Chapter 1

Investigating Early Formation of the Cerebral Cortex by In Utero Electroporation: Methods and Protocols

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Abstract

Cortical development requires a strict balance between neuronal proliferation, differentiation, and cellular migration within restricted developmental stages. The precise spatiotemporal gene manipulation used in developmental studies can be achieved by in vitro or ex vivo experiments or by the generation of transgenic animals. However, these approaches have significant limitations when trying to investigate the origin and molecular regulation of early cortical neurons. In utero electroporation (IUE) is an informative cell labeling technique that provides the ability to label neural progenitor cells and their progeny in vivo with promoter-specific reporter constructs as well as to induce or repress gene expression in a spatially and temporally specific manner. Technical improvements of this method have allowed the targeting of multiple neural cell types, as well as the precise transfection of subpopulations of neurons at increasingly earlier embryonic stages. Furthermore, neuronal projection studies and the use of multiple electroporations in the same embryo have made it possible to examine processes occurring at different developmental stages and/or cortical areas and link their anatomy to their function. In this chapter, we present the latest advances of the in utero electroporation technique for the study of early formation of the cerebral cortex, together with a description of the necessary tools.

Key words In utero, Electroporation, Brain, Cortical development, Transfection, Embryo, Mice

1 Introduction

During brain development, the neocortex arises from a homogeneous sheet of neuroepithelial cells in the telencephalon. Morphogen molecules such as Fgf8, Wnt, Bmps, and Shh secreted by patterning centers within the telencephalon induce the expression of transcription factors in gradients across the cortex, which serve to pattern the neuroepithelium into functional cortical areas [1–4]. Concomitantly, progenitor cells within the neuroepithelium undergo proliferation, differentiation, and migration to generate the six neuronal layers that characterize the mammalian neocortex. Furthermore, each cortical area is distinguished by its functional specificity for the processing of sensory input, motor activity, or cognitive function, as well as its distribution of input and output

David W. Walker (ed.), *Prenatal and Postnatal Determinants of Development*, Neuromethods, vol. 109,
DOI 10.1007/978-1-4939-3014-2_1,

projections which are guided toward their correct targets by specific guidance cues [5]. Although the influence of different morphogens in cortical patterning and subsequent neuronal wiring is not completely understood, sophisticated electroporation techniques have identified some of the mechanisms involved (for example see [6, 7]).

Electroporation is a method of transfection of plasmid DNA into cells that allows for exogenous gene expression via the use of cell-specific promoters, and gene loss-of-function studies via the transfection of small interfering RNAs (siRNAs). Since electroporation was first used in utero [8, 9], this technique has been an important tool for the study of neuronal origin and migration during early mammalian cortical development. By modifying the expression of transcription factors within the cortex in vivo, primarily using mouse and rat models, our knowledge of the migration, differentiation, and growth of multiple subpopulations of neurons originating from the ventricular and sub-ventricular zone zones has expanded [10–13]. Early-born interneurons that populate the cortex originate in the ganglionic eminences (reviewed in [14]), and can be labeled by IUE and then studied separately by their electrophysiological properties [15]. IUE has also been instrumental in the discovery of early-born cortical neurons from the edges of the neocortex which give rise to Cajal-Retzius cells [16, 17], and of new migratory streams of neurons such as those that contribute to the accessory olfactory bulb [18] and the amygdala [19].

The electroporation technique can be modified for the transfection of multiple expression constructs either together or with temporal and/or spatial variation for individual plasmids. The use of IUE at early stages allows the possibility of a second electroporation later in gestation, for example, to differentially label cortical layers generated at different developmental stages (Fig. 1). Alternatively, multiple plasmids can be co-electroporated simultaneously at a single age, and has been recently used to study activity-dependent axon targeting in the primary somatosensory cortex (S1) [20].

In this chapter we describe the methodology required to perform embryonic electroporation in mice at E10 for early cortical development studies. We also describe how to perform an additional electroporation at later developmental stages in order to differentially transfect other cortical layers.

2 Materials

For all equipment and reagents listed, the manufacturers and catalogue numbers are given as a guide only and can be substituted.

2.1 Mice

The IUE procedure in this chapter is described for use in CD1 mice given their relatively large litter size and their ability to cope with the anesthetic and surgical procedure. Other mouse strains such as

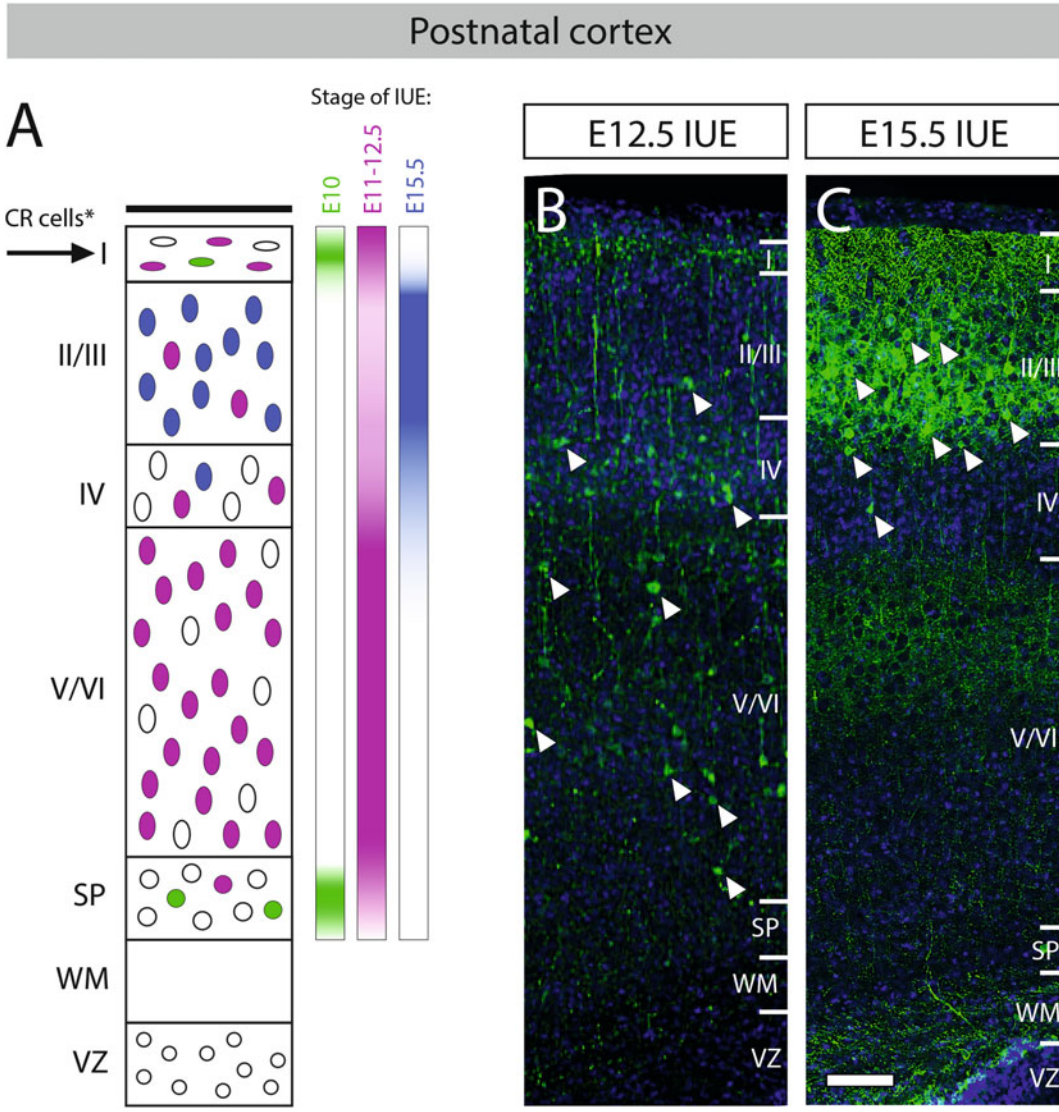

Fig. 1 Cortical layer targeting using in utero electroporation. (**a**) In this schematic of postnatal cortex, cells are represented with different colors according to the developmental stage at which they are electroporated as indicated on the *right*. The cortex develops in an inside-outside manner and electroporation at an early stage of E10 will target cells that eventually occupy the preplate and its derivatives (*green* cells), as indicated previously in cell birth-dating studies [16]. Electroporation at a later stage of E11–12.5 will target cells throughout the cortical plate (greater in layers V/VI) and the subplate, as indicated by the *purple* cells and the *arrowheads* in (**b**). E15.5 electroporation will predominantly target neurons within the superficial cortical layers (II/III), but some electroporated neurons are also present in the underlying layer IV, as indicated by the *blue* cells and the *arrowheads* in (**c**). The colored gradients on the *right* indicate the approximate profile of cell locations in postnatal cortex according to the stage of electroporation. Note that this schematic diagram is representative of IUE in the dorsal telencephalon but includes Cajal-Retzius (CR) cells in layer I (indicated by *). CR cells are generated in the cortical hem and can be targeted with an alternative IUE at E10–12.5 prior to their tangential migration away from this region (*see* Fig. 5h). (**b**) Coronal section of postnatal cortex after electroporation of pCAG-EGFP in E12.5 dorsal telencephalon shows GFP-positive neurons (*arrowheads*) throughout the cortex. (**c**) Electroporation of E15.5 dorsal telencephalon shows GFP-positive neurons (*arrowheads*) predominantly within cortical layers II/III. CR: Cajal-Retzius; I–VI: cortical layers; IUE: in utero electroporation; SP: subplate; VZ: ventricular zone; WM: white matter. Scale bar in (**b**) and (**c**), 100 μm

C57BL/6 can also be used, but may exhibit a lower success rate. The day of appearance of the vaginal plug is embryonic day 0 (E0).

2.2 Anesthesia and Analgesic Solutions

Anesthesia of the pregnant dams is achieved via intraperitoneal injection of a mixture of 0.075 mg/g body weight ketamine, and 0.006 mg/g body weight xylazine, diluted in sterile PBS. Analgesia is achieved via subcutaneous injection of buprenorphine at 0.1 mg/g body weight.

2.3 Plasmid DNA

For effective gene expression it is important that the plasmids transfected via IUE are purified and endotoxin free, which can be achieved using a commercially available preparation kit, and are homogeneous once resuspended in water. If the plasmid solution contains precipitates and is not fully suspended, which can occur in higher concentration preparations, it can be further dissolved by heating the plasmid solution to 45 °C for 30 min.

Purified plasmid can be stored for a long term at −20 °C, and upon thawing can be stored at 4 °C for up to a month. In this protocol, plasmids were prepared using an endotoxin-freeMaxi-prep kit (cat. 12362, Qiagen, Valencia, CA).

2.4 Electroporation and Microinjection Equipment

- ECM 830 Electroporator (BTX Harvard Apparatus, Holliston, MA) with electroporator foot pedal.
- Electrodes, forceps-style, 0.5 mm diameter (cat. CUY650P0.5, NepaGene, Chiba, Japan).
- Borosilicate glass capillaries, 1.2 mm O.D. × 0.9 mm I.D. (cat. TW120F-4, World Precision Instruments, Sarasota, FL).
- Picospritzer II microinjection system (Parker Hannifin, Hollis, NH) with microinjector foot pedal.
- Microcapillary holder (cat. MPH6S12, World Precision Instruments).
- Microloading pipette tips (cat. 0030 001.222, Eppendorf, Hamburg, Germany).
- Micropipette puller (cat. P-97, Sutter Instruments Co., Novato, CA) with square box filament.

2.5 General Equipment and Surgical Instruments

- Light source (cat. LG-PS2, Olympus, Shinjuku, Japan), with a fiber-optic arm for the illumination of E10 embryos through the uterine wall. The fiber-optic arm should be flexible to allow for easy manipulation and positioning against the uterus. Fiber-optic light is ideal since it emits minimal heat and will not damage the uterus or embryos.
- A secondary fiber-optic light (cat. LG-PS2, Olympus) source can also be positioned over the animal to help visualize the uterus and the surgery area.
- Silicone rubber heating pad, set to 37 °C.

- Water bath, set to 37 °C (cat. WB7, Ratek, Melbourne, Australia).
- Germinator 500 glass bead sterilizer, for sterilization of surgical instruments (cat. GRM5-1450, CellPoint Scientific, Gaithersburg, MD).
- Animal recovery chamber (ventilated), set at 37 °C.
- 2× Ring forceps, 6 mm diameter ring tip, 9 cm length (cat. 11106-09, Fine Science Tools, Vancouver, Canada).
- Micro-dissecting forceps with serrated edge, half-curved 1 mm tip, 4 in. length (cat. 160-18, George Tiemann & Co., Hauppauge, NY).
- Suture clamps, Gillies, 6.5 in. length (cat. 222-8-59, George Tiemann & Co.).
- Straight surgical scissors, 12 cm length (cat. 14002-12, Fine Science Tools).
- Syringe 1 ml (cat. SY1SC-TH-ET) and 27 G hypodermic needle (cat. HN-2713-ET) (Nipro, Osaka, Japan).
- Gauze swabs, sterile, 7.5 cm × 7.5 cm, 8 ply (cat. 72502-03, BSN Medical, Hamburg, Germany).
- Cotton tips, 15 cm (cat. 10102121, Surgical & Medical Products, Sydney, Australia).
- Silk sutures (Perma-Hand3-0, Ethicon).
- Pasteur pipettes, polyethylene, sterile, 3 ml (ThermoFisher Scientific, Loughborough, UK).
- Personal protective equipment/surgical attire: gown, face mask, hair net, gloves.
- Adhesive paper tape, 25 mm wide.

2.6 Reagents

- Ketamine-1000 (100 g/ml, Ceva, Lenexa, KS).
- Xylazil-20 (Xylazine, 20 mg/ml, Troy Laboratories, Glendenning, Australia).
- Chlorhexidine (0.5 mg/ml, 0.05 %) and cetrimide (5 mg/ml, 0.5 %) topical antiseptic solution (cat. 16061505, Pfizer, New York, NY).
- Ringer's solution (NaCl 135 mM, KCl 5.4 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1.8 mM, HEPES 5 mM) warmed to 37 °C.
- 1× phosphate-buffered saline (PBS), diluted from a 10× stock of Dulbecco's PBS (cat. 17-515Q, Lonza, Basel, Switzerland) in Milli-Q water, warmed to 37 °C.
- Hair removal cream (Nair, Church & Dwight Co., Sydney, Australia).

- Petroleum Jelly (Vaseline, Unilever, Leatherhead, UK).
- Fast Green (cat. F7258, Sigma, St. Louis, MO).
- Ethanol 70 %.

3 Methods

3.1 Preparation Before Surgery

3.1.1 Animals

Timed-pregnant females are used and the appearance of a vaginal plug is considered E0. For early-stage IUE, the embryos used are E10–10.5.

3.1.2 Surgery Area Preparation

Ensure that a sterile surgical environment is achieved by wearing surgical attire (gown, gloves, face mask, hair net) and wiping down surfaces, the fiber-optic light source, and all equipment with 70 % ethanol. Arrange the surgery area and equipment as shown in Fig. 2a. Turn on the heating pad, animal recovery chamber, glass bead sterilizer, and water bath, and place paper towel on the heat pad to soak up any excess PBS during the surgery. Place Ringer's and PBS solution into the water bath and warm to 37 °C.

Immerse the surgical instruments (forceps, scissors, and the suture clamp) in 70 % ethanol, allow to air-dry, and then further sterilize them by placing each of them into a glass bead sterilizer for several minutes.

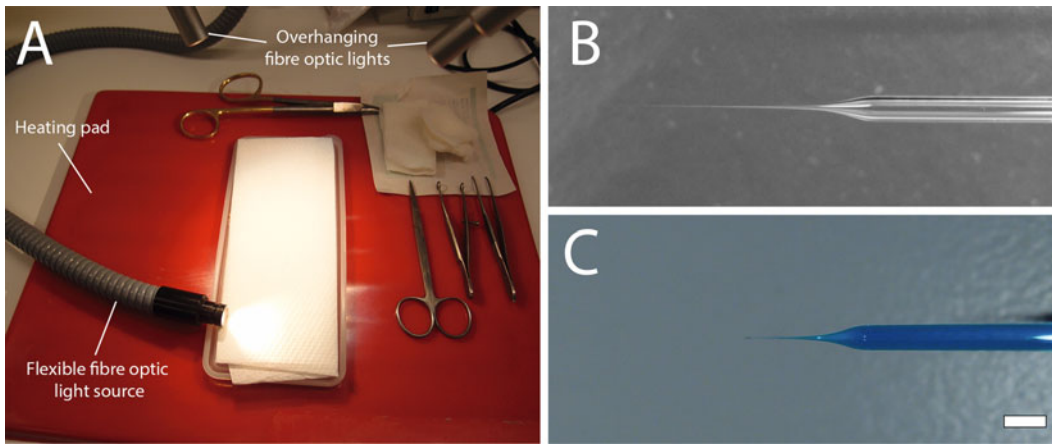

Fig. 2 Setup of surgery area and microcapillaries. (**a**) The surgery area is set up with the heating pad set at 37 °C, the paper towel in the center of the heating pad, the sterilized surgical tools (ring forceps, micro-dissecting forceps, scissors, suture clamps), and the flexible fiber-optic arm and overhanging light sources in position. (**b**) A fine, closed tip with a long taper is formed after the glass microcapillary is pulled. (**c**) The microcapillary tip must be cut at an oblique angle to produce a sharp tip that can penetrate the mouse uterine wall. At least two microcapillaries are cut and filled with plasmid DNA/Fast Green solution before surgery is commenced. Scale bar in (**b**) and (**c**), 2 mm

3.1.3 Plasmid DNA

Prepare the desired concentration of plasmid DNA, typically between 0.5 and 2.0 μg/μl, into 0.025 % Fast Green solution (final concentration) made in sterile PBS. At this concentration, Fast Green is nontoxic to the embryo and allows the plasmid solution to be visualized during injection into the ventricle. The DNA/Fast Green preparation can be stored at 4 °C until needed.

3.1.4 Anesthetic and Analgesic Solutions

Prepare working solutions of a mixture of 15 mg/ml ketamine and 1.5 mg/ml xylazine, and 30 mg/ml buprenorphine, both in sterile PBS.

3.1.5 Microinjector

Connect the Picospritzer II microinjection system to a nitrogen gas line regulated at approximately 100 psi. Regulate the nitrogen pressure from the Picospritzer to the capillary holder at 10–15 psi, and the injection pulse time to 100 ms. Connect the foot pedal for hands-free initiation of microinjection pulse.

3.1.6 Microcapillaries

Using a micropipette puller, pull the ends of several borosilicate glass capillaries to form fine tips with a long (11–14 mm) taper (Fig. 2b). The precise settings to produce these tips depend on the size and age of the box filament, and the size of the glass capillaries, but generally require settings with a high level of heat, pull force, and pull velocity, but minimal (or no) use of air cooling. Using scissors or forceps cut approximately 6 mm off each microcapillary tip at an oblique angle, which will allow them to effectively penetrate through the uterine wall (Fig. 2c).

Load the desired volume of plasmid/Fast Green solution in the back of at least two capillaries using microloading pipette tips. If the capillary is longer than the pipette tip, the plasmid solution can be pushed to the injection end of the capillary by firmly holding the back end and forcefully flicking the capillary. Place one filled capillary in the holder connected to the Picospritzer II, and leave the other filled capillaries aside to allow for a rapid replacement in the event that the existing capillary is broken or more plasmid is required. Test the capillary injection onto a sterile surface to determine if the desired volume of plasmid (0.5–1.0 μl) can be delivered within a few seconds. If the injection capacity is insufficient, cut off an additional millimeter of the capillary tip to increase the tip diameter and its outflow. If different plasmids will be injected into each hemisphere, pull and load separate capillaries for each plasmid prior to beginning the surgery.

3.1.7 Electroporator

Connect the 0.5 mm diameter microelectrodes to the ECM 830 electroporator. Connect the electroporator foot pedal for hands-free activation of square wave pulses.

3.2 Procedure for Surgery and IUE

1. Weigh the animal and anesthetize with approximately 4.5 μl/g body weight of the ketamine/xylazine working solution via intraperitoneal injection; for example for a 30 g pregnant dam, inject approximately 0.14 ml ketamine/xylazine.

   Once the animal is sedated, inject 0.1 ml of the buprenorphine working solution subcutaneously. Place the mouse on the heat pad and, without compromising the animal's respiration, tape down the four limbs to stabilize the animal during surgery (Fig. 3a).

2. Place a liberal amount of Vaseline over the animal's eyes to prevent them from drying during surgery. Apply hair removal cream to the abdomen and wipe this area to remove the hair. Wash the abdomen thoroughly with PBS followed by chlorhexidine antiseptic solution to sterilize the area before incision.

   Cut out a crescent-shaped hole, approximately 30 mm in length, from the center of a sterile gauze swab and place onto the hair-free area of the abdomen (Fig. 3a).

3. Perform a caesarian section via midline laparotomy to reveal the embryos. Grasp the abdominal skin or muscle with the serrated micro-dissecting forceps and use the surgical scissors to make

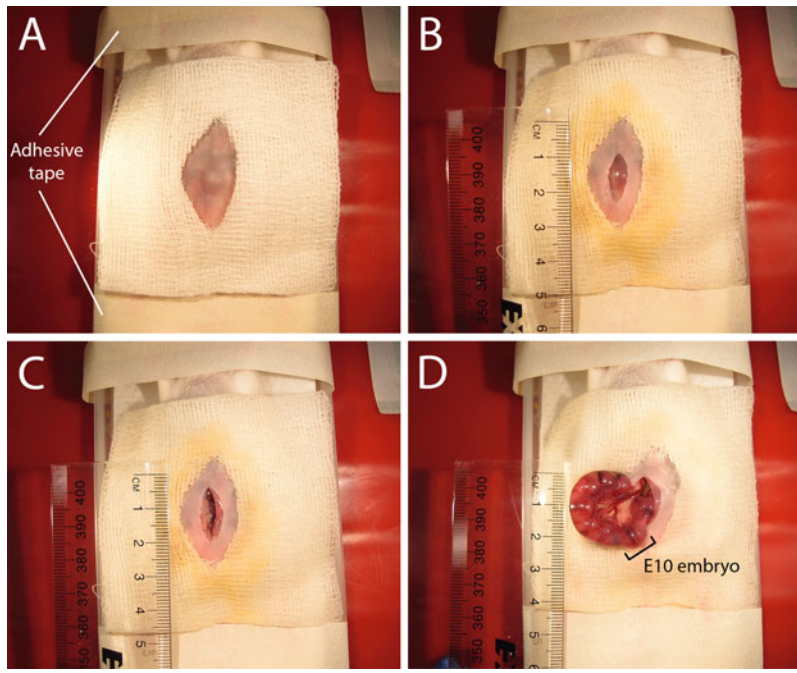

Fig. 3 Midline laparotomy and exposure of right uterine horn at E10. The dam is placed in a supine position on the paper towel and adhesive tape is used to secure the forelimbs and hindlimbs (**a**). A midline laparotomy is performed using serrated micro-dissecting forceps and surgical scissors on the abdominal skin (**b**) and muscle (**c**), and 3–4 embryos from one uterine horn are extracted from the abdominal cavity at a time for electroporation (**d**). The rostral-caudal length of one embryo within the uterus is indicated in (**d**)

two identical midline incisions, each approximately 12–14 mm, through the muscle and skin (Fig. 3b, c). During the muscle incision, take care to avoid piercing major blood vessels and organs such as the intestines or bladder.

4. Gently extract the uterine horns from the abdominal cavity with ring forceps and lay them on the gauze (Fig. 3d). Liberally administer warm PBS over the uterus and vasculature throughout the entire surgery to prevent the tissue from drying out and to maintain the mother at a stable warm temperature. To ensure that the embryos remain within the warm environment of the abdominal cavity for as much as possible during surgery, extract only 3–4 embryos at a time and reposition embryos back inside the abdominal cavity once they are electroporated before extracting the next embryos. Later stage embryos are larger and require extraction of the entire uterine horn to prevent contortion of the uterus and damage or occlusion of the uterine arteries.

   The embryos can be rotated within the amniotic sac by using cotton tips and ring forceps; however, avoid applying too much pressure to each embryo since this could break the amniotic sac and potentially cause the embryo to reabsorb. For further information on handling the embryos during surgery and achieving a high survival rate, *see* **Note 5.1**.

5. At E11 the major cranial dural sinus vessels can be identified and used as a reference for determining the position of the telencephalic hemispheres. However, at E10 these major vessels are not apparent and visualization of the telencephalon at E10 is not possible through conventional overhead light sources. Instead, the telencephalon must be identified by placing the fiber-optic light source directly against the uterus in order to illuminate the embryos through the thick uterine wall and orienting the embryo with the dorsal side of the brain facing up (Fig. 4a, b). Once the position of the hemispheres has been identified (Fig. 4b, c), it is important to stabilize the embryos to avoid damaging the telencephalon during microinjection of the plasmid DNA. A convenient approach is to use the fingers and thumb of one hand to gently hold the light source against the uterus, and place a cotton tip under the uterus for further stabilization and positioning as shown in Fig. 4a. Once the cotton tip is positioned, hold the microcapillary or electrodes with the other hand (Fig. 5a, d).

6. Target the capillary tip to penetrate into the middle of one telencephalic hemisphere (Fig. 5b, c) and microinject 0.5–1.0 μl of plasmid DNA (mixed with 0.025 % Fast Green dye in sterile PBS) into the lateral ventricular space. This injection volume typically requires 3–5 × 100 ms pulses at 10–15 psi. It is important to inject the embryos carefully to avoid causing excess cortical damage and that the injection site is outside the intended region of electroporation (*see* **Note 5.2**).

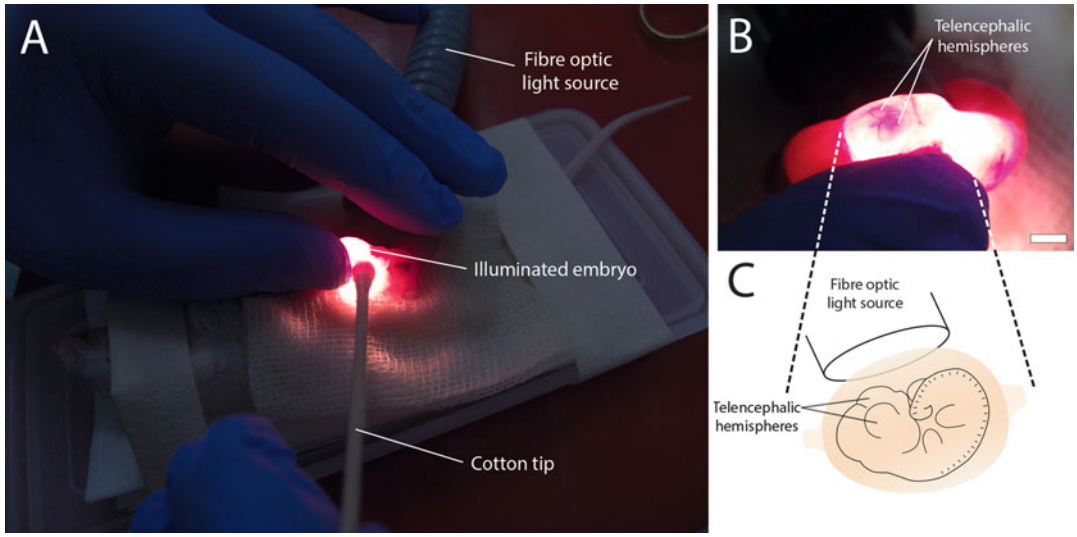

Fig. 4 E10 embryo illumination and stabilization immediately prior to plasmid DNA injection. The flexible fiber-optic light source is positioned directly against the uterus to illuminate each embryo, and a cotton tip is used to stabilize the position of the uterus (**a**). Once the embryo is oriented with the ventral side facing up, the telencephalic hemispheres can be identified (**b**). The orientation of the illuminated embryo and position of the telencephalic hemispheres shown in (**b**) are depicted in the schematic in (**c**). Scale bar in (**b**), 2 mm

Note that the ventricles are more openly connected at early embryonic stages and it is important not to inject excess plasmid; otherwise it may diffuse throughout the ventricular space and into the opposing hemisphere, resulting in undesired labeling. A small plasmid volume (no more than 1.0 μl) will ensure a more restricted area of electroporation and reduce the chance of transfecting cells in unwanted areas.

7. Position the positive electrode above the targeted cortical area and position the negative electrode on the uterus in such a way as to achieve the desired angle of electroporation. Figure 5e, f shows the position of the electrodes for the electroporation of the dorsal lateral telencephalon, and Fig. 5g shows a schematic (in coronal view) of the orientation of the electrodes relative to the embryo.

   Electroporate the plasmid DNA into the target neuroepithelial tissue using five square wave pulses of 20–25 V, each at 50 ms, with 1-s interval. It is important that the electrodes have the correct orientation and are kept steady to ensure that the correct region is electroporated, and that some pressure is applied to the electrodes to ensure that their terminals are in complete contact with the uterine wall (Fig. 5e).

   Medially positioned cortical areas such as the cingulate cortex or hippocampus are more difficult to electroporate because of their position within the developing brain, but can be accessed by an indirect method of electrode placement. For example, the

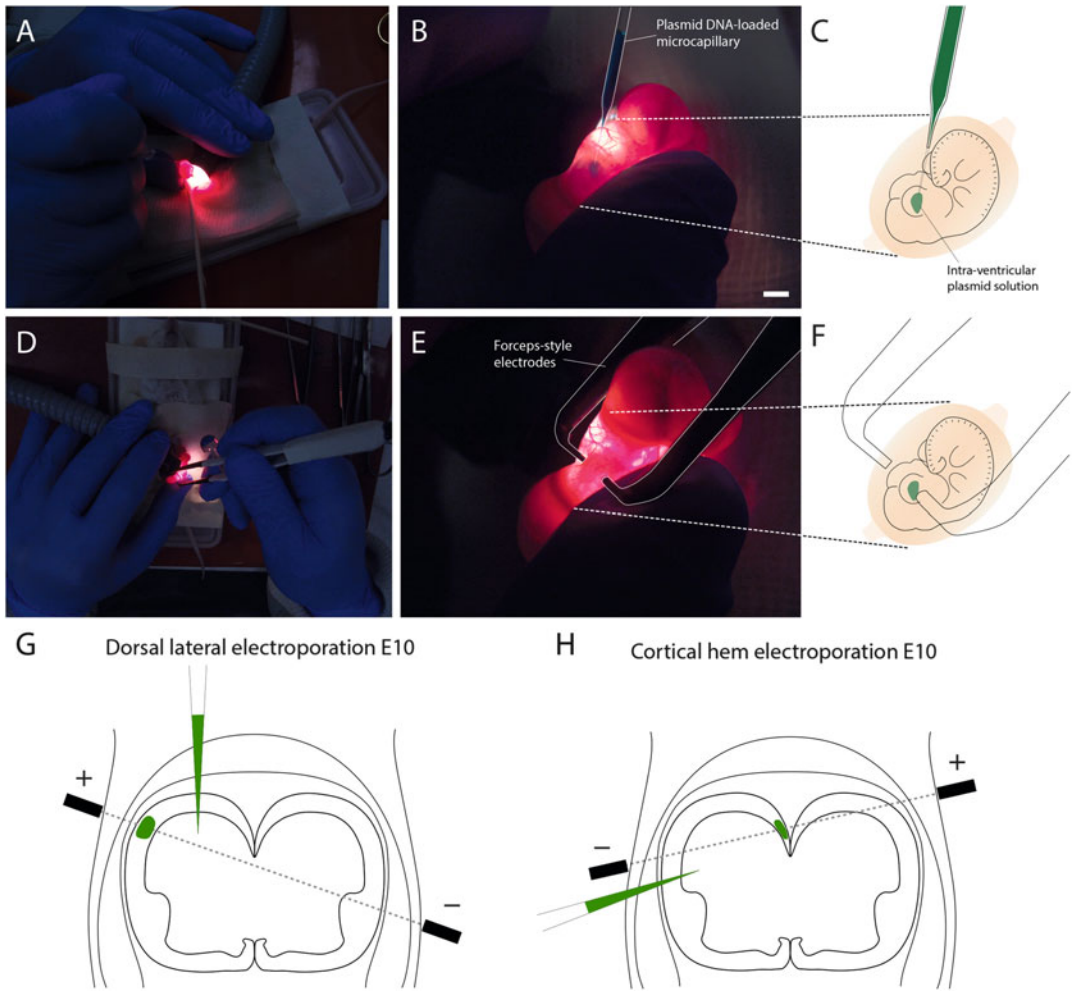

Fig. 5 Plasmid injection and electrode placement on an E10 embryo for the electroporation of dorsal lateral telencephalon. (**a**–**c**) Injection of plasmid DNA. Once the embryo is illuminated and secured in position (**a**), the plasmid DNA is injected into one hemisphere and is visualized with the use of Fast Green (**b**). The position of the injecting microcapillary with respect to the embryo is highlighted in *white outline* in (**b**), and the schematic in (**c**) depicts this microcapillary injection. (**d**–**f**) Electroporation. In order to achieve the desired angle of electrode orientation, the dam can be rotated on the heating pad (**d**). Once the embryo is stabilized, the electrodes are applied with a small degree of pressure to ensure that they are in complete contact with the surface of the uterus (**e**) and the electrical pulses are applied. The position of the electrodes with respect to the embryo is highlighted in *white outline* in (**e**), and the schematic in (**f**) depicts this electrode placement. (**g**) Dorsal lateral telencephalon electroporation. To target this region the positive electrode must be placed above the lateral portion of the same telencephalic hemisphere to where the plasmid DNA is injected. (**h**) Cortical hem electroporation. To target this region the positive electrode must be placed above the telencephalic hemisphere contralateral to where the plasmid DNA is injected. Scale bar in (**b**) and (**e**), 2 mm

cortical hem is located within the medial aspect of the dorsal telencephalon, but can be specifically targeted within one hemisphere using the electrode orientation shown in Fig. 5h.

8. Repeat steps 6 and 7 for each embryo, remembering to stabilize the embryo position prior to plasmid injection.
9. Once all embryos are electroporated, position the uterine horns back inside the abdominal cavity in their original positions and apply some warm PBS within the cavity. Apply a running suture to close the abdominal muscle incision (Fig. 6a), followed by a running suture to close the abdominal skin incision (Fig. 6b). Wipe the sutured skin with chlorhexidine solution.
10. Administer 1–2 ml of Ringer's solution subcutaneously to dilute the anesthetic and prevent dehydration of the animal.
11. Transfer the animal to the recovery chamber heated to 37 °C, monitor its recovery until it is able to walk and easily access its food and water supply, and then transfer back to its animal cage at room temperature.
12. Inspect the dam on the next day and administer analgesic solution subcutaneously if there are signs of pain or suffering. For further information on ensuring survival of the dam after surgery, *see***Note 5.3**.
13. To prepare embryonic electroporated brains for histology, euthanize the dam at the desired stage of embryonic development, and dissect the embryos from the uterus. Dissect the brain from each electroporated embryo and drop-fix in 4 % paraformaldehyde (PFA) overnight at 4 °C. The next day, rinse brains in PBS and prepare fixed tissue for sectioning.

    To prepare postnatal electroporated brains for histology, euthanize pups with an intraperitoneal injection of pentobarbital sodium solution (100 μg/g body weight). Perform transcardial perfusion of 0.9 % sodium chloride solution, and a perfusion of 4 % PFA. Dissect the brain from each electroporated pup and

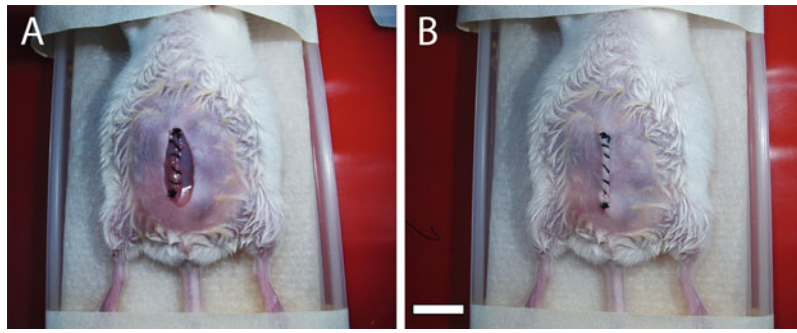

Fig. 6 Suturing of the abdominal muscle and skin. Once the embryos are electroporated, the uterine horns are positioned back inside the abdominal cavity in their original positions and warm PBS is applied into the cavity. The abdominal muscle is then closed using a running suture along the line of incision tied off with a knot at the rostral and caudal ends (**a**). This running suture method is repeated to close the abdominal skin incision (**b**). Scale bar, 10 mm

post-fix in 4 % PFA overnight at 4 °C. The next day, rinse brains in PBS and prepare fixed tissue for sectioning.

3.2.1 Multiple Electroporations

The procedure described above can be used for the electroporation of a cortical area at a single developmental stage; however, an additional electroporation on the same embryos can be performed at a later stage. Multiple electroporations performed over time can be used for the differential transfection of cortical layers generated at different stages. For example, performing electroporations 5 days apart, at E10 and E15, will differentiate cortical layers II/III from the subplate (*see* Fig. 1). For the second surgery, it is recommended to re-weigh the dam to ensure that an appropriately higher volume of anesthetic solution is injected given the relatively larger size of the embryos. In addition, remove the previous suture silk and perform the midline laparotomy along the previous incision to minimize the wound area. The voltage settings must also be increased to ensure efficient electroporation (for example, 30–35 V at E15).

For later stage embryos, it is also possible to perform two electroporations within the same embryo at a single developmental stage, and this technique has been commonly used to transfect different fluorescent protein expression plasmids to investigate or manipulate the development of different areas. For example, Zhou et al. [21] used this technique to electroporate different fluorophores between S1 and the primary motor cortex in E15.5 mice in order to distinguish the axon bundles from these two areas and their relative position within the corpus callosum. In addition, Suárez et al. [20] electroporated the inward-rectifying potassium channel, Kir2.1, into the right and left S1 at E15.5 to disrupt neuronal activity in both hemispheres. To perform multiple spatial electroporations, it is recommended to wait for 30 min between the two electroporations, during which time an additional small volume of anesthetic solution is applied to the dam to prolong the sedation and a PBS-soaked sterile gauze swab is placed over the caesarian section.

4 Outcome of In Utero Electroporation

By 2.5–3.0 days after electroporation of the dorsal telencephalon at E10 using the configuration shown in Fig. 5g, fluorescence is observed in a patch 400–500 μm wide in a lateral portion of the cortex. Transfected progenitor cells in the ventricular zone are observed radially migrating to the preplate layer (Fig. 7a′) and also beginning to extend corticofugal axonal projections to subcerebral targets (Fig. 7a). By 5.5–6.0 days after electroporation, transfected progenitor cells are present in the subplate and cortical plate,

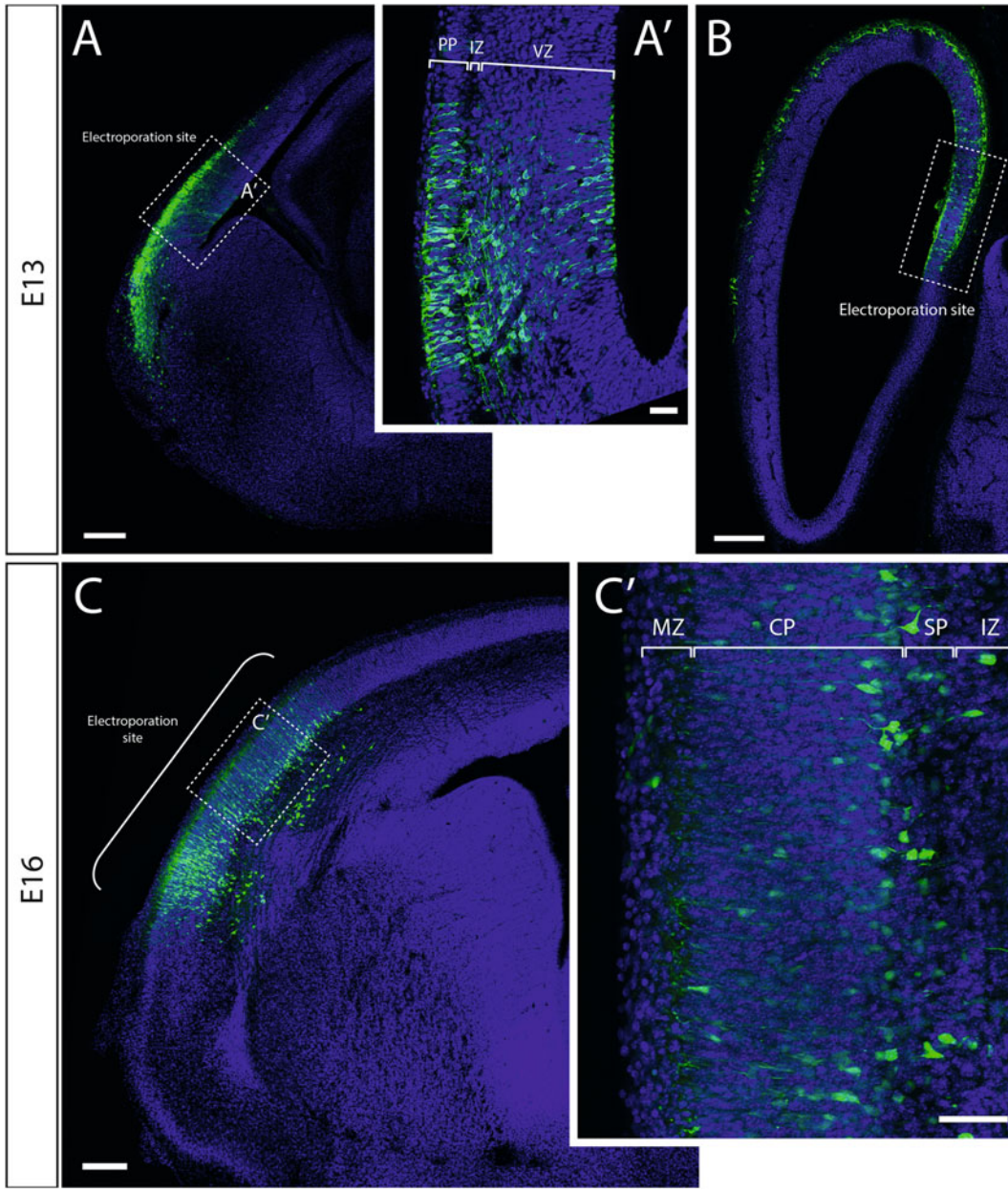

Fig. 7 Outcomes of electroporation of the developing cortex with pCAG-EGFP at E10. (**a**) Coronal view of E13 cortex after electroporation of pCAG-EGFP in E10 dorsal telencephalon shows 400–500 μm wide region of electroporation, and the initial extension of corticofugal projections from these EGFP-positive transfected cells. (**a**′) At higher magnification, the site of electroporation is indicated by the region of radial migration of fluorescent progenitor cells to the PP layer from the VZ. (**b**) A horizontal section of E13 cortex after electroporation of pCAG-EGFP at E10 in the cortical hem shows tangential migration of transfected Cajal-Retzius cells from medial to lateral portions of the cortex. (**c**) A coronal view of E16 cortex after electroporation at E10 in the dorsal telencephalon. The density of fluorescent cells appears to increase at the caudal edge of the electroporation site since this coincides with the change in laminar structure at the edge of the neocortex. (**c**′) Within the region of electroporation, transfected cells are present in the cortical plate and subplate. *CP* cortical plate, *IZ* intermediate zone, *MZ* marginal zone, *PP* preplate, *SP* subplate, *VZ* ventricular zone. Scale bar 200 μm in (**a**)–(**c**), and 50 μm in (**a**′) and (**b**′)

and are observed migrating radially with leading processes extending toward the pial surface (Fig. 7c, c′).

2.5–3.0 days after electroporation of the cortical hem at E10 using the configuration shown in Fig. 5h, tangential migration of transfected Cajal-Retzius cells is observed throughout the cortex. In the horizontal section shown in Fig. 7b, fluorescent cells are observed migrating from the medially located cortical hem to lateral regions of the cortex.

A low or lack of fluorescent signal in the cortical tissue may indicate a problem with the plasmid, microcapillary, injection, or electrodes. See Note 5.4 for troubleshooting these problems.

5 Notes

5.1. Embryo Handling and Survival

Often an initial difficulty with the electroporation of embryos at E10 is the low survival rate of the embryos given their greater vulnerability to external manipulation compared with later stages of gestation. Ensure that the uterus is handled gently using the ring forceps and cotton tips. In particular avoid contact with the placenta of each embryo and the dorsal, vascularized surface of the uterus, and do not place the electrodes on these structures to prevent cauterization of the vasculature. In addition, avoid excessive pulling or twisting of the uterine horn, particularly of the terminal embryos adjacent to the ovaries and fundus/cervix, as this could cause reabsorption of all the embryos in the horn at early stages of gestation. It is also recommended to avoid electroporation of these terminal embryos.

Furthermore, a high embryo survival rate can be achieved by not using an electroporation voltage greater than 25 V, and after surgery, ensuring that the uterine horns are not twisted or contorted when placed back inside the abdominal cavity to prevent occlusion of the uterine blood supply.

5.2. Reducing Cortical Damage During Microinjection

A localized region (approximately 50 μm radius) of cortical damage is normally caused by microcapillary injection. To avoid causing excess cortical damage, ensure that the embryo is stabilized and that the microcapillary tip is not blunt and has an oblique angle cut before each injection. Furthermore, do not inject an excessive volume of plasmid solution into the telencephalon. The injection volume can be monitored by marking the volume increments along the outside of the microcapillary.

5.3. Dam Survival
Monitoring the dam's breathing is required during surgery to ensure that the animal is responding normally to the anesthesia, and should continue to be monitored closely after surgery while in the heated recovery chamber. To ensure that the dam recovers quickly from sedation, it is important that the appropriate dosage of ketamine and xylazine relative to the weight of the dam was given prior to surgery. The dosage recommended here should produce a maximum sedation time of 1 h. It is also important that there is no excessive bleeding during surgery, by ensuring that the laparotomy is performed carefully along the midline of the abdominal skin and muscle and avoiding damage to major blood vessels.

5.4. Troubleshooting a Low or Lack of Fluorescent Signal

- Check the plasmid by verifying the fluorescent protein sequence, and ensuring an appropriate promoter is used. The cytomegalovirus (CMV) early enhancer/chicken beta-actin (CAG) promoter drives high levels of expression in cortical neurons [22] and has been shown to be more efficient than the CMV promoter alone [23]. Plasmid expression can be tested in vitro first by transfection into an appropriate cell culture line.
- Microcapillary blockage: Check that the microcapillary tip is open and that there is outflow of plasmid DNA solution before injection. It should be possible to visualize the Fast Green solution entering the telencephalon during injection.
- Mis-targeting of the intended region of electroporation: Ensure that the embryo is illuminated to assist with the identification of the telencephalic hemispheres and that the microcapillary penetrates the embryonic tissue at the correct depth.
- Check the electrodes: Ensure that these are functional by testing an electric pulse in PBS solution and observing the appearance of bubbles during each pulse. Also avoid cleaning electrodes with ethanol as this may damage the platinum surface and reduce their conductivity.

6 Final Remarks

IUE is a valuable technique for the transfection of cortical cells and can be used to manipulate the expression of many different genes in vivo. While electroporation is often used in later stages of gestation to target the later born cortical plate neurons, this technique can be

performed at early stages of development to transfect cells in the early cortical plate. The protocol described in this chapter involves features optimized for early-stage IUE, such as techniques and equipment for improved visualization, injection, and electroporation of the embryos. Furthermore, the technique can be used for the transfection of multiple plasmids with temporal variation, allowing for the possibility of differential labeling between the subplate and cortical plate layers.

A further modification to the technique to enable cell lineage tracing within a target region is the integration of exogenous genes within the genome of electroporated cells via the incorporation of *Tol2* or *Piggybac* transposition sequences in the expression vector [24, 25]. Genome integration via IUE has previously been used for the expression of advanced reporters such as Brainbow transgenes, which contain multiple fluorophores co-expressed in random combinations upon Cre-mediated recombination. This technique provides a method for the detection of individual neuronal progenitor cells and their lineages, including those of cortical neurons by the differential expression of reporter genes [26].

Overall IUE provides significant advantages over conventional transgenic approaches by providing specific spatial and temporal precision. It provides a means for assaying neurons in vivo and can be used to generate chimeric tissue to investigate gene function. Combining this delivery technique with the sophisticated DNA construct technologies described above greatly extends the capabilities for investigating important questions in cortical development.

Acknowledgements

We thank R. Suárez and L. Fenlon for their input on the manuscript and providing histological images; Queensland Brain Institute (QBI) Microscopy Facility for microscopy assistance; the University of Queensland Biological Resources and QBI animal team for animal support. PK was supported by a National Health and Medical Research Council (NHMRC, Australia) Early Career (CJ Martin) Fellowship, GA was supported by a Becas Chile Fellowship, and LJR was supported by an NHMRC Principal Research Fellowship. IG was supported by NHMRC project grant GNT1048849.

References

1. Monuki ES, Porter FD, Walsh CA (2001) Patterning of the dorsal telencephalon and cerebral cortex by a roof plate-Lhx2 pathway. Neuron 32:591–604
2. O'Leary DDM, Chou S-J, Sahara S (2007) Area patterning of the mammalian cortex. Neuron 56:252–269
3. Borello U, Pierani A (2010) Patterning the cerebral cortex: traveling with morphogens. Curr Opin Genet Dev 20:408–415
4. Caronia-Brown G, Yoshida M, Gulden F et al (2014) The cortical hem regulates the size and patterning of neocortex. Development 141:2855–2865

5. Chédotal A, Richards LJ (2010) Wiring the brain: the biology of neuronal guidance. Cold Spring Harb Perspect Biol 2:a001917
6. Shimogori T, Grove EA (2005) Fibroblast growth factor 8 regulates neocortical guidance of area-specific thalamic innervation. J Neurosci 25:6550–6560
7. Assimacopoulos S, Kao T, Issa NP, Grove EA (2012) Fibroblast growth factor 8 organizes the neocortical area map and regulates sensory map topography. J Neurosci 32:7191–7201
8. Saito T, Nakatsuji N (2001) Efficient gene transfer into the embryonic mouse brain using in vivo electroporation. Dev Biol 240:237–246
9. Tabata H, Nakajima K (2001) Efficient *in utero* gene transfer to the developing mouse brain using electroporation: visualization of neuronal migration in the developing cortex. Neuroscience 103:865–872
10. Britanova O, De Juan RC, Cheung A et al (2008) Satb2 is a postmitotic determinant for upper-layer neuron specification in the neocortex. Neuron 57:378–392
11. Shibata M, Kurokawa D, Nakao H et al (2008) MicroRNA-9 modulates Cajal-Retzius cell differentiation by suppressing foxg1 expression in mouse medial pallium. J Neurosci 28:10415–10421
12. Rouaux C, Arlotta P (2013) Direct lineage reprogramming of post-mitotic callosal neurons into corticofugal neurons in vivo. Nat Cell Biol 15:214–221
13. Srivatsa S, Parthasarathy S, Britanova O et al (2014) Unc5C and DCC act downstream of Ctip2 and Satb2 and contribute to corpus callosum formation. Nat Commun 5:1–15
14. Faux C, Rakic S, Andrews W, Britto JM (2012) Neurons on the move: migration and lamination of cortical interneurons. Neurosignals 20:168–189
15. Borrell V, Yoshimura Y, Callaway EM (2005) Targeted gene delivery to telencephalic inhibitory neurons by directional in utero electroporation. J Neurosci Methods 143:151–158
16. Takiguchi-Hayashi K, Sekiguchi M, Ashigaki S et al (2004) Generation of reelin-positivemarginal zone cells from the caudomedial wall of telencephalic vesicles. J Neurosci 24:2286–2295
17. Bielle F, Griveau A, Narboux-Nême N et al (2005) Multiple origins of Cajal-Retzius cells at the borders of the developing pallium. Nat Neurosci 8:1002–1012
18. Huilgol D, Udin S, Shimogori T et al (2013) Dual origins of the mammalian accessory olfactory bulb revealed by an evolutionarily conserved migratory stream. Nat Neurosci 16:157–165
19. Remedios R, Huilgol D, Saha B et al (2007) A stream of cells migrating from the caudal telencephalon reveals a link between the amygdala and neocortex. Nat Neurosci 10:1141–1150
20. Suárez R, Fenlon LR, Marek R et al (2014) Balanced interhemispheric cortical activity is required for correct targeting of the corpus callosum. Neuron 82:1289–1298
21. Zhou J, Wen Y, She L et al (2013) Axon position within the corpus callosum determines contralateral cortical projection. Proc Natl Acad Sci U S A 110:E2714–E2723
22. Sahara S, Kawakami Y, Izpisua Belmonte J, O'Leary DD (2007) Sp8 exhibits reciprocal induction with Fgf8 but has an opposing effect on anterior-posterior cortical area patterning. Neural Dev 2:1–22
23. Yaguchi M, Ohashi Y, Tsubota T et al (2013) Characterization of the properties of seven promoters in the motor cortex of rats and monkeys after lentiviral vector-mediated gene transfer. Hum Gene Ther Methods 24:333–344
24. Yoshida A, Yamaguchi Y, Nonomura K et al (2010) Simultaneous expression of different transgenes in neurons and glia by combining in utero electroporation with the *Tol2*transposon-mediated gene transfer system. Genes Cells 15:501–512
25. Chen F, Maher BJ, LoTurco JJ (2014) PiggyBac transposon-mediated cellular transgenesis in mammalian forebrain by in utero electroporation. Cold Spring Harb Protoc 2014:741–749
26. Loulier K, Barry R, Mahou P et al (2014) Multiplex cell and lineage tracking with combinatorial labels. Neuron 81:505–520