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TITLE:

Double In Utero Electroporation to Target Temporally and Spatially Separated Cell Populations

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KEYWORDS:

electroporation, brain, cerebral cortex, neurodevelopment, radial glia, cortical projection neurons, neuronal connectivity

SUMMARY:

Double in utero electroporation allows targeting cell populations that are spatially and temporally separated. This technique is useful to visualize interactions between those cell populations using fluorescent proteins in normal conditions but also after functional experiments to perturb genes of interest.

ABSTRACT:

In utero electroporation is an in vivo DNA transfer technique extensively used to study the molecular and cellular mechanisms underlying mammalian corticogenesis. This procedure takes advantage of the brain ventricles to allow the introduction of DNA of interest and uses a pair of electrodes to direct the entrance of the genetic material into the cells lining the ventricle, the neural stem cells. This method allows researchers to label the desired cells and/or manipulate the expression of genes of interest in those cells. It has multiple applications, including assays targeting neuronal migration, lineage tracing, and axonal pathfinding. An important feature of this method is its temporal and regional control, allowing circumvention of potential problems related with embryonic lethality or the lack of specific CRE driver mice. Another relevant aspect of this technique is that it helps to considerably reduce the economic and temporal limitations that involve the generation of new mouse lines, which become particularly important in the study of interactions between cell types that originate in distant areas of the brain at different developmental ages. Here we describe a double electroporation strategy that enables targeting of cell populations that are spatially and temporally separated. With this approach we can label different subtypes of cells in different locations with selected fluorescent proteins to visualize

them, and/or we can manipulate genes of interest expressed by these different cells at the appropriate times. This strategy enhances the potential of in utero electroporation and provides a powerful tool to study the behavior of temporally and spatially separated cell populations that migrate to establish close contacts, as well as long-range interactions through axonal projections, reducing temporal and economic costs.

INTRODUCTION:

The cerebral cortex is a very complex and intricately organized structure. To achieve such a degree of organization, cortical projection neurons go through complex developmental processes that require their temporal generation, migration to their final destination in the cortical plate, and the establishment of short- and long-range connections[1,2]. For a long time, the classical way to study corticogenesis was based on the use of knockout or knock-in murine models of genes of interest. However, this strategy, and particularly the use of conditional knockout mice, is time consuming and expensive, and sometimes presents additional problems regarding the existence of genetic redundancy or the lack of specific CRE drivers, among other issues. One of the approaches that arose to try to address those problems and that is nowadays extensively used to study cortical development is in utero electroporation[3,4]. In utero electroporation is a technique used for somatic transgenesis, allowing in vivo targeting of neural stem cells and their progeny. This method can be used to label cells by the expression of fluorescent proteins[5,6], for gene manipulation in vivo (i.e., gain or loss of function assays)[7–9], for isolating electroporated cortices in vitro and culturing cells[8,10]. Moreover, in utero electroporation permits temporal and regional control of the targeted area. This technique has numerous applications and has been widely used to study neuronal migration, stem cell division, neuronal connectivity, and other subjects[8,9,11,12].

The current manuscript describes the use of an in utero electroporation variant, termed double in utero electroporation, to analyze the interactions of cells in the cerebral cortex with different temporal and spatial origins. These studies are extremely complex to complete when employing murine models because they require the combined use of several transgenic lines. Some of the applications of the protocol described in this paper include the study of close interactions among neighboring cells, as well as interactions between distant cells through long-range projections. The method requires performing two independent in utero electroporation surgeries, separated temporally and spatially, on the same embryos to target different cell populations of interest. The advantage of this approach is the possibility of manipulating gene function in one or both types of neurons using wild type animals. In addition, these functional experiments can be combined with the expression of cytoplasmic or membrane-tagged fluorescent proteins to visualize the fine morphology of targeted cells, including dendrites and axons, and analyze possible differences in cellular interactions in comparison with a control (i.e., cells only labeled with the fluorescent protein).

The protocol delineated here is focused on the study of cellular interactions inside the neocortex, but this strategy could also be used to examine interactions with extracortical areas that can be targeted using in utero electroporation, like the subpallium or the thalamus[13,14], or cell-cell interactions in other structures, like the cerebellum[15]. Targeting of different areas is based on the

orientation of the electrodes and on the ventricle where the DNA is injected (lateral, third, or fourth). With the strategy described here, we can label a substantial number of cells, which is useful to evaluate general changes in connectivity/innervation in functional experiments. Nevertheless, to study fine changes in connectivity, one can use modified versions of in utero electroporation to get sparser labeling and identify single cells[16]. In summary, double in utero electroporation is a versatile method that allows targeting temporally and spatially separated cell populations and studying their interactions in detail, either in control conditions or combined with functional experiments, considerably reducing temporal and economic costs.

PROTOCOL:

The procedure herein described has been approved by the ethical committee in charge of experimentation and animal welfare of the Universidad de Valencia and adheres to the guidelines of the International Council for Laboratory Animal Science (ICLAS) reviewed in the Real Decreto 53/2013 of the Spanish legislation as well as in the Directive 2010/63/EU of the European Parliament and of the Council.

NOTE: This protocol involves two different purposes: 1) the first study, referred to as "strategy A", allows the analysis of the interactions between Cajal-Retzius cells (CR-cells) and early-born cortical projection neurons within the same brain hemisphere; 2) the second study, "strategy B", is carried out in order to examine the innervation of the upper layer callosal projection neurons to the contralateral side of the neocortex.

1. Presurgery preparation

1.1. DNA preparation

1.1.1. Transform chemically or electrocompetent *E. coli* DH5α cells with the plasmids of interest, plate them on LB agar plates with the appropriate antibiotic, and incubate them overnight at 37 °C.

NOTE: All plasmids used here contain the general promoter for chicken β-actin (CAG) driving the expression of a fluorescent-reporter protein (CAG-mCherry and CAG-EGFP for strategy A and CAG-BFP and CAG-nEGFP-2A-mtdTomato for strategy B). All of them contain resistance to ampicillin (AMP).

1.1.2. Pick individual colonies from each plasmid transformation and initiate a starter liquid culture in 2 mL of Luria broth + ampicillin (LB+AMP) in bacterial culture tubes during 3−4 h at 37 °C with vigorous shaking (200 rpm). After, set a larger bacterial culture in a 500 mL Erlenmeyer flask adding 200 mL of LB+AMP and 1 mL of the starter culture. Incubate overnight at 37 °C in the orbital shaker at 200 rpm.

1.1.3. Use an endotoxin-free maxi-prep kit (**Table of Materials**) following the manufacturer's instructions to obtain pure and concentrated plasmid DNA from the liquid cultures. Resuspend

the DNA in ~50−100 µL of endotoxin-free Tris-EDTA (TE) buffer to obtain concentrations of at least 5 µg/µL.

1.1.4. For each surgery, prepare a solution with a final volume of 10 µL containing 1 µL of fast green dye, plasmid DNA solution of interest for a final concentration of 1 µg/µL for each plasmid, and endotoxin-free TE buffer. For example, mix 1 µL of fast green dye, 2 µL of a 5 µg/µL plasmid DNA solution, and 7 µL of TE buffer.

1.2. Pipette pulling

1.2.1. Pull borosilicate glass capillaries (1/0.58 mm outer/inner diameter) in a vertical micropipette puller until the tip reaches a length of 1−1.5 cm and trim it using dissecting forceps at an angle of approximately 30° under a dissecting scope.

1.3. Surgery room setup

1.3.1. Put all the equipment on the operating table (i.e., tweezers, scissors, forceps, micropipettes, needle, and needle holder). Turn on the heating pad and cover it with a sterile surgical absorbent pad. Ensure that the reservoir in the machine for inhalation anesthesia is filled with isoflurane, the oxygen tank contains enough oxygen, and the system functions properly.

NOTE: The surgery room and the material must be kept as sterile as possible (i.e., all the material must be autoclaved before the surgery and surfaces must be sanitized with 70% ethanol). Similarly, temperature and air ventilation must be maintained in optimal conditions.

1.3.2. Prepare 100 mL of 0.9% (w/v) sterile saline solution containing penicillin-streptomycin 1:100 and fill a 10 cm Petri dish. Place the plate on top of the heated pad to warm up the solution.

1.3.3. Fill a 1 mL syringe with 150 µL of an analgesic solution (e.g., 0.1 mg/kg buprenorphine).

1.3.4. Load the pulled pipette with 5 µL of the final plasmid DNA solution prepared in step 1.1.4. Connect the capillary to a mouth-controlled aspirator tube.

2. First in utero electroporation surgery

2.1. Place an E11.5 (strategy A) or E13.5 (strategy B) C57BL/6 pregnant mouse inside a closed induction chamber with 2.5% (v/v) isoflurane at 0.8 L/min and wait until it is anesthetized. Transfer the mouse to the heating pad and put its nose into a mask for constant delivery of isoflurane. Check for the absence of a pedal reflex as an indicator of proper anesthesia.

2.2. Inject the pregnant female with the analgesic solution (0.1 mg/kg buprenorphine) subcutaneously. To prevent the eyes from drying during the procedure, apply one drop of eye ointment in each eye using a cotton swab.

2.3. Shave the mouse abdominal area with an electric razor and wash it 2x with 70% (v/v) ethanol wipes, once with iodine wipes, and one last time with an ethanol wipe.

2.4. Use scissors to make a 30 mm long incision through the skin in the right side of the animal and carefully separate the adjacent skin from the muscle with a blunt spatula. Afterwards, make a second incision in the abdominal wall.

2.5. Cover the abdomen with a piece of folded tissue paper previously disinfected with 70% ethanol containing a 40 mm long slit in its center. Carefully pull the uterus out of the abdominal cavity with ring forceps.

NOTE: The uterus must be kept wet with the warm saline solution prepared in step 1.3.2 during the whole procedure.

2.6. Preload the pulled pipettes with the DNA solution prepared in step 1.1.4. Inject ~0.5 µL of the DNA solution per embryo into the lateral ventricle of the selected hemisphere using the mouth-controlled aspirator tube until the fast green dye is noticeable inside the ventricle.

2.7. Place forceps-type platinum electrodes laterally around the head of the injected embryo (as shown in **Figure 1A** and **Figure 2A**). Orient the electrodes to target the desired brain region. Direct the positive pole towards the medial wall to electroporate the cortical hem (strategy A) or towards the lateral cortex (strategy B) to label cells generated in that area.

NOTE: In all cases, the heart and placenta must be avoided to ensure embryonic survival.

2.8. Apply the specific sequence of electric pulses with a square wave electroporator following the indications shown in **Table 1** (strategy A E11.5 embryos: four pulses of 25 V and 40 ms, separated by 950 ms intervals; strategy B E13.5 embryos: five pulses of 35 V and 60 ms, with 950 ms intervals).

2.9. Carefully place the uterus back into the abdominal cavity with forceps, fill it with warm saline solution, and close the abdominal wall with a needle 6-0 suture. Join the two sides of the initial incision made in the skin either using a needle 6-0 suture or suture clips.

2.10. Maintain the animal on the heating pad and monitor it until its recovery from anesthesia. Provide extra doses of analgesic solution (two doses of 150 µL) in a hydrogel solution placed in its home cage.

2.11. Continue daily monitoring by visual inspection for possible pain and distress. Observe the animal's behavior, test its normal hind limb reflexes, and inspect the suture for possible signs of damage due to licking or scratching of the wound.

3. Second in utero electroporation

3.1. Two days after the initial surgery, repeat steps 2.1−2.3. Although the recovery of pregnant females after the surgery is very good, check that they show normal behavior and present no signs of pain or distress before performing the second surgery (E13.5 embryos for strategy A and E15.5 for strategy B).

3.2. Make a 30 mm long incision through the skin as in step 2.4 and a second incision at the abdominal wall, this time in the left side of the animal. Carefully expose the uterus on top of a disinfected tissue as described in step 2.5.

NOTE: Be careful not to interfere with the incision previously made in the other side.

3.3. Inject around 0.5 µL per embryo of the DNA solution into the lateral brain ventricle of the hemisphere previously electroporated in the case of strategy A and in the lateral brain ventricle of the contralateral hemisphere for strategy B.

NOTE: Use only embryos showing normal development and no signs of reabsorption.

3.4. Place the electrodes around the embryo's head as described in step 2.7, directing the positive electrode towards the lateral cortex and apply the appropriate pulses following the indications shown in **Table 1** (strategy A E13.5 embryos: five pulses of 35 V and 60 ms separated by 950 ms intervals; strategy B E15.5 embryos: five pulses of 50 V and 80 ms separated by 950 ms intervals).

3.5. Continue and finish the surgery as described in steps 2.8−2.10.

4. Tissue harvesting and sectioning

4.1. Strategy A

4.1.1. Four days after the second electroporation (E17.5), perform cervical dislocation of the pregnant female and place it in a supine position.

4.1.2. Using scissors, make a ventral incision to extract the uterine horns and with forceps place them in a Petri dish filled with 1x phosphate-buffered saline (PBS) placed on ice.

NOTE: The low temperature makes the anesthetization of the embryos possible.

4.1.3. Using tweezers, extract the embryos out of the amniotic sac and transfer them with forceps to a new PBS-filled Petri dish under a dissecting scope. Carefully hold the head of the embryos using forceps and conduct brain dissection with tweezers to first remove the skin over the head and then the skull. Use a spatula to pull out the exposed brains.

4.1.4. Collect the brains with a spoon and deposit them in a 48 well plate filled with the fixative solution (4% [w/v] paraformaldehyde [PFA] in 1x PBS). Test immediately for the successful

outcome of both electroporations by examining the brains using an inverted epifluorescence microscope, for example.

4.1.5. Fixate the embryonic brains overnight at 4 °C in an orbital shaker. Wash with 1x PBS to eliminate traces of PFA. Then transfer them to PBS with antifungal preservatives (1x PBS-0.05% (w/v) sodium azide).

CAUTION: PFA and sodium azide are cytotoxic compounds that require special precaution during their utilization.

4.1.6. Embed the fixated brains in 4% (w/v) low melting point agarose in 1x PBS and wait ~10 min until it solidifies. Stick them to the vibratome tissue holder using cyanoacrylate glue with the olfactory bulbs facing upwards to obtain coronal sections.

4.1.7. Initiate the vibratome and select the desired parameters: 100 µm width, 0.60 µm/s speed, and 0.60 mm of amplitude.

4.1.8. Secure the brain inside the vibratome container, fill it with 1x PBS solution, and begin collecting coronal serial sections with the help of a brush in a 48 well plate filled with 1x PBS-0.05% (w/v) sodium azide to have a complete depiction of the brain (e.g., around seven sections per well and six wells per embryo).

4.1.9. Mount the desired sections in microscope glass slides using a fine brush. Cover them with glass coverslips. For long-term storage add mounting medium, which prevents photobleaching and photooxidation. Observe the slides under an upright epifluorescence microscope to assess electroporation efficacy.

4.2. Strategy B

4.2.1. Let the pups previously electroporated at E13.5 and E15.5 be born and wait until P15 to perform transcardial perfusion using the same fixative solution used in step 4.1.4.

4.2.2. Just before perfusion, intraperitoneally administer a dose of 75/1 mg/kg ketamine/medetomidine. When the pedal reflex is lost, secure the mouse in a supine position and make a ventral incision following the middle line using scissors to expose both the rib cage and diaphragm.

4.2.3. Cut the diaphragm and open the rib cage to gain access to the heart and hold it using a hemostat. Make an incision in the right atrium with fine scissors.

4.2.4. Penetrate the left ventricle with a needle connected with a flexible tube to a peristaltic perfusion pump. Start transcardial perfusion, delivering at least 25 mL of 4% PFA at a constant flux of 5.5 mL/min (total time ~5 min).

4.2.5. Dissect the brain of perfused animals. First, remove the skin over the head with scissors and forceps. Start cutting the skull using scissors and carefully pull off sections of the skull bones until they are completely removed. Finally, extract the brain with the help of a spatula.

4.2.6. Transfer the brains to a 24 well plate and fix them with 4% PFA overnight at 4 °C on an orbital shaker. Stop fixation by replacing PFA with 0.05% (w/v) sodium azide in PBS.

NOTE: As indicated in step 4.1.5, it is advisable to carry out an intermediate wash with 1x PBS.

4.2.7. Repeat steps 4.1.6–4.1.9, changing the vibratome parameters for postnatal brains (40 µm width, 1.20 µm/s speed, and 0.5 mm of amplitude).

NOTE: Immunohistochemistry or immunofluorescence can be carried out to detect specific cell markers or enhance the signal of fluorescent proteins used in electroporation.

5. Confocal fluorescence imaging and analysis

5.1. Turn on the confocal microscope, place the microscope slides containing the mounted brain sections onto the microscope slides holder and select the channels at which fluorescence images will be taken (i.e., 420–460 nm for BFP, 490–540 nm for GFP, and 570–620 nm for mCherry and tdTomato).

5.2. Perform sample scanning to obtain map images of each brain section at two different wavelengths for a general view of the double electroporation output. Once finished, select the 10x lens and the multi-area-Z-stack-timelapse observation mode. This will allow programming the automatic acquisition of fluorescence images at different XYZ localizations within brain sections.

5.3. In each of the chosen regions (XY), set proper imaging parameters (i.e., laser intensity, photomultiplier sensitivity, and a minimum resolution of 1,024 x 1,024), as well as the depth of the scanning (Z) according to the planes of the sample where fluorescence is visible.

5.4. Obtain low magnification (10x) images of all the chosen regions and export them from OIF to TIFF format using the microscope viewer software.

5.5. Change to the 60x lens, repeat step 5.3 and capture high magnification images to observe the cell-cell interactions in a more detailed manner. Export them as indicated in step 5.4.

5.6. Open the acquired images with any imaging software (e.g., Fiji) for further analysis.

REPRESENTATIVE RESULTS:

Interactions between neighboring cells originated in distal places and at different times: Cajal-Retzius cells (CR-cells) and early migrating cortical projection neurons (strategy A)

The interaction of CR-cells and early cortical projection neurons was previously described as necessary to regulate somal translocation via nectin and cadherin adhesion molecules using a double electroporation strategy[8]. CR-cells originated from the neuroepithelium at the edges of the pallium and migrated tangentially to populate the most superficial part of the cortex, the marginal zone[17–19], whereas cortical projection neurons were generated in the proliferative zone of the cerebral cortex and migrated radially into the nascent cortical plate[20]. There was a temporal difference in the generation of both types of cells. CR-cells were generated at very early embryonic stages from E10.5[21,22] and cortical projection neurons that migrate by somal translocation were born from E12.5–E13.5[23]. Using double in utero electroporation, the temporal gap between the surgeries allowed CR-cells, targeted at E11.5 in one of its places of origin (the cortical hem), to reach the marginal zone of the lateral neocortex including the somatosensory area in time to establish contacts with cortical projection neurons labeled at E13.5 (**Figure 1A**,**B**). The leading processes of projection neurons expressing enhanced GFP (EGFP) profusely arborized in the marginal zone of the cortex and intermingled with the processes of CR-cells that expressed mCherry (**Figure 1C**). Functional experiments have shown that perturbation of cell-cell adhesion molecules expressed by projection neurons or CR-cells affects the arborization of their processes as a consequence of altered contacts between both cell types[8].

Long-range interactions between distal cells generated at different times: innervation of upper layer callosal projection neurons to the contralateral side of the neocortex (strategy B)

Callosal cortical projection neurons were present throughout the cerebral cortex, being more abundant in upper layers[24]. These neurons projected their axons through the corpus callosum and contacted projection neurons in the contralateral side, mostly located in layers II-III and layer V, but some cells were also present in other layers[25–27]. Upper layer projection is evolutionarily newer than lower layer neurons and have been greatly expanded in primates[28]. These cells are critical for complex thought and higher associative tasks, and dysfunctions in groups of genes specifically expressed by this population of cells have been recently related with autism[29].

In order to study specifically the interactions of the subpopulation of callosal projection neurons located in the upper layers with their target cells distributed throughout the contralateral hemisphere, we developed a double in utero electroporation protocol. To label the target cells of upper layer callosal projection neurons we performed in utero electroporation at E13.5 using a BFP expressing plasmid (**Figure 2A**–**C**). This age was strategically chosen because it allowed not only targeting of a broad population of cortical projection neurons including many layer-V neurons, but also a considerable number of neurons located in upper layers (**Figure 2C**), basically covering all target areas of callosal projection neurons from contralateral hemisphere. A second electroporation in the contralateral side at E15.5 targeted the upper layer callosal projection neurons subpopulation (expressing nEGFP and mtdTomato) (**Figure 2A**,**B**,**D**) but not the lower layer callosal projection neurons born at earlier ages. The need of heterochronic double electroporation was therefore justified because of the different times in the generation of the projecting cells of interest and the cells innervated by them. Those upper layer targeted neurons sent their axons to the contralateral hemisphere with a characteristic arborization pattern (**Figure 2C**). Differences in this typical axonal arborization pattern could be evaluated upon gain or loss of function experiments in target cells using this double electroporation protocol. High

magnification analysis showed in detail the callosal axons innervating targeted projection neurons in the contralateral hemisphere (**Figure 2E**).

FIGURE AND TABLE LEGENDS:

Figure 1: Strategy A. Double in utero electroporation strategy to study close interactions among cells with different spatial and temporal origin. (**A**) Schematics of the protocol used to target CR-cells expressing mCherry and cortical projection neurons expressing EGFP. (**B**) Representative image of a coronal section of a brain after double electroporation in the cortical hem and the lateral cortex. Cells targeted in the cortical hem (red) migrated tangentially, populating the marginal zone of the neocortex. Labeled cells in the ventricular zone of the cortex generated cortical projection neurons (green) that migrated radially to enter the nascent cortical plate. Scale bar = 200 µm. (**C**) Magnification of the area boxed in panel **B** displaying the somatosensory cortex and the two types of cells labeled after the double electroporation protocol. Dashed lines frame the marginal zone and cortical plate. Scale bar = 100 µm. High magnification on the right shows a detail of the marginal zone containing the arborized leading processes of the projection neurons intermingled with CR-cells bodies and processes. Scale bar = 10 µm. Ctx = cortex; Hem = cortical hem; MZ = marginal zone; CP = cortical plate; IZ = intermediate zone.

Figure 2: Strategy B. Double electroporation strategy to study long-range interactions among cells with different spatial and temporal origin. (**A**) Scheme displaying the strategy to target different populations of cortical projection neurons in the lateral neocortex including the somatosensory area in different hemispheres by double in utero electroporation. Projection neurons in the somatosensory cortex of the right hemisphere targeted at E13.5 expressed BFP. Targeted projection neurons in the contralateral side targeted at E15.5 expressed nuclear EGFP (nEGFP) and membrane-targeted tdTomato (mtdTomato). (**B**) Representative image of a coronal section of a brain that underwent double electroporation surgery with the mentioned plasmids in panel **A**. Note the different distributions of the cells labeled by BFP or nEGFP and the intense labeling of the axons of upper layer callosal projection neurons (mtdTomato) labeled at E15.5. Scale bar = 500 µm. (**C**) Image of the somatosensory cortex located in the right hemisphere in a double electroporated brain. Note the broad distribution of the projection neurons (blue) across layers and the profuse arborization of the callosal axons (red) coming from projection neurons targeted in the contralateral hemisphere. Scale bar = 100 µm. (**D**) Image of the somatosensory cortex in the left hemisphere of a double electroporated brain. Note the discrete localization of the targeted projection neurons in the upper part of the cortical plate as shown by the expression of nEGFP (green), as well as the profuse red labeling surrounding cell bodies and all neuronal projections (red). Scale bar = 100 µm. (**E**) High magnification pictures of the somatosensory cortex in the right hemisphere showing details of the arborization of the callosal axons around targeted projection neurons. Scale bar = 10 µm.

Table 1: Summary of the electroporation conditions used in the different experiments.

Table 2: Survival of embryos following double in utero electroporation in Strategy A.

Table 3: Survival of pups following double in utero electroporation in Strategy B compared with

simple electroporation.

DISCUSSION:

The study of cell-cell interactions in vivo in regions with high cellular density like the cerebral cortex is a complex task. Traditional approaches including the use of antibodies to label neurites are not suitable because of the lack of specific markers for different cell populations. The use of transgenic murine models, where a particular cell type expresses a fluorescent protein, is useful to visualize the neuronal processes, but this depends on the availability of such models. This task is even more complicated when trying to visualize possible differences in the interactions between two particular cell types upon perturbation of the genes of interest, because it involves the use of other animal models, like knockout mice. All of these issues made these studies complicated in the past due to economic and temporal costs.

The emergence of new techniques allowing somatic transgenesis in vivo, such as in utero electroporation, offers the possibility to design strategies like the one described in this protocol, which circumvent the use or generation of combined reporter and knockout animals, making this type of experiment more feasible. The results shown in this publication and previously published studies[8] demonstrate that this protocol 1) successfully permits the targeting of cell populations with different temporal and spatial origin; 2) makes possible the visualization of cell-cell contacts with high resolution; and 3) is useful to detect differences in cell contacts after functional experiments.

Despite the wide use of in utero electroporation, this technique requires considerable training in order to safely perform the surgery, manipulate the embryos without damaging them, and assure the correct targeting of the desired region. However, after this training, survival of pregnant females is excellent (around 100% in our hands) and we have found no differences between single and double in utero electroporation. Single and double electroporated pregnant females recover very quickly from the surgery. In the vast majority of the cases, their behavior the day after single or double surgery seem normal and these pregnant females eat, drink, walk, and even climb without difficulties without apparent signs of pain and distress.

Survival of the embryos after the first and the second electroporation is also good overall, and the rate of abortion decreases as the age of the embryos increases (**Table 2** and **Table 3**). The main difficulty is successful targeting in both electroporations. With older embryos from E13.5 onwards the degree of success is very high. Early ages like E11.5 are more challenging because the small size of the embryos makes handling and injection more difficult, which in addition affects their survival. However, embryos surviving the first E11.5 electroporation present very good rates of survival after the second electroporation at E13.5 (**Table 2**). To improve proficiency with this technique, we strongly recommend practicing surgeries in pups at ~E14.5 and progressively trying surgeries at younger ages.

Another challenge is postnatal survival, because mothers undergoing surgery do not always take care of all of their pups, although pregnant females single or double electroporated deliver the pups without problems (**Table 3**) and their behavior and fitness status look normal. In our hands,

and with the timing described here, we find no important differences in postnatal survival after simple and double electroporation (**Table 3**), but to circumvent possible survival problems pups can be transferred to foster mothers upon delivery when real mothers display poor maternal behavior at birth. Providing extra nesting material and rich food to pregnant females previous to the delivery date can also help to increase the survival rates of the pups.

One of the main advantages of this strategy is the use of wild type mice for functional studies, but it can also be applied, for example, to CRE reporter mice or floxed mice for genes of interest, when available. In these mice, electroporation of CRE-recombinase expressing plasmids will allow the permanent expression of the reporter gene or the inactivation of the desired gene, respectively, in the targeted cells. For functional experiments we can also control the expression of the construct only in the cell type of interest. For example, a neuronal promoter can be used to manipulate a candidate gene only in neurons and not in progenitors, hence preventing undesired effects at the progenitor level. All of these considerations, together with the control of the time and the targeted region, make double in utero electroporation a very versatile technique to study cell-cell interactions not only inside the cortex but also in other structures that can be targeted using this technology.

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DISCLOSURES:

The authors have nothing to disclose.

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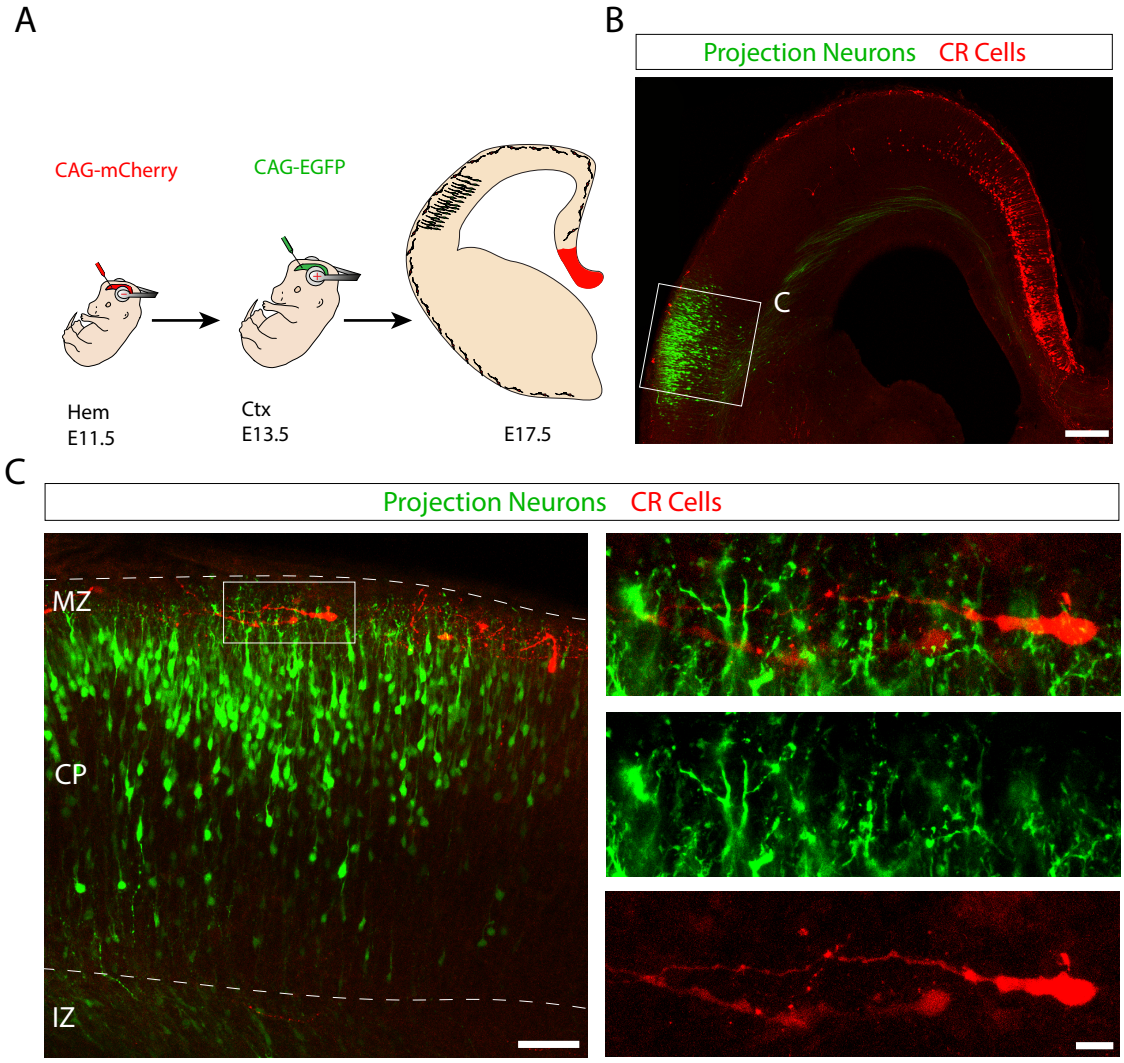


Figure 2

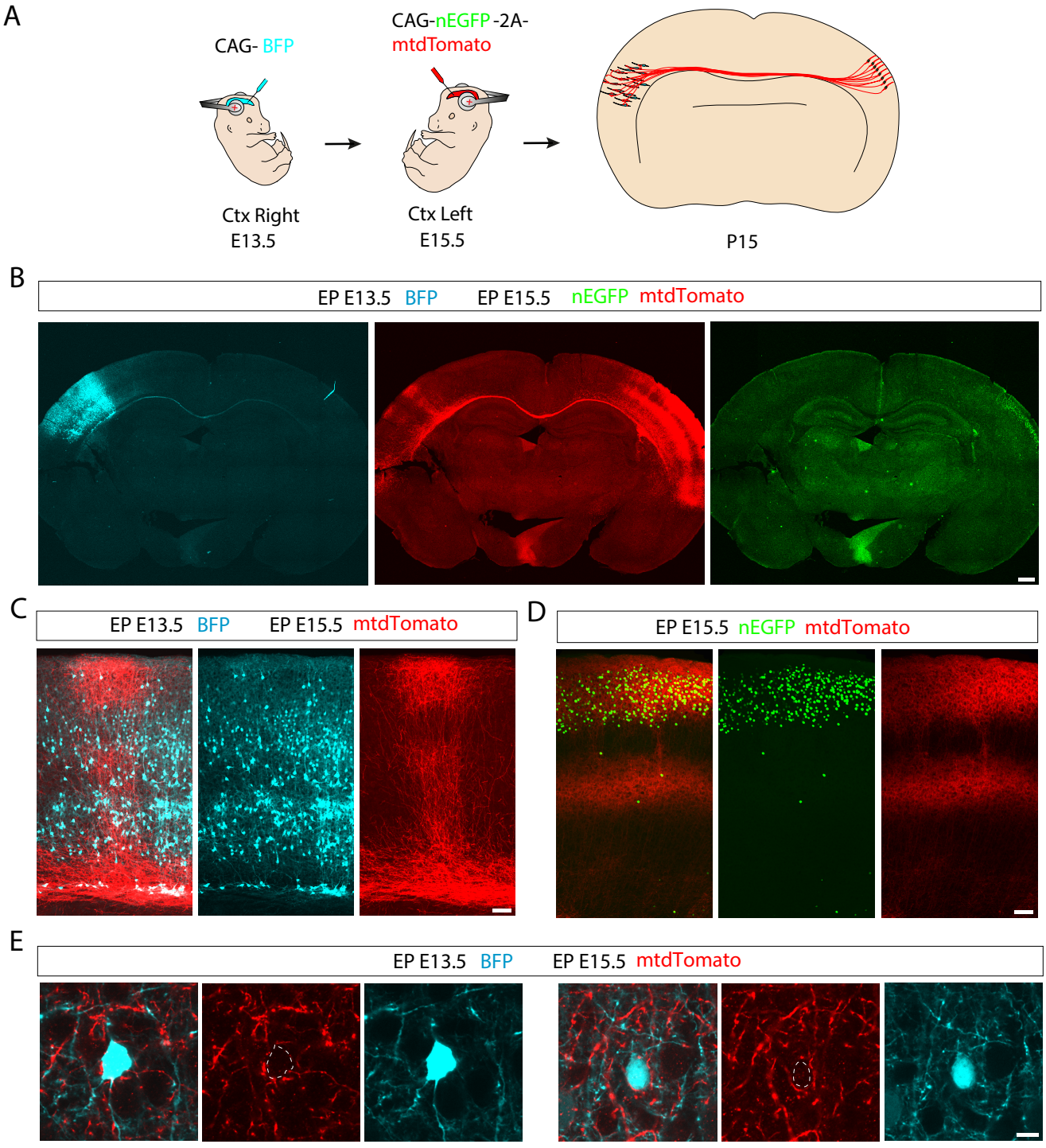


Table 1

Strategy	Order of electroporation	Target cells	Targeted region	Ventricle injected	Location of the electrodes	Voltage and pulses
A	First	CR-cell at E11.5	Cortical Hem	Left	Positive in left hemisphere (directed towards the medial wall)	25 V, 40 ms, 4p
	Second	Cortical Projection neurons at E13.5	Somatosensory Cortex	Left	Positive in left hemisphere (directed towards the cortex)	35 V, 60 ms, 5p
B	First	Cortical Projection neurons at E13.5	Somatosensory Cortex	Right	Positive in right hemisphere (directed towards the cortex)	35 V, 60 ms, 5p
	Second	Cortical Projection neurons at E15.5	Somatosensory Cortex	Left	Positive in left hemisphere (directed towards the cortex)	50 V, 80 ms, 5p

Plasmids used	Age of analysis
CAG-mCherry	E17.5
CAG-EGFP	
CAG-BFP	P15
CAG-nEGFP-2A-mTdTomato	

Table 2 Click here to access/download;Table;Table 2.xlsx

Survival of embryos following double in utero electroporation in Strategy A

Number of litters	Initial number of embryos		Number of embryos surviving first EP		Number of embryos surviving second EP	
9	Total number	(Avg. litter ± SD)	Total number	(Avg. litter ± SD)	Total number	(Avg. litter ± SD)
	64	7.11 ± 2.08	48	5.33 ± 1.22	40	4.44 ± 1.01
	Number of aborted embryos after first EP		Number of aborted embryos after second EP		Total number of aborted embryos	
	Total number	(Avg. litter ± SD)	Total number	(Avg. litter ± SD)	Total number	(Avg. litter ± SD)
	16	1.8 ± 1.09	8	0.88 ± 0.78	24	2.67 ± 1.32

*Survival and abortion calculated considering the embryos that survived first electroporation

% of survival after first EP	% of survival after second EP*	% of global survival rate
75%	83.33%	62.50%
% of abortion	% of abortion after second	% of global abortion
25%	16.66%	37.50%

Table 3 Click here to access/download;Table;Table 3.xlsx

Survival of embryos and pups following Strategy B (double EP E13.5 and E15.5)

Number of litters	Initial number of embryos		Number of embryos surviving first EP		Number of pu double EP
8	Total number	(Avg. litter ± SD)	Total number	(Avg. litter ± SD)	Total number
	52	6.5 ± 2	46	5.75 ± 2.05	43

Survival of embryos and pups following single EP at E13.5

Number of litters	Initial number of embryos		Number of pups born after EP		Number of en P15
11	Total number	(Avg. litter ± SD)	Total number	(Avg. litter ± SD)	Total number
	78	7.1 ± 1.64	70	6.36 ± 1.7	45

ups born after	Number of embryos surviving at P15		% of survival after first EP	% of survival after second EP	% of survival at P15
(Avg. litter ± SD)	Total number	(Avg. litter ± SD)	88.46%	82.69%	55.77%
5.38 ± 1.77	29	3.63 ± 1.6			

nbryos surviving at	% of survival after EP	% of survival at P15
(Avg. litter ± SD)	89.74%	57.69%
4.09 ± 2.07		

Name of Material/Equipment	Company	Catalog Number
Ampicillin sodium salt	Sigma-Aldrich	A9518-25G
Aspirator tube	Sigma-Aldrich	A5177-5EA
Baby-mixter hemostat (perfusion)	Fine Science Tools (FST)	13013-14
Borosilicate glass capillary	WPI	1B100-6
Buprenorphine (BUPREX 0,3 mg/ml)	Rb Pharmaceuticals Limited	921425
CAG-BFP plasmid	Kindly provided by U.Müller Lab	
CAG-EGFP plasmid	Kindly provided by U.Müller Lab	
CAG-mCherry plasmid	Kindly provided by U.Müller Lab	
CAG-mtdTomato-2A-nGFP plasmid	Kindly provided by U.Müller Lab	
Confocal microscope	Olympus	FV10i
Cotton Swabs	BFHCVDF	
Cyanoacrylate glue	B. Braun Surgical	1050044
Dissecting scope	Zeiss	stemi 305
Dumont Forceps #5 Fine Forceps	Fine Science Tools (FST)	11254-20
ECM830 Square Wave Electroporator	BTX	45-0052
Electric Razor	Oster	76998
Endotoxin-free TE buffer	QIAGEN	1018499
Ethanol wipes	BFHCVDF	
Extra Fine Graefe Forceps	Fine Science Tools (FST)	11150-10
Eye ointment	Alcon	682542.6
Fast Green dye	Sigma-Aldrich	F7252-5G
Fine Scissors	Fine Science Tools (FST)	14069-09
Fluorescence LEDs	CoolLED	pE-300-W
Genopure Plasmid Maxi Kit	Roche	3143422001
Halsted-Mosquito Hemostats (suture)	Fine Science Tools (FST)	91308-12
Heating Pad	UFESA	AL5514
Inverted epifluorescence microscope	Nikon	Eclipse TE2000-S
Iodine wipes	Lorsoul	
Isofluorane vaporizer	Flow-Meter	A15B5001
Isoflurane	Karizoo	586259
Ketamine (Anastemine)	Fatro Ibérica SL	583889-2
Kimtech precision wipes	Kimberly-Clark	7252
LB (Lennox) Agar GEN	Labkem	AGLB-00P-500
LB (Lennox) broth GEN	Labkem	LBBR-00P-500
Low-melting point agarose	Fisher Scientific	BP165-25
Medetomidine (Sedator)	Dechra	573749.2
Microscope coverslips	Menel-Gläser	15747592
Microscope Slides	Labbox	SLIB-F10-050
Mounting medium	Electron Microscopy Sciences	17984-25

Mutiwell plates (24)	SPL Life Sciences	32024
Mutiwell plates (48)	SPL Life Sciences	32048
NaCl (for saline solution)	Fisher Scientific	10112640
Needle 25 G (BD Microlance 3)	Becton, Dickinson and Company	300600
Orbital incubator S150	Stuart Scientific	5133
P Selecta Incubator	J. P. Selecta, s.a.	0485472
Paraformaldehyde	PanReac AppliedChem	A3813
Penicillin-Streptomycin	Sigma -Aldrich	P4333
Peristaltic perfusion pump	Cole-Parmer	EW-07522-30
Platinum Tweezertrode, 5 mm Diameter	Btx	45-0489
Reflex Skin Closure System - 7mm Clips, box of 100	AgnThos	203-1000
Reflex Skin Closure System - Clip Applyer, 7mm	AgnThos	204-1000
Ring Forceps	Fine Science Tools (FST)	11103-09
Sodium azide	PanReac AppliedChem	122712-1608
Surgical absorbent pad (steryle)	HK Surgical	PD-M
Suture (Surgicryl PGA 6-0)	SMI Suture Materials	BYD11071512
Syringe 1ml (BD plastipak)	Becton, Dickinson and Company	303172
Tissue Culture Dish 100 x 20 mm	Falcon	353003
Vertical Micropipette Puller	Sutter Instrument Co	P-30
Vertical microscope	Nikon	Eclipse Ni
Vibratome	Leica	VT1200S

Comments/Description

We would like to thank the editor and the reviewers for their constructive criticisms and suggestions that have helped us to improve the manuscript. We have addressed editor requests and the concerns raised by the reviewers and we included additional tables with the survival rates of embryos and pups in single vs double electroporation experiments as requested by reviewer # 1.

Below is our point-by point response to the editor and the reviewers´ comments:

Editorial comments:

Changes to be made by the author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version. We have proofread the manuscript trying to fix possible spelling and grammar issues
2. Authors and affiliations: Please provide an email address for each author in the manuscript. We added this info.
3. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below. We added more detail to the protocol as requested.
4. 1.1.1: Please include the maxi-prep kit used in the Table of Materials. Done Obtain plasmid DNA from what? Please specify. We have provided more information about the obtaining of the DNA at the beginning of the protocol.
5. 1.1.2: What volume of TE buffer is used? 9 µL? The amount of TE depends on the concentration of the DNA, which varies. We have provided an example to explain this “For example, take 2 µl of a 5 µg/µl plasmid DNA solution, 1 µl of Fast Green dye and 7 µl of TE buffer”
6. 1.3.4: Please specify the size of the syringe and the volume of the analgesic solution. Done (1 ml, 150 ul).
7. 1.3.5: For plasmid DNA solution, do you mean the solution prepared in step 1.1.2? Yes, we have now specified it.
8. 2.1: Please specify the strain of mouse used. We apologize for this mistake. The strain is C57BL6 we have now specified it in the manuscript.
9. 4.2.2: For how long does the perfusion last? It last around 5 min, duration of the procedure is now specified. Please specify surgical tools used here. We have added this information in the perfusion section.
10. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step. Please include single line spacing between each numbered step or note in the protocol. Done
11. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text. Done
12. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting. We have tried our best to obtain a cohesive narrative. We excluded notes from the highlighting.
13. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted. We believe we have followed these instructions properly.
14. Please do not highlight any steps describing anesthetization and euthanasia. Done.
15. Figures: Please include a scale bar, ideally at the lower right corner, for all microscopic images to provide context to the magnification used. Define the scale in the appropriate figure Legend. We apologize for forgetting the scale bars. We added them to the figures and defined them on the figure legends
16. Please upload Table 1 to your Editorial Manager account as an .xlsx file. Done
17. Table of Materials: Please sort the materials alphabetically by material name. Done.
18. References: Please do not abbreviate journal titles; use full journal name. Done

Reviewer #1:

1) “The Authors state in the discussion that this new protocol…The protocol is very well described and the experimental examples provided are straightforward. Nevertheless, this protocol fails to show any new procedures; it describes just two consecutive in utero electroporations (IUE) at different time points and at different cortical regions in the same pregnant female.”

We would like first to thank reviewer #1 for his/her comments. We agree that the base of the methodology described here consists in the use of two consecutives in utero electroporations, but electroporation are performed at different ages and places and we believe this is not trivial, because the strategy detailed here provide a powerful tool to study interactions between cells with different spatial and temporal origins that otherwise will be almost impossible to study. The current manuscript offers a detailed explanation of how to successfully perform an *a priori* complex procedure that involve the two *in utero* surgeries at different times and locations and provides valuable examples showing its versatility that we think will be of interest to the scientific community.

2) The major concern is the survival rate of electroporated females after two consecutive IUE. The IUE procedure is very invasive and is usually considered a severe procedure in animal licence protocols. I think that in Europe where animal procedures are very strict, a double IUE would not be accepted by the ethical committee. It is thus very important that the authors shows us a table with the survival rate of the progeny after double IUE, compared to a single IUE procedure, before delivery (thus at E17.5 -E18.5) and after delivery (P5-P7). And also the survival rate and fitness of the electroporated mum. What is the strain of the elelctroporated females? Thus, to better evaluate the strength and efficiency of the method, it is important to add a report on the rate of abortion (strategy A) or delivery (strategy B), as well as the rate of survival at postnatal ages (strategy B) in comparison with the single IUE protocol.

We understand the concerns of reviewer #1 probably due to the incomplete explanations about these aspects in the previous version of the manuscript. Method safety for mother survival was already assessed for its use in several experiments described in a publication leaded by current senior author (Gil-Sanz et al, 2013). IUE is an invasive procedure because requires a laparotomy surgery, but performed under controlled circumstances does not imply risks for the mother survival. Survival of double electroporated females in our hands is the same than simple electroporated females, being around 100%. Females undergoing simple or double IUE surgery equally recover without problems after the surgery and show the same normal behavior the day following the first or the second surgery. Regarding the severity of the technique and according to the Spanish and European animal normative, the laparotomy surgery under anesthesia and with analgesia is considered to be a moderate in severity procedure, although interpretation may vary among ethical committees. About the possible problems on approval of the procedure in Europe, this double IUE procedure has been already approved by the ethical committee of the Universidad de Valencia (Spain) like the rest of the IUE procedures of our laboratory. In the current version of the manuscript we have added additional information regarding the survival of mothers in the discussion section and stated the approval by our animal committee at the beginning of the protocol.

We also want to thank the reviewer for suggesting the addition of tables clarifying embryos’ and pups’ survival rates after single versus double electroporations embryonically and postnatally. We have now included in the discussion more details about these questions in the discussion section and added tables with survival rates (Tables 2 and 3) as requested. Briefly, in our hands no important differences are found either in prenatal/postnatal survival or pup delivery, in double versus single electroporated embryos or pups.

Main difficulties with strategy A are related to the ability to manipulate and successfully target the desired part of the brain in very early embryos (E11.5). This increased difficulty in their manipulation is linked to the higher mortality at that age; however, embryos that survive after the first surgery have not important survival problems after second electroporation. Due to the difficulty to manipulate small embryos, we advise to learn and acquire enough expertise in the IUE technique with older ages E13-E14. We also want to state that efficiency and strength of the method is already proven as demonstrated by our previous publication in which this technique was used in several experiments (Gil-Sanz et al. 2013).

Respecting the strategy B, embryos’ survival after simple or double electroporation is higher than in A because of the increased hardiness of these older embryos (E13-E15). Moreover, no remarkable differences are found after single or double electroporation. No substantial differences in the delivery rate of the pups are found in simple versus double electroporated females. Main difficulties are associated with maternal behavior of the electroporated mothers. Similarly to what happens with single electroporated mothers, a proportion of them (displaying normal behavior and normal fitness status) take care of all the pups whereas others do not. Evaluation of this behavior after delivery helps to distinguish between “good or bad mothers”. When the mother displays poor maternal behavior, the use of foster mothers is helpful for us. Likewise, we provide extra-nesting material and tastier food including sunflowers seeds to electroporated females before the delivery date to try to favor maternal behavior. Again, no important differences have been found between simple or double electroporated females. We added more info in the discussion section and provided a table with survival rates.

The strain we have used for those experiments is the inbreed strain C57BL/6, we apologize for forgetting to provide this info. Better rates of maternal care after surgery are found using mixed background females (C57BL/6;129) or outbreed strains like CD-1 mice. Using those strains can help but differences among strains need to be evaluated before deciding timings for the surgeries.

3) "line 208: sodium azide is not a fixator, but an antibacterial agent"
We apologize for that mistake that has already been fixed.

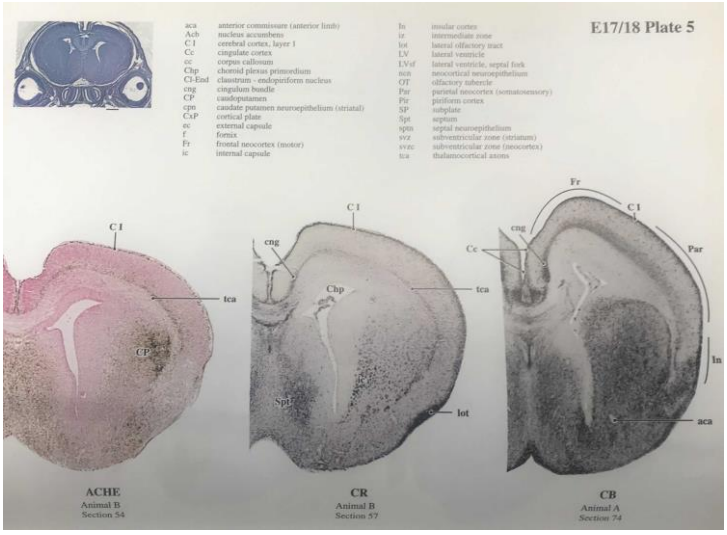
4) "The IUE electroporation performed at E13.5 in the two experiments seems to target all layers and not only deep layers as it should be when IUE is performed at E13.5 (see Figure 1C and Figure 2C, D). If the authors wish to target layer V, then they should electroporate earlier than E13.5"
In utero electroporation at E13.5 targets many layer V projection neurons and also part of upper layer projection neurons, being E13.5 probably one of the ages that allows broader labeling of cortical projection neurons. This is the reason why this age was chosen. Our intention in this particular experiment was to label cells in different layers, because axons sent by upper layer cortical neurons to the contralateral side not only innervate layer V, but also layers II-III. We have avoided the use of transposon versions that would permit broader labeling across layers because it could also make more difficult the observation of fine contacts. We added additional information on the manuscript regarding to the election of the age for the electroporations in the representative results section.

Reviewer #2:

1) Can authors suggest type of mouth-controlled aspirator tube to be used for double in utero electroporation
We use the "Aspirator tube assemblies for calibrated microcapillary pipettes" from Sigma (A5177-5E) referred in the materials table as "Aspirator tube".

2) Figure 1: authors may want to state what does the dashed white line represent in C.
We want to apologize for forgetting to mention what dashed lines mean. Upper dashed line delimits the marginal zone and lower dashed line the edge between intermediate zone and cortical plate. We have incorporated this info in the figure legend.

3) Figure 1.: GFP electroporated area is very lateral and may not be only somatosensory cortex that was transfected, as it is suggested. Authors may want to confirm in developmental atlases available and cite it.
We appreciate reviewer #2 comment regarding the electroporated region in Figure 1. The orientation of electrodes proposed in the scheme is aimed to target the parietal neocortex (somatosensory) although sometimes in early embryos we can also hit more lateral locations including insular, perirhinal or piriform cortex. Concerning this particular picture after the analysis of embryonic atlases of similar ages and rostrocaudal levels we believe the electroporated area is located in the parietal neocortex but it is close to the insular cortex (see attached picture from the Chemoarchitectonic Atlas of the Developing Mouse Brain, Jacobowitz and Abbott). In order to prevent possible misinterpretations, we will use the following term to describe the region targeted at these early ages: "lateral neocortex including somatosensory area".



4) Authors may want to discuss if there is any advantage in using strategy B over double hemisphere electroporation at the same embryonic day E13.5.

It is known that callosal projection neurons are located in both upper and lower layers and that projections sent by these neurons innervate upper- and lower-layer neurons in the contralateral site. In this particular experiment we wanted to study specifically the contacts of a **subpopulation of callosal projection neurons**, **those situated in upper layers**, with targeted projection neurons located in both upper and lower layers of the contralateral cortex. Therefore, the main advantage of the double heterochronic electroporation *versus* double hemisphere electroporation is the possibility to target a subpopulation of callosal projection neurons, because E15.5 electroporation does not target lower layer callosal projection neurons. If we wanted to study callosal projections in general (from upper- and lower-layers projection neurons), double hemisphere electroporation at E13.5 could work. We have tried to further clarify these aspects on the manuscript (representative results section).

5) Authors may want to cite more recent reviews on neocortical development (e.g. Popovitchenko and Rasin, 2017) and possible advanced usage of in utero electroporation in the in vitro studies (e.g. Popovitchenk et al., 2016).

We kindly thank reviewer #2 suggestions about citation of reviews on cortical development and other uses of IUE technique. We have added new references related with these suggestions in the new version of the manuscript.

We want to thank the editor for the revision of the manuscript. We have addressed all the editorial comments on the formatted manuscript.

Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain the same. The updated manuscript is attached and please use this version to incorporate the changes that are requested. We have used the formatted manuscript for our editions.
2. Please address specific comments marked in the attached manuscript. Please turn on Track Changes to keep track of the changes you make to the manuscript. We have addressed all of the editor comments using the Track Changes as requested.