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Efficient Gene Transfer into the Embryonic Mouse Brain Using *in Vivo* Electroporation

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Mouse genetic manipulation has provided an excellent system to characterize gene function in numerous contexts. A number of mutants have been produced by using transgenic, gene knockout, and mutagenesis techniques. Nevertheless, one limitation is that it is difficult to express a gene *in vivo* in a restricted manner (i.e., spatially and temporally), because the number of available enhancers and promoters which can confine gene expression is limited. We have developed a novel method to introduce DNA into *in/exo utero* embryonic mouse brains at various stages by using electroporation. More than 90% of operated embryos survived, and more than 65% of these expressed the introduced genes in restricted regions of the brain. Expression was maintained even after birth, 6 weeks after electroporation. The use of fluorescent protein genes clearly visualized neuronal morphologies in the brain. Moreover, it was possible to transfect three different DNA vectors into the same cells. Thus, this method will be a powerful tool to characterize gene function in various settings due to its high efficiency and localized gene expression. © 2001 Academic Press

Key Words: **electroporation; *in vivo;* DNA transfer; embryonic mouse brain; *in utero; exo utero;* forced gene expression; fluorescent protein; EYFP; DsRed.**

INTRODUCTION

Gene function has been characterized in various organisms using gain- or loss-of-function analyses. The mouse system has been used extensively to characterize gene function in mammals because of a large number of mutant lines and the availability of ES cells, which enable the specific modification of desired genes. Forced gene expression has been performed by making transgenic animals and using various vector systems. However, the number of characterized transcription-control sequences, which confine gene expression, is still limited, because their analysis is time- and labor-consuming. It is not always possible to restrict gene expression both spatially and temporally, although some inducible expression systems (Furth *et al.,* 1994; Feil *et al.,* 1996; No *et al.,* 1996) and viral vectors (Cepko, 1988; Slack and Miller, 1996) have been developed. In addition, the generation of recombinant viruses and transgenic mice is a lengthy process with which there are some limitations.

Electroporation has been used successfully to introduce DNA into cultured cells, and recently into chick embryos (Muramatsu *et al.,* 1997; Itasaki *et al.,* 1999; Swartz *et al.,* 2001), cultured mouse embryos (Itasaki *et al.,* 1999; Swartz *et al.,* 2001), and the mouse brain (Tabata and Nakajima, 2001). Unlike the chick, mouse embryos are more difficult to manipulate, because they develop *in utero.* In comparison with the mouse, however, the chick system has some drawbacks regarding genetic manipulations and behavioral assays. Mouse embryos can be maintained in a culture for only a few days, in which time it is difficult to analyze numerous features of gene function. We have developed a novel *in vivo* electroporation method to introduce DNA in restricted regions of the embryonic mouse brain, using *in utero* and *exo utero* surgeries. After electroporation at various stages, the introduced genes were expressed in regions close to DNA injection sites. Forced expression of the constitutively active form of Notch (caNotch) and the *Hes1* gene demonstrated inhibition of neuronal differentiation, showing that this method can be used for assays of gene function *in vivo.* In comparison with other DNA transfer methods, this *in vivo* electroporation method exhibits several advantages, in that it is: (1) quick and easy, (2) highly efficient, (3) delivers localized and unidirectional transfer of genes, and (4) enables transfection of multiple genes into one cell.

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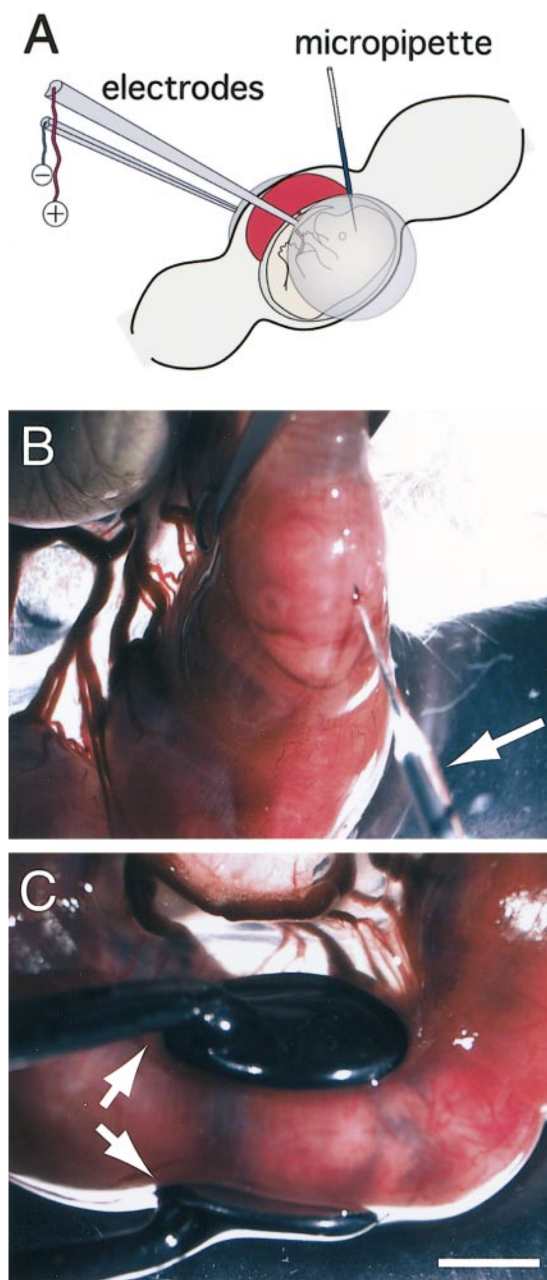

FIG. 1. *In vivo* electroporation. (A) Schematic representation of a micropipette and electrodes. (B) DNA injection into an E15.5 mouse embryo *in utero,* illuminated with a fibre-optics light source. (C) Electroporation by holding the embryo through the uterus with forceps-type electrodes. Arrows indicate a micropipette for DNA injection (B) and electrodes (C). Scale bar, 5 mm.

MATERIALS AND METHODS

Plasmids

All plasmids had reporter genes downstream of the CAG promoter (Niwa *et al.,* 1991). The following fragments were inserted into the end-filled *Eco*RI site of pCAGGS: a 740-bp end-filled *Bam*HI/*Not*I fragment encoding the EYFP protein from pEYFP-Mito (Clontech, Palo Alto, CA) to construct pCAG-EYFP; an 820-bp end-filled *Nhe*I/*Dra*I fragment encoding only the ECFP protein, after deletion of 84 bp between the *Bgl*II and *Bam*HI sites, from pECFP-Nuc (Clontech) to make pCAG-ECFP; an 870-bp end-filled *Bam*HI/*Not*I fragment encoding the d2EGFP protein from pd2EGFP-N1 (Clontech) to make pCAG-d2EGFP. pCAG-DsRed-Mito carried a gene encoding a mitochondrial targeting peptide fused to the DsRed protein (a gift from Dr. Kouichi Hasegawa in our laboratory).

In order to express both a given and the *EYFP* genes in the same cells, pCAG-EYFP-CAG was made by inserting a 2.4-kb *Bam*HI fragment containing an *Eco*RI site downstream of the CAG promoter into the *Bam*HI site downstream of the *EYFP* gene of pCAG-EYFP. pCAG-EYFP-CAG-caNotch was constructed by inserting a 3-kb end-filled *Eco*RI/*Bam*HI fragment encoding the transmembrane region, RAM domain, cdc10/ankyrin repeats, and nuclear localization signal of the Notch1 protein (Ohtsuka *et al.,* 1999) from pME-FNIC (a gift from Dr. Masashi Kawaichi) into the end-filled *Eco*RI site of pCAG-EYFP-CAG. pCAG-EYFP-CAG-Hes1 was constructed by inserting a 1-kb *Eco*RI fragment containing the entire open reading frame of the mouse Hes1 protein from pmHes1 into the *Eco*RI site of pCAG-EYFP-CAG.

In Vivo Electroporation

Plasmids were prepared by using the EndoFree Plasmid Kit (Qiagen, Hilden, Germany). ICR strain mice were purchased from Clea (Tokyo, Japan). Timed pregnant mice were anesthetized with Nembutal. After cleaning the abdomen with 70% ethanol, a 3-cm midline lapratomy was performed, and the uterus was taken out.

For DNA microinjection, 75-mm glass capillary tubes (Drummond Scientific, Broomall, PA) were pulled by using a micropipette

TABLE 1
Effect of Voltage and Pulse Numbers on Survival and EYFP-Positive Rate

Voltage (V)	No. of pulses	Embryo survival (%)	$EYFP^+$ embryos (%)	No. of operated embryos (litters)
0	0	93.3	0	15 (1)
20	5	93.3 ± 0.0	32.2 ± 3.6	30 (2)
30	5	92.8 ± 1.1	53.9 ± 0.6	28 (2)
40	5	90.7 ± 4.7	68.5 ± 3.5	42 (3)
	8	87.5 ± 0.0	71.5 ± 7.2	32 (2)
50	3	76.1 ± 5.8	58.0 ± 4.0	43 (3)
	5	70.6 ± 3.6	84.3 ± 7.9	44 (3)
	8	61.5 ± 7.7	74.6 ± 3.2	26 (2)
60	5	63.9 ± 19.5	71.9 ± 1.7	37 (3)
70	3	58.8 ± 12.6	60.0 ± 10.0	27 (2)
	5	41.7 ± 8.4	73.4 ± 6.7	27 (2)

Note. Two microliters of 0.2 mg/ml of a reporter plasmid, pCAG-EYFP, was microinjected into the cephalic ventricle of E13.5 embryos. Two days after electroporation, embryos were examined for fluorescence. Survival and $EYFP^+$ rates (percentages) were calculated for every litter from the number of surviving embryos/operated and EYFP-positive/surviving embryos, respectively. The data are represented as mean ± SEM.

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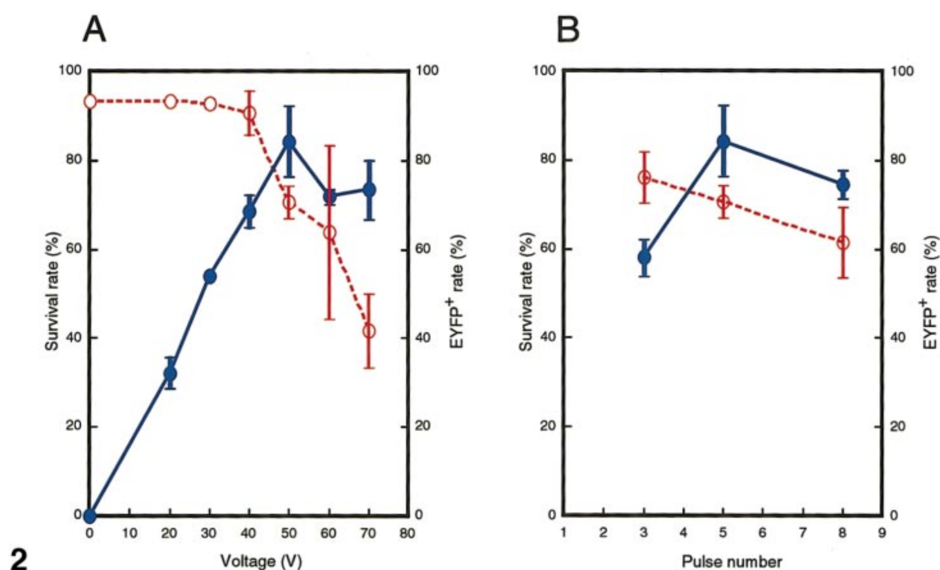

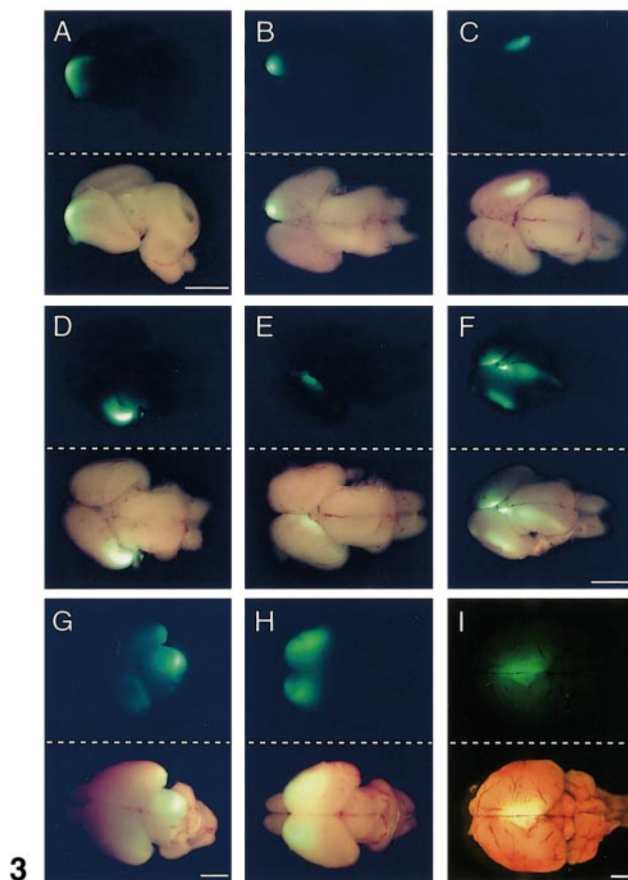

FIG. 2. Effects of voltage (A) and pulse numbers (B) on survival and $EYFP^+$ rates of embryos by electroporation. Five pulses were delivered in (A). Details are described in Table 1. Survival and $EYFP^+$ rates are indicated by dashed red and solid blue lines, respectively. Error bars indicate SEM.

FIG. 3. *EYFP* expression in restricted regions of the brain. Upper and lower photos of each panel were recorded in dark and semi-illuminated conditions to see fluorescence and brain structure, respectively. (A–F) E15.5 brains after electroporation at E13.5. (A–E) Electroporation immediately after DNA injection. (F) Electroporation 10 min after DNA injection. (G, H) P3 brains, 9 days after electroporation at E13.5. (I) P37 brain, 6 weeks after electroporation at E14.5. Similarly, P15 brains after electroporation at E13.5 and E16.5, exhibited fluorescence (data not shown). Rostral is to the left. The same magnifications are used in (A–E) and (G) and (H). Scale bars, 2 mm.

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puller P-97 (Sutter Instrument, Novato, CA) under the following conditions: pressure, 500; heat, 800; pull, 30; velocity, 40; time, 1. Pulled-out pipettes were broken at ~60 μm external diameter (~1.2 cm from the shoulder of the pipette) by pinching with forceps, and tips of broken pipettes were marked with a water-resistant magic marker to see injection sites. Indigo Carmine solution (Daiichi Pharmaceutical, Tokyo, Japan) was used only for practice of injection.

One to three microliters of DNA solution in PBS (1 μl for E11.5, 2 μl for E13.5, 3 μl for E15.5) were injected into the lateral or third ventricle using a mouth-controlled pipette system (Drummond Scientific). DNA (0.1 to 1 mg/ml) was used for injection. Brighter fluorescence was obtained by injection of higher concentrations of DNA, and it seemed that the intensity of fluorescence reaches plateau at 0.5 mg/ml. DNA (0.2 mg/ml) was used here to evaluate the conditions. As for embryos older than E13.5, aimed injection was possible into anterior or posterior points of one of the two lateral ventricles, since the dorsal surface of the telencephalon was visible through the uterine wall by illuminating with a fibre-optics light source. In fluorescence-positive brains, fluorescence was always observed in the regions covering the sites of DNA injection. Square electric pulses were delivered at a rate of one pulse per second to embryos through the uterus by holding them with forceps-type electrodes, while the uterus was kept wet by dropping saline (prewarmed at 37°C) between the electrodes. The electrodes, CY650P10 (Unique Medical Imada, Miyagi, Japan), consisted of a pair of platinum round plates with a 1-cm diameter. The electric pulses were obtained by using ElectroSquarePorator T820 (BTX, San Diego, CA) connected to Enhancer 400 (BTX). Each pulse was for a duration of 50 ms. Then, the uterine horns were repositioned in the abdominal cavity, and the abdominal wall and skin were sewed up with surgical sutures.

The *exo utero* surgery was performed as described (Muneoka *et al.,* 1990) with minor modifications. After taking out the uterus, the uterine wall was cut on both horns along the antiplacental side with angled iridectomy scissors, and DNA was injected as above. Electric pulses were delivered as above by holding the yolk sac with the forceps-type electrodes. Without sewing the uterine wall, embryos inside the yolk sac with the uterus were repositioned in the abdominal cavity. The cavity was filled with prewarmed saline at 37°C, and then the abdominal wall and skin were sewed as above.

In most cases, all embryos in a pregnant mouse were electroporated. It usually took about 40 and 45 min for above *in utero* and *exo utero* procedures, respectively. Supplemental text and troubleshooting guide are available online (http://www.frontier.kyoto-u.ac.jp/rc01/in_vivo_electroporation.html).

Examination of Brains

After electroporation, brains were recovered and photographed using a fluorescent stereo microscope, MZ FLIII (Leica, Nussloch, Germany), and a camera, Axiocam (Zeiss, Oberkochen, Germany). Embryos were then fixed with 4% formaldehyde solution and sliced at 50- to 100-μm thickness with a microslicer DTK-1500 (Dohan, Kyoto, Japan). Slices were viewed under a fluorescent microscope, BH-2 (Olympus, Tokyo, Japan), or a laser scanning microscope, LSM 5 Pascal (Zeiss), using appropriate filter sets (Olympus and Omega Optical, Brattleboro, VT) to see specific signals: XF104 and G520 band pass filter for EYFP, XF114 for ECFP, and U-MNG for DsRed.

In Situ Hybridization

In situ hybridization was performed as described before (Saito *et al.,* 1996). In the presence of digoxygenin-UTP (Roche Diagnostics, Manheim, Germany), sense and anti-sense probes for *EYFP* were synthesized by using T3 and T7 RNA polymerases, respectively, from a linearlized plasmid which carries the end-filled *Bam*HI/*Not*I fragment encoding the EYFP protein in the *Eco*RV site of pBluescript SK (Stratagene, La Jolla, CA). Sections of frozen brains (25 μm thick) were treated and visualized by using anti-digoxygenin antibody conjugated with alkaline phosphatase (Roche Diagnostics) and NBT/BCIP (Bio-Rad, Hercules, CA).

RESULTS

Optimal Conditions for Electroporation

Mouse embryos at embryonic day (E) 13.5 were used to determine optimal conditions for *in vivo* electroporation, because the embryos were visible through the uterine wall and because the forebrain develops significantly after this stage. To analyze the efficiency of transfection, a plasmid carrying the *EYFP* gene, downstream of a ubiquitous CAG promoter (Niwa *et al.,* 1991), was injected into the cephalic ventricle. Then, square electric pulses were applied to embryos through the uterine wall, by holding them in parallel along the anteroposterior axis with forceps-type electrodes (Fig. 1). Since higher voltages killed more embryos, we tried to optimize conditions by reducing the voltage and by increasing the number of pulses (Table 1). Operated embryos survived better at lower voltages, whereas the EYFP-positive rate (percentage of embryos expressing *EYFP*/surviving embryos) increased proportionally with the voltage up to 50 V (Fig. 2A). The intensity of fluorescent signals also seemed to increase and reach plateau at 50 V, suggesting that maximum transfection into each cell could be obtained at 50 V. Five pulses at 40–50 V showed better viability and a higher EYFP-positive rate than three pulses at 70 V, indicating that the voltage and number of pulses are more critical than a simple sum of applied electric power. At 50 V, more than five pulses did not seem to improve the efficiency further (Fig. 2B).

We have also succeeded in efficient gene transfer into embryos at later stages by increasing the voltage (Table 2). For embryos younger than E13.5, *exo utero* surgery was performed to visualize embryos more clearly. The uterine wall was cut, and electric pulses were delivered to embryos through the yolk sac after DNA injection. Significant percentages of embryos expressed *EYFP.*

Localized Gene Expression

EYFP fluorescence was detected in various areas covering the sites of DNA injection (Fig. 3). EYFP-positive regions were relatively confined when electric pulses were delivered immediately after DNA injection (Figs. 3A–3E). On the other hand, the size of EYFP-positive regions expanded when electroporation was 10 min after DNA injection (Fig.

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TABLE 2
Optimal Conditions for Electroporation at Different Stages

Embryonic stage	Electrode gap (mm)	Voltage (V)	Surviving embryos (%)	EYFP$^+$ embryos (%)	No. of operated embryos (litters)
E11.5	5.8 ± 0.1	20	49.9 ± 12.2	71.5 ± 6.9	84 (6)
E13.5	8.2 ± 0.1	40	90.7 ± 4.7	68.5 ± 3.5	42 (3)
E15.5	9.3 ± 0.2	60	88.3 ± 7.3	67.4 ± 7.1	32 (3)

Note. pCAG-EYFP was microinjected as in Table 1. *Exo utero* surgery was performed for E11.5 embryos. Since the gaps between electrodes to hold the yolk sac or uterus were relatively constant at each embryonic stage, voltages were fixed. The data are represented as mean ± SEM.

3F), probably reflecting diffusion of DNA. Fluorescent signals exhibited a higher strength at 2 days, than at 1 day after electroporation (data not shown), as is the case with standard DNA transfection into cultured cells. Strong EYFP fluorescence was detected even after birth, 9 days to 6 weeks after electroporation (Figs. 3G–3I).

EYFP-expressing cells were analyzed on sections (Fig. 4). Fluorescence was detected in the E15.5 telencephalon, where a number of neurons had been produced and had migrated (Figs. 4A and 4B). Fibers were also labeled heavily (arrows). P3 brains, after electroporation at E13.5, contained numerous EYFP-positive cells in many layers of the cortex (Figs. 4C and 4D). The E13.5 dorsal diencephalon exhibited fluorescence in many cells, commissural fibers, and thalamocortical axons (arrow and arrowhead in Fig. 4E, respectively). Other areas of the brain were also labeled: the E15.5 hippocampus (Fig. 4F) and midbrain (Fig. 4G). In all cases, the other side, facing the ventricle, did not express the reporter gene. These results demonstrated that the efficiency of transfection was high, and that expression was localized.

Long-Term Expression of the Transfected Gene

Fluorescence observed in the postnatal brains suggested that expression of the *EYFP* gene persisted for a long time. But the possibility could not be eliminated that the EYFP protein was produced only at embryonic stages and remained active until postnatal stages, because the EGFP protein is stable (Li *et al.,* 1998). In order to clarify whether the *EYFP* gene is expressed at postnatal stages, *in situ* hybridization was performed (Fig. 5). Strong signals were detected in the regions containing EYFP-positive cells not only in embryonic brains (Figs. 5A and 5B) but also in P15 brains three weeks after electroporation (Figs. 5C and 5D). Moreover, postnatal brains transfected with the *d2EGFP* gene, which encodes a destabilized fluorescent protein with a half-life of 2 h, also exhibited strong fluorescence, 3 weeks after electroporation at E13.5 (data not shown). These results indicated that expression of the transfected gene persisted even at postnatal stages.

Visualization of Neuronal Morphologies

The use of fluorescent protein genes visualized neuronal morphologies (Fig. 6). Neurons were clearly seen at P37, even 6 weeks after electroporation (Fig. 6A). At P15, many cortical neurons were labeled with the EYFP and DsRed proteins (Figs. 6B and 6C). The cells labeled by electroporation at E16.5 were localized in upper superficial layers (Fig. 6B), whereas the fluorescence-positive cells by electroporation at E13.5 occupy lower layers (Fig. 6C). The morphology of the pyramidal neuron was visible (Fig. 6D). Expression of the mitochondrial-targeted DsRed protein showed many spots, reflecting sites of the mitochondria in neurons (Figs. 6E and 6F).

Functional Assays of Genes

In order to confirm whether this *in vivo* electroporation method can be used for functional assays of genes, the

FIG. 4. *EYFP*-expressing cells in electroporated brains. (A, B) E15.5 telencephalons after electroporation at E13.5. Arrows show fibers extending from EYFP-positive cells. Dorsal is to the left, lateral to the top. (C) P3 cortex after electroporation at E13.5. An arrow indicates the corpus callosum. Dorsal is to the top, medial to the left. (D) Magnified view of the right part of (C). (E) E13.5 diencephalon after electroporation at E11.5. Commissural and thalamocortical fibers are indicated by arrow and arrowhead, respectively. Dorsal is to the top. (F, G), E15.5 hippocampus (dorsal is to the upper right) and midbrain (dorsal is to the left) after electroporation at E13.5. The same magnifications are used except in (D). Scale bars, 200 μm.

FIG. 5. Long-term expression of the *EYFP* gene. (A, B) E16.5 telencephalon after electroporation at E14.5. (C, D) P15 telencephalon, three weeks after electroporation at E13.5. (A) and (B) or (C) and (D) are adjacent sections. The sections in (B) and (D) were hybridized with anti-sense cRNA probes for *EYFP.* Control hybridization using a sense-strand RNA probe of *EYFP* gave no signal (data not shown). Scale bar, 200 μm.

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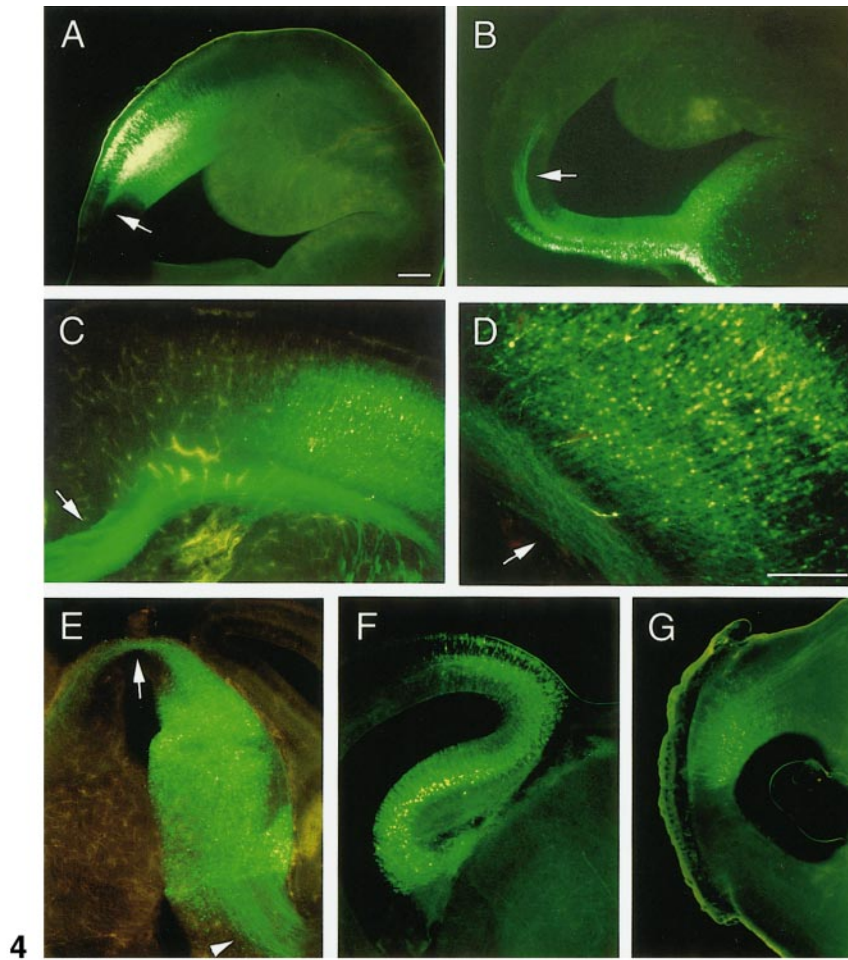

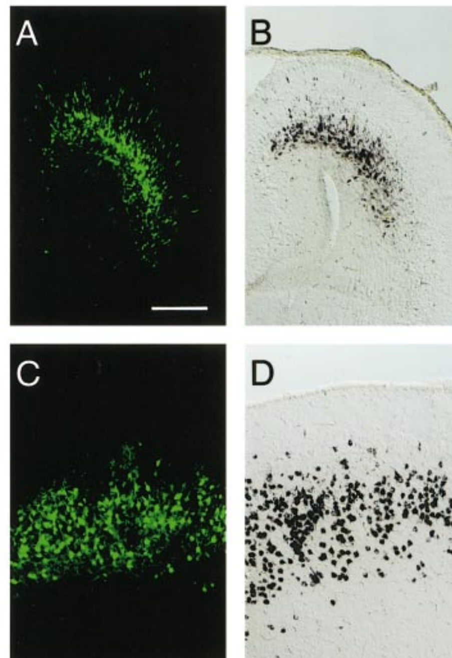

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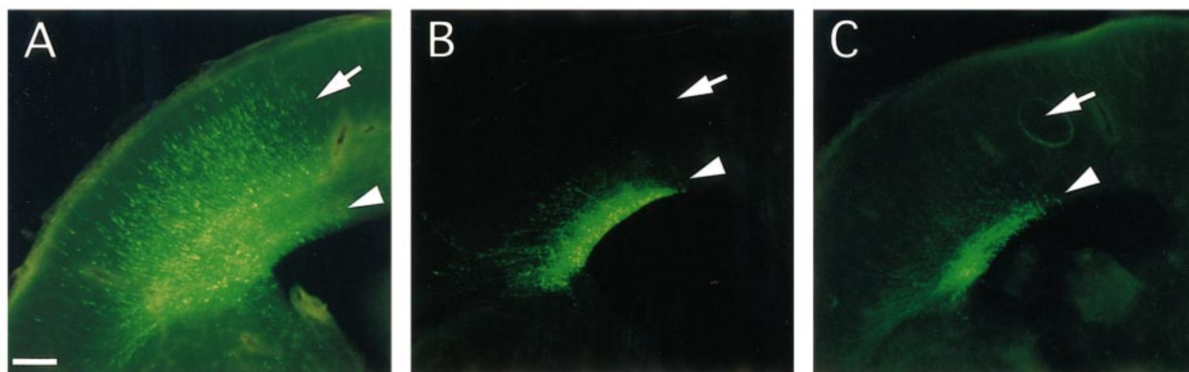

FIG. 6. Neuronal morphologies highlighted with the fluorescent proteins. The EYFP (A, B, D) and mitochondrial-targeted DsRed (C, E, F) proteins were expressed. (A) P37 subicular area after electroporation at E14.5. cc, corpus callosum. (B) P15 cortex after electroporation at E16.5. (C) P15 cortex after electroporation at E13.5. (D), (E), and (F) Magnified views of pyramidal neurons in (B) and (C), respectively. The same magnifications are used in (B) and (C), and (D) and (E). Scale bars: (A), (B), 200 μm; (D), 20 μm; (F), 5 μm.

FIG. 7. Forced expression of *caNotch* and *Hes1* in the developing telencephalon. Brains were sliced at E18.5, 3 days after transfection of plasmids carrying following genes into E15.5 telencephalon: (A), *EYFP;* (B) *EYFP* and *caNotch;* (C) *EYFP* and *Hes1.* Arrowheads and arrows indicate the ventricular zone and cortical plate, respectively. Scale bar, 200 μm.

caNotch and *Hes1* genes were introduced into the embryonic telencephalon (Fig. 7). Cells expressing *caNotch* and *Hes1* were labeled with EYFP, by using plasmids which carried the *EYFP* gene in addition to either the *caNotch* or *Hes1* gene. The number of EYFP-positive neurons in the cortical plate was significantly reduced by forced expression of either *caNotch* or *Hes1,* whereas many EYFP-positive neurons were detected by transfecting only the *EYFP* gene

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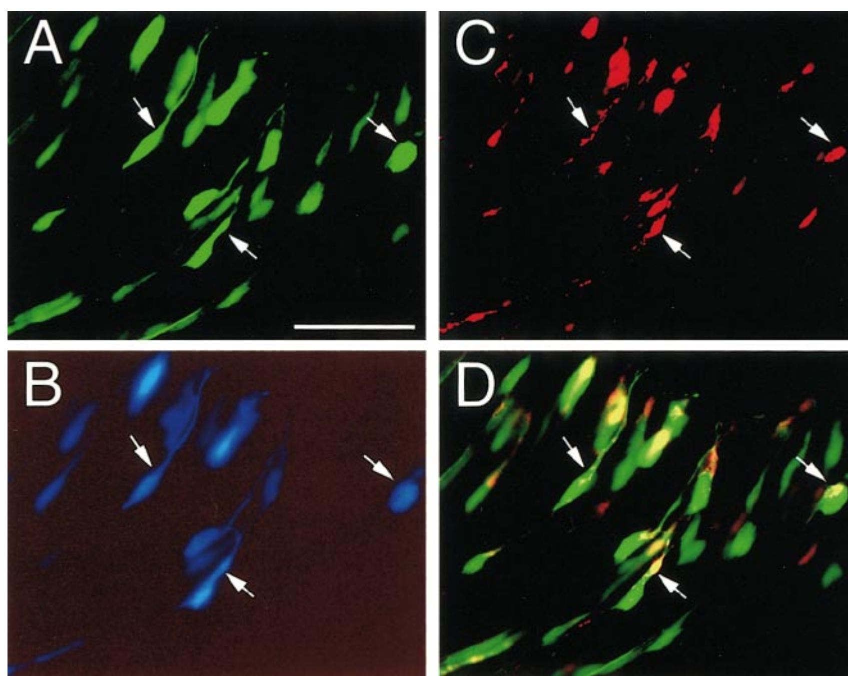

FIG. 8. Triple gene expression in E15.5 telencephalon cells after electroporation at E13.5. Three plasmids were used, which carry the genes encoding the EYFP (A), ECFP (B), and mitochondrial-targeted DsRed proteins (C), respectively. (D) Superimposed image of (A) and (C). Arrows show cells expressing the three proteins. Scale bar, 50 μm.

(arrows). These results were consistent with previous observations obtained by retroviral vectors, that caNotch and its downstream effector gene, *Hes1,* inhibit neuronal differentiation (Ishibashi *et al.,* 1994; Ohtsuka *et al.,* 1999), showing that the *in vivo* electroporation method enables quick assays of gene function.

Transfection of Multiple Genes

In order to further examine another advantage of this method, three plasmids encoding different fluorescent proteins were transfected into the embryonic brain simultaneously: the EYFP, ECFP, and mitochondrial-targeted DsRed proteins (Fig. 8). Many cells expressed all three genes, showing that three different plasmids were introduced into the same cells.

DISCUSSION

Our results demonstrate that this *in vivo* electroporation method is a powerful technique for the introduction of DNA into the embryonic mouse brain. This procedure is simple and performed more easily and quickly than any other transfection method targeting the brain. After determination of optimal conditions, we have obtained several embryos expressing introduced genes routinely from a single pregnant ICR mouse, because there are usually around 14 embryos per litter. This method could be applied to many genes, even those whose expression may cause early embryonic lethality by conventional transgenic techniques. Expression was confined on one side of the ventricle toward the anode, showing that DNA was transferred unidirectionally as in the case of chick *in ovo* electroporation (Itasaki *et al.,* 1999). This assists in the characterization of gene function, because the nontransfected side can be used as a control within the same sample. By contrast, it is difficult to confine the region of transfection when using lipofection and viral vector systems. In addition, transfer of multiple genes is difficult for retroviral systems, which have been extensively used, because the size of the DNA that can be incorporated is limited, and because cells cannot accept multiple rounds of viral infection. Using the *in vivo* electroporation method, we succeeded in transferring three independent plasmids into the same cells, showing that a combination of multiple constructs can be assayed *in vivo.* Large DNA molecules have been introduced into cultured cells using electroporation. This *in vivo* electroporation method has succeeded in transferring a relatively large plasmid of 11 kb. Forced expression of negative regulatory genes of neurogenesis, such as *caNotch* and *Hes1,* using this method demonstrated a clearer view than previous results obtained by retroviral vectors, probably because of high efficiency of transfection of the genes. Thus, our present study clearly demonstrates that this method will be very useful for the analysis of gene function

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in the mouse brain. The number of mouse mutants has been expanded vigorously by transgenesis, gene targeting, and recent mutagenesis (Nolan *et al.,* 2000; Hrabe *et al.,* 2000) techniques. Application of this *in vivo* electroporation method to the mutants will have a great potential to accelerate analyses of function and cascades of genes.

Some genes have been recently knocked out in specific regions of the brain, using the Cre recombinase-loxP system (Tsien *et al.,* 1996; Xu *et al.,* 2000). Since the *in vivo* electroporation method can deliver confined gene expression, this method will be helpful for the generation of region-specific gene knockouts, especially for regions which do not have known specific transcription-control sequences. Region- and cell-specific expression in our method could be enhanced further by the use of specific enhancers and promoters instead of the ubiquitous CAG promoter. Although the embryonic head can be seen through the uterine wall or yolk sac, it is still difficult to inject DNA into an exact position, particularly into the diencephalon and ventral parts of the brain. The use of an Ultrasound Backscatter Microscope (Olsson *et al.,* 1997; Gaiano *et al.,* 2000) will help such DNA injection into a specific location.

At the same stages, the layers containing fluorescence-positive cells were different with the timing of electroporation, consistent with an inside–out pattern of migration and settling of neurons (Angevine and Sidman, 1961; Berry and Rogers, 1965). This suggests that DNAs are transfected into the cells close to the ventricle which are differentiating into or generating neurons at the time of electroporation. Neuronal morphologies were clearly visible using the fluorescent proteins. The expression of the mitochondrial-targeted DsRed protein visualized sites of the mitochondria in neurons. Similar fluorescent protein derivatives which are targeted to some organella will be useful to analyze neuronal cytoarchitecture during development, since genes can be easily transfected into neurons in many CNS areas by this electroporation method.

Surprisingly, fluorescent signals persisted for a long time, even in weaned mice, 6 weeks after electroporation. Fluorescence at postnatal stages was still strong. Existence of the *EYFP* mRNA and fluorescence of the destabilized EGFP protein in postnatal brains showed that introduced genes were expressed even at postnatal stages. In electroporated brains, transfected DNA would not be diluted by cell division in differentiated cells, such as postmitotic neurons. Foreign gene expression has been reported to last for an extended time in muscle cells (Wolfe *et al.,* 1990). Long-term expression of introduced genes may enable us to analyze gene function not only for development at embryonic stages but also for animal behavior.

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