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Manipulating gene expressions by electroporation in the developing brain of mammalian embryos

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Abstract One of the goals of developmental neuroscience in the post-genomic era is to clarify functions of a huge number of anonymous genes of which only DNA sequences are identified. More convenient methods for genetic manipulation in vertebrates, especially mammals, could help us to identify functions of the novel genes. Here we introduce a novel gene transfer technology using electroporation (EP), which is a simple and powerful strategy for genetic analysis. We have applied this method to cultured mammalian embryos in order to understand the function of specific genes in the developing brain. We have also performed EP in developing fetuses *in utero* guided by ultrasound image. The combination of these techniques in addition to analysis of genetic mutants will clarify functions of individual genes, gene interactions, and the molecular mechanisms underlying the brain development.

Key words mammalian brain · electroporation · whole-embryo culture · *in utero* manipulation · ultrasound guided manipulation

Introduction

Recent studies have shown that molecular mechanisms which govern compartmentalization and neuronal differentiation are phylogenically conserved among a variety of vertebrate species (Puelles and Rubenstein, 1993; Inoue et al., 2001b). However, each vertebrate species has specific brain structures as a result of adaptation to the environment. For example, mammals developed the six-layered neocortex for processing numerous environmental stimuli, while a similar structure was developed in the avian optic tectum as a visual center (La Vail and Cowan, 1971). One of the important issues in developmental neurobiology is how genetic mechanisms control the development of such species-specific brain structures. Although genome projects and transcriptome analyses may give us clues to identify the large number of genes expressed during mammalian brain development, the task we face in the post-genomic research era is to analyze functions and interactions of these novel genes.

For a better understanding of the molecular basis for mammalian brain development *in vivo*, we need to manipulate gene expression in mammalian embryos and fetuses. Introduction of exogenous genes into embryos and targeting of given genes by homologous recombination are conventional and standard techniques to analyze gene function. However, several problems need to be overcome for strategies to succeed. First, regulatory elements which control expression of a gene of interest in a spatio-temporal manner during development are not always accessible to genetic manipulation by conventional techniques. Secondly, embryonic lethality of some mutations precludes investigation of gene function at later stages of development. Recently, techniques for conditional gene targeting and gene activation have been designed using the Cre/LoxP system or other genetic tools (see review by Porter, 1998). These methods have overcome some difficulties of conventional gene manipulation. However, a severe limitation of these systems is that much time and labor are needed to generate the transgenic animals upon which these systems rely.

Embryo culture systems have opened the way to investigate mammalian development. For post-implantation embryos, whole-embryo culture (WEC) is the most popular method (Cockroft, 1990; New, 1990; Morriss-

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Kay, 1993). Several surgical manipulations such as cell labeling (Serbedzija et al., 1990; Matsuo et al., 1993), cell grafting (Osumi-Yamashita et al., 1997; Kinder et al., 2000), and injection of antisense-oligonucleotides (Augustine et al., 1993) have been used in conjunction with mammalian WEC as well as in other vertebrate species (Heasman et al., 1991; 1994). However, this technique is not applicable for embryos at later stages because the placenta does not develop *in vitro*. In older mammalian embryos, *in utero* and *ex utero* manipulations are used to inject virus vectors for misexpression of genes (Muneoka et al., 1990; Papaioannou, 1990; Kageyama et al., 1996). *In utero* manipulation guided by ultrasound biomicroscope (UBM) has been used to inject virus solution (Gaiano et al., 2000) and to transplant cells (Olsson et al., 1997; Liu et al., 1998; Turnbull, 2000; Wichterle et al., 2001) into specific regions of the embryo.

Recently, introduction of exogenous genes by electroporation (EP) has proved to be a powerful tool, especially for avian embryologists (Muramatsu et al., 1997). We have adopted EP together with *in utero* manipulation of mammalian embryos/fetuses. Here we show the results of two gene transfer systems, using EP to introduce exongenous genes to the developing brain of rodent embryos or fetuses. We introduce a method that combines WEC with EP and *in utero* manipulation with EP that is guided by ultrasound imaging. We prefer to use rat embryos for *in vitro* manipulations because both their body size and litter size (number of embryos within one pregnant animal) are bigger than mice. The methods shown here are of course applicable for mouse embryos and fetuses. We believe the spatio-temporal targeting of these methods will provide more information on specific genetic interaction than can be ascertained from analysis of traditional knock-out or transgenic rodents alone.

Electroporation into cultured rat embryos

The *in ovo* electroporation (EP) method established originally by Muramatsu et al. (1997) has been widely used in chick embryos for analyses of gene function in a variety of developmental events such as brain patterning and neural crest formation (Yasugi and Nakamura, 2000; Nakamura and Funahashi, 2001; Endo et al., 2002). We applied EP to rodent embryos in WEC to study the mechanism of mammalian brain development (see also Inoue et al., 2001a; Inoue and Krumlauf, 2001; Osumi and Inoue, 2001; Swartz et al., 2001; Takahashi and Osumi, 2002). This method leads to a high efficiency of exogenous gene expression in target tissues without tissue damage due to the use of square pulses for the electroporation. Expression of exogenous genes is transient but commences more quickly than expression mediated by virus vectors. Moreover, the strategy is safer than experiments using virus vectors. Here we show a further application of WEC + EP aimed at revealing genetic interactions in the developing rat hindbrain.

Methods for EP into cultured rat embryos

Our detailed procedures of EP + WEC have previously been described (Osumi and Inoue, 2001). WEC is applicable to E9.0–14.0 rat embryos (corresponding to E7.0–12.0 mouse embryos; Fig. 1); the growth of mammalian embryos at later stages depends on enough nutrients being provided through the placenta *in vivo*, but as mentioned, the placenta does not grow *in vitro*. Here we use EP E11.5 rat embryos, which are subsequently cultured for 1 day prior to expression and function analysis. For culture, the uterus is isolated from anaesthetised females; littermate embryos within the yolk sac with the placenta intact are dissected out and placed in sterile Tyrode's solution where the yolk sac is opened. After a 2-hour preculture in medium (100% rat serum (Charles River Japan, Inc) with 2 mg/ml glucose and antibiotics) the embryos were transferred into a chamber bordered on two sides by electrodes (8 × 20 mm electrodes and 20 mm distance between electrodes; Unique Medical Imada, Miyagi, Japan) which is filled with Tyrode's solution. A solution of 0.25 μl of plasmid DNA dissolved in phosphate buffered saline (PBS) at 5 mg/ml (with

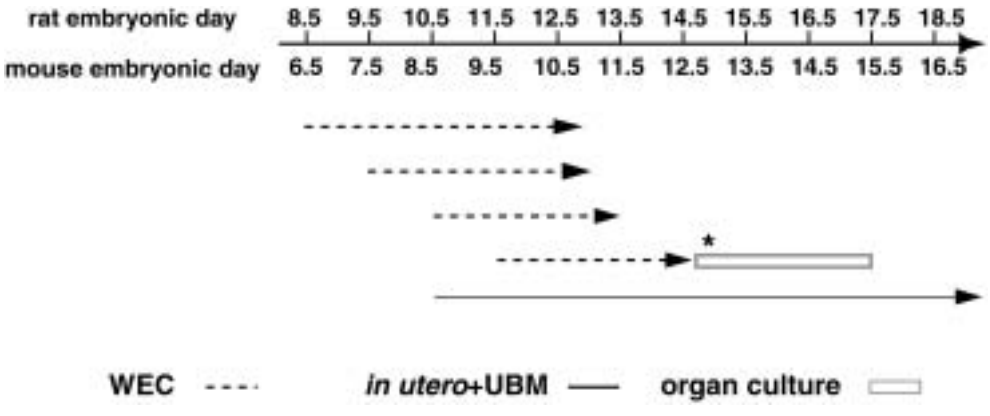

Fig. 1 Time course showing applicable periods for various manipulations and gene transfer into mammalian embryos using EP. *Asterisk* indicates the period in which telencephalon dissected from cultured embryos can further be cultured in organ culture system for 3 days (T.N. and N.O; unpublished data). UBM: Ultrasound Biomicroscope.

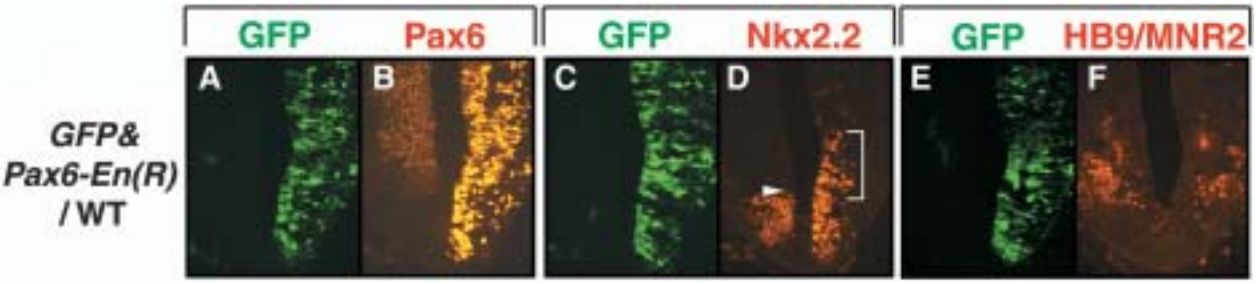

Fig. 2 Loss-of-function study by introduction of dominant-negative *Pax6* construct in cultured mammalian embryos. (**A-F**) *Pax6* dominant negative form, *Pax6-En(R)*, fused with *Drosophila engrailed* repressor domain was transfected into the hindbrain of early E11.5 (somite 22) wild-type rat embryo together with GFP reporter construct. These embryos were cultured for 24 hours after EP. GFP fluorescence and antibody staining were performed. Sets of two panels indicate double images of the same section. (**A, B**) *Pax6-En(R)* fusion protein is detected by an antibody which recognizes the paired domain of Pax6. A very high degree of overlapping expression is observed between the electroporated Pax6 and GFP protein. Endogenous Pax6 protein is expressed in both sides. (**C, D**) Pax6-En(R) induced expansion of Nkx2.2 domain (*bracket* in **D**). (**E, F**) The numbers of HB9/MNR2 positive somatic motor neurons reduce due to indirect inhibition of Pax6 function.

0.05% Fast Green; Sigma Chemical Co., St. Louis, MO) is injected into the embryo's hindbrain with fine glass needles (outer diameter = 1 mm, inner diameter = 0.58 mm, length = 100 mm: B100-58-10; Sutter Instrument Co., Novato, CA). Immediately, square pulses (50 mseconds, 5 pulses at 1second intervals, 70 V, five times) are sent through the chamber using an electroporator (CUY21; NEPPA GENE, Tokyo, Japan). The embryos are returned to the culture medium and incubated at 37°C for one day. By using a general expression vector of one of CMV (cytomegalo virus), beta-actin, or EF-1 (elongation factor-1) promoters and changing direction and shape of electrodes, transgenes are introduced directly into specific brain regions where they are transiently expressed.

Loss of gene function study in cultured mammalian embryos

It has been reported that overexpression of dominant negative forms of a gene induces a loss-of-function phenotype in brain development (Akamatsu et al., 1999). Here, we show analyses on *Pax6* gene function in specification of ventral motor neurons and interneurons in the rodent hindbrain. To clarify gene cascades controlled by *Pax6*, we electroporated the truncated form of *Pax6* combined with *Drosophila engrailed* repressor domain (Yamasaki et al., 2001) into the hindbrain of cultured rat embryos. Together with co-transfected GFP gene product, used as an EP marker (Fig. 2A), strong expression of *Pax6-En(R)* protein was detected in the electroporated side (Fig. 2B). In the side where *Pax6-En(R)* was expressed, upregulation of Nkx2.2 was seen (bracket in Fig. 2D). Additionally, the number of HB9/MNR2 positive somatic motor (SM) neurons decreased (Fig. 2F). It should be noted that this expansion of Nkx2.2 domain and the decrease of SM neurons resemble the phenotype of the *Pax6* homozygous mutant rat (left side in Fig. 4D; also reported in Ericson et al., 1997; Takahashi and Osumi, 2002). These results suggest that EP of a dominant-negative form of a gene is very convenient strategy to elucidate its spatio-temporal roles in brain development.

Here, the fact that Nkx2.2 expands in the presence of dominant negative Pax6 leads to a couple of important conclusions on neuronal specification in the ventral hindbrain. First, several lines of evidence suggest that Pax6 represses Nkx2.2 (Ericson et al., 1997; Briscoe et al., 2000; Mastick and Andrews, 2001; Muhr et al., 2001). Our new result strongly suggests that this repression is not direct in the hindbrain. For example, if Pax6 bound directly to *Nkx2.2* promoter to repress it, then the Pax6-EnR protein would also repress Nkx2.2. Instead, our result implies that Pax6 normally activates some other factor, which then acts more directly to repress Nkx2.2. Second, Nkx2.2 does not turn on in all regions containing the dominant negative Pax6, suggesting that other factors that activate Nkx2.2, or other factors that can also repress Nkx2.2 in the absence of Pax6, must be spatially limited in their function.

Recent studies reported that modified antisense oligonucleotides (Morpholino) efficiently suppress gene expression in zebrafish embryogenesis (Nasevicius and Ekker, 2000). Morpholinos have higher affinity for mRNA than general antisense oligonucleotides and can alter RNA splicing and inhibit mRNA translation (Summerton and Weller, 1997; Summerton, 1999; Ekker and Larson, 2001; Heasman, 2002). It should be noted that the structure of Morpholinos is stable and does not exhibit toxicity for cells and tissues. Misexpression of Morpholino has been shown to knock down gene expression and therefore gene function in chick (Kos et al., 2001; Tucker, 2001), *Xenopus* (Heasman et al., 2000), and sea urchin (Howard et al., 2001) embryos. To obtain

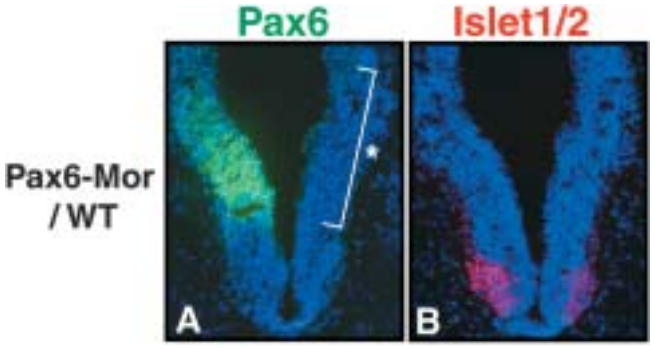

Fig. 3 Loss-of-function study by introduction of *Pax6* Morpholino oligonucleotides. (**A, B**) *Pax6* Morpholino oligonucleotides were transfected into the hindbrain of early E11.5 (somite 22) wild-type rat embryo. These embryos were cultured for 24 hours after EP. (**A, B**) Serial sections of the wild-type hindbrain electroporated with *Pax6*-Morpholino. (**A**) Pax6 immmunoreactivity is normally detected on the control side (*green*) but eliminated in the electroporated side, as indicated by *bracket*. (**B**) In the electroporated side, the number of Islet1/2 positive motor neurons decreases. These sections are counterstained with DAPI (*blue*).

the loss-of-function phenotype of *Pax6*, we performed EP of *Pax6*-Morpholino into the rat hindbrain. We designed *Pax6*-Morpholino complementary to the sequence which covers the first 8 amino acids of mouse Pax6 protein (5′-CACTCCGCTGTGACTGTTCTG-CATG-3′; underline indicates complementary sequences for the initiation codon). We injected approximately 0.25 µl of Morpholino solution dissolved in 1 mM PBS with 0.05% Fast Green into the hindbrain of precultured rat embryos as described above. We electroporated using 5 square pulses (50 mseconds, 5 pulses at 1 second intervals, 65–70 V). Loss of endogenous expression of Pax6 protein was observed in the electroporated side after several hours and up to at least 36 hours (Fig. 3A), indicating *Pax6*-Morpolino inhibited translation of *Pax6* mRNA. Also, the number of Islet1/2 positive motor neurons decreased in the electroporated side (Fig. 3B), being a similar phenotype seen in the *Pax6* mutant hindbrain. These results suggest that EP of Morpholino is a useful and powerful technique for analysis of gene functions during mammalian brain development.

Gain-of-function study in cultured mutant embryos

Inoue et al. (2001a) have shown that ectopic cadherin-6 positive cells in the cortex side of wild-type mouse were sorted into the lateral ganglionic eminence (LGE) region where cadherin-6 is normally expressed. On the other hand, ectopic expression of cadherin-6 in the cortex of the *cadherin-6* mutant did not lead cell sorting to LGE side. We tested by EP in the *Pax6* mutant whether exogenous *Pax6* can rescue the expression pattern of downstream genes that are altered in the *Pax6* mutant. As a result, Nkx2.2 expression was repressed as seen in the wild type (bracket in Fig. 4D; see also Takahashi and Osumi, 2002). Previous studies reported that expression of *Wnt7b* and *En1* is missing in the *Pax6* mutant hindbrain (left side in Fig. 4F; Osumi et al., 1997; Burrill et al., 1997; Ericson et al., 1997; Osumi and Nakafuku, 1998). These molecules are normally expressed in Pax6 positive neuroepithelial cells and their descendants, respectively. EP of *Pax6* into the *Pax6* mutant hindbrain recovered expression of *Wnt7b* (bracket in Fig. 4F) and *En1* (data not shown, see Takahashi and Osumi, 2002). These results suggest that Pax6 acts as a positive regulator for *Wnt7b* and a negative regulator of Nkx2.2. Therefore, gain-of-function studies such as these combined with analysis of specific mutant mice may lead us to deepen our understanding of mechanisms which result in brain patterning.

Electroporation into embryos/fetuses developing *in utero*

To study long-term functions of genes involved in the brain development, another gene transfer system is needed. Such a system will allow embryos and fetuses to be experimentally manipulated throughout embryogenesis and then their development maintained up to delivery. Although the whole embryo culture system is very useful for the analysis of the molecular mechanisms of the developing mammalian brain, as mentioned earlier, the embryonic stages that can be studied are limited (Fig. 1).

Conventional *in utero* EP methods have been useful for the analysis of gene functions at later stages when the embryos are visible through the thin uterine wall (Saito and Nakatsuji, 2001; Tabata and Nakajima, 2001; Fukuchi-Shimogori and Grove, 2001). *In utero* manipulation of mouse embryos guided by ultrasound biomicroscope (UBM) established by Turnbull (2000) enables us to precisely manipulate embryos or fetuses even from early stages when they are veiled by the thicker uterine wall. Additionally, this method optimally maintains the development of the manipulated embryos/fetuses due to minimal invasion of pregnant mothers.

We have established a new gene transfer method using EP in combination with *in utero* ultrasound-guided operation to efficiently express exogenous genes. This method expands the application of *in utero* manipulation to earlier embryonic stages. The ultrasound-guided operation will assist in locating the injection of the DNA solution into a specific location of the embryo. Here we introduce *in utero* EP guided by UBM in rat embryos, but the technique is applicable to mouse embryos as well.

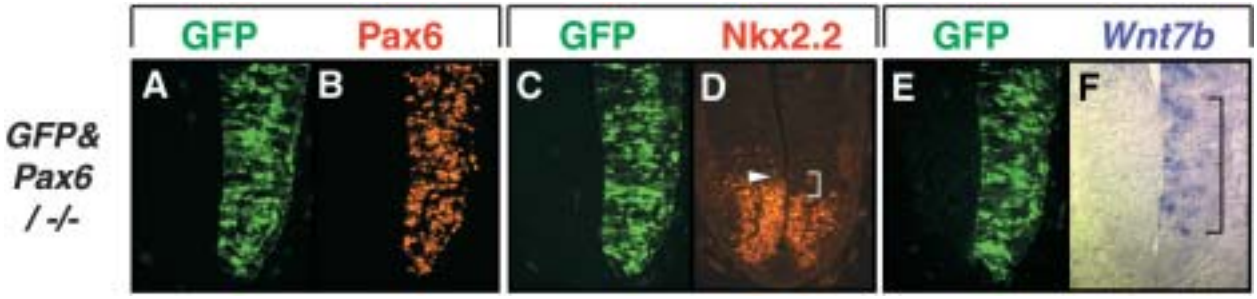

Fig. 4 Gain-of-function study by introduction of exogenous *Pax6*. (**A-F**) Wild-type form of Pax6 was electroporated into the hindbrain of early E11.5 (somite 22) *Pax6* homozygous mutant rat embryo together with GFP reporter construct. These embryos were cultured for 24 hours after EP. Sets of two panels indicate double image of the same section. GFP fluorescence and antibody staining were performed except **E** and **F**. (**E, F**) In **E**, GFP protein was detected by GFP antibody after *in situ* hybridization was performed (**F**). (**A, B**) In the *Pax6* mutant, exogenous Pax6 was only detected in the right side. (**C, D**) the expansion of Nkx2.2 domain in the *Pax6* mutant hindbrain is repressed by exogenous Pax6 (*bracket* in **D**). (**E, F**) In the ventral domain of the neural tube expressing Pax6, *Wnt7b* is coexpressed, while the expression is eliminated in the *Pax6* mutant (*left side* in **F**; see also Osumi et al., 1997). Exogenous Pax6 induced *Wnt7b* expression (*bracket* in **F**).

Methods for *in utero* EP guided with UBM

In utero manipulation guided by UBM is as described by Turnbull (2000) with some modifications. After pregnant rats are anesthetized with sodium pentobarbital (0.7 mg/100 g body weight, injected intraperitoneally), the abdomen is shaved with a razor blade, and a 2-cm midline laparotomy is performed. A 100-mm diameter plastic petri dish with a 25-mm diameter hole sealed with a thin rubber membrane (Silastic L room temperature vulcanization (RTV) silicone rubber; Dow Corning) in the bottom center, is placed on the abdomen. The area of the skin that contacts the rubber is shaved carefully because hairs disrupt the seal of between the rubber membrane and the rat abdomen, leading to leakage of the solution in the Petri dish. The rat should be in a position which allows the abdomen to make good contact with the rubber membrane (Fig. 5A). Inappropriate abdominal positioning will restrict breathing of the animal and create excessive pressure on the uterus. The uterus is gently pulled from the abdomen through a slit in the rubber membrane into the petri dish filled with sterile PBS. We used a high-frequency ultrasound scanner (UBM; Paradigm Medical Industries, Salt Lake City, UT) to perform *in utero* embryo imaging. This imaging system is equipped with a high frequency (40–50 MHz) focused ultrasound transducer that is scanned mechanically to produce two-dimensional images in real time (Fig. 5A, C, D). While monitoring the images, an injection needle made of glass microcapillary pipettes (outer diameter = 1 mm, inner diameter = 0.5 mm, length = 100 mm: BF100-50-10; Sutter Instrument Co., Novato, CA) is inserted into the cerebral vesicle through the uterine wall, and plasmid DNA solution (2.5–5 μg/μl with 0.05% Fast Green) is injected (Fig. 5A, D). The amount of the DNA solution for injection is approximately 1 μl, but varies according to the developmental stage of the embryo. After injection of DNA, EP is performed using tweezer-type electrodes which consist of a pair of round platinum plates of 0.7 mm diameter (Unique Medical Imada, Miyagi, Japan) (Fig. 5B). Optimal conditions of electrical pulses vary according to developmental stages. We performed EP with 40–50 V, 50 mseconds, 5 pulses at 1 second intervals for E13.5 rat embryos. The sterile

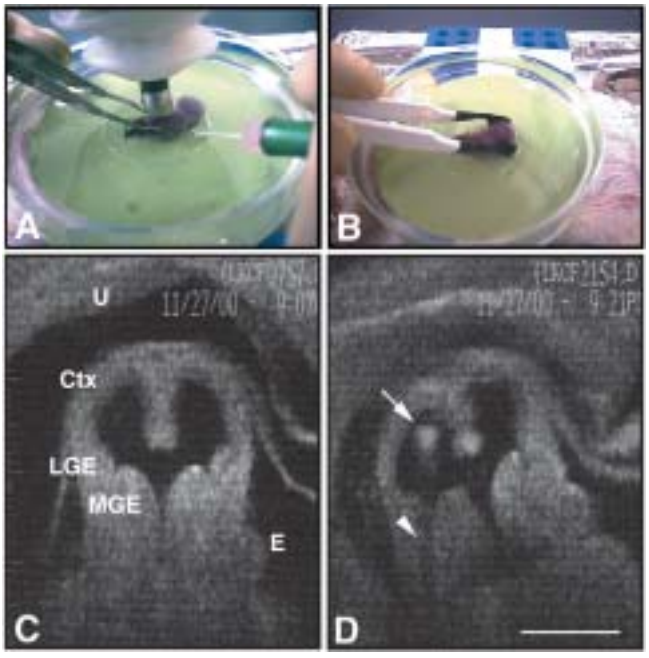

Fig. 5 Procedures for *in utero* EP and frontal section images of the E13.5 rat telencephalon through the UBM. (**A**) The uterus is held with the forceps and injected with a glass needle. (**B**) Electroporation with the tweezer-type electrode. All of these procedures are performed through the UBM imager. Before (**C**) and after (**D**) injection of DNA solution into the hemisphere. White arrow and arrowhead indicate injection needle and its acoustic shadow. *E*, eye; *Ctx*, cortex; *LGE*, lateral ganglionic eminence; *MGE*, medial ganglionic eminence; *U*, uterine wall. *Bar* = 1 mm.

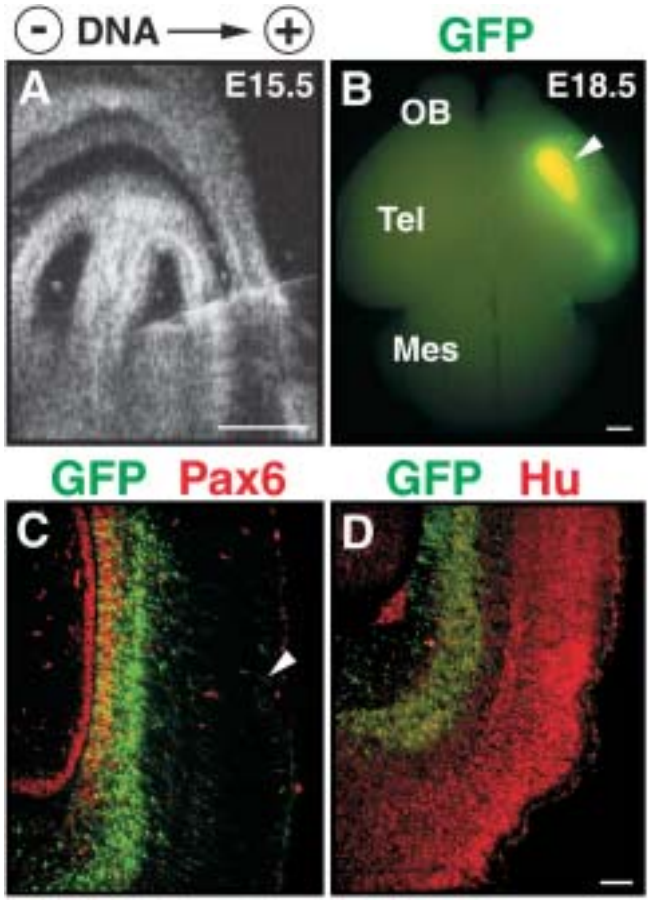

Fig. 6 Expression of the exogenous gene after *in utero* EP. (**A**) GFP expression vector was injected to the right lateral ventricle (LV) of E15.5 rat embryo by monitoring the image. DNA was electroporated toward the plus electrode. The direction of the electric field is indicated by + or −. (**B**) E18.5 whole brain of the same embryo. As expected, the expression of GFP was observed in the right telencephalon (*white arrowhead*). (**C, D**) Behavior of GFP-positive cells in E18.5 rat telencephalon electroporated at E15.5. Image of (**D**) is more ventral to that of (**C**). (**C**) GFP positive cells are *green*, and Pax6 are *red*. GFP positive cells coexpress Pax6 in the ventricular zone (*yellow*). Radial fibers extending toward the pial surface are also present (*white arrowhead*). (**D**) Immunostaining for a neuronal marker, Hu (*red*). In the whole layer except the ventricular zone, GFP positive cells coexpress Hu protein (*yellow*). *Mes*, mesencephalon; *OB*, olfactory bulb; *Tel*, telencephalon. *Bar* = 1 mm (**A, B**), 250 μm (**C, D**).

PBS in the petri dish should be changed after each EP procedure. The procedure should be completed within one hour. The survival rate of embryos is around 60%, but this also depends on the developmental stage of the embryo; the earlier embryos are more vulnerable.

Exogenous gene expression by ultrasound guided electroporation

A GFP expression vector, used as a marker for cells that incorporate the exogenous plasmid, was electropolated to the ventricular zone of the lateral cortex in E15.5 rat embryo (Fig. 6A) by the methods described. Three days later, the expression of GFP was observed in the lateral side of the telencephalon (Fig. 6B). Furthermore, histological analysis showed that a large number of GFP positive cells were located in the ventricular zone, which is positive for Pax6 (Fig. 6C). GFP positive cells were also observed in the subventricular zone and the intermediate zone, where neuronal marker protein Hu is expressed (Fig. 6D). This finding suggests that these cells derived from the GFP-positive neuronal progenitor. Therefore, misexpression of genes of interest or introduction of dominant negative-constructs in the wild-type and mutant embryos could easily be performed using this method allowing for direct analysis of the gene of interest. Furthermore, as evidenced by the expression of GFP in Hu positive cells, this method can also be used to trace migration of neurons in normal and abnormal development (Tomioka et al., 2000; and T.N. and N.O., unpublished results).

Future applications

Gene transfer by EP combined with various manipulations of rodent embryos is a powerful tool for understanding molecular mechanisms of mammalian brain formation. Other applications of EP include analysis of enhancer regions by *in ovo* EP of reporter constructs driven by the enhancer regions in question (Itasaki et al., 1999; Muller et al., 2000; Sakamoto et al., 2000; Yasuda et al., 2000; Timmer et al., 2001). Therefore, introduction of these types of constructs or yeast artificial chromosome (YAC) clone including larger regulatory regions into cultured rodent embryos by EP may provide more quick and simple method to analyze or identify regulatory elements in the mouse genome.

WEC for rat embryos is restricted to E9.0–14.0 (corresponding to E7.0–12.0 in mouse embryos), and growth of embryos can be maintained for only 2–3 days after EP (Fig. 1). In contrast, we can maintain embryos electroporated *in utero* guided by UBM to full term and even analyze at postnatal or adult stages. Another technique available to enable analysis of development over a longer term than WEC/EP allows is organ culture combined with EP (Fig. 1; Tomioka et al., 2000; T.N. and N.O., unpublished data). For example, the whole telencephalon can be taken from cultured embryos, electroporated with a gene of interest, and cultured on the membrane for several days. In conclusion, we have tested various tools to explore mechanisms of brain development at the molecular level. EP combined with UBM, WEC, or organ culture are methods which allow analysis of mammalian specific brain formation, such as the establishment of the six-layered cortex and the subdivisions of the cortical areas.

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