Published in final edited form as:

Genesis. 2008 June ; 46(6): 289–293. doi:10.1002/dvg.20393.

Cre Recombinase-mediated Gene Deletion in Layer 4 of Murine Sensory Cortical Areas

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Abstract

Thalamocortical input to layer 4 carries the major ascending sensory information to the mammalian sensory cortex and is crucial for the function and plasticity of sensory cortical areas. Here we report identification of a *Six3-cre* transgene that is selectively expressed in layer 4 of sensory cortical areas but not in the thalamus. In the mature somatosensory cortex Cre recombinase expressed from the transgene is able to mediate gene deletion in the overwhelming majority of layer 4 neurons, including GABAergic interneurons. The gene deletion in layer 4 mainly occurs during the first postnatal week. This *cre* transgene therefore provides a useful tool for examining the role of proteins expressed in layer 4 neurons.

The mouse *Six3* gene is a vertebrate homologue of *Drosophila sine oculis* and is expressed in the anterior-most neural plate and the developing eye in embryos (Oliver *et al.*, 1995). Its promoter and regulatory elements have been used to generate several *Six3-cre* transgenic mouse lines (Furuta *et al.*, 2000). Many of these *cre* transgenes are able to mediate deletion of a floxed gene in the hypothalamus and the retina (Furuta *et al.*, 2000; Liu *et al.*, 2007). We found that one of the reported lines, #69 (termed *Six3-cre#69* here), was also able to mediate gene deletion in layer 4 of sensory cortical areas.

To monitor the activity of Cre recombinase in the brain of *Six3-cre#69* mice, we crossed *Six3-cre#69* mice to *Rosa26* (*R26R*) mice (Soriano, 1999) to obtain *R26R/+;Six3-cre#69* double heterozygous mice. Once the *R26R* allele is recombined by Cre recombinase, it starts to express β -galactosidase that produces blue color in X-gal staining. To determine where *Six3-cre#69* mediates recombination of the *R26R* locus in the brain, we performed X-gal staining on coronal brain sections from either adult double heterozygous mice or adult *R26R/+* mice. In the absence of a *cre* transgene, no β -galactosidase activity was detected in *R26R/+* brain sections (Fig. 1l). In *R26R/+;Six3-cre#69* brain sections, we did detect β -galactosidase activity in the auditory cortex (Fig. 1g–i), the somatosensory cortex (Fig. 1a–g), and the visual cortex (Fig. 1g–j), which indicates that *Six3-cre#69* is expressed in the sensory cortical areas. There were many more Cre-expressing cells in the somatosensory cortex than in the other two sensory cortical areas. In addition, the Cre activity was also present in some ventral forebrain structures, including the bed nucleus of the stria terminalis, the medial preoptic nucleus, and the anterior hypothalamus (Fig. 1b–f). No Cre activity was observed in the midbrain, the hindbrain, or the cerebellum (Fig. 1). In the sensory cortical areas, X-gal staining seems to be specifically localized to layer 4. The recombination pattern was preserved in all six adult *R26R/+;Six3-cre#69* mice we analyzed.

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To confirm that the Cre activity is restricted to layer 4 in sensory cortical areas, we performed immunohistochemistry with an antibody to NeuN on X-gal stained coronal brain sections prepared from adult *R26R/+;Six3-cre#69* mice. Since the Cre activity is most robust in the somatosensory cortex, we focused our further analyses in the barrel field of the somatosensory cortex. NeuN immunoreactivity marked all neurons in the cortex, making all cortical layers recognizable (Fig. 2a). As expected, X-gal staining was mainly limited to layer 4. Importantly, nearly every neuron ($94.0 \pm 1.5\%$; $n = 3$ mice) in layer 4 of the barrel field had the Cre activity, as indicated by the presence of blue dots (Fig. 2b). To confirm this observation, we examined the colocalization of NeuN and β -galactosidase in layer 4 of the barrel field by using confocal microscopy. The majority of layer 4 neurons in this brain area contained β -galactosidase-immunoreactive particles (Fig. 2d). Because β -galactosidase particles are small, confocal images that have very thin depth likely miss the particles in some β -galactosidase-expressing neurons, thereby underestimating the colocalization of NeuN and β -galactosidase. Indeed, some neurons appear lacking β -galactosidase particles in one confocal image (Fig. 2d, arrows), but do have β -galactosidase particles in confocal images that are $2 \mu\text{m}$ away (Fig. 2c and e). In other cortical layers there were only a few scattered neurons with β -galactosidase activity. There were $6.5 \pm 1.1\%$ in layers 2/3 and $6.9 \pm 0.6\%$ in layers 5/6 NeuN-expressing neurons which were positive for X-gal staining. These observations indicate that the *Six3-cre#69* transgene can be used to delete genes of interest selectively in layer 4.

There are many subsets of GABAergic interneurons in layer 4. To determine if the *Six3-cre#69* transgene is expressed in these neurons, we examined β -galactosidase activity in a few subsets of interneurons by employing the following four specific markers: somatostatin, parvalbumin, calretinin, and neuropeptide Y (NPY). In layer 4 of the barrel field, β -galactosidase activity was detected in all of these subsets of interneurons (Fig. 3). Quantitative analyses in 3 mice revealed that blue dots were found in $77 \pm 2.5\%$ of somatostatin-immunoreactive interneurons, $76 \pm 2.1\%$ of parvalbumin-containing interneurons, $33 \pm 2.9\%$ of calretinin-containing interneurons, and $55 \pm 1.2\%$ of NPY-immunoreactive interneurons in this brain region. This result indicates that Cre is expressed in the majority of layer 4 GABAergic interneurons in the somatosensory cortex.

To determine when the *Six3-cre#69* transgene starts to express Cre recombinase in layer 4, we collected brains from *R26R/+;Six3-cre#69* mice at the ages of 2, 7, 14, and 21 days and examined expression of β -galactosidase in layer 4 of the barrel field using X-gal staining. Very few layer 4 cells expressed β -galactosidase at postnatal day 2 (P2) whereas the majority of layer 4 cells expressed β -galactosidase at the other three time points (Fig. 4). Therefore, recombination of the *R26R* locus mediated by the *cre* transgene in layer 4 mainly occurs during the first postnatal week.

In conclusion, we have identified a *cre* transgenic mouse line that expresses Cre recombinase in layer 4 of sensory cortical areas, especially in the somatosensory cortex. As far as we are aware, this represents the first *cre* transgene that is expressed in a specific cortical layer. Since the *Six3* gene has not been found to be expressed in layer 4 (Bovolenta *et al.*, 1998; Oliver *et al.*, 1995), the expression pattern of the *Six3-cre#69* transgene in the cerebral cortex likely results from an enhancer element located near the insertion site of the transgene. The timing of *Six3-cre#69* expression suggests that the enhancer becomes active only after layer 4 neurons migrate to their final destinations. More importantly, identification of this *cre* transgene makes it possible to delete genes selectively and postnatally in layer 4 of sensory cortical areas. Thus, the *Six3-cre#69* transgene may become a valuable tool for elucidation of the mechanism governing activity-dependent synaptic modifications in layer 4. For example, increasing neuronal activity in the whisker-to-barrel pathway in adult mice induces the expression of brain-derived neurotrophic factor (BDNF) as well as the formation of GABAergic synapses in layer 4 of the barrel field (Knott *et al.*, 2002; Rocamora *et al.*, 1996). The *Six3-cre#69* transgene

can be used to determine whether BDNF expressed in layer 4 is required for the activity-dependent synapse formation in the same layer.

Methods

The *Six3-cre#69* mouse line was kindly provided by Dr. Guillermo Oliver at the St. Jude Children's Research Hospital, Memphis, Tennessee. *ROSA26* mice were obtained from the Jackson Laboratory (Bar Harbour, ME, USA). Mice were maintained on standard mouse chow at 22 °C on a 12-hour light/12-hour dark cycle. The adult *R26R/+;Six3-cre#69* mice used in this study were from more than four different litters. All animal procedures were approved by the Georgetown University Animal Care and Use Committee.

Anaesthetized mice were transcardially perfused with 40 ml of phosphate-buffered saline (PBS), followed by 40 ml of 4% paraformaldehyde. One hour later, the animals were transcardially perfused with another 20 ml of PBS to wash out the paraformaldehyde. Their brains were removed from skulls and soaked in 30% sucrose in PBS at 4 °C until they sank. The brains were sectioned at 50 µm with a sliding microtome. Sections were collected into a 12-well tissue culture plate containing PBS and stored at 4 °C.

For X-gal staining, sections were incubated with X-gal staining solution (1X PBS, 2 mM MgCl₂, 0.02% nonidet P40, 0.01% sodium deoxycholate, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) at 37 °C for 6–16 hours. The sections were rinsed with PBS, fixed with 4% paraformaldehyde for 15 min, and then washed once with PBS. After the sections were mounted onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA, USA), they were counterstained with nuclear fast red, dehydrated with a series of ethanol, cleared in xylene twice (one minute each time), and coverslipped with the DPX mountant.

For combined X-gal staining and immunohistochemistry, X-gal stained sections were incubated with 10% methanol-3% hydrogen peroxide in Tris-buffered saline (10 mM Tris-Cl, 150 mM NaCl, pH 7.5) to quench endogenous peroxidase. Immunohistochemistry was then performed on the sections as described (Gharami *et al.*, 2007). Stained sections were mounted and coverslipped as described above. Antibodies to NeuN (Millipore, 1:500), NPY (Sigma-Aldrich, 1:4000), parvalbumin (Sigma-Aldrich, 1:2000), somatostatin (ImmunoStar, 1:1000), calretinin (Millipore, 1:1000), and β-galactosidase (Cappel Research Reagents, 1:3000) were obtained commercially.

Acknowledgements

We thank Dr. Guillermo Oliver for the *Six3-cre#69* mouse line. This work was supported by grants from the National Institute of Neurological Disorders and Stroke (NS050596), the Whitehall Foundation, and the American Diabetes Association to BX. Correspondence and requests for materials should be addressed to bx3@georgetown.edu.

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Figure 1.

Pattern of *ROSA26* recombination mediated by *Six3-cre#69*. Coronal brain sections from adult *R26R/+;Six3-cre#69* or *R26R/+* mice were stained for β -galactosidase activity (blue staining) and then counterstained with nuclear fast red. Brain images of *R26R/+;Six3-cre#69* mice in **a–k** are arranged in a rostral-caudal order. No blue staining was detected in brain sections from *R26R/+* mice (**l**). Abbreviations: Amy, amygdala; Au, auditory cortex; BS, brainstem; BST, bed nucleus of the stria terminalis; Cbl, cerebellum; DMH, dorsomedial hypothalamus; Hp, hippocampus; M1, primary motor cortex; MPO, medial preoptic nucleus; PAG, periaqueductal gray; Pir, piriform cortex; S1, primary somatosensory cortex; S1BF, S1 cortex barrel field; S1L, S1 cortex limb region; S2, secondary somatosensory cortex; SN, substantia nigra; Stm, striatum; Th, thalamus; V, visual cortex; VMH, ventromedial hypothalamus.

Figure 2.

Restriction of the Cre activity from the *Six3-cre#69* transgene to layer 4 of the somatosensory cortex. (a) X-gal staining (blue) and NeuN immunohistochemistry (brown) indicate that β-galactosidase activity is restricted to layer 4 of the S1 cortex barrel field. (b) The majority of NeuN-positive cells in layer 4 of the S1 cortex barrel field contain β-galactosidase activity (blue spots). Scale bar, 25 μm. (c–e) A series of confocal microscopy images at a 2-μm interval shows colocalization of NeuN (red) and β-galactosidase (green dots) in layer 4 neurons. Arrows denote neurons that appear lacking β-galactosidase particles in (d) but do express β-galactosidase, as shown in (c) or (e). Scale bar, 50 μm.

Figure 3.

Colocalization of Cre activity with markers for GABAergic interneurons in layer 4 of the S1 cortex barrel field. The activity of β -galactosidase resulting from Cre-mediated recombination of the *R26R* locus was detectable in layer 4 interneurons expressing somatostatin (**a**), parvalbumin (**b**), calretinin (**c**), and NPY (**d**). Arrows denote representative neurons expressing β -galactosidase and an interneuron marker. Scale bar, 25 μ m.

Figure 4.

Time course of Cre-mediated recombination in layer 4 of the sensory cortex. X-gal staining shows the activity of Cre recombinase in layer 4 of the S1 cortex barrel field in *R26R/+;Six3-cre#69* mice at postnatal day 2 (P2), 7 (P7), 14 (P14), and 21 (P21). The “4” in panel (a) marks the approximate position of layer 4. Scale bar, 50 μ m.