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Generation of *Frizzled10*-Cre Transgenic Mouse Line: A Useful Tool for the Study of Dorsal Telencephalic Development

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Summary

Wnt signaling plays an important role in regulating cortical and hippocampal development, but many of the other molecular mechanisms underlying dorsal telencephalic development are largely unknown. We are taking advantage of the highly regionalized expression patterns of signaling components of the Wnt pathway to generate new mouse lines that will be useful for studying forebrain development. Here, we describe a transgenic mouse line where Cre is driven by the promoter of the Wnt receptor, *Frizzled10*. In these mice, Cre activity is mainly detected in the dorsal telencephalon during development and is confined to the pyramidal cell fields in the adult hippocampus. The Cre recombinase has very high efficiency when assayed by crossing the transgenic line with the ROSA26 reporter line. Thus, this Cre line will be useful for the study of dorsal telencephalic development and conditional inactivation of target genes in the cortex and hippocampus.

Keywords

Frizzled10; Wnt; Cre; dorsal telencephalon; cortex; hippocampus

Gene targeting techniques have become highly developed to study gene function in mice. However, in conventional gene knockouts, the function of a gene is often difficult to recognize because of embryonic lethality. This is particularly difficult for the brain whose function is controlled by complex gene expression patterns and is made up of numerous distinct cell types. These limitations can be overcome by transgenic expression of Cre recombinase, which catalyses the excision of DNA segments that are flanked by loxP sites (Sauer and Henderson, 1988). With the Cre/loxP recombinase system as a tool, scientists have gotten closer to the level of “no more practical limitations” in tailoring the mouse genome. When Cre is expressed

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under the control of a tissue- or cell type-specific promoter in transgenic mice, precise spatial control of target gene disruption is straightforward (Branda and Dymecki, 2004).

To study the molecular mechanisms of cortical development or aspects of learning and memory, many mouse lines that express Cre in specific areas of the forebrain have been generated (Berger *et al.*, 2004; Casanova *et al.*, 2001; Iwasato *et al.*, 2004; van den Bout *et al.*, 2002). However, compared with the extreme complexity of the brain, the range and specificity of the existing Cre lines remains a significant limitation. We have been taking advantage of the specific regional and temporal expression of members of the Wnt family and their receptors to generate a variety of useful mouse reagents (Kim *et al.*, 2001; Zhao and Pleasure, 2004, 2005; Zhao *et al.*, 2006).

The Wnt family of glycoproteins is involved in numerous developmental and disease processes in higher eukaryotes. They signal by binding to at least two cell surface receptor families: the Frizzled family of receptors and their coreceptor “low density lipoprotein-related proteins” (Logan and Nusse, 2004). Frizzled proteins are cell surface receptors that participate in a wide variety of developmental processes, including cerebellar degeneration, esophageal distension and dysfunction loss of hair cells or other auditory neurons [*Fzd4* (Wang *et al.*, 2001)], macroscopic hair patterning [*Fz6* (Guo *et al.*, 2004)], hippocampal cell survival [*Fz9* (Zhao *et al.*, 2005)], and the development of major brain axon tracts [*Fz3* (Lyuksyutova *et al.*, 2003; Wang *et al.*, 2002)]. In our previous study, we generated a transgenic mouse line in which the TauLacZ reporter gene was expressed in the cortical hem, dorsal thalamus, and dorsal neural tube under the control of *Frizzled10* promoter during embryonic life (Zhao *et al.*, 2006). To take advantage of the regionally specific expression of the *Frizzled10* promoter, we have generated a Cre transgenic mouse line using the same *Frizzled10* promoter. In these mice, Cre was strongly expressed in the developing cortex and hippocampus, and there is very strong recombination activity in these regions. Our transgenic Cre line has Cre expression with the following features:

- i. expressed in cortex, hippocampus, dorsal thalamus, and caudate putamen (CPu)
- ii. strong activity in the developing dorsal telencephalon
- iii. dynamic expression at different development stages

The Cre expression shows prominent differences with that of our previously generated TauLacZ transgene mice, which expressed TauLacZ in a pattern closely resembling the native expression of *Frizzled10*. These differences are probably due to the different insertion site of the transgene. Despite the unique expression pattern of Cre in this mouse line, we believe that this line will be quite useful for the study of brain development and conditional inactivation of target genes in cortex and hippocampus. Additionally, we detected Cre expression in the specific patterns in the cerebellum, retina, and spinal cord.

RESULTS AND DISCUSSION

The construct used to generate the *Frizzled10*-Cre transgenic line contained a 6.7 kb fragment upstream of the transcriptional start site of the mouse *Frizzled10* gene, as we described previously (Zhao *et al.*, 2006), followed by a modified Cre gene carrying a nuclear localization signal and a polyA sequence (Fig. 1a). Construct DNA was then injected into B6/FvB oocytes and all founders were mated to the Cre-reporter line R26R mice. Cre recombination activity was examined by the expression of β -galactosidase upon Cre-mediated deletion of a floxed stop-cassette in R26R mice (Soriano, 1999). Anti-cre immunostaining was used to characterize the protein expression of Cre recombinase. Among seven founders, we obtained one line in which Cre was specifically expressed in the developing dorsal telencephalon.

To characterize the *Frizzled10*-Cre mouse line, we first performed immunohistochemical staining to examine Cre expression. At E10.5, Cre expression was mainly detected at the dorsal telencephalic vesicle and dorsal neural tube (Fig. 1b,c). By E12.5, distinct Cre-immunoreactive cells were widely observed in the dorsal telencephalon (Fig. 1d). We then further studied the dynamic expression of Cre in the cortex and hippocampus at different developmental stages.

In the developing cortex, Cre is expressed in quite discrete layers with a very dynamic pattern. At E12.5, Cre were mainly expressed very strongly in cells in the intermediate zone (IZ), which were presumably migrating neurons (Fig. 1e). From E12.5 to E14.5, Cre was detected in the cortical plate (CP) (Fig. 1f) and was colocalized with Tag-1, a neural cell adhesion molecule expressed in cells with axons in the IZ and the CP (Fig. 1k) (Denaxa *et al.*, 2001). At E16.5, Cre was detected in the subplate, marginal zone, and CP (Fig. 1g). At P0, Cre expression was most prominent observed in cortical neurons in Layer 2/3 and Layer 5 (Fig. 1h), and this pattern persisted through P8 (Fig. 1i). Additionally, Cre was also highly expressed in the cingulate cortex (Cg) of P0 and P8 brains (Fig. 1j and data not shown). However, Cre expression decreased thereafter and was not detectable in the adult neocortex (data not shown).

Cre protein was also highly expressed in the Hippocampal formation during development. At E12.5, Cre was detected in the medial wall that will develop into the hippocampus and dentate gyrus (Dg) (Fig. 1l) and persisted in the hippocampal neuroepithelium until E16.5. High Cre expression was observed in the primordial Dg at E14.5 and E16.5 (Fig. 1m,n), and even more distinctly at P0. At P0, strong Cre activity was observed in Dg and a gradient from CA3> CA1 (Fig. 1o). Cre staining was also detected highly from CA3 to CA1 area at P8 (Fig. 1p) and stronger Cre expression was observed in the ventral Dg (Fig. 1q). After P8, Cre expression in the Dg declined rapidly to an undetectable level, compared with high expression in CA area pyramidal neurons that persisted in the adult (Fig. 1r–t).

To test the recombination activity of Cre, *Frizzled10*-Cre transgenic mice were crossed with ROSA26 reporter mice (Soriano, 1999). In general, X-gal staining showed that recombination was strongest in dorsal structures within the telencephalon and the thalamus (see Fig. 2). At E14.5 and E16.5, LacZ was highly expressed in the cortex, and there was a distinct border between dorsal and ventral structures at E14.5 (Fig. 2b,c). Similarly, LacZ activity was also stronger in dorsal cortical structures at each level from rostral to caudal at P0 and P8 (Fig. 2d,i). In the adult brain, strong LacZ staining was observed in the cortex, hippocampus, and CPu (Fig. 3j–l).

Consistent with the Cre antibody staining, during cortical development, LacZ activity in the CP appeared to be strong at E14.5 and E16.5 (Fig. 3a,b). There was also high LacZ activity in cingulate cortex at P0 (Fig. 3c). From P0 to adult cortex, LacZ expression was the strongest in Layer 2/3 (Fig. 3d–f). Interestingly, high LacZ activity was also observed in Layer 4 of the cortex, including the barrel fields in somatosensory cortex (Fig. 3d–f), despite the lack of clear expression of Cre in Layer 4 cells. In addition, X-gal staining of embryonic day E14.5 and E16.5 embryos showed staining in the MZ and VZ (Fig. 3b), whereas no Cre staining was clearly observed in these regions. These discrepancies, thus, may be due to the earlier developmental expression of Cre, thereby resulting in persistent LacZ expression in the lineal descendants of cells expressing Cre at earlier times. To exclude early embryonic activity of Cre, we performed anti-Cre and X-gal staining at E3.5 and found no detectable signal with either method (data not shown).

High LacZ activity indicating Cre-mediated recombination was also observed in the developing hippocampus (Fig. 3g–l). From E14.5 to E16.5, LacZ activity was observed in the hippocampal neuroepithelium (Fig. 3g,h). From P0 to adult, LacZ was highly expressed in pyramidal cells and the dentate gyrus of the hippocampus (Fig. 3i–l). Importantly, Cre was only transiently

expressed in Dg granule cells during development. Thus, this line offers a novel potential tool to study dynamics of dentate granule cell production and might offer an approach to allow temporally controlled excision of genes in granule cells.

To characterize the cell types expressing Cre during development, we performed double immunostaining in the developing cortex and hippocampus with cell type-specific markers. Strong overlapping expression of Cre with MAP2 in P8 mouse cortex and P0 hippocampus was found (Fig. 4a,b), but no overlap of Cre with GFAP was found in P0 hippocampus and Dg (Fig. 4c). Taken together, these data demonstrated that detectable expression of Cre marks in neurons but is absent from glial cells in the postnatal brain.

The extracellular matrix protein reelin controls neuronal migration by binding to different reelin receptors (Tissir and Goffinet, 2003). In the hippocampus, reelin also control the migration of dentate granule cells to proper position and is expressed by a developmentally important group of neurons called Cajal-Retzius cells (Frotscher *et al.*, 2003). We performed Cre and Reelin double staining in Dg at P0 and found some Cre-positive cells expressing reelin at the border of the Dg (Fig. 4d,e). This colocalization could be observed till P8 (data not shown). This suggested that this transgenic mouse line may be a useful tool for further study of Cajal-Retzius cell function.

Additionally, Cre activity in developing spinal cord, retina, thalamus, cerebellum, heart, liver, and stomach was also observed. High levels of Cre expression could be detected in the dorsal spinal cord and dorsal root ganglia at E14.5 (Fig. 5a,b). Specific Cre expression was also observed at E12.5 in the retina (Fig. 5c) and the developing dorsal thalamus (Fig. 5d-f). In the cerebellum, distinct Cre expression was detected in the deep cerebellum nuclei at P0 (Fig. 5g) as well as in the Purkinje cell layer and external granule cell layer at P8 (Fig. 5h). High LacZ activity was also detected in the adult cerebellum (Fig. 5i). Generally, the strong LacZ activity was mainly detected at neural tube (Fig. 5j), additionally in heart, stomach, and liver (Fig. 5k,l).

Taken together, this *Frizzled10*-Cre mouse line will be a useful tool to achieve the early onset of Cre expression in the dorsal telencephalon. Additionally, in the pyramidal fields of the hippocampus, Cre activity is maintained in the adult brain. This mouse line can be used to specifically inactivate a gene in the dorsal telencephalon during development, or in adult hippocampus CA area. Although the overall pattern of Cre-mediated excision is complex in these mice, we have identified several temporally restricted patterns of Cre expression (notably the layer-specific expression in the cortex, transient expression in dentate granule cells and the adult expression in hippocampal pyramidal cells) that make this a promising line for manipulating some neuronal populations. We believe that with the development of potent new ligand-regulated Cre fusion proteins that this promoter will allow the generation of mice with highly temporally selective patterns of Cre activity.

MATERIALS AND METHODS

Mouse Manipulations

A 7.7 kb fragment amplified by PCR containing promoter region of mouse *Frizzled 10* gene was subcloned into Topo XL vector (invitrogen) (Zhao *et al.*, 2006). Cre gene and a polyadenylation site were excised from β -Actin-Cre pBluescript SK(+) by Kpn and SalI, and inserted into the EcoRV site behind *Frizzled10* promoter. When the transgenic construct was linearized by SalI and NotI, a 6.7 kb fragment upstream of the *Frizzled10* transcriptional start site was used as the promoter. The linearized DNA was then purified by Qiagen kit (Cat No: 20021), microinjected into the pronuclei of oocytes of B6/FVB using standard procedures. All offspring were analyzed by PCR using Cre primers (5'-CATACCTGGAAAATGCTTCTGTCC-3', and 5'-ATTGCTGTCACTTGGTCGTGGC-3').

X-Gal Staining

Embryos or embryonic brains were immersed into freshly prepared 2% PFA at 4°C for 2 h; brains older than P0 were perfused by 2% PFA. Tissues were then cryoprotected in 30% sucrose, embedded in OCT, and sectioned at 16–25 µm (embryonic brains) on a cryostat or 40 µm (older than P8) on a sliding microtome. After washing and permeabilization in PBS containing 0.02% NP-40, 2 mM MgCl₂ for 30 min, tissue was stained with 1 mg/ml X-gal in PBS containing 0.02% NP-40, 5 mM of K₃Fe(CN)₆, 5 mM of K₄Fe(CN)₆, and 2 mM of MgCl₂ at 37°C about 6–16 h. Sections were then rinsed with PBS, refixed in 4% formalin about 4 h, and coverslipped.

Immunohistochemistry

Tissues were fixed in 4% PFA and sections were prepared as described earlier. Sections were then permeabilized with 0.1% Triton in PBS, blocked in 10% calf serum, incubated with first antibody at 4°C overnight, and detected by fluorescent secondary antibodies. The following antibodies and reagents were used for immunostaining: rabbit anti-Cre (Covance, PRB-106C), mouse anti-GFAP (Chemicon, Temecula, CA; MAB360), mouse anti-MAP2&3 (Chemicon, Temecula, CA; MAB378), mouse anti-Reelin (Chemicon, Temecula, CA; MAB536), mouse anti-Tag-1 (DSHB, 4D7), Texas Red goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA; 115-075-146), FITC goat anti-Rabbit IgG (Jackson Immuno-Research, 111-095-144), Texas Red goat anti-Rabbit IgG (Jackson ImmunoResearch, 111-075-144), and FITC goat anti-mouse IgG (Jackson ImmunoResearch, 115-095-146).

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FIG. 1. Schematic drawing of the *Frizzled10*-Cre construct and Cre immunostaining in developing cortex of transgenic mice

(a): transgenic construct of *Frizzled10*-Cre. (b-d): Cre activity was detected in dorsal telencephalon at E10.5 and E12.5 (arrow in b show the telencephalon); (e-j): dynamic Cre expression in developing cortex; Cre was detected in IZ at E12.5 (e), cortical plate at E14.5 (f), marginal zone, cortical plate and subplate at E16.5 (g). From P0 to P8, Cre immunostaining indicated Cre was expressed in Layer 2/3 and Layer 5 in the cortex at P0 (h) and P8 (i) and the Cg (j). (k): Double staining of anti-Cre and anti-Tag-1 showed that Cre protein was mainly expressed at Cortical plate; (l-t): dynamic Cre expression in developmental hippocampus of the *Frizzled10*-Cre transgenic mouse. (l-n): Cre was expressed in the hippocampus

neuroepithelium at E12.5 (l), E14.5 (m), E16.5 (n). Immunodetection of Cre at P0 showed high activity in Dg and CA region with the pattern of CA1<CA3 (o). At P8, high level of Cre expression was maintained in CA area (p) but decreased in dorsal Dg neurons (q, arrows). (r–t): Cre expressed in adult CA area (r). (s): adult CA3. (t): adult CA1. CP, cortical plate; IZ, intermediate zone; PP, preplate; SP, subplate; DG, dentate gyrus; Cg, cingulate cortex; MZ, marginal zone; VZ, ventricular zone; 2/3, Layers 2 and 3, Hp, hippocampus; CA1, hippocampus CA1 area; CA3, hippocampus CA3 area. Scale bars: b, d, p, r: 500 μ m, others: 100 μ m.

FIG. 2. Cre-mediated recombination was mainly detected in the developing dorsal telencephalon as revealed by LacZ reporter gene expression in *Fzd10 Cre* R26R* transgenic mice

(a) Nontransgenic brain control of X-gal staining. (b, c): High LacZ activity was detected in the dorsal telencephalon at E14.5 (b) and E16.5 (c). There is a distinct border (arrow in b) between dorsal and ventral at E14.5. X-gal staining showed lacZ activity was detected at dorsal telencephalon at P0 (d–f) and P8 (g–i) from rostral to caudal levels (arrows showed the border between dorsal and ventral). (d, g): rostral; (e, h): median; (f, i): caudal. (j–l): X-gal staining in adult brain. (j): Sagittal view of LacZ activities mainly detected at cortex, CPu and hippocampus. Lines in (j) indicated coronal levels showed in (k) and (l). (k): Anterior view of

LacZ activities in cortex and CPu. (l): Posterior view of LacZ activity mainly detected in cortex. CPu, caudate putamen. Scale bars: 1 mm.

FIG. 3. Cre-mediated recombination in the developmental cortex and hippocampus

(a, b): LacZ activity was mainly detected in the cortical plate at E14.5 (a) and E16.5 (b) cortex. Section of P0 brain stained with X-gal showing the medial > lateral gradient of LacZ expression in cingulate cortex (c) and Layer 2/3 (d). (e, f): X-gal staining at P8 and adult showed LacZ activity was also mainly detected at Layer 2/3 in transgenic cortex. Interestingly, LacZ expression was observed at the barrel cortex (arrows). (g–l): Strong LacZ activity was detected in the hippocampus at E14.5 (g), E16.5 (h), P0 (i), P8 (j, k), adult (l). CP, cortical plate; IZ, intermediate zone; PP, preplate; SP, subplate; DG, dentate gyrus; Cg, cingulate cortex; MZ, marginal zone; VZ, ventricular zone; 2/3, Layers 2 and 3; Hp, hippocampus; CA1, hippocampus CA1 area; CA3, hippocampus CA3 area. Scale bars: 100 μ m.

FIG. 4. Identification of Cre-positive cells in cortex and hippocampus, and Cre was restricted to neurons but not glia
 (a, b): Strict Cre and MAP2/3 overlap was observed in transgenic mouse cortex (a) and hippocampus (b). (c): Immuno-double staining showed there is no overlapping of Cre with GFAP at P0. (d, e): Double staining of anti Cre and Reelin in Dg at P0. e: High magnification view of boxed area in (d) showing Cre labeled cells are Reelin positive (arrows). Scale bars: 100 μ m.

FIG. 5. Cre activity was also detected at spinal cord, cerebellum, thalamus, retina, heart, liver, and stomach

(a): In developing spinal cord, Cre activity was detected at dorsal spinal cord and some ventral neurons (arrows). (b): Strong Cre expression in E14.5 DRG. (c): Cre activity was detected in retina and eyeball in E12.5 transgenic embryo. (d–f): Cre expression and recombination activity dorsal thalamus. (d): Cre expression in E14.5 dorsal thalamus. (e, f): Recombination took place at P0 (e) and P8 (f) dorsal thalamus, arrows show the border between dorsal and ventral. (g–i): Cre immunostaining and recombination activity in cerebellum. (g): Cre was expressed in deep cerebellum nuclei in P0 cerebellum. (h): Cre activity in external granule cell layer (EGL) and purkinje cell layer at P8. (i): X-gal staining in adult cerebellum, LacZ activity

was mainly detected in EGL. (**j–l**): LacZ activity was mainly detected in spinal cord at E11.5 (**j**), additionally in heart (**k**, arrows), stomach (**l**, arrow), and liver (**l**, arrowheads). CB, cerebellum; DCN, deep cerebellum nuclei; DRG, dorsal root ganglia; EGL, external granule cell layer; Pu, purkinje; SC, spinal cord. Scale bars: e,f,i,j: 1 mm; others: 100 μ m.