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SOX5 SPECIFIES LAYER V SUBCEREBRAL VERSUS CALLOSAL PROJECTION NEURONAL FATES

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by

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Abstract

***Sox5* specifies subcerebral versus callosal projection neuronal fates in layer V**

Sarah Dillon

The precise generation of distinct subtypes of neocortical projection neurons and their proper migration into appropriate laminar positions underlies our most advanced cognitive and perceptual abilities. Recent work suggests that a transcription factor network, including the genes *Sox5, Satb2, Fezf2,* and *Bcl11b,* is responsible for directing the fate specification of subcerebral projection neurons (SCPN) in layer Vb of the cerebral cortex, which is immediately adjacent to laver Va, which is comprised of callosal projection neurons (CPN). Because we observed the absence of SCPN in *Sox5* null cortices, we hypothesized that *Sox5* may specify subcerebral versus callosal neuronal fates in layer V of the cortex. Here, via a series of *in situ* hybridization and immunohistochemistry, and retrograde tracing experiments, we show that in *Sox5* null cortices, layer V neurons do not take on the molecular identity of layer Vb SCPN and instead, they appear to take on the identity of layer Va CPN. I also demonstrate via birthdating experiments that there is a migration defect *Sox5* null cortices, and layer V cells are generated prematurely. Overall, the work presented here provides an in-depth phenotypical characterization of layer V in *Sox5* null cortices.

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Sox5 specifies subcerebral versus callosal projection neuronal fates in layer V

by Sarah Dillon

Introduction

Proper functioning of the neocortex relies upon the precise specification of its distinct subtypes of projection neurons, their appropriate laminar positioning, and their integration into functional neural circuits. The human neocortex is the most evolutionarily recent brain structure, characterized by its six-layered structure; it is a major integrating center in the nervous system and is responsible for complex brain functions such as consciousness, decision-making, and perception. The specification of cortical neurons takes place during neocorticogenesis and is governed by cell-intrinsic factors, such as the expression of transcription factors, and cell-extrinsic factors, such as the sensing of extracellular signals. Improper specification of neuronal subtypes and the consequential inappropriate laminar positioning and connectivity of these neurons can lead to neurological disorders, such as epilepsy and schizophrenia (Selemon et al., 2015; Barkovich 2015). Furthermore, mutations in specific genes that regulate cortical development have been implicated in intellectual deficiencies and autism (Balasubramanian et al., 2011; Docker et al., 2014; Willsey et al., 2013; Notwell et al., 2016). Understanding the molecular mechanisms that underlie cell fate specification and sequential generation of neural circuits can aid in the discovery of potential therapies for neurological disorders.

The process of neocortical development is relatively well-conserved between mice and humans. Although mice are lissencephalic, the neocortical cytoarchitecture, regionalization of major cortical areas, and major developmental processes are similar between mice and humans (Geschwind et al., 2013; DeFelipe, 2011). Additionally, the wealth of genetic tools available allows for precise and rapid genetic manipulation. Therefore, to study cortical development, the Chen lab takes advantage of forward and reverse genetics in the mouse to study gene function.

Gene-expression programs during neocorticogenesis regulate the fates of neural progenitors and influence developing neurons to produce distinct subtypes of cortical neurons during neocorticogenesis. Glutamatergic excitatory neurons are generated from radial glial cells (RGCs) and intermediate progenitor cells (IPCs). The newly generated neurons migrate to their birthdate-appropriate layers and are further specialized to adopt their subtype-specific gene expression and phenotypical characteristics. Much progress has been made toward understanding the molecular mechanisms by which fate specification of cortical neurons is mediated. Several transcription factors have been identified that are critical in the specification of layer V subcerebral projection neurons (SCPNs). For example, *Sox5, Satb2, Fezf2, and Bcl11b* are necessary for directing the fate specification and terminal differentiation of SCPNs in layer V of the murine neocortex (Chen et al., 2008; Kwan et al., 2008; Alcamo et al., 2008; Lai et al., 2008; McKenna et al., 2015; Tsyporin et al., 2021; unpublished data). However, the ways in which this network operate are still being investigated.

Neocorticogenesis and migration

Throughout neocorticogenesis, the development potential of neural progenitors becomes progressively more restricted. Such restriction leads to the specification of distinct subtypes of cortical neurons. At the onset of neurogenesis in the neocortex, neuroepithelial cells (NCs) in the developing telencephalon differentiate into RGCs (Malatesta et al., 2003; Götz and Huttner, 2005). In the ventricular zone (VZ), multipotent RGCs undergo symmetric proliferation and divide asymmetrically to produce cortical excitatory neurons or fate-restricted neural progenitors, IPCs. IPCs migrate to the subventricular zone (SVZ), where they undergo 1-3 cycles of symmetric divisions before beginning to generate postmitotic cortical excitatory neurons. As the newly born neurons migrate along the basal processes of RGCs through the cortical plate, they are subjected to intrinsic and extrinsic factors that influence their birthdate-appropriate laminar positioning.

The neocortex is characterized by its six-layered structure, and this requires the proper migration of newly generated neurons into their birthdate-appropriate layers. The neocortex is formed in an inside-out manner, such that the earliest-born neurons settle in the subplate and deeper layers (V, VI), and the later-born neurons migrate through the cortical plate, past the established neurons, to their respective positions in the upper layers (I-IV). Mutations in genes associated with epilepsy and mental retardation are due to altered patterns of neuronal migration, which leads to a disruption

or of normal laminar organization. For instance, in *reeler* mutants, the neocortex develops in an outside-in manner due to the improper secretion of extracellular matrix protein, REELIN, by Cajal-Retzius cells (D'Arcangelo et al., 1995). Previous studies have shown that subplate cells also play a large role in radial neuronal migration patterns, as well as thalamocortical axon pathfinding in the developing neocortex (Ghosh et al., 1990; Lopez-Bendito and Molnar, 2003; Hoerder-Suabedissen and Molnar, 2015; Maruyama, 2020).

Fate specification and differentiation

The Each layer of the neocortex is composed of a distinct combination of neuronal subtypes, classified by their axonal projections and molecular identities. For example, layer V can be subdivided into layer Va and Vb, where layer Va is composed of corticocortical projection neurons that either extend their axons ipsilaterally (intracortical) or contralaterally (callosal; CPN), and layer Vb is predominantly made up of SCPNs, which project to the midbrain, pons, superior colliculus, and spinal cord. Transcription factors, including *Fezf2, Bcl11b, Sox5,* and *Satb2,* are necessary to specify these neuronal fates, however the precise underlying molecular mechanisms are not completely understood (Chen et al., 2008; Alcamo et al., 2008; Britanova et al., 2008; McKenna et al., 2015; Kwan et al., 2008; Lai et al., 2008; Tsyporin et al., 2021). *Sox5* has been studied in layer VI, but has equally important and underreported roles in layer V. Therefore, we decided to further investigate its function in layer V.

The SOX family

Sox5 is a member of the *SRY-related high-mobility-group (HMG)-box (Sox*) superfamily of genes that encode for transcription factors expressed in a wide variety of developing tissues. The N-terminus of OX5 has a coiled-coil domain, allowing the protein to form homo- and heterodimers with itself or other comembers of the SoxD subfamily. The HMG-box DNA-binding domain, which is present in all SRY-box family members, is located in the C-terminus region of SOX5, and can bind and bend DNA (Angelozzi, 2019). Unlike many transcription factors, SOX5 does not have its own transcriptional inhibition or activation domains, but it can act as a transcriptional repressor or enhancer, perhaps by interacting with other proteins (Lefebrve, 2010).

Approach

To determine the function of *Sox5* in layer V cortical neuron specification and differentiation, I used a conditional knockout strategy in which I utilized a conditional *Sox5* allele ($Sox5^{flox}$) in conjunction with a cortex-specific Cre driver line ($Emx1^{Cre}$) to specifically knock out *Sox5* in the developing cerebral cortex. I performed a series of immunohistochemistry and *in situ* hybridization experiments using layer V specific markers in order to characterize the molecular phenotype associated with the *Sox5 cko*. To determine if there was a cell fate change from SCPN to CPN, my partner, Jeremiah Tsyporin, performed retrograde tracing from the primary somatosensory cortex, as well

as the pyramidal decussation, of *Sox5 wt* and *Sox cko* mice, to label CPN and SCPN, respectively. Finally, I performed a birthdating experiment to determine if there is a migration defect in the *Sox5 cko* at E13.5. I conclude that *Sox5* specifies subcerebral versus callosal neuronal fates in layer V of the developing cerebral cortex.

Results

Layer Va molecular markers expand in the *Sox5 cko*

Because *Sox5* knockout *(ko)* mice die within hours of birth, we generated *Sox5*$^{LacZ/fl}$*; Emx1*$^{Cre/+}$*(Sox5 cko)* mice to delete *Sox5* in dorsal radial glial cells. *Sox5*$^{fl/+}$*; Emx1*$^{+/+}$ mice were used as wildtype (*wt*) controls. Notably, SOX5 is not expressed in neural progenitors of the VZ and SVZ but is expressed in newly born subplate, layer V, and IV cortical excitatory projection neurons (Figure 1).

Previous studies showed that in layer VI, corticothalamic projection neurons are imprecisely differentiated in the *Sox5 ko* (Kwan et al., 2008; Lai et al., 2008). To determine the layer V phenotype in *Sox5 cko* cortices, we performed a series of immunohistochemistry experiments to specifically label populations of layer V projection neurons (Figure 2). Using molecular marker BCL11B with either marker BHLHB5, BCL11A, or FEZF2, we observed a significant reduction in BCL11B; BHLHB5, BCL11A, or FEZF2 double-positive cells within layer V of the P7 *Sox5 cko* primary somatosensory cortex (S1) compared to *Sox5 wt* (Figure 2A-B). While co-expression of BHLHB5 and BCL11B was nearly completely absent in the *cko*, the population of cells co-expressing BCL11B with either BCL11A or FEZF2 shifted from layer V to layer VI (Figure 2A-B). Notably, the population of layer Va FEZF2^{+};BCL11B^{-} cells expanded in the *Sox5 cko* (Figure 2C), suggesting that there may be an increase in layer Va CPN in the *Sox5 cko*.

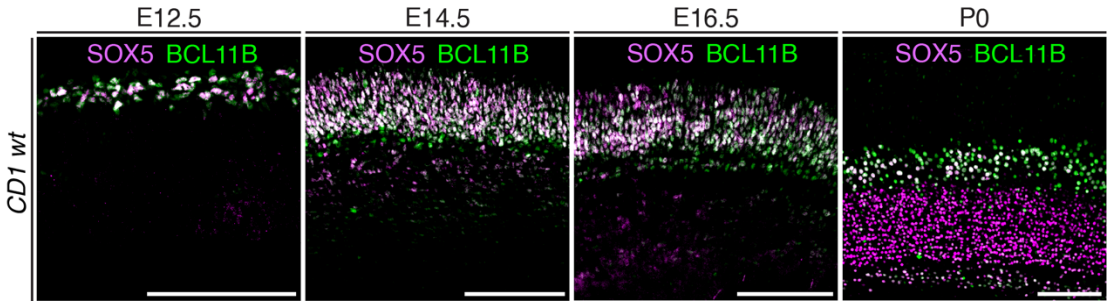

Figure 1. SOX5 is expressed in postmitotic cortical excitatory neurons. Immunostaining for SOX5 and BCL11B on sections from embryonic and postnatal CD-1 wildtype (wt) cortices. Scale bars: 100um.

We then decided to further investigate whether there is an increase in layer Va CPN in the *Sox5 cko*. It has previously been shown that co-expression of BCL11A and SATB2 molecularly identifies deep-layer CPN (Cánovas et al., 2015). We performed immunohistochemistry for BCL11A and SATB2 to label this subpopulation of neurons, along with BCL11B to denote the layer V and VI boundaries (Figure 3A). We observed a slight but significant increase in the number of $SATB2^{+}$;$BCL11A^{+}$ cells in layer Vb of the *cko* (Figure 3B). This suggests that there may, indeed, be an increase in CPN in the *Sox5 cko*. Taken together with the observed increase in layer Va $FEZF2^{+}$;$BCL11B^{-}$ cells (Figure 2C), these results suggest that layer Va may be expanded in the *Sox5 cko*.

Next, we sought to further characterize the layer V phenotype in *Sox5 wt*, *cko* and *Fezf2 ko* (Chen et al., 2008) S1 cortices by performing a series of *in situ* hybridizations with probes targeting layer V molecular markers (Figure 4). Previous results showed that *Fezf2 ko* cortices completely lack layer V SCPNs, but whether there is an expansion or absence of layer Va is unclear. A dramatic expansion

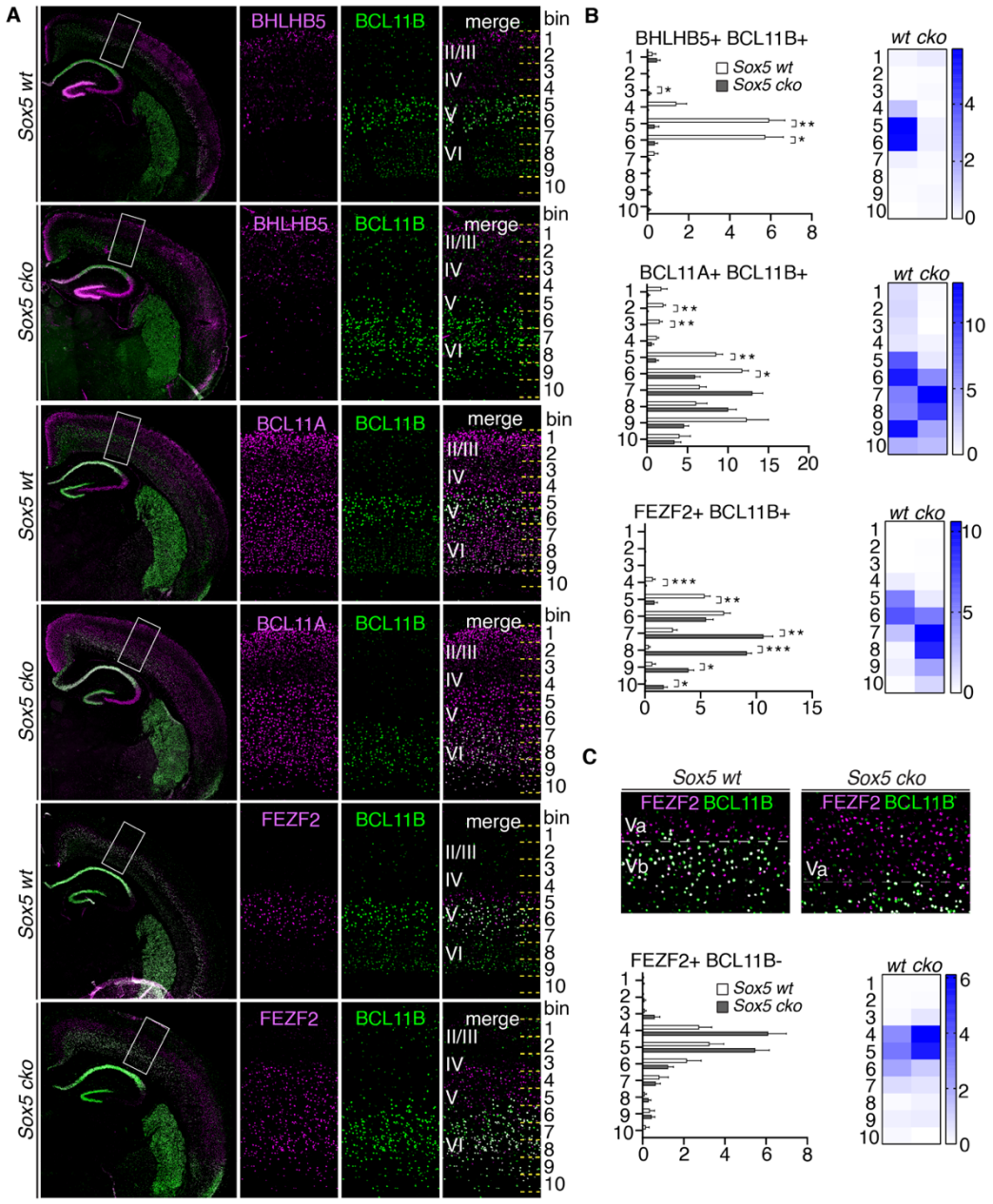

Figure 2. Layer V neurons in *Sox5 cko* S1 cortices do not molecularly identify as SCPN. (**A**) Immunostaining for BHLHB5, FEZF2, BCL11A, and BCL11B on sections from P7 *Sox5 wt* and *Sox5 cko* cortices. Each section was divided into 10 bins (width: 500μm) for the purpose of quantification. Scale bar for low magnification: 1000um. Scale bar for high magnification: 100um. (**B**) Quantifications for double-marker+ neurons per 10,000μm2 for each bin. n=3 of each genotype, 3 sections per brain. Heat maps represent the mean number of double-marker+ neurons per 10,000μm2 for each bin. Error bars in all graphs represent ±SEM. Statistical significance was determined by using a paired t-test (*p < 0.05; **p < 0.01; ***p < 0.001). **Continued on next page.**

Continued: C) High magnification images of cortical layer V immunostaining for FEZF2 and BCL11B derived from merge panels in **A** (bins 4-7). Dashed lines represent the border between layers Va and Vb, as indicated by $FEZF2^{+}$;$BCL11B^{-}$ neuronal expression. Scale bar: 100um. Parameters for the quantification, heatmap, and statistical analysis are the same as outlined in **B**.

of *Kirrel3* was observed in the *Sox5 cko*, and its expression in the *Fezf2 ko* remained unchanged or slightly decreased, demonstrating that *Kirrel3* is expressed in layer Va (Figure 4A). *Etv1* expression also dramatically increased in the *Sox5 cko* (Figure 4A). However, the known layer Va CPN marker, *Plxnd1,* and its ligand, SEMA3E, showed a very slight increase and sharp decrease in expression in the *Sox5 cko* respectively (Molyneaux et al., 2009; Velona et al., 2019; Figure 4B). Although *Kirrel3* and *Etv1* expanded in the *Sox5 cko*, *Plxnd1* and *Sema3e* did not. Therefore, it's unclear based on molecular markers alone if layer Va CPNs expand into layer Vb in the Sox5 cko.

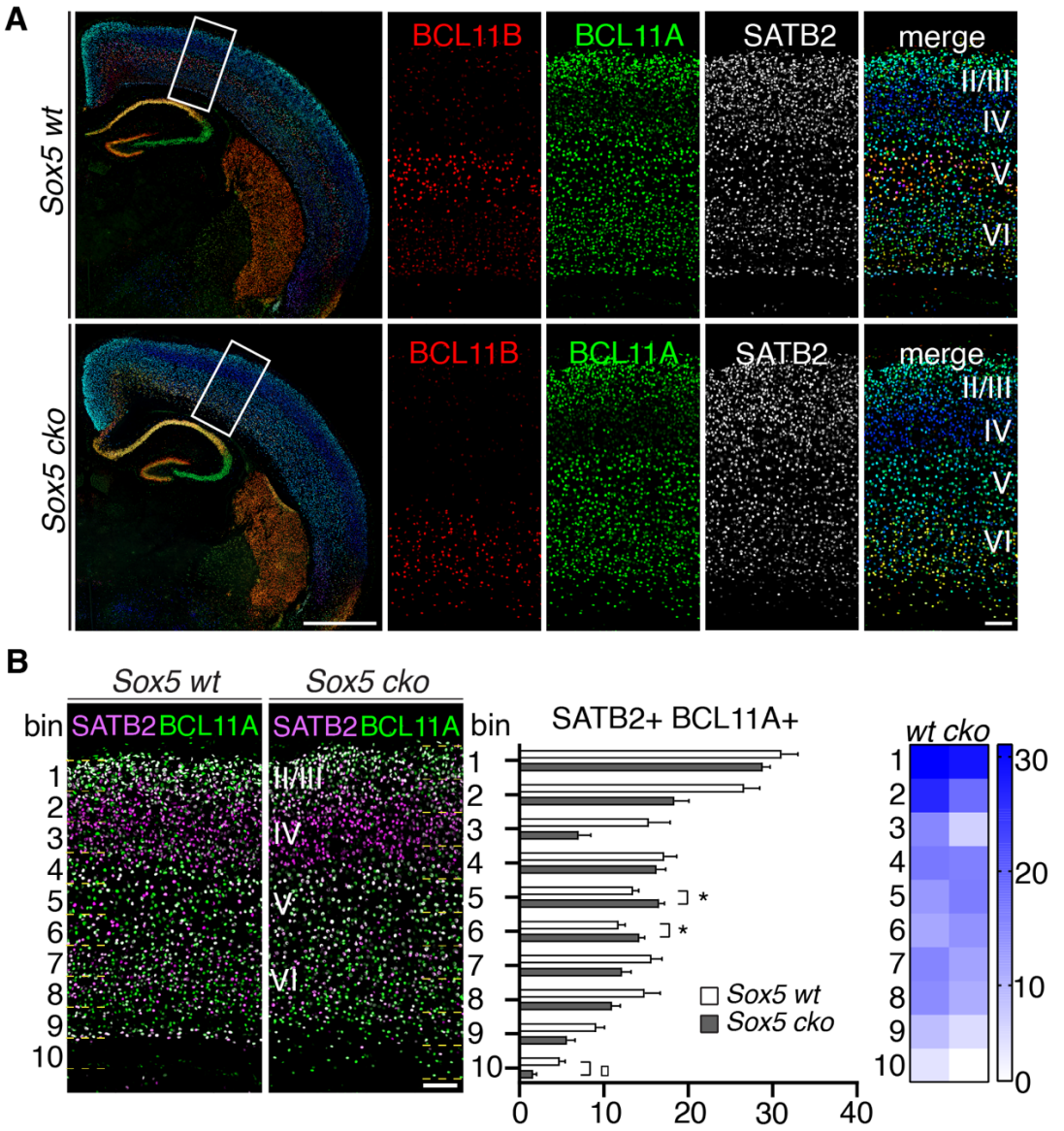

Figure 3. Molecular markers for CPN increase in layer Vb of the *Sox5 cko* (**A**) Immunostaining for SATB2, BCL11A, and BCL11B on sections from P7 *Sox5 wt* and *Sox5 cko* cortices. Scale bar for high magnification: 100um. Scale bar for low magnification: 1000um. **(B)** Higher magnification images of SATB2 and BCL11A immunostaining from cortical sections in **A**. Scale bar: 100um. Quantifications for double-marker$^+$ neurons per 10,000um^2 for each bin (width: 500μm). n=2 of each genotype, 3 sections per brain. Error bars represent +/- SEM. Heat map represents the mean number of double-marker$^+$ neurons per 10,000um^2 in each bin. Statistical significance was determined by using an unpaired t-test (*$p < 0.05$).

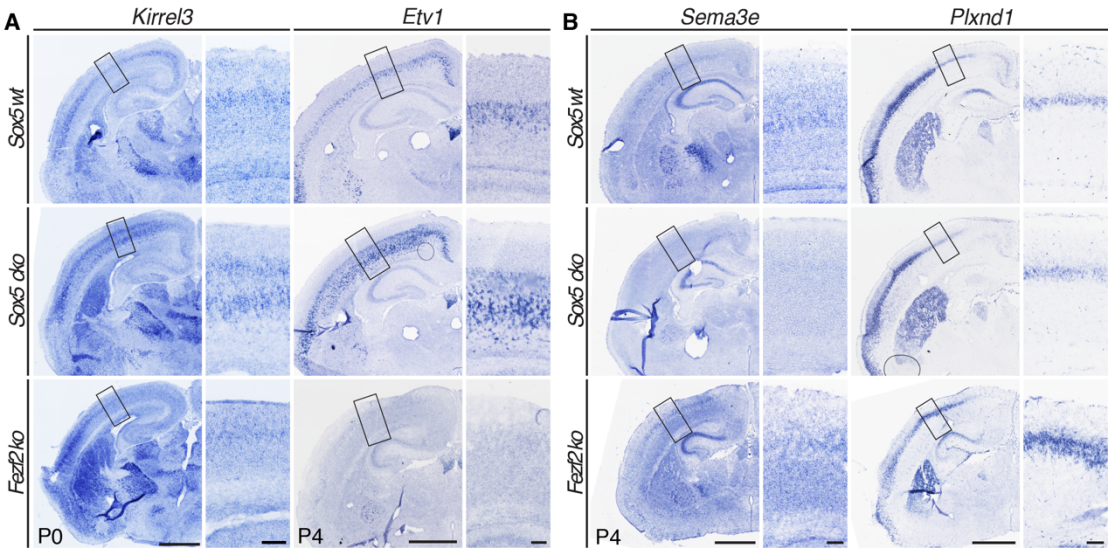

Figure 4. Layer Va markers expand in the *Sox5 cko.* *In situ* hybridizations for *Kirrel3, Etv1* (**A**), *Sema3e,* and *Plxnd1* (**B**) on sections from *Sox5 wt, Sox5 cko,* and *Fezf2 ko* cortices. Scale bars for low magnification: 1000um. Scale bars for high magnification: 100um.

Layer Va CPNs may be present in layer Vb at the expense of SCPNs in the *Sox5 cko* cortex.

To determine if there is indeed an increase in CPN in the *Sox5 cko*, we performed retrograde tracing experiments by injecting fluorophore-conjugated cholera toxin beta (Ctß) into the contralateral S1 cortices of P4 *Sox5 wt* and *cko* mice (Figure 5A-B). In the P7 *Sox5 wt* cortex, we observed a thin band of BCL11B^{-}; Ctß$^{+}$ CPN in layer Va (Figure 5A'), similar to the band of FEZF2^{+}; BCL11B^{-} cells observed in the immunostaining experiments mentioned earlier (Figure 2C). Unlike in the *Sox5 wt,* a tightly organized band of Ctß$^{+}$ CPN was not present in the *Sox5 cko* cortex (Figure 5B"). Instead, Ctß$^{+}$ CPN were more widely distributed throughout layer V, suggesting

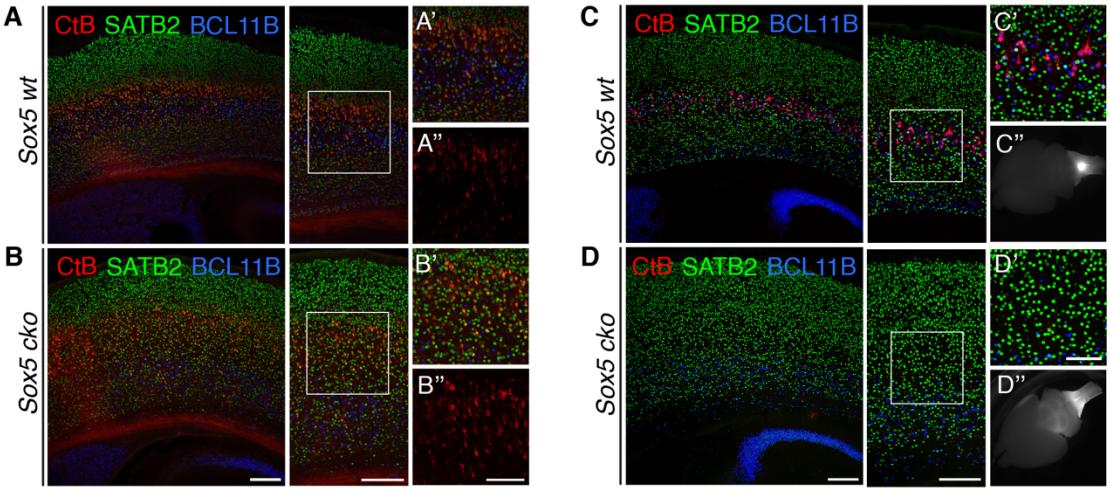

Figure 5. Layer Va cells may be present in the layer Vb at the expense of SCPNs in the *Sox5 cko* cortex. Immunostaining for SATB2 and BCL11B on sections from P7 *Sox5 wt* and *Sox5 cko* mice injected in contralateral S1 cortices (**A-B**) or in the pyramidal decussation (PD) (**C-D**) with fluorophore-conjugated Ct-ß at P4. Scale bars: 200um. (**A'-A"; B'-B"; C'; D'**) Higher magnification images of boxed cells in previous panel. Scale bars 100um. (**C"; D"**) Whole mount P7 brains with injection site in PD.

an expansion of layer Va CPNs into layer Vb. Because only two brains per genotype were looked at, the data are currently inconclusive, and data acquisition is in progress. However, we were able to confirm previous findings that SCPN in *Sox5 cko* cortices are nearly completely absent (McKenna et al., 2015; Kwan et al., 2008; Lai et al., 2008; Figure 5C-D). There is a possibility that in the *Sox5 cko*, more layer Va CPNs are generated at the expense of SCPNs.

Cells born at E13.5 express more layer Va markers in the *Sox5 cko*

It has previously been reported that *Sox5 cko* cortices exhibit a migration defect in which cortical excitatory neurons migrate past their typical laminar targets (Lai et al., 2008; Kwan et al., 2008). However, this migration defect has not been studied

during the developmental time window when SCPNs are being generated (E13.5). Therefore, we injected EdU into pregnant dams at E13.5 and analyzed the cortices of P7 *Sox5 wt* and *cko* progeny (Figure 6). The results show that, indeed, there is a shift upwards in the laminar positioning of neurons born at E13.5 in the *Sox5 cko* (Figure 6A-C). Whereas ~80% of EdU^+ cells in the *wt* are relatively equally distributed between layers IV/V and V/VI boundaries (Figure 6C; bins 4-7), ~60% of EdU^+ cells are positioned at, or immediately adjacent to, the layer IV/Va boundary (Figure 6C; bins 3-4) in the *cko*.

In conjunction with EdU-labeling, we performed immunohistochemistry with combinations of molecular markers that identify SCPN ($FEZF2^+$;$BCL11B^+$), layer Va neurons ($FEZF2^+$; $BCL11B^-$), and CPN ($SATB2^+$; $BCL11A^+$; Figure 6A-B). We observed an increase in layer Va marker expression in cells born at E13.5 in the *Sox5 cko* as a percent of total EdU^+ cells (Figure 6A-B, E). We also observed that nearly no cells born at E13.5 molecularly identified as SCPN (Figure 6A, D, E).

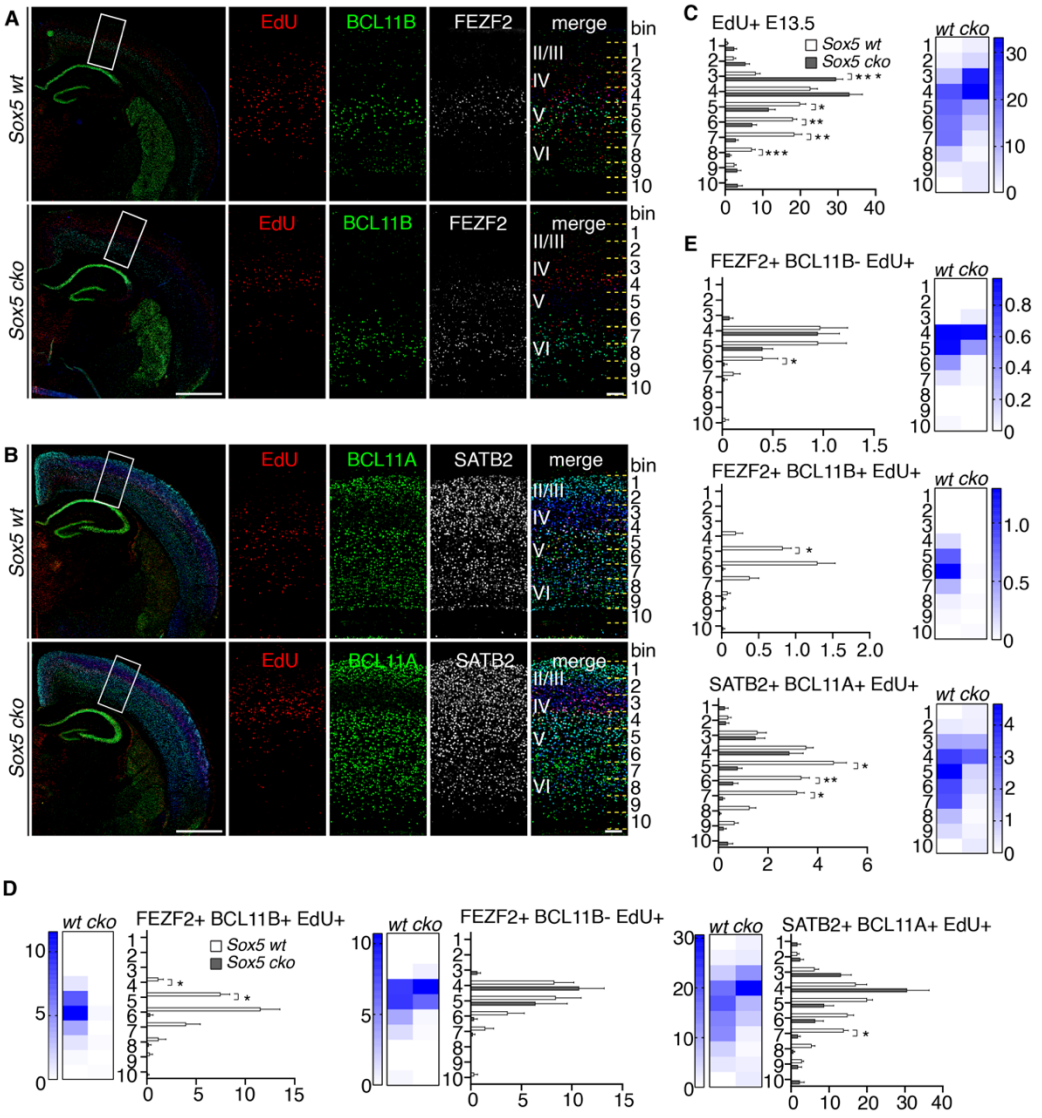

Figure 6. Cells born at E13.5 express more layer Va markers in the *Sox5 cko*. (**A-B**) Immunostaining for FEZF2 and BCL11B (**A**) or SATB2 and BCL11A (**B**) on cortical brain sections from P7 *Sox5 wt* and *Sox5 cko* mice injected with EdU (red) at E13.5. Each section was divided into 10 bins (width: 500μm) for the purpose of quantification. Scale bar for low magnification: 1000um. Scale bar for high magnification: 100um. (**C-E**) Quantifications for EdU$^+$ neurons (**C**) and EdU$^+$;marker$^{+/-}$ neurons per bin as a percentage of total EdU$^+$ neurons (**D**) or number of EdU$^+$;marker$^{+/-}$ neurons per 10,000μm^2 in each bin (**E**). For EdU$^+$ quantifications (**C**), n=3 per genotype, 6 sections per brain. n=3 per genotype, 3 sections per brain for EdU$^+$;marker$^{+/-}$ quantifications (**D-E**)**.** Heat maps represent the mean number of EdU$^+$ and EdU$^+$;marker$^{+/-}$ neurons in each bin as a percentage of total EdU$^+$ neurons (**C-D**) or the mean number of EdU$^+$;marker$^{+/-}$ neurons per 10,000μm^2 in each bin (**E**). Error bars in all graphs represent ±SEM. Statistical significance was determined by using a paired t-test (*p < 0.05; **p < 0.01; ***p < 0.001).

Discussion

Frequently, it is observed in the cerebral cortex that cell fate specification occurs via transcriptional repressive mechanisms in post-mitotic neurons. For example, FEZF2 promotes subcerebral identity in layer V by repressing genes associated with corticothalamic and callosal projection neurons (Tsyporin et al., 2021). Within layer V, there are two distinct subpopulations of projection neurons: layer Va CPN and layer Vb SCPN. SOX5 has been proposed to function as a transcriptional repressor (Kwan et al., 2008). Similar mechanisms may be at play in the specification of cell types within different layers of the cortex. One of the functions of Sox5 may be to promote layer Vb identity through the repression of genes associated with layer Va. Further mechanistic studies using CUT&RUN and single-cell-RNAseq may elucidate the precise function of SOX5.

Retrograde tracing from S1 in *Sox5 wt* and *cko* cortices showed a difference in the spatial distribution of CPN in layer V (Figure 5A-B). Although our initial retrograde tracing results support our hypothesis that SCPN switch to CPN in layer Va, further experiments need to be conducted to confirm this result.

We observed a migration defect in the *Sox5 cko* cortex, where neurons generated at E13.5 migrated past their typical laminar target in layer V (Figure 4). An earlier report showed that the subplate is missing or severely defective in the *Sox5 ko* (Lai et al., 2008). Recent findings indicate that subplate neurons are critical for the proper radial neuronal migration in mice (Ohtaka-Maruyama et al., 2018, Ohtaka-

Maruyama et al., 2020). These findings suggest a possible mechanism for the migration defect seen in the *Sox5 cko*: in the absence of subplate neurons, radially migrating cortical excitatory neurons might not pause, causing them to transition into locomotion mode prematurely. This may explain why we observed an upward shift in EdU^+ cells in the *Sox5 cko;* however, this proposed mechanism needs to be investigated further.

Methods

Mice

Experiments were performed in accordance with protocols approved by Institute of Animal Care and Use Committee at University of California, Santa Cruz, as well as with institutional and federal guidelines. The following mouse lines were used in this study: *Sox5*$^{Lacz/+}$ (Lefebvre et al., 2008), *Sox5*$^{fl/fl}$ (Lefebvre et al., 1998), *Emx1*^{Cre} (Gorski et al., 2002), and *Fezf2 ko* (Chen et al., 2005).

Immunohistochemistry

Anesthetized mice were perfused transcardially with 4% paraformaldehyde (PFA). Brains were stored in 4% PFA, 0.1% saponin, and PBS overnight at 4°C and then cryoprotected with 30% sucrose in PBS. Brains were embedded in optimal cutting temperature (OCT) compound and stored at -80°C until future use.

The general immunohistochemistry protocol was as follows: 20μm-thick brain sections mounted on slides were washed in PBS and then immersed in citrate buffer (10mM citric acid monohydrate, 0.05% Tween-20, pH 6.0), brought to boil 3 times in a microwave, and then steamed for 30 minutes. Slides rested at room temperature (RT) for 1.5 hours. Slides were then incubated for 45 minutes in a blocking buffer solution (5% horse serum, 0.03% Triton X-100, PBS). The blocking solution was removed and slides were incubated in primary antibodies diluted in blocking buffer solution at 4°C

overnight. Primary antibodies used in this paper are listed in Table 1. The sections were washed and then incubated in secondary antibodies conjugated to Alexa 488, Alexa 549, and Alexa 647 (Jackson Laboratories and Invitrogen) for 1.5 hours at RT.

Table 1: Primary antibodies used in this study

Antibodies	Concentration	Source	Identifier
Mouse-anti-SATB2	1:200	Santa Cruz Biotechnology	sc-81376
Goat-anti-BHLHB5	1:250	Santa Cruz Biotechnology	sc-6045
Rat-anti-BCL11B	1:2000	Abcam	ab31490
Rabbit-anti-FEZF2	1:500	IBL	F441
Rabbit-anti-BCL11A	1:1000	Abcam	ab191401
Mouse-anti-TLE4	1:100	Santa Cruz Biotechnology	sc-365406
Rabbit-anti-TBR1	1:1000	Abcam	ab31940
Rabbit-anti-SOX5	1:500	Abcam	ab94396

EdU Birthdating

EdU (25mg/kg of bodyweight; Thermo Fisher Scientific, E108187) was administered via a single abdominal injection into pregnant *Sox5*$^{fl/fl}$ dams at E13.5. Brains from P7 *Sox5 wt* and *cko* progeny were collected at P7.

EdU detection was performed by incubating slides for 45 minutes at RT in a click-chemistry reaction solution containing (per 1 mL): 950µL 100mM Tris-HCl (pH 7.4), 40 uL 100mM $CuSO_4$, 10 µL 200 mg/mL sodium ascorbate, and 0.5 µL azide 555 (#). Brains from control and cKO littermates were analyzed.

In situ hybridization

In situ hybridizations were performed using a protocol identical to that detailed in Tsyporin et al., 2021.

Table 2: Primers used to generate probes

Gene	**Forward Primer 5'→ 3'**	**Reverse Primer 3'→ 5'**
Kirrel3	GCCTCCTCTTCCCACCAT	AGGAAGGGAGAACACGGG
Plxnd1	CCACTACAAGATACCTGAGGGC	GTGAGAGATGTGGGGAAGAAAC
Sema3a	GGAAGGGGCAGATGTCCT	CATACTGGCCATCCTCCG
Etv1	GTGCCTCTGTCTCACTTTGATG	CTACTGGCCTGTGACTCAGTTG

Retrograde tracing

Retrograde tracings were performed using cholera toxin ß-subunit (Ct-ß) conjugated to AlexaFluor-555 (Invitrogen, C22843). The Ct-ß solution (4mg/mL in PBS) was injected through pulled glass pipets attached to a Picospritzer III (Parker). P4 mice of either sex were anesthetized and 0.5 uL of the Ct-ß solution was injected at three proximal locations in S1 of the cerebral cortex (coordinates: A/P -1.3mm, M/L +3mm, Z -0.8mm) in order to label CPN. To label SCPN, ~1.0 uL of the Ct-ß solution was injected in the pyramidal decussation (visually identified). Injections sites were inspected after brains were collected at P7.

Image acquisition and quantification

The Zeiss 880 confocal microscope was used for image acquisition. The laser power and gain for each channel were chosen such that <1% of pixels were saturated. For analysis, single Z-slices were analyzed in FIJI. Images of sections were divided into 500μm-wide areas, and images were divided into 10 equally sized bins, ranging from the top of the ventricle to layer I/II boundary. Care was taken to match the region for all sections. Each channel was manually adjusted for brightness/contrast. In particular, the channel associated with EdU was adjusted so that only the brightest EdU^+ cells were visible. To quantify, the auto threshold "Moments" was applied to all channels. The number of double-$marker^+$ and triple-$marker^+$ cells in each bin were quantified for n=3 different brains of each genotype and n=3 sections from each brain. GraphPad Prism Version 8.1 was used to perform statistical analysis.

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