Transcription factor Lmo4 defines the shape of functional areas in developing cortices and regulates sensorimotor control

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ABSTRACT

Proper formation of the shape and size of cortical functional areas is essential for complex brain function, including sensory perception and motor control. Our previous work identified the transcription factor Lim domain only 4 (Lmo4), a regulator in calcium-dependent gene transcription, that has unique, region-specific expression in postnatal mouse cortices with high expression anteriorly and posteriorly but very low expression in between. Here we report that Lmo4 expression coincides with the timing of the development of the somatosensory barrel field. Lmo4 cortical deletion causes changes in expression patterns of cortical regional markers and results in rostro-medial shrinkage but not rostral or caudal shift of the somatosensory barrel subfield. Fine regulation of accurate shape of the barrel subfield by Lmo4, as well as Lmo4-mediated calcium-dependent gene expression, is critical for normal brain functions, as Lmo4-deficient mice display impaired sensorimotor performance. Moreover, even though Lmo4 has broad expression in the central nervous system, it plays a subtle role in the development of non-cortical regions. Our results reveal a new mechanism of cortical area formation and normal sensorimotor control that is regulated by genes with region-specific expression in the developing cortex.

Introduction

The human cerebral cortex was first mapped into more than 47 areas by histological descriptions such as cell shapes and packing density (Brodmann, 1909; Vogt, 1919). Recent studies, using modern imaging techniques, have demonstrated the existence and correlation of different anatomical and functional areas in the cortex (Bartels and Zeki, 2005; Toga and Thompson, 2001). By combining functional magnetic resonance imaging (MRI) with structural MRI, more than 100 anatomical and functional subdivisions are predicted in the human cerebral cortex (Van Essen et al., 1998). It is unclear how the size and shape of cortical functional areas are accurately organized in order to conduct complex behaviors.

The organization of anatomical and functional areas in mice is relatively simple but strikingly similar to that of humans (Rakic, 1988; Van Essen, 2002). For instance, there are motor (M), primary (S1) and secondary (S2) somatosensory, auditory (A) and visual areas (V) in the mouse cerebral cortex. This simple and parallel cortical organization makes the mouse an excellent model for the investigation, using genetic and molecular approaches, of how cortical areas are formed in humans.

A critical step in the formation of functional areas is to organize the developing forebrain into a three dimensional structure with anterior–posterior (A–P), dorsal–ventral (D–V) and left–right (L–R) features (Grove and Fukuchi-Shimogori, 2003; Levitt and Eagleson, 2000; O'Leary et al., 2007; O'Leary and Nakagawa, 2002; Rakic, 1988; Sun and Walsh, 2006; Sur and Rubenstein, 2005). This patterning event is partly controlled by molecules from patterning centers; for example, Fgf8 is secreted from the anterior cortical region (Fukuchi-Shimogori and Grove, 2001; Garel et al., 2003). These molecules induce downstream gene expression, usually with gradients along the A–P axis in the cortex (Monuki and Walsh, 2000). Specifically, Pax6 is expressed in an anterior-high and posterior-low gradient, while Emx2 and an orphan nuclear receptor COUP-TFI are expressed in an anterior-low and posterior-high gradient (Bishop et al., 2002; Liu et al., 2000; Zhou et al., 2001). Mutations in Pax6, and Emx2 and Coup-TF1 produce expansion and shrinkage of posterior areas of the cortex, respectively (Armentano et al., 2007; Bishop et al., 2000; Fukuchi-Shimogori and Grove, 2003; Hamasaki et al., 2004; Mallamaci et al., 2000).

Proper cortical area formation is essential for normal brain function, which is realized through connections within the cortex

and between the cortex and other brain regions. For example, in Fgf8 hypomorphic mutants, in which the anterior cortical areas are shrunken, neurons located in the posterior cortical areas ectopically project axons into the anterior (Huffman et al., 2004). In cortices where Fgf8 is misexpressed, the thalamic axons follow ectopic area cues and innervate a duplicated somatosensory cortical region (Shimogori and Grove, 2005). Moreover, altering the organization of functional areas at embryonic stages, and perhaps subsequently the intra-cortical and thalamocortical connections, can affect mouse behaviors in the adult. A recent study has shown that a larger or smaller visual cortex, modified by Emx2 expression levels, can cause impaired sensory detection and motor control (Leingartner et al., 2007).

Extensive studies have revealed the role of transcription factors that have gradient cortical expression, for instance Pax6 and Emx2, in the organization of functional areas (O'Leary et al., 2007). In our previous work, we identified a transcription factor Lim domain only 4 (Lmo4) that has high expression in the anterior and posterior cortical region but very low or no expression in between in murine postnatal day 1 (P1) cortices (Sun et al., 2005). Lmo4 cortical region-specific expression is different from other transcription factors that normally have gradient expression. In this study we analyzed Lmo4 function in the organization of cortical functional areas. Using mouse genetics, we show that deleting Lmo4 cortical expression disrupts expression patterns of cortical regional markers, alters the shape of the barrel subfield and results in impaired sensory perception and motor coordination. Lmo4 function appears to be cortex-specific, since Lmo4 deficiency in other regions in the central nervous system (CNS) does not show strong defects, for example in the cerebellum. Thus, transcription factor Lmo4, with unique cortical region-specific expression, likely cooperates with those having gradient expression and defines the precise shape and size of cortical functional areas.

Materials and methods

Mouse lines and genotyping

The floxed Lmo4 line was maintained in a C57BL/6×129 background (Hahm et al., 2004) and the Nestin-Cre and Emx1-Cre lines were maintained in a C57/B6 background. They were bred to produce homozygous conditional knockout mice (Lmo4$^{Cre/flox}$). Mouse tail DNA was used for genotyping by PCR reactions.

The floxed Lmo4 allele was detected by the primer A (5′-CGAGCTGAAATTGTCAGCAGCAAG-3′) and the primer B (5′-CGAGCTGCTGCCCGGATTCAC-3′), which produces a 500 bp product. The wild type allele produces a 350 bp product. The Nestin-Cre and Emx1-Cre lines were genotyped by PCR reactions using the primer-F: 5′-TAAAGATATCTCACGTACTGACGGTG-3′ and the primer-R 5′-TCTCTGACCAGAGTCATCCTTAGC-3′, which produce a 350 bp product.

Heterozygous mice did not show any phenotype and were used as controls. For staging of embryos, midday of the day of vaginal plug formation is considered as embryonic day 0.5 (E0.5), the first 24 h after birth are defined as postnatal day 0 (P0).

Tissue preparation and in situ hybridization

Collected brain samples were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) and soaked in 25% Sucrose in PBS. Coronal and sagittal sections were collected using a cryostat.

Digoxygenin (DIG)-labeled sense and antisense mRNA probes were produced by in vitro transcription. The in situ hybridization was performed as described (Sun et al., 1998). Briefly, the sections were hybridized at 65 °C overnight and then washed. After blocking for 2 h, sections were labeled with anti-DIG antibody (1:1500 dilution, Roche) at 4 °C overnight and then washed and stained with BM purple (Roche) at room temperature until ideal intensity. The images of in situ hybridization were collected using a Leica digital camera under a dissection scope (Leica, MZ16F).

Cytochrome oxidase (CO) histochemistry

Mice at P14 were perfused with 4% PFA and postfixed in 4% PFA overnight. The cerebral cortices were flattened during the fixing process by gently pressing them between two clean, distance-adjustable microscope slides. 200–300 μm sections were tangentially collected by a vibratome (Leica). Coronal sections in the brain stem were collected using a vibratome. Sections were incubated in the CO staining buffer: 4 g sucrose, 50 mg diaminobenzidine (DAB) (Sigma), 30 mg cytochrome oxidase C (Sigma), 15 mg catalase (Sigma) in 100 ml 0.1 M (0.2 M stock) phosphate buffer (pH 7.4). Sections were stained at room temperature in the dark until barrels became visible.

Thalamocortical axonal tracing

P0 mice were perfused and postfixed in 4% PFA overnight at 4 °C. Single crystals of the fluorescent carbocyanide dye DiI (1,1′-dioctadecyl 3,3,3′,3′-tetramethylindocarbocyanine perchlorate; Invitrogen) were placed in the somatosensory region in the cortex or in the dorsal thalamus. To allow the DiI to diffuse, brains were kept in 4% PFA in the dark at 37 °C for 4–7 weeks. 60–120 μm coronal sections were collected using a vibratome and counterstained with Hoechst 33258. Images of DiI-labeled thalamic cell bodies or axons were captured using a Leica digital camera under a fluorescent microscope (Leica DMI6000B).

Mapping the position and the shape of the barrel subfield

The left and right hemispheres were separated at the midline. Tangential sections were collected from flattened cortices and stained with the CO staining buffer. Using NIH ImageJ software, the length from the anterior edge of the barrel subfield to the most anterior cortex, and the length from the posterior edge of the barrel subfield to the most posterior cortex were normalized by the full length of the cortex to form ratios. The shape of the barrel subfield was assessed by measuring the distance between barrels e4 to a4 and the distance between barrels β to e12. The circumferences of the barrels (α, β and γ) in the most posterior row in the barrel subfield were measured. The ratios of the circumferences between the control and Lmo4 knockout mice were calculated.

Behavioral tests

Adult mice (at least ten weeks old) of Lmo4 conditional knockouts created with Nestin-Cre and Emx1-Cre lines and normal controls (wild type or heterozygous littermates) were used. Body weight for each mouse was measured before each test. At least 5 male and 5 female animals were randomly chosen in each genotyping group for performing the following tests:

1. The hanging wire test: The mouse was placed on a wire cage lid and the lid was gently waved in the air to induce the mouse to grip the wire. The lid was then turned upside down, approximately 6 in. above a surface of soft bedding material. Latency time to fall onto the bedding was recorded.
2. The rotarod test: Mice were put on a rotating cylinder of 3 cm diameter. Mice were trained at low rotational speeds (12 rpm) up to 5 times or until they could stay on the rod for 2 min. They were next tested on a rod that accelerates from 8 to 45 rpm in 3 min. Mice were tested in three trials. The mean latency time and speed at drop-off for the three trials were statistically analyzed using the unpaired Student's t test, one-way ANalysis Of VAriance between groups (ANOVA) and post hoc multiple comparison tests.

3. *The adhesive removal test*: To avoid distraction, mice were tested in their home cage without cage mates, food or water supplies. Adhesive patches (6 mm diameter) were placed on the dorsal surface of each hindpaw. Mice were then replaced in the home cage. Latency to contact and remove the patch on either hindpaw was recorded. Mice were tested for four trials with one minute for each trial and a one-hour rest period between two trials. The unpaired Student's *t* test was performed for statistical analyses.

4. *Footprint analysis*: Mice were trained three times to walk along a 50-cm-long, 10-cm-wide paper (with 15-cm-high walls on either side). Before the fourth trial, their hind paws were stained with black ink. Stride length and width were measured from seven consecutive steps on a paper covering the floor of their walking path as previously described (Patel and Hillard, 2001).

Results

Transcription factor Lmo4 has dynamic and region-specific expression in mouse developing cortices

Our previous work identified the transcription factor *LMO4* that shows high expression in the perisylvian region with an asymmetric pattern in human fetal brains (Sun et al., 2005). To test the role of *LMO4* in the formation of distinct cortical functional areas in mice, we examined its expression pattern in developing mouse brains.

In coronal sections of an embryonic day 12.5 (E12.5) brain, *Lmo4* expression was detected in the preplate in the cortex (Fig. 1A). *Lmo4* was also expressed in the striatum in the ventral forebrain. At E15.5 *Lmo4* expression was detected in the cortical subventricular zone (SVZ) and in the hippocampus (Fig. 1B). At postnatal day 0 (P0) *Lmo4* was mostly expressed in postmitotic neurons in the cortical plate (Fig. 1C).

Lmo4 cortical expression is restricted to distinct regions with well-defined boundaries at P0 when thalamic axons just reach the cortical plate in the somatosensory area (Lopez-Bendito and Molnar, 2003). *Lmo4* was strongly expressed in anterior and posterior regions with clear boundaries but not the medial area in between, as shown in sagittal sections of P0 cortices (Fig. 1C, arrowheads). The anterior and posterior *Lmo4* expression regions corresponded approximately to regions that would later become the motor cortex and the visual cortex, respectively (Fig. S1). The region (gap) between the zones of high *Lmo4* expression appears to correspond approximately to the presumptive somatosensory cortex (Fig. 1C and Fig. S1). *Lmo4* cortical region-specific expression suggests that it may play a role in controlling cortical functional area formation.

Interestingly, while the gap between *Lmo4* anterior and posterior expression was still detectable, *Lmo4* expression emerged in layer 3/4 in the medial cortical region at P5, a time when thalamic axons form synaptic connections with layer 4 neurons in the cortex (Fig. 1D) (Lopez-Bendito and Molnar, 2003). Later on, at P10 when the initial development of the somatosensory cortex is complete (Lopez-Bendito and Molnar, 2003), *Lmo4* was expressed in the deeper and upper layers across the entire cortex (Fig. 1E). The dynamic and region-specific expression of *Lmo4* during a critical period of connections of thalamocortical axons indicates that Lmo4 may control somatosensory cortical development.

The boundaries of cortical functional areas are perturbed in CNS-specific Lmo4 knockout mice

Previous studies reported that *Lmo4* null mutation resulted in embryonic lethality (Hahm et al., 2004; Lee et al., 2005; Tse et al., 2004). In agreement with previous reports, we found that most *Lmo4* null mice (*Lmo4$^{-/-}$*) died during embryogenesis. Even though a few *Lmo4* null mice were born, they died within two hours. *Lmo4* mutation resulted in exencephaly during brain development (Fig. S2). It is not
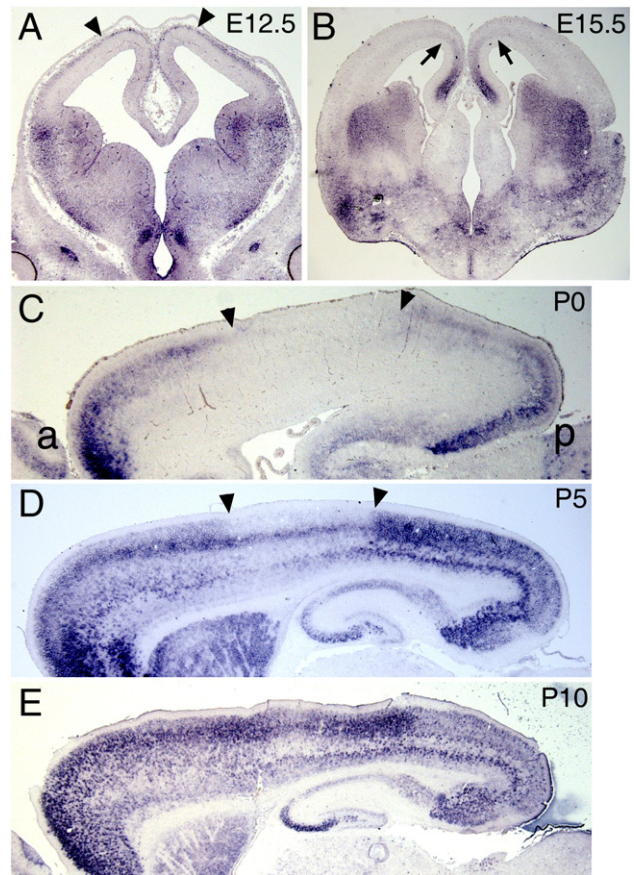

Fig. 1. Lmo4 has dynamic and region specific expression in mouse developing cortices. (A) *Lmo4* was weakly expressed in the preplate in the cerebral cortex (arrowheads) and in the striatum in the ventral cortical region at E12.5, as detected by *in situ* hybridization in coronal sections. (B) *Lmo4* expression was detected in the subventricular zone (arrows) in E15.5 cortices. (C) In P0 sagittal sections, *Lmo4* expression was observed in the anterior (a) and posterior (p) cortical regions with clear boundaries but not in between (arrowheads). (D, E) While *Lmo4* region-specific expression was still detectable at P5 (arrowheads), its expression appeared in layer 3/4 in the medial cortical region, and later in the entire cerebral cortex at P10.

clear whether the closure defect in the dorsal brain is caused by abnormal neural crest development or abnormal brain formation.

To overcome the severe phenotype of *Lmo4* null mice, which prevent further study of Lmo4 function in regulating cortical functional area formation, we generated *Lmo4* conditional knockout mice using the *Cre-loxp* system. To delete *Lmo4* expression only in the central nervous system (CNS), we bred floxed *Lmo4* mice with the *Nestin-Cre* line (Figs. S3A and B) (Zimmerman et al., 1994).

If Lmo4 controls the organization of cortical functional areas, deleting *Lmo4* in the CNS should alter the expression pattern of known cortical regional markers, such as Cdh8 and Id2 (Bishop et al., 2002). In sagittal sections of E18.5 control cortices (wild type or heterozygote), *Cdh8* was expressed in the deeper layer across the entire cortex and in the upper layer in the anterior region (Fig. 2A). However, *Cdh8* anterior expression in the anterior upper layer was greatly reduced in *Lmo4* conditional knockout cortices (*Lmo4$^{Nestin-Cre/flox}$*) (Fig. 2B, arrowhead). *Id2* expression can be divided in three patterns in E18.5 sagittal cortical sections: anterior-medial expression in the upper layers (asterisk), posterior-medial expression in the deeper layers (layer 5) (arrowhead) and posterior expression in the upper layers (arrow) (Fig. 2C). In *Lmo4$^{Nestin-Cre/flox}$* cortices, while *Id2* anterior and posterior expression in the upper layers did not change, *Id2* posterior-medial expression in layer 5 expanded rostrally (Fig. 2D, arrowhead).

We next examined the expression pattern of *EphrinA5* and its receptor *EphA7*, which also show region-specific expression in developing cortices (Miller et al., 2006; Rash and Grove, 2006; Torii and Levitt, 2005). *EphrinA5* and its receptor *EphA7* have opposite expression in E18.5 cortices with *EphrinA5* expression in the medial and *EphA7* in the anterior and posterior cortical region (Figs. 2E and G). While the posterior boundary between the *EphrinA5* and *EpahA7* expression did not change significantly, the anterior boundary expanded rostrally in *Lmo4^Nestin-Cre/flox* cortices (Figs. 2F and H, red arrowheads). Thus *Lmo4* cortical deletion causes a rostral shift of cortical regional markers.

The shape of somatosensory barrel subfields is abnormal in CNS-specific Lmo4 knockout mice

Because *Lmo4* is expressed in specific cortical regions and CNS *Lmo4* deletion alters expression patterns of cortical regional markers (Figs. 1 and 2), we examined whether Lmo4 affects the position and shape of the somatosensory barrel field in the cortex.

Barrels are specialized structures in layer 4 of the primary somatosensory cortex that correspond to representations of individual whiskers on the mouse face with clear and well-defined patterns (Armstrong-James and Fox, 1987; Simons, 1978; Welker and Woolsey, 1974; Woolsey and Van der Loos, 1970). Individual barrels consist of the barrel center, formed by afferents from the ventrobasal (VB) complex of the thalamus, surrounded by layer 4 neurons arranged in a circular pattern that form the barrel "walls". Since the thalamic afferents can be preferentially stained for markers such as cytochrome oxidase (CO), the pattern of barrels in the barrel fields can be used as landmarks in the primary somatosensory cortex.

Barrel fields were readily detected in the *Lmo4^Nestin-Cre/flox* cortex using CO histochemistry at P14 when the barrel field development is complete (Fig. 3). We mapped the relative position of the posteromedial barrel subfield (PMBSF) in flattened cortical sections, because the barrel numbers and patterns in the PMBSF are well defined. The length from the anterior edge of the barrel subfield to the most anterior cortex, and the length from the posterior edge of the barrel subfield to the most posterior cortex were normalized by the full length of the cortex to form ratios (Figs. 3A–C). The relative position of the PMSBF in the cortices did not show significant differences between control and *Lmo4^Nestin-Cre/flox* mice, indicating that *Lmo4* cortical deletion does not shift the barrel subfield rostrally or caudally (Fig. 3C).

We next examined the barrel morphology and the shape of the PMBSF. While the individual barrels were solid and had clear edges in controls, they were blurry in *Lmo4^Nestin-Cre/flox* cortices (Figs. 3D and E). Barrels β and γ in the most posterior row were also enlarged in *Lmo4^Nestin-Cre/flox* cortices (Figs. 3E and H). We further mapped the shape of the barrel subfield by measuring the length and the width of the PMBSF. We calculated the ratio of the distance between barrels e4 to a4 and the distance between barrels β to e12 (Fig. 3D). The shape of the PMBSF was altered in *Lmo4^Nestin-Cre/flox* cortices, with narrowed width but unchanged length, resulting in decreased PMBSF area (Figs. 3F and G).

Lmo4 anterior and posterior expression has clear boundaries with an angle against the cortical midline, leaving a trapezoid-shaped *Lmo4* non-expression region (Fig. S1 and Fig 3I). *Lmo4* deletion, instead of altering the cortical position of the barrel subfield along the anterior–posterior axis, caused shrinkage of the PMBSF with an angle along the rostral–medial and caudal–lateral axis (Fig. 3I, arrows). Thus, although *Lmo4* cortical deletion does not shift the entire barrel subfield rostrally or caudally, it alters the shape of the somatosensory barrel subfield.

CNS-specific Lmo4 deletion causes weak somatosensory connections

Individual whiskers on the mouse face are innervated by neurons that reside in the trigeminal ganglion. Sensory inputs from distinct facial regions are conveyed to the somatosensory cortex via the brain
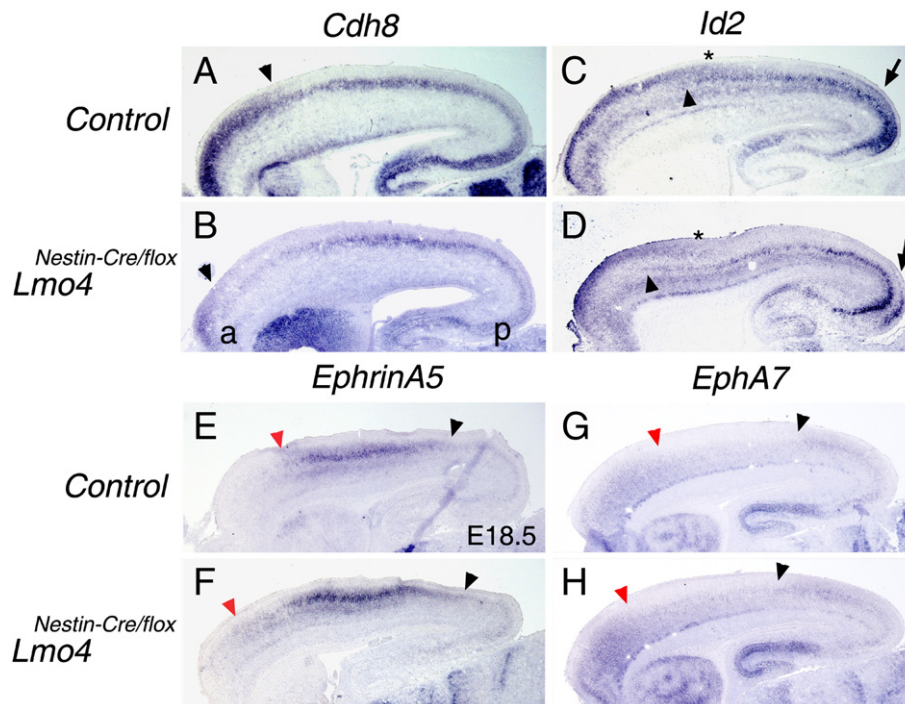

Fig. 2. Expression patterns of cortical regional markers are altered in E18.5 *Lmo4* conditional knockout mice bred with the *Nestin-Cre* line (*Lmo4^Nestin-Cre/flox*). (A, B) Compared to normal controls (wild type or heterozygote) in sagittal sections, *Cdh8* anterior expression (arrowheads) in the upper layer was reduced in *Lmo4^Nestin-Cre/flox* cortices. (C, D) *Id2* expression in layer 5 (arrowheads) was shifted rostrally in *Lmo4^Nestin-Cre/flox* cortices, while its expression in the anterior (asterisk) and posterior (arrow) region in upper layers did not change. (E–H) The boundaries of anterior expression (red arrowheads) of *EphrinA5* and its receptor *EphA7* were shifted rostrally in *Lmo4^Nestin-Cre/flox* cortices.
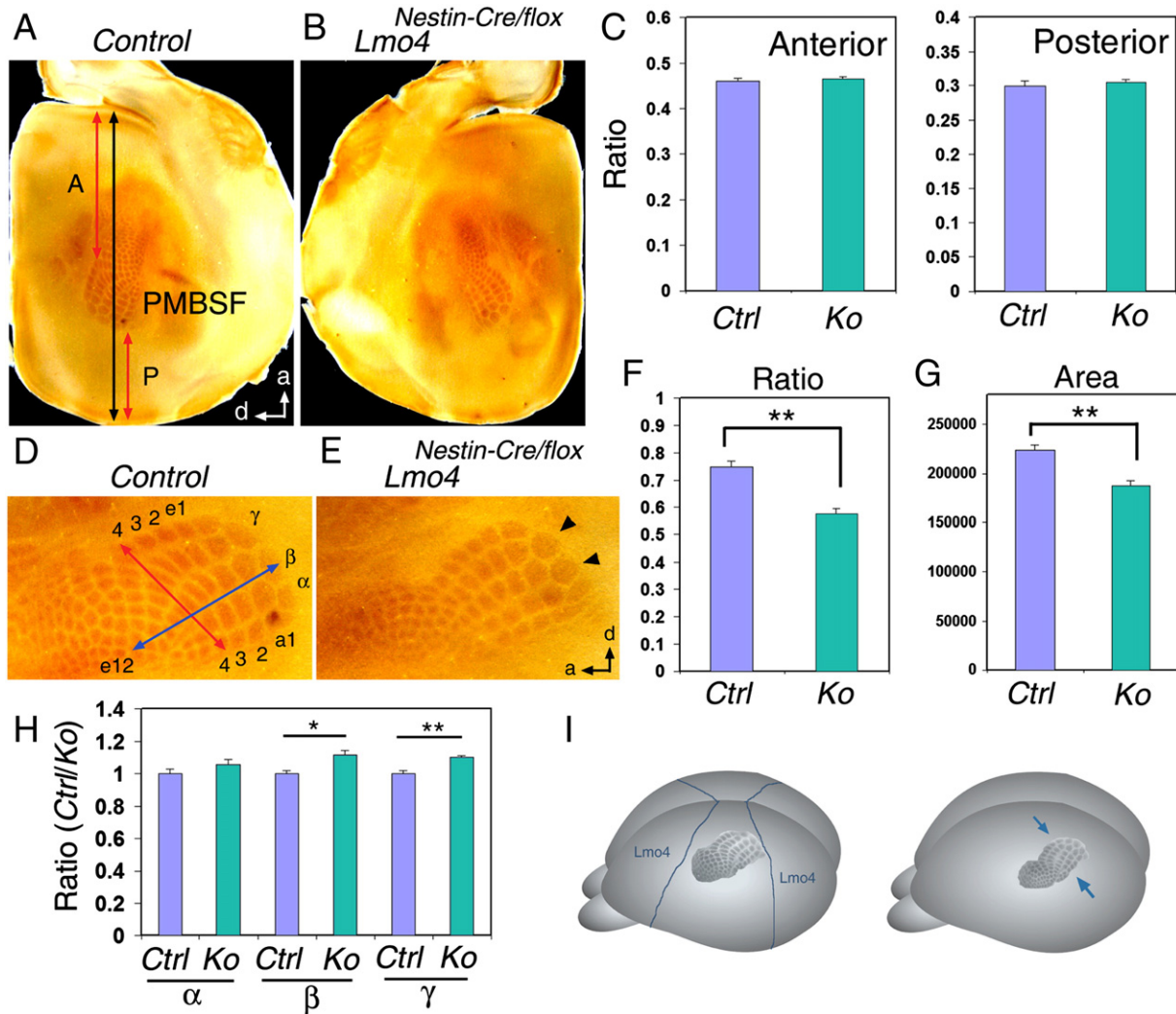
Fig. 3. The shape of the somatosensory barrel subfield is altered in P14 $Lmo4^{Nestin-Cre/flox}$ cortices. (A, B) $Lmo4$ cortical deletion does not change the position of the barrel subfield in the cortex. The barrel field was visualized in flattened, tangentially sectioned sections stained with cytochrome oxidase (CO) histochemistry. The length of the anterior edge of the posteromedial barrel subfield (PMBSF) to the most anterior cortex was measured and normalized with the full length of the cortex, defined as the ratio of A (anterior). Similarly, the posterior location of the PMBSF was mapped and defined as the ratio of P (posterior). (C) Neither the anterior nor posterior ratios showed significant differences between the control (Ctrl, $n=6$) and $Lmo4^{Nestin-Cre/flox}$ (Ko, $n=6$) mice. (D, E) In flattened tangential sections stained with CO histochemistry, the barrel patterns in $Lmo4^{Nestin-Cre/flox}$ cortices were not as clear as in controls. (F) The ratio of the distance between barrels e4 to a4 and the distance between barrels β to e12 in the PMBSF were calculated in Ctrl ($n=8$) and Ko ($n=10$) mice. While the length of the PMBSF was not changed, the width was narrowed in Ko cortices (**: $p<0.0001$ in unpaired Student's t test). (G) The area of the PMBSF in Ko ($n=10$) mice was smaller than that in Ctrl ($n=8$) (**: $p<0.0003$). (H) Barrels β and γ in the most posterior row were enlarged in $Lmo4^{Nestin-Cre/flox}$ cortices (arrowheads in E). The ratio of circumferences of each barrel in Ctrl and Ko was calculated. $n=5$, *: $p<0.02$; **: $p<0.009$. (I) A model of Lmo4 function in regulating the shape of the somatosensory barrel subfield. $Lmo4$ anterior and posterior expression has an angle against the midline. $Lmo4$ region-specific expression defines the shape of the presumptive barrel subfield from the cortical midline toward the lateral cortex. $Lmo4$ deletion causes shrinkage of the PMBSF along the rostral–medial and caudal–lateral axis (arrows). The dorsal (d) and anterior (a) cortical regions are labeled in panel A and E.

stem and thalamus (Armstrong-James and Fox, 1987; Simons, 1978; Welker and Woolsey, 1974; Woolsey and Van der Loos, 1970). Because $Lmo4$ is deleted in the entire CNS, we examined whether the somatosensory input pathway is affected by $Lmo4$ deletion. In coronal sections of the control brain stem, five rows of large barrelettes and a cluster of small barrelettes were detected using CO histochemistry (Fig. 4A). However, both large and small barrelettes were blurry and the barrelette pattern was not clearly detectable in the brain stem of $Lmo4^{Nestin-Cre/flox}$ mice (Fig. 4B). These results suggest that $Lmo4$ deletion affects accurate connections of whisker sensory inputs in the brain stem.

The whisker sensory inputs from the brain stem reach the ventrobasal (VB) complex in the dorsal thalamus (Lopez-Bendito and Molnar, 2003). The axons from the VB then project sensory information to layer 4 neurons in the barrel cortex (Welker and Woolsey, 1974; Woolsey and Van der Loos, 1970). We mapped the thalamocortical projections by placing a DiI crystal in the VB of P0

control and $Lmo4^{Nestin-Cre/flox}$ mice. The thalamic axons reached the cortical plate and formed connections with layer 4 neurons in both control and $Lmo4^{Nestin-Cre/flox}$ cortices at P0 (Figs. 4C and D) and P14 (data not shown). Similarly axon projections from the cortex, retrogradely labeled by placing the DiI crystal in the somatosensory region, also reached the VB in the thalamus of $Lmo4^{Nestin-Cre/flox}$ mice (Figs. 4G and H). Thus CNS $Lmo4$ deletion does not affect the projections of thalamocortical axons. However, innervation of thalamic axons in $Lmo4^{Nestin-Cre/flox}$ cortices was not as clear as in controls, implying that the strength of synaptic connections is likely affected by $Lmo4$ deletion (Figs. 4E and F).

CNS-specific Lmo4 deletion causes impaired sensorimotor control

Cortical functional areas control various physiological functions, including motor and sensory functions (Grove and Fukuchi-Shimogori, 2003; O'Leary et al., 2007; Rakic, 1988; Sur and Rubenstein, 2005).

Because CNS *Lmo4* deletion results in altered expression of cortical regional markers and narrowed barrel subfields, we examined whether the lack of *Lmo4* expression in embryonic stages causes behavioral changes in the adult.
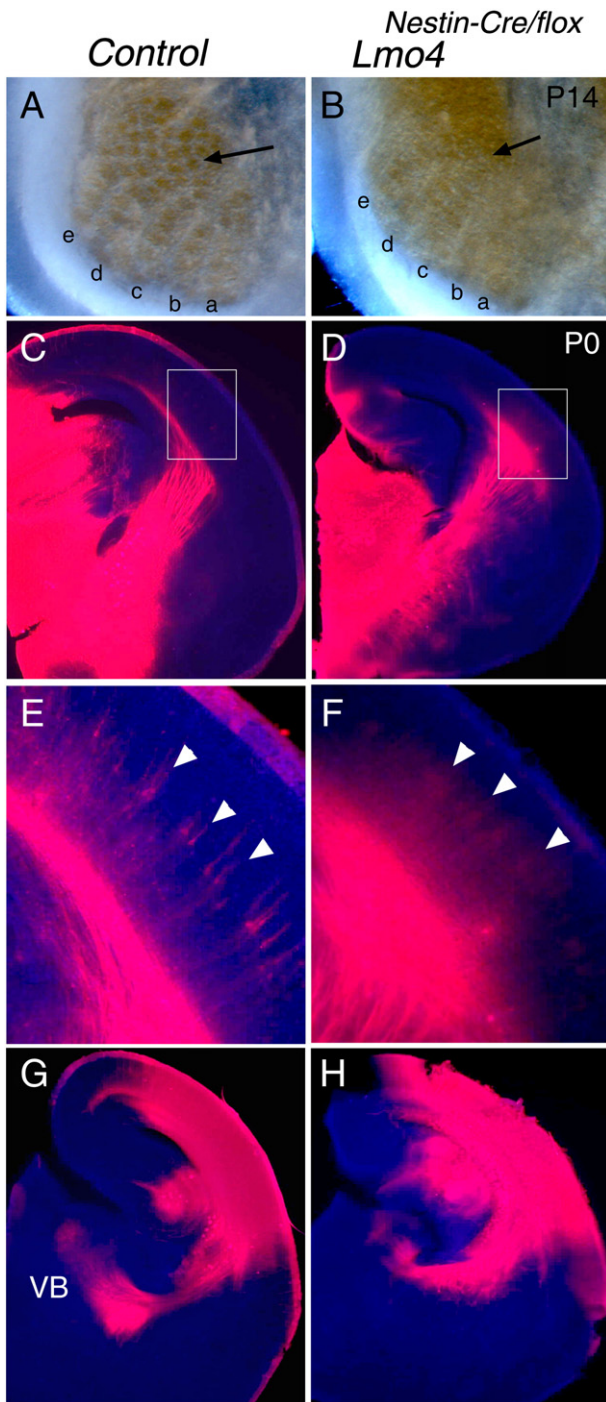

Fig. 4. Weak somatosensory connections in *Lmo4^Nestin-Cre/flox* mice. (A, B) *Lmo4* deletion affects the formation of barrelettes in the brain stem. In coronal sections of P14 brain stems with CO histochemistry, the large barrelettes (row-a to row-e) and small barrelettes (arrows) were detectable in the control but not in the *Lmo4^Nestin-Cre/flox* mice. (C, D) The thalamic axons reached the cortical plate and formed connections with layer 4 neurons. The thalamic axons were visualized by placing a DiI crystal in the ventrobasal (VB) complex of the thalamus of both control and *Lmo4^Nestin-Cre/flox* brains. (E, F) Innervation of thalamic axons (arrowheads) in *Lmo4^Nestin-Cre/flox* cortices was not as clear as in controls under a high power view of highlighted regions in C and D. (G, H) Axon projections from the cortex, retrogradely labeled by placing the DiI crystal in the somatosensory region, also reached the VB in the thalamus of control and *Lmo4^Nestin-Cre/flox* mice.
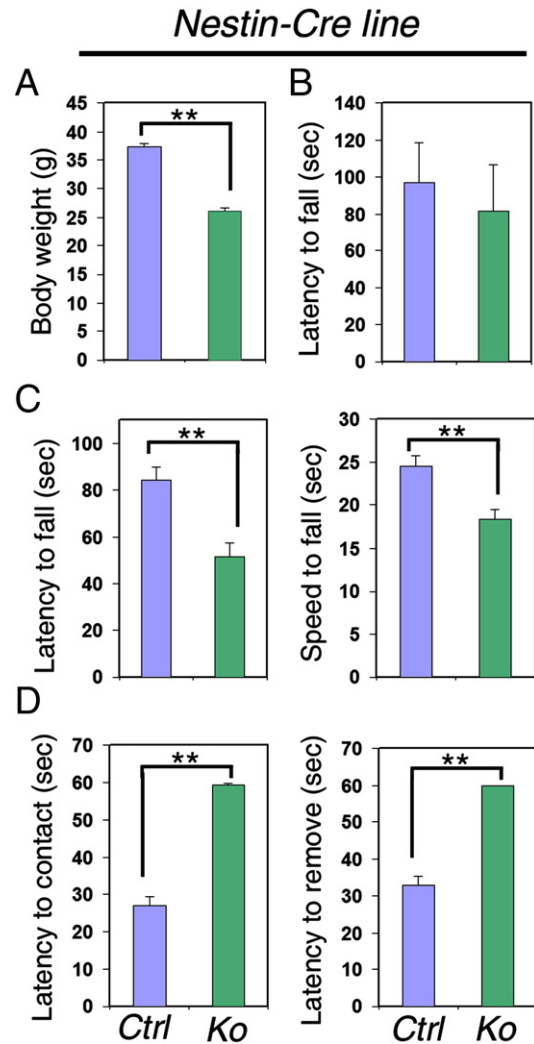

Fig. 5. CNS-specific *Lmo4* deletion causes abnormal mouse sensorimotor control. (A) *Lmo4^Nestin-Cre/flox* mice (*Ko*, $n = 10$) had smaller body weights when compared to control mice (*Ctrl*, $n = 10$) (*Ctrl*: 37.39±0.53 grams (g); *Ko*: 26.17±0.56 g, **: $p < 0.0001$). (B) The hanging wire test: *Lmo4^Nestin-Cre/flox* mice had normal muscle strength, detected by the latency to fall (*Ctrl*: 96.73±22.02 seconds (s); *Ko*: 81.58±25.17 s, $p < 0.6559$). (C) The rotarod test: *Lmo4^Nestin-Cre/flox* mice had poor motor coordination. Latency to fall off: *Ctrl*: 84.17±5.86 s; *Ko*: 51.37±6.12 s, **: $p < 0.0007$. Speed to fall off: *Ctrl*: 24.58±1.16 rpm; *Ko*: 18.4±1.19 rpm, **: $p < 0.0007$. (D) The adhesive removal test: *Lmo4^Nestin-Cre/flox* mice had impaired sensory perception and motor control. Latency to contact: *Ctrl*: 26.97±2.45 s; *Ko*: 59.43±0.44 s, **: $p < 0.0001$. Latency to remove: *Ctrl*: 32.83±2.41 s; *Ko*: 60±0 s, **: $p < 0.0001$.

We found that *Lmo4* conditional knockout mice (*Lmo4^Nestin-Cre/flox*) had low mobility, even though they can survive after birth (Fig. S4). *Lmo4* conditional knockout mice had smaller body weights than control littermates (wild type and heterozygote) (Fig. 5A). They tended to hide in the corner of the cage instead of moving around. The eyelids of *Lmo4^Nestin-Cre/flox* mice were closed most of time (Fig. S4).

We performed the hanging wire test to assess the muscle strength. The *Lmo4^Nestin-Cre/flox* mice displayed normal muscle strength, even though they had smaller body weights (Figs. 5A and B). We then conducted the rotarod test to examine motor coordination. In the training sessions, *Lmo4^Nestin-Cre/flox* mice learned to stay on a rotating cylinder with a constant 12 rpm speed just like their littermate controls. However, during the test sessions, on the rotating cylinder with speed accelerating from 8 to 45 rpm, *Lmo4^Nestin-Cre/flox* mice fell off the moving rod after a shorter latency time and at a lower speed than did the controls (Fig. 5C).

We next performed the adhesive removal test (Leingartner et al., 2007). By placing adhesive patches (6 mm diameter) on the dorsal
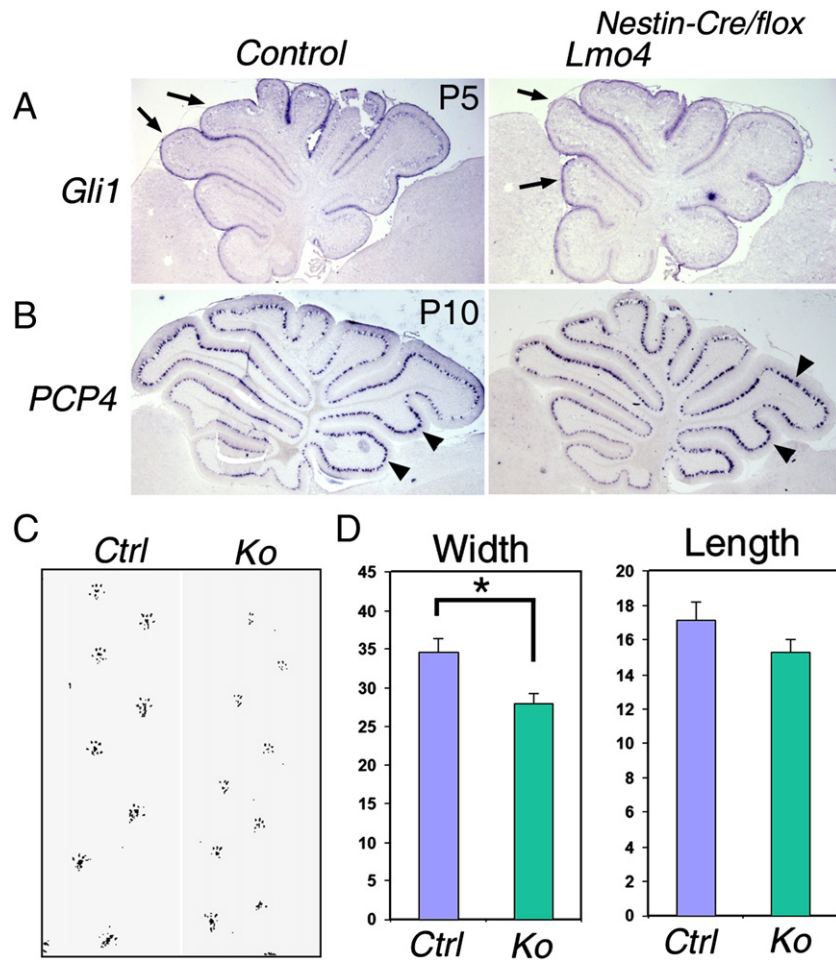
Fig. 6. The development and function of the cerebellum is normal in *Lmo4* conditional knockout mice bred with the *Nestin-Cre* line (*Lmo4*^{*Nestin-Cre/flox*}). (A, B) The expression pattern of *Gli1*, labeling granule neuron progenitors in the external germinal layer (arrows), and *PCP4*, labeling Purkinje cells (arrowheads), was normal in sagittal sections of the P5 *Lmo4*$^{Nestin-Cre/flox}$ cerebellum compared to controls. (C, D) The footprint test: The gait pattern was indistinguishable between *Lmo4*$^{Nestin-Cre/flox}$ (*Ko*, *n*=10) and control (*Ctrl*, *n*=10) mice, even though the average step width was reduced in *Lmo4*$^{Nestin-Cre/flox}$ mice. Step width: *Ctrl*: 34.53±1.81 millimeter (mm); *Ko*: 27.92±1.41 mm, *: $p<0.01$; step length: *Ctrl*: 17.17±1.04 mm; *Ko*: 15.27±0.74 mm, $p<0.1552$.

surface of each hindpaw, a mouse will contact the patch with its snout and eventually use its teeth to remove it. *Lmo4*$^{Nestin-Cre/flox}$ mice took a longer time to contact and remove the patches than controls (Fig. 5D). Our results show that CNS *Lmo4* deletion results in behavioral abnormalities related to sensorimotor functions.

Lmo4 plays a subtle role in the development of non-cortical regions in the CNS

Lmo4 conditional knockout mice generated by the *Nestin-Cre* line had smaller body weights, lower mobility and poorer sensorimotor performance (Fig. 5 and Fig. S4). To test whether these defects are caused by *Lmo4* deletion in non-cortical regions in the CNS, we first examined *Lmo4* expression patterns in the developing CNS. *Lmo4* expression was detected in various CNS regions (Fig. S5). In sagittal sections of the E11.5 brain, *Lmo4* was highly expressed in the most anterior region and weakly expressed in the posterior region of the cerebral cortex (Fig. S5A). At E15.5, *Lmo4* was expressed in the anterior and posterior cortical region, in the hippocampus and in the subcortical regions such as the striatum (Fig. S5B). *Lmo4* was expressed in the developing cerebellum, with highest expression in Purkinje cells at E15.5 and P5 (Figs. S5C and D).

We next examined whether *Lmo4* CNS deletion affects normal development of the cerebellum. In sagittal sections of the P5 cerebellum, the dividing granule neuron progenitors, labeled with

Gli1, were detected in the external germinal layer of both controls and knockouts (Fig. 6A). The Purkinje cells, labeled with *PCP4* (Purkinje cell protein 4), were also observed in the *Lmo4*$^{Nestin-Cre/flox}$ cerebellum at P10 (Fig. 6B). Moreover, the cerebellum of *Lmo4*$^{Nestin-Cre/flox}$ had normal morphology compared to controls in the adult (data not shown).

Because the cerebellum is essential for motor coordination, to further assess the proper function of the cerebellum, we performed the footprint analysis. The footprint test measures walking gait patterns by letting mice whose hind paws are stained with black ink walk across a piece of paper (Fig. 6C). The average step length and the gait pattern appeared normal in *Lmo4*$^{Nestin-Cre/flox}$ mice, even though the average step width was reduced (Figs. 6C and D). The reduction of step width is likely due to the smaller body size of the *Lmo4* knockout mouse and not because of defects in the cerebellum. Thus CNS *Lmo4* deletion does not appear to affect cerebellum development and function.

Strong *Lmo4* expression was also detected in the hindbrain region in the E15.5 brain (Fig. S5B). To examine whether proper *Lmo4* expression is essential for sensory neuron development in the hindbrain, we looked at the expression pattern of three markers in *Lmo4* null (*Lmo4*$^{-/-}$) and *Lmo4*$^{Nestin-Cre/flox}$ mice. A homeobox gene *Drg11* is critical for pain-related sensory neuron formation, and transcription factor *Phox2b* and *Rnx* are important for noradrenergic neuron development, which is essential for autonomic reflexes such as

cardiovascular and respiratory functions (Chen et al., 2001; Pattyn et al., 1999; Qian et al., 2001). We found that the *Rnx* expression level was decreased in *Lmo4*^{−/−} and *Lmo4*^{Nestin-Cre/flox} hindbrains but its expression pattern was not altered (Figs. S6A and B). Moreover, we did not detect significant difference in *Drg11* nor *Phox2b* expression at both early (E11.5) and late (E18) developmental stages (Fig. S6). Lmo4 may modify sensory neuron development but its function is not required for sensory neuron formation in the developing hindbrain.

In addition to *Lmo4* expression in the cerebral cortex, *Lmo4* is also expressed in other CNS regions including the cerebellum and the hindbrain. However, *Lmo4* function in the development and function of non-cortical regions in the CNS is subtle.

Lmo4 plays a unique role in the development and function of the somatosensory cortex

Lmo4 CNS deletion results in a shift of cortical regional markers, abnormal barrel field development and poor sensorimotor control. To further distinguish whether these abnormalities are caused by *Lmo4* deletion in the cortex or in other CNS regions, we created a second *Lmo4* conditional knockout line using *Emx1-Cre* mice (*Lmo4*^{Emx1-Cre/flox}), because the activity of the *Emx1* promoter is restricted in the cortex (Gorski et al., 2002).

In *Lmo4*^{Emx1-Cre/flox} brains, Lmo4 expression was absent only in the cerebral cortex but not in the ventral subcortical region, suggesting a cortical-specific Lmo4 deletion (Figs. S3C and D). We then mapped the expression pattern of cortical regional markers in P0 *Lmo4*^{Emx1-Cre/flox} cortices (Fig. S7). Similar to *Lmo4*^{Nestin-Cre/flox} mice, the expression pattern of cortical markers was altered in *Lmo4*^{Emx1-Cre/flox} cortices. The *Cdh8* anterior expression in the upper layer was greatly reduced and the *Id2* expression in layer 5 was expanded rostrally (Figs. S7A–D). The anterior boundaries of *EphrinA5* and *EphA7* expression were also shifted forward (Figs. S7E–H).

We next investigated the barrel field development in *Lmo4*^{Emx1-Cre/flox} cortices. While the position of the PMBSF did not shift either rostrally or caudally, the shape of the PMBSF was also altered (Fig. S8). The length of the PMBSF did not change but its width was narrowed with an angle against the cortical midline (Figs. S8D–F). Therefore, *Lmo4* region-specific expression in the cerebral cortex is necessary for defining the shape of the somatosensory barrel subfield.

To further analyze whether cortical-specific *Lmo4* deletion may cause defects of sensory and motor functions, we performed behavioral tests on *Lmo4*^{Emx1-Cre/flox} mice. *Lmo4*^{Emx1-Cre/flox} mice survived into adulthood and, unlike *Lmo4*^{Nestin-Cre/flox} mice, they had similar body weights to control littermates (Fig. 7A). They also had normal muscle strength assessed by the hanging wire test (Fig. 7B). However, similar to *Lmo4*^{Nestin-Cre/flox} mice, mice with cortical-specific *Lmo4* deletion had poor performance in the rotarod test (Fig. 7C). *Lmo4*^{Emx1-Cre/flox} mice also displayed impaired sensory perception and motor control, as detected by the adhesive removal test (Fig. 7D).

Our results demonstrate that Lmo4 plays a specific role in regulating the development of the somatosensory barrel subfield and sensory and motor functions.

Discussion

The human and rodent cerebral cortices consist of distinct anatomical and functional areas. The developmental programs that control the size and shape of these areas are poorly understood. We show here that the transcription factor Lmo4 has unique region-specific expression in the mouse cortex that coincides with the critical developmental period of the somatosensory barrel field. *Lmo4* cortical expression is essential for accurately defining the shape of the barrel subfield and for eliciting proper sensorimotor performance. Our results demonstrate that genes with cortical region-specific expression are essential for the fine regulation of functional area formation

during development. Thus, the accurate organization of cortical functional areas is likely orchestrated by precise expression and interaction of patterning molecules (i.e. Fgf8), transcription factors with gradient expression (Emx2 and Pax6) and genes with region-specific expression (Lmo4).

Lmo4 defines the accurate shape of cortical functional areas

The transcription factor *Lmo4* has a unique expression pattern in P0 mouse cortices. *Lmo4* has very high expression in the anterior and posterior region but no expression in between. The *Lmo4* non-expression region corresponds to the presumptive somatosensory barrel area (Fig. 1 and Fig. S1). Interestingly, both CNS- and cortex-specific deletions of *Lmo4* result in an altered shape of the somatosensory barrel subfields (Fig. 3 and Fig. S8).

How does Lmo4 affect the barrel field development even though it is not expressed there? We found that the dynamic and region-specific *Lmo4* expression corresponds to the critical timing of the
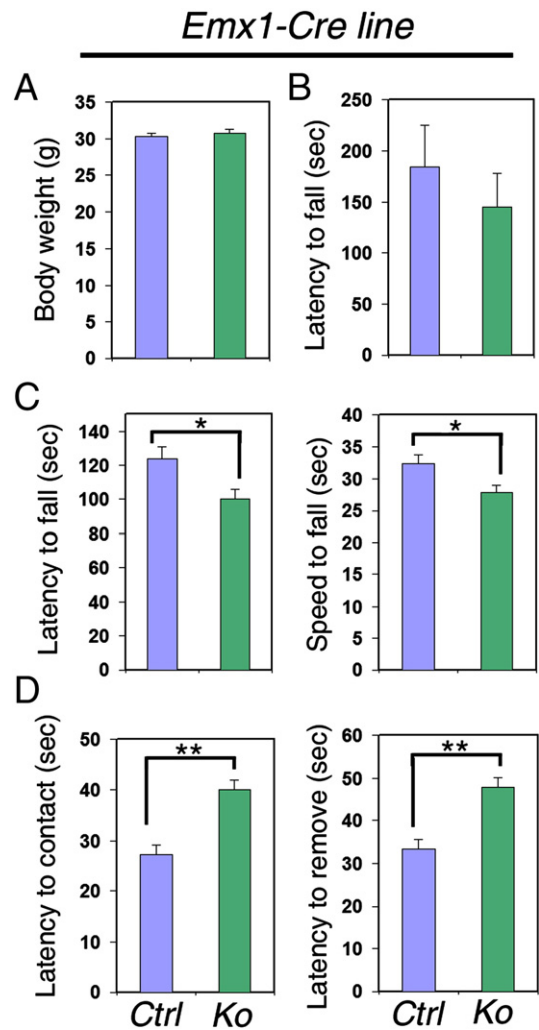

Fig. 7. Cortical specific *Lmo4* deletion results in impaired mouse sensorimotor control. (A) *Lmo4*^{Emx1-Cre/flox} mice (*Ko*, n=11) had normal body weights when compared to control mice (*Ctrl*, n=11) (*Ctrl*: 30.38±0.4 grams (g); *Ko*: 30.82±0.52 g, $p<0.6095$). (B) The hanging wire test: *Lmo4*^{Emx1-Cre/flox} mice had normal muscle strength. Latency to fall: *Ctrl*: 183.91±41.18 seconds (s); *Ko*: 145.11±32.79 s, $p<0.4697$. (C) The rotarod test: *Lmo4*^{Emx1-Cre/flox} displayed poor motor coordination. Latency to fall off: *Ctrl*: 123.95±7.04 s; *Ko*: 100.14±5.76 s, *: $p<0.0112$. Speed to fall off: *Ctrl*: 32.32±1.41 rpm; *Ko*: 27.76±1.15 rpm, *: $p<0.0135$. (D) The adhesive removal test: *Lmo4*^{Emx1-Cre/flox} had poor performance in sensory perception and motor control. Latency to contact: *Ctrl*: 27.18±2.05 s; *Ko*: 40.09± 1.87 s, **: $p<0.0002$. Latency to remove: *Ctrl*: 33.43±2.28 s; *Ko*: 47.79±2.38 s, **: $p<0.0003$.

barrel field development: there is no *Lmo4* expression in the medial cortical region at P0 when thalamic axons just reach the cortical plate. Later on, *Lmo4* is expressed in the entire cortex when the formation of barrels is complete by P10 (Fig. 1) (Lopez-Bendito and Molnar, 2003). We propose that Lmo4 may define the shape of the barrel field during the important period of barrel formation. When *Lmo4* anterior and posterior expression regions are altered at early embryonic stages, the shape of the presumptive somatosensory barrel region will be changed accordingly.

Lmo4 deletion causes an anterior shift of cortical regional markers. Previous work suggests that Lmo4 functions as a transcription adaptor (Retaux and Bachy, 2002). Lmo4 may directly or indirectly specify expression domains of cortical regional markers such as Id2 and Cdh8. In cortices where Lmo4 expression is deleted, the inhibition of anterior expression of cortical regional markers is released, resulting in the anterior shift of these markers and subsequently altered shape of the barrel subfield. It is unclear why only the anterior region is affected, although *Lmo4* is specifically expressed in both anterior and posterior cortical regions.

Notably, unlike transcription factors that normally have gradient expression along the anterior–posterior cortex, such as Pax6 and Emx2 (Bishop et al., 2000; Mallamaci et al., 2000), *Lmo4* expression regions in the anterior and posterior have an angle against the cortical midline and leave a trapezoid-shaped *Lmo4* non-expression region in between (Fig. S1). The alteration of cortical regional markers in *Lmo4* deficient cortices does not shift the barrel field rostrally or caudally. Instead, *Lmo4* deletion causes shrinkage of the barrel subfield along the rostral–medial and caudal–lateral axis (Figs. 3D–I). Therefore, transcription factors with gradient expression (Pax6 and Emx2) may control the overall size and position of cortical functional areas while genes with region-specific expression (Lmo4) may regulate the specific fine shape of functional areas.

A recent report has shown that Lmo4 is important in calcium-dependent gene transcription and the presynaptic and postsynaptic organization was disrupted in the barrel field of *Lmo4* conditional knockout mice created by the *Annexin (nex)-Cre* line (Kashani et al., 2006). In our study, the abnormal barrel morphology and diffused thalamocortical axonal projects in *Lmo4* conditional knockout mice generated by the *Nestin-Cre* line are consistent with the previous report but show less severe defects (Figs. 3 and 4) (Kashani et al., 2006). We found that *Lmo4* is first expressed in the anterior and posterior regions and in early born neurons in the preplate in E11.5 and E12.5 cortices (Fig. 1 and Fig. S5A). *Lmo4* is also transiently expressed in cells residing in the subventricular zone at E15.5 and mostly in postmitotic neurons after E18.5 (Fig. 1 and data not shown). The two *Cre* lines will temporally delete Lmo4 cortical expression in distinct cells at different developing stages, because the *Nestin-Cre* line is active in most progenitor cells from E10.5 and the *Annexin-Cre* is active in postmitotic neurons from E14.5 (Gorski et al., 2002; Schwab et al., 1998). The mouse genetic background may contribute to the severe barrel field defects in *Lmo4*$^{Annexin-Cre/flox}$ mice, and less severe defects in *Lmo4*$^{Nestin-Cre/flox}$ and *Lmo4*$^{Emx1-Cre/flox}$ mice. Moreover, the sensorimotor defects in our mouse models may reflect both Lmo4 function in A–P functional area formation and the role of Lmo4 in calcium-dependent gene transcription regulation.

Accurate size and shape of cortical function areas are essential for mouse normal sensorimotor control

Proper cortical functional area formation is essential for normal behaviors (Grove and Fukuchi-Shimogori, 2003; O'Leary et al., 2007; Rakic, 1988; Sur and Rubenstein, 2005). Recent work has shown that the precise gene regulation is critical for governing accurate formation of distinct functional areas. For example the growth factor Fgf8 is essential for controlling the size of the motor cortex and the transcription factor Emx2 plays an important role in governing the

size of the visual cortex (Bishop et al., 2000; Fukuchi-Shimogori and Grove, 2001; Garel et al., 2003; Mallamaci et al., 2000). Altering cortical expression levels of Emx2 alone results in a larger or smaller visual cortex and causes impaired sensory detection and motor coordination (Leingartner et al., 2007).

The size and shape change of one functional area during early development, for instance the visual cortex, may subsequently alter other functional representative areas, for example those that control motion and/or sensation. Once the functional areas are altered, the intra-cortical and thalamocortical connections will be changed accordingly (Huffman et al., 2004; Shimogori and Grove, 2005). As a result, impaired sensorimotor control will be detected in the adult. Interestingly, recent work showed that in cortical specific *Pax6* mutant mice, even though the expression of regional markers is altered, the thalamocortical projections remain normal (Pinon et al., 2008). It is unclear whether the size and shape of the barrel subfield are altered in cortical specific *Pax6* mutant mice. Our studies showed that the thalamocortical projections reach the somatosensory cortex in *Lmo4* conditional knockout mice (Fig. 4). However, the shape of the barrel subfield is altered. Moreover, *Lmo4* conditional knockout mice displayed abnormal sensorimotor control. Our results suggest that even though the thalamocortical connections have developed, altered shape and size of one functional area, for instance the somatosensory barrel subfield, may subsequently cause changes of functional representative areas in other part of the cortex, for example the motor cortex. A precise detection of cortical functional area formation relies on a convergent effort, including an accurate measurement of cortical regional markers and anatomical landmarks such as the barrel field, a fine mapping of functional representative areas and a serial of well developed behavioral tests.

When a cortical functional area, for example the somatosensory area, is enlarged in only one hemisphere, it can lead to lateralized sensorimotor control such as paw preference in mice (Barneoud and Van der Loos, 1993). Our previous work showed that *LMO4* cortical region-specific expression is asymmetric between the two hemispheres in humans (Sun et al., 2005). Moreover, *Lmo4* expression in mice tends to be slightly asymmetric in each mouse, but without a consistent pattern of being always higher in right or left hemisphere (Sun et al., 2005). Similarly, while a mouse prefers to use the left or right front paw for reaching food (Collins, 1991), paw preference in rodents is random at a population level (Biddle et al., 1993; Signore et al., 1991). In this study we demonstrated that Lmo4 defines the shape of the somatosensory barrel subfield and regulates mouse sensory perception and motor coordination. Therefore asymmetric *Lmo4* expression in mouse cortices may modify lateralized sensorimotor control (Sun and Walsh, 2006), though we have as yet no direct evidence that *Lmo4* expression and paw preference are causally related. Unilaterally altering *Lmo4* cortical expression levels in one hemisphere will further reveal its function in cortical area formation and lateralized behaviors.

Unique expression and function of Lmo4 in the development of the cerebral cortex

Lmo4 expression in the developing cortex is dynamic and region-specific. In P0 cortices, *Lmo4* is highly expressed in the anterior and posterior with clear boundaries, but not in the middle region (Fig. 1). How are boundaries between *Lmo4* expression and non-expression regions established? *Lmo4* anterior and posterior expression is detectable even at E11.5, suggesting an early onset of *Lmo4* region-specific cortical expression (Fig. S5A). Because Fgf8 is strongly expressed in the anterior neural ridge as early as E10.5 (Fukuchi-Shimogori and Grove, 2001), Fgf8 may induce early *Lmo4* expression in the anterior cortical region. We found that mis-expression of Fgf8 in a cortical region where *Lmo4* is not normally expressed using *in utero* electroporation can induce ectopic *Lmo4* expression, suggesting that

Fgf8 may directly or indirectly regulate *Lmo4* anterior expression (Huang and Sun, unpublished observations). How *Lmo4* posterior expression is regulated remains unknown. Moreover, the lack of *Lmo4* expression in the medial cortical region suggests that molecules repressing *Lmo4* activity may be expressed there. This repression may be released by axonal connections projected from the thalamus and allow *Lmo4* expression in the medial cortical region by P5 (Fig. 1D). Identifying regulatory elements for *Lmo4* expression, especially enhancer elements, will help understand temporal and spatial regulation of *Lmo4* expression during cortical development.

Beside the cerebral cortex, *Lmo4* has broad expression in the entire CNS, including the cerebellum and the hindbrain. We found that CNS deletion of *Lmo4* expression does not cause obvious defects in the non-cortical regions. On the other hand, the development and function of the cortical barrel field are significantly affected in both CNS- and cortex-specific *Lmo4* deletion mice. These results suggest that *Lmo4* function is subtle in the development of the cerebellum and the hindbrain. Mice with CNS *Lmo4* deletion displayed more severe defects than those with cortical-specific *Lmo4* deletion. These differences may be a result of subtle Lmo4 function in the non-cortical regions of the CNS. In an E12.5 spinal cord, *Lmo4* was mostly expressed in postmitotic cells along the ventricular zone (Fig. S5E). Strong *Lmo4* expression was detected in a subtype of the motor neurons (Fig. S5E, arrowheads). We examined motor neuron development in the E12.5 *Lmo4* null ($Lmo4^{-/-}$) spinal cord but did not detect obvious defects (Huang and Sun, unpublished observations). A recent report shows that Chx10-expressing interneurons are increased in the *Lmo4* null spinal cord (Lee et al., 2008). Ectopic production of interneurons in the spinal cord may contribute to more severe motor impairment in $Lmo4^{Nestin-Cre/flox}$ than in $Lmo4^{Emx1-Cre/flox}$ mice.

Lmo4 is a cysteine-rich, two-LIM-domain-containing protein. Lmo4 does not have a DNA binding domain but forms a protein complex with other transcription factors that bind to DNA sequences (Retaux and Bachy, 2002). Lmo4 may interact with DNA binding proteins and function as a transcriptional adaptor during development. Thus, the expression pattern (or the availability) of Lmo4 binding partners in the cortex and non-cortical regions may limit the ability of Lmo4 to form a protein complex, even though Lmo4 is expressed in that tissue, for instance the cerebellum. Identifying Lmo4 binding proteins will further reveal its unique function in cortical development.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.12.003.

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