Develop. Growth Differ. (2008) **50**, 499–506 doi: 10.1111/j.1440-169x.2008.01045.x

Review

Gene application with *in utero* electroporation in mouse embryonic brain

Tomomi Shimogori* and Masaharu Ogawa

RIKEN, Brain Science Institute, Saitama 351-0198, Japan

Mouse genetic manipulations, such as the production of gene knock-out, knock-in, and transgenic mice, have provided excellent systems for analysis of numerous genes functioning during development. Nevertheless, the lack of specific promoters and enhancers that control gene expression in specific regions and at specific times, limits usage of these techniques. However, progress in *in utero* systems of electroporation into mouse embryos has opened a new window, permitting new approaches to answering important questions. Simple injection of plasmid DNA solution and application of electrical current to mouse embryos results in transient area- and time-dependent transfection. Further modification of the technique, arising from variations in types of electrodes used, has made it possible to control the relative size of the region of transfection, which can vary from a few cells to entire tissues. Thus, this technique is a powerful means not only of characterizing gene function in various settings, but also of tracing the migratory routes of cells, due to its high efficiency and the localization of gene expression it yields. We summarize here some of the potential uses and advantages of this technique for developmental neuroscience research.

Key words: electroporation, gene transfer, *in utero*, microelectrode, mouse.

Introduction

One key approach to understanding the genetic mechanisms underlying embryonic development includes overexpression or misexpression of target genes in specific regions and at specific times. The mouse gene knockout system has been used extensively for loss-of-function studies because of the large number of mutant lines available and its technical advantages. In contrast, gain-of-function analyses have been carried out by production of knock-in animals and transgenic animals and with the use of various viruses (Cepko 2001; Jakobsson *et al*. 2003; Hashimoto & Mikoshiba 2004). However, it is not always possible to express or suppress genes in spatially and temporally restricted fashions, and the generation of genetically modified mice and recombinant viruses is time-consuming and labor-intensive. To solve this problem, many attempts have been made to apply the electroporation technique to developmental biology. The avian embryo has been used as a classical model system for the study of developmental events in vertebrates because of the accessibility of this type of embryo. Successful use of a novel gene delivery technique in chick embryos, *in ovo* electroporation, has been established, and this appears to be an excellent method for gene introduction, permitting quick and direct examination of the function of delivered genes (Muramatsu *et al*. 1997; Itasaki *et al*. 1999; Momose *et al*. 1999; Nakamura *et al*. 2000). Adaptation of this technique to the mouse embryo seems reasonable, and would permit analysis of the function of genes more quickly than generation of knock-out or transgenic mouse lines. However, the inaccessibility of mammalian embryos in the uterus has made it difficult or impossible for *in utero* manipulations to target precise regions at most stages of development. Many efforts have been made by different labs to establish *in utero* electroporation, and there have been several reports of successful gene delivery systems in mouse embryo with time- and region-specific expression (Saito & Nakatsuji 2001; Tabata & Nakajima 2001; Borrell *et al*. 2005). The establishment of paddle-type electrodes has made possible delivery of current from outside the wall of

*Author to whom all correspondence should be addressed.
Email: tshimogori@brain.riken.jp
Received 22 March 2008; revised 25 May 2008; accepted 24 March 2008.

the uterus, aiming at a relatively specific region. This technique is useful for analysis of gene function over relatively wide regions. Moreover, further refinement of electrodes and application of current have made this approach useful for examination of smaller regions. The generation of fine wire-type electrodes (micro-electrodes) has made it possible to insert electrodes into the uterus and place them closer to the target region (Fukuchi-Shimogori & Grove 2001). This method has made more precise targeting possible, in addition to control the size of the region of transfection. It also permits different electrode orientations, and thus gene transfer along different axes (Fukuchi-Shimogori & Grove 2003). Furthermore, introducing of a new light source and a new method of holding of the uterus during the surgical procedure has yielded better visibility of embryos through the uterus, permitting gene transfer as early as mouse embryonic (E) day 9.5 (Shimogori *et al*. 2004). This allows study of many aspects of neural differentiation and patterning in time-dependent fashion. Detection of the expression of fluorescent reporter genes, which are co-electroporated with target genes, allows visualization of successfully transfected embryos under the fluorescence microscope. Although *in utero* electroporation involves transient gene delivery, transfection of marker genes such as fluorescent reporter genes (e.g. enhanced green fluorescent protein [EGFP], and enhanced yellow fluorescent protein [EYFP]) or alkaline phosphatase work as transient lineage tracers in fate-mapping and migration experiments. In addition, the combination of the Cre recombinase-loxP system and electroporation enables permanent gene delivery (Tsien *et al*. 1996). For instance, introduction of a Cre construct into the R26R reporter mouse line (Soriano 1999) can be used for permanent cell lineage analysis. Furthermore, loss-of-function analysis can be carried out by introducing dominant-negative molecules, antisense morpholinos, and siRNA (small interfering RNA) constructs. Combinatorial experiments with wild-type or mutant embryos can directly demonstrate various types of cell behaviors and phenotypes. In this review, we describe how to carry out microelectroporation and demonstrate some of the results obtained with this unique technique.

Results and discussion

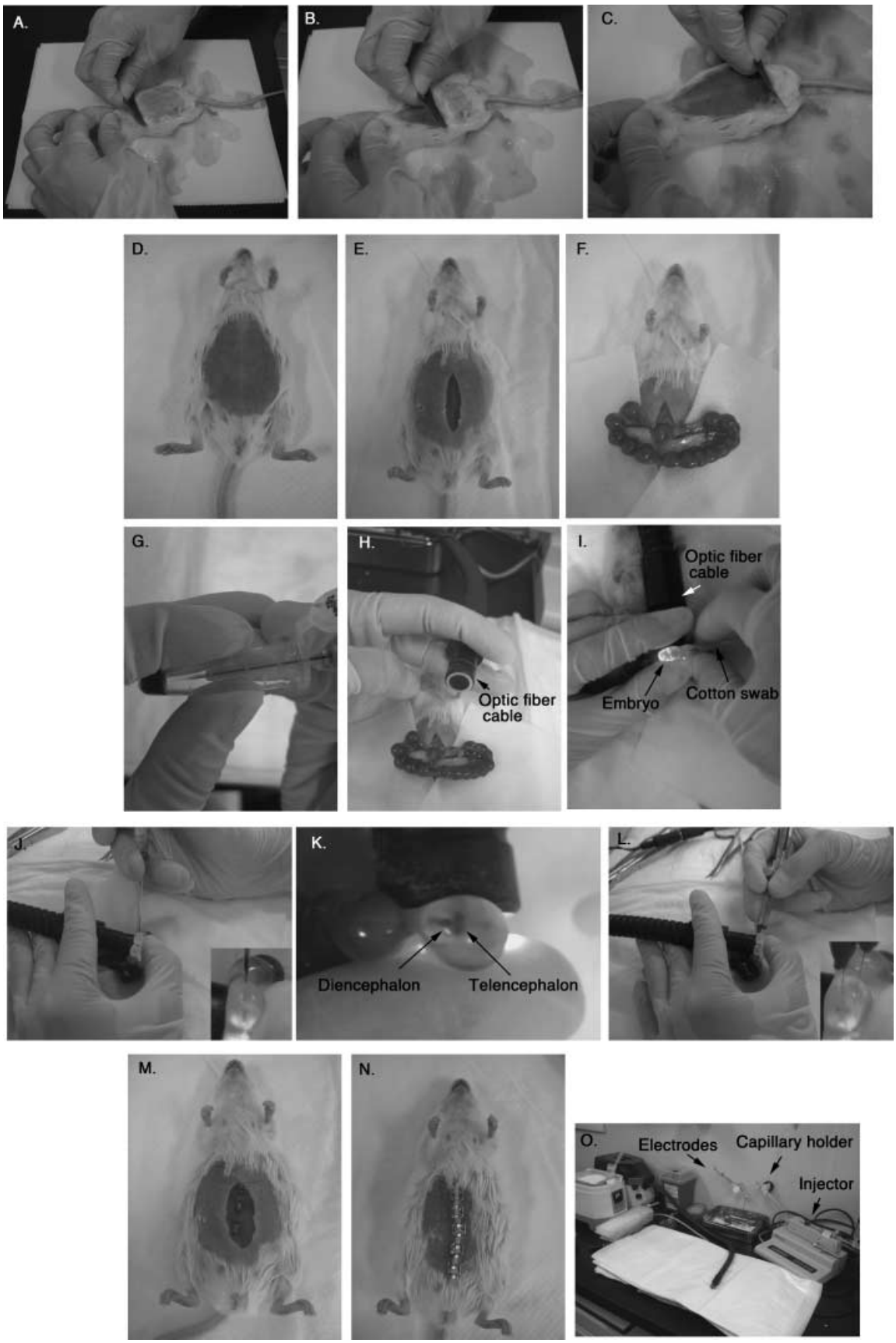
Theory of electroporation

The electrically mediated DNA transfer into tissues involves the formation of transient electropores in the membrane, allowing uptake of large molecules such as DNA into cytoplasm (Neumann *et al*. 1982). Because DNA is negatively charged, the electroporation fields move DNA from one side to the other. The electropores last longer when higher voltage and longer pulse duration are applied. However, higher voltage and longer pulse duration risk induction of non-reversible cell membrane damage, which will cause cell death. For higher efficiency and less damage to tissue, lower voltage with minimum pulse duration with usage of a square-wave electric pulse generator is recommended.

Surgery

After the induction of anesthesia with sodium pentobarbital (50 μg per gram body weight, intraperitoneally), the hair is shaved from the abdomen using a razor blade and 50% EtOH (Fig. 1A–C). A pregnant mouse (embryonic day [E]9.5–15.5) is subjected to abdominal incision with fine scissors (ROBOZ RS-5865, Gaithersburg, MD) (Fig. 1D,E), and all of its uterine horns are carefully exposed onto phosphate-buffered saline (PBS)-moistened cotton gauze, which is placed around the wound (Fig. 1F). The uterus is kept moist with PBS all of the time. For visualization of embryos, a flexible fiber cable (Leica, Wetzlar, Germany) is held between the index and middle fingers (Fig. 1H) and placed under the uterine horn (Fig. 1I). No microscope or magnifier is required for visualization. The tip of the fiber cable and the uterus are moistened with warm (37°C) PBS. The uterus is positioned between the optic fiber light and the thumb (Fig. 1I), and squeezed gently to push up the embryo closer to the uterine wall. When the embryos have been positioned, 1 μL of DNA (1 μg/μL) solution, mixed with the non-toxic dye fast green (SIGMA, Tokyo, Japan), is injected into the ventricle via a pulled glass capillary with the use of a micromanipulator

Fig. 1. Surgical procedure for microelectroporation. After anesthesia with sodium pentobarbital (50 μg per gram body weight, intraperitoneally), the hair is shaved from the abdomen using a razor blade and 50% EtOH (A–C). A pregnant mouse (E10.5) is subjected to abdominal incision, and all of the uterine horns are carefully pulled out onto phosphate-buffered saline (PBS)-moistened cotton gauze, which is placed around the wound (D–F). The capillary is filled with DNA solution directly from the tube (G). A fiber optic cable is held between the index and middle fingers (H). The uterine horn is positioned by using a cotton swab to prevent damage, and placed between the tip of the light source and thumb to hold (I). A 1-μL portion of DNA solution is injected into the ventricle (J). The outline of the brain become more visible with injection of fast green-containing DNA solution (K). A fine tungsten negative electrode and a platinum positive electrode are inserted into the left and right hemispheres, respectively (L). The surgical incision closed with sutures in the case of the wall (M) and with Autoclip for the skin (N). Overview of the electroporation set-up (O).



(KD Scientific, Holliston, MA, KDS310) (Fig. 1J and inset). In this fashion, brain will become easier to distinguish from outside of the uterus (Fig. 1K). Subsequently, a fine tungsten negative electrode and a platinum electrode are inserted into the uterus (Fig. 1L). However, since the injected DNA solution does not easily diffuse away from the point of injection because the neural cavity is closed, immediate electroporation is recommended after the injection of DNA solution. The targeted region is then placed between the two electrodes (Fig. 1L and inset). A series of three square-wave current pulses (7–10 V, 100 ms) is passed three times at 1-s intervals using a pulse generator (A-M Systems Model 2100, Victoria, BC, Canada). Shorter surgical time will yield better viability (it should be less than 30 min per mother). The uterine horn is then placed back in its original location with some PBS (about 500 μL). The surgical incision is sutured closed (3–0 DEXON II, Norwalk, CT) in the case of the wall (Fig. 1M) and with Autoclip 9 mm (ROBOZ RS-9260) for the skin (Fig. 1N). Animals are kept warm until they recover from anesthesia by placement of a heating pad under them (around 2 h). The viability of embryos and efficiency of cell transfection varies depending on the age of embryo and the location of electroporation. In the case of E11.5 electroporation, 60% of embryos survived and 50% of surviving embryos exhibited effective electroporation. Conditions should be optimized for each electroporation depending on the stage and part of the embryo.

Electrodes

Electrode shape is of critical importance for successful targeted electroporation. One end of the tungsten (A-M systems, bare tungsten wire, diameter 125 μm, for negative electrodes) and platinum (A-M systems, bare platinum wire, diameter 125 μm, for positive electrodes) wires is coiled on gold-plated pins (WPI, 5482) and fixed with parafilm (Fig. 2B,C). The wire

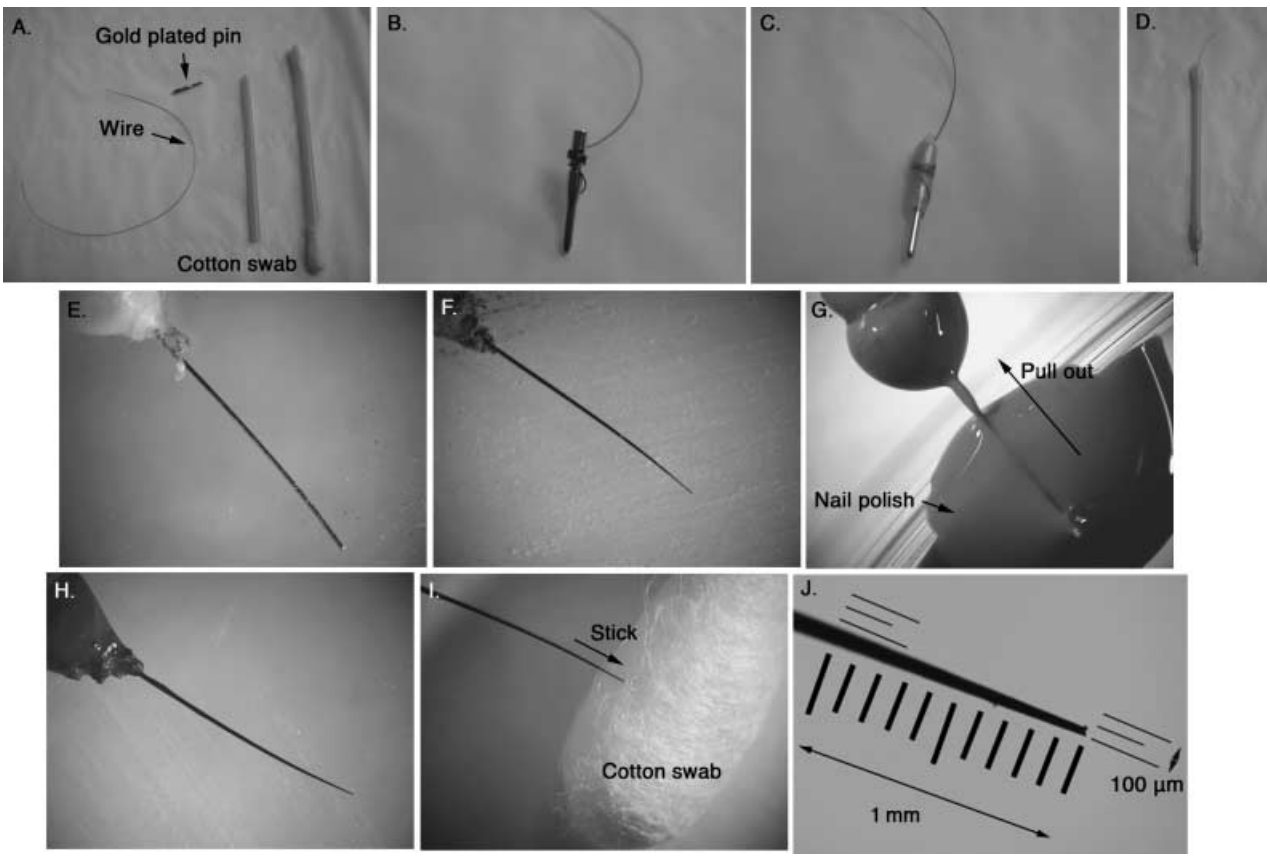


Fig. 2. Shape of microelectrodes. Materials used for making microelectrodes (A). One side of the end of wire is coiled on gold-plated pins (B) and fixed with parafilm (C). The wire is inserted up to the stem in a cotton swab and rolled up with parafilm on both ends (D). Tip of the wire before grinding (E). The tip is ground with sandpaper under a microscope (F). The electrode is soaked in nail polish and slowly pulled out backwards (G). The electrode is left to stand until the nail polish has dried completely (H). The electrode tip is placed in an acetone-soaked cotton swab to remove nail polish from its tip (I). The tip of the electrode is 30–40 μm in external diameter and 60–70 μm in external diameter 1 mm from the tip (J).

is inserted to the stem of a cotton swab to the end and wrapped with parafilm on both ends (Fig. 2D). The tip of the wire is sharpened with sandpaper evenly until it reaches 20–30 μm external diameter and 60 μm from 1 mm of the tip (Fig. 2E,F). Then a thin coat of nail polish is applied for insulation (Fig. 2G). After the nail polish has dried, it is removed from the tip with an acetone-soaked cotton swab (about 200 μm from the tip) (Fig. 2I). In order to prevent damage to the uterus, the portion within 1 mm of the tip should be no more than 70 μm in diameter (Fig. 2H,J). A glass capillary tube (Stoelting Co. no. 50611) is pulled using a micropipette puller and its tip is pinched off by forceps. The tip is 20–30 μm external diameter and 60 μm external diameter 1 mm from the tip. The capillary is connected to the micromanipulator, which is filled with mineral oil (Fig. 1O), and DNA solution is sucked up directly from an Eppendorf tube (Fig. 1G) before surgery.

Targeted electroporation

For electroporation into a desired region of the telencephalon, the configuration of the two electrodes is changed. For example, to electroporate wide regions of cortex, insulation is widely removed from the tip of the electrode. It is prepared by placing it deeply into an acetone-soaked cotton swab to remove nail polish (Fig. 2I). In contrast, a smaller region of electroporation can be obtained by preparing wire electrodes with tips with small exposure. Selection of the correct size of electrode and adequate arrangement of electrodes are of key importance for successful electroporation. We describe here some of the results obtained with different types of electrodes and electrode positions. To observe the pattern of migration of cortical neurons, we introduced an EGFP-expression vector driven by the human EF1 promoter into the telencephalon of E11.5 embryos, which were subsequently fixed and examined at E16.5. Because the expression vectors were injected into the lateral ventricle of the embryos, transfection occurred only at the cortical VZ (Tabata & Nakajima 2001), making it possible to trace neuronal migration from the VZ. When the site of electroporation is a wide area of medial telencephalon (using widely exposed electrodes, with ~300 μm of the tip placed in both lateral ventricles), plasmid DNA is transfected into most medial structures of the 'cortical hem' and also into the dorsal cortex (Fig. 3A). In embryos electroporated in the hem and cortex, 5 days after electroporation, GFP-expressing cells were found widely in the intermediate zone (IMZ), subplate (SP), marginal zone (MZ), and the cortical plate (CP) (Fig. 3B,C). When electroporation is limited to the dorsal cortex or cortical hem alone using highly insulated electrodes (with only ~100 μm of the tip exposed), a different configuration of electrodes is used. To aim at the dorsal cortex, the negative electrode is inserted into the side of the telencephalic ventricle with DNA solution injected and the positive electrode is placed outside of the brain. In the cortex of embryos with electroporation of the dorsal cortex, GFP-expressing cells were found in the IMZ and CP, but not in the MZ (Fig. 3E,F). In contrast, electroporation into the cortical hem is carried out by inserting both electrodes into the telencephalic ventricle. The negative electrode is inserted into the side of the telencephalic ventricle with DNA solution injected and the positive electrode is inserted into the other side of the telencephalic ventricle. In the embryos with hem electroporation, GFP-expressing cells are restricted to the MZ (Fig. 3H,I). Furthermore, some corticothalamic axons are visualized when CP cells are electroporated (Fig. 3A,D; arrow) but not in hem electroporated brain (G). Thus, differences in the distribution of GFP-expressing cells in different sites of electroporation provide information on the birthplace of individual cells. These findings were consistent with those of cell lineage analysis using specific knock-in or transgenic mice (Yoshida *et al*. 2006; Zhao *et al*. 2006).

Cellular localization of EYFP proteins

After cells become postmitotic, they start to migrate and to project axons to their target area. To trace axonal projections, tracers such as plant lectin, wheat germ agglutinin (WGA), and tau-lacZ reporter are delivered selectively to specific types of neurons. However, EYFP, a protein delivered to axons, permits detection of the projections of specific neurons. To demonstrate this, CAG-derived EYFP and Cre (encoding Cre recombinase) plasmids were co-electroporated into E11 embryonic diencephalon from the R26R reporter mouse line (Jackson Laboratory, Bar Harbor, Maine) and harvested at postnatal (P) day 18. However, the electroporation was restricted to the diencephalon, and the cortical area map was clearly distinguished by fluorescence from YFP (Fig. 4A). This demonstrates transfection into functional nuclei of the thalamus, via diffusion of fluorescent protein to the thalamocortical axons, which project to and illuminate their target areas in the cortex. Furthermore, expression of β-galactosidase visualized with X-gal histochemistry, which permanently marks the region of electroporation, demonstrated successful transfection to functional thalamic nuclei, such as the ventrobasal nuclei (VB, a somatosensory nucleus) and dorsal lateral geniculate

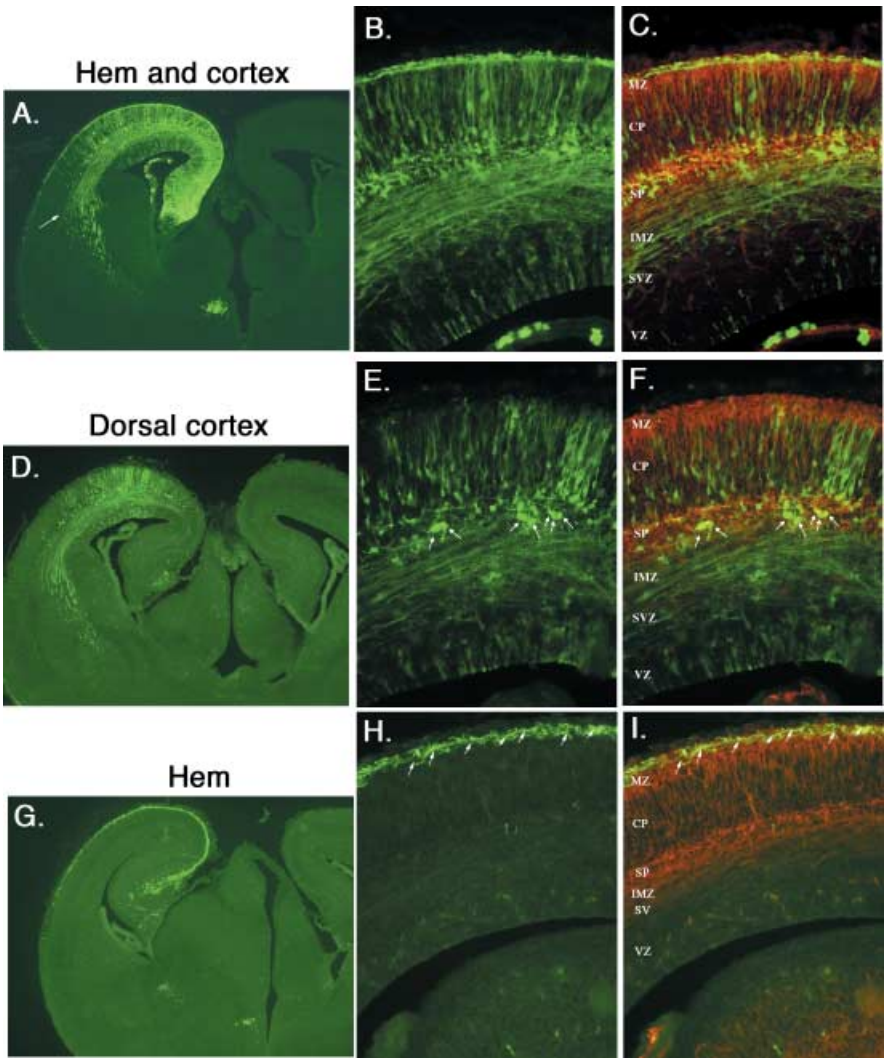


Fig. 3. Targeted electroporation into developing telencephalon. Electroporation is carried out in different region of E11.5 telencephalon, with harvesting at E16.5 and staining with immunohistochemistry using anti-green fluorescent protein (GFP) antibody (A,B,D,E,G,H) and anti-MAP2 antibody (C, F, I) and merged with anti-GFP antibody staining. Electroporation is carried out with wide region of medial and dorsal cortex (A–C). Electroporation is carried out in dorsal telencephalon at E11.5 and harvested at E16.5 (D–F). Cells in cortical plate (CP) and subplate (SP, arrows) are labeled, but no GFP positive cells are obtained in the marginal zone (MZ). The site of electroporation is aimed at the hem in the medial wall of the telencephalon at E11.5, and brains are collected at E16.5 (G–I). GFP positive cells are obtained only in MZ (arrows). Some of corticothalamic axons are stained with anti-GFP antibody when CP cells are electroporated (A, D arrow) but not in hem electroporated brain (G).

nucleus (dLGN, a visual nucleus) (Fig. 4B,C). When electroporation into the diencephalon is carried out at E10.5, area-specific transfection into dLGN occurs according to birth date (Altman & Bayer 1988). Immunostaining of YFP using anti-GFP antibody revealed strong localization of YFP in dLGN and primary visual cortex (Fig. 4D,E). Immunohistochemical labeling also revealed YFP-labeled fiber arbors on detailed examination of the visual cortex (Fig. 3F).

Further applications

In this review, all experiments described were carried out using plasmid DNA. However, dyes, chemical reagents, and antibodies can also be transferred into specific groups of cells, just as any charged macromolecules can be targeted using electroporation.

The most commonly used promoters for forced expression are the CMV promoter, human EF1 promoter, and CAG promoter. Although they exhibit distinct preferences in cells to be transfected, they are ubiquitously transfected within a few days after electroporation (Tabata & Nakajima 2001). To determine expression in a target region with precision, a plasmid can be constructed carrying the promoter region of genes expressed in the target region.

Notably, although electroporation can be carried out any time during embryonic development, it should be carried out when the target cells are generated. In cases in which the target gene will alter cell differentiation and plays different roles in postmitotic neurons, expression of gene products needs to be delayed. This problem can be solved by combined use with tamoxifen-inducible Cre-ER(T) recombinases (Feil *et al.* 1996) and reporter mice (Indra *et al.* 1999).

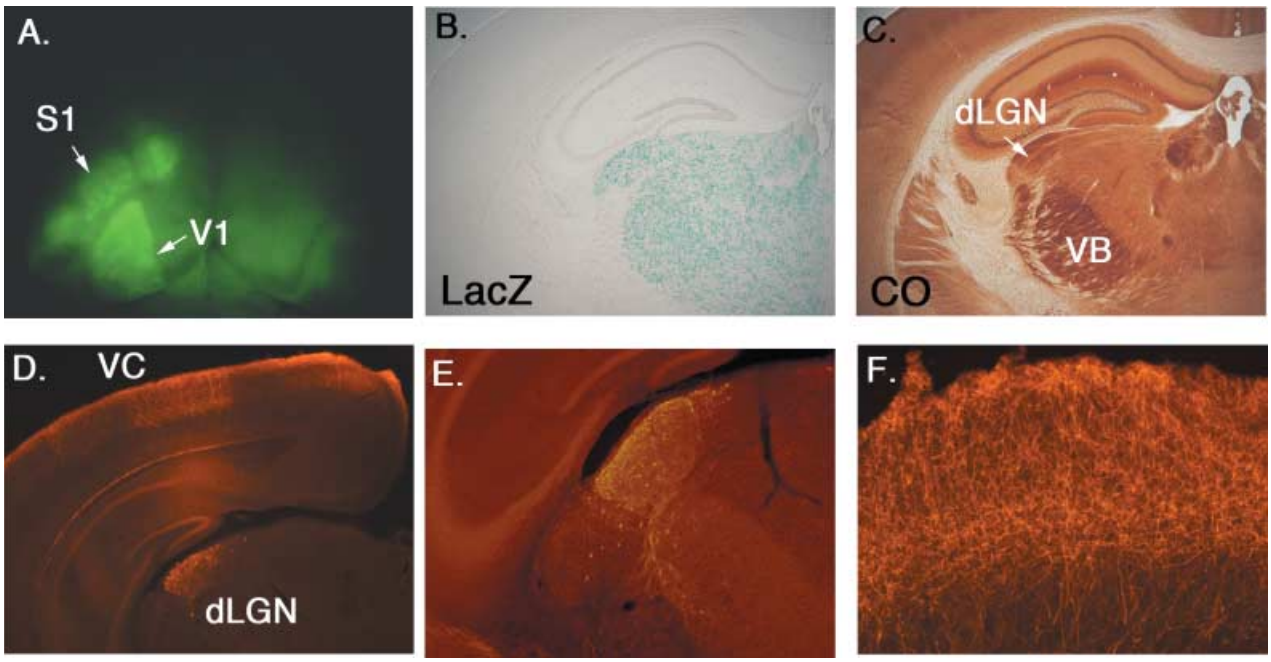


Fig. 4. Localization of enhanced yellow fluorescent protein (EYFP) protein in TC (Thalamo cortico) axons. EYFP and Cre plasmids are co-electroporated into E11 embryonic diencephalon from the R26R reporter mouse line and harvested at P18 (A–C). Cortical area maps, such as S1 (primary somatosensory area) and V1 (primary visual area), are illuminated by thalamocortical axons from the thalamus (A). Anterior to the top (A). Eletctroporated cells are stained with X-gal histochemistry (B), and sensory nuclei such as VB (ventrobasal nucleus) and dLGN (dorsal lateral geniculate nucleus) are stained with cytochrome oxidase (CO) histochemistry in adjacent sections to B (C). Electroporation into the diencephalon is carried out at E10.5 with harvesting at P18, and immunohistochemistry for GFP is carried out (D–F). Area-specific transfection into dLGN and VC (Visual cortex) is observed.

This will allow control of ectopic gene expression after electroporation.

Removal of target cells is one of the best methods for determination of the effects of cells on surrounding tissues rather than of a single molecule. Expression of a cellular toxin gene, diphtheria toxin subunit A (DTA), which is effective in cell killing, is a powerful method of ablation of specific tissue (Maxwell *et al*. 1986).

Blocking of synaptic transmission via expression of the tetanus toxin light chain (TeNT-LC) has also recently been used to silence neuronal activity (Wang *et al*. 2007). Electroporation of TeNT-LC plasmid with fluorescent marker proteins, which stain axonal projections and also dendrites, makes it possible to examine the behavior and morphology of neurons whose activity is simultaneously suppressed.

This broad accessibility of tissues will drastically change the experimental designs used in neuroscience.

Acknowledgments

This work was supported by the RIKEN Brain Science Institute.

References

Altman, J. & Bayer, S. A. 1988. Development of the rat thalamus: II. Time and site of origin and settling pattern of neurons derived from the anterior lobule of the thalamic neuroepithelium. *J. Comp. Neurol.* **275**, 378–405.

Borrell, V., Yoshimura, Y. & Callaway, E. M. 2005. Targeted gene delivery to telencephalic inhibitory neurons by directional *in utero* electroporation. *J. Neurosci. Methods* **143**, 151–158.

Cepko, C. 2001. Preparation of a specific retrovirus producer cell line. *Curr. Protocol. Mol. Biol.* **Chapter 9**, Unit 9.10.

Feil, R., Brocard, J., Mascrez, B., LeMeur, M., Metzger, D. & Chambon, P. 1996. Ligand-activated site-specific recombination in mice. *Proc. Natl Acad. Sci. USA* **93**, 10 887–10 890.

Fukuchi-Shimogori, T. & Grove, E. A. 2001. Neocortex patterning by the secreted signaling molecule FGF8. *Science.* **294**, 1071–1074.

Fukuchi-Shimogori, T. & Grove, E. A. 2003. Emx2 patterns the neocortex by regulating FGF positional signaling. *Nat. Neurosci.* **6**, 825–831.

Hashimoto, M. & Mikoshiba, K. 2004. Neuronal birthdate-specific gene transfer with adenoviral vectors. *J. Neurosci.* **24**, 286–296.

Indra, A. K., Warot, X., Brocard, J. *et al*. 1999. Temporally-controlled site-specific mutagenesis in the basal layer of the epidermis: comparison of the recombinase activity of the tamoxifen-inducible Cre-ER (T) and Cre-ER (T2) recombinases. *Nucleic Acids Res.* **27**, 4324–4327.

Itasaki, N., Bel-Vialar, S. & Krumlauf, R. 1999. 'Shocking' developments in chick embryology: electroporation and in ovo gene expression. *Nat. Cell Biol.* **1**, 203–207.

Jakobsson, J., Ericson, C., Jansson, M., Björk, E. & Lundberg, C. 2003. Targeted transgene expression in rat brain using lentiviral vectors. *J. Neurosci. Res.* **73**, 876–885.

Maxwell, I. H., Maxwell, F. & Glode, L. M. 1986. Regulated expression of a diphtheria toxin A-chain gene transfected into human cells: possible strategy for inducing cancer cell suicide. *Cancer Res.* **46**, 4660–4664.

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**, 335–344.

Muramatsu, T., Shibata, O., Ryoki, S., Ohmori, Y. & Okumura, J. 1997. Foreign gene expression in the mouse testis by localized *in vivo* gene transfer. *Biochem. Biophys. Res. Commun.* **233**, 45–49.

Nakamura, H., Watanabe, Y. & Funahashi, J. 2000. Misexpression of genes in brain vesicles by *in ovo* electroporation. *Dev. Growth Differ.* **42**, 199–201.

Neumann, E., Schaefer-Ridder, M., Wang, Y. & Hofscneider, P. H. 1982. Gene transfer into mouse lyoma cells by electroporation in high electric fields. *EMBO J.* **1**, 841–845.

Saito, T. & Nakatsuji, N. 2001. Efficient gene transfer into the embryonic mouse brain using *in vivo* electroporation. *Dev. Biol.* **240**, 237–246.

Shimogori, T., Banuchi, V., Ng, H. Y., Strauss, J. B. & Grove, E. A. 2004. Embryonic signaling centers expressing BMP, WNT and FGF proteins interact to pattern the cerebral cortex. *Development* **131**, 5639–5647.

Soriano, P. 1999. Generalized lacz expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21**, 70–71.

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**, 865–872.

Tsien, J. Z., Chen, D. F., Gerber, D. *et al.* 1996. Subregion- and cell type-restricted gene knockout in mouse brain. *Cell* **87**, 1317–1326.

Wang, C. L., Zhang, L., Zhou, Y. *et al.* 2007. Activity-dependent development of callosal projections in the somatosensory cortex. *J. Neurosci.* **27**, 1 1334–1 1342.

Yoshida, M., Assimacopoulos, S., Jones, K. R. & Grove, E. A. 2006. Massive loss of Cajal-Retzius cells does not disrupt neocortical layer order. *Development* **133**, 537–545.

Zhao, C., Guan, W. & Pleasure, S. J. 2006. A transgenic marker mouse line labels Cajal-Retzius cells from the cortical hem and thalamocortical axons. *Brain Res.* **1077**, 48–53.