*g* for 5 min at RT.

10. During centrifugation, prepare the re-suspension medium by mixing 2.7 mL EBSS + 300 μL reconstituted albumin-ovomucoid inhibitor (vial 4) + 150 μL of DNase solution (vial 3) + 30 μL APV (5 mM) in a 15 mL conical tube.

11. After centrifugation, remove the supernatant and resuspend the cell pellet in the solution prepared in the previous step using a siliconized Pasteur pipette. Resuspend the pellet by gently pipetting up and down with a siliconized Pasteur pipette.

12. Prepare a discontinuous density gradient by carefully layering the resuspended cell suspension on top of albumin-ovomucoid inhibitor (vial 4). The boundary between the two layers of the gradient should be clearly visible.

13. Centrifuge at 200 ×g for 5 min at RT, remove the supernatant and resuspend the pellet in 2 mL of NB medium.

14. Count the number of viable cells by mixing 50 μL of Trypan blue + 40 μL of NB medium + 10 μL of cell suspension in a tube and put 10 μL of this mix into a hemocytometer (see troubleshooting 2).

15. Plate the cells at low density (50,000 cells/cm^2) in NB medium supplemented with 2% B27, 100 U/mL penicilin, 100 μg/mL streptomycin, amphotericin 0.25 μg/mL and L-glutamine 0.5 mM in adherent 24 multiwell cell culture plates (for time-lapse imaging) or coverslips (for electrophysiology recordings) (see troubleshooting 4).

Live time-lapse video-microscopy imaging

⏱ Timing: 4–5 days

16. Power up the computer, CO_2 and temperature control systems, the microscope and the hardware.

17. Adjust the temperature to 37°C and the CO_2 to 5%.

18. Wait 1–2 h for the CO_2 and temperature levels to stabilize.

19. Make a small mark on the bottom of a well that does not contain cells.

Note: This mark will be used to set the xyz coordinates.

20. Place the culture plate in the microscope chamber.

⚠ CRITICAL: Tightly fix the plate to the microscope to prevent any movement during the video-microscopy experiment.

21. Wait 20 min until the temperature of the cell culture medium has equilibrated in the chamber.

Note: This step will avoid any loss of focus due to the dilation of any component.

22. Run the software and set up the time-lapse experiment.

23. Select the length of the experiment and the image acquisition cycles. We use cycles of 5 min and the experiments typically last 4 or 5 days.

Note: The temporal resolution improves if higher image cycle frequencies are used and the experiment can be finished before the final cycle.

⚠ CRITICAL: Determine the frequency necessary to avoid the cell death potentially produced by phototoxicity of the transmitted light.

24. Search for the mark made previously in the plate and define it as the reference point, (position 1, equivalent to xyz zero coordinates).

⚠ CRITICAL: Take a photo of the zero position to be able to reset the coordinates if necessary.

25. Select the rest of the positions (x and y coordinates) and adjust the focus (z coordinate) for each position.

26. Choose the type of acquisition and the exposure time in the "wavelength selection tab menu". In our experiments we only select brightfield with an exposure time of 10–50 ms.

> △ CRITICAL: To avoid the toxicity induced by light exposure, the shutters for the transmitted light and epifluorescence must be closed between cycles and positions.

27. Name the experiment and the folder where the data will be stored to be able to reload the experiment at your convenience, save the list of positions.

28. Click on the "run now" button to start the experiment.

29. Pause the experiment once a day to re-adjust the focus conditions by employing the "overwrite z button" (if it is necessary). If the medium needs to be changed, pause the experiment and take the plate out of the chamber. Change the medium under sterile conditions and put the plate back into the chamber (see step 21). Finally, re-adjust the focus and continue with the experiment.

> *Note:* We recommend maintaining the plates at constant temperature to avoid changes in focus during the experiment. Alterations to the pH of the medium caused by over-proliferation or cell death might also affect the focus.

Post-imaging immunocytochemistry

> ⊙ Timing: 2 days

30. After finishing the experiment, click the "finish" button and remove the plate to perform post-imaging immunocytochemistry (PIIC).

31. Remove the medium from the wells and add 500 μL of sterile Phosphate Buffered Saline (PBS) to wash the cells. Repeat this step twice.

32. Add 500 μL of 4% (wt/vol) paraformaldehyde (PFA) in PBS and fix the cells at RT for 10 min.

> △ CRITICAL: Handle the PFA carefully and avoid contact with the eyes or skin as PFA is a strong fixative. PFA should be used inside a fume hood.

33. Wash the cells 3 times with 500 μL of sterile PBS.

34. Add 500 μL per well of the blocking solution: PBS containing 2% (wt/vol) BSA and 0.2% (vol/vol) Triton X-100. Incubate for 1 h at RT.

35. Remove the blocking solution, add 200–400 μL of the primary antibody solution and incubate 18 h at 4°C. The primary antibody solution contains the primary antibodies used in this experiment diluted in blocking solution: GFAP (1:200), βIII-tubulin (1:800), SOX2 (1:100), VGLUT1 (1:500) and VGAT (1:100).

36. Remove the antibody solution and wash the wells 3 times with PBS (5 min each).

37. Add 200–400 μL of the secondary antibody solution prepared by diluting the secondary antibodies in the blocking solution and incubate for 1–2 h at RT in the dark: Anti-Rabbit IgG (H+L) 594 (1:500), Anti-Rabbit IgG (H+L) 488 (1:500), Anti-Mouse IgG2b 488 (1:1000), Anti-Mouse IgG1 (1:500).

38. Wash three times with 200–400 μL of PBS (5 min each).

> *Note:* To amplify the VGAT signal it is necessary to use the biotin-streptavidin amplification system. Incubate for 1–2 h at RT in the dark with the diluted biotin Anti-Mouse (1:500) secondary antibody, then perform step 39 and incubate for 1 h at RT in the dark with Streptavidin 594 (1:500) diluted in the blocking solution. Repeat step 39.

39. Maintain the cells in 1 mL of PBS prior to performing the next steps.

   Note: we recommend removing the PBS and adding two drops of the aqueous mounting medium Aqua-Poly/Mount to preserve the immunocytochemistry for longer.

40. Place the plate back in the microscope chamber and fix it tightly.
41. Reload the zero position using the indication made previously and re-set the rest of the positions to this reference point by clicking the "Offset All X, Y, Z" button. Re-set the focal distance (z coordinate).

   Note: Wait around 20 min to allow the temperature of the plate to equilibrate in the chamber, thereby avoiding the appearance of bubbles and a loss of focus.

42. To detect the antigens, configure the conditions for fluorescence emission in the "wavelength tab menu" and obtain a final round of images.

   Note: Adjust the fluorescence exposure to acquire the best quality images.

43. Save the data and export using the software's file/export to Tagged Image File Format (Tiff) option.

Data processing

   ⏱ Timing: 3–4 days

44. Rename the image channels (c). For brightfield images change c1 to c0, for FITC images change it to c2, for CY3 images to c3 and CY5 images to c4.
45. Convert the images to a PNG format.
46. Start the "tTt Converter tool" software, define the input and output folder, and the markers used for each of the channels (c), positions (xy) and time-points (t).
47. Click the "convert images" button.

   Note: Each position of the experiment should be stored in individual folders.

Single-cell tracking

   ⏱ Timing: Approximately 4 h for each position

48. Once the data is renamed, run the tTt software (Hilsenbeck et al., 2016), selecting a user name and tTt work folder.

   Note: All the data analyzed and the results exported will be stored in the tTt folder named as tTt export, which will contain the following subfolders: "AVIexport", "Configs", "TreeExport", and "tTtfiles".

49. In the "select experiment folder window", choose the experiment and indicate the path of the tTt export folder. Then, click the "Load experiment" button.
50. Execute the log file converter to modify the images into a format that can be read by the tracking software.

   Note: This will be automatically requested by the software when you load an experiment for the first time.
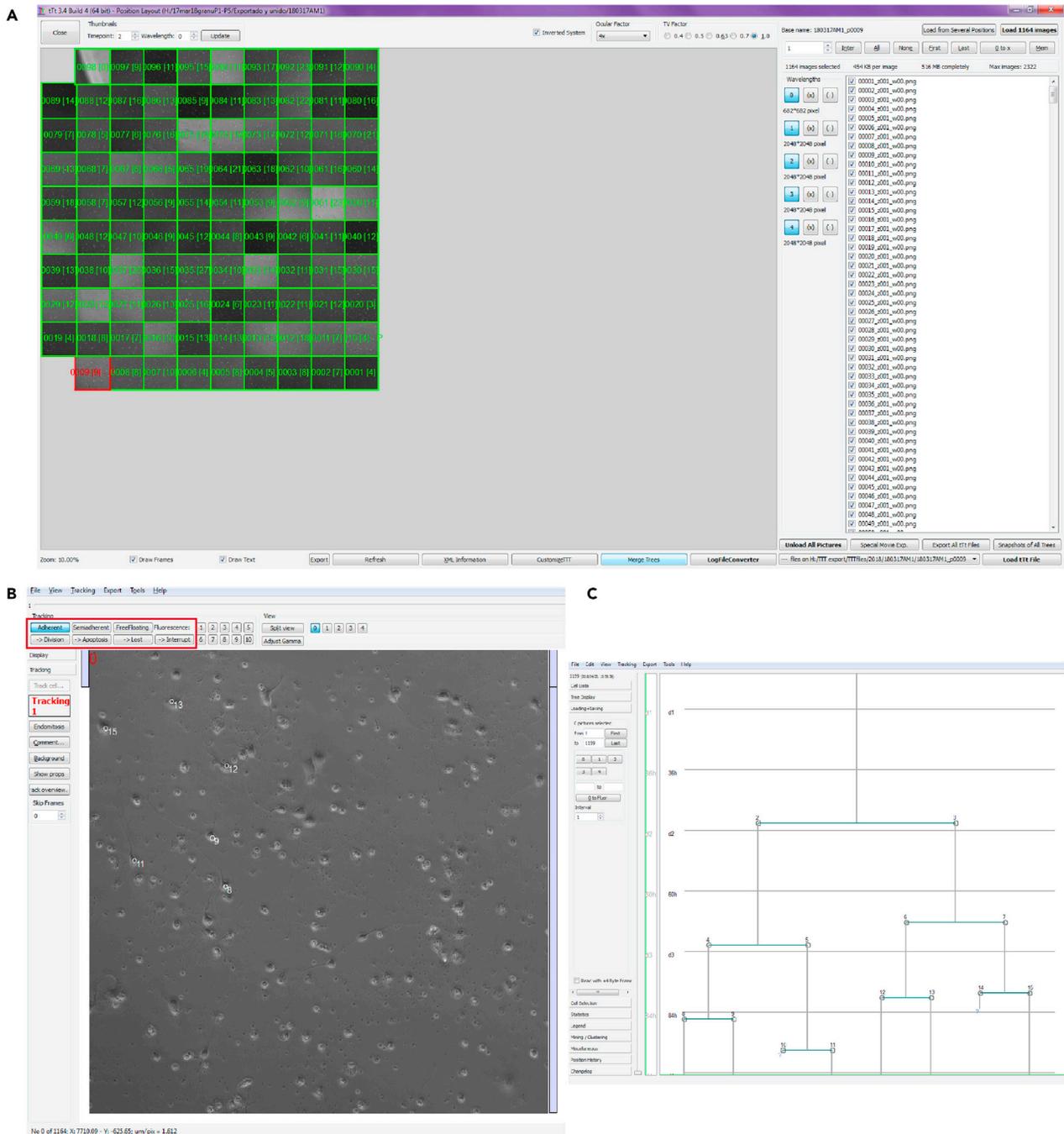
Figure 2. Layout of the single-cell tracking software
(A–C) The images show an overview of the different windows available at the TTT software, including: (A) the general experiment window with a scheme corresponding to the different positions monitored during the experiments; (B) the cell movie window where cells can be tracked; and (C) the cell editor window where the lineage tree is automatically displayed according to the tracking options selected in (B).

51. Select a position, which will be shown as a picture of the position and its corresponding number, and then pick the list of available images that appear on the right side of the window and click the ''Load images'' button (Figure 2A).
52. Select the wavelength 0 that corresponds to the brightfield image and click twice on the position to be tracked in the ''Cell Editor Window'', adjusting the contrast and brightness (Figure 2B).

53. Press the F2 key to start tracking.

   Note: In the course of tracking situate the mouse pointer on the cell and press the "0" key. Specific cell events like cell division, apoptosis or cell loss are among the options available in the "Cell Editor Window". These events will then appear in the lineage tree (Figures 2B and 2C).

54. Match the brightfield images to the immunofluorescence image in order to define the nature of the cell progeny.
55. Once all the positions are tracked, save the experiment and export the results through the "Export menu" which appears in the "Cell Editor Window", exporting the cell data and the lineage tree to the tTt export folder.

Whole-cell patch-clamp recording in cell cultures

⊙ Timing: 6–8 h

Live imaging by time-lapse video microscopy revealed that after completing 3–5 rounds of amplification divisions, some cerebellar cells adopted a neuron-like morphology. Furthermore, immunocytochemistry indicated that cells with a small ovoid soma and bipolar processes express the vesicular glutamate transporter (VGLUT1), while cells with a polygonal soma and multipolar cytoplasmic processes express the vesicular GABA transporter (VGAT), indicative of their glutamatergic and GABAergic nature, respectively. We performed whole-cell patch-clamp recordings on these cells to investigate the functional phenotype of these morphologically distinct cells (Figure 3).

56. Make the patch-clamp recording pipettes for the day.
   a. Pull the glass electrodes using a two-step pipette puller having adjusted the settings so that the resistance is about 4 MΩ (Figure 3D).
   b. Polish the pipette's tip with a microforge so that the resistance becomes 4.5–5 MΩ.
   c. Make the drug application pipettes similarly.
   d. Place the pipettes in a closed container to prevent the pipette tip from being soiled by airborne dust.

   Note: A good compromise must be reached between the ease of seal formation, membrane-patch rupture and a suitable series resistance during recording (see troubleshooting 6).

57. Thaw an aliquot of the internal solution and keep it on ice, protected from the light.
58. Prepare dilutions of the stock drug solutions in the external solution (see steps 10a–d in "before you begin").
59. Switch on all the apparatus and run PATCHMASTER, the software for cell stimulation and electrophysiological data acquisition. Open a "new experiment" in the program's menu (Figures 3B and 3E).
60. Feed the gravity-driven perfusion line with external solution and check there are no air bubbles in the line (Figure 3A).

   ⚠ CRITICAL: Since the external solution contains glucose, flush the perfusion line and recording chamber with ddH$_2$O at the end of each recording day to avoid bacterial contamination. If lipophilic compounds are used in the experiments, rinse the pipelines with ethanol before washing them with ddH$_2$O.

61. With straight forceps, transfer a poly-L-lysine coated coverslip loaded with cells to the recording chamber (Figure 3C).
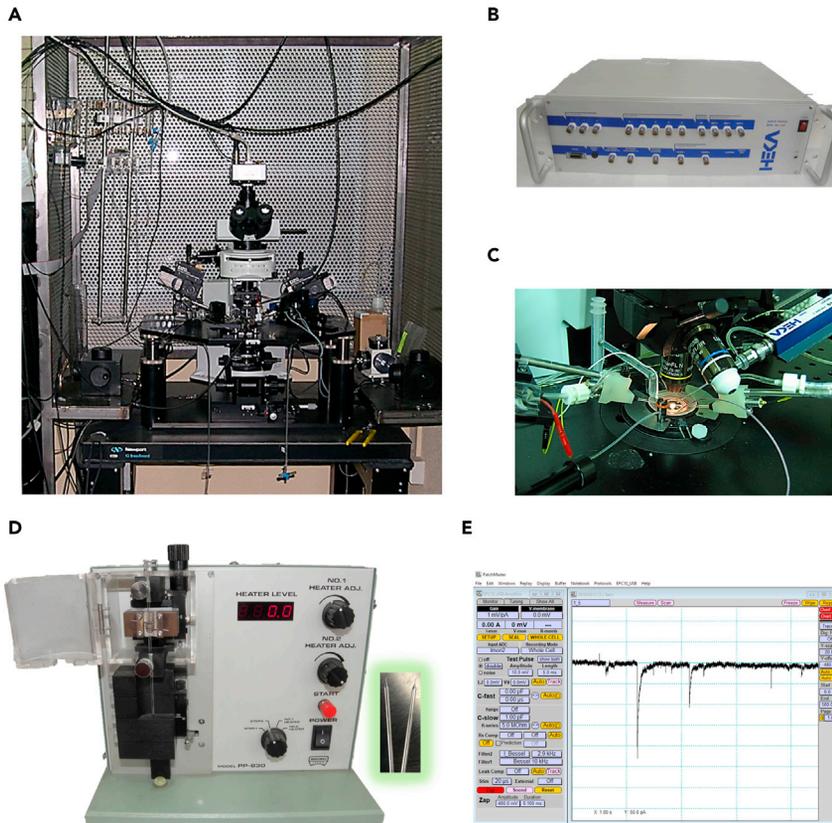
Figure 3. Equipment for electrophysiology recording
(A) Patch-clamp set-up.
(B) Patch-clamp amplifier.
(C) Microscope stage and recording chamber.
(D) Vertical pipette puller and patch-clamp pipettes.
(E) The PATCHMASTER program window.

62. Connect the ground electrode to the head stage of the patch-clamp amplifier and secure the plate in the recording chamber (Figure 3C).
63. Start perfusion of the external solution at a rate of 1 mL/min and turn on the vacuum aspiration so that the solution can be removed from the recording chamber through an outlet connected to an Erlenmeyer container. Check that the level of liquid in the recording chamber stays above the cell's plane.

   Note: Experiments are performed at room temperature (24°C–26°C) and there is therefore no need to heat the external solution.

64. Use a low power objective (10×) and move the stage of the microscope to situate some cells in the center of the field of view. Under IR-DIC, the cells appear to be bi-refringent with a smooth and clear outline.
65. Fill a glass-pipette with the external solution containing a drug (see step 58), in accordance with the experimental protocol.
    a. First, dip the pipette tip into a 1 mL Eppendorf tube with the external solution containing the drug for a few seconds such that the solution enters the pipette by capillarity.
    b. Then, use a needle attached to a 2 mL syringe to backfill the pipette with the same solution.
    c. Remove any air bubbles from the pipette's interior by shaking the pipette as if it were an old mercury thermometer.

66. Fit the drug application pipette to a pipette holder and use a micromanipulator to orientate the pipette towards the recording chamber. Steps 65 and 66 can be repeated 2 or 3 times for other drug application pipettes, depending on the experimental design (Figure 3C).

67. Fill a recording pipette with a filtered internal solution to 60%–75% of capacity, as described for the drug application pipettes, intercalating a 0.22 µm filter between the syringe and the needle.

68. Insert the glass-pipette into the electrode holder so that the chlorinated electrode, which is connected through a gold pin to the amplifier head stage, is placed into the internal solution, and secure the pipette in the holder.

69. Activate the "SET-UP" mode in the PATCHMASTER and configure the recording conditions:
    a. Set the amplifier gain to 5 mV/pA
    b. Set digital oscilloscope gain and time resolution depending on the expected response size
    c. Set the capacitance cancellation and series resistance compensation to 0
    d. Set the current filter bandwidth to 2.9 Hz
    e. Set the test pulse parameters to 5 mV for 5 ms every 30 ms from a holding potential of 0 mV.
    f. Set the baseline correction to 0.

    Note: Since the electrical circuit is still open (e.g.: there is no electrical continuity between the recording and the ground electrodes), no current is measured.

70. Using a micromanipulator, lower the recording electrode into the bath and visualize the pipette current offset and the current response to test pulses in the oscilloscope screen;djust the current offset to zero in the amplifier.

    Note: The current response to voltage pulses allows the pipette's resistance to be calculated by applying Ohm's law: R = V/I (Figure 4A).

71. Move the pipette tip into the field of view (the pipette can be readily visualized under a 10× objective).

72. Again, lower the pipette to permit the 10× objective to be exchanged for an objective with a higher magnification (63×) and a shorter working distance (1.5 mm). Focus the plane immediately above the cell of interest and lower the pipette until its tip can be clearly seen. Focus the cell and capture an image of it (Figure 9A).

    Note: This image will be used to relate the electrophysiological data to the cell morphology.

73. While continuously applying test pulses, bring the electrode tip close to the cell and press it up against the cell membrane. Start to apply negative pressure inside the pipette and monitor the reduction in the current amplitude due to the increase in the pipette resistance.

    Note: Negative pressure is applied by suction through a side port of the pipette holder connected to a 10 mL syringe by a silicone tube when the plunger is retracted. Alternatively, negative pressure can be applied by sucking through the tube. Pressure is carefully adjusted for each cell to achieve a more effective reduction of the current amplitude.

74. Gradually adjust the holding potential to negative values up to −70 mV at ≈3 s intervals (−10 mV, −20 mV, −30 mV, ...), and monitor the formation of a seal with high mechanical and electrical resistance between the pipette tip and the plasma membrane (Figure 4B; see troubleshooting 6).

    Note: Pipette resistance should increase to a minimum of 1 GΩ ("giga-seal"). Depending on the cell, this might occur rapidly after coming into contact with the cell and applying negative pressure, or it may require tens of s of continuous negative pressure at negative potentials in the pipette. The formation of the seal implies a dramatic reduction of the current to values close to 0 pA during the voltage pulse. This indicates that the "cell-attached" configuration has been achieved and a patch of membrane is encircled beneath the pipette's tip
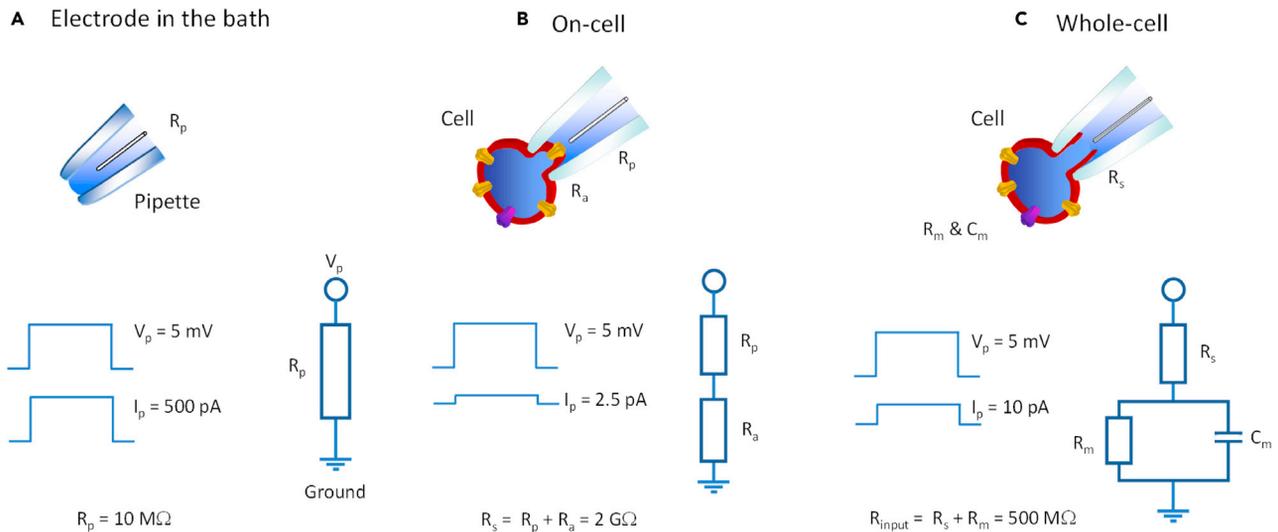
Figure 4. Scheme of the steps needed to achieve whole-cell recording

(A) Electrode in the bath. In this situation the current is determined solely by the pipette resistance (R_p). Below (in each panel), the voltage (V_p; test pulses) and current (I_p) traces are shown on the left, and the corresponding electrical circuit is depicted on the right. The R_p can be calculated using Ohm's law (R = V/I).

(B) On-cell. In this configuration, the current is determined by the R_p in series with the access resistance (R_a), which comprises both the patch and seal resistances. Note that as the R_a is very high ($G\Omega$), the current across this resistance is negligible.

(C) Whole cell. In this configuration, the current is determined by the series resistance (R_s), in series with a parallel circuit of the membrane resistance (R_m) and the membrane capacitance (C_m). The input resistance (R_{input}) is the sum of R_s and R_m, and it can be calculated using Ohm's law (R=V/I). In (B) and (C), the pipette and cell capacitative current transients are canceled.

75. Cancel the pipette capacitive transients that are apparent upon giga-seal formation due to current charging/discharging the pipette's capacitor.

   Note: To best perform this task, it is advisable to increase the gain of the amplifier and/or the oscilloscope screen. Depending on the amplifier, oscilloscope and software used, this can be done manually or automatically (e.g., by pressing the "On-cell" key in PATCHMASTER)

76. Set the holding voltage to −70 mV to bring the pipette potential closer to the resting potential of the cell and to prevent cell depolarization when the membrane patch is broken.
77. Apply brief, intense negative pressure pulses to break open the membrane patch enclosed in the pipette. Do this with care, as excess suction can also disrupt the giga-seal and the cell will no longer be useful. Once you break the patch, electrical access to the cell cytoplasm is achieved and the membrane potential becomes the pipette potential, thereby reaching the "whole-cell" configuration of the patch-clamp technique (Figure 4C).

   Note: The whole cell configuration is manifested by a sudden reduction in membrane resistance and the appearance of large capacitive transients.

78. Cancel the cell capacitive transients (due to the charging/discharging of the membrane capacitor), and adjust the amplifier gain and oscilloscope screen parameters to the expected recording conditions (i.e., the size of the currents to be measured). Depending on the amplifier, oscilloscope and software used, this can be done manually or automatically by the software ("Whole-cell" in PATCHMASTER).

   Note: In the whole-cell configuration, current flows mostly through the cell membrane as long as the seal resistance is preserved. If the pipette potential is set close to the cell's resting potential before breaking the patch, very little current would be expected during the test pulses

and symmetrical current transients will become apparent at the beginning and end of the pulses. These transients reflect both the cell membrane capacitance and the series resistance between the pipette and the cell cytoplasm.

79. Compensate a proportion (70%–90%) of the series resistance for a better voltage-clamp when fast and large currents are to be measured (e.g., voltage-gated Na^+ currents responsible for action potential generation). It is also convenient to monitor series resistance throughout the experiment.

    Note: Ideally, series resistance should be less than twice the pipette resistance. The series resistance should remain low during the whole-cell recording to favor dialysis of the cytoplasm with the internal solution and to prevent voltage-changes caused by large transmembrane currents. Recordings in which series resistance rises by more than 20% with respect to the initial values are usually discarded.

80. Wait several minutes (3–10) to complete the cell dialysis with the internal solution, while monitoring the leak current amplitude and series resistance to assess the stability and voltage-clamp suitability of the seal.

81. Configure or select (if previously configured) a stimulation protocol and then apply it to the cell under study. Typical protocols include step depolarizations (e.g., a 100 ms pulse to +10 mV) capable of activating voltage-gated ion channels, such as the Na^+ and K^+ channels involved in action potential firing. The currents produced in response to voltage-stimulation are recorded and stored for subsequent display and analysis (Figure 9C).

    Note: Most protocols to measure voltage-activated currents include the so-called "P/n" procedure to subtract the leak and capacitive currents evoked by any voltage pulse. These are voltage-related currents that are not due to ion flux through membrane ion channels. Since leak and capacitive currents are linearly related to voltage (as they follow Ohm's law), in the P/n procedure they are triggered by small voltage pulses that are unable to activate ion channels (e.g., one quarter or one sixth of the test pulse), and subsequently scaled to the current size produced by the test pulse (e.g., multiplying their amplitudes by four or six). Subtracting the scaled leak and capacitive currents from the current responses evoked by the test pulse allows the voltage-activated ion currents to be isolated. Their presence is taken as evidence of the functional expression of the ion channels mediating them.

82. Carefully bring the pipette close (5–15 μm) to the cell being recorded while monitoring the holding (leak) current as an indicator of seal integrity. Repeat this procedure for every pipette you plan to use in the experiment (Figure 9B).

    Note: It is convenient to clear external solution from the pipette tip by applying several pressure pulses when it still is far from the cell under study.

83. Apply a pulse of pressure to the interior of the application pipette to deliver the drug (1 mM glutamate, 100 μM GABA or 100 μM 5-HT) used to activate ionotropic receptors. The currents produced in response to agonist-stimulation are recorded and stored for subsequent analysis and display (Figure 9D)

    Note: Sustain drug application for the time needed to produce a clear response and its eventual decline and then, cease the application and washout the drug by perfusion of the external solution. Once the membrane current recovers the baseline, a second drug from a different pipette can be applied.

84. In cells with a leak current of a few pA at a voltage close to the resting membrane potential, it is possible to switch from the voltage-clamp to the current-clamp mode. This allows:

a. To record the resting potential (when I_p = 0 pA).

b. To assess changes in membrane potential when current is injected (e.g., a current step of +75 pA for 250 ms) or a receptor agonist is administered (e.g., 100 μM 5-HT). Positive current steps and ligand-gated inward currents evoke depolarizing responses and, occasionally, elicit action potentials (Figure 9E).

Note: The leak current should remain low during voltage-clamp recordings and particularly during current-clamp recordings. Cells with a leak current larger than −100 pA are usually discarded.

85. Try to record synaptic currents evoked by transmitters released from a cell making a synaptic contact with the recorded cell (Figure 9F).

a. Apply 5-HT (100 μ) to an unclamped bipolar cell to elicit glutamate release which, in turn, could activate synaptic-like currents in a multipolar voltage-clamped cell.

b. Start local application of MK-801 (10 μM), a NMDA antagonist, to this latter may reduce the amplitude of the currents, thereby allowing the receptors responsible for them to be identified and the glutamatergic nature of the bipolar cell to be inferred.

Note: Synaptic-like currents can be recognized by their rapid onset and brief duration.

86. Once the experiment is finished, proceed to stop recording, save the file and pull the recording pipette away from cell. Finally, lift the electrode out of the bath and remove the glass pipette from the electrode holder.

Note: An experiment is finished when the experimental protocol has been completed or when the recording conditions (leak current, series resistance, current run-down, etc.) make the results unreliable or difficult to interpret.

87. If attempting to perform a new experiment on the same coverslip, repeat steps 67–83. If you decide to use a fresh coverslip, start the procedure again from step 61.

Note: The decision as to whether the coverslip should be changed will depend on the time it has been kept in the recording chamber (a maximum of 1 h is advisable), the number of cells previously used, and whether the local drug application and washes are performed effectively.

⚠ CRITICAL: Never reuse patch pipettes.

88. Basic current measurements and processing of electrophysiological data can be performed within the PATCHMASTER software. This software allows replaying data and measuring peak currents or voltage amplitudes in the digital oscilloscope window, which provides a readout of the current or voltage traces by placing a cursor on any sampled value. Likewise, baseline currents or potentials can be manually or automatically subtracted from the peak values.

EXPECTED OUTCOMES

Following the fundamental premises of the protocol on which this preparation is inspired (Costa et al., 2011; Ortega et al., 2011), the cultures are maintained at low cell density with 50,000 cells/cm^2 plated in a 24 well plate under adherent conditions. It is important to bear in mind that typical yields of cerebellar cells are 1–1.2 × 10^6 per pup for these cell preparations. This low density allows the mode of division and lineage progression of cerebellar NSCs and progenitors to be followed *in vitro*. Different lineage trees with distinct level of complexity can be tracked during the experiment (e.g., Figures 5 and 6), although the vast majority of these trees are based on a founder cell that generates two neuroblasts through a symmetric terminal cell division. The trees then involve two, three,
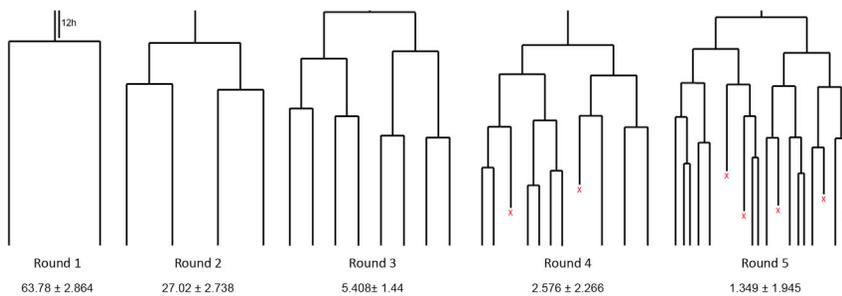
Figure 5. Prototypic lineage trees observed by single-cell tracking of the cerebellar cultures
The representative lineage trees and their relative frequencies (as a percentage below each tree) are shown.
Figure adapted from Paniagua-Herranz et al. (2020b).

four or five rounds of division prior to differentiation, indicating that the cell preparation contains progenitor cells isolated at different stages of progression within the neural lineage.

Differentiated neurons are characterized by (i) the expression of neuronal markers as βIII-Tubulin, (ii) the presence of neuronal processes, (iii) the progressive acquisition of neuronal electrophysiological properties, (iv) positive labeling for nucleotide vesicular transporters (VGLUT or VGAT, respectively), and (v) a postmitotic nature, as indicated by the absence of expression of proliferative markers as Ki67 (Figures 7, 8, and 9).

Remarkably, this cell preparation allows simultaneous monitoring of all the cell populations in the three neurogenic niches of the postnatal cerebellum: the external granular layer (EGL), the Purkinje cell layer (PCL), and the prospective white matter (PWM: (Leto et al., 2016). Accordingly, it is possible to monitor the lineages generating glutamatergic neurons, the main population produced from the EGL (Hallonet et al., 1990; Alvarez Otero et al., 1993), as well as the GABAergic interneurons, parenchymal astrocytes and oligodendrocytes produced in the PWM (Leto et al., 2012; Parmigiani et al., 2015). In addition, it is also feasible to monitor the behavior of astroglial progenitors, a population generated in the PCL (Buffo and Rossi, 2013). The progenitors isolated *in vitro* generate glutamatergic and GABAergic neuronal progeny in proportions that mimic those that arise *in vivo* (Figure 8).

Both neuronal populations can be identified and characterized by either PIIC (Figure 8) or by patch-clamp recordings (Figure 9). So, electrophysiological recording allows the expression of voltage- and neurotransmitter-gated ion channels by cerebellar cells to be determined, as well as their electrical excitability. Furthermore, the use of serotonin (5-HT) allows glutamatergic cerebellar neurons to be easily identified, as functional 5-HT$_3$ ionotropic receptors are expressed only by this cell population in early postnatal life (Oostland et al., 2011) So, most cells with bipolar processes produce membrane currents in response to 5-HT, confirming their glutamatergic nature. Likewise, synaptic transmission in cultured cells can be analyzed.

Finally, it is possible to monitor cerebellar NSCs characterized by the expression of GFAP and SOX2, their cell growth prior to division, and their capability to undergo asymmetric divisions that give rise to a neurogenic branch that becomes progressively smaller and a self-renewal branch to generate new GFAP/SOX2 cells. All these features constitute the stereotypic behavior of NSCs (Temple, 1989; Kriegstein and Alvarez-Buylla, 2009; Costa et al., 2011; Ortega and Costa, 2016; Ortega et al., 2013a, 2013b; Neumuller et al., 2008; Paniagua-Herranz et al., 2020a)

Altogether, this cell culture preparation exhibits several intrinsic features that makes it an ideal tool to study the behavior of the postnatal cerebellar NSCs and how niche factors, or pathological situations may modify this behavior. Amongst them it is worthy to highlight that: (i) cerebellar NSCs isolated in this preparation maintain their neurogenic nature even in the absence of niche
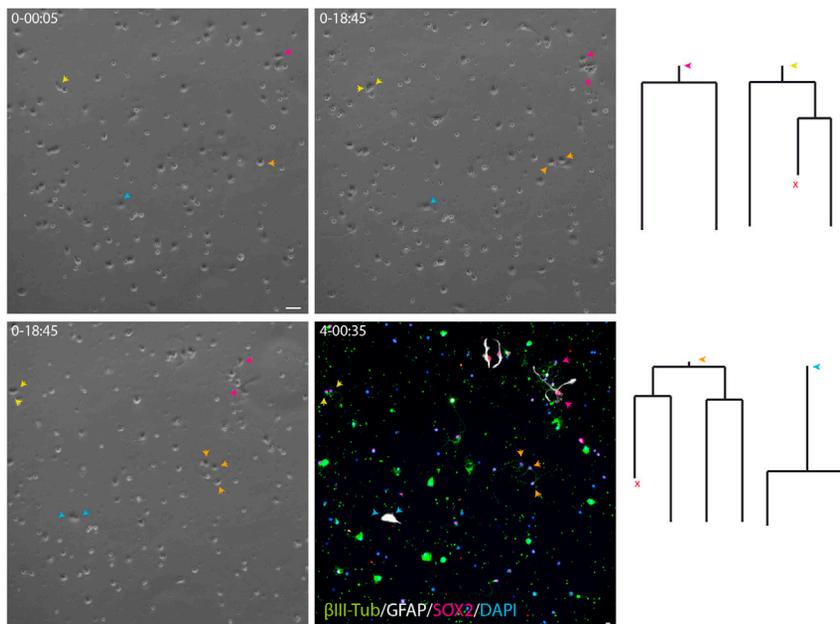
Figure 6. Representative field images and lineage trees from a live imaging experiment using a P0 cell preparation
Phase contrast images represent the lineages trees obtained by time-lapse microscopy at different time points (day-h:min). The last image corresponds to the post-imaging immunocytochemistry for βIII-Tubulin (green), GFAP (white), SOX2 (magenta) and DAPI (blue). Scale bar 30 μm.

or mitogenic signals, (ii) it is feasible to monitor the cell populations generated by the three post-natal cerebellar neurogenic niches simultaneously, and (iii) the isolation from their niche enables to study the effect of each niche factor, individually.

LIMITATIONS

This preparation is ideal to perform analysis at the single cell level due to the low cell density employed. However, precisely due to this low cell density the protocol significantly hampers the application of biochemical assays that require large cell numbers. Another inherent limitation regards the long-term maintenance of the cells in culture. As this is a primary culture, differentiation of neural progenitors is restricted to a specific time window (4–5 days), and the neurons generated can also be maintained for a limited period. In our experience we have imaged cerebellar NSCs and their progeny for a maximum of 14 days (average experiments are limited to 7 days), after which the survival of the cells might be compromised.

Another hallmark of this cell preparation is the absence of the signals from the specific neurogenic niches, which may represent both an advantage and a limitation. Interestingly, isolated cerebellar NSCs and progenitors maintain their neurogenic nature even in the absence of such extrinsic signals. This facet is consistent with a considerable part of the cerebellar NSC lineage progression, and that of progenitors, being reproducible in the absence of the signals from their niche. Conversely, in our preparation we did not find the bi-potent progenitors previously described in the PWM that produce both and an astroglial and a GABAergic neuron progeny (Fleming et al., 2013; Parmigiani et al., 2015). Whether this is due to the scarcity of GFAP/SOX2 NSCs in the PWM and/or the absence of extrinsic signals from the niche is something that will have to be determined in the future.

TROUBLESHOOTING
Problem 1
Difficulty in dissociating the cerebellum, probably due to insufficient enzymatic digestion (step 6).
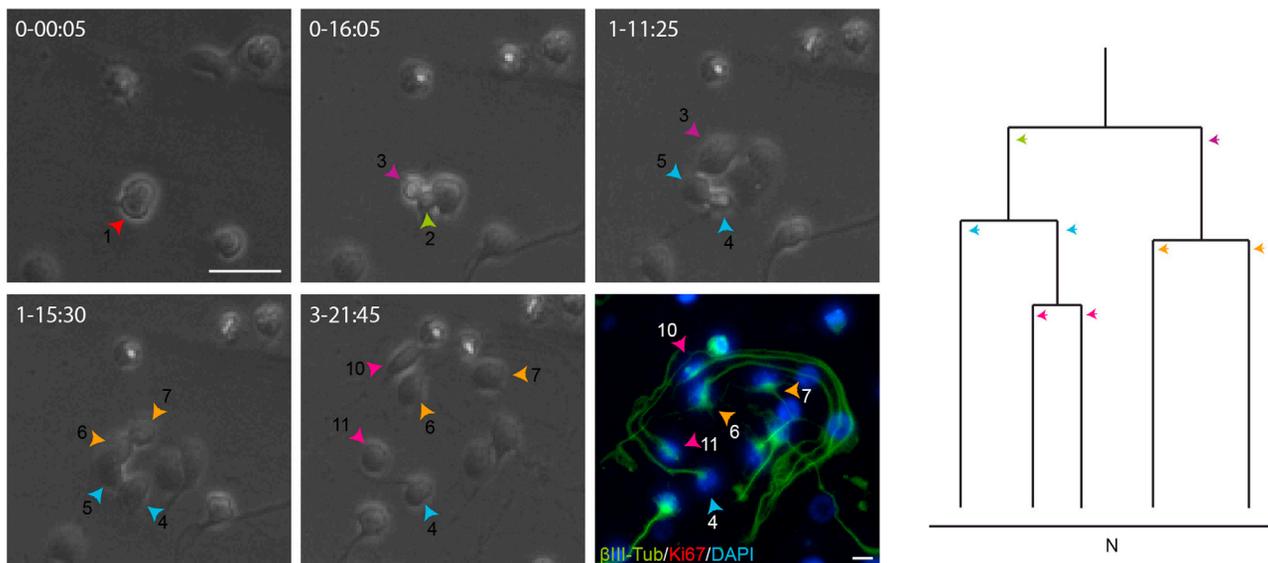
Figure 7. Representative images of symmetric neurogenic trees giving rise to differentiated neurons

Phase contrast images depicts a symmetric neurogenic lineage tree generating βIII-tubulin positive cells that are negative for the proliferation marker Ki67 and, hence, postmitotic neurons (N, neuron;). The lineage tree is detailed as follows: phase contrast images acquired by time-lapse video microscopy at different time points (day-h:min), image of the post-imaging immunocytochemistry for Ki67 (red) and βIII tubulin (green). Scale bar 30 μm.

Potential solution

Use fresh papain, and ensure the correct concentration and incubation times are employed. The time of incubation can be increased up to 1 h.

Problem 2

An excessively large number of dead cells. A decrease in cell viability might be due to harsher trituration or an extended period between removing tissue and its trituration (step 14).

Potential solution

Solution 1: To fix the reduced cell viability due to the harsh trituration, dissociate the cells more gently and avoid the formation of bubbles.

Solution 2: If the loss of cell viability is due to the extended time between removing and triturating the tissue, try to reduce the dissection time by practicing the technique. Speed is essential to maintain cell viability.

Problem 3

Low cellular yields, possibly due to the formation of cell aggregates (step 8).

Potential solution

Ensure the adequate, gentle trituration of cells. Increase the number of smooth up and down strokes with the pipette in step 8.

Problem 4

Cells do not adhere to coverslips or Petri dishes when plated (steps 1 b and 15).

Potential solution

Ensure an adequate volume and concentration of poly-L-lysine is used to coat the coverslips and Petri dishes.
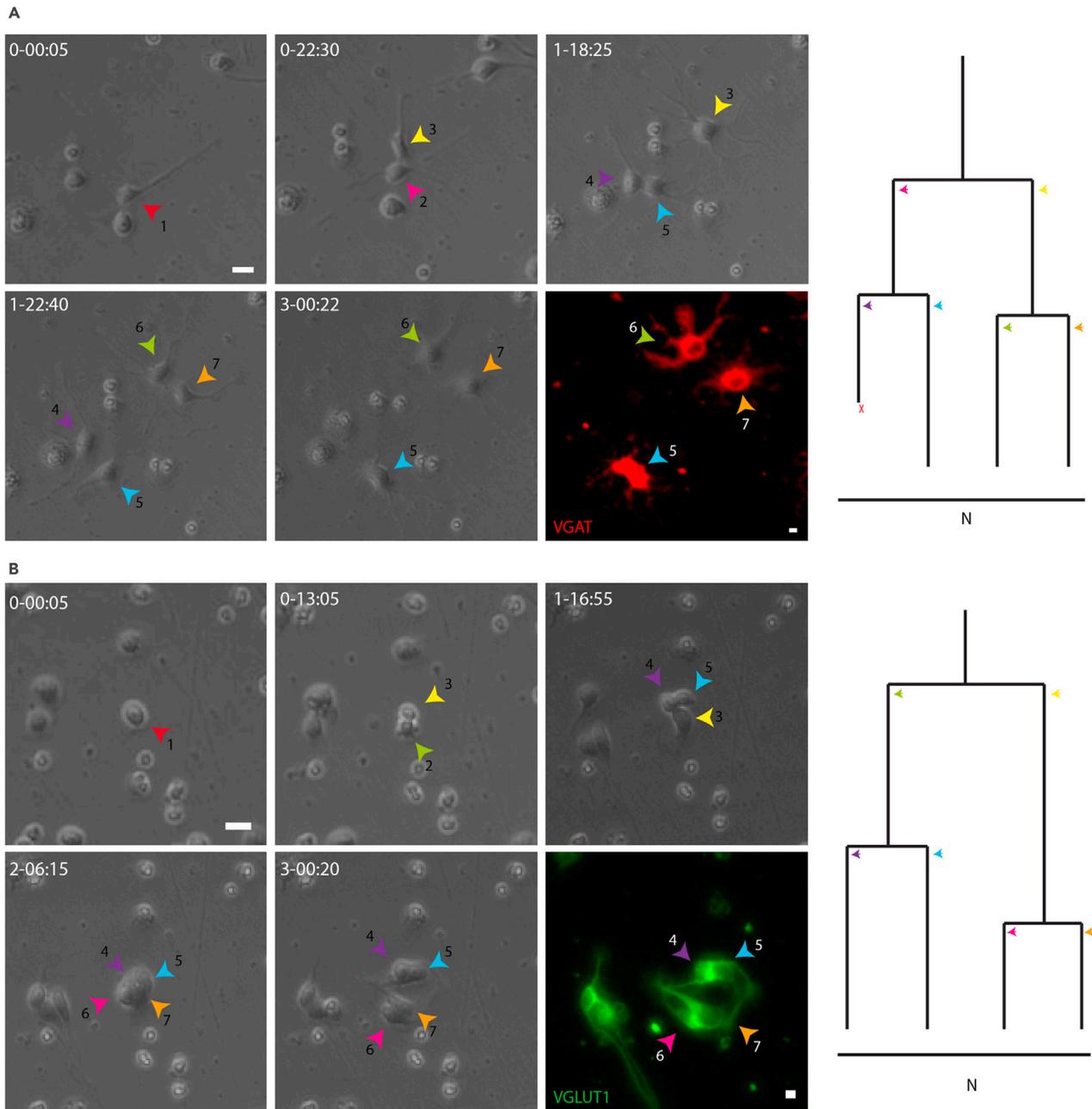
Figure 8. Example of the GABAergic and Glutamatergic neurons generated in the P0 cell preparation

(A) Symmetric lineage generating GABAergic neural progeny (N, neuron; X, cell death).

(B) Symmetric lineage generating glutamatergic neural progeny (N, neuron; X, cell death). Both lineage trees are detailed as follows: phase contrast images acquired by time-lapse video microscopy at different time points (day-h:min), image of the post-imaging immunocytochemistry for VGAT (red) and VGLUT1 (green). Scale bar 15 μm.

Problem 5

Contamination due to non-sterile conditions (steps 2–8).

Potential solution

Sterilize the dissection tools by autoclaving and clean all the working surfaces areas with 70% (vol/vol) ethanol.
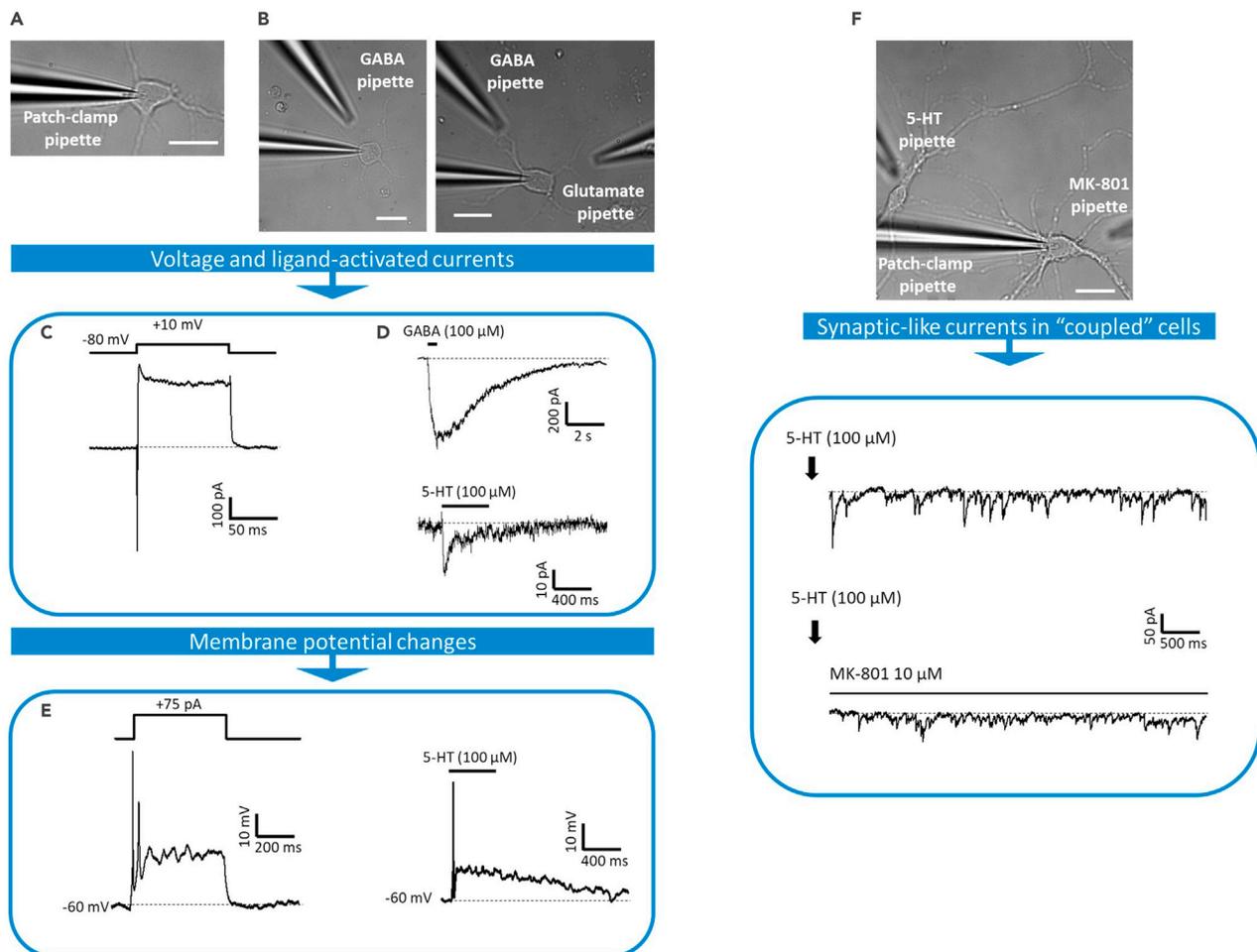
Figure 9. Patch-camp recordings of voltage- and ligand-activated currents, and electrical excitability in cerebellar cell cultures

(A) Bright-field image of a cerebellar cell. A patch-clamp recording pipette placed onto the cell soma is shown. This image will be used to relate electrophysiological data to cell morphology.

(B) Depending on the stimulation protocol, one or two drug application pipettes can be lowered to the vicinity (5–15 μm) of the cell being recorded.

(C) Voltage-gated currents recorded in voltage-clamp mode (from a Vh of −80 mV) after applying a step depolarization (a 100 ms pulse to +10 mV).

(D) Ligand-gated currents responses elicited by pressure application of GABA (100 μM) and 5-HT (100 μM). The black lines above the current traces show the time of drug application.

(E) Membrane potential changes recorded in current-clamp mode in response to current injection (75 pA for 250 ms) or 5-HT application (100 μM). The depolarizing responses occasionally elicit action potentials.

(F) Upper panel: bright-field image of a bipolar and a multipolar cell. The location of the patch pipette and two drug application pipettes (5-HT and MK-801) is indicated. Lower panel: application of 5-HT (100 μM; see arrows) to the bipolar unclamped cell evokes synaptic-like currents in the neighboring patch-clamped multipolar cell. Currents were sensitive to the NMDA antagonist MK-801 (10 μM). Scale bar 20 μm.

Problem 6
Difficulty to seal and/or to break in (steps 56, 74, and 77)).

The cells do not seal when applying negative pressure and/or the patch does not break or the seal is lost when trying to break into the cell.

Potential solution
Check the osmolarity of internal solution, try another batch of internal solution or prepare a new batch.

Check the osmolarity of the external solution.

Pull the pipettes with slightly higher resistance to improve the seals, or with lower resistance to improve patch rupture.

Apply lower negative pressure to prevent the loss of the seal.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Felipe Ortega, fortegao@ucm.es

Materials availability
This study did not generate new unique reagents

Data and code availability
This study did not generate unique databases.

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AUTHOR CONTRIBUTIONS

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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