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TITLE:

In Vivo Targeting of Neural Progenitor Cells in Ferret Neocortex by In Utero Electroporation

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in utero electroporation, ferret, neocortex development, neural progenitor cells, genetic manipulation, in vivo

SUMMARY:

Presented here is a protocol to perform genetic manipulation in the embryonic ferret brain using in utero electroporation. This method allows for targeting of neural progenitor cells in the neocortex in vivo.

ABSTRACT:

Manipulation of gene expression in vivo during embryonic development is the method of choice when analyzing the role of individual genes during mammalian development. In utero electroporation is a key technique for the manipulation of gene expression in the embryonic mammalian brain in vivo. A protocol for in utero electroporation of the embryonic neocortex of ferrets, a small carnivore, is presented here. The ferret is increasingly being used as a model for neocortex development, because its neocortex exhibits a series of anatomical, histological, cellular, and molecular features that are also present in human and nonhuman primates, but absent in rodent models, such as mouse or rat. In utero electroporation was performed at embryonic day (E) 33, a midneurogenesis stage in ferret. In utero electroporation targets neural progenitor cells lining the lateral ventricles of the brain. During neurogenesis, these progenitor cells give rise to all other neural cell types. This work shows representative results and analyses at E37, postnatal day (P) 1, and P16, corresponding to 4, 9, and 24 days after in utero electroporation, respectively. At earlier stages, the progeny of targeted cells consists mainly of

various neural progenitor subtypes, whereas at later stages most labeled cells are postmitotic neurons. Thus, in utero electroporation enables the study of the effect of genetic manipulation on the cellular and molecular features of various types of neural cells. Through its effect on various cell populations, in utero electroporation can also be used for the manipulation of histological and anatomical features of the ferret neocortex. Importantly, all these effects are acute and are performed with a spatiotemporal specificity determined by the user.

INTRODUCTION:

The neocortex is the outer sheet of the mammalian cerebrum and the seat of higher cognitive functions[1-5]. In order to achieve an acute genetic manipulation in the mammalian neocortex in vivo during the embryonic development, two different methods have been explored: viral infection[6] and in utero electroporation[7]. Both methods allow efficient targeting of neocortical cells but suffer from some limitations. The major advantage of in utero electroporation compared to viral infection is the ability to achieve spatial specificity within the neocortex, which is achieved by regulating the direction of the electrical field.

Since electroporation was first shown to facilitate the entry of DNA into the cells in vitro[8], it has been applied to deliver DNA into various vertebrates in vivo. In developmental neuroscience, in utero electroporation of the mouse neocortex was first reported in 2001[9,10]. This method consists of an injection of the DNA mixture in the lateral ventricle of the embryonic brain and subsequent application of the electric field using tweezer electrodes, which allows spatial precision[7,11]. In utero electroporation has since been applied to deliver nucleic acids in order to manipulate the expression of endogenous or ectopically added genes in the mouse neocortex. Important progress has been made recently by applying the methodology of CRISPR/Cas9-mediated genome editing via in utero electroporation in the mouse neocortex to perform (1) gene disruption in postmitotic neurons[12,13] and neural progenitor cells[14], and (2) genome[15] and epigenome[16] editing.

Very soon after the first report in mouse, in utero electroporation was applied to the embryonic rat neocortex[17,18]. Non-rodents remained a challenge until the first in utero electroporation of ferrets , a small carnivore, was reported in 2012[19,20]. Since then, in utero electroporation of ferrets has been applied to study the mechanisms of neocortex development by labeling neural progenitors and neurons[20-23], manipulating the expression of endogenous genes, including the use of CRISPR/Cas9 technology[24], and by delivering ectopic genes[21,22,25], including human-specific genes[26]. Furthermore, in utero electroporation of ferrets has been used to address features of human neocortex development in pathological conditions[27,28].

In the context of neocortex development, the advantages of using ferrets as a model organism compared to mice are due to the fact that ferrets better recapitulate a series of human-like features. At the anatomical level, ferrets exhibit a characteristic pattern of cortical folding, which is also present in human and most other primates, but is completely absent in mice or rats[4,29-31]. At the histological level ferrets have two distinct subventricular germinal zones, referred to as the inner and outer subventricular zones (ISVZ and OSVZ, respectively)[32,33], separated by the inner fiber layer[23]. These features are also shared with primates, including humans, but not with

mice[34]. The ISVZ and OSVZ in ferrets and humans are populated with abundant neural progenitor cells, whereas the subventricular zone (SVZ) of mice contains only sparse neural progenitors[21,32,35,36]. At a cellular level, ferrets exhibit a high proportion of a subtype of neural progenitors referred to as basal or outer radial glia (bRG or oRG, respectively), which are deemed instrumental for the evolutionary expansion of the mammalian neocortex[34,37,38]. bRG are hence highly abundant in the fetal human and embryonic ferret neocortex, but they are very rare in the embryonic mouse neocortex[35,36]. Furthermore, ferret bRG shows morphological heterogeneity similar to that of human bRG, far superior to mouse bRG[21]. Finally, at a molecular level, developing ferret neocortex shows gene expression patterns highly similar to those of fetal human neocortex, which are presumed to control the development of cortical folding, among other things[39].

The cell biological and molecular characteristics of ferret bRG renders it highly proliferative, similar to human bRG. This results in an increased production of neurons and development of an expanded and highly complex neocortex[34]. These characteristics make ferrets excellent model organisms for studying human-like features of neocortex development that cannot be modelled in mice[26,40]. To take full advantage of the ferret as a model organism the presented method was developed. It consists of in utero electroporation of E33 ferret embryos with a plasmid expressing GFP (pGFP) under the control of a ubiquitous promoter, CAG. The electroporated embryos can then be analyzed embryonically or postnatally. In order to reduce the number of sacrificed animals, female ferrets (jills) are sterilized by hysterectomy and donated for adoption as pets. If the targeted embryos are harvested at embryonic stages, a second surgery is performed and the embryos are removed by a caesarian section, whereas the jills are hysterectomized. If the targeted embryos are analyzed at postnatal stages, the jills are hysterectomized after the pups have been weaned or sacrificed. Hence, a protocol for the hysterectomy of jills is also presented.

PROTOCOL:

All experimental procedures were conducted in agreement with the German Animal Welfare Legislation after approval by the Landesdirektion Sachsen (licenses TVV 2/2015 and TVV 21/2017).

1. Preparation for in utero electroporation

1.1. Prepare the DNA mixture. In this protocol a final concentration of 1 µg/µL of pGFP is used. Dissolve DNA in PBS and supplement with 0.1% Fast Green to facilitate visualization. Once prepared, mix the DNA mixture by pipetting up and down several times or by finger tapping. Store at room temperature until use.

NOTE: For coelectroporations, the DNA mixture is prepared to contain a final concentration of 1 µg/µL of plasmid encoding a gene of interest along with 0.5 µg/µL of pGFP diluted in PBS.

1.2. Prepare the surgery table with all the required tools and instruments.

1.3. Pull glass capillaries using the micropipette puller. Adjust the diameter of the capillary tip by cutting off the distal part of the capillary using forceps as previously described[41].

2. Preparation of ferrets for surgery

2.1. Keep the pregnant jills with embryos at E33 fasting for at least 3 h before the surgery to reduce the risk of vomiting.

2.2. Prior to the surgery sterilize all the tools by autoclaving. Perform the surgery in a specially assigned room in aseptic conditions to eliminate potential sources of contamination.

2.3. Place the pregnant jill in the narcosis box with 4% isoflurane.

2.4. When anesthetized, place the ferret on the operation table with a heat pad and attach the narcosis mask with a constant 2–3% isoflurane flow to the nose. To ensure the appropriate level of anesthesia, check for the lack of the following reflexes.

2.4.1. The palpebral reflex by touching the periocular skin

2.4.2. The flexor reflex by pinching the skin between the 2nd and 3rd, or 3rd and 4th toe of both hind limbs

2.4.3. If the palpebral reflex is absent and flexor reflex is clearly reduced, check for the pain reflex by pinching one toe of each hind limb.

NOTE: Ensure to have a stethoscope at hand to monitor heart rate and rhythm if required.

2.5. Inject the ferret subcutaneously with analgesic (0.1 mL of metamizol, 50 mg/kg of body weight), antibiotic (0.1 mL/kg of body weight of 20 mg/kg of amoxicillin + clavulanic acid), and glucose (10 mL of 5% glucose solution).

2.6. Place a drop of eye ointment solution on the eyes to prevent eye dehydration during the surgical procedure.

2.7. Shave the ferret belly using a shaver.

2.8. Clean the skin with water and soap, disinfect the belly with 70% ethanol scrub, disinfect 2x with iodine, and let it dry.

3. In utero electroporation of ferrets

3.1. Ensure that the surgical area is sterile: place sterile surgical tools on sterile tissues, and change coat and gloves.

3.2. Place a sterile surgical drape on the animal.

3.3. Using a scalpel, surgically open the belly at the linea alba with a ~5 cm long cut.

3.4. Cut the muscle layer using scissors.

3.5. Place gauze swabs around the incision site and wet them with PBS. The gauze swabs will absorb the additional PBS that will be added during the surgery.

NOTE: Maintain heat and PBS support throughout the surgery.

3.6. Expose the ferret uterus and place it on the gauze swabs.

3.7. Load a glass capillary with the injection solution using a pipette with a long loading tip. Ensure that the loading volume is approximately between 5–20 µL depending on the number of embryos and number of different experimental conditions. The average injected volume per embryo is 3–5 µL. Attach the loaded glass capillary to a holder and connect the other side of the holder to a tube and a mouthpiece.

3.8. Inspect the first embryo and find the head.

3.9. Place the fiber optic light source next to the embryo's head to facilitate visualization.

NOTE: Ferret uterine walls are very dark, and it is difficult to see through them unless light is shined directly on them.

3.10. Perform a single intraventricular injection into the ventricle of one of the cerebral hemispheres and keep the other hemisphere intact to serve as an internal control. The ventricle itself is not easily distinct by eye, so its location is estimated based on the location of the pigmented iris, which is visible. The intraventricular injection is done by taking the holder attached to the glass capillary and penetrating the skin, skull, and cerebral tissue with the tip of the glass capillary.

NOTE: Make sure to not damage the placenta, which is darker than murine placentas.

3.10.1. When the tip of the glass capillary is in the ventricle, inject approximately 3–5 µL of the injection solution by mouth-pipetting using the mouthpiece connected to the glass capillary holder. Because the injected solution contains 0.1% Fast Green, the injected ventricle will now turn dark green, and will be visible as a kidney-shaped structure.

3.11. Place the tweezer electrodes on the uterus above the embryo's head so that the positive pole is placed above the area that will be targeted and the negative pole below the injected area.

3.12. Set the following electroporation conditions on the electroporator: Pulse length = 50 ms;

Pulse voltage = 100 V; Pulse interval = 1 s; Number of pulses = 5. Then, press the "**Pulse**" button on the electroporator.

3.13. Quickly drop several drops of warm 1x PBS on the electroporated embryo.

3.14. Repeat the procedure for all the embryos. Keep the uterus constantly wet with warm PBS.

NOTE: If the first embryos facing the vagina are not easily accessible, it is best to not electroporate them.

3.15. When all the embryos are electroporated, place the uterus back into the peritoneal cavity.

3.16. Suture the muscle layer with the peritoneum using a 4-0 suture.

3.17. Suture the skin using the same thickness and spray the wound with aluminum spray.

3.18. Place the animal in a cage and keep it warm with a heat source. Carefully monitor the animal until it wakes up.

4. Postoperative care and housing of targeted animals

4.1. For 3 days after the surgery ensure that the animals are undergoing the following postoperative care: 10 mg/kg amoxicillin (antibiotic) 2x daily; 25 mg/kg metamizol (analgesic) 3x daily.

4.2. Ensure that the ferrets are housed individually.

4.3. Ensure that the ferrets are examined by a veterinary at least 1x per day.

4.4. Ensure that the ferrets are disturbed as little as possible during delivery.

4.5. After the pups are weaned or sacrificed, prepare the jills for hysterectomy.

5. Hysterectomy of ferrets

NOTE: A hysterectomy is performed to reduce the number of sacrificed animals so that the sterilized animals can be donated for adoption as pets. The presurgical procedure for hysterectomy is the same as in step 2.

5.1. Shave and sterilize the ferret belly as described in steps 3.1 and 3.2.

5.2. Surgically open the belly at the linea alba. Cut the muscle layer. Lift the muscle layer and shelter the gut with a finger while fully opening the muscle layer.

5.3. Place gauze swabs around the incision site and wet them with sterile PBS. Expose the ferret uterus and the ovaries and place them on the gauze swabs.

5.4. Start the hysterectomy on one side by ligating the arteria ovarica and vena ovarica cranial to the mesovar. Put a clamp on each side of the ligation and perform another ligation cranial to the clamps. Attach the third clamp at the ends of the ligation to save them. Repeat on the other side.

5.5. Cut between the clamps and detach the cornua uteri from the mesentery on both sides.

5.6. Ligate the arteria uterina on both uterine sides caudal to the ostium uteri. Then ligate the vagina and fix the ligature. Attach the clamp at the ends of the ligations to save them. Put two clamps cranial to the ligature. Cut between the clamps and detach the uterus from vagina. Remove the uterus and ovaries and dispose of them. Scrape the residual mucous membrane from the uterus butt.

5.7. Detach all the remaining clamps and shorten the ligature ends. Make sure to control for bleeding after the clamps have been removed.

5.8. Suture the muscle layer and the skin as described in steps 3.16 and 3.17. Follow the postoperative protocol as in steps 3.18 and 4.1–4.3. Keep the animals in the animal facility for at least 2 weeks with regular veterinary visits. After the animals are fully recovered, they can be donated for adoption.

REPRESENTATIVE RESULTS:

In utero electroporation of ferrets at E33 resulted in targeting of the neural progenitor cells lining the ventricular surface of the embryonic neocortex (**Figure 1**). These cells are called apical progenitors and are highly proliferative, giving rise to all other cell types during development. Upon asymmetric division, apical progenitors generated another apical progenitor and a more differentiated cell, typically a basal progenitor (BP), which delaminated from the ventricular surface. BPs migrated into the secondary germinal zone, the SVZ. When the electroporation was performed at E33, many newborn BPs migrated to the basal-most part of the SVZ, where they formed the OSVZ[22].

When the effects of electroporation were examined 4 days later at E37, most of the targeted cells and their progeny were still in the germinal zones (VZ, ISVZ, and OSVZ, see **Figure 2A**) and cells were seldom present further basally in the cortical plate (CP). The progeny of targeted cells mainly consisted of neural progenitor types and newborn neurons. The progenitor identity could be examined by immunofluorescence for markers of cycling cells, such as PCNA[26] (**Figure 2B**, **C**), whereas a subset of progenitors undergoing mitosis could be shown by markers such as phosphohistone 3 (PH3)[21] (**Figure 2D**).

At P0, 8 days after electroporation, the progeny of targeted cells spread in all histological layers (**Figure 3A**, **B**). At this stage, BPs were particularly abundant and bRG were readily identifiable. Using a combination of transcription factor markers, different BP populations could be revealed.

Sox2 is a marker of proliferative progenitor cells, including bRG[26]. Tbr2 is a marker of neurogenic BPs, which are mainly intermediate progenitors[26] (**Figure 3B**). Because the embryos were co-electroporated with a plasmid encoding GFP, the morphology of neural progenitors could be examined by tracking the GFP signal. This is particularly important in the context of BPs, which come in two major morphotypes: multipolar cells, which are largely intermediate progenitors, and radial cells, which are bRG[21]. Hence, Sox2+ Tbr2– radial cells in the OSVZ are the key cell population of interest for studying bRG[26].

By P16, a majority of targeted cells stopped dividing and differentiated into neurons and glia. Therefore, these cell types are best examined at this stage. In addition to various neuronal and glial subtypes, ferret P16 neocortex exhibited the characteristics pattern of folding (**Figure 3C**). At this stage most major gyri and sulci were already present and prospective brain areas could be identified[31]. Ferret brain continued maturing after P16, when processes such as myelination and synaptogenesis take place.

FIGURE AND TABLE LEGENDS:

Figure 1: Targeting neural cells by in utero electroporation of the ferret neocortex. In utero electroporation of the ferret neocortex at E33 resulted in targeting of apical progenitors. During development these cells give rise to all other cell types. Basal progenitors were best studied at later embryonic (E37) and perinatal (P0) stages. Neurons were best studied at postnatal stages, such as P16. Cortical layers: VZ = ventricular zone; ISVZ = inner subventricular zone; OSVZ = outer subventricular zone; IZ = intermediate zone; CP = cortical plate; GZ = germinal zones (VZ+SVZ); WM = white matter.

Figure 2: Example of E37 ferret neocortex after in utero electroporation at E33. (**A** and **B**) Section of the E37 ferret neocortex; green, progeny of electroporated cells (GFP); blue (**A**) cell nuclei (DAPI); magenta (**B**), cycling cells (PCNA). Box (width = 777 µm) indicates area shown at higher magnification in (**C**). Scale bar = 1 mm. Note the lack of GFP signal in the contralateral (nonelectroporated hemisphere), which serves as an internal control. (**C** and **D**) Higher magnifications of the targeted area; green, progeny of electroporated cells (GFP); magenta (**C**), cycling cells (PCNA); red (**D**), mitotic cells (phosphohistone 3, PH3). Note that the majority of the progeny of targeted cells was in the SVZ at this stage. Cortical layers as in **Figure 1**.

Figure 3: Examples of P0 and P16 ferret neocortex after in utero electroporation at E33. (**A** and **B**) Section of the P0 ferret neocortex; green, progeny of electroporated cells (GFP); blue (**A**) cell nuclei (DAPI); cyan (**B**), Sox2; magenta (**B**), Tbr2. (**B**) Higher magnification of the electroporated area. Scale bars = 1 mm (**A**), 100 µm (**B**). Cortical layers as in **Figure 1**. (**C**) Section of the P16 ferret neocortex; green, progeny of electroporated cells (GFP); grey, cell nuclei (DAPI); magenta, astrocytes (GFAP). Scale bar = 1 mm. This figure has been modified from Kalebic et al.[25].

DISCUSSION:

In utero electroporation in ferret is an important technique, with advantages and disadvantages with respect to other methods. There are critical steps and limitations to this method, as well as potential modifications and future applications to keep in mind.

Since the pioneering work of Victor Borrell and colleagues on genetic manipulation of the postnatal ferret neocortex via electroporation or viral injection[35,42,43], the ferret has become a genetically accessible model organism. Establishing genetic manipulation during embryonic development via in utero electroporation[19,20] opened up new research possibilities by allowing targeting of neural progenitor cells at earlier developmental stages. In comparison to postnatal manipulation, in utero electroporation enables targeting of larger areas of the neocortex and less differentiated neural progenitors that sequentially generate all other cell types of the neocortex. Importantly, compared to viral targeting, in utero electroporation allows for spatial precision of targeting.

The most critical part of the method is the surgery itself. In utero electroporation of the ferret neocortex is significantly more complex and difficult in comparison to the procedure in mice. The uterine walls are darker, and the embryos are more difficult to distinguish. Additionally, adult ferrets have greater husbandry and veterinary requirements. Particularly challenging is the period around the birth of the pups. Ferrets are very sensitive in that period and are best not disturbed unnecessarily. The major limitations of the approach itself are related to the efficiency of targeting. In utero electroporation always results in targeting of a mosaic of neural progenitors. This is ideal for studying the cell biological aspects of neural progenitors or neurons, but it is suboptimal for causing large histological and anatomical perturbations, such as a change in neocortical folding. If this is required, the best approach is to move the electrodes along the rostrocaudal axis during the procedure in order to cover large parts of the neocortex. However, for whole organ analysis the best approach is generation of transgenic ferrets starting from the zygote stage[44].

The embryonic stage at which the in utero electroporation was performed (E33) is ideal for studying basal progenitors. Indeed, electroporations and viral targeting at this stage have been applied to reveal the timing of the onset of the OSVZ[22] as well as various cell biological features of basal progenitors pertinent to their morphology and proliferation[21,25,26]. However, depending on the scientific purpose of a study, the timing of electroporation can be easily changed without significant modifications to the method[19,20]. Apart from temporal specificity, in utero electroporation allows for easy modifications of the spatial specificity. The dorsolateral neocortex at the rostromedial position along the rostrocaudal axis was targeted, which resulted in labeling of the motor and somatosensory areas. Other neocortical areas can also be targeted by adjusting the placement of the electrodes and the direction of the electrical field. In mouse, the medial neocortex[45] and ventral telencephalon[46] have been targeted using in utero electroporation, suggesting that similar approaches could be used in ferrets.

Finally, in utero electroporation can easily be combined with the most recent genome and epigenome editing techniques[13,14,16,25], where CRISPR/(d)Cas9 components can be delivered as a plasmid or as a complex of recombinant Cas9 protein and guide RNAs[14], with the latter shortening the time required for genome editing to take place. It is likely that further technological improvements in genome editing will be combined with in utero electroporation in both mice and ferrets in order to generate precise genomic mutations important for understanding normal

brain development and particularly to model human pathological conditions. In this context, in utero electroporation is being increasingly used as the targeting method of choice for subsequent various single-cell omics approaches and live imaging to understand the molecular signatures and dynamic behavior of the targeted cells and their progeny.

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DISCLOSURES:

The authors have nothing to disclose.

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514

Figure

Figure 1

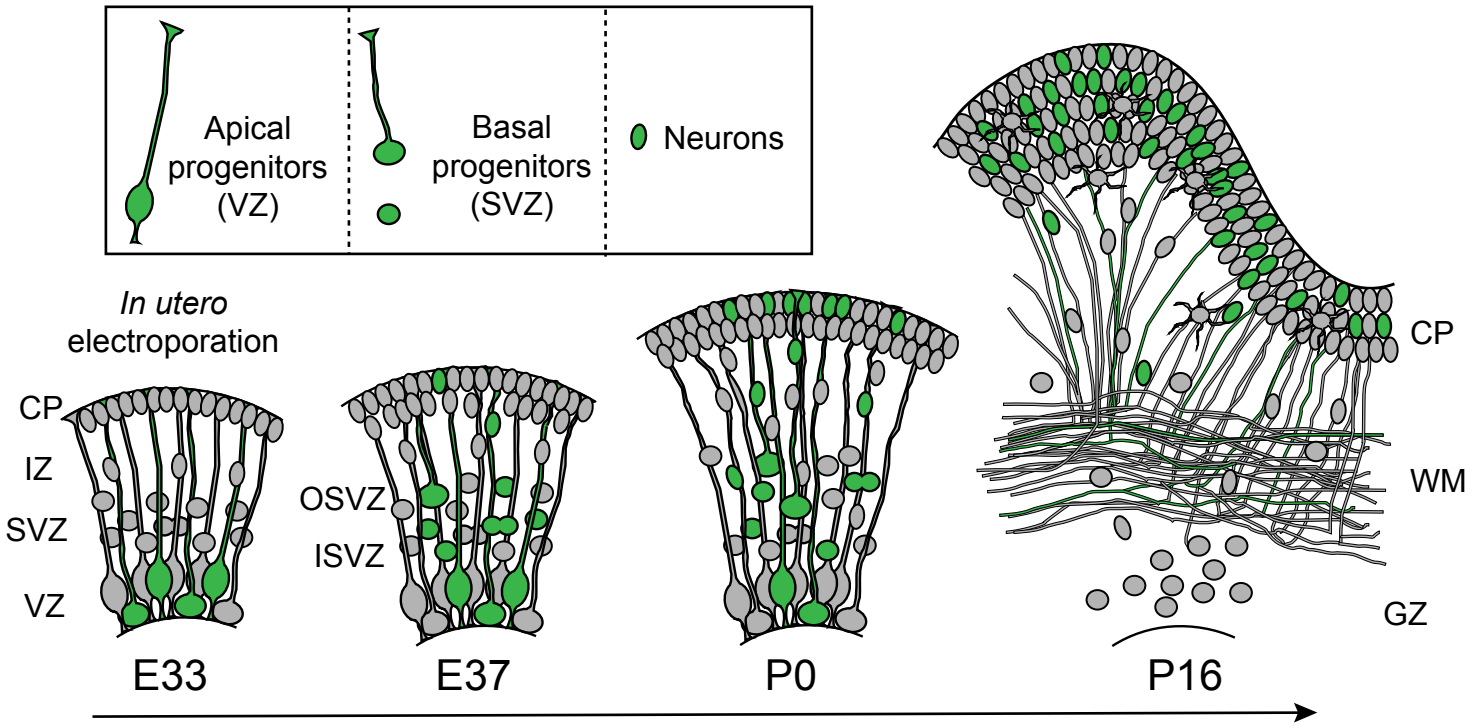
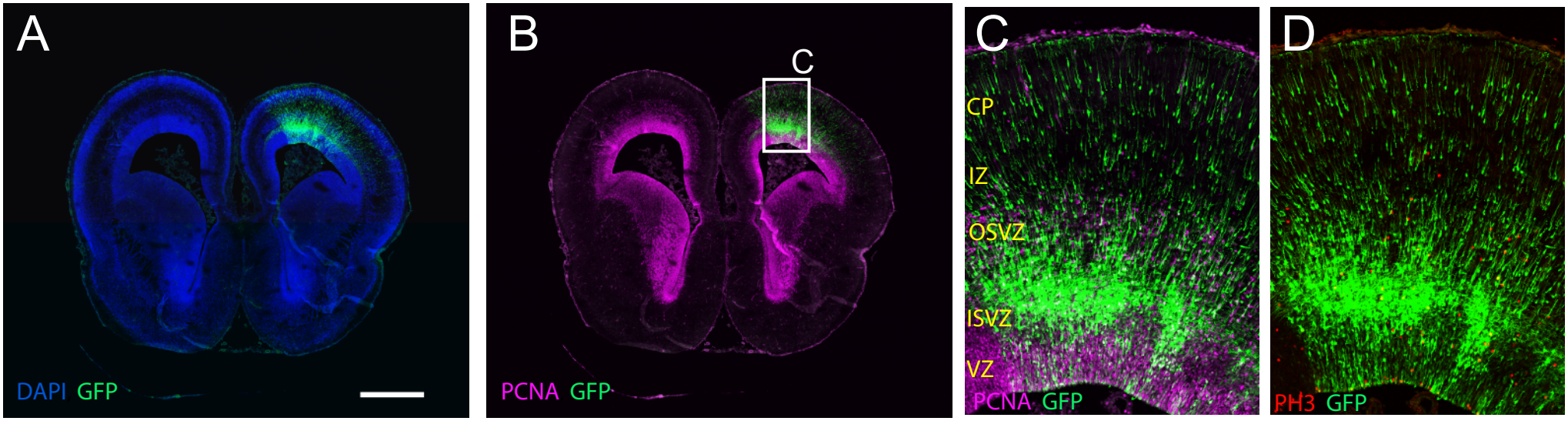
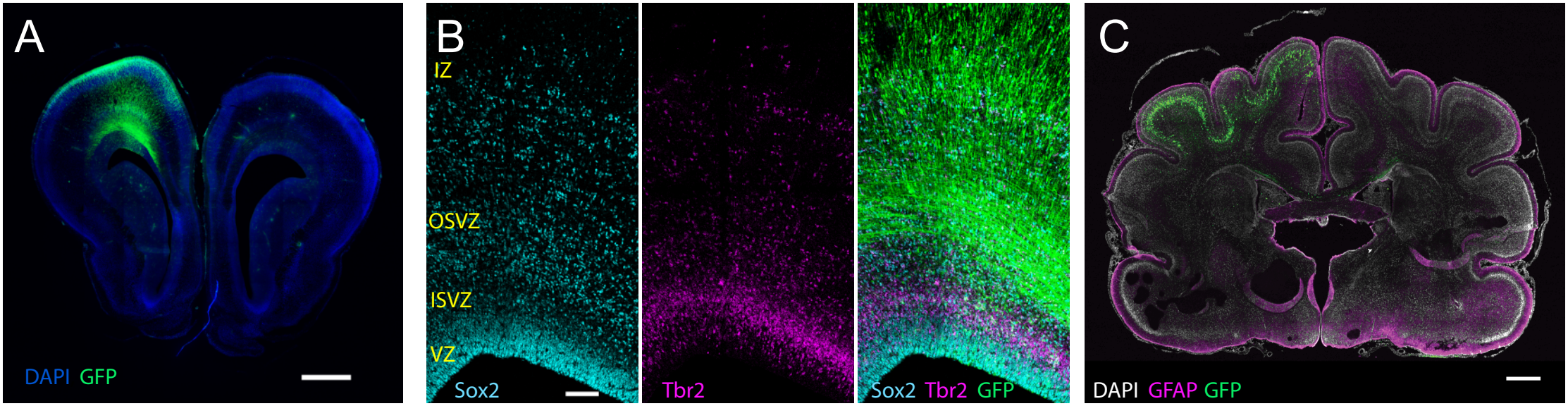


Figure 2



Figure

Figure 3



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1ml syringe	BD	309628	Electroporation
4-0 Vicryl suture	Ethicon	V392ZG	Surgery
Aluminium spray	cp-pharma	98017	Surgery
Amoxicilin+clavulanic acid (Synulox RTU)	WDT	6301	Surgery
Cappilary holder	WPI	MPH6S12	Electroporation
Dexpanthenol Ointment solution	Bayer	6029009.00.00	Surgery
Drape sheet 45x75cm	Hartmann	2513052	Surgery
Electrode Tweezer, platinum plated 5mm	BTX	45-0489	Electroporation
Electroporator	BTX	ECM830	Electroporation
Fast Green	Sigma	F7258-25G	Electroporation
Ferret *Mustela putorius furo*	Marshall	NA	Experimental organism
Fiber optic light source	Olympus	KL1500LCD	Electroporation
Forceps	Allgaier instrumente	08-033-130	Surgery
Forceps 3C-SA	Rubis Tech	3C-SA	Surgery
Forceps 55	Dumostar	11295-51	Surgery
Forceps 5-SA	Rubis Tech	5-SA	Surgery
Gauze swabs large	Hartmann	401723	Surgery
Gauze swabs small	Hartmann	401721	Surgery
GFAP antibody	Dako	Z0334	Antibody
GFP antibody	Aves labs	GFP1020	Antibody
Glass cappilaries (Borosilicate glass with filament, OD:1.2mm, ID: 0.69mm, 10cm length)	Sutter Instrument	BF120-69-10	Electroporation
Glucose	Bela-pharm	K4011-02	Surgery

Heat pad	Hans Dinslage	Sanitas SHK18	Surgery
Iodine (Betadine solution 100 mg/ml)	Meda	997437	Surgery
Isofluran	CP	21311	Surgery
Loading tips 20µl	Eppendorf	#5242 956.003	Electroporation
Metamizol	WDT	99012	Surgery
Metzenbaum dissecting scissors	Aesculap	BC600R	Surgery
Micropipette puller	Sutter Instrument	Model P-97	Electroporation
pCAGGS-GFP	NA	NA	From Kalebic et al., eLife, 2018
PCNA antibody	Millipore	CBL407	Antibody
pH3 antibody	Abcam	ab10543	Antibody
Scalpel	Aesculap	294200104	Surgery
Shaver	Braun	EP100	Surgery
Sox2 antibody	R+D Systems	AF2018	Antibody
Surgical clamp 13cm	WDT	27080	Surgery
Surgical double spoon (Williger)	WDT	27232	Surgery
Surgical drape	WDT	28800	Surgery
Surgical scissors small	FST	14090-09	Surgery
Suturing needle holder	Aesculap	BM149R	Surgery
Tbr2 antibody	Abcam	ab23345	Antibody
Transfer pipette 3ml	Fischer scientific	13439108	Surgery
Water bath	Julabo	TW2	Surgery

Response to Editor

Editor's Comment:
Editorial comments:
1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached version for revision.

Authors' Response:
We have done as requested.

Editor's Comment:
2. Please adjust the highlight to show only the steps of in utero electroporation.

Authors' Response:
We have done as requested.

Editor's Comment:
3. Please address all specific comments marked in the manuscript.

Authors' Response:
We have done as requested.

Editor's Comment:
4. We do not schedule the filming date until the manuscript is accepted for publication.

Authors' Response:
Thank you for informing us about this now. After the acceptance of the manuscript please inform us whether the 24th March suits you well for the filming. The only other date we could potentially arrange is 23rd March. The next available dates are going to be only in Summer. Please consider to allow two months from the moment we decide to order the ferrets for mating until the embryonic stage when *in utero* electroporation is performed.