



EFFICIENT *IN UTERO* GENE TRANSFER SYSTEM TO THE DEVELOPING MOUSE BRAIN USING ELECTROPORATION: VISUALIZATION OF NEURONAL MIGRATION IN THE DEVELOPING CORTEX

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Abstract—We report a novel gene transfer system using electroporation. We used this technique to introduce a marker gene plasmid containing enhanced green fluorescent protein into mouse brains at embryonic day 12–17 without removing the embryos from the uterus. The embryos were allowed to continue to develop *in utero*, and more than 80% were born normally expressing the exogenous gene. Enhanced green fluorescent protein driven by the cytomegalovirus promoter was strongly expressed in the ventricular zone, radial fibers and migrating neuroblasts, but not in mature neurons, suggesting that the cytomegalovirus promoter is silenced after the cells differentiate into mature neurons. Since there is still no convenient way of visualizing the migrating neuroblasts, especially of distinguishing them from the surrounding mature neurons in the cortical plate, this system should provide a good tool for analysing neuronal migration. In the postnatal lateral cortex, neuroblasts migrated almost “tangentially” along the obliquely running “radial” fibers beneath the cortical plate, and after entering the cortical plate, turned towards the marginal zone and migrated radially. Neurons with primitive dendrites were observed only along the border between the marginal zone and the cortical plate, and never at other sites, such as in the middle of the cortical plate. These results imply that the neuroblasts do terminate migration and start differentiation to mature neurons when they encounter the marginal zone, as has long been suggested. By contrast, when elongation factor 1 α promoter was used, prominent fluorescence allowed visualization of the entire mature neurons as well. The labeled neurons were observed to send axons to the contralateral cortex where they arborized extensively.

Thus, this system is much easier and more efficient than virus-mediated gene transfer, and is useful for gain-of-function analysis of neural cell fate determination, migration, positioning and axon path-finding in mouse embryos. © 2001 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: CMV promoter, EF1 α promoter, *in vivo* gene transfer, neuronal morphology, radial fiber, reelin.

One of the most common approaches to elucidate the functions of a particular gene in the developmental pathway is misexpression or overexpression of the gene *in vivo* using virus vectors, but the several months that it takes to construct and prepare the virus stock is a problem. Recently, an *in ovo* gene transfer system that uses electroporation was established in chicks.^{6,9,11,12,14,18} In this system, the effect of recombinant plasmid vectors can be examined directly *in vivo*. Its simplicity is a great advantage over virus vectors, and permits rapid investigation of various forms of a particular gene, such as a dominant negative form, constitutive active form and deletion mutants. In addition, several factors must be considered when constructing expression vectors, namely, the promoter/enhancer region and, if desired,

fusion of an epitope-tag or green fluorescent protein, or other options such as the internal ribosome entry site, the Cre-loxP system and the tetracycline responsive element. The electroporation system, in contrast, allows the optimal combination of these factors to be determined easily. This system has other unique features. Since DNA is negatively charged, the injected plasmid moves towards the anode in the electronic field, allowing it to be selectively introduced into a specific region, which is difficult to achieve with virus vectors. Because this technique is based on a physical phenomenon, injected DNA can be introduced into almost any type of cell at the site of injection. Application of the electroporation system to mouse embryos has already been tried,¹ but the mouse embryos were removed from the uterus at embryonic day (E) 8.25 or 9, electroporated and subjected to whole embryonic culture. Although this system is very useful for analysing the early development of the CNS, the normal course of development cannot be maintained for a long time under culture conditions. Hence, the previously reported system is not applicable to studies of late developmental events, such as neuronal migration and axon path-finding. To overcome this

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Abbreviations: BrdU, bromodeoxyuridine; CMV, cytomegalovirus; E, embryonic day; EF1 α , elongation factor 1 α ; EGFP, enhanced green fluorescent protein; MAP2, microtubule-associated protein 2; P, postnatal day; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate.

problem, we performed all steps of gene transfer without removing the mouse embryos from the uterus. This system should be very useful for gain-of-function analysis of various genes that are involved in late developmental events of the CNS.

EXPERIMENTAL PROCEDURES

Electroporation

The surgery on pregnant mice and embryo manipulation in the uterus were performed as previously described,¹³ and conformed to the guidelines on animal experiments by the Japan Neuroscience Society. All efforts were made to minimize the number of animals used and their suffering. Pregnant ICR mice were provided by Japan SLC (Shizuoka, Japan). At E14 or 15, pregnant mice were deeply anesthetized with sodium pentobarbitone at 50 μg per gram of body weight, and the uterine horns were exposed. Plasmid DNA purified with the QIAGEN (Hilden, Germany) plasmid maxi kit was dissolved in 10 mM Tris-HCl (pH 8.0) at a concentration of 5–10 $\mu\text{g}/\mu\text{l}$, and Fast Green solution (0.1%) was added to the plasmid solution in a ratio of 1:10 to monitor the injection. Approximately 1–2 μl of plasmid solution (0.8 μl for E12.5–13, 1.2 μl for E14–15 and 2 μl for E16–17) was injected into the lateral ventricle with a glass micropipette made from a microcapillary tube (GD-1; Narishige, Tokyo, Japan). The embryo in the uterus was placed between the tweezers-type electrode, which has disc electrodes of 5 mm in diameter at the tip (CUY650-5; Tokiwa Science, Fukuoka, Japan). Electronic pulses (30 V for E12.5–13, 32–35 V for E14, and 35 V for E15 and later; 50 ms) were charged five times at intervals of 950 ms with an electroporator (CUY21E; Tokiwa Science). The uterine horns were placed back into the abdominal cavity to allow the embryos to continue normal development. Several enhanced green fluorescent protein (EGFP) expression vectors, pEGFP-N1 (Clontech, Palo Alto, CA), pEF-BOS-EGFP (the coding sequence of EGFP from pEGFP-N1 recombined with pEF-BOS¹⁰), pT α 1-EGFP (the same cDNA fragment of EGFP from pEGFP-N1 recombined with plasmid 253, which contains the T α 1 α -tubulin promoter^{7,8}) and pDsRed1-N1 (Clontech) were examined.

Tissue preparation and observation

All animals were anesthetized on ice or with sodium pentobarbitone at 50 μg per gram of body weight, and fixed by perfusion of 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) at embryonic or postnatal stages. The brain was dissected out and postfixed in 4% paraformaldehyde for 2–16 h at 4°C. After washing in phosphate-buffered saline (PBS) for 1 h, the samples were equilibrated in 30% sucrose in PBS. Brain samples, except for postnatal day (P) 21 brains, were embedded in OCT compound (Sakura, Tokyo, Japan) and frozen in liquid nitrogen. Frozen sections were cut coronally with a cryostat (CM1900; Leica) at 20 μm and mounted onto silane-coated glass slides (Matsunami, Japan). After removing the OCT compound in PBS containing 0.01% Triton X-100 (Sigma), the sections were coverslipped with PermaFluor (Immunon; Pittsburgh, PA) and directly observed under a fluorescence microscope. When microtubule-associated protein 2 (MAP2) or Reelin staining was performed, the sections were immunostained with anti-MAP2 (1:100, Chemicon, Temecula, CA) or CR-50 (anti-Reelin) antiserum¹⁵ and then with tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-mouse IgG (1:10, Cappel, Aurora, OH). The P21 brains were frozen on specimen discs of cryostat and sliced at 50 μm . The slices were put into PBS, and subjected to immunohistochemistry by the floating method. Immunostaining was performed with anti-NeuN (1:100, Chemicon) and TRITC-conjugated anti-mouse IgG (1:10, Cappel), and the stained slices were mounted on silane-coated slides. Fluorescent images were obtained with a CCD camera (C5810; Hamamatsu, Shizuoka, Japan) or a confocal laser microscope (LSM410; Zeiss, Germany).

RESULTS

In order to allow the electroporated embryos to continue normal development, all gene transfer procedures in this study were performed through the uterine wall. The plasmid vectors were injected into the lateral ventricle of E12.5–E17 mouse embryos, and the electronic pulses were applied from outside the uterus. Good viability with efficient gene transfer was obtained when 1–2 μl of 5–10 $\mu\text{g}/\mu\text{l}$ of plasmid was injected and 30–35 V was applied with 5 mm diameter electrodes, (for example, when 58 embryos were injected at E14, 47 pups were born normally with exogenous gene expression, approximately 81%). Under these conditions, the electronic resistance was 600–1000 m Ω .

This system was used to introduce several plasmid vectors at various times, and the embryos were examined at various stages. We first tested pEGFP-N1 (Clontech), which expresses EGFP under the control of cytomegalovirus (CMV) promoter. In the brain injected with the pEGFP-N1 at E14 and fixed at E15, EGFP fluorescence was observed in the ventricular zone and the radial fibers (Fig. 1A). At the subpial site, the end-feet of the radial fibers were strongly labeled (Fig. 1F). Two days after electroporation (electroporated at E14 and fixed at E16), many migrating neuroblasts in the intermediate zone were also labeled (Fig. 1B). After three days, abundant migrating neuroblasts were seen in the developing cortical plate (data not shown). A similar experiment with pDsRed1-N1 (Clontech), in which the modified red fluorescent protein, DsRed, is driven by the CMV promoter, is shown in Fig. 1E, “red cells”). We then examined pEFBOS-EGFP, in which the EGFP gene is driven by the human elongation factor 1 α (EF1 α) promoter. In the brains injected with pEFBOS-EGFP at E14 and fixed one to three days later, the migration pattern of the labeled cells was similar to the pattern with pEGFP-N1 (Fig. 1C–E, “green cells”). Further observations were performed on the embryos injected with pEGFP-N1 at E15 and fixed at P3 (Fig. 2). The EGFP-labeled migrating neuroblasts were still observed in the cortical plate and in the intermediate zone at this stage (Fig. 2A, C). Radial fibers, including their end-feet, were also clearly visualized (Fig. 2A, B). In addition to these cells, many EGFP-positive neuroblasts were observed to align immediately beneath the marginal zone (Fig. 2A, D–F), where the neuroblasts should terminate migration and start to differentiate into mature neurons by developing primitive dendrites.^{4,16} EGFP permeated into the entire cell structures, so that the morphological features of these cells were clearly visualized. Sections were immunostained with anti-MAP2 to define the border between marginal zone and cortical plate (Fig. 2E). The merged image showed that the primitive dendrites had formed into the marginal zone (Fig. 2F). This type of labeled cell with primitive dendrites was never found in the deeper portions of the cortical plate or in the intermediate zone. In the lateral cortex, where EGFP-labeled radial fibers were observed more frequently than in the medial cortex, EGFP fluorescence visualized bundles of radial fibers. The immunostaining with anti-MAP2 to

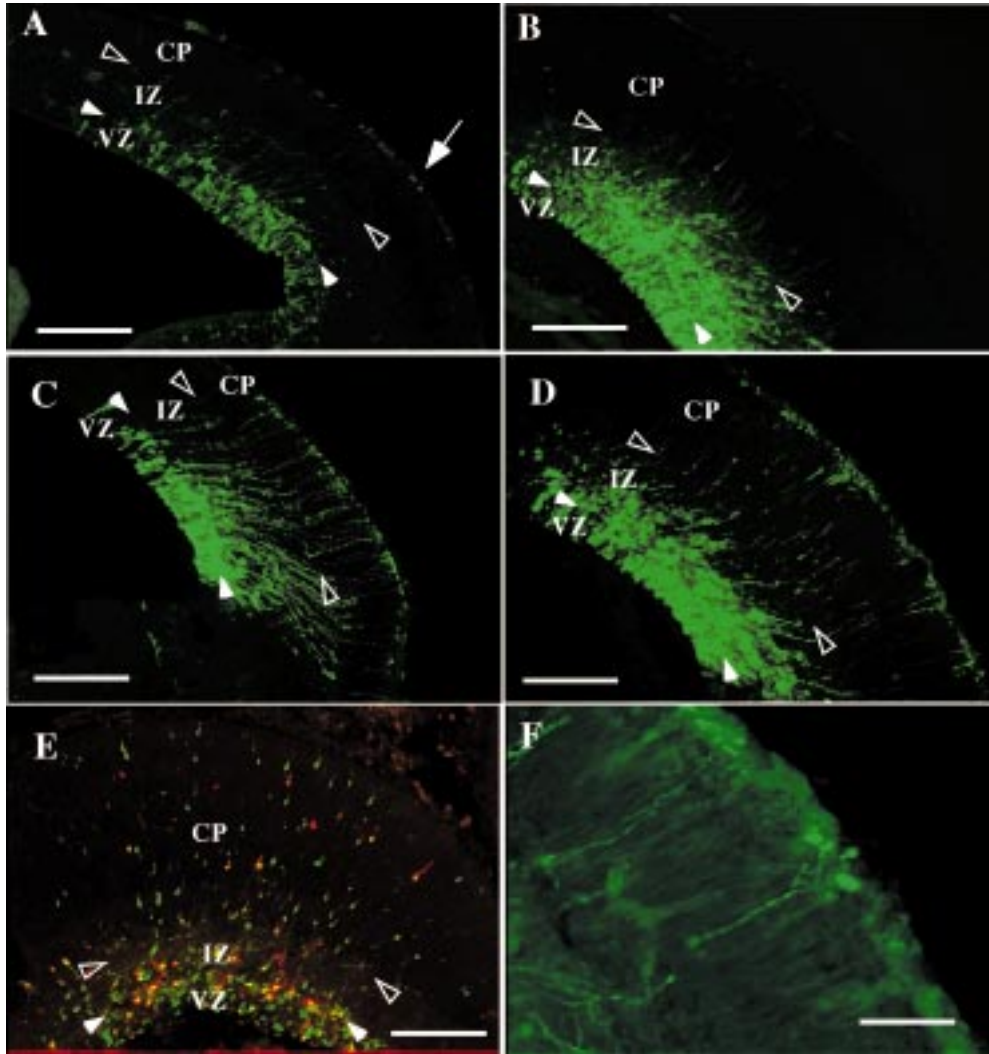


Fig. 1. Initial migration pattern of the transfected neuroblasts in the developing neocortex. Plasmid vectors that express EGFP under the control of CMV promoter (A, B) or EF1 α promoter (C, D) were injected into the lateral ventricle of E14 mouse embryos, and electroporation was performed. EGFP fluorescence was observed one day (A, C) or two days (B, D) after the electroporation. (E) The brain that was co-transfected with the DsRed expression vector driven by CMV promoter and the EGFP expression vector driven by EF1 α was examined three days later. The closed and open arrowheads indicate the boundaries of the ventricular zone (VZ) and intermediate zone (IZ) and of the IZ and cortical plate (CP), respectively. A higher magnification of the area pointed to by the arrow in (A) is shown in (F). Scale bars = 100 μ m (A–E), 50 μ m (F).

identify the border between cortical plate and intermediate zone revealed that these radial fibers were running obliquely beneath the subplate and sharply turning towards the marginal zone (Fig. 2G, H; see also Fig. 2A, B). Some migrating cells along these fiber structures were also observed. In the P3 neocortex, neuroblasts migrated almost “tangentially” along the obliquely coursing “radial” fibers beneath the cortical plate, and then turned towards the marginal zone and migrated radially after entering the cortical plate (Fig. 2G, H). Immunostaining of Reelin, which is secreted in the marginal zone and known to control neuronal positioning,^{5,15} showed the tips of the leading processes of the migrating neuroblasts seeming to enter the Reelin-positive region far before their cell bodies reached the marginal zone, while the cell bodies themselves terminated migration just beneath the Reelin-positive region

(Fig. 2I). No EGFP-positive mature neurons were ever found in the postnatal brains that were injected with pEGFP-N1 in embryonic stages. Moreover, all mature neurons (and neuroblasts, if any) in the embryos injected at E15 and fixed at P11 were EGFP negative, and only some cells that exhibited the morphological features of glial cells and settled beneath the cortical plate were positive (Fig. 3A, B). The most likely interpretation of this is silencing of the CMV promoter after the neuroblasts differentiated into mature neurons. The expression pattern of pEFBOS-EGFP in embryonic stages was similar to that pEGFP-N1, but the results obtained with pEFBOS-EGFP on the postnatal days were completely different from those of pEGFP-N1. Figure 4A and B shows the patterns of different sections of a P21 brain that had been injected with pEFBOS-EGFP at E14.5. EGFP expression was clearly maintained at P21, almost

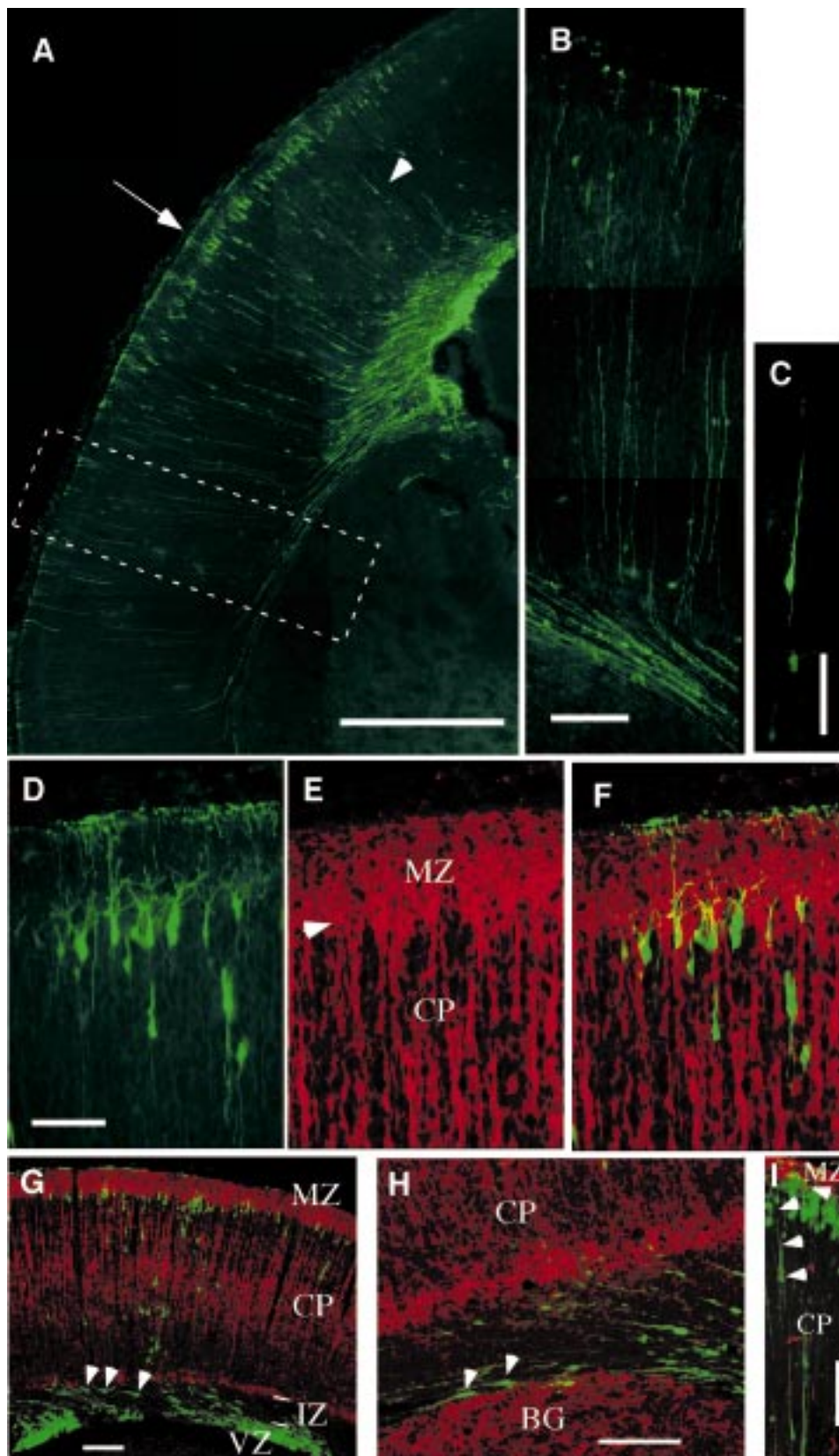


Fig. 2.

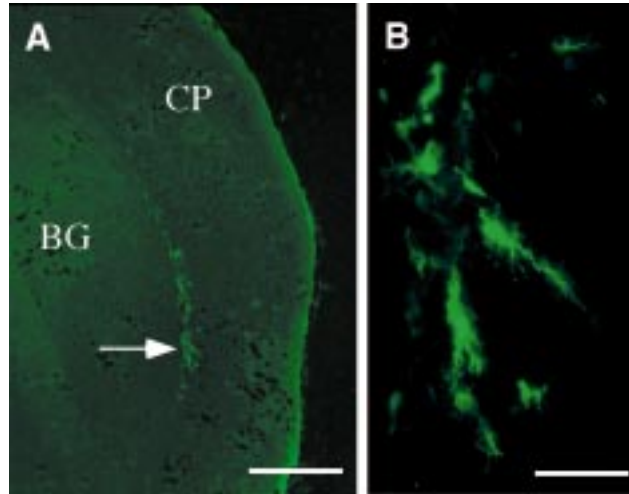


Fig. 3. Fluorescent image of a P11 brain that had been injected with the EGFP-expressing plasmid driven by CMV promoter at E15. (A) No EGFP fluorescent was found in mature cortical neurons. The high-magnification image (B) of the labeled cells indicated by the arrow in A revealed that the EGFP-expressing cells exhibited glial cell morphology. Scale bars = 0.5 mm (A), 50 μ m (B).

one month after transfection. Strong expression of EGFP was found in the NeuN-positive mature neurons (Fig. 4C–E) and the labeled neurons were mainly located in layer II/III of the cerebral cortex, with some in layer IV (Fig. 4A, B, F–H). In the more medial portion of the cortex, the labeled neurons were found in a deeper position (Fig. 4A, B, F–H). This difference probably reflects the neurogenetic gradient in the cerebral cortex.⁵ EGFP fluorescence was seen not only in the soma of the neurons, but in their dendrites and axons. Their apical dendrites were directed towards the marginal zone, and axons arborizing in the deeper layer were visualized clearly (Fig. 4A–H). The commissural fibers running through the corpus callosum (Fig. 4A, B, I) and arborizing in the contralateral cortex (Fig. 4J) were also clearly visualized. When the promoter of the early panneuronal marker, $T\alpha 1$ α -tubulin,^{7,8} was used to drive the EGFP, a similar pattern of callosal fibers was observed at P5 (Fig. 4K).

DISCUSSION

In this study we developed a novel, convenient gene transfer system for use in developing mammalian brains. The ease and high efficiency (>80%) of obtaining living animals with exogenous gene expression should allow

application of this technique to studies of various aspects of brain development. In this report, we described mainly our observations of neuronal migration in the developing cerebral cortex.

The CMV promoter and the EF1 α promoter yielded similar patterns during the first three days after gene transfer (Fig. 1A–E), and they roughly corresponded to the time-course of neuroblast migration revealed by bromodeoxyuridine (BrdU) or ³H thymidine uptake analysis.^{2–4,17} In the P3 neocortex, the labeled neuroblasts migrated almost “tangentially” along the “radial” fibers, and then turned and migrated radially in the cortical plate (Fig. 2A, B, G, H). Using the CMV promoter allowed easy and clear observation of the migrating neuroblasts in the cortical plate, because no mature neurons were labeled at all (Fig. 2A, C–F). The labeled cells with primitive dendrites, which had probably just completed migration and started to differentiate into mature neurons, were only located along the border of the marginal zone and the cortical plate (Fig. 2A, D–F). These findings strongly suggest that the neuroblasts indeed terminate migration when they encounter the Reelin protein in the marginal zone (Fig. 2I), as proposed previously.^{5,15} Interestingly, however, the tips of the leading processes of the migrating neuroblasts seemed to enter the Reelin-positive region far before their cell

Fig. 2. EGFP expression pattern driven by CMV promoter. The brain was injected with pEGFP-N1 plasmid at E15, electroporated and fixed at P3. An overall view is shown in A. The radial fibers enclosed by the dotted line in A are shown at higher magnification in B. The structure of a neuroblast indicated by the arrowhead in A is shown in C. The leading process and the tail process are clearly observed. At this stage, many neuroblasts were aligned immediately beneath the marginal zone. The morphological features of these cells indicated by the arrow in A are shown in D. The distribution of MAP2 revealed by immunohistochemistry on the same section is shown in E. The merged image of D and E is shown in F. The other sections of the same experimental series were immunostained with anti-MAP2 antibody (G, H) or CR-50 (anti-Reelin antibody) (I) followed by TRITC-conjugated anti-mouse IgG. The merged images of EGFP (green) and TRITC (red) are shown in G to I. (G, H) The neuroblasts that have left the ventricular zone (VZ) are migrating almost “tangentially” (arrowhead) along the obliquely running “radial” fibers beneath the cortical plate, which is labeled with anti-MAP2 (red). When the radial fibers enter the cortical plate, they turn towards the marginal zone and run radially in the cortical plate. The neuroblasts migrate radially along these fibers in the cortical plate. BG: basal ganglia. (I) Reelin protein (red), which plays the pivotal role in positioning cortical neurons, is secreted in the marginal zone (MZ). The migrating neuroblasts have a long leading process (arrowhead), which may make contact with the Reelin protein while the cell body is still migrating. The cell bodies terminate migration immediately beneath the marginal zone (arrow). Scale bars = 0.5 mm (A), 100 μ m (B), 50 μ m (C–F), 100 μ m (G–I). BG, basal ganglia; CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone.

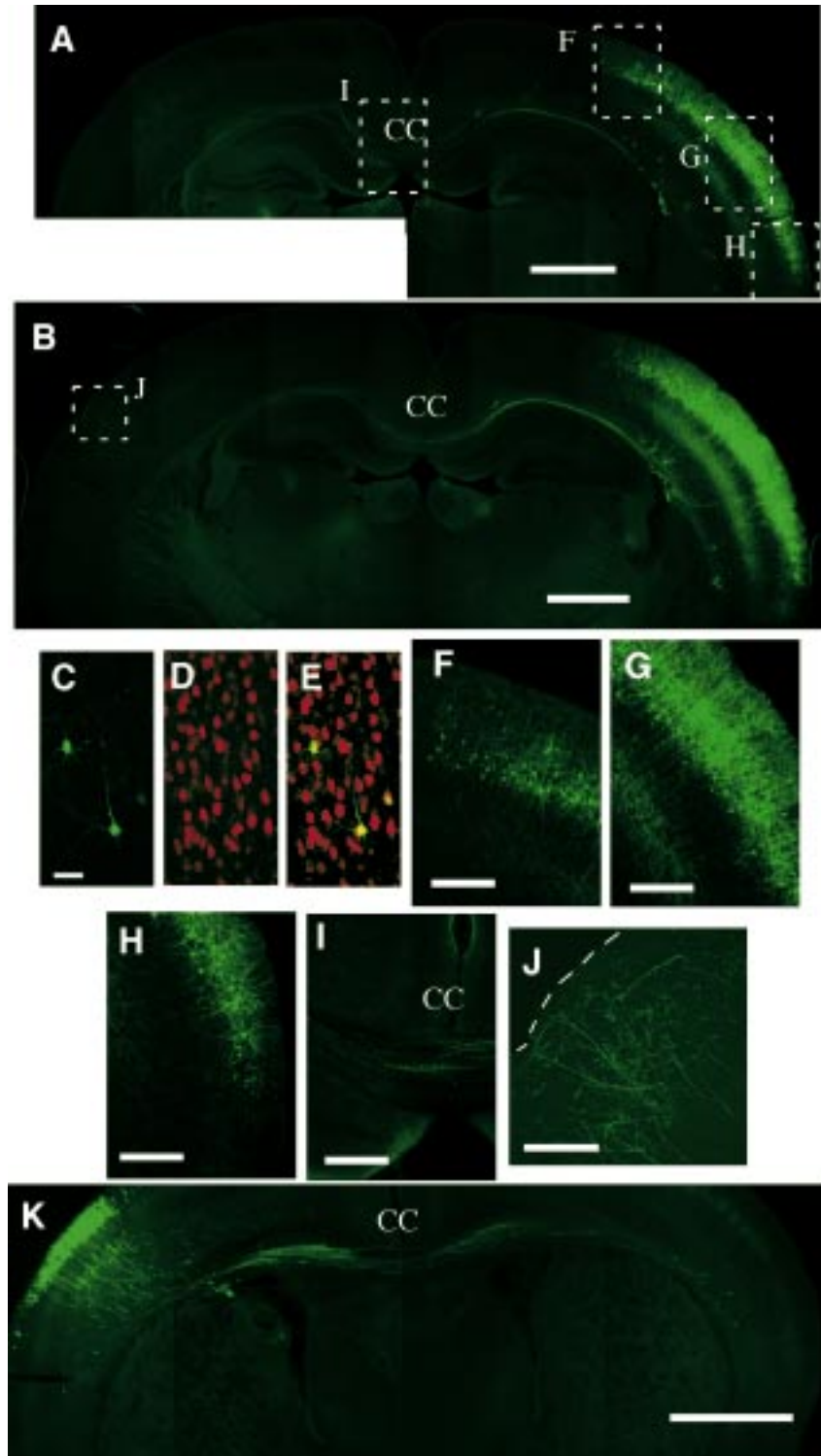


Fig. 4. The EGFP expression pattern in later stages. The fluorescence of EGFP driven by EF1 α promoter remained in the P21 brain, which had been injected with the plasmid at E14.5 (A–J). Overall view of two different levels of the same brain specimen are shown in (A) and (B). (C–E) Confocal images of the brain section immunostained with anti-NeuN followed by TRITC-conjugated anti-mouse IgG. The fluorescence of EGFP (C) and TRITC (D) was observed by a confocal laser microscope. The merged image of C and D is shown in (E). (F–I) Higher magnification images of the boxed regions in A. (J) Higher magnification of the boxed region in B. (K) P5 brain that had been injected with the EGFP expression plasmid driven by T α 1 α -tubulin promoter at E14.5. Scale bars = 1 mm (A, B), 25 μ m (C–E), 250 μ m (F–I), 200 μ m (J), 1 mm (K). CC, corpus callosum.

bodies reached the marginal zone, while the cell bodies themselves completed migration just beneath the Reelin-positive region (Fig. 2I). It is important to clarify whether the Reelin receptors are located on the leading processes to understand the signaling mechanism of Reelin action.

The silencing of the CMV promoter when the cells differentiate into mature neurons is useful for distinguishing migrating neuroblasts from the surrounding mature neurons. The migrating cells labeled with the CMV promoter were probably born within a few days before the date of observation, because cells lose their fluorescence when they stop and differentiate into neurons. This would explain why we found labeled young neurons only immediately beneath the marginal zone and not in the deeper regions of the cortical plate at P3, even though the plasmid was introduced at E15 (Fig. 2D–F, I). By contrast, the cells labeled with the EF1 α promoter should visualize cells born at the time of gene transfer. When the EF1 α plasmid was introduced at E14.5, the mature labeled neurons were located mainly in layer II/III of the P21 cerebral cortex, with some in layer IV (Fig. 4A, B, F–H). Previous BrdU or ³H-thymidin uptake analysis showed that the neurons born at E14.5 were mainly distributed in layer IV,^{2,4,17} suggesting that our electroporation method labels cells that are born slightly later than those labeled with BrdU or ³H-thymidin. This discrepancy may at least in part be attributable to the difference in labeling mechanisms. Although BrdU and ³H-thymidin label only the cells in the S phase of the cell cycle, electroporation should introduce genes irrespective of the phase of the cycle. In other words, BrdU and ³H-thymidin uptake analyses indicate only the population that was in the S phase prior to the final mitosis, whereas the electroporation method may label all cells in any phase of cell cycle. Since the G1 phase is much longer than the G2 phase,¹⁷ the cells labeled by the latter method probably represent cells in a later phase of the cell cycle than those labeled with BrdU or ³H-thymidin.

Finally, the clear observation of expression of the transfected exogenous gene as long as one month after transfection was an unexpected finding (Fig. 4: transfected at E14.5 and examined at P21). Although it was difficult to estimate the approximate lifetime of the plasmid in the transfected cells, the long duration of expression may mean that some of the DNA was integrated into the host cell chromosome.

CONCLUSIONS

This novel electroporation system should be very useful for gain-of-function analysis of genes involved in late developmental events, including determination of cell fate, neuronal migration, positioning and axon guidance in mouse embryos. The great advantage of this system is its simplicity. Multiple constructs of several genes can be easily examined *in vivo*. Moreover, EGFP-expressing plasmid vectors allow visualization of the entire morphology and targeting pattern of each neuron. Hence, this system should help in analysing mutant mouse phenotypes. It also enables the investigation of the morphological changes resulting from over-expression of a particular gene by using a co-expressing vector of EGFP and the gene of interest. This system should greatly contribute to the study of the molecular mechanisms involved in late-stage CNS development.

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