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C57BL/6-specific conditions for efficient *in utero* electroporation of the central nervous system

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HIGHLIGHTS

- We show individually adapted conditions for *in utero* transfection in C57BL/6 mice.
- We outline a detailed angle-map, which allows specific and efficient transfections.
- Histogram analysis objectifies and accelerates postmitotic neural migration assays.

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ABSTRACT

Background: *In utero* electroporation is a fast an efficient tool to specifically address gene expression in the murine central nervous system. This technique was originally established in ICR/CD-1 outbred mice. Neuroanatomical differences between the different mouse strains and variations in gestation length require the optimization of the conditions for each strain to avoid severe complications. Furthermore the relevant position information is currently only scarcely standardized and not always easy to transfer to C57BL/6 mice.
New method: In this study we present an improved method for *in utero* electroporation of C57BL/6 including a detailed atlas that allows for specific and efficient *in vivo* transfection. Further we introduce histogram analysis as a tool for neural migration assays.
Results: We report individually adapted conditions for *in utero* electroporation in C57BL/6 mice that differ from the previously published data for ICR/CD-1 mice. Furthermore, this article outlines a detailed angle-map that allows for the specific and efficient *in vivo* transfection of different regions of the C57BL/6 mouse central nervous system. We also show that histogram analysis is a valuable tool for objectifying and accelerating postmitotic neural migration assays.
Comparison with existing methods: Until now, conditions for *in utero* electroporation of C57BL/6 mice are sparsely defined. Further, compared with time-consuming cell body counting histogram analysis allows objectified and accelerated postmitotic neural migration assays.
Conclusion: Together, our results provide a manual for the *in utero* electroporation of specific regions of the central nervous systems C57BL/6 mice and objectified data analysis.

1. Introduction

We are still far from understanding the enormous complexity of the human brain. Indeed, ongoing neuroanatomical research is still seeking to shed some light on this issue. *In utero* electroporation is a fast an efficient method of specifically addressing gene expression in the murine brain (Fukuchi-Shimogori and Grove, 2001; Saito and Nakatsuji, 2001; Tabata and Nakajima, 2001). *In utero* electroporation allows for the localized insertion of particular genes and siRNAs into specified brain regions and is therefore a powerful technique for studying the development and function of the brain (Kolk et al., 2011; Nishimura et al., 2012; Saito and Nakatsuji, 2001). Transfected progenitors and their descendant cells express the inserted genes, which enable observation of the resulting cell morphology, proliferation and migration at later stages (Tabata and Nakajima, 2008). Different plasmids can be simultaneously transfected into one cell (Saito and Nakatsuji, 2001). Depending on the embryonic

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stage and the transfected region, different cells migrating in specific brain regions can be transfected (Swartz et al., 2001). This technique can even be expanded to postnatal stages (Boutin et al., 2008). Different areas of the mouse brain can be manipulated by varying the position of the positive electrode (Kolk et al., 2011; Langevin et al., 2007; Navarro-Quiroga et al., 2007; Saito and Nakatsuji, 2001). The combination of *in utero* electroporation with transgenic mouse strains additionally enhances the number of possible applications for elucidating the molecular mechanisms of brain development and function (Trimbuch et al., 2009). *In utero* electroporation was primarily established in ICR/CD-1 outbred mice (Fukuchi-Shimogori and Grove, 2001; Saito and Nakatsuji, 2001; Tabata and Nakajima, 2001). However, even within this model, there is a lack of uniformity regarding the optimal voltages (Chen et al., 2005; Saito and Nakatsuji, 2001; Tabata and Nakajima, 2001). Many transgenic mouse models used in neuroanatomical research are based on the C57BL/6 background. Although several publications regarding the *in utero* electroporation of C57BL/6 mice exist (Andrews et al., 2013; Breuss et al., 2012; Cang et al., 2005), an exact protocol for this mouse strain has yet to be published. The importance of adapted protocols is underpinned by the known neuroanatomical differences between the ICR/CD-1 and C57BL/6 strains (Chen et al., 2006). Importantly, the genetically determined gestation period varies between 18 and 22 days depending on the strain (Murray et al., 2010). Thus, the exact embryonic stages might not be exactly comparable. Furthermore, there are known differences in vascular anatomy in different mouse strains (Barone et al., 1993), and there are further general differences in susceptibilities to brain injury between C57BL/6 and ICR/CD-1 mice (Sheldon et al., 1998). Moreover, high voltages negatively influence the endometrium (Letterie et al., 1993), and the sensitivity of the endometrium might also vary across different mouse strains. Ignorance of these basic facts might lead to severe complications such as vascular lesions and abortions caused by *in utero* electroporation. Indeed, our experiences show that there are differences in the reactions of C57BL/6 mice to *in utero* electroporation compared to the first data published for the ICR/CD-1 mice (Fukuchi-Shimogori and Grove, 2001; Saito and Nakatsuji, 2001; Tabata and Nakajima, 2001). The aims of this study were twofold: first, we sought to establish a standardized protocol that provides detailed descriptions of the steps necessary to efficiently transfect certain brain regions of C57BL/6 mice; second, we sought to investigate whether histogram analysis is a valuable tool for accelerating the reliable and objective analysis of neuronal migration.

2. Materials and methods

2.1. Mice

Mice were kept on a regular 12 h light/12 h dark cycle. Timed-pregnant C57BL/6 mice were purchased from commercial suppliers. The day the plug was detected was considered day 0. The handling of the mice and the experimental procedures were conducted in accordance with European, national and institutional guidelines for animal care.

2.2. Plasmid preparation

To obtain endotoxin-free advanced transfection-grade plasmids, the plasmids were purified using the QIAGEN (Venlo, Limburg, Netherlands) EndoFree Plasmid Maxi Kit according to the manufacturer's protocol. Purified plasmids were handled with care to avoid pathogenic contamination.

2.3. In utero electroporation

Timed pregnant mice were anesthetized subcutaneously with ketamine hydrochloride (40 mg/kg, Ketavet, Pfizer New York City, NY, USA) and xylazine (5 mg/kg, Rompun 2%, Bayer, Leverkusen, Germany). The entire length of the anesthesia was kept to a maximum of 35–45 min, and the length of Stage III (stage of surgical anesthesia according to Guedel, 1937) was kept to a maximum of 25–30 min. After fixation of the limbs, the surgical area was sterilized with 70% ethanol. The mother was covered with sterile gauze with only the surgical area exposed (Supplementary Fig. 1b–e). The gauze was moistened with a physiological saline solution containing the bacteriostatic agent benzyl alcohol as a 0.9% solution. Subsequently, the abdominal cavity was cut open (skin incision: 1.5–2 cm, muscle incision: 1–1.5 cm), and the uterine horns were carefully extracted using ring forceps (Supplementary Fig. 1a–c). The age of the embryos in the exposed uterine horns were visually verified using the Theiler stages (e.g. TS 20 = E12) (Theiler, 1972). The abdominal cavity, especially the exposed uterine horns, was kept moist with warmed 0.9% benzyl alcohol solution throughout the surgery.

2.3.1. Injection of DNA and electroporation

Specially designed glass capillaries are required for effective injection of the DNA solution. Borosilicate glass capillaries (0.8–0.9 mm in diameter, World Precision Instruments Inc., Sarasota, FL, USA) were pulled using a P-97 Micropipette Puller (Sutter Instrument Company, Novato, CA, USA). The tips were grinded at 35° angles (Microgrinder EG-44, Narishige, London, GB). Unity was carefully reviewed with the use of a template (8× enlargements). The maximum outer tip diameter was kept to 60 μm. To define the exact injected volume, defined volumes were drawn up into the capillary and the capillary length per μl was calculated. The DNA-solution was colored by adding 1 μl of Fast Green solution (0.01 g/15 ml TE Carl Roth, Karlsruhe, Germany) per 10 μl of DNA solution, thereby allowing for the visual verification of successful injections by the overall green color of the relevant lateral ventricle (Supplementary Fig. 1c). Between 0.5 μl and 3 μl (E12: 0.5–1 μl, E13: 1.5 μl, E14: 1.5–2 μl, E15: 2 μl, E16: 2.5 μl, E17: 3 μl) of colored DNA solution containing 3–4 μg of the GFP-encoding plasmid (pCAGGS (Addgene, Cambridge, UK)) was injected into the lateral ventricle. The injection volume was kept low to reduce the spread of the solution to other ventricles, thereby enhancing the specificity. The low injection volume is especially important because the application of the DNA solution was performed slowly (1 μl in 10–20 s). For the injection, the capillaries were positioned at the defined coordinates using a binocular microscope. The injection depth was 0.5 mm.

The appropriate voltage was applied *via* specialized platinum electrodes (Supplementary Fig. 1d; Nepagene CUY 650P (Nepagene, Ichikawa, Japan), interval cycle length 50 ms, interval pause 950 ms). The voltage was supplied by two types of electroporators (Nepagene CUY 21 (Nepagene, Ichikawa, Japan) or BTX ECM 830 (Harvard Apparatus Inc., Holliston, MA, USA)) that delivered identical results. The size of the electroporation paddle was adapted to the embryonic stage (E12: 0.5 mm; E13 and E14: 3 mm; E15 and E16: 5 mm; and E17: 10 mm). Especially in the younger embryonic stages, using electrodes that are too large reduces specificity (Supplementary Fig. 4 and LoTurco et al., 2009), because the electrodes cover too large of a section of the embryo (Supplementary Fig. 3). Additionally, too large of an electrode size also negatively influences the survival because the electrodes are located closer to the embryo's heart, which can affect the heart rhythm with the electric pulses (Saito, 2006). Transfections of different areas of the murine central nervous system were achieved by adjusting the positive pole in the desired direction. Larger electrode sizes also negatively