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# Cre-Mediated Cerebellum- and Hippocampus-Restricted Gene Mutation in Mouse Brain

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**Using the phage P1-drived Cre/loxP recombination system, we have created a line of cre-transgenic mice in which the Cre-mediated gene deletion is restricted to granule cells of cerebellum and dentate gyrus of hippocampus. Low levels of deletion were also present in pyramidal cells of hippocampal CA1 and CA3 fields. The Cre/loxP recombination occurred prenatally. The recombination efficiencies in the granular layer of the cerebellum, the granular layer of the dentate gyrus, and the CA1 and CA3 pyramidal cells of the hippocampus were 34.0%, 23.1%, 3.0%, and 9.8%, respectively. This line of cre-transgenic mice should be conducive to studies of the effect of a gene mutation upon brain development and plasticity.** © 2000 Academic Press

***Key Words:*** **cerebellum; hippocampus; Cre/loxP; transgenic mice; CA1; CA3; granule cells; dentate gyrus; Emx1; β-galactosidase.**

Gene targeting in embryonic stem cells allows the production of mice containing a deletion in a predefined gene of interest, which is also called gene knockout technology (1). Conventional gene knockout techniques produce animals that inherit genetic mutations in all cell types. This regionally and temporally unrestricted genetic manipulation may lead to severe developmental defects or premature death (2), which can preclude analysis of post-developmental functions. To enhance the utility of gene knockout technology, it is important to develop techniques that impose regional and/or temporal restrictions, to genetic deletions, One method to accomplish cell type- or tissue type- restricted gene knockout is to exploit the Cre/loxP system, a phage P1-derived site-specific recombination system in which the Cre recombinase catalyzes recombination between 34 bp loxP recognition sequences (3). The Cre/loxP system has been applied successfully *in vivo* to delete the mouse DNA polymerase β gene (4, 5), a N-acetylgalactosaminyltransferase gene (6), and the NR1 gene (7). From these studies, it is clear that the key to the cell type-specific gene inactivation is the creation of the cre transgenic mice, which express the cre gene in a cell type-specific manner. However, very few cre transgenic lines are available to inactivate genes in specific regions of the brain. To alleviate the problem, we have initiated a study to characterize the putative promoters derived from a homeobox gene, the Emx1 gene, which specifically expresses in the developing brain.

Emx1 is a mouse homologue of the Drosophila homeobox gene *empty spiracles*. Its expression is exclusively confined to the dorsal telencephalon (8). Its expression begins around embryonic day 9.5 (E9.5) and occurs in virtually every neuron of the developing embryonic and postnatal cerebral cortex. Emx1 is expressed throughout the period of neurogenesis for the murine cerebral cortex, which commences around E10 and ends around E18. Regionally, not only in the cells in the ventricular zone is Emx1 expressed, but also in post-mitotic neurons that will later form different layers of the adult cortex. No expression of the Emx1 gene has been detected in glial cells (9). Taken together, the promoter of the Emx1 gene, if it could be characterized, would be extremely useful for directing gene manipulation to the developing cerebral cortex.

We report here the creation of lines of cre transgenic mice using the upstream DNA fragments from the Emx1 gene and the analysis of their potential to delete genes in the mouse brain. Although we did not succeed in creating cre mice mimicking endogenous Emx1 gene expression, one of the lines we have created could induce cre-mediated gene recombination restricted to the cerebellum and hippocampus.

## MATERIALS AND METHODS

*Transgenic construct and production of transgenic mice.* Oligonucleotides (5′-GTCTCGGAGAGGCTGAGGCTGCCTGCCAGCTG-3′; 5′-CAGCACCGGGACCCTCTCCATTTCTACCCCTG-3′) were used to amplify Emx1 genomic DNA using PCR amplification from a

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mouse-tail DNA preparation derived from a wild type mouse (with a mixed background of C57BL6, BALB/c and 129/Sv). Cloning of the amplified fragment and the determination of its boundary sequences were done according to standard procedures (10).

Library screen, subcloning, mapping and construction of plasmids were performed essentially as described previously (11). Briefly, a 15-kb SalI fragment from a positive phage was cloned into Bluescript plasmid (Stratagene) and mapped by restriction digestion and PCR. The 11-kb SalI–NotI fragment was linked directly using a synthetic linker (5′-GGCCGCGACCATGGTCGC-3′) to a nuclear-localized lacZ coding region derived from the plasmid lacF′ (12). The lacZ coding region was linked to an internal ribosomal entry site (IRES; 13) followed by a nuclear localized Cre coding region. PCR-mediated mutation of the cre was carried out essentially as described (14) to incorporate signal sequences optimized for eukaryotic translational initiation and nuclear localization using a synthetic primer (5′-ATTACCATGGGACCAAAGAAGAAGAGAAAAGTGTCCAATTTACTGACC-3′). SV40 poly(A) was derived from pMSG plasmid (Pharmacia). The sequences of the mutated Cre-coding region were confirmed by sequencing. The final plasmid was purified by CsCl ultra centrifugation. The DNA insert (about 17 kb) was released using restriction digestion and purified by gel electrophoresis. The isolated DNA was diluted to about 2–4 ng/$\mu$l and then injected into fertilized eggs prepared from FVB mice. The injection was performed at the Biotechnology Center of the University of Illinois at Urbana-Champaign directed by Dr. Matt Wheeler.

*Reporter mice.* LoxPlacZ reporter mice (7) were kindly provided to us before publication by Dr. David Anderson of California Institute of Technology.

*Genotyping of transgenic mice.* Genotypes of all offspring were analyzed by either PCR or Southern blot as described (11). Founder mice were identified using PCR with two pairs of the primers. One pair of the primers would amplify a fragment from the wild type NMDAR1 gene (11) to serve as an internal control. The other pair of primers (5′-CTGATGAATGGGAGCAGTGGTGGAA-3, 5′-CTGATGAATGGGAGCAGTGGTGGAA-3′) would produce an amplified fragment from the SV40 poly(A) sequence in the transgene. Later PCR analysis was based on a different pair of primers specific for cre gene (5′-CCCGATATCATTTACGCGTTAATG-3′ and 5′-CACTCATGGAAAATAGCGATC-3′). The primers for the loxPlacZ reporter mice were 5′-CTGATGAATGGGAGCAGTGGTGGAA-3′ and 5′-GCTTACTTACCATTGTCAGATC-3′, respectively. Mouse tail DNA was amplified 30 cycles (60 s, 94°C; 45 s, 55°C; and 60 s, 72°C). For Southern blot analysis, tail DNA samples were digested with XbaI and the blotted DNA were hybridized with a probe derived from the lacZ-coding region.

*Histology.* Staining for the lacZ activity was done essentially according to the standard protocol using either whole mount or brain sections (15). Briefly, transgenic and wild type mice at various ages (postnatal day 0 = P0, P3, P6, P10, P15, P28, and P49) were perfused with ice-cold PBS buffer followed by 4% paraformaldehyde in PBS. The brains were dissected out and post-fixed for 15 min. They were then washed 3 times in a rinse solution comprised of 0.1 M phosphate buffer, pH 7.3, 2 mM $MgCl_2$, 0.01% sodium deoxycholate and 0.02% NP-40 (IGEPAL CA-630, Sigma Chemical Company). The brains were then left overnight at 37°C in X-gal staining solution: 1 mg/ml X-gal, 4 mM $K_4Fe(CN)_6$, 4 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$ in 0.1 M phosphate buffer, pH 7.5. Stained brains were washed twice with PBS, cryoprotected in 30% sucrose overnight, and embedded. Cryostat sections (20 $\mu$m) were prepared and post-fixed for 10 min in 0.2% paraformaldehyde in PBS buffer (pH 7.5). The fixed sections were washed with ice-cold PBS and stained with the X-gal staining solution overnight for up to 20 h at 37°C. The X-gal-stained slides were then washed with PBS twice and counterstained with 0.1% Pyronin Y (Nissl staining).

*Determination of Cre-mediated recombination efficiency.* Brain sections (20 $\mu$m) that were double-stained by X-gal and Pyronin Y were examined under an Olympus BH-2 microscope equipped with a motorized stage and the number of neurons was counted using Stereo Investigator Confocal 3.18 software (MicroBrightField, Inc., USA). In the hippocampus, all of the neurons in CA1 and CA3 fields were counted while in dentate gyrus and cerebellum, and the whole granular layer was divided into adjacent grids (each grid is 200 $\mu$m × 200 $\mu$m) and counted with the aid of the software so that in every grid, all of the neurons in a 100 $\mu$m × 100 $\mu$m counting frame in the upper left corner of the grid were counted. The percentage of deletion was calculated from the number of lacZ-positive neurons and the total number of neurons in each specific brain sub-region. The data were obtained from three P28 tg5cre/loxPlacZ double-transgenic (tg5cre+/−loxPlacZ+/−) mice, 13–18 sections per animal.

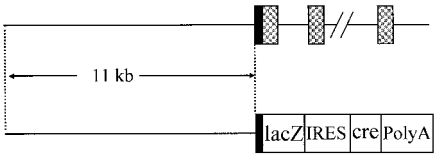


**FIG. 1.** Genomic organization of the Emx1 gene (top) and the transgenic construct (bottom). Filled boxes denote exon sequences of the Emx1 gene. LacZ: $\beta$-galactosidase with a nuclear localization signal. PolyA: SV 40 polyA sequence.

## RESULTS

*Generation of Emx1-cre-transgenic mice.* According to the published partial cDNA sequences of mouse and human Emx1 genes (8), we designed synthetic primers and amplified mouse Emx1 genomic DNA using PCR amplification from a tail DNA preparation. One of the two primers is located within the conserved homeodomain and the other is based on a highly conserved sequence (RDXXXXYPW) between the Emx and Otx genes located immediately before the homeodomain. The amplified fragment was cloned and its boundary sequence was determined. Sequence analysis of the genomic DNA clones indicated that clone 34 contained the correct ends and therefore clone 34 was used for further study (data not shown).

Using the cloned genomic DNA as a probe, we screened a mouse 129/Sv genomic library made from D3 ES cells (11). We have isolated 12 λDNA clones, which cover exons 1 and 2 as determined by sequence analysis. Mapping of these clones and PCR analysis indicated that the Emx1 gene contains three exons and two introns. Restriction mapping and sequencing results demonstrated that one of the λDNA clones we isolated contained about 11 kb of the genomic sequence upstream of the putative ATG translational start site. This 11-kb fragment was fused to a lacZ-coding region containing a nuclear localization signal (12) followed by an internal ribosomal entry site and Cre-coding region, and ended with a SV40 polyA sequence (Fig. 1). The presence of IRES would ensure the efficient translation of bicistronic mRNAs in mammalian cells, leading to the production of both $\beta$-galactosidase and Cre protein (13, 16). We have also mutated cre to incorpo-

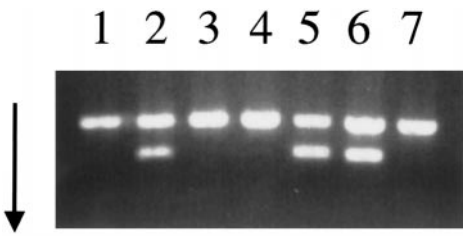


**FIG. 2.** PCR analysis of transgenic founder mice made from the construct shown in Fig. 1. Lanes 1–7 contain PCR samples of tail DNA. The top band (550 bp) was generated with a pair of primers specific for the NR1 wild-type gene (11). This serves as an internal control for the PCR reaction. The bottom band (450 bp) was from a pair of primers specific for the SV 40 polyA sequence, which is part of the transgenic construct. The vertical arrow indicates the direction of electrophoresis.

rate the consensus eukaryotic translational initiation signal (17) and a nuclear localization signal (18).

Next, we tested whether the 11-kb fragment contained the necessary control elements to direct gene expression in the cerebral cortex. The DNA fragment containing the construct was injected into the fertilized eggs to produce transgenic mice. The injection generated 3 founder mice (Fig. 2) among the 31 pups weaned. All of the founder mice transmitted to germline. Staining results for the lacZ activity using X-gal, a substrate for $\beta$-galactosidase, indicated that all of these lines have a small number of neurons stained blue in the brain. Blue staining is restricted to the nucleus because of the nuclear localization signal we used. However, only one of the three lines (line 6; tg6) we have tested had a moderate level of blue staining in the cerebral cortex; the other two lines had scattered blue staining outside the cerebral cortex. Staining of the E12.5 embryo derived from line 2 (tg2) revealed two symmetrical strips of neurons stained in the hindbrain. At 4 weeks of age, the lacZ expression could be detected in the pontine nuclei and in the cochlear nucleus (data not shown). In 3-week-old animals, the lacZ expression of the tg6 line was restricted to the cerebral cortex and certain part of the hippocampus, consistent with the expression pattern of Emx1 in adult animals (9). However, only a small fraction of neurons in the cortex and hippocampus showed blue staining (data not shown). Animals derived from transgenic line 5 (tg5) had only a few lacZ-positive neurons from an entire section. Taken together, none of the transgenic mice derived from these lines displayed an X-gal staining pattern similar to the endogenous expression pattern of the Emx1 gene. These results suggest that there is a core promoter inside the 11 kb of the Emx1 sequence, and that the translation frame in which we fused the lacZ was correct. However, from the limited analysis above, it appeared that the 11-kb sequence does not contain the regulatory sequences responsible for the correct spatial and temporal expression of the Emx1 gene.

*Cre-mediated recombination in cerebellum and hippocampus from tg5 line.* The lacZ expression study conducted above was useful for determining whether the transgenic animals mimicked endogenous expression patterns of the Emx1 gene. However, it was not useful to define the actual deletion specificity of the cre transgene, since transient expression of the cre gene during the development could change deletion specificity as long as the level of cre expression achieved a sufficiently high level to mediate recombination. Recently, reporter mice have been used extensively to define the tissue and cell specificity of the deletion (7, 19, 20, 21). We crossed the cre transgenic mice with lacZ transgenic report mice (loxPlacZ+/−; 7) and analyzed whole brain and brain sections of the cre/loxPlacZ, cre+/−, loxPlacZ+/−, and wild type mice using X-gal staining. For animals derived from tg2 and tg6 lines, the distributions of the lacZ-positive cells in double transgenic mice were essentially the same as their original single cre transgenic mice, indicating that no additional deletions occurred inside the brain. However, for brains derived from the tg5 line, the staining pattern for the double transgenic brains was dramatically different from that of the tg5cre+/− mice. Blue staining could be detected in the cerebellum and the hippocampus in the double transgenic mice and these blue staining were not found in brains of tg5cre+/− mice, loxPlacZ+/− mice, or wild type mice after whole brain X-gal staining (Fig. 3A, data not shown for wild type).

In brain sections stained for $\beta$-galactosidase activity, Cre-mediated recombination mainly occurred in the granular layer of the cerebellum and the granular layer of the dentate gyrus with a deletion efficiency of 34.0 $\pm$ 9.8% and 23.1 $\pm$ 8.6%, respectively. A low level of recombination happened in CA3 (9.8 $\pm$ 2.4%) and CA1 (3.0 $\pm$ 1.0%) pyramidal cell layers of the hippocampus (Fig. 3B). The distribution of the blue cells was not uniform across the lateral medial axis of the cerebellum; higher percentages of the deletion were observed near the lateral parts of the cerebellum (Fig. 3C). No blue cell was found in sections of the wild type, tg5cre+/−, and loxPlacZ+/− mice after X-gal and Pyronin Y staining.

*Cre-mediated recombination occurred as early as P0 in the cerebellum.* To determine the time course of the tg5cre-mediated deletion, we examined the Cre-mediated recombination in double transgenic mice at various ages (P0, P3, P6, P10, P15, P28, P49) using X-gal staining. At P0, blue staining could be detected using whole brain staining. The staining was restricted to the developing cerebellum and the hippocampus (Fig. 3D). In stained sections, blue cells could be seen in the external germinal layer (EGL), the inner cell zone beneath the EGL in the cerebellum (Fig. 3E), the granular layer in the dentate gyrus, and the CA1 and CA3 pyramidal layers in the hippocampus (Fig. 3F). Scat-

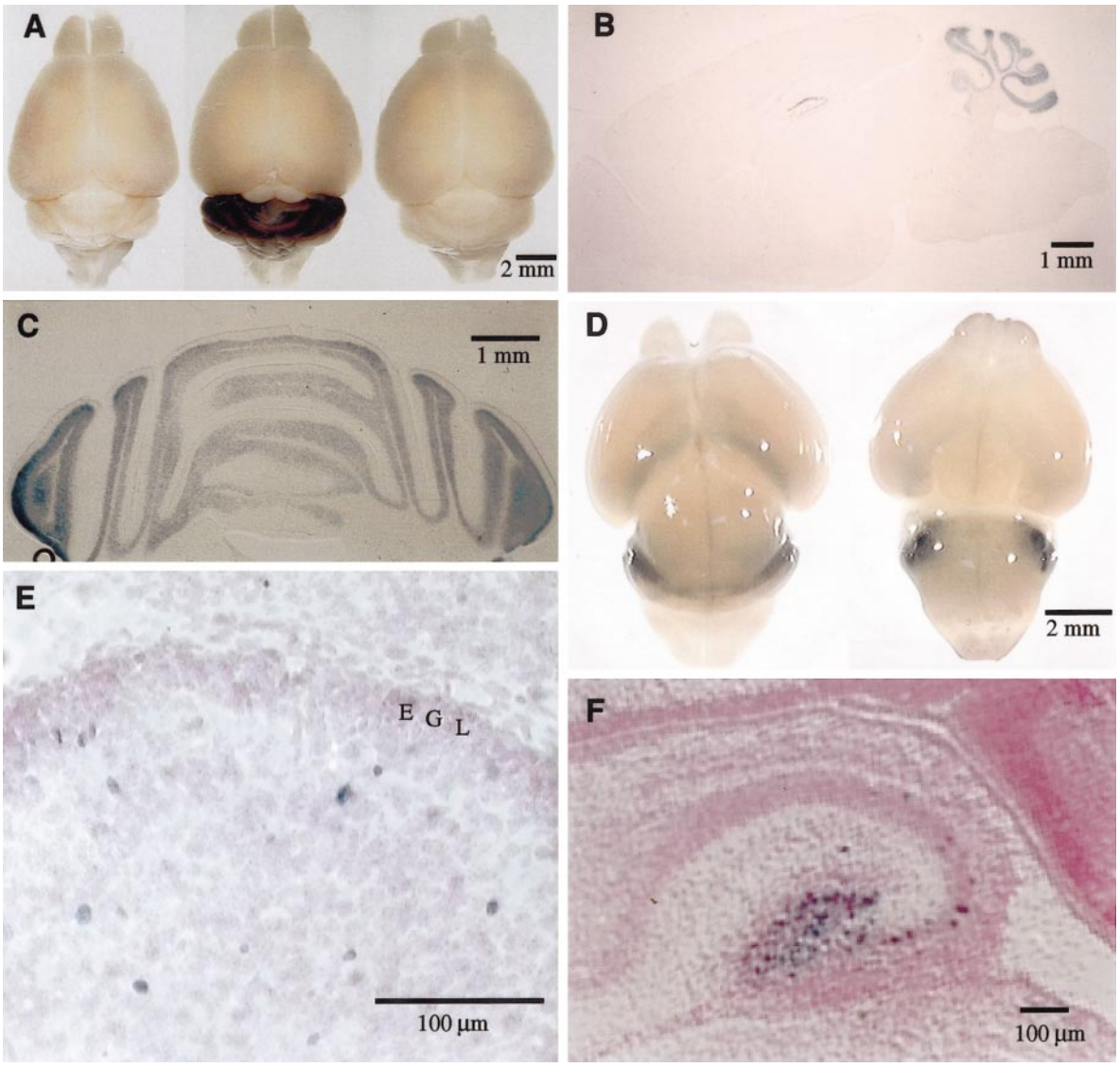


**FIG. 3.** Cre/loxP recombination as detected by X-gal staining in whole brains and brain sections from 4-week-old mice. (A) High β-galactosidase activity could be detected in the cerebellum of the tg5cre+/−loxPlacZ+/− mouse brain (middle). No blue staining was found in brains from tg5cre+/− (left) and loxPlacZ+/− (Right) mice. (B) X-gal-stained sagittal section from a tg5cre+/−loxPlacZ+/− mouse. Blue cells were present in granular layers of cerebellum and dentate gyrus, and pyramidal cell layers of hippocampus. (C) X-gal-stained coronal section from a tg5cre+/−loxPlacZ+/− brain. The distribution of the Cre-mediated recombination was not uniform across the lateral medial axis of the cerebellum of tg5cre+/−loxPlacZ+/− mice. High levels of recombination were evident on the lateral side of the cerebellum. Dorsal is up. Cre/loxP recombination at P0. (D) Dorsal (left) and ventral (right) views of a P0 tg5cre+/−loxPlacZ+/− brain stained with X-gal. LacZ-positive cells could be detected in developing cerebellum and hippocampus. (E, F) Sagittal sections from a tg5cre+/−loxPlacZ brain. The sections were stained with X-gal and counterstained with Pyronin Y. (E) Blue cells were present in the external germinal layer (EGL) and in differentiating neurons beneath the EGL. Dorsal (outer EGL) is up. (F) LacZ-positive cells in the granular layer of the dentate gyrus, CA1 and CA3 pyramidal layers of the hippocampus. Dorsal is up and anterior is right.

tered blue cells could also be found in the brainstem at P0, P3, and P6 (data not shown). However, the majority of these blue cells disappeared when the animals were older than P10. Starting at P6 and through P15, although stained cells appeared in the molecular layer and EGL of the cerebellum, most of the lacZ-positive cells were in the granular layer. At P28 and P49 stained cells were only detected in the granular layer within the cerebellum. No staining was detected in wild type, loxPlacZ+/− and tg5cre+/− animals at all ages examined (n = 3 for each age group).

## DISCUSSION

We have cloned the mouse Emx1 gene and used its 11 kb upstream sequence to produce transgenic animals bearing a cre gene that is useful for conditional mutagenesis in mice. Although none of the transgenic

lines produced faithfully replicated endogenous expression of the Emx1 gene, one of the line produced, tg5, did show an interesting deletion pattern restricted to the developing and adult cerebellum and hippocampus, two brain regions prominent for development and plasticity studies in recent years. In both the hippocampus and cerebellum, the Cre-mediated deletion could be detected as early as P0. Since no blue cells were detected in both the tg5cre+/− and the loxPlacZ+/− mice at P0, the actual deletion time could be much earlier, i.e., cre could be expressed transiently during prenatal development. The tissue- and cell type-specificity of tg5cre+/− mice probably is related to its insertion site.

It is known that granule cells initially are generated from the EGL and later, migrate through the molecular layer and finally settle in the granular layer (22). As described in the Results section, at P6, P10, and P15 stained cells appeared in the granular and molecular layers, and the EGL but at P28 and P49 stained cells were present only in the granular layer of the cerebellum. These results suggest that the stained cells in the EGL and molecular layer in tg5cre/loxPlacZ double-transgenic mice should be differentiating and migrating granule cells.

Cre-mediated recombination occurred mainly in granule cells in tg5cre+/− loxPlacZ+/− double-transgenic mice. In some lateral lobule areas of the cerebellum, the percentage of X-gal stained granule cells was as high as 85%. Therefore, the tg5cre mice could be used to develop animal models to study the development and function of the granule cells *in vivo.* More specifically, since Cre-mediated recombination could be seen in the EGL as early as P0, the tg5cre mouse could be used to study the migration of granule cells or the effect of gene mutation on the migration of granule cells. The cell that underwent cre-mediated recombination could be conveniently marked by the activity of lacZ or use other reporter lines such as reporter mice utilizing green florescent protein.

Although in some lobule areas the percentage of X-gal stained cells was high, the overall percentages of X-gal stained cells to total granule cells were 34.0% in the cerebellum and 23.1% in the dentate gyrus. The low, overall percentage of deletion was probably not due to the inhomogeneous expression of the transgenic loxPlacZ promoter, since we have obtained two additional types of reporter mice (19, 20) recently, and the results of crossing tg5cre with these reporter mice were consistent with the data presented here (H. G., Y.-T. T. and Y. L., unpublished results).

We obtained tg5cre transgenic mice serendipitously when we tried to create transgenic mice that mimic the endogenous expression of the Emx1 gene promoter. The deletion patterns of all the transgenic mice generated did not match the endogenous expression pattern of the Emx1 gene, indicating that the 11-kb sequence located upstream of the Emx1 gene does not contain the regulatory sequences responsible for the correct spatial and temporal expression of the Emx1 gene. A knock-in approach (23) would probably lead to a line of cre transgenic mice with deletion specificity that matches the expression pattern of the Emx1 gene.

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