High-performance and reliable site-directed in vivo genetic manipulation of mouse and rat brain by in utero electroporation with a triple-electrode probe.

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Method Article

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Abstract

One of the challenges for modern neuroscience is to understand the role of specific genes in the determination of cellular fate, and in the formation and physiology of neuronal-circuits. Techniques for genetic manipulation _in vivo_ such as _in utero_ electroporation are fundamental tools to address these issues. Here, we describe an established protocol for _in utero_ electroporation in mouse and rat for reliable targeting of the hippocampus, the motor, prefrontal, and visual cortices, and the Purkinje cells of the cerebellum. The method is based on an electroporation configuration entailing commonly used forceps-type electrodes plus an additional third electrode. This configuration allows highly consistent direction of the electric field to the different neurogenic areas by simple and reliable adjustment of relative positions, polarities and/or dimensions of the electrodes. More than 70% of electroporated embryos survive to postnatal ages and around 60-90% express the electroporated vector, depending on the targeted area. By a single electroporation episode, the protocol enables for symmetric transfection in both brain hemispheres. The procedure requires 4 hours of preparation on the first day and it lasts 1 hour, including a surgery time of 30 mins, on the second day.

Introduction

In utero electroporation was first described in 2001 as a simple and quick procedure to efficiently perform _in vivo_ genetic manipulation of pyramidal neurons of the rodent somatosensory cortex[1-3]. The technique takes advantage of the fact that, by addressing neural progenitors at the epithelium of the ventricular system, one can genetically manipulate specific populations of newborn neurons that will migrate to different brain areas[5]. Therefore, _in utero_ electroporation has theoretically tremendous potentiality for addressing cells in many brain regions by targeting progenitors at their diverse neurogenic areas at the proper developmental stage. Over the past ten years, the number of laboratories using the technique to target the somatosensory cortex has risen exponentially, as confirmed by the increasing number of publications in the field[4]. Nevertheless, it is clear that the experimental conditions required to target other brain areas are not reliable, as demonstrated by the incredibly low number of publications with _in utero_ electroporation targeting brain locations other than the somatosensory cortex[4]. Moreover, some brain areas theoretically attainable have never been successfully electroporated, likely because of the physical impossibility to target the corresponding neurogenic regions with the electric field generated by _two_ forceps-type electrodes, as initially indicated for the technique. In the present manuscript, we describe an _in utero_ electroporation configuration based on the usage of _three_ electrodes, which allows easy and exceedingly reliable transfection at brain locations only sporadically targeted before, just by varying the relative position, polarities and/or dimensions of the electrodes. **Advantages of the three-electrode configuration** The tripolar configuration for _in utero_ electroporation presents the following advantages: It allows extremely easy transfection of many different brain areas \(hippocampus, visual cortex, motor cortex, prefrontal cortex, cerebellum), with a degree of reliability comparable to that attained in the somatosensory cortex with the bipolar electrode \(BOX1). It allows transfection efficiency higher than the conventional two-electrode configuration, and it is proved to enable for the use of lower voltages

for comparable outcomes[4,6]. It allows symmetric electroporation of both brain hemispheres by a single electroporation episode in virtue of the symmetrical electrical field generated by the three electrodes \(BOX4)[4]. **Limitations of the three-electrode configuration** The main drawback of the three-electrode configuration is that it requires two operators during the electroporation phase of the procedure. One person will hold the embryo and the forceps-type electrodes and another person will hold the third spare electrode. Nevertheless, a trained operator and an ergonomic design of the tool can overcome this limitation[4,6].

Reagents

• Mice and rats \(see REAGENT SETUP) • Endofree plasmid kit \(Qiagen cat. no. 12362) • 1 x PBS: 137mM NaCl, 2.7mM KCl, 8mM Na2HPO4, 1.46mM KH2PO4 • 70% Ethanol/30% H2O \(vol/vol) • Betadine \(Sanco, cat. no. SZ-020) • Isoflurane \(Vet Merial, Italia) • Painkiller \(Diclofenacum natricum) • Antibiotic for injection– Baytril \(Bayer, 2,5% Enrofloxacin, injection solution) • Antibiotic topic cream - Iruxol \(Smith&Nephew, 30g 1% + 60 U.I. ointment; chloramfenicol, collagenas, cat. no. 66117030) • Eye cream –Lacrigel \(Farmigea, cat. no. 032038010) • Neutracon solution \(Decon Laboratories Limited, 1-3% in distilled water) • Fast Green \(Sigma-Aldrich; cat. no. F7252) • Sterile water \(ROMIL-SpA Super Purity Reagent; cat. no. H951)

Equipment

• Electrodes: 5mm and 10mm forceps-type electrodes \(Sonidel, cat. nos. CUY650P5 and CUY650P10) and additional custom-made 3rd single electrode and Y-connector \(Fig. 1, 2; BOX 2). • Electroporator CUY21 Edit \(Nepa Gene) or ElectroSquarePorator \(BTX; Fig. 1) • Pneumatic Pico Pump PV 82 \(World Precision Instruments; Fig. 1) • Laboratory Animal Anesthesia System \(VetEquip; cat. no. 901806; Fig. 1) • Oxygen cylinders \(95% O2 – 722mmHG; 5% CO2 – 38 mmHg) • Surgery tools \(Fig. 3a; BOX 3) \(i) Scissors with flat shanks – angular \(Fine Science Tools, cat. no. 14037-10) \(ii) Scissors with flat shanks – straight \(Fine Science Tools, cat. no. 14088-10) \(iii) Ring forceps \(Fine Science Tools, cat. no. 11106-09) \(iv) Shark-tooth tissue forceps \(Fine Science Tools, cat. no. 11023-10) \(v) Olsen-Hegar Needle holders with scissors \(Fine Science Tools, cat. no. 12002-12) \(vi) Scalpel \(Fine Science Tools, cat. no. 10003-12) \(vii) Scalpel blades \(Fine Science Tools. Blade no. 11, cat. no. 10011-00) • Heating platform \(Fine Science Tools, cat. no. 21061-10, Fig. 1) • Bottle warmer: any incubator that is able to warm at 37oC \(Fig. 1) • Fiber Optic Illuminator \(Schott; Agar Scientific, cat. no. AGO6400, Fig. 1) with exchangeable: double branch swan-neck light guide \(hippocampus, cortex; Agar Scientific, cat. no. AGO6402) single swan-neck light guide \(cerebellum electroporation; Agar Scientific, cat. no. AGO6401) • Syringe needles: 32G \(mouse; Mesorelle, cat. no. 1125) and 30G \(rat; BD Microlance, cat. no. 304000; BOX7) • Sterile disposable gloves \(Spermed Classic, Fig. 1) • Gauzes \(4cmx4cm) • Self-sealing autoclave pouches \(140 mm X 250 mm; Secureline, cat. no. 1020/2011; Fig. 3b) • Autoclavable surgical drapes \(Mon&Tex, cat. no. 120320) • Plastic connectors between the syringe needle for injection and the tubing to the picospritzer \(Crisel Instruments, cat. no. F7252-25G; Fig. 4d and e) • Needled suture thread \

(Ethicon Vicryl; 5-0 Gauge, mouse, cat. no. V397H and 4-0 Gauge, rat, V385H) • Electrical shaver \(Fig. 1) • Portable Vacuum cleaner \(Fig. 1) • 0.5 mL syringes • Benchguard \(Barloworld Scientific, cat. no. BG50) • Cotton balls • Plastic box for sterile surgical tools • Cauterizing pen \(Fine Science Tools, cat. no.18010-00, Fig. 1) • Hot Bead Sterilizer \(Fine Science Tools, cat. no. 18000-45, Fig. 1)

Procedure

Preparation of tools. TIMING 4hrs, 1d before surgery 1 Autoclave: a. Surgical Tools \(Fig. 3a and b). CRITICAL STEP Avoid using forceps with sharp tips for surgery. You could easily damage the uterus, causing bleeding and eventually embryos' death. b. 1X PBS. c. Plastic connectors, one for each plasmid that will be used \(Fig. 4e) d. Surgical drapes \(mouse: 12cm x 20cm; rat: 15cm x 25cm). Since during autoclaving drapes may melt and stick together, wrap each piece into separate paper towels. e. Gauzes \(4 x 4 cm). 2 Immerge the electrodes in a container with 2% Neutracon overnight for sterilization. 3 Cover the heated surgery platform with bench-guard to avoid burns to the animal. 4 Into two separate boxes, prepare cotton balls soaked with 70% ethanol or betadine. **Preparation of animals for _in utero_ electroporation.** TIMING 15min 5 Place the animal in the anesthesia-induction box and set the appropriate level of isoflurane and oxygen to anesthetize the animal \(Tab. 1). 6 Place the animal on its back on the heated surgery pad. Set the temperature at 32°C. \! CAUTION Setting temperature above 32°C can cause burns to the animal. 7 Apply the Lacrigel on the eyes of the animal to prevent them from drying during anesthesia. 8 Inject painkiller intramuscularly into one hind paw and the antibiotic into the other. 9 Shave the fur with shaver and vacuum it with the vacuum cleaner. \!CAUTION avoid hurting the nipples of the dam. This will result in poor feeding of the pups. 10 Clean the animal's skin with cotton balls soaked with either Betadine or ethanol. Make circular movements starting in the center of the abdomen and moving progressively toward the periphery to avoid contamination of the wound. Repeat the procedure 6 times alternating first Betadine and then ethanol. 11 Load the DNA into the needle for injection \(Fig. 4a). CRITICAL STEP Place the tip of the pipette at the very bottom of the needle and load the DNA very slowly to prevent air bubbles. Injection of bubbles will likely results in the embryo's death. 12 Wear the sterile gloves. 13 Release all the sterile tools from the self-sealing autoclave pouch into the sterile plastic surgery box. CRITICAL STEP Remember to preserve maximum of sterility. If you have an assistant, ask him/her to unpack tools for you. If you are alone, wear only one sterile glove, open the packages with the free unsterile hand, and release the sterile tools from the pack using the hand with the sterile glove. ? TROUBLESHOOTING **_In utero_ electroporation.** TIMING 30min 14 Use scissors and cut a hole in the surgical drape with flat shanks-straight and place the drape onto the rodent abdomen. The drape will ensure a sterile environment to the embryos. 15 Cut the skin of the animal using scissors \(mouse) or scalpel blade \(rat, blade #11). Cut close to the "linea alba", but not on it, to avoid excessive bleeding. CRITICAL STEP Do not use scalpel blades for cutting the mouse abdomen. The skin and abdominal wall are very thin. There would be risk of stabbing the embryos and/or internal organs by using the blade. ? TROUBLESHOOTING 16 Using shark-tooth tissue forceps, grab the abdominal muscles and make sure with fingers that there are no embryos below the point where you intend to start cutting the abdomen. Then, make a small hole in the abdominal wall using scissors with flat angular shanks and continue

cutting until the hole is big enough for and easy exposure of the uterus. 17 Using ring forceps, start to pull out the embryos from the dam abdomen. CRITICAL STEP Be extremely careful during pulling out of the embryos. With the ring forceps, grab the uterus wall in correspondence of the gap between the yolk sacks of two neighboring embryos. Pull out the first 2/3 embryos with the ring forceps, and then gently use your fingers for pulling out the rest of them. Do it slowly without using force, which can cause damage of the uterus, bleeding, squeezing and eventually death of the embryos. ? TROUBLESHOOTING 18 Immediately after uterus exposure and before electroporation, wet embryos with warm sterile PBS to avoid excessive cooling of the embryos and to favor current flow by PBS-saline solution during electroporation \(Fig. 5). Repeat the procedure before each electroporation. 19 Inject DNA into one of the brain lateral ventricles using the picopump connected to a foot switch. Keep the needle perpendicular to the head surface. Stop injecting the DNA when the whole ventricle is full and the Fast Green dye is clearly visible through the uterine wall as a half-moon shaped spot \(Fig. 4a and b). Electroporation of the cerebellum requires injection in the later ventricle and filling of DNA until reaching the fourth ventricle. For more specificity of transfection, we recommend direct injection into the fourth ventricle \(Fig. 4c). \! CAUTION Before injecting the DNA in the embryo's ventricles, release some DNA from the needle to make sure there are no air bubbles at the tip of the needle. Air bubbles may kill the embryo. CRITICAL STEP Additional third electrode and injection of both ventricles allow for bilateral transfection during a single electroporation episode \(BOX 4). CRITICAL STEP It is very important to have a sharp needle. If the needle is not perforating the yolk sack easily, replace it with a new one. Never use force to sting the embryo. It will damage their brain. When holding the needle, use sterile gauze to prevent contamination. CRITICAL STEP Never manipulate embryos excessively. If the injection appears too difficult, leave the embryo as non-injected and move to the next embryo. ? TROUBLESHOOTING 20 Electroporate the embryos soon after injection to prevent DNA diffusion to the other ventricle, and decrease of electroporation efficiency. Use appropriate electroporation parameters \(Tab.2). \! CAUTION Usage of three electrodes increase the risk of current short circuits, which can kill the embryo. Remember to keep sufficient distance among all electrodes to avoid their physical contact. CRITICAL STEP The size of the electrodes can significantly influence the efficiency of the electroporation \(BOX 1, Fig. 6). To electroporate different brain structures use the following electrode configurations: A. HIPPOCAMPUS Use the forceps-type electrodes connected by a Y- connector to the positive pole and gently grab both sides of the embryo's head. Connect the third electrode to the negative pole placed at 0o with respect to the horizontal plane right above bregma \(Fig. 7) B. MOTOR CORTEX Use the forceps-type electrodes connected by a Y- connector to the negative pole, and gently grab both sides of the embryo's head. Connect the third electrode to the positive pole, and place as for electroporation of the hippocampus \(Fig. 8) C. PREFRONTAL CORTEX Use the forceps-types electrode connected by a Y-connector to the negative pole and place it on both sides of the embryo's head. Connect the third electrode to the positive pole, and place it on the front of the embryo's head \(Fig. 9) D. VISUAL CORTEX Use the tweezer type electrode connected by a T- connector to the negative pole and place it on both sides of the embryo's head. Connect the third electrode to the positive pole, and place it right on \(rat) or below \(mouse) lambda \(Fig. 10) E. CEREBELLUM Use the forceps-type electrodes connected by a T- connector to the negative pole and place it on both sides of the neck at the level of the fourth ventricle. Connect the third electrode to the positive pole, and place it on top of the fourth ventricle

\(Fig. 11) CRITICAL STEP To avoid embryo's death avoid squeezing the uterus and yolk sack containing the embryo during electroporation of all brain areas ? TROUBLESHOOTING 21 Use extreme care to handing back the embryos into the dam's abdominal cavity, and to suture the abdominal wall and skin. For detailed instructions on how to efficiently stitch the abdominal wall and skin, see BOX 5. CRITICAL STEP Stitches must be tight, otherwise the animal will open its wound. ? TROUBLESHOOTING 22 On the stitches, use topic antibiotic cream. 23 After surgery, place the animal belly-down \(to favor breathing) into a clean cage with wet food inside the cage \(to favor feeding and hydration). Place the cage under the heating lamp \(see after-surgery care, BOX 6)

Timing

The procedure requires 4 hours of preparation on the first day and it lasts 1 hour on the second day. Detailed timing of single procedures: • preparation of tools: 4hrs, 1d before surgery. • preparation of animals for surgery: 15 min. • surgery, injection and _in utero_ electroporation: 45 min.

Troubleshooting

See troubleshooting table for details \(Table 3).

Anticipated Results

Neuronal fate of transfected progenitors and migration of newborn-neuron to different brain areas can be easily followed when _in utero_ electroporation of a fluorescent protein is performed[4,6]. Bilateral transfection of both brain hemispheres is achievable with a single electroporation episode, allowing for efficient electrophysiological and behavioral experiments \(BOX 4). Comparison of possibly different functions of a same gene in various brain areas characterized by different cellular contexts is possible thanks to the high reliability of transfection at various brain locations \(BOX 1). Transfection efficiency for different brain areas depends on how accurately one follows this protocol. Accurate matching of the dimension of the forceps-type electrodes to that indicated in the protocol is crucial for high reliability of electroporation \(BOX 1). Increase of the survival rate can be achieved by lowering the electroporation voltage during surgery, due to the higher efficiency of the three-electrode configuration in comparison to bipolar _in utero_ electroporation[4]. Survival rate is mostly improved by pup fostering and after-surgery care, as indicated in the protocol and boxes. Moreover, the high efficiency and reliability of transfection allows for reduced number of dams required \(in agreement with EU guidelines), and decreased costs of animal housing. We conclude that the three-electrode electroporation offers a conceptual advance to the field by paving the way to targeting brain areas never electroporated before by simply varying the number, together with the polarities and dimensions of the electrodes.

References

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Figures

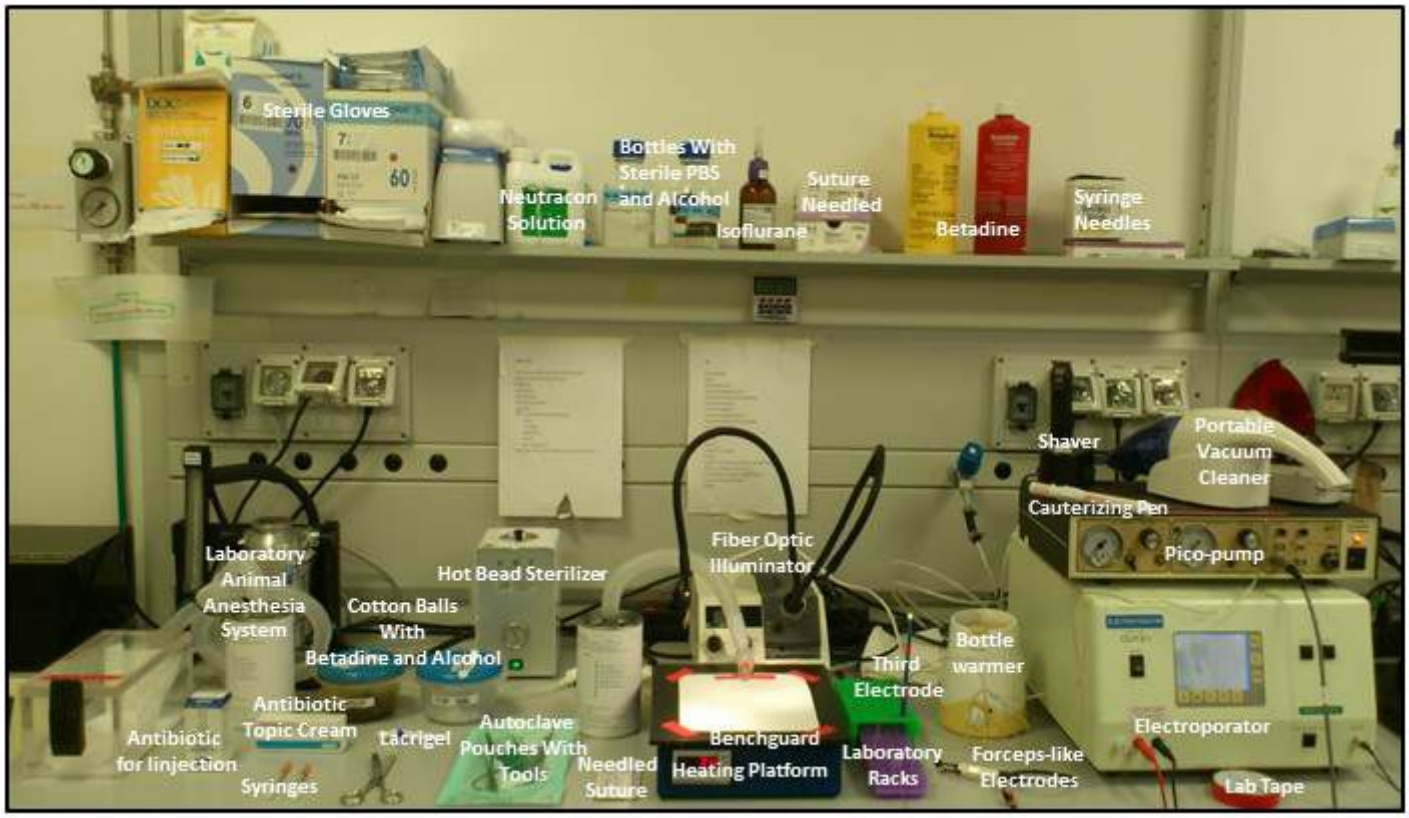

Figure 1

Equipment for _in utero_ electroporation.

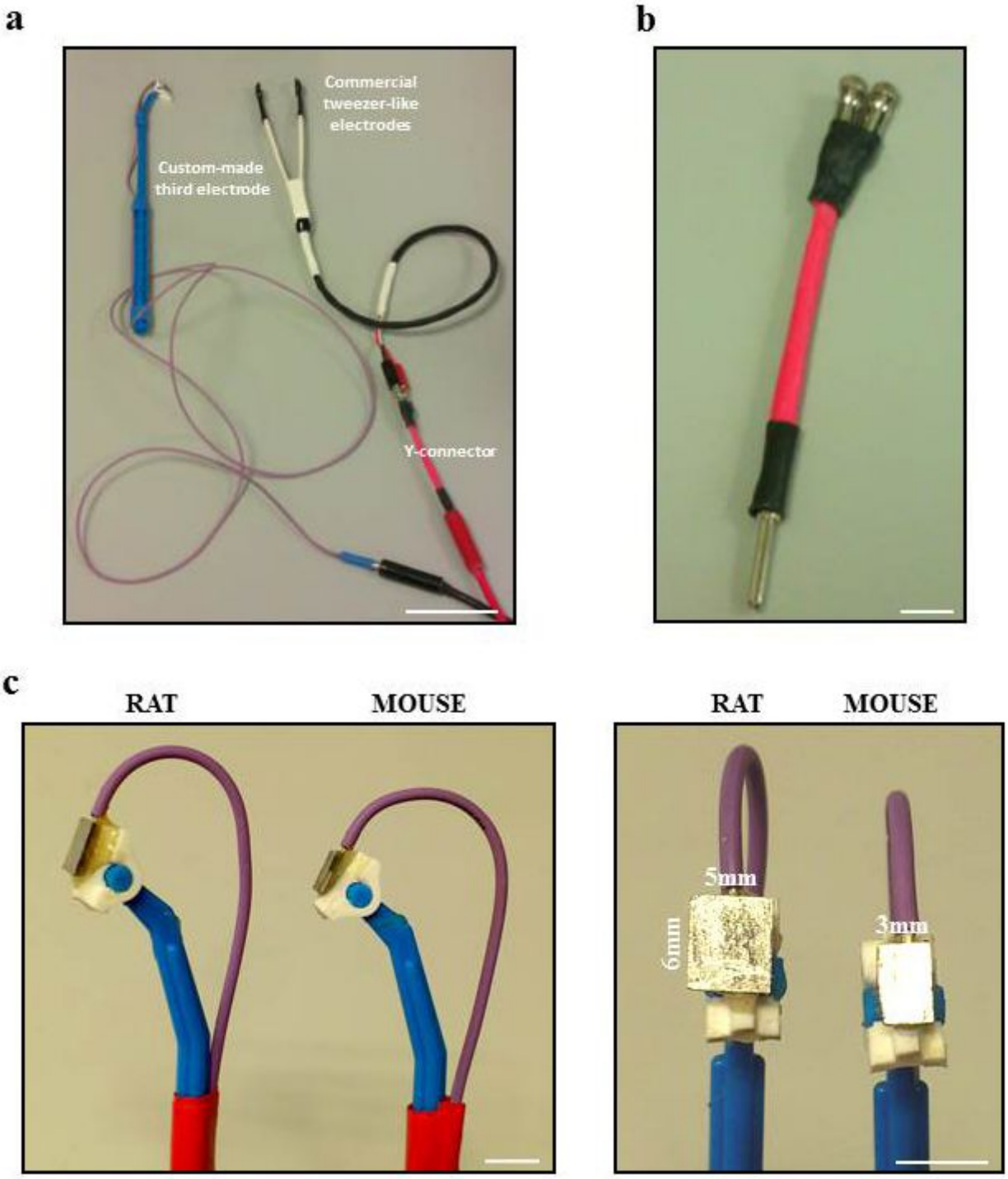

Figure 2

Three-electrode configuration for _in utero_ electroporation. (a) Tripolar _in utero_ electroporation configuration entails two conventional forceps-type electrodes connected to a single polarity by a Y-connector and an additional third custom-made electrode. Scale bar: 5cm. (b) High magnification of Y-connector for connection of commercial forcepsr-like electrodes to a same pole. Scale bar: 1cm. (c) Side (left) and front (right) views of additional third electrodes for electroporation of rat and mouse. Third

electrodes are made from a commercial cell scraper and a platinum plate. Optimal sizes of platinum plates are reported in the figure. Scale bars: 5mm.

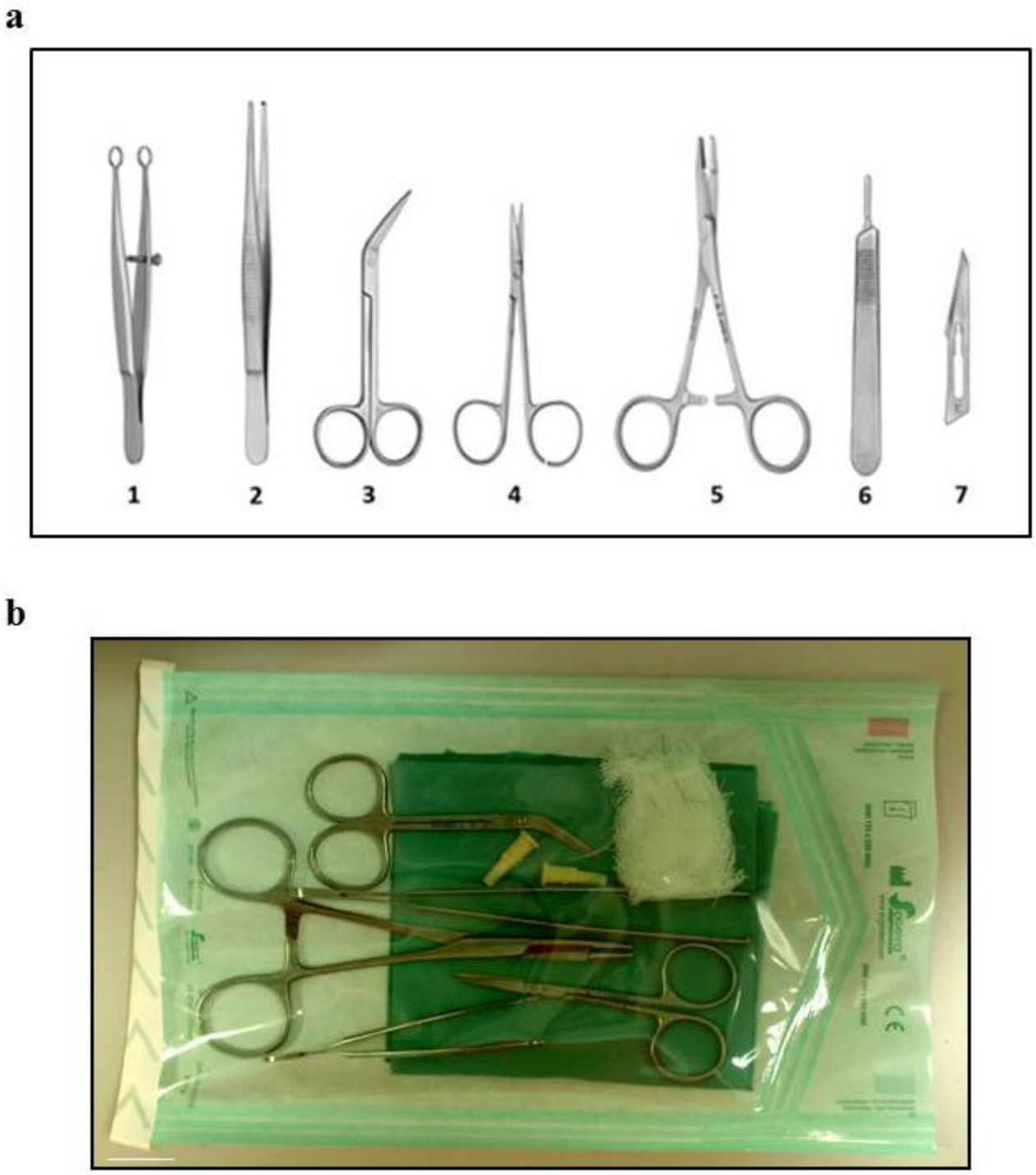

Figure 3

Surgical tools for _in utero_ electroporation. (a) Ring forceps (1); Shark-tooth tissue forceps (2); Scissors with flat shanks –angular (3); Scissors with flat shanks – straight (4); Olsen-Hegar needle holders with scissors (5); Scalpel (6); Scalpel blade (7). (b) Surgical tools, plastic connectors and gauzes in self-sealing autocavable pouch.

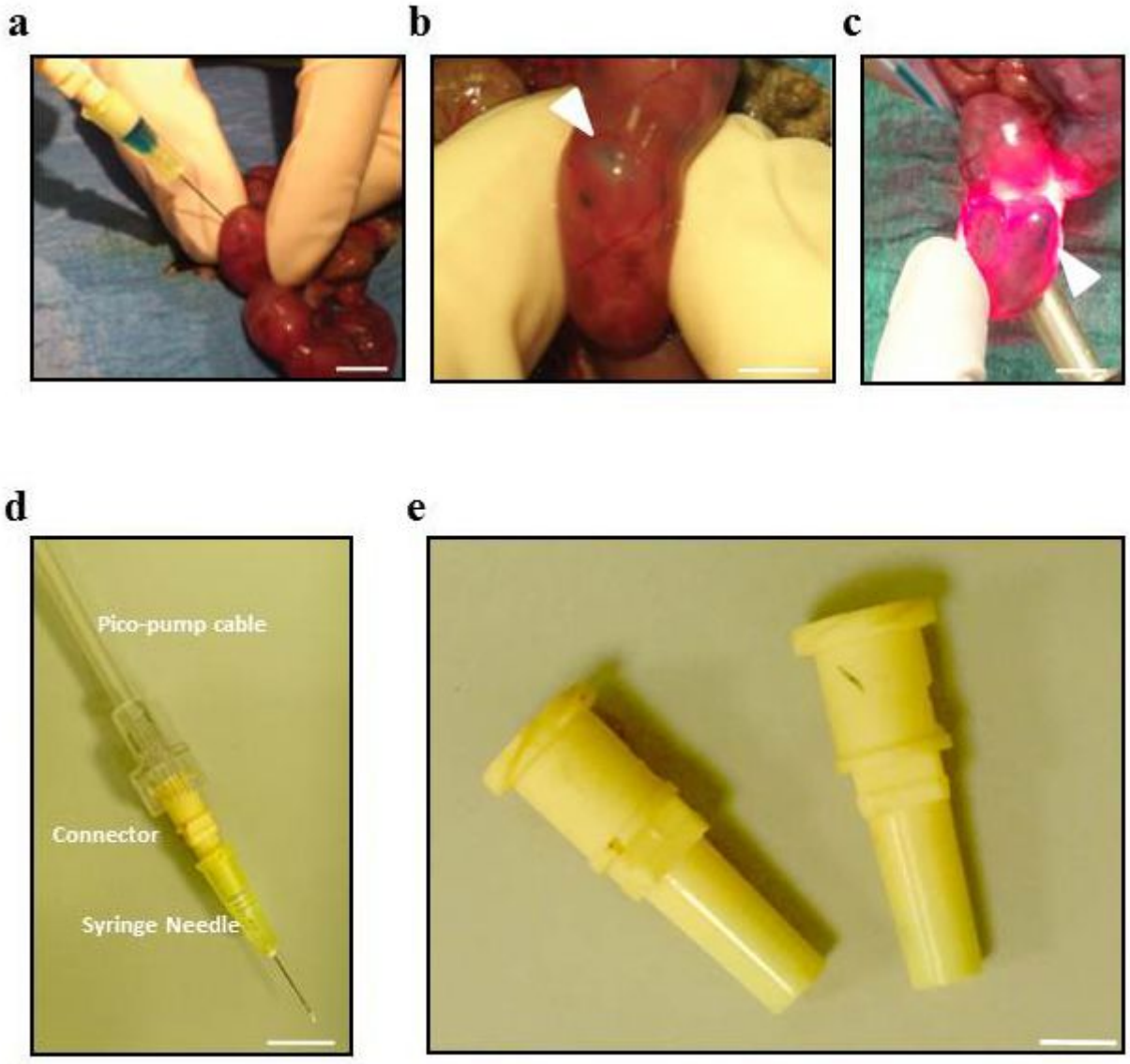

Figure 4

DNA injection in the embryo by a commercial needle. Examples of a mouse embryo (E15.5) during (a) and after (b) DNA injection in the lateral ventricle by the syringe needle filled with DNA and Fast Green dye for vizualization (blue). Note the green half-moon shaped spot, indicating complete filling of the lateral ventricle (arrow). (c) DNA injection of a rat embryo (E14.5) in the fourth ventricle. White arrow indicates filling of the fourth ventricle. (d) Syringe needle connected to the tubing of the pico-pump by a plastic connector. Scale bar: 1cm. (e) High magnification of commercial plastic connectors that connects the syringe needle and the picospritzer. Scale bars: 1cm and 5mm.

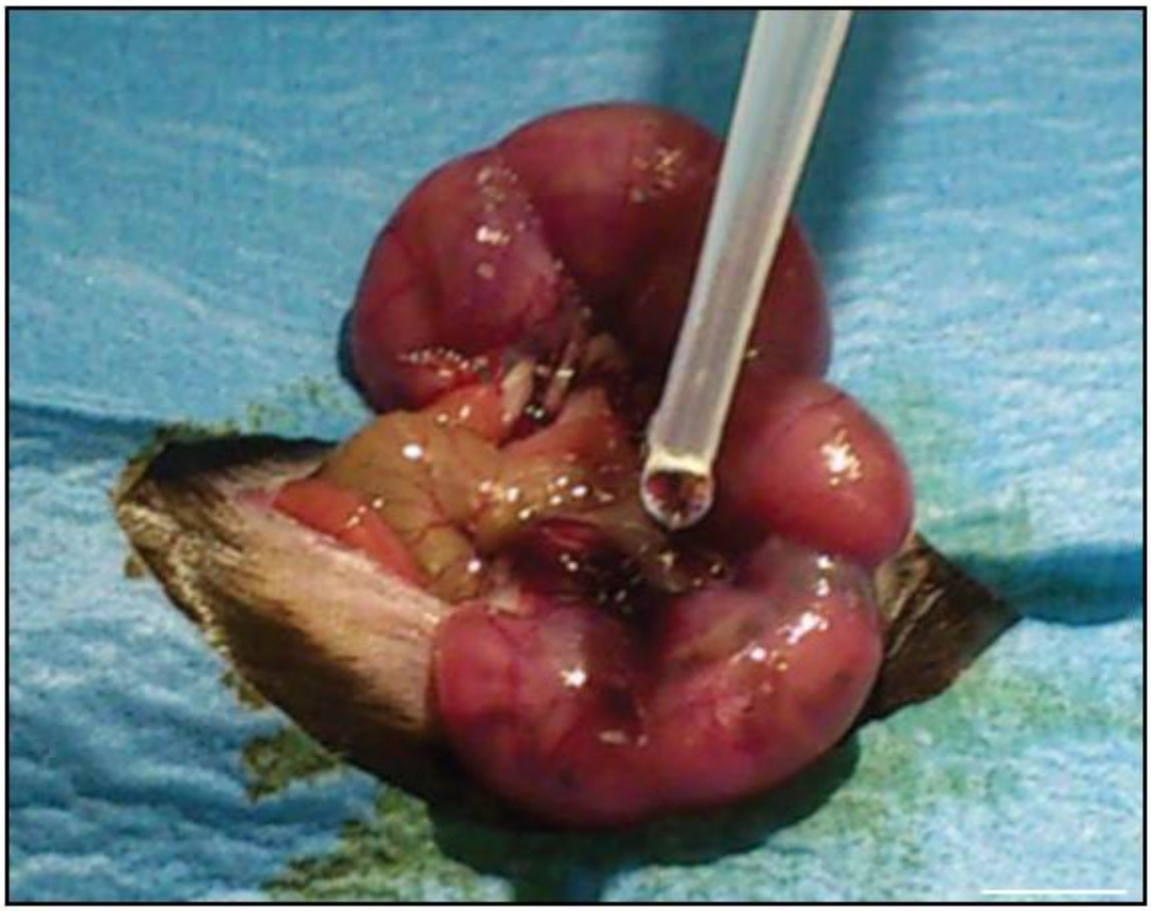

Figure 5

The exposed uterus is wet with warm and sterile PBS before electroporation. Scale bar: 1cm.

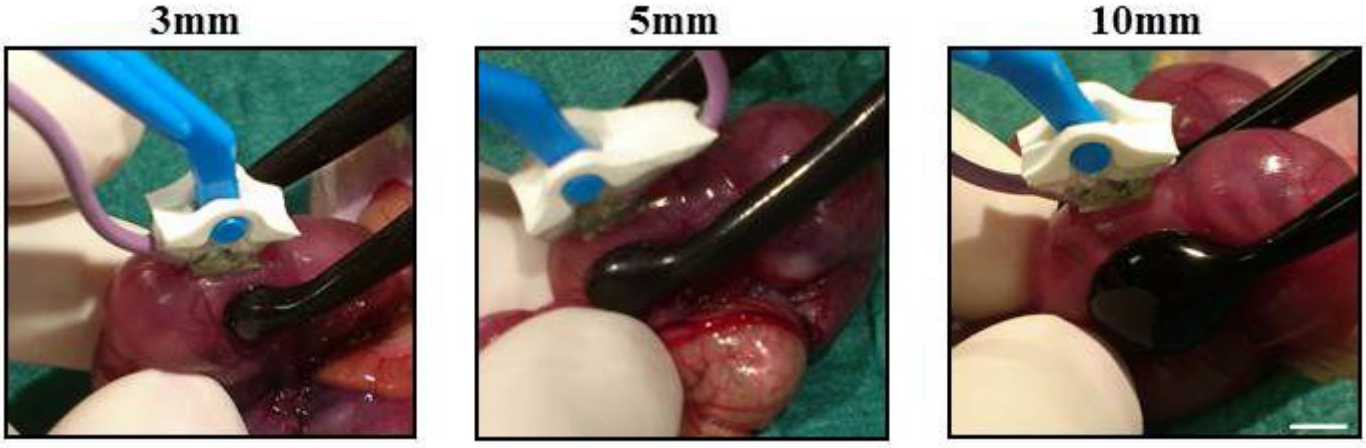

Figure 6

Example of _in utero_ electroporation of mouse embryos (E15.5) with the usage of the additional third electrode and different sizes of forceps-type electrodes. Addition of the third electrode and usage of different sizes of the forceps-type electrodes increase _in utero_ electroporation efficiency. Scale bar: 5 mm.

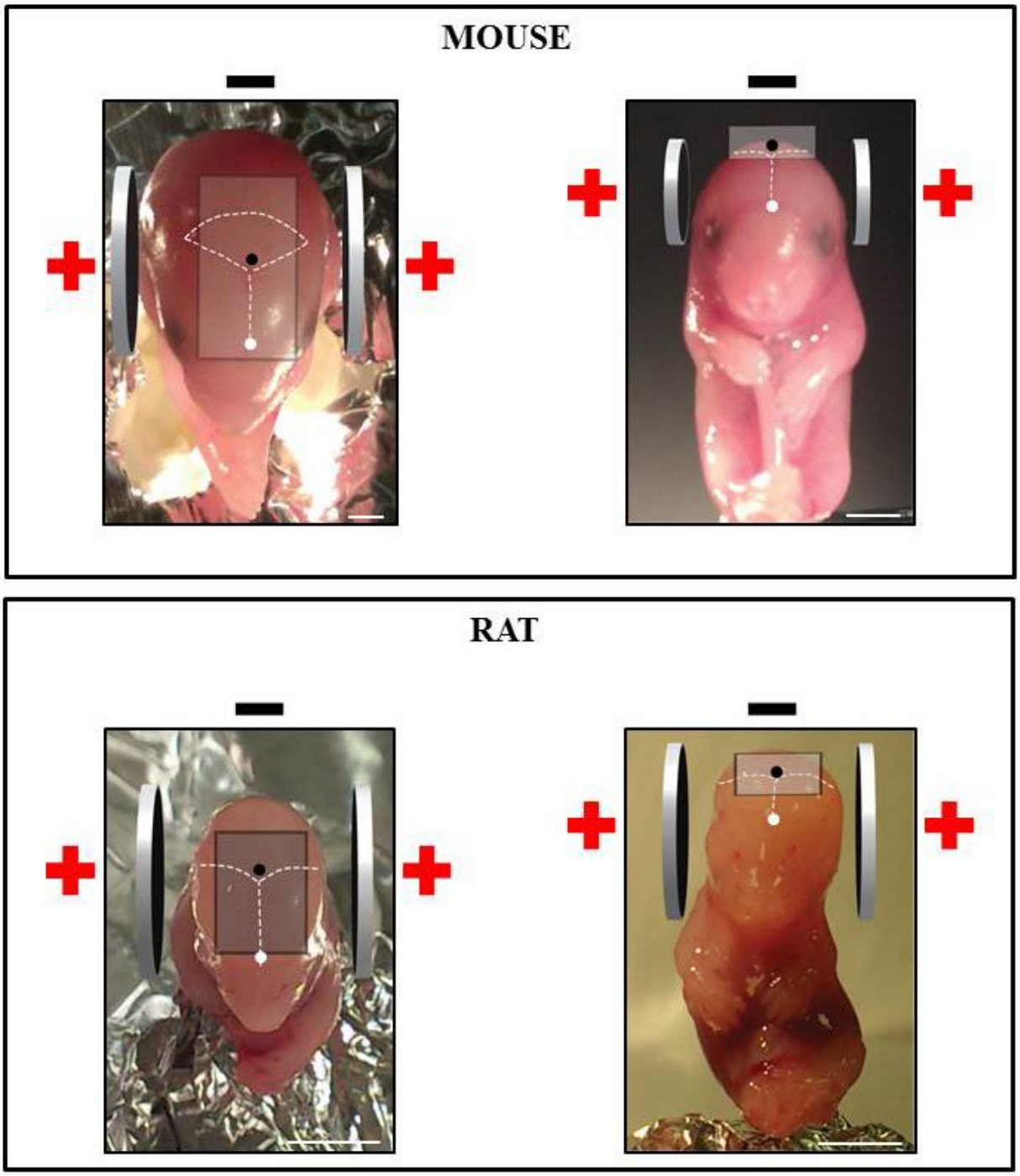

Figure 7

Top (left) and front (left) views of the three-electrode configuration for _in utero_ electroporation of mouse and rat embryos in the hippocampus. – and + indicate polarities of the additional third and forceps-type electrodes, respectively. White dotted lines indicate coronal and sagital sutures on the scull. The white dot indicates Bregma and the black dot indicates Lambda. Scale bars: 3mm (mouse), 5mm (rat).

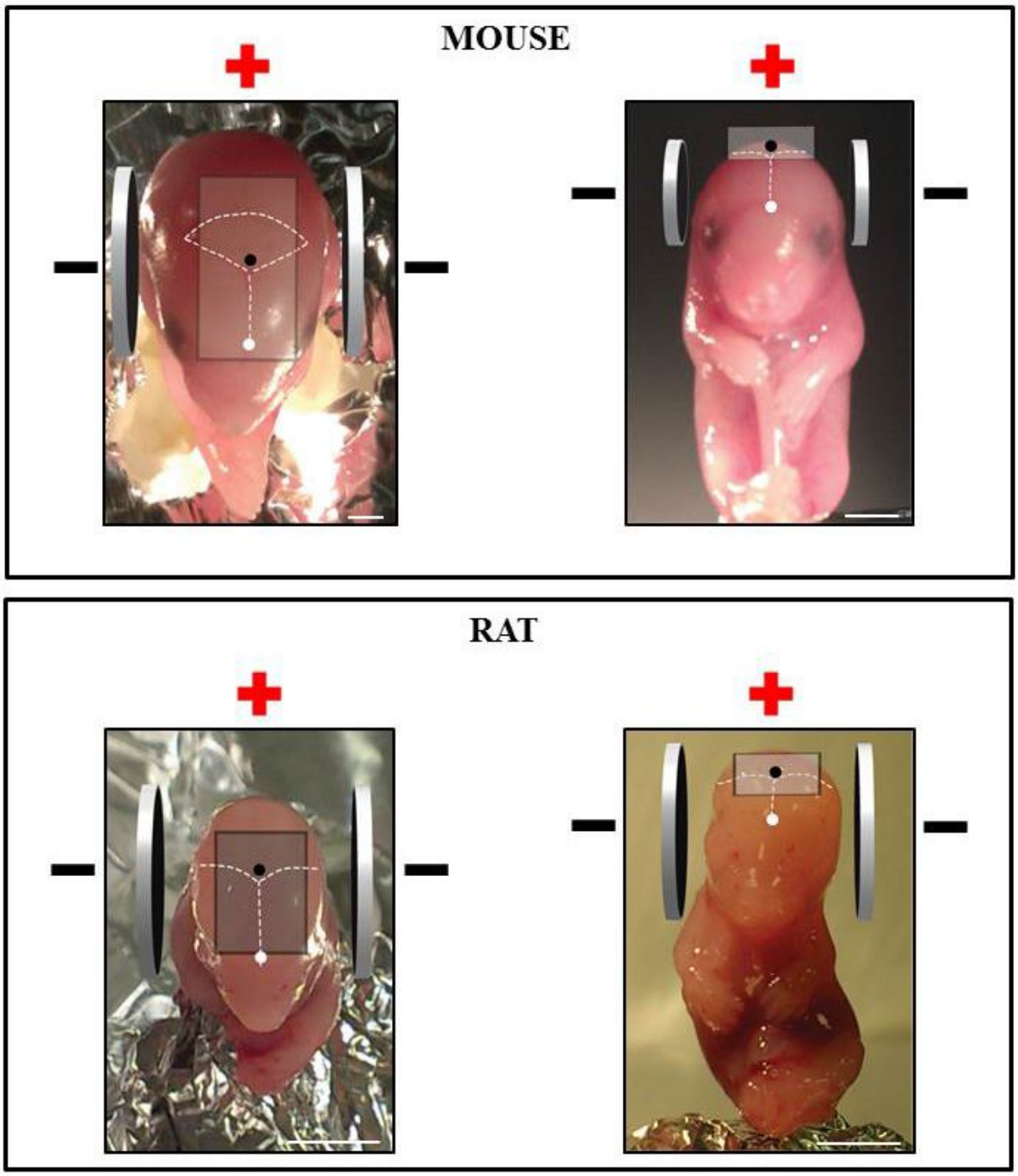

Figure 8

Top (left) and front (left) views of the three-electrode configuration for _in utero_ electroporation of mouse and rat embryos in the motor cortex. + and – indicate polarities of the additional third and forceps-type electrodes, respectively. White dotted lines indicate coronal and sagital sutures on the scull. The white dot indicates Bregma and the black dot indicates Lambda. Scale bars: 3mm (mouse), 5mm (rat).

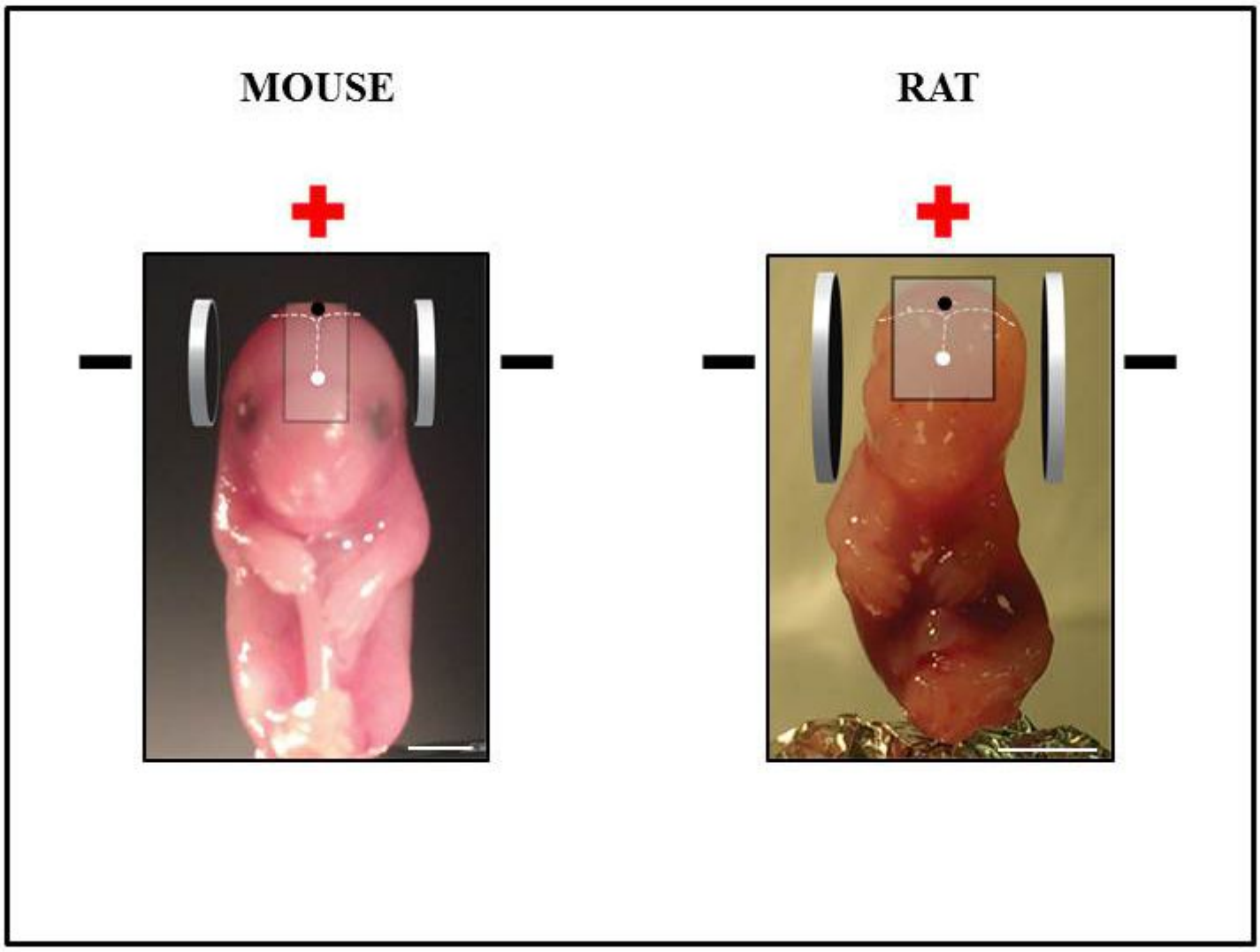

Figure 9

Front views of three-electrode configuration for _in utero_ electroporation of mouse (left) and rat (right) embryos in the prefrontal cortex. + and – indicate polarities of the additional third and tweezer-like electrodes, respectively. White dotted lines indicate coronal and sagital sutures on the scull. The white dot indicates Bregma and the black dot indicates Lambda. Scale bars: 3mm (mouse), 5mm (rat).

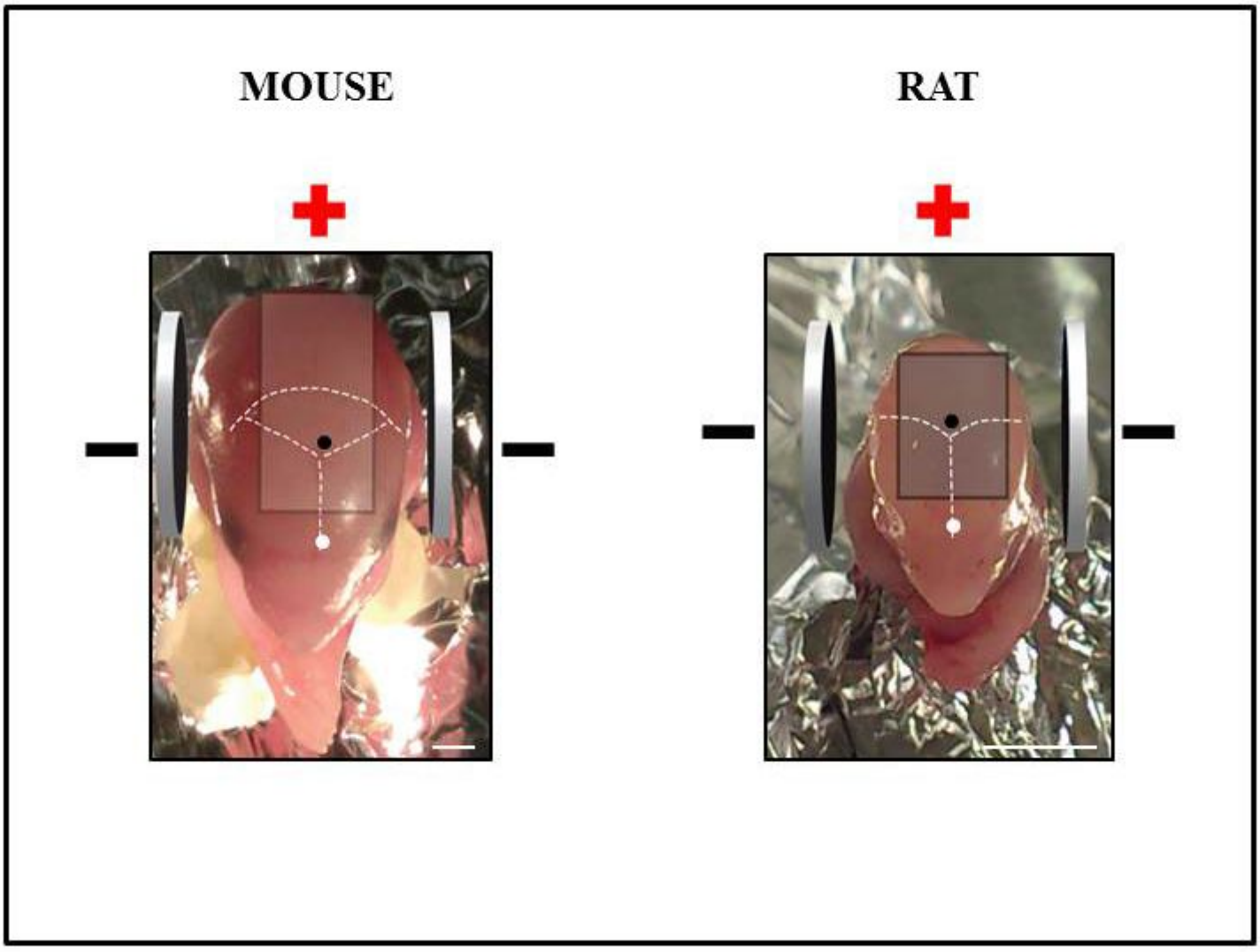

Figure 10

Top views of the three-electrode configuration for _in utero_ electroporation of mouse (left) and rat (right) embryos in the visual cortex. + and – indicate polarities of the additional third and forceps-type electrodes, respectively. White dotted lines indicate coronal and sagital sutures on the scull. The white dot indicates Bregma and the black dot indicates Lambda. Scale bars: 3mm (mouse), 5mm (rat).

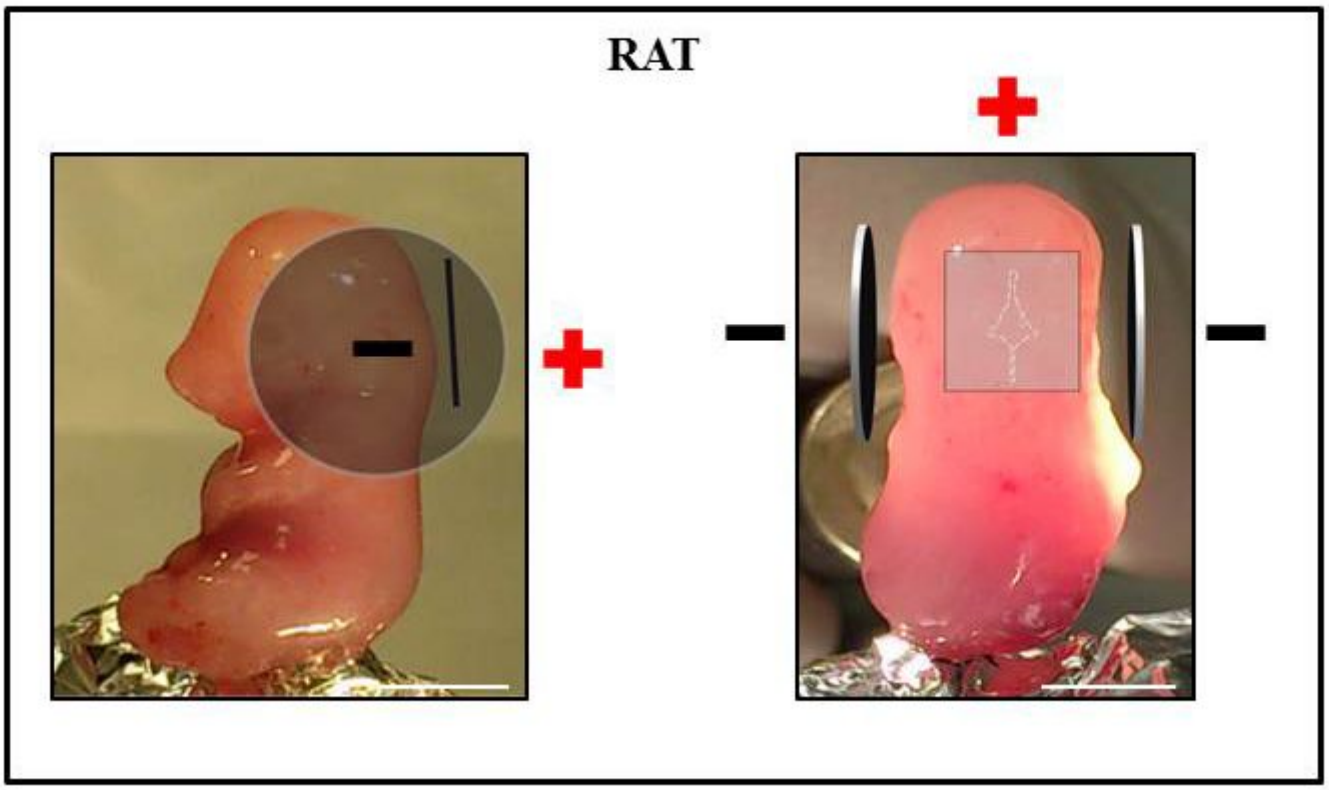

Figure 11

Side (left) and back (right) views of the hree-electrode configuration for _in utero_ electroporation of rat embryos in the cerebellum. + and – indicate polarities of the additional third and forceps-type electrodes, respectively. White dotted lines indicate location of the fourth ventricle. Scale bars: 5mm.

	Anesthesia Induction		Surgery	
	Isoflurane (%)	Oxygen (mL/min)	Isoflurane (%)	Oxygen (mL/min)
Mouse	4,0	1,5	2,0	1,5
Rat	3,5	1,5	2,5	0,8

Figure 12

Table 1 Proper isoflurane and oxygen levels for _in utero_ electroporation on mouse and rat. ! CAUTION All experiments must be performed in accordance with relevant institutional and governmental guidelines and regulations.

Parameter/Animal	MOUSE	RAT
Intensity	30V	50V (hippocampus, cortex-layer II/II) 35V (cerebellum)
Length	50ms	50ms
Interval	1s	150ms

Figure 13

Table 2 Standard parameters for tripolar _in utero_ electroporation of mouse and rat.

Step	Problem	Possible reason	Solution
13	Dam or embryos died	Bacteria contamination	Always use sterile tools and wear sterile gloves.
15	Damage of the embryos or internal organs during opening	Inappropriate choice of surgical tools	Ensure selection of the proper surgical tool. Never use the scalpel for opening the mouse abdominal cavity.
	Bleeding		Always stop the bleeding as soon as possible by applying pressure with curved mosquito-forceps and/or by using the cauterizing pen. Never remove the blood clot.
17	Damage of the uterus	Harsh pulling of the embryos out of the dam's abdominal cavity	Be gentle while pulling the embryos out of the dam's abdominal cavity. Use the ring forceps first and then your own fingers. Never use sharp tools.
19	Damage of the brain	Blunt tip of the injection needle	Ensure that the tip of the needle is sharp. Change the needle immediately in case of blunting.
	Damage of the yolk sac and placenta. Embryos do not float in their sac. Embryos look like all aligned with no space between them. Uterus becomes opaque.	Harsh and prolonged manipulation of the embryos	Never squeeze the embryo with forceps, fingers or electrodes. If the embryo is positioned in a difficult position for injection, gently tap onto it through the uterine wall until the embryo flips while floating in its yolk sac. In case the flipping attempt does not work, skip that embryo and directly go to the next to avoid excessive manipulation
20	Electroporation of the wrong structure	Wrong polarity or position of the electrodes	Ensure that you connected the electrodes to the correct polarities. Use correct configuration of the electrode's positions. Hold the embryo in a stable position with the forceps-type electrode.
		Wrong embryonic stage	Ensure that you are performing surgery at the correct developmental stage to target progenitors cells of desired brain structure (BOX 7)
	Low transfection efficiency (BOX 8)	Wrong electroporation parameters	Ensure that you used correct electroporation parameters for rat or for mouse. See Table 2 for details.
		DNA diffusion	Electroporate the embryo soon after injection.
		Low-quality DNA	Use high-quality endotoxin-free DNA. Prepare fresh DNA.
		Wrong concentration of DNA	Measure the DNA concentration again.
21	The dam died	Wrong stitching	Ensure you put correct and tight stitches or surgical staples. See BOX 5 for details.

Figure 14

Table 3 Troubleshooting

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplement0.pdf