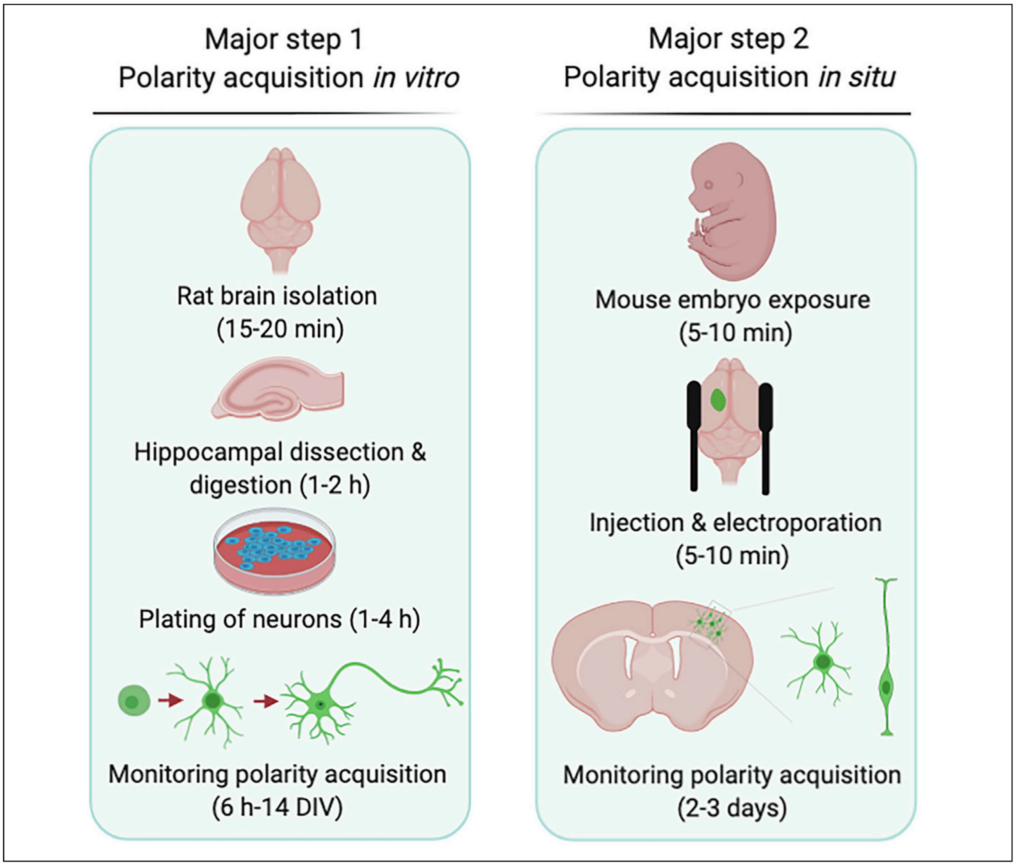
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for Evaluating Neuronal Polarity in Murine Models



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.

Carlos Wilson, Victoria Rozés-Salvador, Alfredo Cáceres

carlos.wilson@iucbc.edu.ar, cwilson@immf.uncor.edu (C.W.)
acaceres@immf.uncor.edu (A.C.)

HIGHLIGHTS

Dissection, isolation, and digestion of embryonic (E18.5) rat hippocampi

Culturing isolated hippocampal neurons and monitoring polarity acquisition *in vitro*

In utero electroporation of embryonic (E15.5) mouse brains with GFP plasmids

Visualization of migration and polarization of E17.5–E18.5 cortical neurons *in situ*

Wilson et al., STAR Protocols 1, 100114
December 18, 2020
https://doi.org/10.1016/j.xpro.2020.100114

Please cite this article in press as: Wilson et al., Protocol for Evaluating Neuronal Polarity in Murine Models, STAR Protocols (2020), https://doi.org/10.1016/j.xpro.2020.100114

Protocol

Protocol for Evaluating Neuronal Polarity in Murine Models

Carlos Wilson,[1,2,3,4,*] Victoria Rozés-Salvador,[2,3] and Alfredo Cáceres[1,2,3,5,*]

[1]Centro de Investigación en Medicina Traslacional "Severo R Amuchástegui" (CIMETSA), Instituto Universitario Ciencias Biomédicas Córdoba (IUCBC), Naciones Unidas 420, 5016 Córdoba, Argentina

[2]Instituto de Investigación Médica Mercedes y Martín Ferreyra (INIMEC-CONICET-UNC) Friuli 2434, 5016 Córdoba, Argentina

[3]Universidad Nacional de Córdoba (UNC), Av. Haya de la Torre s/n, 5000 Córdoba, Argentina

[4]Technical Contact

[5]Lead Contact

*Correspondence: carlos.wilson@iucbc.edu.ar or cwilson@immf.uncor.edu (C.W.), acaceres@immf.uncor.edu (A.C.)
https://doi.org/10.1016/j.xpro.2020.100114

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. Immerse 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 is 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 in this backbone.

11. Load the anesthetic device with isoflurane and open the flux of oxygen at 3 cm^3/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	AB5622; 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 (50×) 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	S5761
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*)

Continued

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-1205S
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/B071KXJDC5#detail_bullets_id)	7105
Glass coverslips (12;25 mm diameter)*,**	Marienfeld Superior	0111520; 0111650

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

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

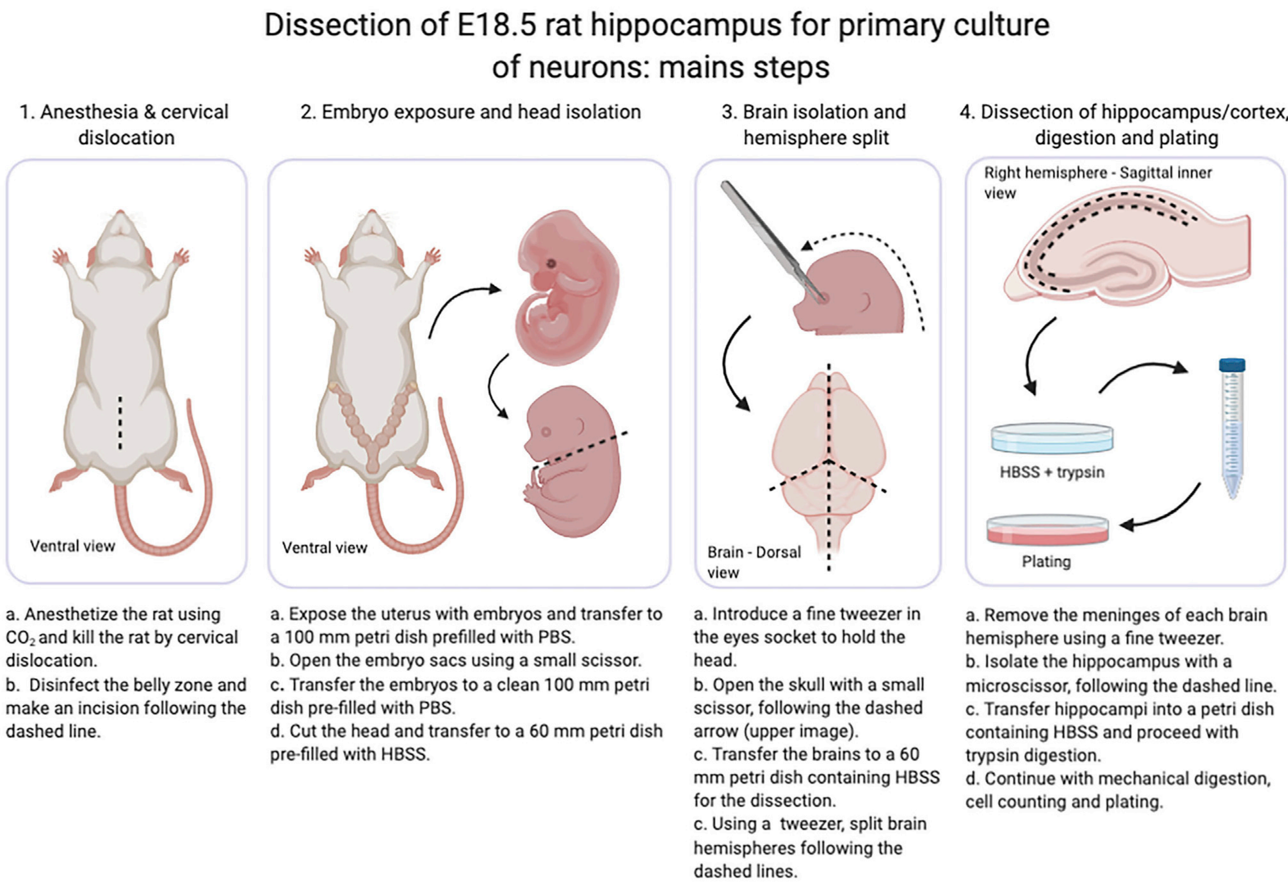


Figure 1. Dissection of Hippocampus of Embryonic (E18.5) Rat Neurons

The scheme summarizes main steps involved on culturing hippocampal (or cortical) primary neurons, including embryo isolation, hippocampal and cortical dissection, enzymatic/mechanical digestion and plating of neurons. Immediately after, neurons will undergo polarization, evidenced by neurite growth and axo-dendritic specification.

a. Place E18.5 pregnant rat inside the CO_2 euthanasia chamber for rodents. Open the CO_2 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 CO_2 may affect the dissection, reducing the yield and viability of neurons. Therefore, be cautious with CO_2 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 $NaHCO_3$.

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 × 10^6 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/cm^2
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; Kaech 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 sub-divided (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 (ß3-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 (ß3-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 (ß3-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).

Please cite this article in press as: Wilson et al., Protocol for Evaluating Neuronal Polarity in Murine Models, STAR Protocols (2020), https://doi.org/10.1016/j.xpro.2020.100114

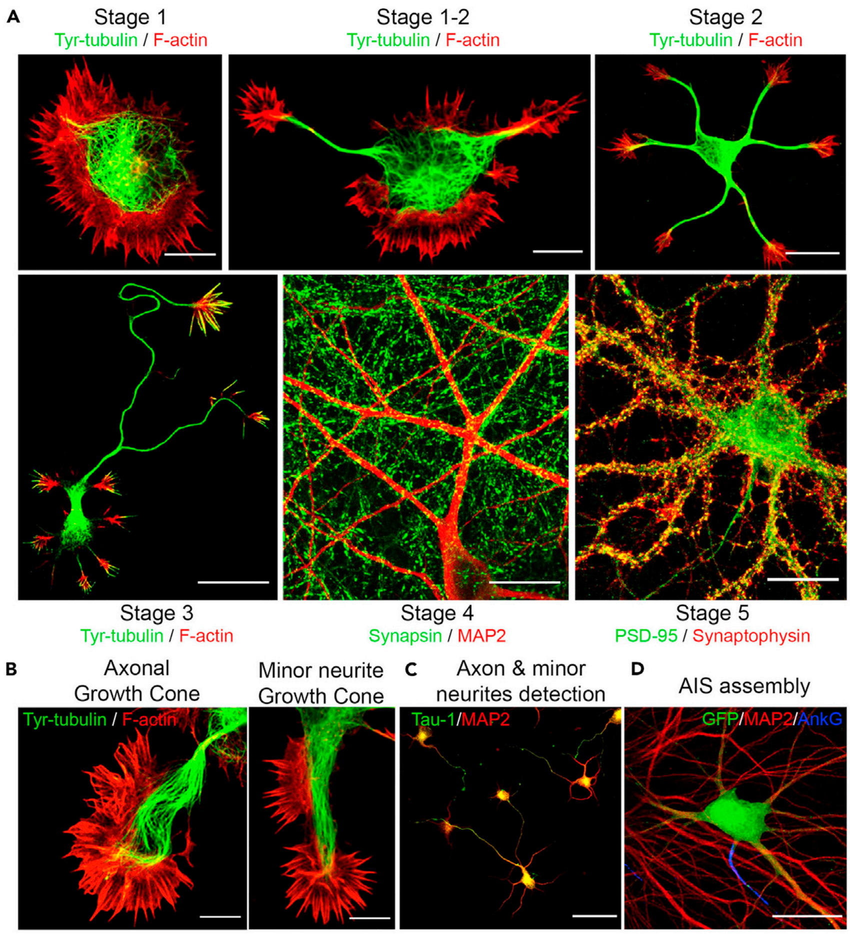


Figure 2. Morphological Stages and Molecular Markers Visualized during the Establishment of Hippocampal Neurons in Culture

(A) Representative images showing cultured hippocampal neurons in stage 1 (6 h after plating), stage 2 (18 h after plating), stage 3 (2–3 DIV after plating), stage 4 (4–5 DIV) and stage 5 (14 DIV). Stage 1 and stage 1–2 scale bar, 10 μm; stages 2-5 scale bar, 20 μm.

(B) Representative images showing the GC of axon-like process and minor neurites in stage 2 neurons. Scale bar, 10 μm.

(C) Representative image showing stage 3 neurons stained by immunofluorescence (IF) with MAP2 and Tau-1 antibodies. The Tau-1 epitope is enriched in the distal part of the axon, whilst MAP2 is mostly segregated in the soma and minor neurites. Scale bar, 50 μm.

(D) Representative 10 DIV neuron (stage 5), stained by IF to detect the dendritic marker MAP2, and the clustering of Ankyrin G (AnkG) in the axon initial segment (AIS). Neurons were transfected with a GFP-encoding plasmid to be able to visualize single neurons at this stage. Scale bar, 20 μm.

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 Püschel, 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., 2008)
Stage 3	Acetylated α-tubuin	Axon	(Ferreira and Cáceres, 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	TfR	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	AnkG	Axon initial segment	(Hedstrom et al., 2008; Galiano 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; Tfr: transferrin receptor; GOPS: Golgi outposts; AnkG: 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.

Table 2. Cytoskeleton Dynamics, Vesicle Trafficking and Organelle Distribution Parameters Sustaining Axo-Dendritic Specification

Polarity stage	Cellular Parameter	Observation	Reference
Stage 1	Centrosome localization	Centrosome in the base of the prospective axon	(De Anda et al., 2005)
Stage 2–3	Growth cone (GC) area	Axonal GC size > minor neurites GC size	(Bradke and Dotti, 1999; Kunda et al., 2001)
Stage 2–3	F-actin dynamics in growth cones (GC)	Axonal GC actin dynamics > MN GC actin dynamics	(Bradke and Dotti, 1999)
Stage 2–3	Vectorial cytoplasmic flow	Bulk flow to the nascent axon	(Bradke and Dotti, 1997)
Stage 2–3	MT stability	Microtubules stabilization defines early neuronal polarization	(Ferreira and Cáceres, 1989; Witte et al., 2008)
Stage 3	Microtubule polarity	Polarized in axons (+ end oriented to GC); mixed polarity in dendrites	(Baas et al., 1988)
Stage 3	Ribosome localization and RNA transport	Enriched in dendrites	(Bartlett and Banker, 1984; Davis et al., 1987)

The list contains critical cell biology parameters defining axonal formation and dendritic growth at early stages of neuronal polarization (stage 1 – stage 3).

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

Timing: 2–3 days

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
   a. Place the pregnant mouse inside the chamber containing the isoflurane + oxygen mix for anesthesia. The anesthesia procedure consists of two phases:
      i. Induction: 4% isoflurane flow is needed to achieve full anesthesia in short time (visible by a reduction on the breath frequency).
      ii. 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.
   b. Make a subcutaneous injection of 1 mg/kg tramadol (or equivalent).
   c. Sterilize the animal:
      i. Shave the abdomen using a razor blade or an electric razor.
      ii. Disinfect the zone with iodine solution and 70% v/v ethanol.
7. Embryo exposure

Please cite this article in press as: Wilson et al., Protocol for Evaluating Neuronal Polarity in Murine Models, STAR Protocols (2020), https://doi.org/10.1016/j.xpro.2020.100114

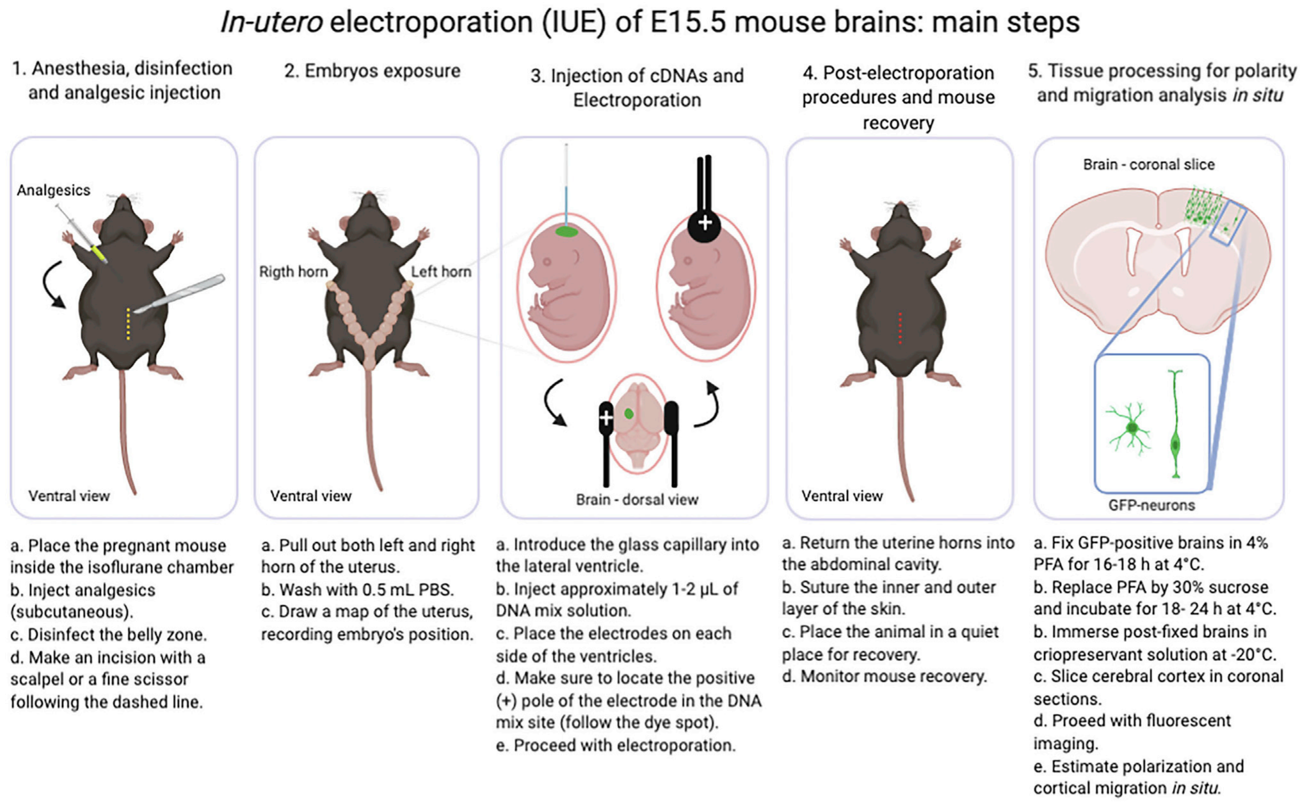


Figure 3. DNA Injection and IUE of Mouse Brains (E15.5)

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.

a. 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).

b. Pull out the uterine horns carefully (Figure 3; second step). Expose embryos one by one and avoid twisting the uterus.
c. Make sure to expose all embryos.
d. Keep the uterus moist with warm PBS (37°C) during the whole surgery.
e. 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.

8. Injection & Electroporation
   a. Injection of the DNA mix solution.
      i. Carefully, insert the glass capillary into the lateral ventricle of each brain (Figure 3; third step).
      ii. 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).
      iii. Record the embryos injected in the map drawn in the step 7e.

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

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

Please cite this article in press as: Wilson et al., Protocol for Evaluating Neuronal Polarity in Murine Models, STAR Protocols (2020), https://doi.org/10.1016/j.xpro.2020.100114

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:
ΔV = 39 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 μL 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.

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

   f. Two days after (at E17.5), sacrifice the mouse using a CO_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×–20× magnification).

10. Tissue processing and imaging of polarity acquisition *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.

⚠ 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 μm 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.

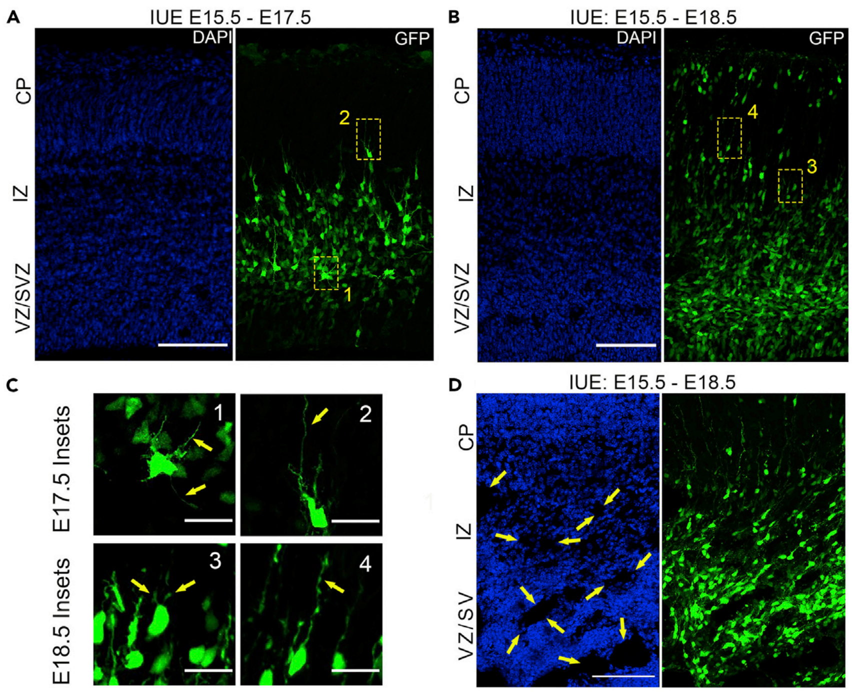


Figure 4. Migration and Cortical Neurons and Polarization *In Situ* by IUE Mouse Brains

(A and B) Representative images showing E17.5 and E18.5 brain cortices *in utero* electroporated (IUE) at E15.5. Scale bar, 200 μm.

(C) Magnification of insets 1–4, showing bipolar and multipolar neurons at E17.5 and E18.5. Scale bar, 20 μm.

(D) Representative image showing an over-fixed cortical slice. The presence of "holes" in the tissue (arrows) reveals either over-fixation or inefficient post-fixation procedures. Scale bar, 200 μm.

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,

Table 3. Molecular Markers Used to Identify Multipolar and Bipolar Polarizing Neurons *In Situ*

Morphology	Marker	Cortical layer	Reference
Round	Tbr2; Sox2; Nestin	VZ/SVZ	(Englund et al., 2005; Barnes et al., 2007; Fuentes et al., 2012)
Multipolar	TAG-1	Lower IZ	(Barnes et al., 2007; Namba et al., 2014; Xu et al., 2015)
Multipolar-Bipolar transition	TAG-1	Efferent axons at lower IZ	(Barnes et al., 2007; Namba et al., 2014; Xu et al., 2015)
Bipolar	N-cadherin; Trb1; LKB1	Upper IZ	(Englund et al., 2005; Barnes et al., 2007; Xu et al., 2015)
	SMI-312	Afferent and efferent axons at lower and upper IZ	(Namba et al., 2014)
	MAP2	Upper IZ/CP	(Barnes et al., 2007)

Molecular markers currently used to identify the main cellular morphologies detected in cortical layers of the mouse brain cortex after IUE procedures. LKB1: serine/threonine kinase; TAG-1: transient axonal glycoprotein 1; Trb1: telomerase repeat binding factor 1; Tbr2: T-box brain protein 2; MAP2: microtubule associated protein 2; SMI-312: neurofilament marker.

Figure 2). Later, at 18 h in vitro, neurons will develop a symmetric array of neurites, without significant differences in length among them (Figure 2A, stage 2). Then, after 36 h in culture, neurons will exhibit an axon-like process, 2-times longer than minor neurites. Of note, the size of GC of the nascent axon is significantly larger than the GC of minor neurites (Figure 2B). After 2 days in vitro (DIV), neurons will display an axonal process of 100 μm length (usually 3-times longer than minor neurites; Figure 2A, stage 3). At this stage, MAP2 is enriched in the somato-dendritic region, whilst a distal gradient of the Tau-1 signal is observed in the nascent axon (Mandell and Banker, 1996) (Figure 2C). Stages 1–3 represent the early stages of polarity, where axonal specification occurs. Of note, the actin-rich lamella detected in stage 1 (using phalloidin-Alexa Fluor 546) will progressively disappear, leading the growth of neurites (Figure 2A, stage 1–2 neuron). After stage 3, growth cones (Figure 2A, stage 2 and 3, in red) will be reduced to minimal structures. Then (4–5 DIV), minor neurites will grow into dendrites, developing secondary and tertiary processes (dendritic branching) (stage 4, Figure 2). Finally, neurons will reach stage 5 (7–10 DIV and beyond), visualized by dendritic spine development and the assembly of the axon initial segment (AIS) (Figures 2A and 2D). Of note, AnkG is expressed early in cultured neurons (3–4 DIV), although its localization is confined to the AIS later during axonal maturation (above 7 DIV in middle-low culture densities (Galiano et al., 2012). At this stage, neurons have accomplished morphological and biochemical requirements for neurotransmission.

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

Please cite this article in press as: Wilson et al., Protocol for Evaluating Neuronal Polarity in Murine Models, STAR Protocols (2020), https://doi.org/10.1016/j.xpro.2020.100114

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 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/xylazine, 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.

ACKNOWLEDGMENTS

This work was funded by the Consejo Nacional de Investigación Científica y Tecnológica (CONICET) and Agencia Nacional de Promoción de la Investigación, el Desarrollo Tecnológico y la Innovación (grants PICT 2015-1436 and PUE to A.C.; grant PICT-2018-01684 to C.W). C.W. and V.R.-S. are postdoctoral fellows of CONICET; A.C. is staff scientist of CONICET. Authors acknowledge Centro de Microscopía y Nanoscopía de Córdoba (CEMINCO) for technical assistance. Graphical abstract, Figures 1 and 3 were created using the BioRender App.

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.

REFERENCES

Arregui, C., Busciglio, J., Caceres, A., and Barra, H.S. (1991). Tyrosinated and detyrosinated microtubules in axonal processes of cerebellar macroneurons grown in culture. J. Neurosci. Res. *28*, 171–181.

Baas, P.W., Deitch, J.S., Black, M.M., and Banker, G.A. (1988). Polarity orientation of microtubules in hippocampal neurons: Uniformity in the axon and nonuniformity in the dendrite. Proc. Natl. Acad. Sci. U S A *85*, 8335–8339.

Banker, G.A., and Cowan, W.M. (1977). Rat hippocampal neurons in dispersed cell culture. Brain Res. *126*, 397–442.

Barnes, A.P., Lilley, B.N., Pan, Y.A., Plummer, L.J., Powell, A.W., Raines, A.N., Sanes, J.R., and Polleux, F. (2007). LKB1 and SAD kinases define a pathway required for the polarization of cortical neurons. Cell *129*, 549–563.

Barnes, A.P., and Polleux, F. (2009). Establishment of axon-dendrite polarity in developing neurons. Annu. Rev. Neurosci. *32*, 347–381.

Bartlett, W.P., and Banker, G.A. (1984). An electron microscopic study of the development of axons and dendrites by hippocampal neurons in culture. I. Cells which develop without intercellular contacts. J. Neurosci. *4*, 1944–1953.

Bisbal, M., Conde, C., Donoso, M., Bollati, F., Sesma, J., Quiroga, S., Díaz Añel, A., Malhotra, V., Marzolo, M.P., and Cáceres, A. (2008). Protein kinase D regulates trafficking of dendritic membrane proteins in developing neurons. J. Neurosci. *28*, 9297–9308.

Bradke, F., and Dotti, C.C. (1999). The role of local actin instability in axon formation. Science *283*, 1931–1934.

Bradke, F., and Dotti, C.G. (1997). Neuronal polarity: Vectorial cytoplasmic flow precedes axon formation. Neuron *19*, 1175–1186.

Burack, M.A., Silverman, M.A., and Banker, G. (2000). The role of selective transport in neuronal protein sorting. Neuron *26*, 465–472.

Caceres, A., Binder, L.I., Payne, M.R., Bender, P., Rebhun, L., and Steward, O. (1984a). Differential subcellular localization of tubulin and the microtubule-associated protein MAP2 in brain tissue as revealed by immunocytochemistry with monoclonal hybridoma antibodies. J. Neurosci. *4*, 394–410.

Caceres, A., Banker, G., Steward, O., Binder, L., Payne, M., et al. (1984b). MAP2 is localized to the dendrites of hippocampal neurons which develop in culture. Dev. Brain Res. *13*, 314–318.

Caceres, A., Banker, G.A., and Binder, L. (1986). Immunocytochemical localization of tubulin and microtubule-associated protein 2 during the development of hippocampal neurons in culture. J. Neurosci. *6*, 714–722.

Cáceres, A., Ye, B., and Dotti, C.G. (2012). Neuronal polarity: Demarcation, growth and commitment. Curr. Opin. Cell Biol. *24*, 547–553.

Chuang, J.Z., Yeh, T.Y., Bollati, F., Conde, C., Canavosio, F., Caceres, A., and Sung, C.H. (2005). The dynein light chain tctex-1 has a dynein-independent role in actin remodeling during neurite outgrowth. Dev. Cell *9*, 75–86.

De Anda, F.C., Pollarolo, G., Da Silva, J.S., Camoletto, P.G., Feiguin, F., and Dotti, C.G. (2005). Centrosome localization determines neuronal polarity. Nature *436*, 704–708.

Di Tella, M.C., Feiguin, F., Carri, N., Kosik, K.S., and Cáceres, A. (1996). MAP-1B/TAU functional redundancy during laminin-enhanced axonal growth. J. Cell Sci. *109* (Pt 2), 467–477.

Dotti, C.G., Sullivan, C.A., and Banker, G.A. (1988). Establishment of polarity by hippocampal neurons in culture. J. Neurosci. *8*, 1454–1468.

Englund, C., Fink, A., Lau, C., Pham, D., Daza, R.A., Bulfone, A., Kowalczyk, T., and Hevner, R.F. (2005). Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. J. Neurosci. *25*, 247–251.

Ferreira, A., and Cáceres, A. (1989). The expression of acetylated microtubules during axonal and dendritic growth in cerebellar macroneurons which develop in vitro. Dev. Brain Res. *49*, 205–213.

Fletcher, T.L., Cameron, P., De Camilli, P., and Banker, G. (1991). The distribution of synapsin I and synaptophysin in hippocampal neurons developing in culture. J. Neurosci. *11*, 1617–1626.

Fuentes, P., Cánovas, J., Berndt, F.A., Noctor, S.C., and Kukuljan, M. (2012). CoREST/LSD1 control the development of pyramidal cortical neurons. Cereb. Cortex *22*, 1431–1441.

Galiano, M.R., Jha, S., Ho, T.S., Zhang, C., Ogawa, Y., Chang, K.J., Stankewich, M.C., Mohler, P.J., and Rasband, M.N. (2012). A distal axonal cytoskeleton forms an intra-axonal boundary that controls axon initial segment assembly. Cell *149*, 1125–1139.

Gonzalez-Billault, C., Avila, J., and Cáceres, A. (2001). Evidence for the role of MAP1B in axon formation. Mol. Biol. Cell *12*, 2087–2098.

Goslin, K., Schreyer, D.J., Skene, J.H., and Banker, G. (1988). Development of neuronal polarity: GAP-43 distinguishes axonal from dendritic growth cones. Nature *336*, 672–674.

Goslin, K., Schreyer, D.J., Skene, J.H., and Banker, G. (1990). Changes in the distribution of GAP-43 during the development of neuronal polarity. J. Neurosci. *10*, 588–602.

Halpain, S., Hipolito, A., and Saffer, L. (1998). Regulation of F-actin stability in dendritic spines by glutamate receptors and calcineurin. J. Neurosci. *18*, 9835–9844.

Hedstrom, K.L., Ogawa, Y., and Rasband, M.N. (2008). AnkyrinG is required for maintenance of the axon initial segment and neuronal polarity. J. Cell Biol. *183*, 635–640.

Horton, A.C., Rácz, B., Monson, E.E., Lin, A.L., Weinberg, R.J., and Ehlers, M.D. (2005). Polarized secretory trafficking directs cargo for asymmetric dendrite growth and morphogenesis. Neuron *48*, 757–771.

Kaech, S., and Banker, G. (2006). Culturing hippocampal neurons. Nat. Protoc. *1* (5), 2406–2415.

Kennedy, M.B. (1997). The postsynaptic density at glutamatergic synapses. Trends Neurosci. *20*, 264–268.

Kriegstein, A.R., and Noctor, S.C. (2004). Patterns of neuronal migration in the embryonic cortex. Trends Neurosci. *27*, 392–399.

Kunda, P., Paglini, G., Quiroga, S., Kosik, K., and Caceres, A. (2001). Evidence for the involvement of Tiam1 in axon formation. J. Neurosci. *21*, 2361–2372.

Davis, Lauren, Banker, G.A., and Steward, O. (1987). Selective dendritic transport of RNA in hippocampal neurons in culture. Nature *330*, 477–479.

Lein, P.J., Banker, G.A., and Higgins, D. (1992). Laminin selectively enhances axonal growth and accelerates the development of polarity by hippocampal neurons in culture. Dev. Brain Res. *69*, 191–197.

Lochter, A., and Schachner, M. (1993). Tenascin and extracellular matrix glycoproteins: from promotion to polarization of neurite growth in vitro. J. Neurosci. *13*, 3986–4000.

Mandell, J.W., and Banker, G.A. (1996). A spatial gradient of tau protein phosphorylation in nascent axons. J. Neurosci. *16*, 5727–5740.

Masliah, E., Mallory, M., Hansen, L., Alford, M., DeTeresa, R., and Terry, R. (1993). An antibody against phosphorylated neurofilaments identifies a subset of damaged association axons in Alzheimer's disease. Am. J. Pathol. *142*, 871–882.

Namba, T., Kibe, Y., Funahashi, Y., Nakamuta, S., Takano, T., Ueno, T., Shimada, A., Kozawa, S., Okamoto, M., Shimoda, Y., Oda, K., Wada, Y., et al. (2014). Pioneering axons regulate neuronal polarization in the developing cerebral cortex. Neuron *81*, 814–829.

Paglini, G., Pigino, G., Kunda, P., Morfini, G., Maccioni, R., Quiroga, S., Ferreira, A., and Cáceres, A. (1998). Evidence for the participation of the neuron-specific CDK5 activator P35 during laminin-enhanced axonal growth. J. Neurosci. *18*, 9858–9869.

Quassollo, G., Wojnacki, J., Salas, D.A., Gastaldi, L., Marzolo, M.P., Conde, C., Bisbal, M., Couve, A., and Cáceres, A. (2015). A RhoA signaling pathway regulates dendritic Golgi outpost formation. Curr. Biol. *25*, 971–982.

Schwamborn, J.C., and Püschel, A.W. (2004). The sequential activity of the GTPases Rap1B and Cdc42 determines neuronal polarity. Nat. Neurosci. *7*, 923–929.

Shi, S.H., Jan, L.Y., and Jan, Y.N. (2003). Hippocampal neuronal polarity specified by spatially localized mPar3/mPar6 and PI 3-kinase activity. Cell *112*, 63–75.

Szebenyi, G., Bollati, F., Bisbal, M., Sheridan, S., Faas, L., Wray, R., Haferkamp, S., Nguyen, S., Caceres, A., and Brady, S.T. (2005). Activity-driven dendritic remodeling requires microtubule-associated protein 1A. Curr. Biol. *15*, 1820–1826.

Takano, T., Funahashi, F., and Kaibuchi, K. (2019). Neuronal Polarity: Positive and Negative Feedback Signals. Front Cell Dev. Biol. *7*, https://doi.org/10.3389/fcell.2019.00069.

Wilson, C., Giono, L.E., Rozés-Salvador, V., Fiszbein, A., Kornblihtt, A.R., and Cáceres, A. (2020). The histone methyltransferase G9a controls axon growth by targeting the RhoA signaling pathway. Cell Rep. *31*, 107639.

Witte, H., Neukirchen, D., and Bradke, F. (2008). Microtubule stabilization specifies initial neuronal polarization. J. Cell Biol. *180*, 619–632.

Xu, C., Funahashi, Y., Watanabe, T., Takano, T., Nakamuta, S., Namba, T., and Kaibuchi, K. (2015). Radial glial cell–neuron interaction directs axon formation at the opposite side of the neuron from the contact site. J. Neurosci. *35*, 14517–14532.

Zuccaro, E., Bergami, M., Vignoli, B., Bony, G., Pierchala, B.A., Santi, S., Cancedda, L., and Canossa, M. (2014). Polarized Expression of p75NTR specifies axons during development and adult neurogenesis. Cell Rep. *7*, 138–152.