博士論文

論文題目　**Sox11 negatively regulates dendritic morphogenesis in the developing cerebral cortex**

(発生期大脳皮質において Sox11 は樹状突起形成を抑制的に制御する)

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Table of Contents

Abstract

The coordinated mechanisms balancing promotion and suppression of dendritic morphogenesis are crucial for the development of the cerebral cortex. Although previous studies have revealed important transcription factors that promote dendritic morphogenesis during development, those that suppress dendritic morphogenesis are still largely unknown. Here I found that the expression levels of the transcription factor Sox11 decreased dramatically during dendritic morphogenesis. Knockdown of Sox11 using postnatal electroporation promoted dendritic morphogenesis of layer 2/3 pyramidal neurons. Overexpression of Sox11 using *in utero* electroporation partially inhibited morphogenesis of basal dendrites of layer 2/3 pyramidal neurons. Interestingly, I found that precocious suppression of Sox11 expression caused precocious branching of neurites and a neuronal migration defect. These findings are consistent with the idea that suppression of dendritic morphogenesis by Sox11 during radial migration is crucial for the formation of the cerebral cortex.

Introduction

Dendritic morphology has profound impacts on neuronal information processing. Appropriate dendritic morphology is essential for receiving inputs from other neurons, and branching patterns of dendrites affect the efficiency in transmitting information from the synapse to the soma [1]. Because abnormal development of dendrites has been associated with several neurological disorders [2,3], it is important to understand the molecular mechanisms regulating dendritic morphogenesis during development.

During development, dendritic morphogenesis consists of several steps [3-5]. First, newly generated neurons are polarized. One of the neurites of pyramidal neurons differentiates into an axon, another one differentiates into an apical dendrite, and the others differentiate into basal dendrites. This step seems to be regulated mainly by intrinsic genetic programs because dissociated neurons obtain an apical dendrite distinguishable from basal dendrites in culture [6,7]. In the second step, dendrites extend and form their branches. This step is governed by multiple mechanisms including transcription factors, neuronal activities and cell-cell interactions mediated by secretory proteins and/or cell surface molecules. Finally, dendrites stop growing. This step is regulated by cell surface receptors for cues derived from other cells or other dendrites.

In dendritic morphogenesis, transcription factors play important roles not only in intrinsic genetic programs of dendritic morphogenesis, but also as mediators of extrinsic signals such as growth factors and neuronal activity [3-5,8]. For example, the transcription factor CREST is necessary for promoting activity-dependent dendritic morphogenesis in the developing brain [9]. CREST was reported to contribute to the dendritic complexity of neurons in the cerebral cortex, including dendritic extension and branching in pyramidal neurons [9]. Another transcription factor involved in dendritic morphogenesis is Zfp312,

which is expressed in deep layers of the cerebral cortex and is necessary for dendrite elongation and branching [10]. In contrast to transcription factors promoting dendritic morphogenesis, those suppressing dendritic morphogenesis are still largely unknown (Fig. 1) [11].

Recent pioneering studies proposed that inhibition of dendritic morphogenesis is crucial for the formation of the cerebral cortex during development [12-15]. p35 and Cdk5 are required for maintaining the simple morphology of migrating neurons, and the loss of p35 and Cdk5 results in abnormally branched leading processes and severe migration defects [14,15]. I therefore searched for transcription factors suppressing dendritic morphogenesis. Here, I focused on Sox11, which is a member of group C of Sry-related high-mobility group (HMG) box-containing (Sox) transcription factors. Sox transcription factors in vertebrates are involved in numerous developmental processes, including sex determination, maintenance of the pluripotency of stem cells, and development of the central nervous system [16-21]. Sox11 contains a Sry-related HMG-box DNA-binding domain, which is located in the N-terminal third, and a transactivation domain located in the C-terminal third [22]. During embryonic development, Sox11 is transiently expressed in many tissues, including neural precursor cells and differentiated neurons in the developing brain, and is rarely expressed in the adult brain outside of the neurogenic niches [22-26]. Using Sox11 knockout mice, it has previously been shown that Sox11 plays important roles in the embryonic development of many organs [27]. In the central nervous system, Sox11 plays crucial roles in early stages of neurogenesis. In the developing chick spinal cord, Sox11 induces pan-neuronal genes and promotes the establishment of neuronal property [28]. In the developing mouse cerebral cortex and hippocampus, Sox11 is required for promoting proliferation of neurogenic precursor cells. Furthermore, deletion and mutations of the SOX11 gene are associated with a

neurodevelopmental disorder that has features of Coffin-Siris syndrome [29]. Although these previous studies suggested important roles of Sox11 in neurogenic precursor cells, the roles of Sox11 in postmitotic neurons had not been fully uncovered. In this study, I found that Sox11 was necessary for inhibiting apical and basal dendrite morphogenesis, and sufficient for inhibiting basal dendrite morphogenesis in layer 2/3 pyramidal neurons during development. Interestingly, I also found that precocious suppression of Sox11 expression caused a neuronal migration defect. These findings suggest that the timing of dendritic morphogenesis is genetically fine-tuned at appropriate time points by positive and negative transcriptional factors.

Materials and Methods

Animals

All procedures were performed in accordance with protocols approved by the University of Tokyo Animal Care Committee and the Kanazawa University Animal Care Committee. Either male or female ICR mice (SLC, Japan) and *Sox11* knockout mice (kind gifts from Drs. Elisabeth Sock and Michael Wegner, Universität Erlangen-Nürnberg) [27] were reared on a normal 12 h light/dark schedule. *Sox11* knockout mice are missing the entire exon of Sox11, lacking genomic DNA from its start codon to 400 bp downstream of the end of its open reading frame. The day of conception and that of birth were counted as embryonic day 0 (E0) and postnatal day 0 (P0), respectively. Experiments were repeated at least three times and gave consistent results.

Plasmids

The pCAG plasmid vector, pCAG-EGFP, pCAG-mCherry, pThy1S-EGFP and pCAG-FloxedSTOP-EGFP were described previously [30-32]. pCAG-NLS-Cre was a kind gift from Dr. Mikio Hoshino (National Institute of Neuroscience, Tokyo, Japan). pCAG-Sox11 was constructed by inserting mouse Sox11 (NM_009234.6) into the pCAG plasmid vector.

The Sox11-shRNA-expression vector (shSox11) was constructed using a pSUPER.basic vector (Oligoengine, Seattle, WA). The sequence used for shRNA against Sox11 was designed using the web-based software siDIRECT (http://sidirect2.rnai.jp/) and is 5'-GAGAAGATCCCGTTCATCA-3'. The specificity of this sequence was confirmed using NCBI-BLAST. The sequence used for negative control of shRNA (5'-CAACAAGATGAAGAGCACC-3') was designed based on a commercially available non-mammalian shRNA control plasmid (Sigma) that should not target any known mammalian genes. Using NCBI-BLAST, I confirmed the sequence of the negative control shRNA does not share homologies to other known mammalian genes.

Plasmids were purified using an Endofree plasmid Maxi kit (Qiagen, Germany). For control experiments, appropriate amounts of empty pCAG vector or the non-mammalian shRNA control plasmid were used instead of pCAG-Sox11 or shSox11, respectively. Prior to electroporation experiments, plasmid DNA was diluted to less than 3 mg/mL in PBS, and Fast Green solution was added at a final concentration of 0.1% to monitor the injection.

In utero *electroporation*

In utero electroporation was performed as described previously with slight modifications [30-35]. Pregnant ICR mice (E15.5) were deeply anesthetized, and the uterine horns were exposed. Approximately 1–3 μL of DNA solution was injected into the lateral ventricle of the

embryo using a pulled glass micropipette. Each embryo within the uterus was placed between tweezer-type electrodes with a diameter of 5 mm (NEPA Gene, CUY650P5). Square electric pulses (45 V, 50 ms) were passed 5 times at 1 Hz using an electroporator (BTX). Care was taken to quickly place embryos back into the abdominal cavity to avoid excessive temperature loss. The wall and skin of the abdominal cavity were sutured.

Postnatal electroporation

Postnatal electroporation was performed as previously described with modifications [36]. Neonatal ICR mouse pups at P1.5 were anesthetized with hypothermia, and craniotomy was performed. The capillary tube (Drammond, #3-000-203-G/X) was pulled using a two-stage vertical puller (Narishige, PC-10) and was inserted into the primary somatosensory cortex at 700 μm in depth from the surface of the cranial bone. After DNA solution was injected, square electric pulses (80 V, 50 ms) were passed five times at 1 Hz using an electroporator (BTX). The scalp was bonded with Vetbond (3M), and the pups were warmed for recovery from anesthesia.

Immunohistochemistry

Immunohistochemistry was performed as described previously with modifications [37,38]. Mice were deeply anesthetized and transcardially perfused with 4% paraformaldehyde (PFA) in PBS. To make coronal sections, the brains were dissected, post-fixed by overnight immersion in the same fixative, cryoprotected by two-day immersion in 30% sucrose in PBS and embedded in OCT compound. Sections of 50 μm thickness were made using a cryostat, permeabilized with 0.1–0.5% Triton X-100 in PBS and incubated overnight with primary antibodies. After being incubated with Alexa 488-, Cy3-, and/or Alexa 647-conjugated

secondary antibodies and 1 μg/mL Hoechst 33342, the sections were washed and mounted. The primary antibodies used here include goat anti-Sox11 (Santa Cruz), rabbit anti-GFP (Medical & Biological Laboratories, Japan), rabbit anti-RFP (Medical & Biological Laboratories, Japan), goat anti-Brn2 (Santa Cruz), rat anti-Ctip2 (Abcam) and goat anti-FOXP2 (Abcam) antibodies.

Imaging and morphometric measures

Epifluorescence microscopy was performed with an AxioImager A1 microscope (Carl Zeiss), a BIOREVO BZ-9000 (Keyence), and a fluorescence stereomicroscope (MZ16 F, Leica). Confocal microscopy was performed with an LSM510 microscope (Carl Zeiss), an LSM700 microscope (Carl Zeiss) and an FV10i FLUOVIEW microscope (OLYMPUS). For accurate neuron reconstructions, thin optical sections (i.e. less than 0.53 μm) were obtained. For each animal, 3-4 neurons per one section which had both cell bodies and the largest dendritic trees were analyzed. For morphometric measures, quantifications of the total length of the dendrites of each neuron, the number of the dendritic branches, the number of the dendritic ends, and the number of primary dendrites per cell were performed using either the Simple Neurite Tracer plug-in of ImageJ software [39] or Neurolucida360 (MBF Bioscience). During tracing, when a dendrite was running very close to a dendrite derived from another neuron, it was often difficult to distinguish these two dendrites clearly. In this case, to separate these dendrites clearly, I examined the dendrites carefully with a 360-degree view using the pivot point tool of Neurolucida360. Before statistical comparisons, homogeneity of variances was confirmed by F-test. Statistical comparisons were performed using Student's *t*-test.

Quantification of Sox11 expression levels in EGFP-positive nuclei

Confocal microscopic images were analyzed using ImageJ software. To select the areas of EGFP-positive transfected cell nuclei in the images, Hoechst and EGFP double-positive areas were extracted using the Image Calculator tool of ImageJ software. EGFP-positive cell nuclei were registered to ROI manager using the Analyze particle tool. Sox11 signal intensities in the area of EGFP-positive nuclei were measured. After background signals were subtracted from the Sox11 signal intensities, statistical comparisons were performed using Welch's *t*-test.

Quantification of the position of shSox11-transfected cells

Epifluorescence microscopic images of the cerebral cortex at E18.5 were analyzed using ImageJ software. The cortical plate (CP) and the intermediate zone (IZ) were selected using Hoechst images, and the numbers of mCherry-positive cells in the CP and the IZ were manually and blindly counted by using the ImageJ cell count plug-in. Statistical comparisons were performed using Welch's *t*-test.

Results

The expression levels of Sox11 in the mouse cerebral cortex during development

I focused on Sox11 as a candidate for a transcription factor suppressing dendritic morphogenesis. To test the expression levels of Sox11 using immunohistochemistry, I first tested the specificity of the anti-Sox11 antibody used in this study using coronal sections of the mouse cerebral cortex. Consistent with previous reports and the Allen Brain Atlas [22,24], immunohistochemistry showed that this antibody widely recognized many cells in the cortical plate and the subventricular zone at embryonic day 18.5 (E18.5), while it did not recognize

cells in the ventricular zone (Fig. 2*A*). Sox11 signals in the intermediate zone were relatively low, presumably because of low cell density in the intermediate zone (Fig. 2*A*). Importantly, Sox11 immunoreactivity was totally lost in the cerebral cortex of *Sox11* knockout mice (Fig. 2*A*). Consistent with the fact that Sox11 is a transcription factor, higher magnification of Hoechst-stained cells revealed that Sox11 immunoreactivity was found in the nucleus (Fig. 2*B*). Sox11 immunoreactivity was also lost in higher magnification images of *Sox11* knockout mice (Fig. 2*B*). These results clearly indicate that the anti-Sox11 antibody used here specifically recognizes Sox11 protein.

I next examined Sox11 expression in the mouse cerebral cortex during development. I found that the expression levels of Sox11 decreased dramatically soon after birth (Fig. 3*A*,*B*). While cortical neurons expressed high levels of Sox11 at postnatal day 0 (P0), Sox11 expression rapidly decreased by P3 (Fig. 3*A*,*B*). At P3 and P5, Sox11 expression was still detected weakly in layer 2/3, where newly generated young neurons were located, but the expression of Sox11 was lost in all cortical layers at P11 (Fig. 3*A*,*B*). These results suggest that Sox11 expression in the cortical neurons decreases soon after cortical neurons arrive at the cortical plate.

I hypothesized that Sox11 regulates dendritic morphogenesis of cortical neurons because Sox11 reduction and dendritic morphogenesis proceed simultaneously soon after birth of mouse pups [40]. To visualize the morphology of single layer 2/3 neurons in the mouse cerebral cortex, I expressed EGFP in a small number of cortical neurons by introducing pCAG-FloxedSTOP-EGFP plus a very low concentration of pCAG-NLS-Cre (1.5 μg/mL) using *in utero* electroporation at E15.5 (Fig. 3*C*) [41,42]. My observation of sparsely labeled neurons confirmed that the dendritic complexity of layer 2/3 neurons increased during the two weeks after birth (Fig. 3*D*). While the dendrites of layer 2/3 neurons were relatively

simple at P3, apical and basal dendrites of labeled neurons became longer, and the number of primary basal dendrites was increased at P5. Apical and basal dendrites became much longer and more complex by P15. Because dendritic morphogenesis of layer 2/3 neurons proceeds soon after the reduction of Sox11 expression, I hypothesized that Sox11 suppresses dendritic morphogenesis, and that the reduction of Sox11 expression leads to dendritic morphogenesis.

Precocious Sox11 knockdown is sufficient to promote dendritic morphogenesis of layer 2/3 neurons

To examine the role of Sox11 in dendritic morphogenesis, I tested whether precocious reduction of Sox11 expression promotes dendritic morphogenesis in layer 2/3 neurons. Because *Sox11* knockout mice die soon after birth, it was impossible to use them to examine dendritic morphogenesis. Instead, to suppress Sox11 expression, I designed a Sox11-shRNA-expression vector (shSox11), and tested its ability to suppress Sox11 expression using *in utero* electroporation (Fig. 4*A*). Immunostaining and a quantitative analysis confirmed that my shSox11 construct significantly reduced Sox11 expression (Welch's *t*-test, $p = 0.002$; Fig. 4*B*,*C*). To introduce EGFP plus shSox11 into layer 2/3 neurons, I performed postnatal electroporation at P1.5 (Fig. 5*A*,*B*) [36]. The results showed that Sox11 knockdown resulted in complex dendritic morphology at P5 (Fig. 5*C*). Quantitative analyses revealed that Sox11-down-regulated neurons had longer dendrites (n = 3 animals per each experimental group. 9-10 transfected cells per each experimental group were analyzed. Student's *t*-test, $p = 0.035$; Fig. 5*D*), larger numbers of dendritic ends (p = 0.041; Fig. 5*F*) and larger numbers of primary dendrites per cell ($p = 0.008$; Fig. 4*G*) than those of control neurons. My result also showed that Sox11-down-regulated neurons tended to have larger numbers of dendritic branches ($p = 0.075$; Fig. 5*E*). It should be noted that I have

not tested if Sox11 expression is indeed suppressed by introducing shSox11 by postnatal electroporation. It would be important to test this point in my future experiments. These results suggest that the precocious reduction of Sox11 expression is sufficient for promoting dendritic morphogenesis of layer 2/3 neurons.

The reduction of Sox11 is required for the formation of dendrites of layer 2/3 pyramidal neurons

I next tested the necessity of the reduction of Sox11 expression for dendritic morphogenesis. I co-expressed Sox11 and EGFP in layer 2/3 pyramidal neurons using *in utero* electroporation at E15.5, and brain samples were obtained at P15 (Fig. 6*A*). My immunohistochemical analysis showed that ectopically expressed Sox11 was indeed detected in EGFP-positive transfected layer 2/3 neurons at P15, when endogenous Sox11 was undetectable (Fig. 6*B*,*C*). To examine the dendritic morphology of Sox11-overexpressing neurons, I sparsely labeled layer 2/3 neurons with EGFP under the control of the Thy1S promoter (Fig. 7*A*,*B*) [31] and found that dendrites of Sox11-overexpressing neurons were much simpler than those of control neurons (Fig. 7*B*). To quantitatively analyze the dendritic morphology, I traced the basal dendrites of layer 2/3 neurons, because it was difficult to trace entire apical dendrites of layer 2/3 neurons at P15. Quantitative analyses of the basal dendrites showed that Sox11-overexpressing neurons had shorter dendrites (n = 3 animals per each experimental group. 9 transfected cells per each experimental group were analyzed. Welch's t-test, p = 0.040; Fig. 7*C*), and smaller numbers of branches (Student's t-test, p = 0.007; Fig. 7*D*) and dendritic ends (p = 0.007; Fig. 7*E*) than those of control neurons. I did not detect any differences in the number of primary dendrites per cell (p = 0.376; Fig. 7*F*). These results suggest that the reduction of Sox11 expression is necessary for promoting morphogenesis of

basal dendrites of layer 2/3 pyramidal neurons.

Although my results showed that Sox11 overexpression suppressed morphogenesis of basal dendrites of layer 2/3 pyramidal neurons, it remained possible that Sox11-overexpressing neurons had become neurons other than layer 2/3 neurons, and as a result, their dendritic morphology was changed by Sox11 overexpression. To exclude this possibility, I examined layer markers of the cerebral cortex such as Brn2, Ctip2 and Foxp2 [43] (Fig. 8). Brn2 is expressed in layers 2/3 and 5 (Fig. 8*A*), and Ctip2 and Foxp2 are expressed in layers 5 and 6 (Fig. 8*B*,*C*). The expression patterns of Brn2, Ctip2 and Foxp2 were not affected by expressing Sox11 (Fig. 8*A*-*C*), suggesting that the identity of Sox11-overexpressing layer 2/3 neurons is not affected by Sox11.

Embryonic knockdown of Sox11 induces precocious neurite elongation and a migration defect

I have shown that Sox11 knockdown after neurons arrived at the cortical plate resulted in precocious dendritic morphogenesis (Fig. 5). I next examined what would happen if Sox11 expression were suppressed before neuronal radial migration. I introduced pSUPER-shSox11, pCAG-mCherry and pCAG-FloxedSTOP-EGFP with a very low concentration of pCAG-NLS-Cre (1.5 μg/mL) into layer 2/3 neurons using *in utero* electroporation at E15.5 and prepared coronal sections at E18.5 (Fig. 9*A*). This mixture of plasmids enabled us to suppress Sox11 expression and visualize the morphology of single neurons. Interestingly, I found that radial migration of mCherry-positive transfected neurons was markedly inhibited by Sox11 knockdown (Fig. 9*B*). Quantitative analyses showed that some of control cells had already reached the cortical plate, while significantly fewer shSox11-transfected cells had done so (Welch's *t*-test, $p = 0.033$; Fig. 9*E*,*F*). To address the reason of this migration defect,

I examined the morphology of each transfected cell (Fig. 9*C*,*D*). In control conditions, EGFP-positive cells were still migrating and had a simple morphology with leading processes and trailing processes. In contrast, when shSox11 was expressed, EGFP-positive cells had intricately branched processes, suggesting that branching of processes proceeded precociously. It seemed plausible that this complex branching resulted in difficulty in migration. This is consistent with previous reports associating precocious branching with radial migration defects [12-15]. Thus, my findings suggest a biological importance of the suppression of dendritic morphogenesis during migration by Sox11-mediated mechanisms.

Discussion

In this study, I have shown that the reduction of Sox11 expression is required and sufficient for morphogenesis of basal dendrites during development. I also showed that precocious inhibition of Sox11 expression resulted in a radial migration defect, presumably due to precocious branching of neurites. My findings underline a biological importance of the Sox11-mediated mechanisms suppressing dendritic morphogenesis during radial migration.

Sox11 is a transcription factor suppressing dendritic morphogenesis during development

Previous studies have identified transcription factors promoting dendritic morphogenesis in the mammalian cerebral cortex [8,11]. Neurogenin2 and Otx1 regulate cell type-specific morphology by preferentially promoting development of either apical or basal dendrites [44,45], and CREST and CREB contribute to regulating the timing of dendritic morphogenesis by responding to neural activity or other extrinsic signals [9,46-48].

Although previous findings have been primarily related to the roles of transcription factors in promoting dendritic morphogenesis, my findings indicate that the transcription factor Sox11 plays a crucial role in suppressing dendritic morphogenesis, especially during radial migration. It has already been established that transcription factors are important for promoting and suppressing neurogenesis in neural stem cells [49]. My results indicate that, as in the case of neurogenesis, transcription factors play important roles in balancing the suppression and promotion of dendritic morphogenesis during development.

Sox11 maintains simple morphology of migrating neurons

Recent pioneering studies proposed that precocious branching of neurites leads to radial migration defects in post-mitotic neurons. For example, when the GTPase activating protein srGAP2 is overexpressed in migrating neurons, branching of the leading process occurs, and radial migration is inhibited [13]. p35 and Cdk5 are required for maintaining the simple morphology of migrating neurons, and loss of p35 and Cdk5 results in abnormally branched leading processes and severe migration defects [14,15]. Apart from these molecules regulating the cytoskeleton, transcription factors that ensure radial migration by suppressing branch formation had been unclear. My study showed that Sox11 is a transcription factor that negatively regulates dendritic morphogenesis and ensures radial migration. Although the mechanisms downstream of Sox11 are unclear, it seems possible that srGAP2, p35 and Cdk5 are involved in the effect of Sox11. It was also reported that an increase in spontaneous neural activity resulted in premature branching of the leading process [12]. It would be intriguing to investigate whether Sox11 suppresses dendritic morphogenesis by regulating spontaneous neural activity.

Roles of Sox11 in the central nervous system

Previous studies have shown that Sox11 is required for promoting early stages of neurogenesis. Sox11 is required for neuronal differentiation of neural precursors, and cell-fate specification, differentiation and survival in the spinal cord and the retina [28,50,51]. In the peripheral nervous system, Sox11 promotes cell proliferation [52]. In addition to the previously identified roles of Sox11 in early stages of neurogenesis, my findings revealed a novel role of Sox11 in suppressing dendritic morphogenesis in post-mitotic neurons. The reduction of Sox11 expression was observed not only in the cortical neurons, but also in other tissues including sympathetic ganglia and retina [50,52,53]. Thus, it seems reasonable that Sox11 also regulates dendritic morphology in these tissues. Interestingly, Sox11 is also expressed in the adult brain regions where neurogenesis is observed, such as the dentate gyrus and the subventricular zone [26,54,55]. Therefore, Sox11 may also play similar roles in adult neurogenesis.

Possible roles of Sox11 in inhibiting neuronal maturation

In this study, I focused on the role of Sox11 in dendritic morphogenesis because the expression levels of Sox11 decrease when dendritic morphogenesis proceeds. However, this decrease coincides not only with dendritic morphogenesis of layer 2/3 neurons but also with other neuronal maturation processes including gene expression and axon growth. In fact, my experiments showed that Sox11 also inhibited callosal axon projection (data not shown). Therefore, it seems possible that Sox11 regulates not only dendritic morphogenesis but also other neuronal maturation processes.

Although my results are consistent with the idea that Sox11 promotes radial migration by inhibiting dendritic morphogenesis [12-15], it is also possible that Sox11

directly regulates radial migration independently of dendritic morphogenesis. Uncovering the signaling molecules of dendritic morphogenesis downstream of Sox11 would make it possible to determine whether these molecules are shared by dendritic morphogenesis and radial migration.

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Figures

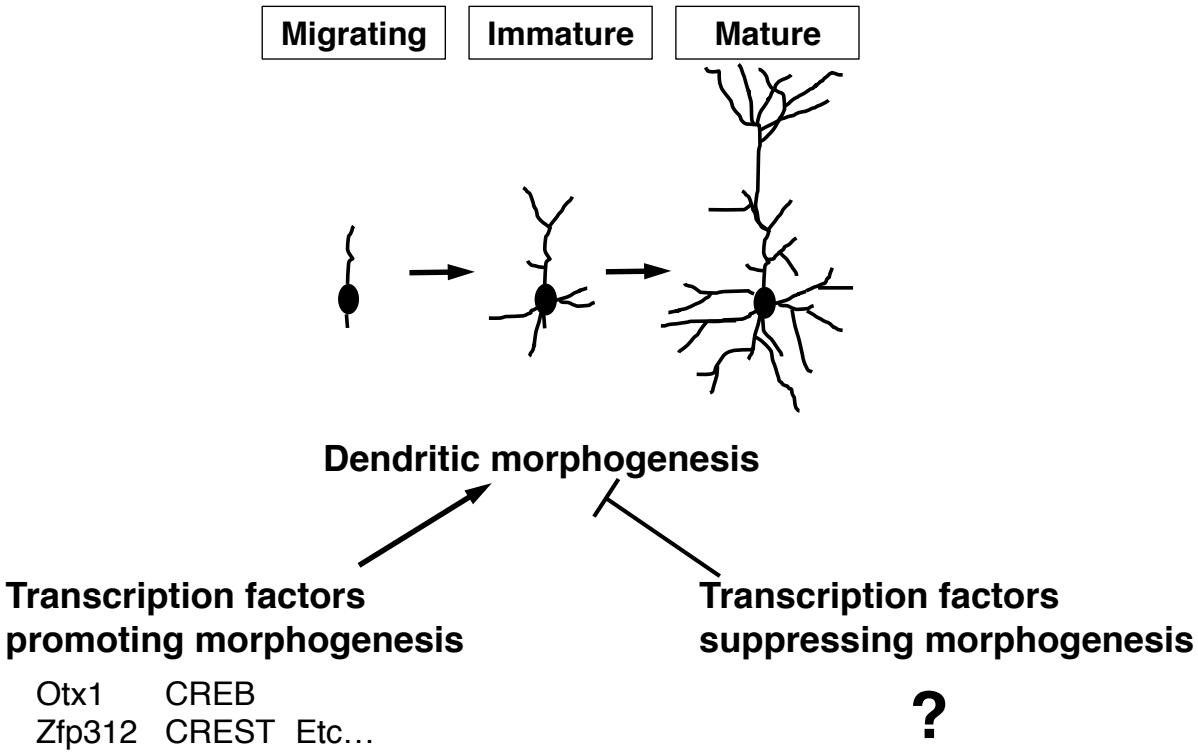

Figure 1. Transcriptional regulation of dendritic morphogenesis in the mouse cerebral cortex during development. During development, dendritic morphology of cortical neurons changes dramatically. Newly generated young neurons have bipolar morphology during radial migration and their processes rarely have branches (left). Once neurons reach their position in the cortical plate, dendritic morphogenesis occurs, and the number of primary dendrites increases, the dendrites become longer and develop their branches (middle and right). Although several transcription factors promoting dendritic morphogenesis has been reported, those suppressing dendritic morphogenesis are largely unknown.

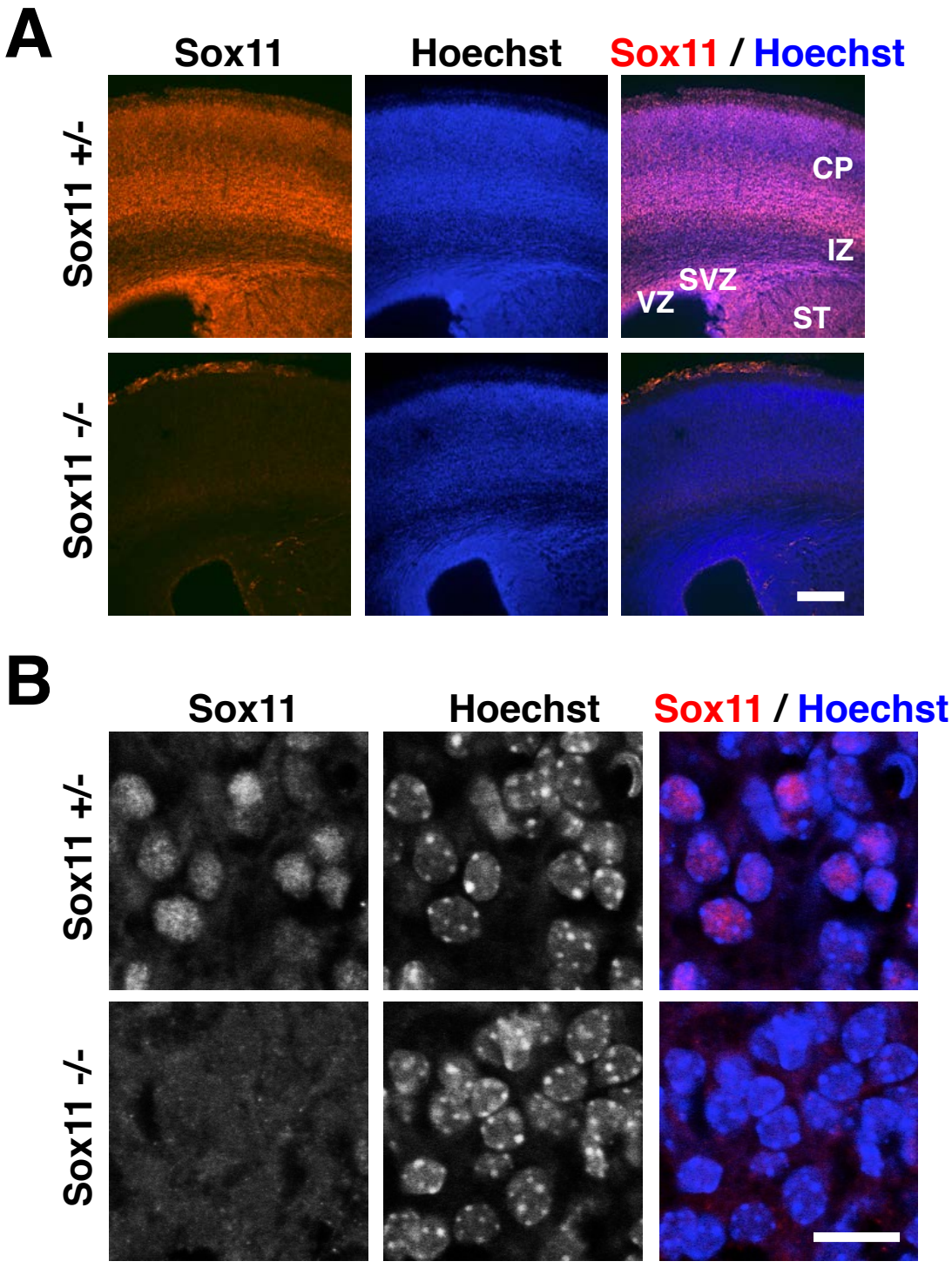

Figure 2. Characterization of anti-Sox11 antibody. ***A***, Coronal sections of the mouse cerebral cortex were prepared from Sox11-deficient (-/-, bottom) and heterozygous (+/-, top) embryos at E18.5. The sections were stained with anti-Sox11 antibody (red) and Hoechst 33342 (blue). CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone; ST, striatum. Scale bar = 200 μm. ***B***, High magnification confocal microscopic images of a deep layer in the CP which will become layer 6 at later stages. Note that Sox11 immunoreactivity was lost in the sections derived from Sox11-deficient mice. Scale bar = 10 μm.

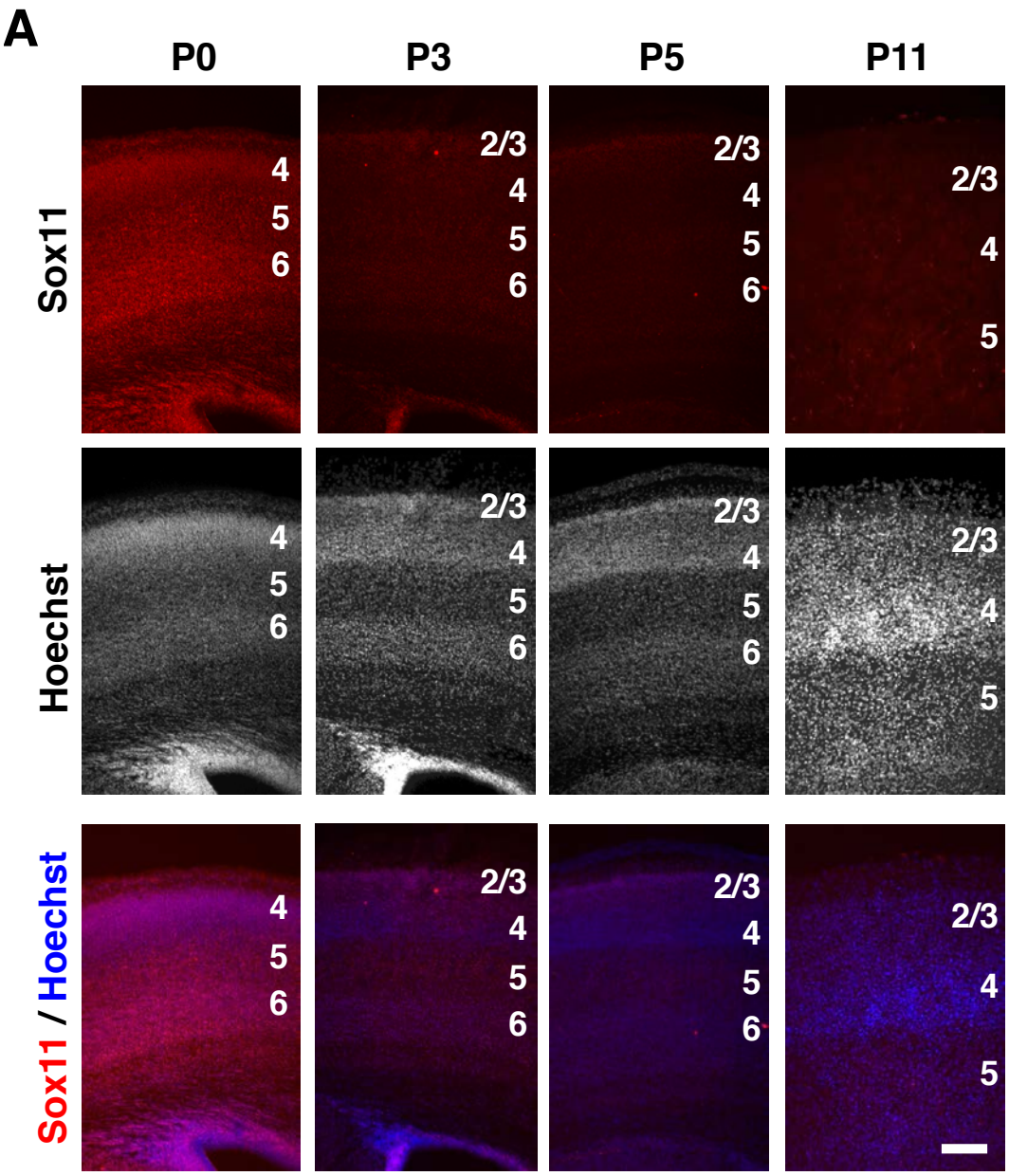

Figure 3. Expression levels of Sox11 and dendritic morphogenesis in the mouse cerebral cortex during development. ***A***, Sox11 immunohistochemistry and Hoechst 33342 staining of coronal sections of the mouse cerebral cortex at P0, P3, P5 and P11. Numbers indicate the corresponding layers in the cerebral cortex. Scale bar = 200 μm.

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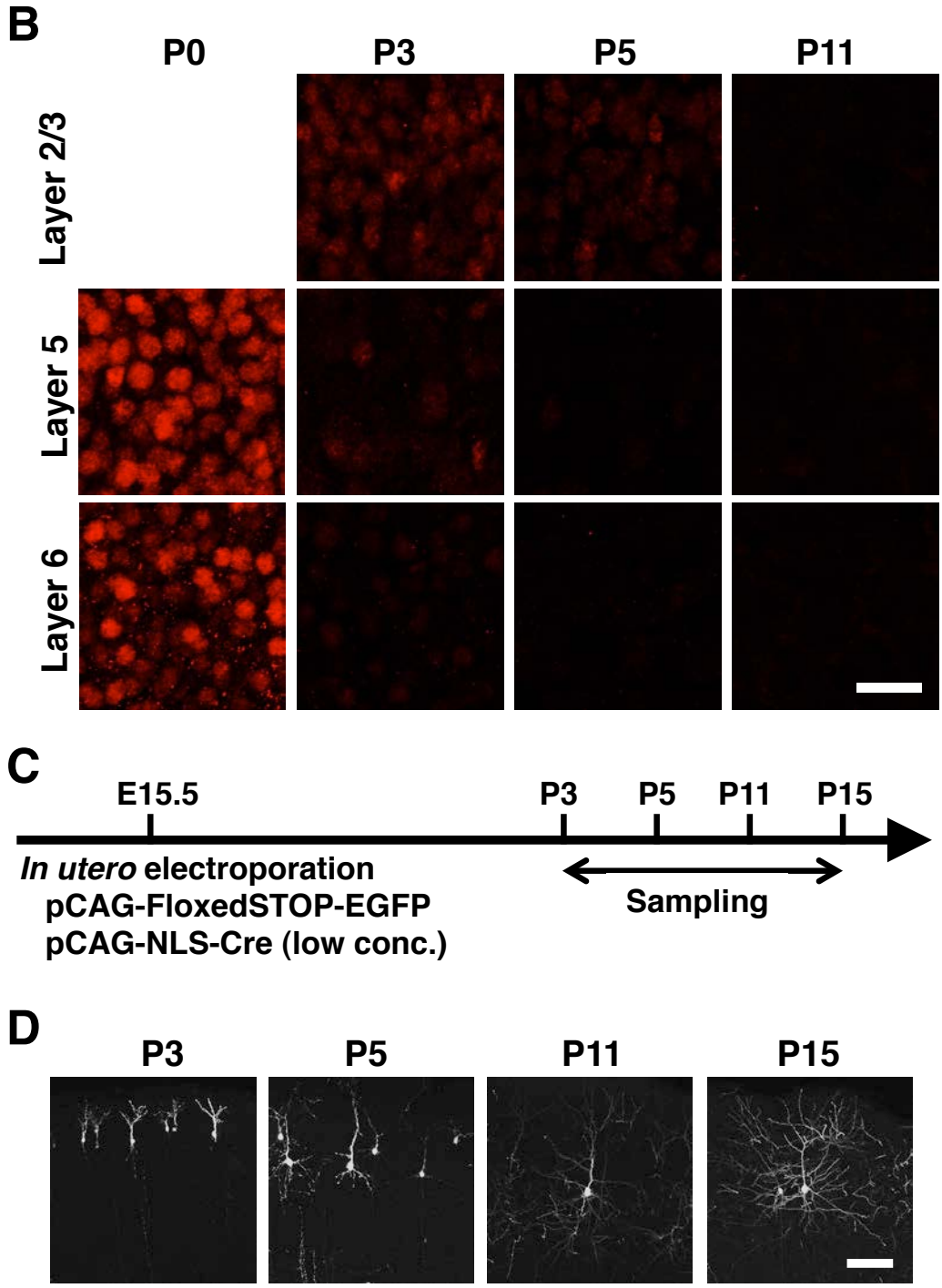

(Figure 3. continued)

B, Magnified confocal microscopic images of Sox11 immunohistochemistry. Layer 2/3 has not been formed at P0. Scale bar = 20 μm. ***C***, Experimental procedure of *in utero* electroporation to reveal the dendritic morphology of small numbers of layer 2/3 neurons. pCAG-FloxedSTOP-EGFP (1 mg/mL) and a very low concentration of pCAG-NLS-Cre (1.5 μg/mL) were co-transfected into layer 2/3 neurons using *in utero* electroporation at E15.5, and coronal sections were prepared at the indicated time points. EGFP signals were enhanced by immunostaining. ***D***, Confocal microscopic images of EGFP-positive layer 2/3 neurons in coronal sections. Scale bar = 100 μm.

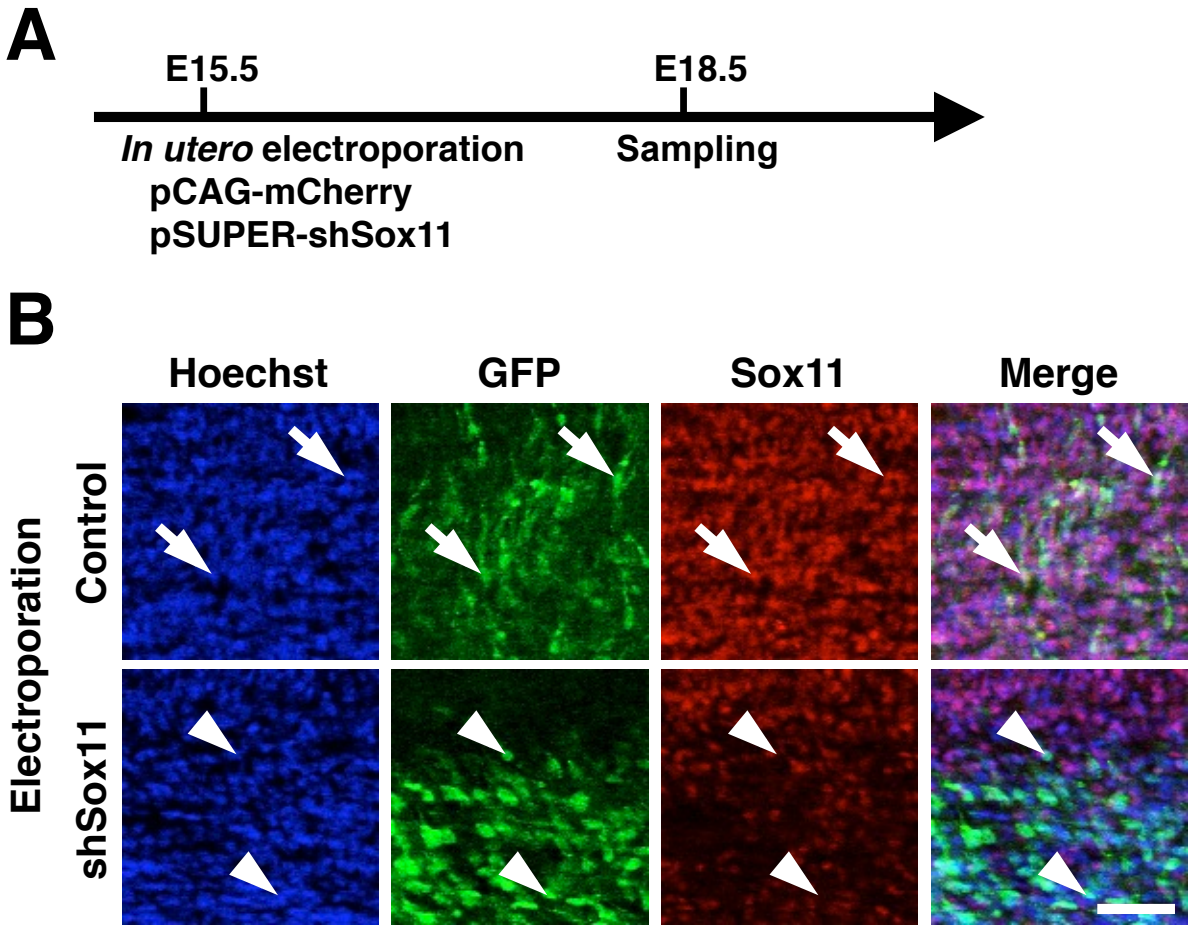

Figure 4. Characterization of the Sox11-shRNA-expression vector. ***A***, Experimental procedure. pCAG-mCherry (0.3 mg/mL) and pSUPER-shSox11 (1.65 mg/mL) were co-transfected into layer 2/3 neurons using *in utero* electroporation at E15.5, and coronal sections were prepared at E18.5 and stained with anti-Sox11 antibody and Hoechst 33342. ***B***, Confocal microscopic images of cells transfected with either control (arrow) or shSox11 (arrowhead) vectors. mCherry-positive transfected neurons in the intermediate zone are shown. Note that density of cell nuclei labeled with Hoechst 33342 was not affected. Scale bar = 50 μm.

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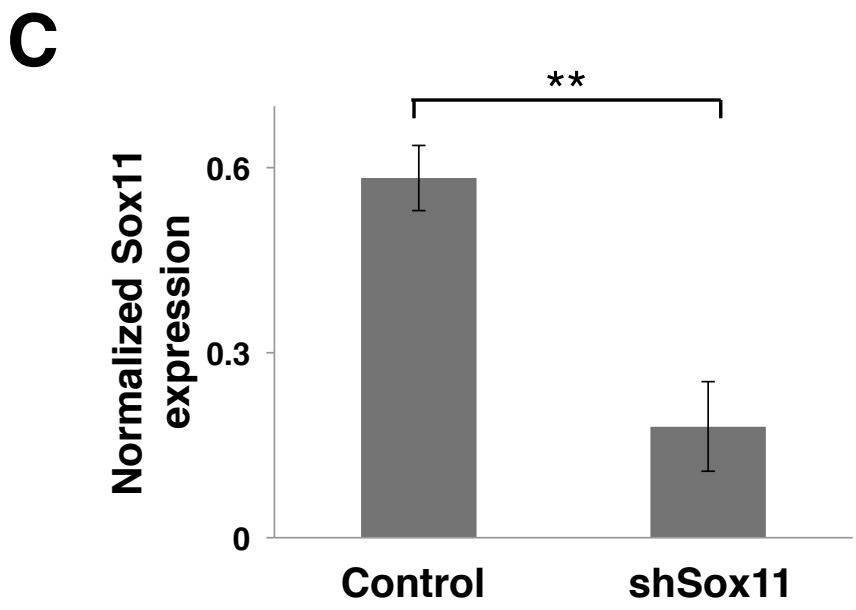

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C, Quantification of the expression levels of Sox11 in EGFP-positive neurons normalized with those in EGFP-negative neurons in a deep layer in the CP which will become layer 6 at later stages. Data are presented as mean ± s.e.m. **, $p < 0.01$. $n = 7$ control embryos and $n = 4$ shSox11-transfected embryos.

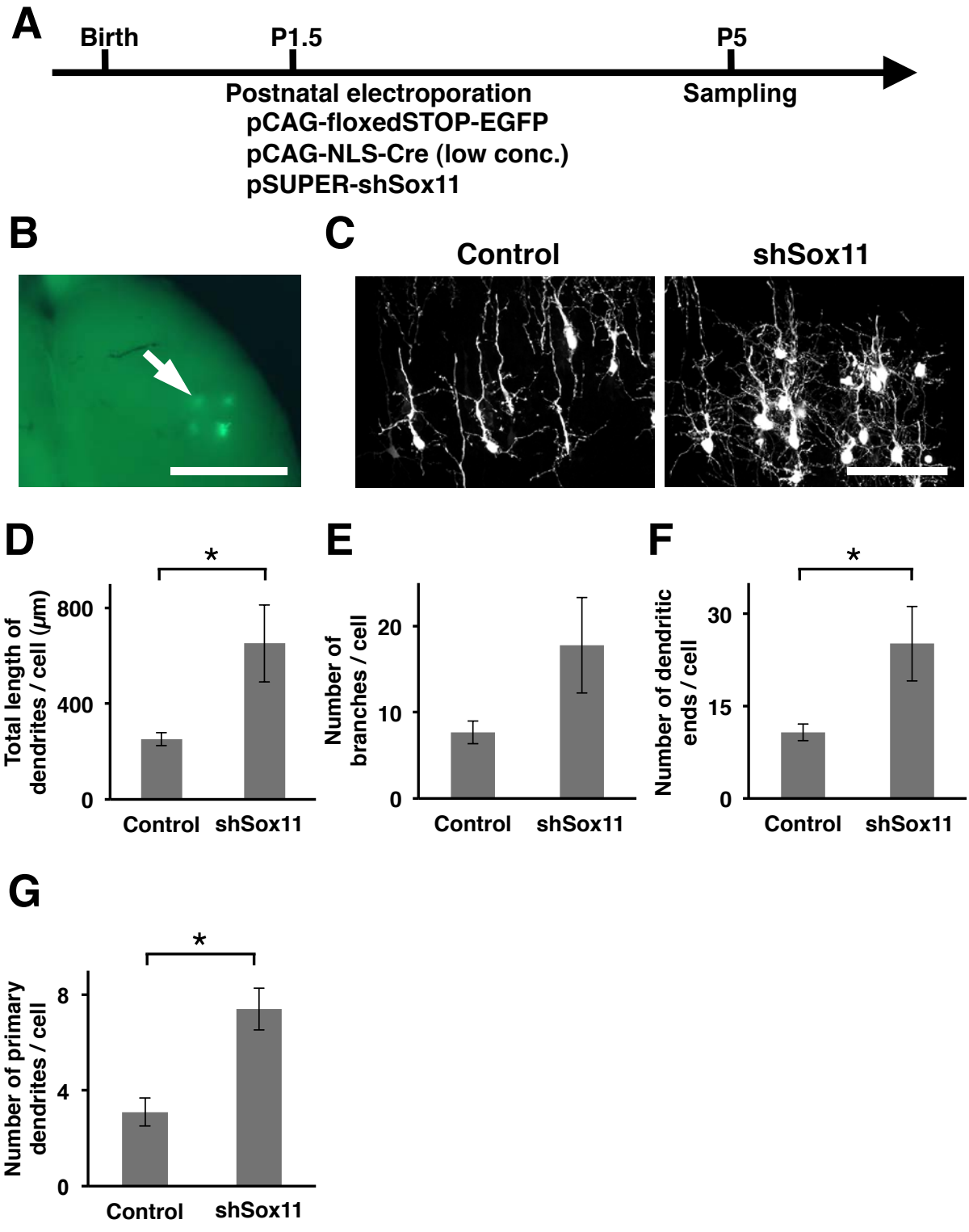
A
Birth
P1.5
P5
Postnatal electroporation
pCAG-floxedSTOP-EGFP
pCAG-NLS-Cre (low conc.)
pSUPER-shSox11
Sampling
B
C
Control
shSox11
D
Total length of dendrites / cell (μm)
800
400
0
*
Control
shSox11
E
Number of branches / cell
20
10
0
Control
shSox11
F
Number of dendritic ends / cell
30
15
0
*
Control
shSox11
G
Number of primary dendrites / cell
8
4
0
*
Control
shSox11

Figure 5. Precocious Sox11 knockdown promotes dendritic morphogenesis in layer 2/3 neurons. ***A***, Experimental procedure of postnatal electroporation to investigate the dendritic morphology of Sox11-down-regulated layer 2/3 neurons. pCAG-FloxedSTOP-EGFP (0.75 mg/mL), a very low concentration of pCAG-NLS-Cre (2.0 μg/mL) and pSUPER-shSox11 (1.25 mg/mL) were co-transfected into layer 2/3 neurons using postnatal electroporation at P1.5, and coronal sections were prepared at P5. EGFP signals were enhanced by immunostaining. ***B***, A low magnification image of the cerebral cortex in which postnatal electroporation was performed. EGFP signals (arrow) were observed at four plasmid injection sites. Scale bar = 2.0 mm. ***C***, Confocal microscopic images of layer 2/3 neurons transfected with either control (left) or shSox11 (right) vectors. Coronal sections are shown. Scale bar = 50 μm. ***D***-***G***, Quantification of the total length of dendrites per cell (***D***), the number of dendritic branches per cell (***E***), the number of dendritic ends per cell (***F***) and the number of primary dendrites per cell (***G***). All values were obtained using 3-4 transfected cells per one animal, and were averaged. Averaged values from 3 animals were used for statistical analyses. Data are presented as mean ± s.e.m. *, $p < 0.05$.

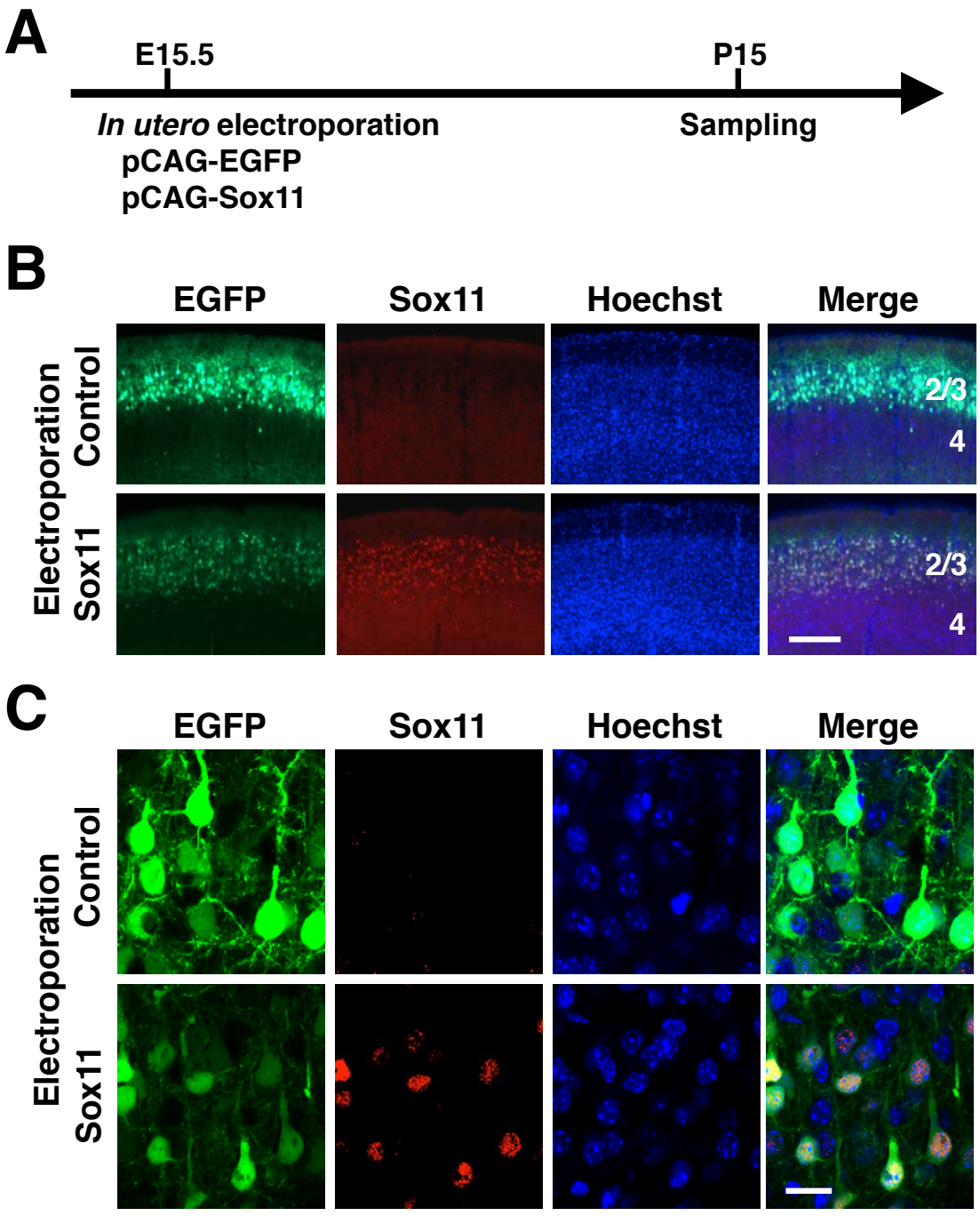

Figure 6. Characterization of the Sox11 expression vector. ***A***, Experimental procedure. pCAG-EGFP (0.5 mg/mL) and pCAG-Sox11 (1.0 mg/mL) were co-transfected into layer 2/3 neurons using *in utero* electroporation at E15.5, and coronal sections were prepared at P15 and immunostained with anti-Sox11 antibody and Hoechst 33342. ***B***, Sox11 immunoreactivity was detected in the cerebral cortex of the pCAG-Sox11-electroporated brain, whereas no signal was detected in that of the control brain. Numbers indicate the corresponding layers in the cerebral cortex. Scale bar = 200 μm. ***C***, High magnification confocal microscopic images of layer 2/3 neurons in ***B***. Scale bar = 10 μm.

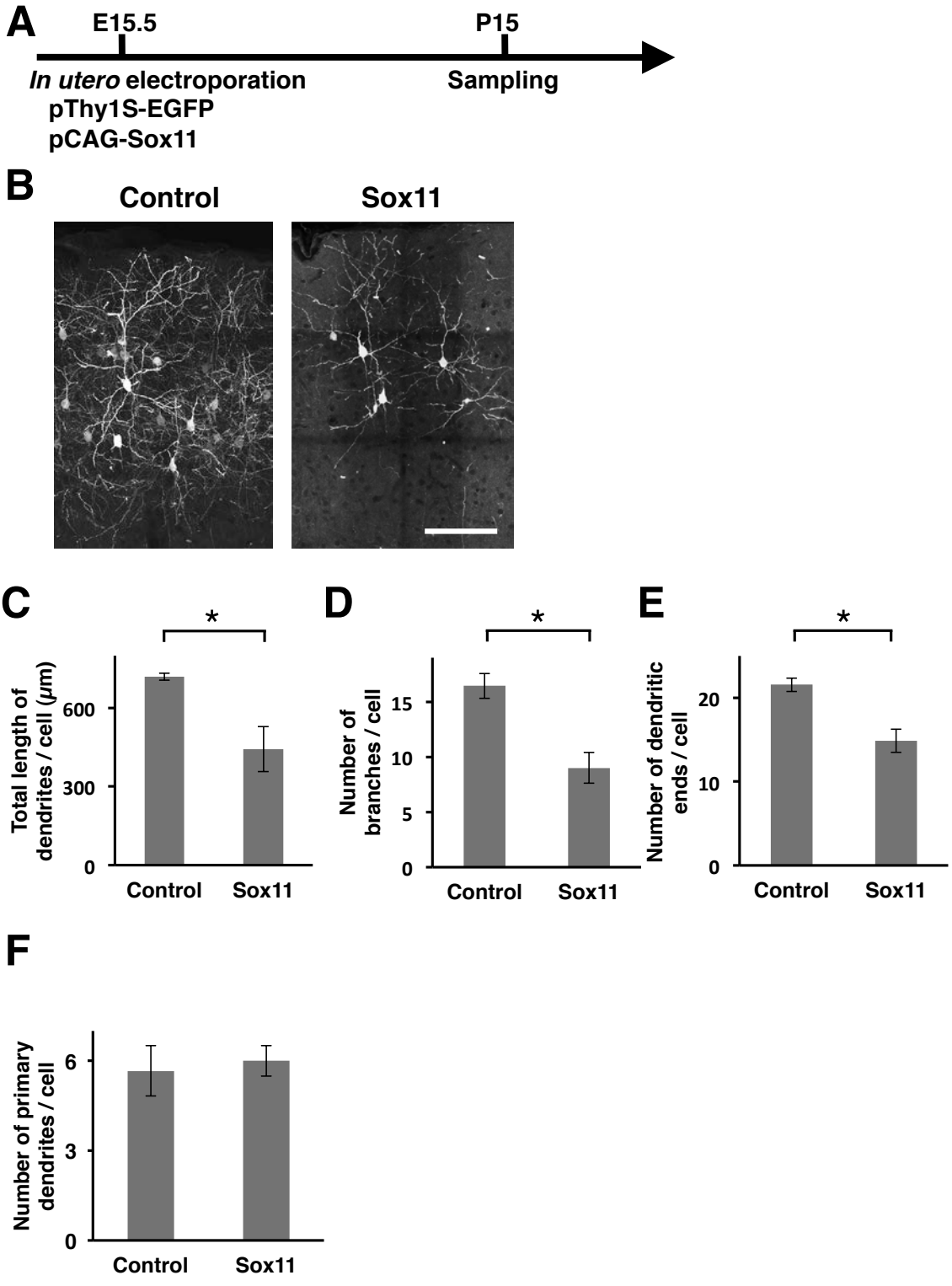

Figure 7. The reduction of Sox11 expression is required for dendritic morphogenesis of layer 2/3 neurons. ***A***, Experimental procedure of *in utero* electroporation to investigate the dendritic morphology of Sox11-overexpressing layer 2/3 neurons. pThy1S-EGFP (1.75 mg/mL) and

pCAG-Sox11 (1.0 mg/mL) were co-transfected into layer 2/3 neurons using *in utero* electroporation at E15.5, and coronal sections were prepared at P15. EGFP signals were enhanced by immunostaining. ***B***, Confocal microscopic images of layer 2/3 neurons transfected with either control (left) or Sox11 (right) vectors. Scale bar = 100 μm. ***C***-***F***, Quantification of the total length of basal dendrites per cell (***C***), the number of branches of basal dendrites per cell (***D***), the number of dendritic ends of basal dendrites per cell (***E***) and the number of primary basal dendrites per cell (***F***). All values were obtained using 3-4 transfected cells per one animal, and were averaged. Averaged values from 3 animals were used for statistical analyses. Data are presented as mean ± s.e.m. *, $p < 0.05$.

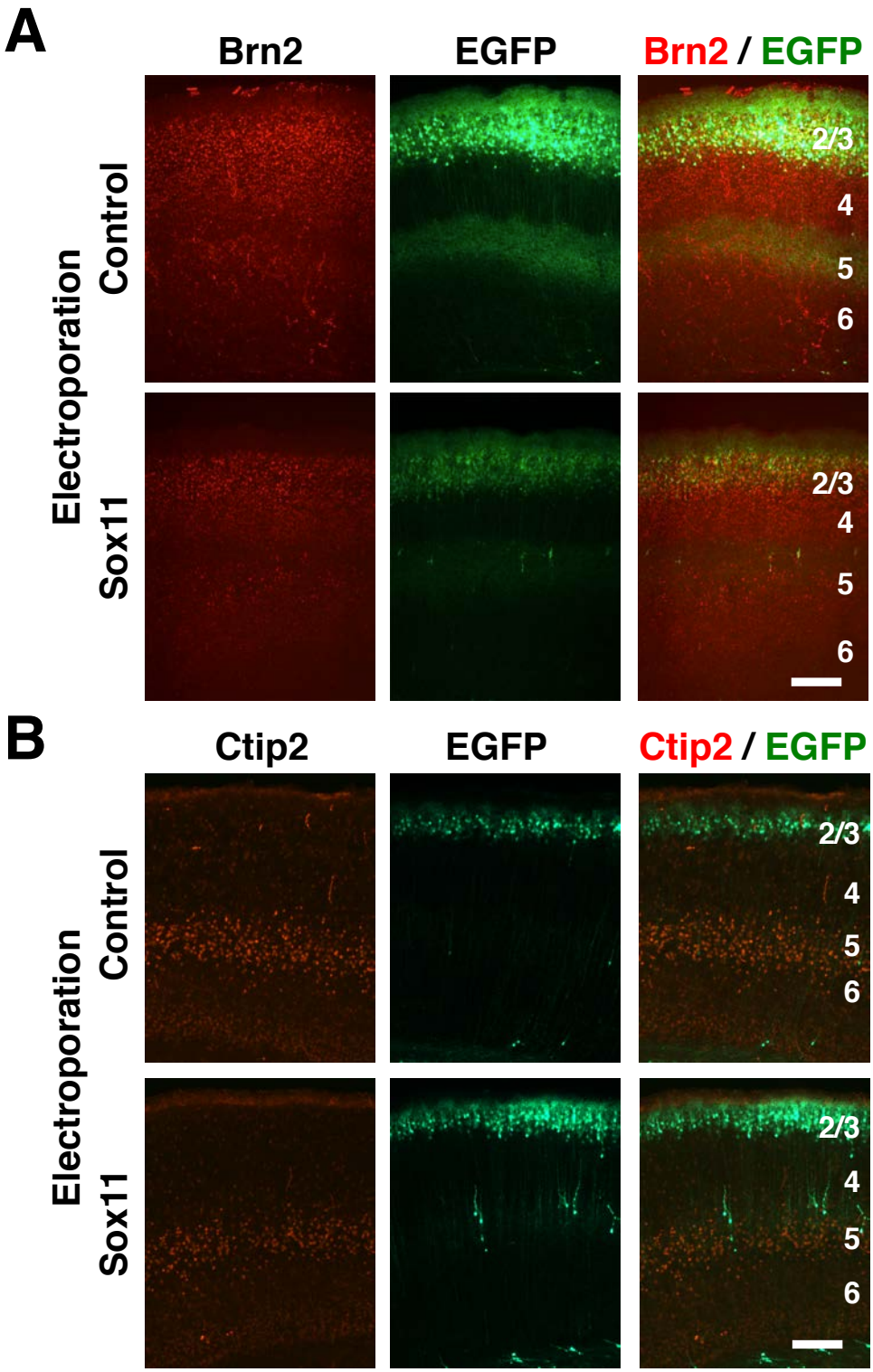

Figure 8. Sox11 overexpression in layer 2/3 neurons does not affect the identities of cortical neurons. EGFP and Sox11 were co-transfected by using *in utero* electroporation at E15.5, and sections were prepared at P5 (Ctip2) or P15 (Brn2, Foxp2). The sections were stained with anti-Brn2, Ctip2 and FOXP2 antibodies. ***A***, Brn2 immunostaining at P15. ***B***, Ctip2 immunostaining at P5.

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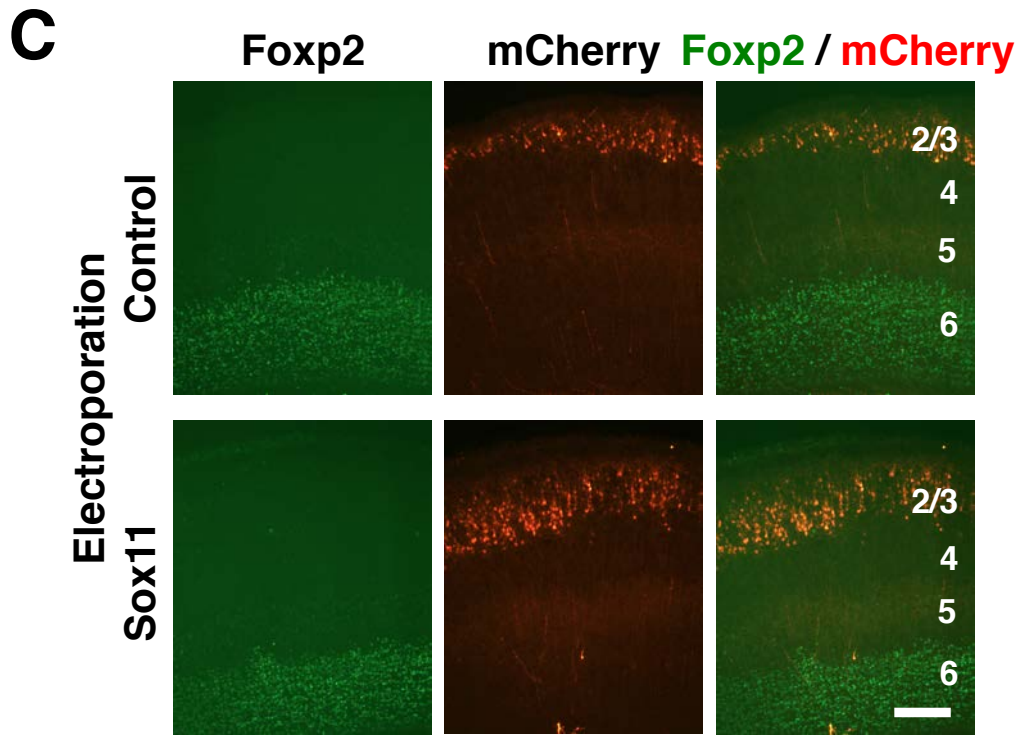
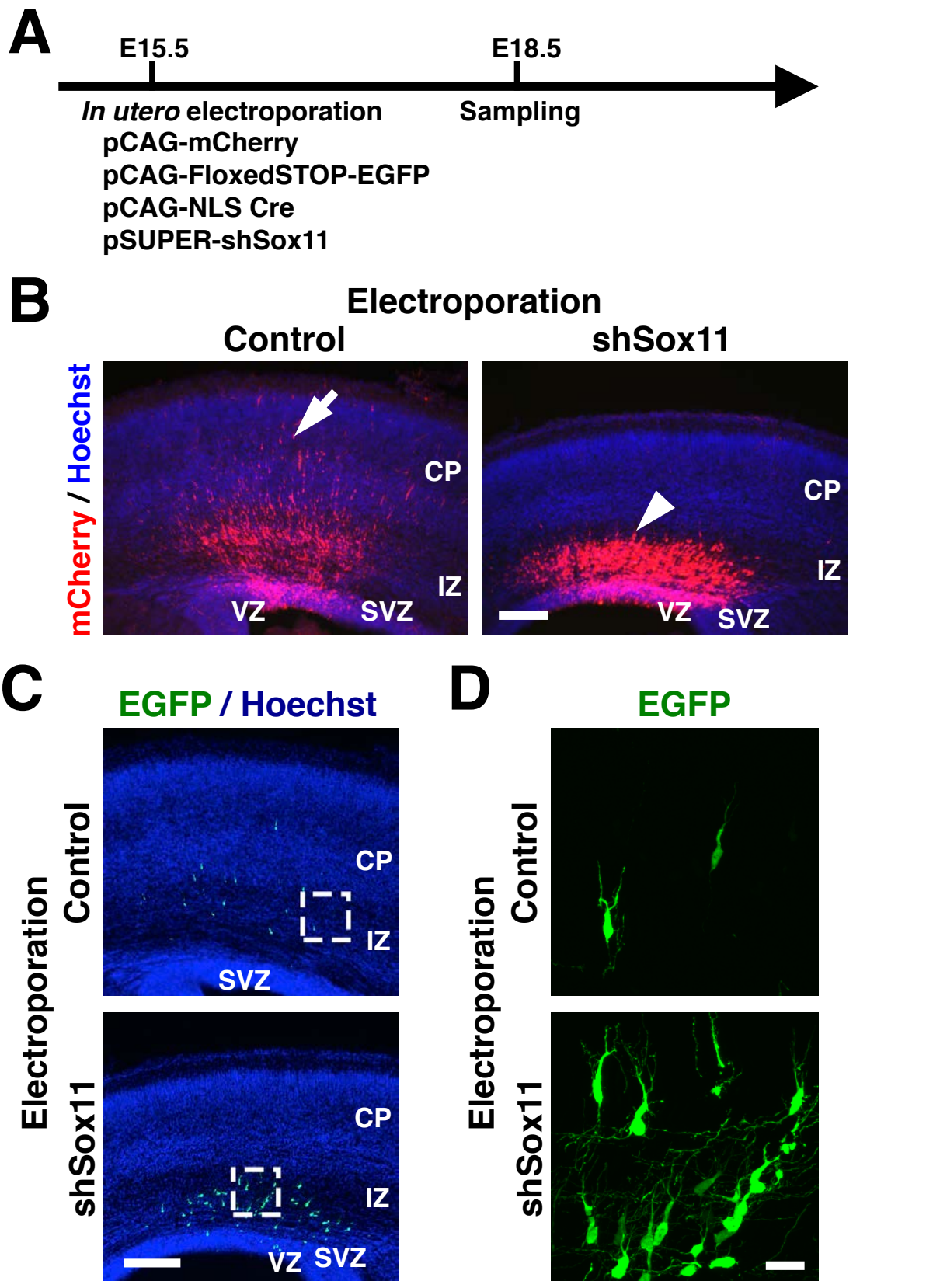


(Figure 8. continued)

C, Foxp2 immunostaining at P6. Numbers indicate the corresponding layers in the cerebral cortex. Scale bars = 250 μm.



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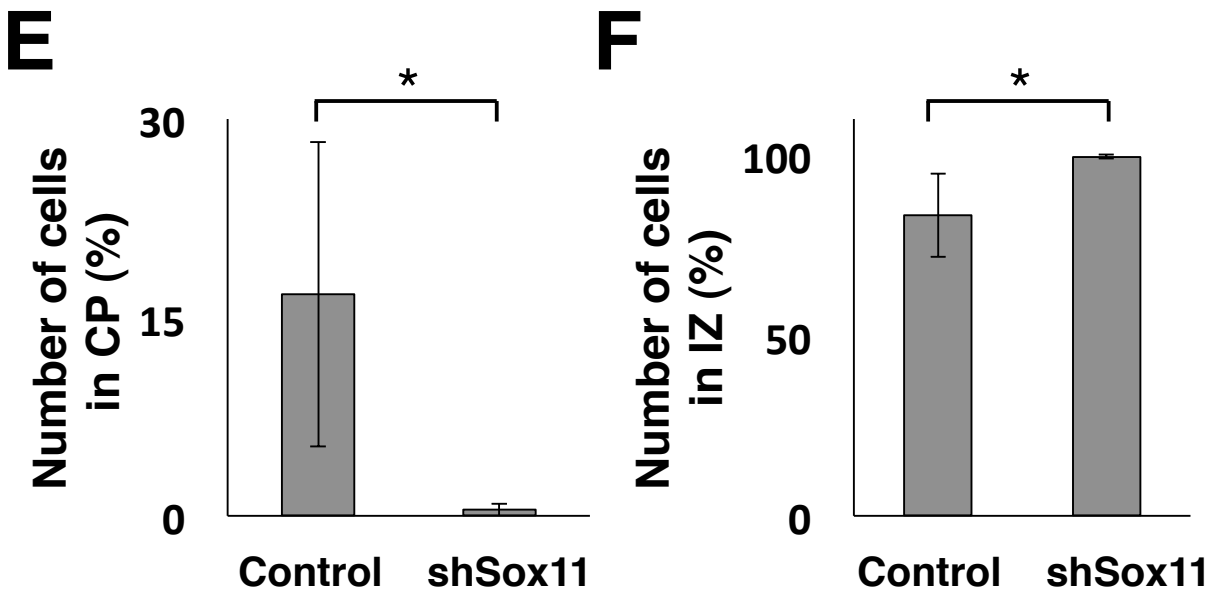

Figure 9. Embryonic knockdown of Sox11 induces precocious dendrite branching and a radial migration defect. ***A***, Experimental procedure. pCAG-mCherry (0.3 mg/mL), pSUPER-shSox11 (1.65 mg/mL), pCAG-FloxedSTOP-EGFP (1.0 mg/mL), and a very low concentration of pCAG-NLS-Cre (1.5 μg/mL) were co-transfected into layer 2/3 neurons using *in utero* electroporation at E15.5, and coronal sections were prepared at E18.5. EGFP signals were enhanced by immunostaining. ***B***, The distribution of mCherry-positive transfected neurons. Note that many mCherry-positive control neurons had reached the cortical plate (CP) at E18.5 (arrow), whereas shSox11-transfected neurons were unable to reach cortical plate (arrowhead). Scale bar = 200 μm. ***C***,***D***, The morphology of neurons transfected with either control (top) or shSox11 (bottom) vectors (green). ***C***, Low magnification images of the cerebral cortex. Scale bar = 400 μm. ***D***, High magnification confocal microscopic images of the boxed areas in **c**. Scale bar = 25 μm. ***E***,***F,*** Quantification of mCherry-positive cells located in the CP (***E***) and the IZ (***F***). 3-4 embryos were used for statistical analyses. Data are presented as mean ± s.d. *, $p < 0.05$. CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone.