Video Article
Subtype-selective Electroporation of Cortical Interneurons

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Abstract

The study of central nervous system (CNS) maturation relies on genetic targeting of neuronal populations. However, the task of restricting the expression of genes of interest to specific neuronal subtypes has proven remarkably challenging due to the relative scarcity of specific promoter elements. GABAergic interneurons constitute a neuronal population with extensive genetic and morphological diversity. Indeed, more than 11 different subtypes of GABAergic interneurons have been characterized in the mouse cortex[1]. Here we present an adapted protocol for selective targeting of GABAergic populations. We achieved subtype selective targeting of GABAergic interneurons by using the enhancer element of the homeobox transcription factors *Dlx5* and *Dlx6,* homologues of the Drosophila distal-less (Dll) gene[2,3], to drive the expression of specific genes through *in utero* electroporation.

Video Link

The video component of this article can be found at http://www.jove.com/video/51518/

Introduction

The bulk of cortical GABAergic interneurons originate from two transient embryonic structures named the medial and caudal ganglionic eminences (MGE and CGE respectively)[4]. Parvalbumin and somatostatin expressing interneurons originate in the MGE whereas Calretinin (Cr), Vasointestinal peptide (VIP) and Reelin (Re) expressing interneurons originate from the CGE. These interneuron subtypes can be distinguished by their birthdates. MGE derived subtypes are born between embryonic day 9.5 (e9.5) and e16.5[5,6]. In contrast, CGE derived interneurons are born from e12.5 through e18.5 with their production peaking at e15.5[6]. The genetic targeting of this late born population, however, remains elusive.

The murine distal-less (Dlx) genes are exclusively expressed in the developing ventral forebrain[3]. GABAergic interneurons and striatal projection neurons but not cortical pyramidal cells express *Dlx1,2,5,* and *6* genes at early developmental stages[3]. Indeed, the *Dlx* genes are expressed in the MGE and CGE subventricular zone (SVZ) in all GABAergic progenitors. Expression of these genes becomes restricted to select subtypes at postmitotic stages[7-9]. Previous experimental evidence showed that the *Dlx5/6* enhancer element allows for the selective targeting of GABAergic lineages in transgenic mouse models[2]. We tested the use of one of these enhancer elements in the context of episomal expression in the developing mouse brain. We sub cloned the Dlx5/6 enhancer element together with a minimal promoter and the enhanced green fluorescent protein (eGFP) in a bluescript (BS) backbone plasmid (**Figure 1**). We introduced the plasmid by means of *in utero* electroporation at e15.5 to selectively target Cr-, VIP and Re- subtypes[3,8,10]. Our technique allows for sparse electroporation, which facilitates the reconstruction of morphological features of singe cells. In addition, the exceptionally high levels of gene expression in cortical GABAergic neurons allows for functional studies. We carried out loss and gain of function studies using several wild type and dominant negative genes[11].

Protocol

All animals were treated in accordance with the regulations and guidelines of the Institutional Animal Care and Use Committee of the NYU School of Medicine.

Mouse Strains
Swiss Webster female mice provided by TACONIC were used for these experiments. In order to specifically target superficial layer interneurons, e15.5 embryos were used.

Note: The plasmid used in this work (*Dlx5/6.eGFP* plasmid 3 μg/μl) was generated using standard cloning techniques. The *eGFP* cDNA was cloned into a *Dlx5/6-Pmin-polyA* plasmid. This plasmid is available upon request (demarn02@nyumc.org).

1. Preparation of Microinjection Pipettes

1. Set the puller Heat #2 to 860.
2. Place and secure the glass capillary near the filament. Check that the outer diameter (OD) of the pipette is roughly 30 µm.
3. Press the "pull" button.
4. Carefully remove the capillary. The bottom part is the needle. Discard the top part.

2. Anesthesia Procedure

1. Prepare an isoflurane containing chamber. Note: Isoflurane is the preferred anesthetic due to its fast action and low incidence of side effects. Pentobarbital is another option; however, tolerance of this drug is variable and may lead to a higher mortality rate than isoflurane.
2. Set the flow of isoflurane to 4-5% in 0.5-1 L/min O_2.
3. Make sure that the valve for the chamber is open whereas the valve for the head adaptor is closed.
4. Place the pregnant mouse in the chamber.
5. Allow the mouse to go under the effect of anesthesia. This will take about 2-3 min. Check the breathing rate. If the mouse begins hyperventilating, lower the dose of isoflurane.
6. After the mouse is anaesthetized, promptly remove it from the chamber. Place it ventral side up on the heating pad and insert the head in the mask that will continue to supply isoflurane throughout the surgery. Make sure to switch the flow of isoflurane from the chamber to the tubing connecting the head adaptor piece. Put a drop of eye lubricant in each eye and close the eyes.
7. Set the heating pad to 36 °C degrees to minimize hypothermia.
8. Check for the absence of foot and tail reflexes by pinching the rear paws and the tail.

3. Surgery

1. Cleanse the skin covering the abdomen with 70% ethanol.
2. Use forceps to pull the skin upwards. After each surgery, wash all instruments with detergent and water, and sterilized at 72 °C O/N.
3. Make a small vertical incision (about 2 cm) along the midline starting midway between the third and fourth pairs of mammary glands. Use fine surgery scissors. Take care to not damage the abdominal wall.
4. Gently separate the skin from the peritoneum.
5. Visualize the arteries and veins on the abdominal wall. Make another 2 cm vertical incision through the peritoneum (avoiding the vessels) to expose the abdominal cavity.
6. Clamp the skin and abdominal wall on each side avoiding clamping the arteries. Note: If the arteries are accidentally punctured, sacrifice the animal immediately by performing cervical dislocation or isoflurane overdose.
7. Pre warm the sterile PBS to 37 °C. Cover the clamps with PBS moist Kim wipes.
8. Moisturize the exposed abdominal cavity with PBS. Repeat this step several times while performing the surgery.
9. Using round forceps, gently pull the embryonic chain (e15.5) away from the cavity. Let the chain rest on the wet wipes. Start by exposing one half of the uterus first. Once the embryos in it are electroporated, bring it back inside, then work on the second half.
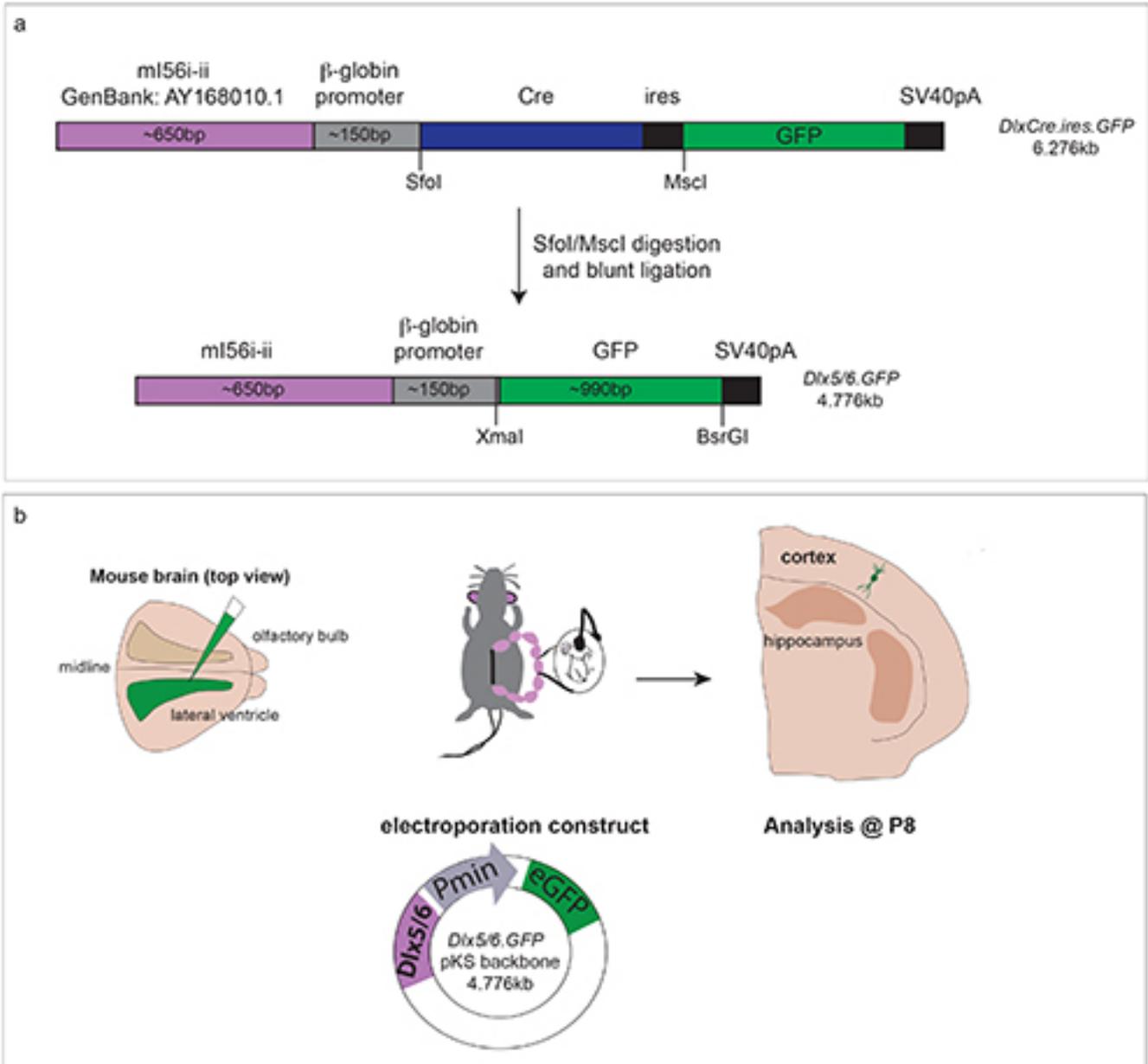
4. Electroporation

1. Continue to use round forceps to manipulate the embryos.
2. Visualize the lateral ventricles. The lateral ventricles run along the midline (**Figure 1b**).
3. Add Fast Green (Stock: 10% w/v) to the DNA solution for a 1:20 mixture to visualize it during the injection. Prepare the DNA solution by dissolving the pellet obtained from a standard maxi-prep purification protocol in distilled water.
4. Use pre pulled micropipettes with a plunger to inject 1 ml of DNA solution (*Dlx5/6-eGFP,* 3 µg/µl) with Fast Green. Cut the tip of the micropipette with a fine forcep #5 (Dumont) to leave 6 mm length from the beginning of the shoulder to the end of the tip of the needle before loading the pipette. Hold the head of the embryo with round tweezers. Enter the brain with the pipette at a 45° angle aiming for the lateral ventricle located near the midline. If the needle penetrates through the ventricle, slowly retract the pipette as you use the plunger to inject DNA. The ventricle should turn green when the solution begins to fill it. At this point, stop moving the pipette and inject 1 ml of DNA. Gently, withdraw the pipette.
5. Set the CUY21 electroporator to five 45V pulses of 50 msec spaced by 950 msec.
6. Use 5 mm paddle electrodes for e15.5 embryos. Dip the electrodes in PBS to ensure efficient current transmission.
7. Place the electrode paddles around the embryo's head with the positive paddle on the ventricle containing the DNA solution. Slightly lower the positive paddle with respect of the negative one to create a ventral current through the ganglionic eminences. This manipulation will minimize ectopic expression in pyramidal cells.
8. After placing the electrodes, deliver a pulse by pressing the foot pedal once.
9. Remove the electrodes and wipe them clean. Repeat steps 4.6-4.9 with each embryo.
10. After electroporating all the embryos, pour 3 ml of PBS into the abdominal cavity and return them to the abdominal cavity.
11. First suture the abdominal wall with a curved needle and 5-0 silk suture and then the skin. Apply Lidocaine (4%) on the wound. Administer 120 mg/kg of Aspirin intra peritoneum for analgesia.
    Note: The entire procedure should be performed in 20 min or less. It is advisable that beginners only electroporate a few embryos to keep the operation time short. Prolonged surgeries will decrease the survival rate of the embryos.
12. Remove the animal from the anesthesia tubing and allow recovery on the heating pad on a paper wipe.
13. Return animal to the cage, keep it on the heating pad at 37 °C and monitor the health status. The animals generally tolerate the surgery well and begin to move slowly soon after they are removed from the anesthesia tubing. If excessive bleeding or lethargy is observed, sacrifice the animal immediately by performing IACUC approved euthanasia procedures such as cervical dislocation or anesthesia overdose.

14. Return cage to the vivarium. The females will give birth naturally.

Representative Results

We adapted the *in utero* electroporation technique to achieve cell type specific targeting of maturing neurons. To drive the expression of eGFP in CGE-derived interneurons, we used the *Dlx5/6* enhancer element and restricted our injections to e15.5, the stage when the majority of CGE-derived interneurons are generated. We carried out the analysis at P8 and P15 [11] (**Figures 1** and **2**). We confirmed the ventral origin of electroporated neurons by co electroporating a *CAG-mCherry* and a *Dlx5/6-eGFP* plasmids at equimolar concentrations at e15.5. In this experiment, we observed that only ventral progenitors located in the subventricular zone (SVZ) co expressed both proteins[11]. This spatio temporal expression of fluorescent proteins coincides with the normal developmental expression of the *Dlx5* and *6* genes, which are not expressed in the ventricular but subventricular zone[4]. Furthermore, we assessed the GABAergic identity of *Dlx5/6-mCherry* electroporated interneurons in a *GAD67-eGFP* knockin mouse line. We found that the vast majority of electroporated neurons expressed GAD67, an enzyme expressed by all GABAergic cells[11]. In addition, we determined the subtype identity of electroporated interneurons by performing immunohistochemistry and electrophysiological analysis at P15. The pattern of expression of subtype specific markers was revealed by immunofluorescence in cryostat sections[11] (**Figure 2**). We found that interneurons electroporated at e15.5 almost exclusively express NPY, Reelin, VIP and Cr, previously described CGE-derived interneuron markers[6]. Furthermore, the intrinsic electrophysiological properties of these neurons are in agreement with the one previously described in fate mapping studies[6,11]. All together, these results indicate that electroporation with the *Dlx5/6* enhancer element at e15.5 selectively target CGE-derived interneuron subtypes.

Figure 1. Schematic representation of the electroporation experiments. a) Subcloning strategy for the *Dlx5/6-eGFP* plasmid. **b)** Diagram illustrating the experimental strategy.
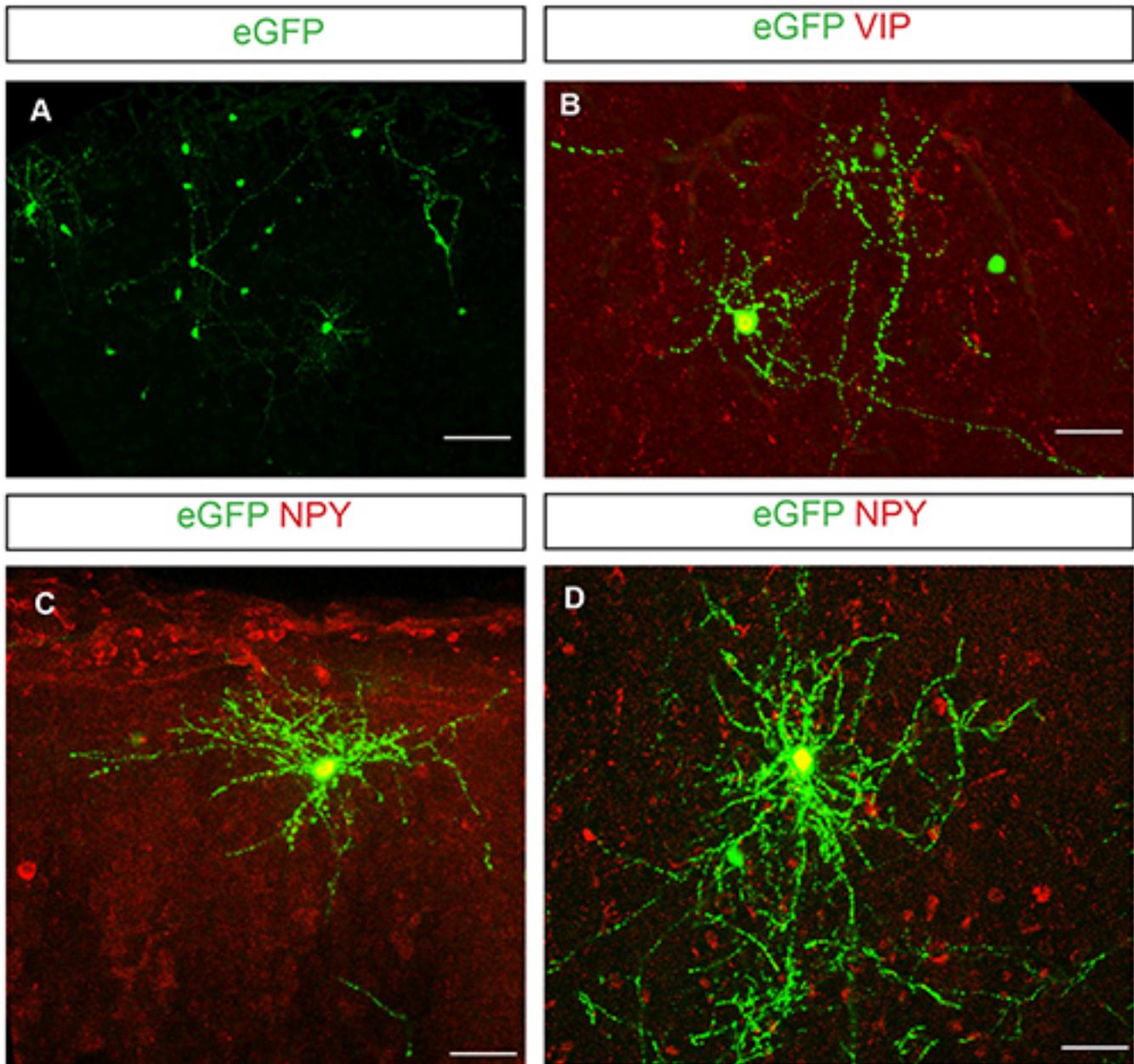
Figure 2. Electroporated interneurons are delineated by the expression of CGE-derived subtype markers. A) CGE-derived interneurons electroporated with a *Dlx5/6-eGFP* plasmid. Note the sparse electroporation of diverse CGE subtypes. **B)** A VIP-expressing interneuron at P8. eGFP expression delineates the entire dentritic tree and axonal arbor of single neurons. **C)** An NPY-expressing interneuron at P8. NPY expression delineates neurogliaform cells. **D)** An NPY-expressing interneuron at P15. Scale bar A, 100 mm; B-D, 50 mm

Discussion

Limitations of the Technique

While this technique allows for cell autonomous analysis of cell processes, it is not suitable for population analysis. The electroporations are very sparse with less than a thousand cells electroporated per brain. As a consequence, the technique cannot be used to assess behavioral consequences arising from the genetic manipulation of CGE-derived interneurons.

While electroporations carried out at e13.5-e14.5 target MGE-derived subtypes, the efficiency is low[10]. We speculate that the *Dlx5/6* enhancer is less active in postmitotic MGE subtypes at least in the context of episomal expression.

Significance with Respect to Existing Methods

The technique represents advancement from previously electroporation methods[12,13] for the study of interneurons. Due to their relative scarcity and ventral embryonic origin, this population of neurons has proven difficult to target. Our technique provides the means to carry out cell-

autonomous analysis of CGE-derived interneurons. The high levels of eGFP expression allow for the visualization and analysis of single interneurons.

Future Applications

By combining the use of specific plasmids and genetically modified mouse lines it is possible to achieve temporal and spatial control in the expression of genes of interest. For example, we used a *Dlx5/6-Tta* plasmid to turn on the expression of tetO inducible transgenes. In these experiments, we were able to silence the transgene expression by administrating Doxycycline[8]. We are currently developing a protocol to use the technique in combination with the CreER system.

Critical Steps within the Protocol

To achieve efficient electroporation and survival, try to avoid excessive manipulation of the embryos and/or organs. In addition, avoid pinching arteries and veins. The mouse will quickly become hypovolemic if bleeding occurs. After the electroporation is completed, place the embryos and organs in the same positions where you found them to avoid creating kinks that could cause hypoxia. Aim for 20 min or less surgery time. Moisturize the abdominal cavity with PBS numerous times during the surgery. During the electroporation, try to puncture the ventricle only once with the capillary needle. Repetitive puncturing will decrease the survival rate. After the training period, the survival rate should be 80% or higher.

Disclosures

The authors have nothing to disclose.

Acknowledgements

References

1. Fishell, G., & Rudy, B. Mechanisms of inhibition within the telencephalon: "where the wild things are". *Annual review of neuroscience.* **34**, 535-567, 061010-113717 (2011).
2. Stenman, J., Toresson, H., & Campbell, K. Identification of two distinct progenitor populations in the lateral ganglionic eminence: implications for striatal and olfactory bulb neurogenesis. *J Neurosci.* **23**, 167-174, (2003).
3. Eisenstat, D. D. *et al.* DLX-1, DLX-2, and DLX-5 expression define distinct stages of basal forebrain differentiation. *J Comp Neurol.* **414**, 217-237 (1999).
4. Batista-Brito, R., & Fishell, G. The developmental integration of cortical interneurons into a functional network. *Curr Top Dev Biol.* **87**, 81-118, (2009).
5. Miyoshi, G., Butt, S. J., Takebayashi, H., & Fishell, G. Physiologically distinct temporal cohorts of cortical interneurons arise from telencephalic Olig2-expressing precursors. *J Neurosci.* **27**, 7786-7798, (2007).
6. Miyoshi, G. *et al.* Genetic fate mapping reveals that the caudal ganglionic eminence produces a large and diverse population of superficial cortical interneurons. *J Neurosci.* **30**, 1582-1594, (2010).
7. Bulfone, A. *et al.* The mouse Dlx-2 (Tes-1) gene is expressed in spatially restricted domains of the forebrain, face and limbs in midgestation mouse embryos. *Mechanisms of development.* **40**, 129-140 (1993).
8. Bulfone, A. *et al.* Spatially restricted expression of Dlx-1, Dlx-2 (Tes-1), Gbx-2, and Wnt-3 in the embryonic day 12.5 mouse forebrain defines potential transverse and longitudinal segmental boundaries. *J Neurosci.* **13**, 3155-3172 (1993).
9. Robinson, G. W., Wray, S., & Mahon, K. A. Spatially restricted expression of a member of a new family of murine Distal-less homeobox genes in the developing forebrain. *The New biologist.* **3**, 1183-1194 (1991).
10. Close, J. *et al.* Satb1 is an activity-modulated transcription factor required for the terminal differentiation and connectivity of medial ganglionic eminence-derived cortical interneurons. *J Neurosci.* **32**, 17690-17705, (2012).
11. De Marco Garcia, N. V., Karayannis, T., & Fishell, G. Neuronal activity is required for the development of specific cortical interneuron subtypes. *Nature.* **472**, 351-355, (2011).
12. Petros, T. J., Rebsam, A., & Mason, C. A. *In utero* and *ex vivo* electroporation for gene expression in mouse retinal ganglion cells. *Journal of visualized experiments : JoVE.* (31), (2009).
13. Walantus, W., Castaneda, D., Elias, L., & Kriegstein, A. *In utero* intraventricular injection and electroporation of E15 mouse embryos. *Journal of visualized experiments : JoVE.* (6), e239, (2007).