The establishment of polarity is crucial for the physiology and wiring of neurons. Therefore, monitoring the axo-dendritic specification allows the mechanisms and signals associated with development, growth, and disease to be explored. Here, we describe major and minor steps to study polarity acquisition, using primary cultures of hippocampal neurons isolated from embryonic rat hippocampi, for \textit{in vitro} monitoring. Furthermore, we use \textit{in utero} electroporated, GFP-expressing embryonic mouse brains for visualizing cortical neuron migration and polarization \textit{in situ}. Some underreported after-protocol steps are also included.
Protocol for Evaluating Neuronal Polarity in Murine Models

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SUMMARY
The establishment of polarity is crucial for the physiology and wiring of neurons. Therefore, monitoring the axo-dendritic specification allows the mechanisms and signals associated with development, growth, and disease to be explored. Here, we describe major and minor steps to study polarity acquisition, using primary cultures of hippocampal neurons isolated from embryonic rat hippocampi, for in vitro monitoring. Furthermore, we use in utero electroporated, GFP-expressing embryonic mouse brains for visualizing cortical neuron migration and polarization in situ. Some underreported after-protocol steps are also included. For complete details on the use and execution of this protocol, please refer to Wilson et al. (2020).

BEFORE YOU BEGIN

Polarity Acquisition In Vitro: Primary Culture of Hippocampal Neurons

© Timing: 1–2 days

1. Make sure to sterilize all materials that will be used during the culture, including buffers, culture media, glassware, and surgical instruments.

2. If needed, prepare PBS, HBSS and MEM supplemented with 10% horse serum (HS). Preferably, use cold PBS and HBSS during the dissection step.

3. Prepare glass coverslips for microscopy:
   a. Immerses coverslips in nitric acid (pure) for 16–18 h (overnight, ON) in ceramic racks.
   b. Next day, transfer racks to a beaker containing distilled water.
   c. Wash 3 times (20–30 min/wash) using a stirring bar.
   d. Dry coverslips using a stove at 180°C (2–3 h).
   e. Proceed with Poly-L-Lysine (PLL)-coating or store coverslips under sterile conditions.

Alternatives: To replace cleaning with acid, immerse coverslips in ethanol and flame using a burner in the hood.

4. To coat culture plates, prepare a 1 mg/mL poly-L-lysine (PLL) solution:
   a. Prepare borate buffer (0.05 M boric acid and 0.01 M sodium tetrahydroborate in, preferably, milli-Q water).
   b. Dissolve 100 mg of PLL in 100 mL of borate buffer (scale up for larger volumes).
Adjust pH to 8.9 and sterilize by filtration (0.2 μm pore size).

**PLL Preparation for Cell Culture Dish Coating**

Amounts (mL or mg) are expressed considering 100 mL of 1 mg/mL PLL solution. Scale up or down for alternative volumes. Make sure to adjust pH to 8.9.

| Reagent               | Stock Solution | Amount for 100 mL | Final Concentration |
|-----------------------|----------------|-------------------|---------------------|
| Acid boric            | 0.2 M          | 25.0 mL           | 0.05 M              |
| Sodium tetrahydroborate| 0.05 M       | 21.3 mL           | 0.01 M              |
| Poly-L-lysine         | n/a            | 100 mg            | 1 mg/mL             |
| milli-Q water         | n/a            | 53.7 mL           | n/a                 |

5. PLL coating:
   a. Coat culture dishes with 1 mg/mL PLL ON at 37°C, using suitable volume to cover the surface properly. In case of coverslips, use a drop (150 μL) of PLL per glass.
   b. Discard PLL and wash the dishes with sterile water for 20 min at RT (20°C–25°C). Repeat 2 times.

**Alternatives:** other laboratories use laminin rather PLL, although expected outcomes may slightly vary (we have included a brief discussion about this in troubleshooting, problem 3).

**Note:** PLL can be recycled a couple of times before discarding.

   c. Add 10% HS MEM to the culture dishes and incubate at 37°C until plating neurons.

**Pause Point:** If needed, PLL-coated dishes can be stored in water up to 1 week at 4°C.

6. Prepare the set of Pasteur pipettes for the digestion step:
   a. Using a burner, polish pipettes to obtain:
      i. Half of diameter tip pipette
      ii. Quarter diameter tip pipette
   b. If needed, autoclave polished pipettes for long-term storage.

7. Prepare the workplace before isolating the embryos. In the bench/hood, prepare the stereo-microscope and embed surgical tools in 70% v/v ethanol for at least 10 min. During this time, prepare petri dishes for dissection (see major step 2g) and pre-warm 10% HS MEM at 37°C.

**Polarity Acquisition In Situ: In Utero Electroporation of E15.5 Mouse Brains**

© Timing: 1–2 days

8. Disinfect the surgical tools by immersing in 70% v/v ethanol.

9. Prepare glass capillaries for DNA injection.
   a. Polish several (10–20) capillaries (75 mm) using a micropipette puller (P-97 Flaming/Brown type - Sutter Instruments or equivalent) with a 2.5 mm square filament.

10. Prepare DNA mix solution.
    a. If needed, purify plasmid DNA using an endotoxin-free maxi-prep kit to obtain a yield of 2–3 μg/μL.
    b. DNA mix solution (20 μL):
       i. Take a volume of plasmid containing 20 μg of DNA. Of note, DNA concentration in the mix should be approximately 1 μg/μL.
       ii. Add Fast Green dye to the DNA (work concentration = 0.1% v/v).
iii. If needed, add PBS to complete 20 μL.

⚠ CRITICAL: Make sure your cDNAs are cloned under a strong promoter. In this protocol we recommend the pCAGIG-GFP plasmid (CAG U6 promoter). If needed, subclone your cDNAs into this backbone.

11. Load the anesthetic device with isoflurane and open the flux of oxygen at 3 cm³/L. The oxygen is required to allow the survival of the mother during the anesthesia procedure.

12. Prepare the analgesic mix:
   a. Estimate the animal weight using a scale. Of note, the bodyweight of a pregnant E15.5 mouse is close to 25–30 g. This value may vary depending on age and pregnancy status.
   b. Check the stock concentration of tramadol and estimate the volume needed to inject 1 mg per 1 kg body weight.

Note: Tramadol stock concentration (and vehicle) may vary depending on suppliers and local regulations. In any case, make sure to administrate 1 mg of tramadol per 1 kg animal body weight.

   c. A suitable injection volume for the analgesics is 150 μL. If needed, dissolve in physiological solution (0.9% w/v NaCl) up to reach this volume.

Alternatives: Physiological solution may be replaced by sterile PBS.

13. Load the DNA mix into the glass capillaries (sucking is the most effective way).

14. Set the animal warming system at 37°C.

15. For fixation and post-fixation procedures:
   a. Prepare fresh 4% Paraformaldehyde (PFA), dissolved in PBS. Adjust pH to 7.4.
   b. Prepare a 30% v/v sucrose solution in water.

16. Prepare 2% w/v gelatin:
   a. For 100 mL, dissolve 2 g of gelatin in cold water.
   b. Then, add remaining 50% of hot water until dissolve.

17. Coat glass slides with 2% w/v gelatin.
   a. Immerse the glass slides in gelatin for 10 min RT.
   b. Dry the glass slides in a stove at 37°C ON.
   c. If needed, store the slides at 4°C for up to 1 month.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-tubulin class III | Abcam    | 78078; RRID:AB_2256751 |
| Anti-Tau1           | Lester Binder’s laboratory (Binder et al., 1985) | N/A |
| Anti-MAP2           | Merck Millipore | ABS622; AB_91939 |
| Anti-Ankyrin G      | Santa Cruz | sc-12719; AB_626674 |
| Alexa Fluor 546 Phalloidin | Thermo Fisher | A22283 |
| Anti-Tau1 (commercially available) | Merck Millipore | MAB3420; AB_94855 |
| DAPI                | Sigma-Aldrich | D9542 |
| Chemicals, Peptides, and Recombinant Proteins |        |            |
| Poly-L-lysine*      | Sigma-Aldrich | P2636 |

(Continued on next page)
Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| MEM*                | Gibco – Thermo Fisher | A1451801 |
| Horse Serum*        | Gibco-Thermo Fisher   | 16050122 |
| Neurobasal*         | Gibco – Thermo Fisher | 21103049 |
| Glutamax*           | Gibco – Thermo Fisher | 35050061 |
| B27 (50x) serum free* | Gibco – Thermo Fisher | 17504044 |
| Trypsin-EDTA (0.5%)* | Gibco – Thermo Fisher | 15400054 |
| DNase 10x*          | Thermo Fisher         | AM8170G  |
| HBSS* **            | Thermo Fisher         | 14025092 |
| PBS* **             | Sigma-Aldrich         | D8537    |
| Nitric acid*        | Merck                | 438073   |
| Ethanol (technical grade is enough)** | Merck (or equivalent from local suppliers) | 117271000 |
| Sodium tetrahydroborate | Sigma-Aldrich | 452882   |
| Boric acid*         | Sigma-Aldrich         | 1001651000 |
| Sodium bicarbonate  | Sigma-Aldrich         | 55761    |
| Pen/Strep*          | Thermo Fisher         | 15070063 |
| Fast green FCF dye** | Sigma-Aldrich        | F7252    |
| Isoflurane**        | Abcam                | ab145581 |
| Tramadol**          | Laproff              | TDEL-11599 |
| Paraformaldehyde* ** | Sigma/Merck         | 441244   |
| Sucrose* **         | Sigma/Merck          | 84100    |
| Gelatin**           | Sigma/Merck          | G1393    |
| Crioplast**         | Biopack              | 2000120400 |
| Mowiol*             | Sigma/Merck          | 9002-89-5 |

Critical Commercial Assays

Endotoxin-free Maxi Prep Kit | Qiagen (or equivalent) | 12362

Experimental Models: Organisms/Strain

| Rat: Wistar | Produced in the animal facility of Instituto de Investigación Médica Mercedes y Martin Ferreyra (Córdoba, Argentina); originally from Charles River, USA | N/A |
| Mouse: C57BL/6N | Produced in the animal facility of Instituto de Investigación Médica Mercedes y Martin Ferreyra (Córdoba, Argentina); originally from Universidad Nacional de la Plata (La Plata, Argentina) | N/A |

Recombinant DNA

| pCAGiG-GFP | AddGene | Plasmid #11159 |

Software and Algorithms

| Fiji | Open source community maintained by the Eliceiri/LOCI group at the University of Wisconsin-Madison, and the Jug and Tomancak labs at the MPI-CBG in Dresden | www.fiji.sc |

Other

| BTX™ ECM™ 830 Electroporation Generator** | Thermo Fisher | 15427230 |
| Tweezers w/3 mm platinum disk electrodes** | Nepagene | CUY650P3 |

(Continued on next page)
**STEP-BY-STEP METHOD DETAILS**

**Polarity Acquisition In Vitro: Primary Culture of Hippocampal Neurons**

© Timing: 6 h–14 days

The embryonic hippocampus is enriched in pyramidal neurons, a homogeneous population of neural cells exhibiting the cell body (soma), several dendrites and one single axon. In this regard, the culture of hippocampal neurons, isolated from embryonic rat brains, is a well-characterized cellular model widely used to study neurons at the single-cell level, in physiological or pathological contexts.

Culturing hippocampal neurons involves 4 main steps: embryo isolation, hippocampal dissection, digestion, and plating. The yield by E18.5 brain is approx. 300,000 neurons per hippocampus (600,000 hippocampal neurons by brain). In case of needing larger amounts of neurons, especially for biochemical studies, neurons isolated from the brain cortex can be used, which is also enriched in pyramidal neurons. Nevertheless, additional neural types may also be found. In any case, both hippocampal and cortical neuronal polarization are almost (if not completely) identical.

**Note:** Major steps 1–2 do not need sterile conditions.

1. Isolation of E18.5 rat embryos.

© Timing: 15–20 min

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### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Surgical scissors (straight; curve)** | BrainTree Scientific (or equivalent) | SCT-S 511; SCT-S 508 |
| Forceps (straight; curved)** | BrainTree Scientific (or equivalent) | FC003-7; FC003-8 |
| Ring-shaped special tweezers** | Carl Roth (or equivalent) | LL00.1 |
| Scapel Handle** | BrainTree Scientific (or equivalent) | SSS-11-CS |
| Silk Suture straight tapered** | BrainTree Scientific (or equivalent) | SUT-9403 |
| Glass capillaries** | Sutter Instrument | BF100-78-10 |
| P-97 Flaming/Brown type micropipette puller** | Sutter Instrument | P-97 |
| Anesthesia gas machine (vaporizer for small animals)** | Kent Scientific Corporation (or equivalent) | VetFlo-12055 |
| Sliding top chamber for vaporizers** | Kent Scientific Corporation (or equivalent) | VetFlo-0530XS |
| Stereo microscope* | Olympus (or equivalent) | SZX7 |
| Cryostat** | Leica CM 1850 (not commercialized anymore, alternatives will work as well) | Phased out |
| Neubauer chamber* | Electron Microscopy Sciences (or equivalent) | 68052-14 |
| Borosilicate glass pasteur pipets 5 3/4 inch* | Thomas Scientific (or equivalent) | P0458-5 |
| 15, 50 mL centrifuge tubes* | Fisher Scientific (or equivalent) | 14-959-53A; 10788561 |
| Microscope glass slides (75 × 25 × 1.4 mm)*** | Generic (https://www.amazon.in/Microscope-Glass-Slides-Pack-slides/dp/B071XXUDC5#detail_bullets_id) | 7105 |
| Glass coverslips (12.25 mm diameter)*** | Marienfeld Superior | 0111S20; 0111650 |

* For primary culture of embryonic rat neurons (major step 1)
** For in utero electroporation of mouse brains (major step 2)

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a. Place E18.5 pregnant rat inside the CO2 euthanasia chamber for rodents. Open the CO2 flux until notice a reduction on breath frequency and the rat is completely asleep (usually 8–10 s in a chamber for rats). Once vital signs are no longer detectable, proceed with cervical dislocation.

CRITICAL: Overdose of CO2 may affect the dissection, reducing the yield and viability of neurons. Therefore, be cautious with CO2 over-exposure to avoid unnecessary carbonation of brain tissue. In any case, euthanasia must be adjusted to local and international guidelines for animal care and use.

b. Sterilize the belly zone with 70% v/v ethanol.
c. Make an incision in the middle zone of the belly to expose embryos (Figure 1; second step).

d. Transfer embryos to a 100 mm petri dish, partially filled with pre-chilled PBS (4°C).

CRITICAL: Check the pregnancy (E18.5) and size of embryos (total length should be close to 1.5–1.8 cm).

2. Hippocampal and cortical dissection.
Timing: 0.5–2 h

a. Open embryo sacs with a small scissor and transfer each embryo to a new 100 mm petri dish, partially filled with pre-chilled PBS (4°C).
b. Isolate heads by doing an incision at the neck using a small scissor (Figure 1; third step).
c. Open the skull using a micro-scissor, cutting the dorsal line of the head from the neck to the front. To hold heads, introduce a fine tweezer (No.5 Dumont or equivalent) through the eye sockets (Figure 1; third step).
d. Transfer the embryonic brains to a 60 mm petri dish (or equivalent), previously filled with 3–4 mL HBSS supplemented with 0.35 g/L NaHCO₃.

Note: You will need a stereo-microscope to continue with the dissection.

e. Under scope, split hemispheres with a tweezer.
f. Using a fine tweezer (No.5), remove and discard meninges from each hemisphere.
g. Isolate the hippocampus using a micro-scissor and transfer to a new 60 mm petri dish (pre-filled with 4.5 mL HBSS) (Figure 1; fourth step).
h. If needed, transfer cerebral cortices to a petri dish pre-filled with 4.0 mL HBSS.

3. Digestion

Timing: 20–40 min

a. Hippocampal neurons
   i. Add 0.5 mL of 10× trypsin to the petri dish containing hippocampi (pre-filled with 4.5 mL HBSS). Incubate 20 min at 37°C.
   ii. Transfer digested hippocampi to a 15 mL centrifuge tube.
   iii. Make 3 quick washes with 2 mL HBSS.
   iv. Discard HBSS and add 2–3 mL of 10% HS MEM, pre-warmed at 37°C.
   v. Resuspend hippocampi with a glass Pasteur pipette, repeating several up-and-down (using a pipette filler, bulb or Finnpipette) movements until obtaining a homogenous suspension. The classical pipette rubber bulb may confer more sensitivity.
   vi. Repeat the procedure with a “half-diameter tip” pipette.
   vii. Finally, repeat “up-and-down” movements using a “quarter-diameter tip” pipette, until homogenate. Avoid making foam.

Alternatives: Tissue homogenization can be done using micropipettes (200–1,000 µL). Repeat “up-and-down” movements until obtain a homogenous suspension, following the rule of using, first, a large tip (1,000 µL) and then a small one (200 µL). The goal is to isolate neurons gradually, avoiding cell clumps, without - or the less possible - mechanical stress.

   viii. Count cells using a Neubauer chamber and estimate the yield [cells / (µL or mL)]. If needed, dilute cells 1/10. Of note, if hippocampi are resuspended in 2–3 mL 10% HS MEM (step v), the yield should be close to 1–2 × 10⁶ neurons/mL (considering 10–12 brains; 20–24 hippocampi).

b. Cortical neurons
   i. Add 0.5 mL of 10× trypsin + 0.5 mL of 10× DNase to the petri dish containing cortices (pre-filled with 4.0 mL HBSS). Incubate 25 min at 37°C.
   ii. Transfer cortices to a 15 mL centrifuge tube.
   iii. Make 3 quick washes with 4–5 mL of HBSS.
   iv. Discard HBSS and replace by 5 mL of 10% HS MEM (or suitable volume), pre-warmed at 37°C.
   v. Repeat several “up and down” movements using a 1,000 µL micropipette until obtain a homogeneous suspension of cells. Avoid making foam.
vi. Count cells using a Neubauer chamber and estimate the yield [cells / (μL or mL)]. For counting, dilute cells 1/100. Of note, if cortices are resuspended in 5 mL 10% HS MEM (step iv), the yield should be close to $10^7$ neurons/mL (considering 10–12 brains; 20–24 cortices)

**CRITICAL:** In both cases (hippocampal and cortical neurons), should get isolated cells after digestion. The presence of clumps reflects inefficient enzymatic/mechanical digestion. If needed, repeat mechanical digestion until obtain isolated cells or check trypsin.

4. Plating neurons

**Timing:** 1–4 h

- a. Estimate the volume needed to plate neurons at the required density:

| Cell Density | N° of Neurons/cm² |
|--------------|-------------------|
| Low          | 5,000 – 20,000    |
| Medium       | 20,000 – 40,000   |
| High         | 40,000 – 80,000   |

- b. Plate hippocampal or cortical neurons in pre-coated PLL culture dishes, using 10% HS MEM.
- c. Once neurons are attached (1–2 h after plating), replace 10% HS MEM by Neurobasal medium supplemented with B27, Glutamax and Pen/Strep.

**CRITICAL:** If needed, change the culture medium every 3–4 days, but always preserving 1/3 of the volume. Neurons will enrich the medium by secreting growth factors.

5. Monitoring polarity acquisition in vitro

**Timing:** 6 h–14 DIV

**Note:** After plating, hippocampal and cortical neurons will transform from symmetric cells (stages 1–2; 0.5–1 DIV) to neurons with well-defined axonal and dendritic compartments (stages 3–5; 3–14 DIV (Dotti et al., 1988; Kaeck and Banker, 2006; Cáceres et al., 2012). Of note, culture times indicated below are applicable to middle-low cell culture densities. It is important to highlight that timing suggested below may vary depending on neuronal confluency after plating, culture media composition and coating (for extended comments, please see problem 3 in troubleshooting). In addition, later stages (3–5) could be subdivided (e.g., early and late-stage 3; 2 and 4 DIV, respectively).

- a. Prepare a fresh solution of 4% w/v paraformaldehyde (PFA) and 4% w/v sucrose, dissolved in PBS. Adjust pH to 7.4.
- b. Fix neurons with 4% PFA/sucrose solution for 20 min at RT (20°C–25°C). Then, wash with PBS for immunofluorescence (IF) staining.
  - i. Stage 1 (6 h after plating). Stain with tubulin (β3-tubulin or Tyr-tubulin) antibody and phalloidin to visualize neurons and their actin organization by IF (Figure 2A).
  - ii. Stage 2 (18 h after plating). Stain with tubulin (β3-tubulin or Tyr-tubulin) antibody and phalloidin to visualize neurons, neurites, and growth cones by IF (Figure 2A, B).
  - iii. Stage 3 (2–5 DIV after plating). Stain neurons with tubulin (β3-tubulin or Tyr-tubulin) antibody and phalloidin to visualize minor neurites and axons by IF (Figure 2A). In addition, stain with MAP2 and Tau-1 antibodies to identify somatodendritic and axonal compartments, respectively (Figure 2C).
iv. Stage 4 (5–7 DIV after plating). Stain neurons with synapsin and MAP2 antibodies to visualize dendrites and branching at this stage IF (Figure 2A).

v. Stage 5 (7–14 DIV (and beyond) after plating). Stain neurons with PSD-95 and synaptophysin to visualize post and pre-synaptic terminals, respectively (Figure 2A). Alternatively, stain with MAP2 and AnkG antibodies, to detect dendrites and the assembly of the axon initial segment (AIS) of mature axons, respectively (Figure 2D).

Note: Currently, several molecules have been identified as markers, enriched in either the axonal or dendritic domain. Accordingly, Table 1 summarizes a battery of markers to identify these compartments during the acquisition of neuronal polarity.
Table 1. Molecular Markers Currently Used to Characterize Neuronal Polarity Stages, Axons and Dendrites Throughout Development

| Polarity stage | Marker         | Compartment                        | Reference                                      |
|---------------|----------------|------------------------------------|------------------------------------------------|
| Stage 2       | MAP2           | Minor neurites                     | (Caceres et al., 1986)                         |
| Stage 2-3     | Tiam1          | Nascent axon                       | (Kunda et al., 2001)                          |
| Stage 2-3     | MAP2           | Minor neurites and nascent axon    | (Caceres et al., 1986)                         |
| Stage 2-3     | GAP-43         | Axonal growth Cones                | (Goslin et al., 1988; Goslin et al., 1990)    |
| Stage 2-3     | p75 NTR        | Nascent axon                       | (Zuccaro et al., 2014)                        |
| Stage 2-3     | PI-3 kinase    | Nascent axon                       | (Shi et al., 2003)                            |
| Stage 2-3     | mPar3-mPar6    | Nascent axon                       | (Shi et al., 2003)                            |
| Stage 2-3     | Rap1B          | Nascent axon                       | (Schwamborn and Puschel, 2004)                |
| Stage 3       | Tau-1          | Distal axon                        | (Mandell and Banker, 1996)                    |
| Stage 3       | Tiam1          | Axon                               | (Kunda et al., 2001)                          |
| Stage 3       | p75 NTR        | Axon                               | (Zuccaro et al., 2014)                        |
| Stage 3       | SMI 312        | Axon                               | (Masliah et al., 1993)                        |
| Stage 3       | MAP2           | Minor neurites and axon            | (Caceres et al., 1984b; Caceres, et al., 1986) |
| Stage 3       | Cdc42          | Axon                               | (Chuang et al., 2005)                         |
| Stage 3       | Tyrosinated α-tubulin (Tyr-Microtubules, MT) | Axon and minor neurites | (Gonzalez-Billault et al., 2001) |
| Stage 3       | Detyrosinated α-tubulin (Glu-MT) | Axon                             | (Arregui et al., 1991; Gonzalez-Billault et al., 2001; Witte et al., 2009) |
| Stage 3       | Acetylated α-tubulin | Axon                             | (Ferreira and Caceres, 1989)                  |
| Stage 3       | MAP1B          | Axon                               | (Gonzalez-Billault et al., 2001)              |
| Stage 4       | MAP1A          | Dendrites                          | (Szebenyi et al., 2005)                       |
| Stage 4       | MAP2           | Dendrites                          | (Caceres et al., 1984a; Caceres et al., 1984b; Caceres et al., 1986) |
| Stage 4       | TIR            | Dendrites                          | (Burack et al., 2000; Bisbal et al., 2008)    |
| Stage 4       | GOPS           | Major dendrite                     | (Horton et al., 2005; Quassollo et al., 2015) |
| Stage 5       | Synapsin; Synaptophysin | Axon                             | (Fletcher et al., 1991)                       |
| Stage 5       | AnG            | Axon initial segment               | (Hedstrom et al., 2008; Galano et al., 2012)  |
| Stage 5       | PSD-95, NR2B   | Post-synaptic densities            | (Kennedy, 1997; Halpain et al., 1998)         |

Tiam1: T-cell lymphoma invasion and metastasis-inducing protein 1 (Rac1 guanine exchange factor); GAP-43: growth associated protein 43; p75 NTR: p75 neurotrophin receptor; Tau-1: dephosphorylated epitope of Tau protein; SM-31: phosphorylated MAP1B; SMI-312: neurofilament protein; MAP2: microtubule associated protein 2; MAP1A: microtubule associated protein 1A; TIR: transferrin receptor; GOPS: Golgi outposts; AnG: ankyrin G; PSD-95: post-synaptic density 95; NR2B: NMDA-receptor subunit 2B.

Note: In addition, cytoskeleton dynamics, vesicle trafficking and organelle distribution are instrumental to predict the axo-dendritic specification. The following table (Table 2) enlists events to be considered during the acquisition of neuronal polarity.
The development of the brain cortex starts early during embryonic life. Firstly, neurogenesis begins at E11.5-E13.5 in the ventricular zone (VZ), a cortical layer highly enriched in neural precursors (Kriegstein and Noctor, 2004). During this time, precursors experience their last mitotic division to then differentiate into post-mitotic neurons. After that, neurons undergo polarization and migration; both phenomena occur simultaneously and represent the best-characterized parameters to evaluate embryonic neuronal development in situ. Of note, embryonic cortical neurons exhibit (mostly) a pyramidal phenotype, although additional types of neurons can be found.

The analysis of polarity acquisition in situ involves 4 main steps: anesthesia of the animal, in utero electroporation (IUE), post-electroporation procedures, and imaging. The expression of plasmids encoding fluorescent proteins, such as GFP, allows monitoring the migration and polarization of cortical neurons during embryonic corticogenesis (as well as in post-natal stages). In this protocol, we focus on E15.5-E17.5 days, because within this time-frame neurons undergo morphological transformations that resemble the first stages of polarity acquisition in culture (Kriegstein and Noctor, 2004; Barnes and Polleux, 2009; Takano et al., 2019).

### 6. Anesthesia and sterilization of the animal

- Place the pregnant mouse inside the chamber containing the isoflurane + oxygen mix for anesthesia. The anesthesia procedure consists of two phases:
  - **Induction:** 4% isoflurane flow is needed to achieve full anesthesia in short time (visible by a reduction on the breath frequency).
  - **Maintenance:** Place the animal in the warming system at 37°C. Make sure to introduce the mouth into the anesthesia device and set isoflurane flow to 2%–2.5% for the surgery. To confirm full anesthesia, pinch the toes with a tweezer and confirm the lack of response.

- Make a subcutaneous injection of 1 mg/kg tramadol (or equivalent).

- Sterilize the animal:
  - Shave the abdomen using a razor blade or an electric razor.
  - Disinfect the zone with iodine solution and 70% v/v ethanol.

### 7. Embryo exposure
**In-utero electroporation (IUE) of E15.5 mouse brains: main steps**

1. **Anesthesia, disinfection and analgesic injection**
   - Place the pregnant mouse inside the isoflurane chamber.
   - Inject analgesics (subcutaneous).
   - Disinfect the belly zone.
   - Make an incision with a scalpel or a fine scissors following the dashed line.

2. **Embryo exposure**
   - Pull out both left and right horns of the uterus.
   - Wash with 0.5 mL PBS.
   - Draw a map of the uterus, recording embryo’s position.

3. **Injection of cDNAs and Electroporation**
   - Introduce the glass capillary into the lateral ventricle.
   - Inject approximately 1-2 µL of DNA mix solution.
   - Place the electrodes on each side of the ventricles.
   - Make sure to locate the positive (+) pole of the electrode in the DNA mix site (follow the dye spot).
   - Proceed with electroporation.

4. **Post-electroporation procedures and mouse recovery**
   - Return the uterine horns into the abdominal cavity.
   - Suture the inner and outer layer of the skin.
   - Place the animal in a quiet place for recovery.
   - Monitor mouse recovery.

5. **Tissue processing for polarity and migration analysis in situ**
   - Fix GFP-positive brains in 4% PFA for 16-18 h at 4°C.
   - Replace PFA by 30% sucrose and incubate for 18-24 h at 4°C.
   - Immerse post-fixed brains in cryopreservant solution at -20°C.
   - Slice cerebral cortex in coronal sections.
   - Proceed with fluorescent imaging.
   - Estimate polarization and cortical migration in situ.

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**Figure 3. DNA Injection and IUE of Mouse Brains (E15.5)**

In utero electroporation (IUE) of E15.5 mouse brains involves several steps, including anesthesia with isoflurane, embryo exposure, DNA injection & electroporation, recovery after surgery, histology, and imaging of GFP-positive brains. The scheme summarizes the main steps of this procedure.

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- Make and incision with fine scissors in the abdomen, along the alba line, of both skin and muscle layers. An incision of 5 mm length is enough to expose embryos (Figure 3; first step).

- Pull out the uterine horns carefully (Figure 3; second step). Expose embryos one by one and avoid twisting the uterus.

- Make sure to expose all embryos.

- Keep the uterus moist with warm PBS (37°C) during the whole surgery.

- Draw a map of the exposed uterus, reproducing embryos in the left and right horns. This will be critical to determine possible embryo resorption after surgery.

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**8. Injection & Electroporation**

- Injection of the DNA mix solution.
  - Carefully, insert the glass capillary into the lateral ventricle of each brain (Figure 3; third step).
  - Inject approx. 1–2 µL of DNA mix solution. A successful injection will be evident if a small and well-defined spot of the dye appears in the brain (Figure 3; third step).
  - Record the embryos injected in the map drawn in the step 7e.

  △ **CRITICAL:** Do not inject the same embryo more than once.

- **In Utero Electroporation (IUE) of the embryos**
  - Immerse electrodes in saline solution (PBS) to improve conductivity.
  - One at time, hold the head of each embryo with a fine tweezer to position the electrodes on each side of the ventricles.

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iii. Match the positive (+) pole of the electrode with the side of the injection (Figure 3; third step).

iv. Run the electroporation. We recommend starting with the following setting:
   \[ \Delta V = 39 \text{V} \]
   Pulse time: 50 ms
   No. of pulses: 5
   Resting time between pulses: 950 ms
   Polarity: Unipolar

v. Repeat the procedure with the remaining embryos.

9. Post-electroporation procedures
   a. Using tweezers, return embryos to the abdominal cavity. Avoid twist the horns.
   b. Clean the area with 500 \( \mu \text{L} \) PBS (pre-warmed at 37°C).
   c. Suture the inner and outer layer (muscular and skin layers, respectively) with surgical suture.
   d. Place the animal in a quiet place to allow the recovery from anesthesia.
   e. Monitor mouse recovery after surgery.

   Δ CRITICAL: The surgery time should not exceed 30 min per mouse.

f. Two days after (at E17.5), sacrifice the mouse using a CO\textsubscript{2} chamber, followed by cervical dislocation.

Optional: If needed, extend experimental IUE times above E17.5 depending on your experimental conditions.

g. Expose embryos (steps 7.a-c), compare with the map (step 7.e) and check (if any) resorptions.

h. Remove and sacrifice embryos by decapitation.

i. Isolate brains and transfer to a petri dish containing pre-chilled PBS.

j. Check GFP-positive brains using a fluorescent/stereo microscope (10\( \times \)–20\( \times \) magnification).

10. Tissue processing and imaging of polarity acquisition \textit{in situ}

a. Transfer GFP-brains to a 15 mL centrifuge tube and fix them in 4% v/v PFA overnight (ON; 16–18 h) at 4°C, with gentle agitation. For a proper fixation, the volume of PFA must be at least 10 times the volume of the brain.

   Δ CRITICAL: Over-fixation will damage the brain, affecting tissue staining (if applicable) and imaging (see an example of over-fixed tissue in Figure 4D).

b. Next day, place the fixed brains in a 1.5 mL centrifuge tube (Eppendorf or equivalent) with 30% v/v sucrose solution and incubate at 4°C for 18–24 h.

Optional:Brains can be store for longer periods of time once in sucrose solution. In any case, avoid long-term storage to avoid contamination.

c. Remove from sucrose and immerse brains in criopreservant solution (Crioplast Biopack or equivalent) at –20°C. Brains will be ready once look like a solid white block.

d. Using a cryostat (Leica CM 1850), slice cerebral cortex into 40 \( \mu \text{m} \) coronal sections.

e. Transfer brain into gelatin pre-coated glass slides.

f. Permeabilize cortical slices with 0.3% v/v Triton X-100-PBS solution for 15 min at RT.

g. Incubate DAPI for 15 min at RT (20°C–25°C). Make 3 washes of 5 min with PBS at RT.

h. Seal samples using Mowiol (or equivalent).

i. Image cortices using a confocal microscope.

Alternatives: A properly set wide-field microscope may also work.
j. Perform z-stack imaging of GFP-positive cortices at the magnification needed.
k. If needed, take several fields to reconstruct the whole cortex during post-imaging.
l. Using Fiji, go to z-project and project the maximal intensity of the fluorescence.
m. If needed, stitch images to reconstruct the whole cortex using the Fiji’s plug-in “stitching”.
n. Divide the cortex in 4 layers (from the bottom to the top): ventricular zone (VZ), subventricular zone (SVZ), intermediate zone (IZ), cortical plate (CP) (Figures 4A and 4B).
o. Use the Fiji’s plug-in “cell counter” to quantify the number of GFP-positive cells in each layer.
p. Estimate, by visual inspection and cell counting, the number of rounds, multipolar and bipolar cells in each layer (Figure 4C).

Note: Neuronal polarization in situ occurs in the IZ of the cortex (Namba et al., 2014; Xu et al., 2015). In this zone, multipolar neurons acquire a bipolar morphology, developing the leading neurite (apical dendrite) and a trailing neurite (axon). Therefore, morphological parameters are commonly used to discriminate between polarized (bipolar) and unpolarized neurons (multipolar or round cells). Nevertheless, molecular markers available to identify axonal and dendritic compartments in situ are less abundant than in vitro markers. In any case, we have summarized several markers currently used to identify each phenotype during polarization of neurons in situ (Table 3).

EXPECTED OUTCOMES

Major Step 1. Polarity Acquisition In Vitro: Primary Culture of Hippocampal Neurons

After plating, neurons will undergo polarization (Dotti et al., 1988; Kaech and Banker, 2006; Cáceres, et al., 2012; Wilson et al., 2020). Briefly, 6 h after plating most of neurons will look like symmetrical cells surrounded by an actin-rich structure (lamella), from which neurites will emerge (stage 1,
Major Step 2. Polarity Acquisition In Situ: In Utero Electroporation of E15.5 Mouse Brains

Successful IUE E17.5 embryos will express the green fluorescent protein (GFP). Accordingly, imaging GFP-positive cortical slices will show neurons undergoing polarization and cortical migration in situ. For morphometrical purposes, embryonic mouse cortex will be divided in 4 layers (from the bottom to the top): ventricular zone (VZ), subventricular zone (SVZ), intermediate zone (IZ) and cortical plate (CP), according to (Kriegstein and Noctor, 2004). At E17.5, most of GFP-positive neurons (60%–70%) will be located at the IZ; these neurons will exhibit a bipolar morphology, with a leading neurite oriented to the surface and a trailing process in the opposite direction. Of note, the trailing neurite is usually hard to image at this stage and further processing is required, including electron microscopy imaging. The remaining neurons (30%–40%) will be mostly distributed between VZ and SVZ, displaying a symmetrical and/or multipolar morphology (Wilson et al., 2020). Figures 4A–4C shows representative E17.5 and E18.5 IUE mouse GFP-cortices showing morphological patterns detected during migration.

LIMITATIONS

Polarity Acquisition In Vitro

- The yield of primary hippocampal neurons is limited (500,000 hippocampal neurons/brain, approximately). Therefore, biochemical assays needing large amounts of cells, especially in early
stages (1–3; within the first 48 h of culture), may represent a limitation. For these purposes, use cortical neurons to obtain larger amounts of cells.

- In neurons, the efficiency of transfection of plasmid DNAs, is low (below 5%). Alternatively, neurons can be electroporated in suspension before plating (the efficiency may increase up to 20%). For higher efficiencies, viral particles are recommended. However, the timing needed to express DNA using this methodology could be incompatible with the analysis of early polarity (stages 1–3; first 72 h in culture).
- Single cell analysis in long-term cultures (5 DIV or more) could represent a limitation. At this stage, the culture will look like a mesh of neurites, making it hard to distinguish axons and dendrites. To overcome this limitation, try transfection with GFP (or equivalent) to visualize neurons at the single-cell level.

**Polarity Acquisition In Situ**

- Successful IUE will depend, among other factors, on the plasmid backbone used. We recommend using the pCAGIG-GFP under U6 CAG promoter. If needed, subclone your cDNAs in this plasmid.
- GFP and HcRed/RFP are usually used to visualize neurons after electroporation. This will limit the imaging of more than 2 markers in the same slice.
- The expression of electroporated DNAs last up to 3 weeks post-IUE. This could represent a limitation in case of experiments considering post-natal stages.

**TROUBLESHOOTING**

**Problem 1**  
*In vitro* neurons: Inefficient digestion/ cell clumps. (step 3)

**Potential Solution**

- Check trypsin activity
- If needed, re-set time of enzymatic digestion. In any case, this time should not exceed 30 min.
- Enhance mechanical digestion (increasing the number of “up-and-downs”)
- Check that Pasteur pipettes be properly polished.
- Alternatively, resuspend neurons using a cell strainer.

**Problem 2**  
*In vitro* neurons: Neurons do not attach to plastic dishes or glass coverslips (or detaching). (step 4)

**Potential Solution**

- Check PLL solution, including borate buffer and pH.
- Make sure PLL is properly washed.
- Extend plating time in 10% HS MEM up to 3–4 h.
- Make sure to wash and clean glass coverslips (if applicable).
- With training, improve the timing of the dissection steps.

**Problem 3**  
*In vitro* neurons: Neurons do not polarize within the timeframe suggested. (step 5)

**Potential Solution**

The timing for polarity acquisition may vary depending on several factors, including the coating, culture media composition and cellular confluency after plating. In this protocol, we recommend PLL for coating, although other laboratories prefer laminin. We use PLL because it is an inert coating agent, enhancing neuronal attachment to the culture dish/coverslip by electrostatic forces with the plasma membrane of neurons, accordingly to the classical culture of hippocampal neurons (Banker and
Cowan, 1977; Dotti et al., 1988; Kaech and Banker, 2006). Of note, PLL coating requires plating neurons in MEM 10% HS for 1 h, which may add some noise. However, HS is washed early after plating to avoid artifacts and glial proliferation.

In contrast, laminin-cultured neurons will polarize faster than PLL-cultured neurons (Lein et al., 1992; Lochter and Schachner, 1993; Di Tella et al., 1996; Paglini et al., 1998), developing longer axons, a phenomenon most likely due to the activation of laminin/integrin-dependent pathways. Other laboratories use laminin, without HS plating, and still have very good cultures. In our experience, the coating step is a gamble between technical efficiency and neuronal development.

Additional factors, such as confluency and culture media composition, will also affect the timing suggested in this protocol. In any case, it is important to notice that differences on coating agents, confluency and culture media composition will modify the outcomes. Therefore, our suggestion is to be consistent with the protocol chosen and be aware of these concerns to avoid artifacts and misinterpretations.

**Problem 4**

*In situ IUE:* Embryo resorption after IUE. (steps 6 and 7)

**Potential Solution**

- Avoid unnecessary manipulation of embryos during the surgery.
- The optimal timing to complete the surgery is 30 min.
- Verify the recovery of the mouse after surgery and administrate/change the analgesics (especially if pain signs are visible).

**Problem 5**

*In situ IUE:* GFP-negative brains (unsuccessful IUE). (steps 8 and 9)

**Potential Solution**

- If needed, subclone cDNAs into pCAGIG-GFP (or HcRed). Alternatively, other plasmids encoding strong promoters may work upon experimental validation.
- Check quality and concentration of plasmid cDNAs. Make sure concentrations are 1.5–2 μg/μL.
- Resuspend the purified DNA in PBS or water and avoid TE (Tris-EDTA) since it seems to affect the survival of embryos.
- Check electroporation parameters and adjust if needed.
- Make sure to immerse electrodes in PBS before and in-between electroporation of each embryo.

**Problem 6**

*In situ IUE:* Failures on DNA injection. (step 8)

**Potential Solution**

- Check the pulling of the capillaries. After pulling, the size of the capillary should be:
  - Pulled tip diameter: 20–30 μm
  - Pulled tip length: 800–1,000 μm
  - Non-pulled region diameter: 60 μm

**Problem 7**

*In situ IUE:* Anesthesia and pain relief. (step 6)
Potential Solution

- The anesthesia protocol is critical for the IUE. Although some protocols recommend ketamine/xylocaine, we have had better results using isoflurane.
- If needed, check the oxygen flow and isoflurane load. It is crucial to mix properly isoflurane with oxygen for mother's survival.
- Make sure both entry and exit routes of isoflurane are clean.
- Increase up to 3% the flow of isoflurane during the maintenance phase.
- With training, improve timing to complete the surgery in no more than 30 min.

RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Alfredo Cáceres (acaceres@immf.uncor.edu).

Materials Availability
Plasmids used in this protocol are available under request to the Lead Contact, Alfredo Cáceres.

Data and Code Availability
This protocol did not generate/need datasets.

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AUTHOR CONTRIBUTIONS

C.W., V.R.-S., and A.C. designed and wrote this protocol. Representative neurons shown in Figures 2A and 2B were cultured and imaged by A.C.; neurons shown in Figure 2C and IUEs of Figure 4 were cultured and imaged by V.R.-S.; neurons shown in Figure 2D were cultured and imaged by C.W.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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