genesis 32:209–217 (2002)

ARTICLE

Cre/*loxP* Recombination-Activated Neuronal Markers in Mouse Neocortex and Hippocampus

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Received 12 October 2001; Accepted 31 December 2001

A new strategy for visualizing neuronal cell morphology of mouse brain based on Cre/*loxP* recombination-activated gene expression is described. A "reporter" transgenic line was generated which expressed a fusion gene encoding a dendrite-targeted green fluorescent protein (MAP2-GFP) upon deletion of a transcription/translation STOP (transcription and translation stop signal) cassette. Cre transgenic "deleter" lines were established that activated reporter gene expression at various frequencies in pyramidal neurons in the forebrain. A deleter line was identified which activated a MAP2-GFP reporter gene at very low frequency (less than 0.1% of pyramidal neurons) and allowed the visualization of dendritic structures of individual neocortical and hippocampal pyramidal neurons. In addition, vertical "columns" of pyramidal neurons in the neocortex were labeled in these mice. In a second deleter line, a MAP2-GFP reporter gene was selectively activated in pyramidal neurons of the CA-1 subregion of the hippocampus in young mice. With its combinatorial property, this binary recombination-activated neuronal marker system should facilitate the study of detailed morphology, connectivity, and plasticity of defined classes of live neurons in vitro and in vivo. genesis 32:209–217, 2002. © 2002 Wiley-Liss, Inc.

Key words: Cre/*lox*P; recombination; neocortex; green fluorescent protein (GFP)

The enormous complexity of the neocortex is derived from hundreds of neuronal cell types and extensive synaptic connections between them. Studies of the development and function of the neocortex would be facilitated by the availability of techniques that permit visualization of detailed morphology of individual or defined class of live neurons in vitro and in vivo. Traditionally, intracellular injection of fluorescent dyes has been used to visualize cortical neurons at high resolution (e.g., Markram *et al.*, 1997). However, this procedure is technically demanding and laborious. More recently, ballistic gene transfer (e.g., of green fluorescent proteins) was shown to efficiently transfect 1–10% of cortical neurons in brain slices (Dunaevsky *et al.*, 1999; Lo *et al.*, 1994). But this technique can only be applied to young brain slices and is not selective for specific classes neurons. A variety of viral vectors have been used to introduce marker genes into neurons both in brain slice and in vivo (Moriyoshi *et al.*, 1996; Okada *et al.*, 1999). However, viral infections do not distinguish specific neuronal cell types and often lead to cellular damage and toxicity. In addition, most viral promoters lose their activity several days after the infection of neurons and therefore preclude their application for the study of a prolonged developmental process.

Transgenic expression of GFP-based markers provides another promising approach for labeling neocortical neurons in vivo. In the conventional transgenic approach, strong transcriptional promoters are employed to drive high levels of expression of a marker gene. In one striking example, transgenic lines were identified that expressed GFP markers in a small subset of motorneurons of the sternomastoid muscle and in a subset of retinal ganglion cells and neocortical neurons (Feng *et al.*, 2000). In most cases, however, such strong promoters tend to be active broadly in a given brain tissue and therefore preclude the study of the detailed morphology of single neurons or defined neuronal cell types in the central nervous system (Feng *et al.*, 2000; Okabe *et al.*, 1997). On the other hand, the activity of cell-type specific promoters may be weak and regulated developmentally and physiologically, leading to variable levels of marker gene expression. To overcome these limitations,

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Grant sponsor: National Institutes of Health, Grant number: NIHMH58880 (to ST).

DOI: 10.1002/gene.10054

we incorporated Cre/*loxP* recombination-activated gene expression (Lakso *et al.*, 1992; Zinyk *et al.*, 1998) into the transgenic approach to develop a binary neuronal marker system. In this system, the level of reporter gene expression is expected to be high due to the use of a strong and ubiquitous promoter, and yet it is restricted to certain cell types because of the specificity of Cre/*loxP* recombination. Furthermore, the proportion of labeled cells within a given cell type can be kept low by a judicious choice of the Cre transgenic line. This would allow labeling of individual neurons in the background of unlabeled cells. Thus, this method may allow marker gene expression at high cellular concentration and in defined cell types and cell density. Such a binary system should be generally useful in studying the detailed morphology, connectivity, and plasticity of defined classes of neurons in vitro and in vivo.

RESULTS

Low-Frequency Cre/*loxP* Recombination-Activated Gene Expression in the Forebrain

The strategy of Cre/*loxP* recombination-activated gene expression has previously been described (Lakso *et al.*, 1992) (Fig. 1). Briefly, in a transgenic "reporter" line the expression of a reporter gene is driven by a strong and ubiquitous promoter but is blocked by the presence of a transcription/translation STOP sequence inserted between the promoter and the reporter gene. Crossing of this reporter transgenic line with a Cre transgenic "deleter" line removed the STOP-mediated blockade of the reporter gene expression in cells in which the Cre transgene was expressed. The distribution and frequency of the reporter-positive cell was dictated by, among other parameters, the levels of the expression of the Cre transgene. In order to apply this strategy to the visualization of neuronal dendrites of single neurons in living brain tissue, we set out to: 1) generate reporter mice expressing a dendrite-targeted GFP marker, and 2) establish deleter lines that give rise to low-frequency recombination in the brain so that the detailed dendritic structures of individual neurons can be resolved.

We have previously reported low-frequency Cre/*loxP* recombination in the neocortex of the αCaMKII promoter-Cre (CaMKII-Cre) deleter lines that had been crossed with a nucleus-targeted lacZ gene reporter line (Tsien *et al.*, 1996). The recombination frequency in the forebrain varied among different CaMKII-Cre transgenic lines and increased significantly with age (Huang and Tonegawa, unpubl. data; Tsien *et al.*, 1996). This variation of recombination frequency most likely reflected the variation of the level and distribution of Cre expression in different transgenic lines due to the different genomic integration loci of the CaMKII-Cre transgene. In order to establish more deleter lines with low-frequency recombination in the brain, we further explored this integration site-dependent recombination pattern by generating additional deleter lines using the same CaMKII-Cre transgenic construct (Fig. 1A). One of 12 new transgenic lines characterized, M34, was chosen for this study.

When bred to the nls-lacZ reporter line (Zinyk *et al.*, 1998), M34 activated low-frequency recombination in the neocortex, hippocampus, and striatum. Recombination-positive neurons detected by their blue nuclei after X-gal staining were scattered and well separated in the neocortex and hippocampus (Fig. 2A,B). In the neocortex, recombination-positive neurons were found in all cortical areas including visual, somatosensory, auditory, motor, and frontal cortex. To estimate the recombination frequency in young mice (4–6 weeks of age), we counted the numbers of X-gal-stained and Nissl-stained neurons in 20-μm thick brain sections treated with both dyes. The overall frequency of X-gal-stained neurons in the neocortex was less than 0.1%. An accurate quantification is difficult since variations among different sections and among different cortical areas in the same section was rather large. In the hippocampus, the frequency was approximately 1.9% of the pyramidal neurons in CA1 region (20 lacZ positive neurons out of 1,014 total neurons counted in four sagittal sections from two mice), 0.9% of the pyramidal neurons in the CA3 region (11 lacZ positive neurons out of 1,168 total neurons counted in four sagittal sections from two mice), and 0.7% of granule cells in the dentate gyrus (eight lacZ-positive neurons out of 1,078 total neurons counted in four sagittal sections from two mice). Recombination did not occur in other brain regions, including thalamus, brain stem, and cerebellum (data not shown). The low frequency of X-gal-stained cells is not due to a spontaneous excision of the STOP cassette in the reporter gene construct because no stained cells were detected in the nls-lacZ reporter mice in the absence of the CaMKII-Cre transgene (n = 4 mice). The frequency of X-gal-stained cells increased with age. At 7 months of age the frequency of stained cells in the forebrain was approximately 2–3-fold higher than those in the 4–6-week-old animals. However, even at 7 months the frequency of stained cells in the neocortex was still sufficiently low so that they were still well separated. Therefore, this M34 transgenic line should be useful to activate dendritic or other neurite markers to visualize the detailed morphology of individual neocortical and hippocampal neurons.

Neocortical "Columns" Revealed by Cre/*loxP* Recombination in M34

Interestingly, a substantial proportion of X-gal-stained cells in the M34/nls-lacZ mice appeared in clusters or "columns" in the neocortex (Fig. 2C). These columns usually spanned all layers of the cortex and were 50–100 μm in width. Within each column, the number of labeled neurons varied from a dozen to over 50 in a 50-μm thick section. There were usually a dozen or more such columns in a given animal. These columns were found in the visual, somatosensory, motor, and frontal cortex in both hemispheres (Fig. 2C–I and data not shown). The occurrence of such a columnar pattern of recombination

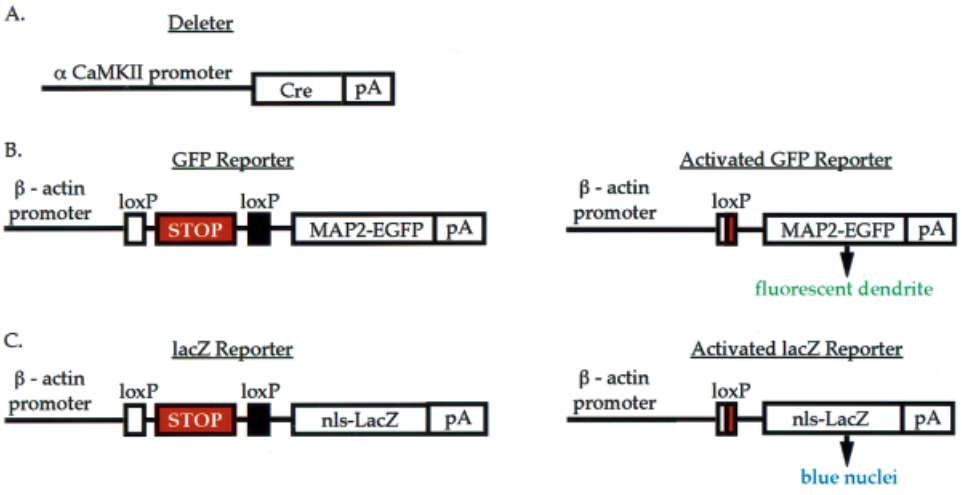

FIG. 1. Schematics of DNA constructs used for generating transgenic mice. **A**: In the deleter transgenic construct, Cre is linked to the αCaMKII promoter (Tsien *et al.*, 1996) and a polyadenylation signal (pA). **B**: MAP2-GFP reporter construct (left) and its activated form (right). STOP: transcription and translation stop signal. **C**: Nuclear lacZ reporter (left) and its activated form (right) (Zinyk *et al.*, 1998).

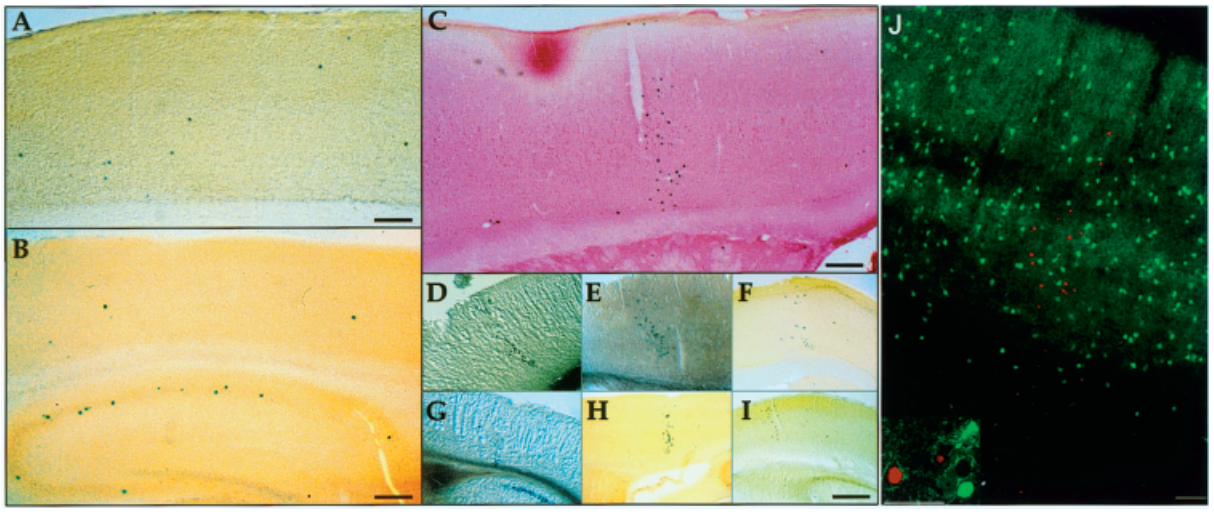

FIG. 2. nls-LacZ reporter expression in neocortex activated by the deleter line M34. **A,B**: X-gal-stained sagittal brain sections from 5-week-old mice showing low-frequency recombination in neocortex and hippocampus. **C**: A column of cortical neurons labeled by Cre/*loxP* recombination-activated nls-lacZ expression. This section was double-stained with X-gal and nuclear fast red. **D–F**: Nls-lacZ labeled columns in the frontal cortex in three different animals. **G–I**: Nls-lacZ labeled columns in the somatosensory cortex in three different animals. The rough appearance of tissues in **D** and **G** were fixation artifacts. **J**: Nls-lacZ positive neurons were pyramidal neurons. The section was double-labeled with antibodies against β-gal (red) and parvalbumin (green) and imaged with laser scanning confocal microscopy. No overlap between the two cell populations was detected. Inset: The pyramidal shaped soma of a β-gal positive nuclei (red) was revealed by parvalbumin positive GABAergic boutons (green). Thickness of brain sections: **D,E,G**: 100 μm; all others: 50 μm. Scale bars: **A–C**: 100 μm; **D–I**: 240 μm; **J**: 60 μm, inset: 40 μm.

was quite reproducible, since we observed them in 10 out of 12 double transgenic mice studied.

Are the recombination-positive neurons in these columns pyramidal neurons or GABAergic interneurons? In the double-labeling experiment, there was no overlap between recombination-positive neurons revealed by antibodies against β-gal and GABAergic interneurons detected by antibodies to parvalbumin (Fig. 2J) or GABA (data not shown). The nuclei of recombination-positive neurons were frequently surrounded by GABAergic terminals that formed a pyramidal-shaped puncta-ring (Fig. 2J, inset). Therefore, the recombination-positive neurons were pyramidal neurons and not GABAergic interneurons.

A simple explanation for the columnar pattern of reporter gene expression is that neurons in such clusters/columns are clonally related and inherit the activated reporter gene from a common progenitor neuron that underwent recombination prior to or during cortical neurogenesis. These progeny neurons may have migrated into and within the cortex in a predominantly radial but not tangential direction, thereby forming a column. This would predict that: 1) such columns appear early during embryonic cortical development, and

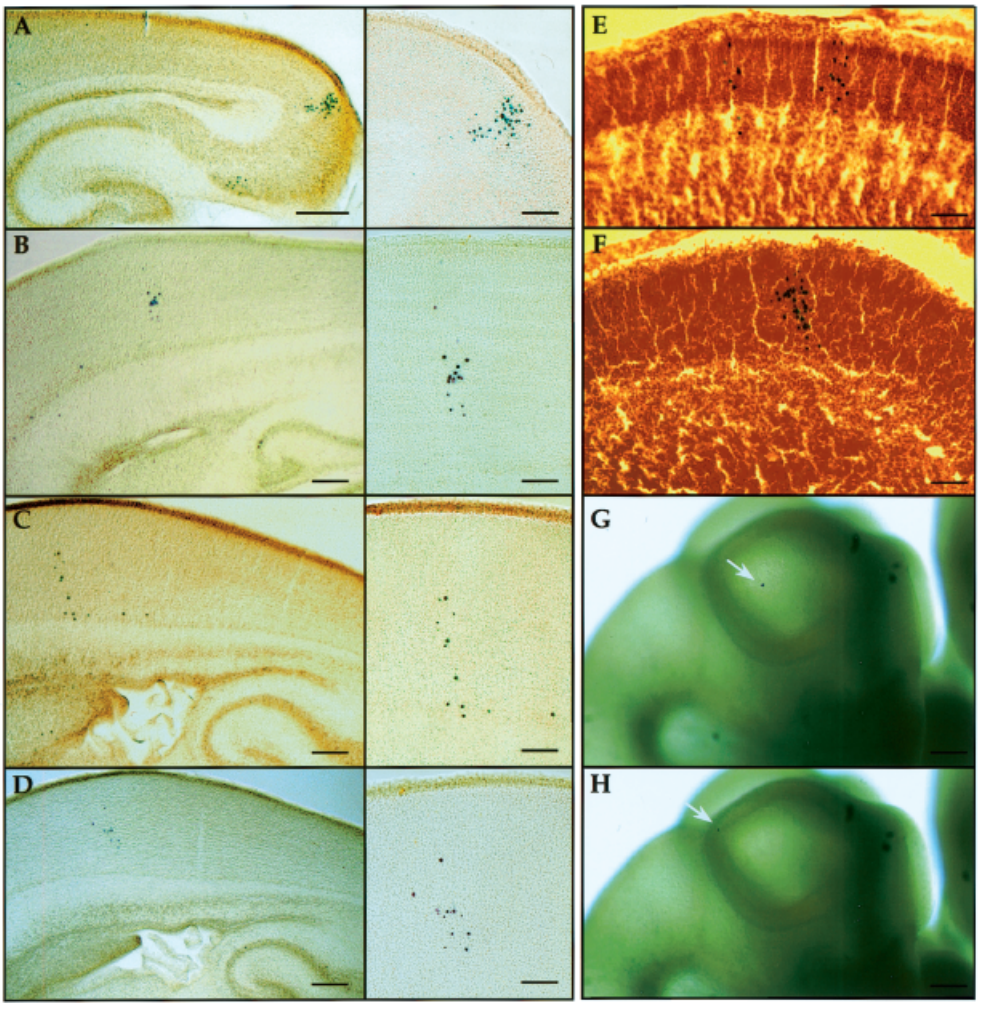

FIG. 3. Patterns of nls-lacZ reporter activation by the M34 deleter during early postnatal and embryonic development. **A–D**: X-gal-stained brain sections at postnatal day 3 showing nls-lacZ labeled columns in visual (**A**) and somatosensory (**B–D**) cortex. The right panel is a higher magnification view. **E,F**: Tissue section at embryonic day 15 double-stained with X-gal and nuclear fast red. **G,H**: Two lacZ-positive nuclei at different focal planes of the same sample at E11. Scale bars in left panels of **A–D**: 250 μm; Scale bars in right panels of **A-F**: 100 μm; Scale bars in **G,H**: 400 μm;

2) recombination occurs in the cortical progenitor cells at the stage of neurogenesis of the neocortex. Indeed, we observed such columns in brain sections of mice at postnatal day 3 (Fig. 3A–D) and E15 (Fig. 3E,F). At E11, individual and well-separated recombination-positive cells were observed in the neuroepithelium in whole-mount preparations (Fig. 3G,H). These results are consistent with, although do not prove, the notion that lacZ-positive neurons in a cluster/column inherit the activated reporter gene from common progenitor neurons and are therefore clonally related.

Regardless of the mechanism of the columnar pattern of reporter gene expression, these data suggest that the M34 Cre transgenic line, when crossed with an appropriate GFP-based reporter line, may allow visualization of individual or a "column" of live cortical neurons for detailed morphological and functional studies.

A Dendrite-Targeted GFP Reporter Line

To generate reporter mice expressing a dendrite-targeted GFP marker, a MAP2GFP fusion gene was constructed and linked to the chicken β-actin promoter and the transcription and translation STOP cassette (Fig. 1B, LSLMAP2GFP, see Material and Methods). Twelve MAP2-GFP reporter transgenic founders were produced by pronuclear injection of linearized LSLMAP2GFP. Of the eight transgenic lines analyzed, five gave MAP2-GFP expression when mated with the CaMKII-Cre deleter mice. One of the reporter lines, F29, was characterized further. F29 was estimated to carry 5–10 copies of the LSLMAP2GFP transgene (data not shown).

To test the expression level of MAP2-GFP, F29 was first bred with T50, a CaMKII-Cre deleter line which activated Cre/*loxP* recombination at low frequency in

young neocortex (Tsien *et al.*, 1996) but at relatively high frequency in adult neocortex (Huang and Tonegawa, unpubl. data). In 4-month-old T50/F29 double transgenic mice, soma and proximal dendrites of neocortical pyramidal neurons were visible but faint when examined using an epifluorescence microscope. When imaged using a confocal microscope, soma and dendritic structures of neocortical pyramidal neurons were clearly visualized (Fig. 4E,F). As expected, dendritic spines and axons were not labeled since MAP2c does not enter these neuronal compartments (Bernhardt and Matus, 1984). However, labeled neuronal dendrites were highly intermingled and overlapping due to the high cell density of MAP2-GFP expression. Dendrites of individual neurons could not be resolved.

F29 was then bred with the low-frequency recombination line M34. In 6-week-old double transgenic mice, the detailed dendritic structures of individual, well-separated cortical pyramidal neurons were resolved (Fig. 4A,B). These neurons were detected in all layers in the visual, somatosensory, motor, and frontal cortex. It was usually not possible to reconstruct the entire dendritic tree of labeled neurons in 50–100-μm thick brain sections using confocal microscopy. However, small dendritic branches were frequently found in layer one, suggesting that the entire dendritic trees were labeled by MAP2-GFP. Interestingly, clusters or columns of MAP2-GFP labeled pyramidal neurons were also observed (Fig. 4C,D). In hippocampus, MAP2-GFP was expressed in a small number of pyramidal neurons (Fig. 5A–C). These results suggest that the M34 deleter line activated similar patterns of recombination in the MAP2-GFP reporter mice compared to the nls-lacZ reporter mice (Fig. 2).

To further examine whether and to what extent the pattern of reporter gene expression activated by the same deleter line may be influenced by the genomic integration site of the reporter transgene among different reporter lines, we compared the activation of MAP2-GFP and nls-lacZ reporters in hippocampus by the T50 deleter line. The T50 line was shown to activate the nls-lacZ reporter at a high frequency in pyramidal neurons in the CA-1 subfield of the hippocampus of young mice (Tsien *et al.*, 1996). We therefore examined MAP2-GFP expression in the hippocampus of T50/F29 double transgenic mice at 4 weeks of age. Pyramidal neurons in the CA-1 region were again selectively labeled, while no neurons were labeled in the adjacent CA3 field and the dentate gyrus (Fig. 5D). Both the apical dendrites and the basal dendrites were intensely labeled (Fig. 5E). We also bred T50 with F35, another independently derived MAP2-GFP reporter line. We again observed preferential labeling of CA-1 pyramidal cells in 4-week-old T50/F35 double transgenic mice (data not shown). Therefore, it is feasible to use a deleter line with a known recombination pattern determined from one reporter line to express a different reporter gene in the same neuronal population by breeding the deleter line with a different reporter line.

The activation of MAP2-GFP reporter in the hippocampus by the T50 deleter also increased with age. At 4 months of age significant MAP2-GFP expression appeared in the CA3 area (Fig. 5F). However, recombination did not occur in other nonpyramidal neurons, such as GABAergic interneurons, since antibodies against GABA (data not shown) or parvalbumin (Fig. 5F) did not label any MAP2-GFP-expressing neurons. The labeled

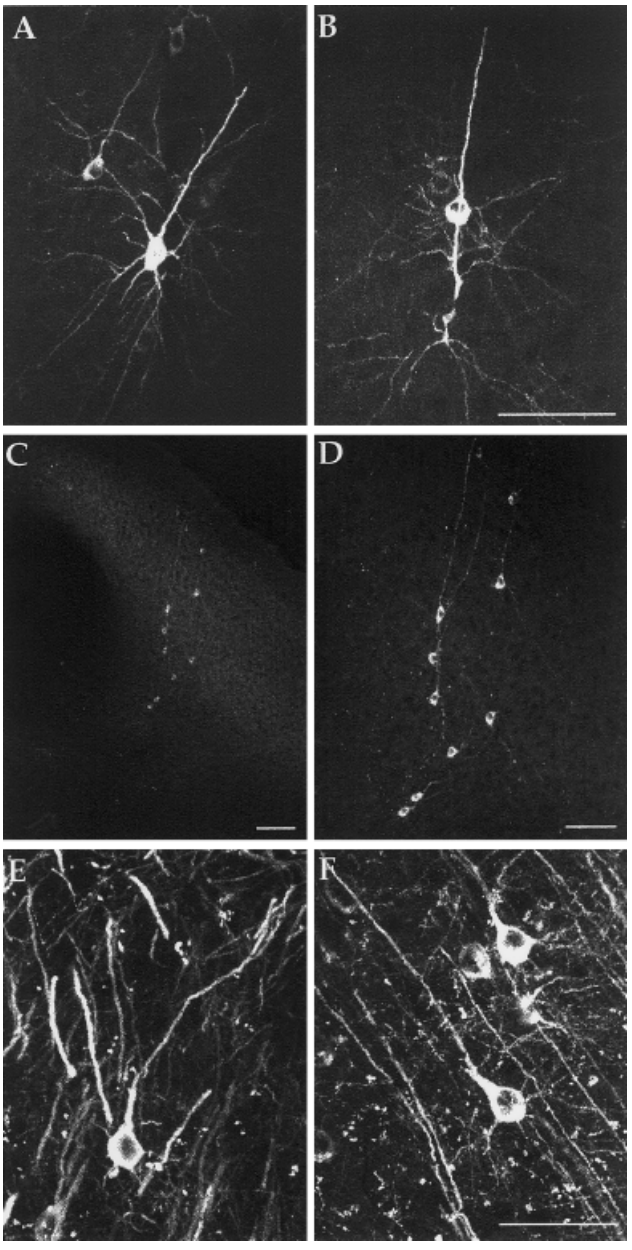

FIG. 4. MAP2-EGFP reporter expression in the neocortex imaged by laser scanning confocal microscopy. **A,B**: Dendritic structure of single pyramidal neurons in the visual cortex revealed by low-frequency recombination in a 6-week-old M34/F29 double transgenic mouse. **C,D**: A "column" of pyramidal neurons expressing the MAP2-GFP marker in the visual cortex of a 4-week-old M34/F29 double transgenic mouse. **E,F**: MAP2-GFP expression at high cell density label intermingled and overlapping dendrites in the visual cortex of a 4-month-old T50/F29 mouse. Scale bars: 50 μm in **A,B,E,F**; 100 μm in **C**; 60 μm in **D**.

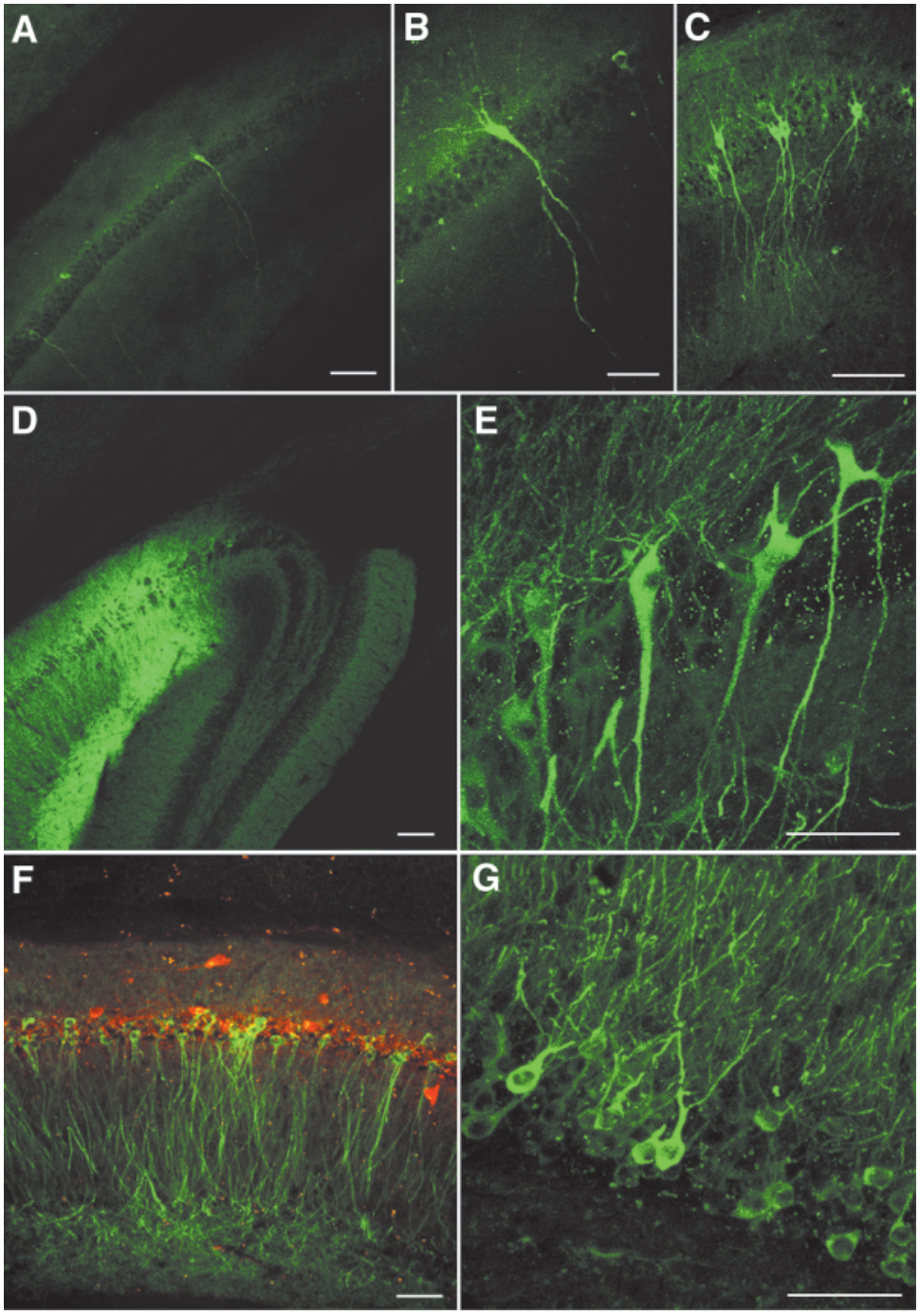

FIG. 5. MAP2-EGFP reporter expression in hippocampus. **A–C**: Low-frequency recombination labeled single pyramidal neurons of a 5-week-old M34/F29 mouse. **D,E**: The T50 deleter activated MAP2-GFP expression in the CA-1 field of the hippocampus in a 4-week-old animal. At 4 months of age, the T50 deleter line also activated MAP2-GFP in the CA-3 region (**F**) and dentate gyrus (**G**). **F**: Brain section was labeled with antibodies against parvalbumin. No overlap between MAP2-GFP expression neurons and parvalbumin positive GABAergic neurons was detected. Scale bars: 100 μm in **A**; 50 μm in **B,C**; 200 μm in **D**; 40 μm in **E,F,G**.

neurons did not show any obvious morphological abnormality (Fig. 5E,F). Recombination also appeared in the granule cells of the dentate gyrus at this age (Fig. 5G). In all cases, only the dendrites, but not the axons and dendritic spines, were labeled by MAP2-GFP.

DISCUSSION

The activity of the endogenous CaMKII promoter is restricted to the postnatal forebrain (Burgin *et al.*, 1990; Mayford *et al.*, 1995). Indeed, of the 12 CaMKII promoter –Cre transgenic lines that we have newly generated, most produced patterns of recombination that were largely restricted to the postnatal forebrain (Lovett and Tonegawa, unpubl. results). On the other hand, it is well known that the exact spatial and temporal expression pattern of a transgene could be profoundly influenced by the genomic sequences surrounding the site of its integration (Cohen-Tannoudji *et al.*, 1994). Therefore, it is not entirely surprising that the pattern of recombi-

nation in the M34 line is very different from the pattern of the endogenous CaMKII promoter activity. It is possible that the Cre transgene in the M34 line integrated near a genomic transcriptional regulatory element that was active in a limited number of neuronal progenitor cells in the neuroepithelium or in a small number of pyramidal neurons and "columns" of pyramidal neurons in the neocortex. For technical reasons, we were not able to test these possibilities directly since low levels of Cre expression in transgenic mouse brain could not be detected by in situ hybridization or immunohistochemistry.

The columnar pattern of reporter gene activation in the M34 deleter line might have occurred via two different mechanisms. In the first scenario, reporter activation in each neuron in a cluster/column is the result of an independent recombination event. In this case, the CaMKII-Cre transgene may be selectively expressed at a higher level in pyramidal neurons within such columns. However, it is difficult to explain how neurons expressing higher levels of the Cre gene are clustered together. In the second scenario, the columnar pattern of reporter activation arises from a single recombination event in a progenitor cell in the neuroepithelium. During subsequent cortical neurogenesis, the progenies of this progenitor cell inherit the activated reporter gene. They then migrate radially and in an "inside-out" manner into the cortex and form the observed clusters or columns. A number of studies involving different species indicate that radial movement is the predominant migratory event undertaken by most but not all of the neuroblasts exiting the ventricular zone (Kornack and Rakic, 1995; Rakic, 1995). However, nonradial migration of neurons has also been reported (O'Rourke *et al.*, 1995) and was thought to contribute to the tangential dispersion of clonally related neurons (Walsh and Cepko, 1992). Recently, it has been shown that distinct progenitor neurons are committed to generating neuroblasts that disperse in either radial or tangential direction (Tan *et al.*, 1998). In addition, radially dispersed neurons are glutamatergic pyramidal neurons. In contrast, progenitors that are committed to generate tangentially dispersed neuroblasts give rise to predominantly GABAergic neurons. Interestingly, in the M34 deleter line the columnar pattern of reporter gene expression occurred exclusively in the pyramidal neurons (Fig. 1J). It is thus likely that Cre/*loxP* recombination in M34 line takes place only in progenitors that are committed to generate pyramidal neurons which later form columns through radial migration into the cortex. The number of lacZ-positive neurons within the column would depend on the timing of the recombination event during neurogenesis: the earlier the recombination event takes place, the more lacZ-positive cells in a column.

In contrast to the lacZ-positive cells in columns, the sparsely labeled neurons in the neocortex most likely result from independent recombination events that occurred within those neurons postnatally. It is possible that Cre/*loxP* recombination in pyramidal neurons requires a certain threshold level of the Cre protein. In the M34 line, this threshold is reached stochastically only in a small proportion of neurons. However, we cannot exclude the possibility that some of these sparsely labeled pyramidal neurons outside of the columns are in fact also clonally related to neurons within the columns. They may have subsequently been dispersed from the column by tangential migration.

Although we show that different transgenic reporter mice could be activated with the same deleter line to give rise to similar patterns of reporter expression, it is usually necessary to generate and characterize multiple transgenic lines for each reporter construct to identify a useful reporter line. An alternative approach is to use homologous recombination in embryonic stem cells to target a reporter gene to a strong and ubiquitous endogenous promoter and produce a "knockin" reporter line (Soriano, 1999). The knockin approach has the advantage in that each reporter gene is present as a single copy at identical genomic loci. This should minimize the variation of reporter gene expression intrinsic to the transgenic approach.

In the MAP2-GFP reporter mice, GFP was selectively targeted to dendrites of pyramidal neurons but not axons or spines. Overexpression of MAP2-GFP did not significantly alter the dendritic morphology. This is not surprising, since in transgenic mice overexpressing MAP2c at levels higher than the endogenous level there were no detectable effects either on the arrangement or the morphology of neurons in the brain (Marsden *et al.*, 1996). Therefore, the MAP2-GFP reporter mice should be generally useful to study the morphological alterations of dendritic structures that occur during development or in response to sensory experience. However, the current MAP2-GFP reporter line may need to be improved to increase the expression level of MAP-GFP. This may be achieved by "knocking-in" the MAP2-GFP fusion gene to a highly and ubiquitously expressed genomic locus, such as Rosa26 (Soriano, 1999).

With its combinatorial property, the Cre/*loxP* deleter/reporter binary system is poised to take advantage of the increasing reagents generated by the mouse genetics researchers. The identification of additional transcriptional promoters in specific cortical cell types will allow the construction of more cell-type-specific Cre deleter lines. Subcellular delivery of GFP to other neuronal compartments, such as axons and spines, can be achieved by producing reporter lines expressing fusion genes encoding GFP and relevant cytoskeleton protein motifs such as Tau (Callahan and Thomas, 1994; Mombaerts *et al.*, 1996) or Drebrin (Hayashi *et al.*, 1996). Finally, an alternative recombination-activated marker system can also be constructed using the yeast site-specific recombinase flp/frt system (Dymecki, 1996).

MATERIALS AND METHODS

Generation of Transgenic Mice

CaMKII-Cre deleter mice

The 11.2 Kb DNA fragment containing the αCaMKII promoter and the Cre transgene was obtained by digestion of pJT-CRE with Sal I as previously described (Tsien *et al.*, 1996). Transgenic founders were produced by pronuclear injection of the linearized DNA into zygotes of C57B/6 mice.

MAP2-GFP reporter mice

To construct MAP2EGFP, a 1.4 Kb EcoRV fragment of MAP2c from pβactin2C/GFP (a gift from Andrew Matus) was ligated to the SmaI site of EGFP-N2 (Clontech, Palo Alto, CA). To construct chicken β-actin promoter/ *loxP*Stop*loxP*/MAP2EGFP (LSLMAP2GFP), a 3.1 Kb lacZ fragment from cAct-XstopXnZ (a gift of Mercer and Anderson) (Zinyk *et al.*, 1998) was first excised by EcoRI partial digestion. The 8.3 Kb fragment containing the chicken β-actin promoter/ *loxP*Stop*loxP*/ and the protamin polyA was gel purified, blunted, and dephosphorylated. A 2.1 Kb ApaI and Not I fragment from MAP2EGFP was then blunted and ligated to the above 8.3 Kb EcoRI fragment. Ligation junctions were verified by DNA sequencing. A 7.2 Kb NotI fragment was purified from LSLMAP2GFP and used for pronuclear injection.

Genotyping

Tail DNAs were analyzed by PCR using the following primer sets. CaMKII-Cre transgene (120 bp amplified): 5′-ACCTGATGGACATGTTCAGGGATCG-3′, 5′-TCCGGTTATTCAACTTGCACCATGC-3′. LSLMAP2GFP transgene: 5′-CTGGTCGAGCTGGACGGCGACG-3′,5′-GTAGGTCAGGGTGGTCACGAG-3′ nls-lacZ transgene: 5′-TCGGCGGTGAAATTATCGATGAGC-3′, 5′-CCACAGCGGATGGTTCGGATAATGC-3′.

Histology and Immunohistochemistry

Embryonic day 11 (E11) embryos were harvested and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight for whole-mount X-gal histochemistry. E15 embryos were fixed in the same way and 50 μm sagittal whole-embryo sections were cut in a cryostat (Leica). Postnatal day 3 pups were decapitated and fixed in the same manner and 50 μm sagittal head sections were cut in a cryostat. Adult mice were anesthetized and perfused intracardially with cold saline (0.9% NaCl) followed by freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were postfixed in the same solution for less than 2 h at 4°C and equilibrated first in 15% sucrose and then 30% sucrose in PBS overnight. Twenty μm or 50 μm sagittal brain sections were cut in a cryostat and stored at −80°C. Sections were subjected to X-gal staining for 12–16 h at 37°C, washed with PBS, and mounted. Some sections were then counterstained with nuclear fast red or cresyl violet.

For immunohistochemistry, a monoclonal antibody against parvalbumin (Sigma, St. Louis, MO) was used at 1:5,000 dilution in 10% normal horse serum (NHS) and 0.2% triton in PBS. The secondary Alexa 594 conjugated Goat antimouse IgG (Molecular Probes, Eugene, OR) was used at 1:200 dilution. For double-labeling, monoclonal antibodies against parvalbumin (Sigma) and polyclonal antibodies to β-gal (Cappel, Malvern, PA) were used at 1:2,000 and were visualized with Alexa 594 (for β-gal) and Alexa 488 (for parvalbumin) (Molecular Probe, 1:200 dilution). A MAP2 monoclonal antibody (Boehringer-Mannheim Biochemicals, Indianapolis, IN) was used at 1:1,000 and visualized with Alexa 594 (1:200 dilution).

To estimate Cre/*loxP* recombination frequency, 20 μm brain sections were double-stained with X-gal and cresyl violet and the number of neurons counted. In the hippocampus, all neurons in CA1, CA3, and dentate gyrus were included. In the neocortex, neurons in five adjacent field (each field is 400 × 400 μm) that, as a whole, covered all six cortical lamina were counted. In the striatum, neurons in two randomly selected fields (400 × 400 μm) were counted. The percentage of X-gal-positive neurons of the total number of neurons from each brain region was calculated.

Confocal Microscopy

Brain sections were examined under a 20× objective or a 40× water immersion objective. Laser excitation wavelengths and emission filter were 488 nm and 522DF35 for GFP and Alexa 488, and 568 nm and HQ598DF40 for Alexa 594. For GFP imaging, 10–30 images were acquired at 1.5-μm intervals in a z-series. For double-labeling of GFP and parvalbumin or lacZ and parvalbumin, 10–30 images were acquired in the double-labeling mode at 1.0-μm intervals in a z-series. The detection aperture was kept the same for the two channels. Images from each channel were collected in sequential series mode and subsequently merged. Care was taken to use the lowest laser power and no bleed-through was visible between the Alexa 488 to the Alexa 594 channels even in nonsequential mode. To produce a 3D reconstruction of the z-series, the optical sections from each scanned and merged image were projected and compiled into a single image. They were saved as TIFF files and processed in Adobe PhotoShop 5.0.

ACKNOWLEDGMENTS

Z.J.H. was a postdoctoral fellow of the Damon-Runyon Walter-Winchell Foundation.

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