

Chapter 14

In Utero Electroporation: Assay System for Migration of Cerebral Cortical Neurons

Hidenori Tabata and Kazunori Nakajima

1 Introduction

During the development of the cerebral cortex, the majority of cortical neurons are generated in the ventricular zone (VZ) facing the lateral ventricle and then migrate toward the pial surface to form the highly organized 6-layered cerebral cortex. Detailed profiles of these processes and their molecular mechanisms had been largely unknown because of the absence of an efficient assay system. The *in vivo* electroporation system was initially devised for use within chick embryos (Funahashi et al., 1999; Itasaki et al., 1999; Momose et al., 1999; Muramatsu et al., 1997), and we and other groups have used that system as a basis for developing an *in utero* electroporation system, which allows plasmid DNA to be introduced into cortical progenitor cells in developing mouse embryos in the uterus (Fukuchi-Shimogori and Grove, 2001; Saito and Nakatsuji, 2001; Tabata and Nakajima, 2001; Takahashi et al., 2002). *In utero* electroporation of other sites in the brain, including the hippocampus (Navarro-Quiroga et al., 2007), cerebral basal ganglia (Borrell et al., 2005; Nakahira et al., 2006), cortical hem (Takiguchi-Hayashi et al., 2004), and dorsal thalamus (Bonnin et al., 2007), has recently been reported. Introducing green fluorescent protein (GFP) enables the entire processes of migration and layer formation to be visualized (Ajioka and Nakajima, 2005; Sasaki et al., 2008; Tabata and Nakajima, 2002, 2003), and the role of any gene involved in these processes can be easily assessed by overexpressing the proteins or their mutants (Ohshima et al., 2007), or by knocking down the genes by the RNA interference technique (Bai et al., 2003). Furthermore, the Tet-On/Off system and/or other plasmid-vector-based technologies will expand the potential of the analyses. In this section we review the principles and methods of gene transfer into the cortical wall of mouse embryos by means of the *in utero* electroporation system.

H. Tabata and K. Nakajima(✉)
Department of Anatomy, Keio University School of Medicine,
Shinjuku-ku, Tokyo 160-8582, Japan
e-mail: kazunori@sc.itc.keio.ac.jp

2 Materials

2.1 Instruments

- Electroporator: CUY21E or CUY21SC (Nepa gene, Chiba, Japan)
- Tweezers-type electrode: CUY650P3 for embryonic day (E) 12.5, or CUY650P5 for E13 or older (Nepa gene)
- Aspirator tube assembly (Drummond)
- 1-mm diameter glass capillary tubes (GD-1; Narishige, Tokyo, Japan)
- Optical fiber light
- Sterile gauze: 7.5 × 7.5 cm
- Surgical instruments: fine forceps × 2, surgery scissors × 2, ring forceps, needle holder
- Surgical tape
- Nylon suture
- Silk suture

2.2 Reagents

- HEPES-buffered saline (HBS): Dilute 2 × concentrated HBS (51558, Sigma) with autoclaved water at 1/2
- 0.1% FastGreen solution: Dissolve the powder (F7258, Sigma) with autoclaved water
- Autoclaved phosphate-buffered saline (PBS)
- Diluted Nembutal solution: Dilute Nembutal solution (5% Pentobarbital sodium salt solution) with autoclaved water at 1/10

2.3 Preparation of Plasmid DNA (See Also Comments 1 and Fig.14.3b)

Plasmid solution prepared by conventional methods, such as with the Qiagen kit, can be used for *in utero* electroporation. If precipitates form in the plasmid solution, further purification with phenol/chloroform followed by chloroform extraction is required, because such precipitates may cause hydrocephalus. Plasmids are dissolved with HBS. The location of the DNA solution is monitored by adding 0.1% Fast Green solution at 1/10 volume to the aliquot of DNA solution to be injected.

2.4 Preparation of Micropipettes

The micropipettes for injection are made with a puller (PC-10, Narishige, Tokyo, Japan) from 1-mm diameter glass capillary tubes (GD-1; Narishige). The tip of the

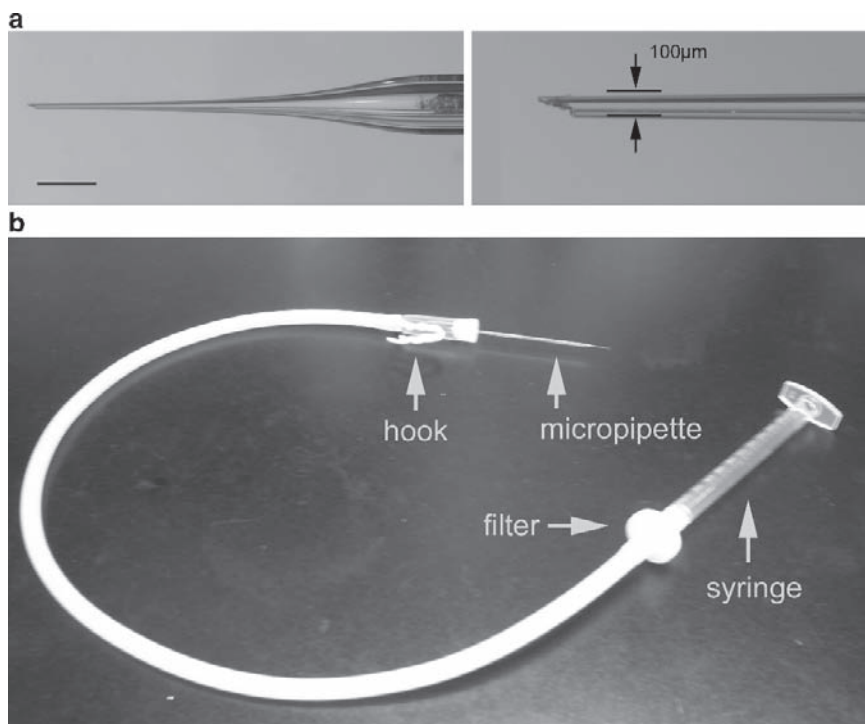


Fig. 14.1 Preparation of the aspirator tube assembly. **(a)** Shape of micropipette. A high-power view of the tip is shown in the right panel. The tip of the needle has been cut obliquely. **(b)** The aspirator tube assembly. The mouthpiece of the original product has been replaced with a $0.22\ \mu\text{m}$ filter and 1 ml syringe, as a new mouthpiece. Scale bar in **(a)**, 1 mm

micropipettes is cut obliquely with a fine forceps under a dissecting microscope (Fig. 14.1a). The micropipettes are attached to the aspirator tube assembly (Drummond) (Fig. 14.1b).

3 Procedure

1. A pregnant mouse is intraperitoneally injected with a $12\ \mu\text{l/g}$ body weight dose of 1/10 diluted Nembutal solution (see Comments 2).
2. Ten minutes later the mouse is placed on its back on a dissecting board, and its limbs are held in place with a surgical tape.
3. After disinfecting the abdominal skin with 70% ethanol, an approximately 2 cm midline skin incision is made extending anteriorly from the level of the midpoint between the most posterior two pairs of nipples using one set of forceps and scissors (Fig. 14.2a).
4. A 2 cm midline incision is then made in the abdominal wall along the linea alba using another set of forceps and scissors. A piece of sterile gauze with a hole

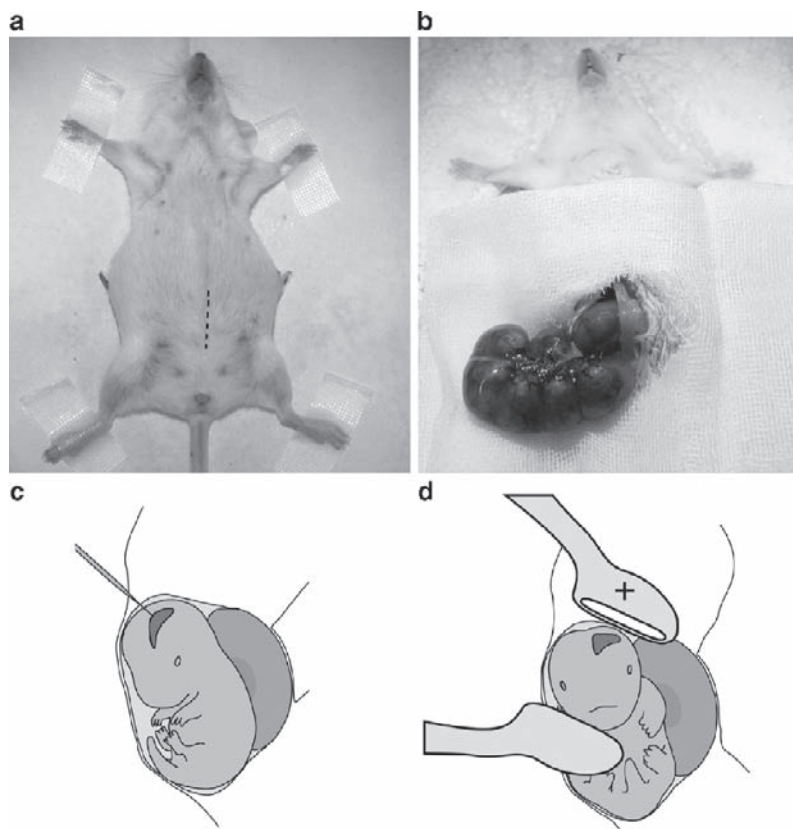


Fig. 14.2 *In utero* electroporation procedure. (a) Anesthetized pregnant mouse placed on a dissecting board. The broken line indicates the site of the incision. (b) One uterine horn is drawn out through a hole in a piece of sterile gauze. (c) Plasmid DNA is injected into one or both lateral ventricles through the uterine wall. (d) Electronic pulses are applied from outside the uterine wall

cut in the center is placed over the incision, and one uterine horn is drawn out through the hole in the gauze (Fig. 14.2b).

5. After observing the orientation of the embryos through the wall of the uterine horn, a micropipette is inserted into the lateral ventricle, and 1–2 μ l of plasmid solution is injected by expiratory pressure using the aspirator tube assembly (see Comments 3–6, Fig. 14.2c).
6. After soaking the uterine horn with PBS, the head of embryo is pinched with a tweezers-type electrode (CUY650P3 or CUY650P5), and electronic pulses are applied with a CUY 21E electroporator (see Comments 7, Fig. 14.2d). The electroporation conditions are shown in Table 14.1. The voltage should be adjusted so that the actual current would become 40–60 mA.
7. After the procedure is completed on the first uterine horn, it is replaced in the abdominal cavity, and the other uterine horn is exposed and subjected to the same procedure.

Table 14.1 The electroporation conditions

Age	Electrode diameter	Voltage	Pulse-on	Pulse-off	Number of pulses
E12.5	3 mm	33 V	30 ms	970 ms	4
E13 ~	5 mm	30~35 V	50 ms	950 ms	4

Pulse-on; the duration of square pulse.

Pulse-off; the interval between pulses.

8. When the procedures on the second uterine horn have been completed, the abdominal cavity is filled with PBS.
9. The abdominal wall and skin are closed with nylon sutures and silk sutures, respectively.
10. Since the body temperature of the mouse decreases as a result of the exposure of uterus and the effect of anesthetization, the mouse should be warmed with hands or on a plate warmer (see Comments 8).
11. After surgery, the mice should give birth to the electroporated pups normally. If CAG-promoter-driven expression vector of enhanced GFP (CAG-EGFP) (Niwa et al., 1991) has been injected, the GFP fluorescence would be visible through the skin under a fluorescence stereomicroscope on postnatal day 1.

4 Application and Results

4.1 Optimization of Electroporation Conditions

Several conditions must be optimized to make *in utero* electroporation more efficient.

4.1.1 Number of Pulses

A 4 $\mu\text{g}/\mu\text{l}$ dose of CMV promoter-driven *luciferase*-expression vector (CMV-luc) was injected into the lateral ventricle at E14.5, and one, two, or four electronic pulses were applied. The transfected brains were sampled 24 h later, and the efficiency of gene-transfer was evaluated on the basis of luciferase activity (Fig. 14.3a). Application of one pulse was insufficient to achieve significant gene transfer. When more than one pulse was applied, the efficiency of gene-transfer increased almost linearly with the number of pulses. The electric pulses create tiny pores in the plasma membrane, and move DNA toward the anode in the electric field. One pulse might be enough to create the pores, not to drive the DNA toward the anode.

4.1.2 Plasmid Concentration

CMV-luc was injected in dose of 0.5, 2, or 5 $\mu\text{g}/\mu\text{l}$ at E15.5 and four pulses were applied. The brains were sampled 24 h later, and the luciferase activity of each

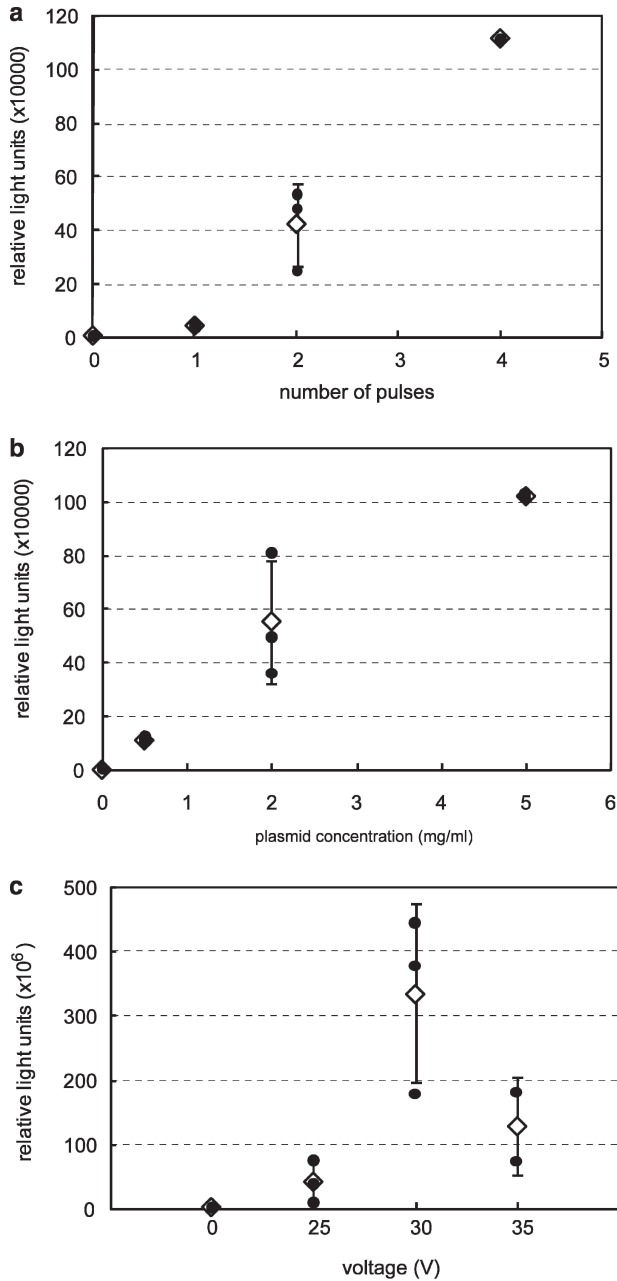


Fig. 14.3 Optimization of various factors for more efficient *in utero* electroporation. Effect of number of pulses (**a**), plasmid concentration (**b**), and voltage (**c**) on transfection efficiency. (**a**) A 4 $\mu\text{g}/\mu\text{l}$ dose of CMV-luc was injected into the lateral ventricle at embryonic day 14.5 (E14.5), and different numbers of 30 V pulses were applied. The transfected brains were sampled 24 h later, and luciferase activity was measured. (**b**) CMV-luc plasmid prepared in various concentrations was injected at E15.5 and four 30 V pulses were applied, and transfected brains were sampled 24 h later. (**c**) 4 $\mu\text{g}/\mu\text{l}$ dose of CMV-luc was injected at E15 and electric pulses of various voltages were applied four times. The brains were sampled at E18. The duration of the square pulses applied in all experiments was 50 ms, and the interval between pulses was 950 ms

sample was analyzed (Fig. 14.3b). The efficiency of gene-transfer increased almost linearly with the concentration of plasmid DNA injected.

4.1.3 Voltage

CMV-Luc was injected in dose of $4\mu\text{g}/\mu\text{l}$ at E15 and four pulses were applied. The brains were sampled at E18, and the luciferase activity of each sample was analyzed (Fig. 14.3c). Luciferase activity was detected when a voltage of around 30V was applied, and lower voltages did not bring about effective gene transfer. This finding seems to indicate that a high level of energy is needed to produce the pores in the plasma membrane. When a voltage of 35V was applied, the expression of the plasmid that was introduced tended to reduce. It may have resulted from cell death.

4.2 Tracing the Lineage of GFP-Positive Cells

CAG-EGFP ($5\mu\text{g}/\mu\text{l}$) (Niwa et al., 1991) was injected into both hemispheres at E12.5. After fixing the embryos at E14.5, the brains were dissected out and examined for GFP fluorescence under a fluorescence stereomicroscope (Fig. 14.4a). Fluorescence was observed in the lateral region of the hemisphere onto which the anode had been placed and in the medial region of the opposite hemisphere. GFP-positive cells were observed in the VZ 12h after electroporation. Accumulation of GFP-positive cells in the subventricular zone (SVZ) and scattered distribution of GFP-positive cells in the intermediate zone (IZ) were observed 1.5 days after

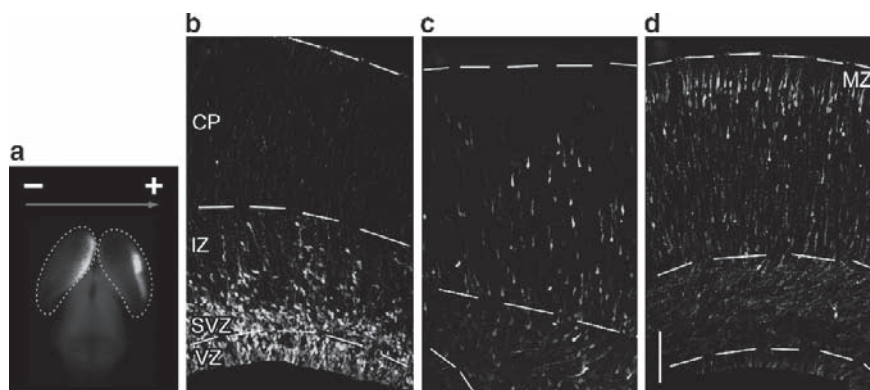


Fig. 14.4 GFP expression after electroporation. E12.5 mouse embryos were subjected to *in utero* electroporation and fixed 2 days later. The brains were removed and examined under a fluorescence stereomicroscope (a). Changes in the position and morphology of GFP-positive cells after *in utero* electroporation (b–d). E14.5 (b,c) or E15 (d) mouse embryos were transfected with CAG-EGFP and then fixed 1.5 days (b), 3 days (c), and 6 days (d) after electroporation. Abbreviations: VZ; ventricular zone, SVZ; subventricular zone, IZ; intermediate zone, CP; cortical plate, MZ; marginal zone. Scale bar, 50 μm

electroporation (Fig. 14.4b). By 2.5 days, the number of GFP-positive cells in the IZ had increased, but only a few GFP-positive cells were found within the cortical plate (CP) at that time. By 3 days, many GFP-positive cells assuming bipolar morphology with their leading process pointing toward the pial surface were found in the CP (Fig. 14.4c). At 6 days after electroporation, the major population of GFP-positive cells was observed beneath the marginal zone (MZ) (Fig. 14.4d), and the cells were extending primitive dendrites into the MZ.

5 Comments

1. The efficiency of electroporation is highly dependent on the DNA concentration. The higher the concentration of DNA used, the higher the level of expression became (Fig. 14.3b). When CAG-EGFP is used, a concentration of $1\ \mu\text{g}/\mu\text{l}$ is sufficient to visualize the migrating neurons. Overexpression of unstable or large proteins requires higher concentrations, such as 5 or $10\ \mu\text{g}/\mu\text{l}$. When a mixture of two different plasmids is injected, all of the transfected cells should contain both plasmids.
2. Sometimes the anesthesia is inadequate to carry out surgery at the dose of Nembutal described in Procedure Step1. In this case, half the original dose should be applied intraperitoneally.
3. Do not grasp the uterus too tightly. Hook the uterus with ring forceps first, and then draw the uterus out with the fingers.
4. Fast Green enables the distribution of the DNA solution in the lateral ventricle to be seen through the uterine wall.
5. Pushing the back of the head of the embryos so that the head is pressed against the uterine wall facilitates the injection process. The micropipette should be inserted with momentum but should not deeply.
6. We avoid injecting the embryos closest to the vaginal duct, because of the possibility of inducing abortion if they are damaged.
7. The plasmid can be introduced into a larger region of the cortex by placing the cathode on the chin of the embryo and the anode at the center of the injected hemisphere.
8. A long operation time decreases the embryo survival rate. The abdominal cavity should be open for no more than 30 min.

6 Troubleshooting

Gene transfer by electroporation has the major advantages of being a simple procedure and achieving a high level of expression of the gene introduced. However, the risk of damages to the brain may be higher than with other gene transfer systems. The troubleshooting was listed below (Table 14.2).

Table 14.2 Troubleshooting

Problem	Cause	Solution
Dam dies	Operation time too long	Not every embryo needs to be injected. Select embryos to inject and record their location
	Excessive bleeding due to abortion	See “high abortion rate” below
High abortion rate	Operation time too long	See above
	Severe embryo injury	Handle embryos gently. We recommend getting used to handling embryos by starting with E14 or E15 embryos, the ages at which they are easiest to inject
	Disruption of blood flow to the uterus	Make larger skin and abdominal wall incisions. Make sure blood flow is not obstructed by twisting of blood vessels during the operation
	Delayed recovery from the anesthesia	Keep mouse warm during and after the operation
Hydrocephalus	Embryo injury	See solution to “high abortion rate”
	Poor DNA quality	Remove all precipitates by centrifugation or by phenol/chloroform treatment
	Voltage too high	Determine appropriate voltage
	Volume of DNA injected too large	Do not inject more than 2 μ l of DNA into one lateral ventricle of an E15 embryo
	Tip of the micropipette blunt	Cut tip obliquely

References

- Ajioka I, Nakajima K (2005) Birth-date-dependent segregation of the mouse cerebral cortical neurons in reaggregation cultures. *Eur J Neurosci* 22 (2): 331–42.
- Bai J, Ramos RL, Ackman JB, Thomas AM, Lee RV, LoTurco JJ (2003) RNAi reveals doublecortin is required for radial migration in rat neocortex. *Nat Neurosci* 6 (12): 1277–83.
- Bonnin A, Torii M, Wang L, Rakic P, Levitt P (2007) Serotonin modulates the response of embryonic thalamocortical axons to netrin-1. *Nat Neurosci* 10 (5): 588–97.
- Borrell V, Yoshimura Y, Callaway EM (2005) Targeted gene delivery to telencephalic inhibitory neurons by directional in utero electroporation. *J Neurosci Methods* 143 (2): 151–8.
- Fukuchi-Shimogori T, Grove EA (2001) Neocortex patterning by the secreted signaling molecule FGF8. *Science* 294 (5544): 1071–4.
- Funahashi J, Okafuji T, Ohuchi H, Noji S, Tanaka H, Nakamura H (1999) Role of Pax-5 in the regulation of a mid-hindbrain organizer’s activity. *Dev Growth Differ* 41 (1): 59–72.
- Itasaki N, Bel-Vialar S, Krumlauf R (1999) ‘Shocking’ developments in chick embryology: electroporation and in ovo gene expression. *Nat Cell Biol* 1 (8): E203–7.
- Momose T, Tonegawa A, Takeuchi J, Ogawa H, Umesono K, Yasuda K (1999) Efficient targeting of gene expression in chick embryos by microelectroporation. *Dev Growth Differ* 41 (3): 335–44.
- Muramatsu T, Mizutani Y, Ohmori Y, Okumura J (1997) Comparison of three nonviral transfection methods for foreign gene expression in early chicken embryos in ovo. *Biochem Biophys Res Commun* 230 (2): 376–80.
- Nakahira E, Kagawa T, Shimizu T, Goulding MD, Ikenaka K (2006) Direct evidence that ventral forebrain cells migrate to the cortex and contribute to the generation of cortical myelinating oligodendrocytes. *Dev Biol* 291 (1): 123–31.

- Navarro-Quiroga I, Chittajallu R, Gallo V, Haydar TF (2007) Long-term, selective gene expression in developing and adult hippocampal pyramidal neurons using focal in utero electroporation. *J Neurosci* 27 (19): 5007–11.
- Niwa H, Yamamura K, Miyazaki J (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108 (2): 193–9.
- Ohshima T, Hirasawa M, Tabata H, Mutoh T, Adachi T, Suzuki H, Saruta K, Iwasato T, Itoharu S, Hashimoto M, Nakajima K, Ogawa M, Kulkarni AB, Mikoshiba K (2007) Cdk5 is required for multipolar-to-bipolar transition during radial neuronal migration and proper dendrite development of pyramidal neurons in the cerebral cortex. *Development* 134 (12): 2273–82.
- Saito T, Nakatsuji N (2001) Efficient gene transfer into the embryonic mouse brain using in vivo electroporation. *Dev Biol* 240 (1): 237–46.
- Sasaki S, Tabata H, Tachikawa K, Nakajima K (2008) The cortical subventricular zone-specific molecule Svet1 is part of the nuclear RNA coded by the putative Netrin receptor gene *Unc5d* and is expressed in multipolar migrating cells. *Mol Cell Neurosci* 38: 474–483.
- Tabata H, Nakajima K (2001) Efficient in utero gene transfer system to the developing mouse brain using electroporation: visualization of neuronal migration in the developing cortex. *Neuroscience* 103 (4): 865–72.
- Tabata H, Nakajima K (2002) Neurons tend to stop migration and differentiate along the cortical internal plexiform zones in the Reelin signal-deficient mice. *J Neurosci Res* 69 (6): 723–30.
- Tabata H, Nakajima K (2003) Multipolar migration: the third mode of radial neuronal migration in the developing cerebral cortex. *J Neurosci* 23 (31): 9996–10001.
- Takahashi M, Sato K, Nomura T, Osumi N (2002) Manipulating gene expressions by electroporation in the developing brain of mammalian embryos. *Differentiation* 70 (4–5): 155–62.
- Takiguchi-Hayashi K, Sekiguchi M, Ashigaki S, Takamatsu M, Hasegawa H, Suzuki-Migishima R, Yokoyama M, Nakanishi S, Tanabe Y (2004) Generation of reelin-positive marginal zone cells from the caudomedial wall of telencephalic vesicles. *J Neurosci* 24 (9): 2286–95.