SOX5 Controls the Sequential Generation of Distinct Corticofugal Neuron Subtypes

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SUMMARY

The molecular mechanisms controlling the development of distinct subtypes of neocortical projection neurons, and CNS neuronal diversity more broadly, are only now emerging. We report that the transcription factor SOX5 controls the sequential generation of distinct corticofugal neuron subtypes by preventing premature emergence of normally later-born corticofugal neurons. SOX5 loss-of-function causes striking overlap of the identities of the three principal sequentially born corticofugal neuron subtypes: subplate neurons, corticothalamic neurons, and subcerebral projection neurons. In Sox5−/− cortex, subplate neurons aberrantly develop molecular hallmarks and connectivity of subcerebral projection neurons; corticothalamic neurons are imprecisely differentiated, while differentiation of subcerebral projection neurons is accelerated. Gain-of-function analysis reinforces the critical role of SOX5 in controlling the sequential generation of corticofugal neurons—SOX5 overexpression at late stages of corticogenesis causes re-emergence of neurons with corticofugal features. These data indicate that SOX5 controls the timing of critical fate decisions during corticofugal neuron production and thus subtype-specific differentiation and neocortical neuron diversity.

INTRODUCTION

Projection neurons of the adult neocortex consist of two main classes: corticofugal (CFu) neurons, which send their axons away from the cortex, and intracortical projection neurons, which send their axons to the ipsi- or contralateral cortex. CFu neurons can be further divided into subplate (SP) neurons and corticothalamic (CTh) neurons (together, subcortical projection neurons), which project to the thalamus, and subcerebral projection neurons (corticospinal and related corticobrainstem), which project more caudally to the tectum, pons, and spinal cord. These distinct subtypes of cortical projection neurons are born in a tightly orchestrated sequence and reside at distinct laminar locations in the neocortex (Gupta et al., 2002; Molyneaux et al., 2007). Subcortical and subcerebral projection neurons are confined to deep cortical layers (layers V, VI, SP), while intracortical projection neurons are found mostly within superficial cortical layers (layers II/III), as well as in layer V.

CFu neurons are exclusively generated during the first few days of murine cortical development. Neurons born around embryonic day (E) 11.5 in the mouse form the deepest cortical layer, the SP, and send pioneering subcortical projections toward the thalamus, forming the first corticofugal tract (Allendoerfer and Shatz, 1994; McConnell et al., 1989). The thalamus is also the target of layer VI CTh neurons, which are born around E12.5. Although SP neurons and CTh neurons share the same target, these two neuronal subtypes have distinct functions and fates. SP neurons die postnatally in most species but are thought to play a critical developmental role in instructing corticothalamic and thalamocortical connectivity (Allendoerfer and Shatz, 1994; Deng and Elberger, 2003). CTh neurons, on the other hand, persist throughout life in layer VI and establish permanent connections between the cortex and the sensory and motor thalamus.

The next-born CFu neurons are the subcerebral projection neurons, whose peak time of generation is E13.5 (Angeline and Sidman, 1961); these migrate to layer V. A subset of subcerebral projection neurons project to the spinal cord (corticospinal motor neurons [CSMN]) and are of particular clinical importance, since these are the brain neurons that specifically degenerate in amyotrophic lateral sclerosis (ALS) and whose damage in spinal cord injury contributes centrally to loss of motor function.

Intracortical projection neurons are born together with and reside alongside subcerebral projection neurons. While CFu neuron production ceases after E14.5, intracortical projection neurons continue to be generated and populate more superficial...
cortical layers. The latter neurons are phylogenetically younger and are thought to originate from progenitors distinct from those giving rise to CFu neurons (Molyneaux et al., 2007; Zimmer et al., 2004; Abotiz and Montiel, 2003; Gupta et al., 2002; Tarabykin et al., 2001; Desai and McConnell, 2000; McConnell and Kaznowski, 1991).

Despite substantial advances in our understanding of the molecular mechanisms regulating general aspects of precursor specification during early telencephalic development (Guillenot et al., 2006), only a few genes have been shown to play a layer-specific role in the differentiation of neocortical neurons. Examples include Tbr1, whose loss affects SP formation and corticothalamic connectivity, and Otx1, which plays a role in the refinement of subcerebral projections (Hevner et al., 2001, 2006; Weimann et al., 1999). Understanding of the mechanisms underlying specification of individual subtypes of projection neurons is even more limited.

By isolating pure populations of projection neurons, our laboratory has recently identified what appear to be combinatorial programs of gene expression that specifically control the development of distinct subtypes of projection neurons: subcerebral projection neurons (including CSMN), callosal projection neurons (CPN), and striatal medium spiny neurons (Arlotta et al., 2005, 2008; Molyneaux et al., 2005, 2007). The first two genes belonging to the subcerebral subtype-specific developmental program reported so far are central to proper CSMN development. Ctip2 is specifically expressed in subcerebral projection neurons in layer V and is critical for their axonal fasciculation and pathfinding, and Fez2 (previously called Fez1 or ZFP312) is both necessary and sufficient for the birth and specification of subcerebral projection neurons from cortical progenitors (Arlotta et al., 2005; Molyneaux et al., 2005; Chen et al., 2005a, 2005b).

Here, we investigate the molecular mechanisms that control the generation of distinct cortical projection neuron subtypes by investigating the role of Sox5, a transcription factor belonging to the Sox-box (Sox)-containing gene family. Sox5 is expressed at high levels in developing CFu neurons and is excluded from CPN of layer V and superficial layers. We find that Sox5 controls the sequence and pace of generation of the different subtypes of corticofugal neurons during corticogenesis, leading to a model of progressive withdrawal of Sox5 repression over coordinately regulated subtype-specific genes.

The function of Sox5 has been best characterized in chondrogenesis, where it is required for the proper differentiation of prechondrocytes into early chondroblasts by preventing premature differentiation (Smits et al., 2004, 2001; Lefebvre, 2002). Members of the Sox family of transcription factors are highly conserved and have precisely defined temporal and spatial patterns of expression in many tissues during embryonic development, including in the neuroectoderm (Hong and Saint-Jeannet, 2005; Wegner and Stolt, 2005; Bylund et al., 2003; Wegner, 1999). Sox5 itself is expressed in the chick neural crest and controls the duration of segregation of this structure (Perez-Alcala et al., 2004). More recently, Sox5 was shown to regulate oligodendrocyte differentiation in the spinal cord by maintaining precursor immaturity (Stolt et al., 2006). The specific expression of Sox5 in developing CFu neurons, together with the convergent function of Sox genes in other systems, prompted us to investigate whether this transcription factor plays a role in the development of these neurons.

We find that Sox5 controls the differentiation of postmitotic CFu neurons by repressing subcerebral projection neuron fate during early corticogenesis. In Sox5-null mutants, the normal sequential generation of subtypes of CFu neurons is accelerated, leading to the premature generation of subcerebrally projecting neurons. As a consequence, the delineation between the distinct CFu neuron subtypes is lost in Sox5−/− cortex, as earliest-born SP neurons inappropriately express genes, molecular characteristics, and projections of normally later-born subcerebral projection neurons. Similarly, subcerebral projection neurons prematurely differentiate before they reach their final positions in layer V and arrest their migration heterotopically in the white matter and within deep cortical layers. Thus, the cellular, anatomic, and functional diversity of the output of the neocortex are critically controlled by Sox5.

In strong support for Sox5’s central role in setting the proper sequence of generation of CFu neurons, gain-of-function experiments demonstrate that overexpression of Sox5 past the normal period of generation of CFu neurons is sufficient to disallow callosal projection neuron differentiation and cause the re-emergence of neurons with corticofugal projections.

We conclude that Sox5 fine tunes the identity of the closely related, yet distinct, CFu neuron subtypes during development by controlling the pace of generation of the sequentially born neurons, preventing the premature and inappropriate emergence of later-born CFu neuron features in early-born neurons.

RESULTS

SOX5 Is Specifically Expressed in Corticofugal Neurons

We previously found that Sox5 mRNA is expressed at high levels in subcerebral projection neurons at embryonic and early postnatal stages, while expression remains low throughout development in intracortical projection neurons (Figure 1A and Arlotta et al., 2005). We therefore sought to investigate whether this transcription factor might function in the development of subcerebral and other CFu neurons.

We first examined the temporal course and cell-type-specific expression of SOX5 protein in the neocortex during embryonic corticogenesis. We find that SOX5 is first expressed at about E12.5 in the emerging cortical plate (CP). At E13.5, during CFu neuron generation, SOX5 is expressed at high levels in the CP and subplate (SP). SOX5 is expressed in postmitotic cells, but not in the dorsal ventricular zone or subventricular zone where neural progenitors are located (Figure 1B and data not shown). By P0, neocortical SOX5 expression is confined to neurons of deep cortical layers (SP, VI, and V), where CFu neurons reside (Figures 1C, 1D, and 1G), and decreases rapidly after P3 (data not shown). SOX5 is not expressed in astrocytes or interneurons (Figures 1E and 1F). Outside of the neocortex, SOX5 is expressed at low levels in the subpallial ventricular wall, as well as in the ventral olfactory nucleus, and in the dorsal thalamus (Figure 1D). In all of these locations, levels of expression decrease rapidly postnatally.

In the SP and layer VI, SOX5 is present in TBR1-positive CTH neurons (Figures 1G, 1H, 1K–1K*, and 1L–M*). Within layer V,
SOX5 Controls Development of Corticofugal Neurons

Figure 1. SOX5 Is Specifically Expressed in Corticofugal Neurons of the Subplate, Layer VI, and Layer V

(A) Normalized levels of Sox5 mRNA in CSMN and CPN determined by microarray experiments during development (from Arlotta et al., 2005). (B–D) SOX5 immunocytochemistry (ICC). Coronal brain sections at E13.5 (B) and P0 (C) and a sagittal section at P0 (D) reveal SOX5 expression throughout deep cortical layers. (E and F) SOX5 (red, solid arrows) is not expressed in neocortical astrocytes (E: S100β-positive cells, green, empty arrows) or interneurons (F: GABA-positive cells, green, empty arrows). (G) SOX5-expressing neurons are located in layers V, VI, and SP at P0. (H) CTIP2 is expressed in layer V and at lower levels in layer VI and SP. (I) TBR1 labels neurons in layer VI and SP. (J–J') Magnified views show that SOX5 and CTIP2 are coexpressed at high levels in layer V neurons. (K–K') Magnified views show that SOX5 and TBR1 are coexpressed in layer VI and SP. (L–M') Fluorogold (FG) injection into the thalamus at P0 retrogradely labels CTh neurons (green cells) in layer VI and SP neurons at P3. Boxed region in (L) is magnified in (M–M'). FG-labeled CTh neurons (M) are SOX5 positive (M'; red, solid arrows). (M') Merged.
SOX5-positive neurons express CTIP2, a specific marker for subcerebral projection neurons in this layer (Arlotta et al., 2005) (Figures 1G, 1H, and 1J–J’). Each subtype of CFu neurons, therefore, has a characteristic combinatorial expression of the transcription factors SOX5, CTIP2, and TBR1: (1) SP neurons express an intermediate level of SOX5, a high level of TBR1, and a low level of CTIP2; (2) CTh neurons in layer VI strongly express SOX5 and TBR1 and little CTIP2; and (3) subcerebral projection neurons in layer V express a high level of CTIP2, an intermediate level of SOX5, and little TBR1 (Figures 1G–1I).

In order to confirm the specific expression of SOX5 in CFu neurons, we retrogradely labeled neurons from their axonal projection sites. We find that CTh neurons and SP neurons express SOX5 (retrograde labeling by FluoroGold [FG] injection in the thalamus; Figures 1L–1M’). Strikingly, in layer V, SOX5 is expressed by subcerebral projection neurons (labeled by injection in the cerebral peduncle) (Figures 1N–O’), but not by callosal (intracortical) projection neurons (labeled by injection in the contralateral hemisphere) (Figures 1P–1Q’). The temporal and subtype-specific patterns of SOX5 expression therefore suggest that SOX5 might be involved in the postmitotic differentiation of the three principal corticofugal neuron subtypes.

Loss of SOX5 Function Specifically Impairs Early Differentiation of Corticofugal Neurons

We find that SOX5 is expressed across the distinct CFu neuron subtypes and that each subtype expresses a characteristic complement of transcription factors. This suggests that these neuronal subtypes might share a common lineage, within which SOX5 could act in a combinatorial manner to coordinate regulatory fate decisions. To test this hypothesis, we analyzed Sox5 null mutant mice in which a lacZ/neo cassette is knocked into the Sox5 locus, yielding β-galactosidase (β-gal) expression in a SOX5-specific pattern (Smits et al., 2001). β-gal and SOX5 strictly colocalize in Sox5+/− mice, and β-gal and SOX5-expressing CFu neurons are confined to cortical layers V, VI, and SP at P0 (Figures 2A, 2C, and 2E–E’). Using β-gal immunoreactivity to trace the fate of CFu neurons in null mutants, which die at birth (Smits et al., 2001), we find that loss of SOX5 dramatically affects the laminar distribution of these neurons at P0 (Figures 2B and 2D). First, there is seemingly a failure of neurons of layers VI and V to segregate from each other, with subcerebral projection neurons nearly absent from layer V. Instead, β-gal-expressing neurons are found in dense clusters within layer VI and form heterotopias in the subcortical white matter (Figures 2D and 2F, open arrowheads). Second, despite the high density of β-gal-expressing neurons in layer VI, the SP is not discernable as an individual layer and is cell-sparse (Figure 2D and Figures S1A and S1B available online). Instead, in the superficial layers of Sox5+/− cortex, there is an aberrant population of heterotopic β-gal-expressing neurons (Figure 2D, solid arrowheads). These findings indicate that CFu neurons in developing Sox5+/− cortex are unable to assume proper laminar position, a critical and very early differentiation step of postmitotic neurons.

We next examined how each of the three sequentially born subtypes of CFu neurons is specifically affected by loss of SOX5. Nissl staining shows absence of a morphological SP in Sox5−/− cortex (Figures S1A and S1B). Using the SP-specific marker Ctgf (Heuer et al., 2003), we observed a lack of Ctgf-expressing neurons in the SP and abnormal Ctgf-expressing neurons in superficial layers (Figures 2G and 2H). Although there are fewer Ctgf-positive neurons in Sox5+/− cortex, the number of cells labeled by BrdU at E11.5 (the peak of SP neuron birth) is similar in WT and Sox5−/− cortex (data not shown). This strongly suggests that the reduced number of Ctgf-positive neurons reflects an abnormal differentiation of SP neurons rather than an absolute decrease in the size of this cell population. We observed a similar distribution of SP neurons using another SP/layer V marker, Crim1 (Figures S1K–S1N) and Nurr-1 (a deep layer VI/SP marker, data not shown; see Molyneaux et al., 2007). The lack of a morphological SP in Sox5−/− cortex, therefore, appears to result from the reallocation of SP neurons to more superficial locations, suggesting that at least some of the abnormal β-gal-expressing neurons within these layers are SP derived.

We next examined the fate of CTh neurons in Sox5−/− cortex. While TBR1-expressing neurons remain mostly confined to layer VI, there is a subtle but distinct local dispersion of these neurons and blurred laminar borders (Figures 2I and 2J). Additional analysis using TLE4, a marker for layer VI and V neurons (Yao et al., 1998), further confirms an intralaminar redistribution of layer VI neurons in Sox5−/− neocortex (Figures S1E–S1H).

Finally, we used the layer V subcerebral projection neuron specific transcription factor Fez2 (Molyneaux et al., 2005) to examine the fate of these neurons in Sox5−/− cortex (Figures 2K and 2L). We find that Fez2-positive neurons are almost completely absent from layer V in Sox5−/− cortex, but instead accumulate within layer VI and the white matter (Figure 2L). Analysis using another subcerebral projection neuron specific transcription factor, CTIP2, confirms this redistribution of neurons (Figures 2M–2Q). Additionally, CTIP2 labeling reveals a heterotopic neuronal population in superficial cortical layers, reminiscent of the abnormally located Ctgf-positive SP neurons (Figure 2P, solid arrowheads). These results are further confirmed using a series of recently identified markers of subcerebral projection neurons (Arlotta et al., 2005; Molyneaux et al., 2005), including Cry-mu, Crim1, and Diap3 (Figures 5D and 5E and Figures S1K–S1N and data not shown), indicating that these neurons are lacking from layer V. Instead, neurons with molecular characteristics of subcerebral projection neurons are found in the white matter and in the deepest and most superficial layers of Sox5−/− cortex. Of note, lateral to motor cortex, the neocortex is almost completely devoid of CTIP2-positive neurons, resulting in a medialization of CTIP2-expressing neurons.
in Sox5−/− cortex (Figures 2N and 2Q), and suggests that SOX5 functions in an areal-specific manner in combination with other factors.

The laminar and areal redistributions of projection neurons in the absence of SOX5 function are restricted to CFu neurons; non-CFu and superficial neuron populations are unaffected. In particular, expression of the layer IV marker RORβ (Figures 2R and 2S), the layer II/III marker Cux2 (Figures 2T and 2U), and the marginal zone (MZ, layer I) marker reelin (Figures 2V and 2W) are all unchanged in Sox5 null mutants. Similarly, β-gal and CTIP2 expression by non-neocortical neuron populations in striatum, thalamus, and piriform cortex appear unchanged in Sox5−/− brains (data not shown), reinforcing the specificity of SOX5 function in CFu neuron development.

In summary, loss of SOX5 function highly selectively causes a loss of distinction between the centrally important and normally sequentially differentiating classes of CFu neurons, indicating a critical role for this transcription factor in the generation of diversity of cortical output neurons.

Abnormal Differentiation of Corticofugal Neurons Leads to Aberrant Subcerebral Projections in Sox5−/− Brain

We next examined whether the early abnormal differentiation of CFu neurons would have consequences on their later
developmental processes leading to establishment of proper axonal connectivity.

To investigate whether heterotopic CTIP2-positive neurons in Sox5−/− cortex send axons to subcerebral targets, we retrogradely labeled neurons from the cerebral peduncle using ultrasound-guided in utero injections of FG into this subcerebral target at E17.5 and examined brains at P0 (Figure 3A). We find that, in contrast to WT mice, in which subcerebral projection neurons are sharply confined to layer V (Figure 3B), subcerebrally projecting neurons (all still CTIP2 positive) are abnormally positioned throughout all laminae in the neocortex of Sox5−/− mice (Figures 3C–3F†). This demonstrates that the abnormal neurons in Sox5−/− neocortex, which heterotopically express CTIP2 and other subcerebral projection neuron markers, have a corresponding aberrant connectivity and send their axons subcerebrally.

We directly visualized subcerebral projections by placing small crystals of DII in the motor cortex of WT and Sox5−/− mice (Figures 3G–3H†). Though many efferent axons in Sox5 null mutants have a rostrocaudal course comparable to that of WT mice and reach the pons, there is substantial caudal defasciculation in null mutants (Figures 3H and 3H†). Quite notably, there is an additional subcerebral projection tract in Sox5−/− mice, which runs aberrantly within the external capsule and reaches the cerebral peduncle and pons (Figures 3H′–3H†). In contrast, the course of corticothalamic axons is seemingly unaffected in Sox5−/− mice (Figure S2). These data suggest that these two subpopulations of CFu neurons are differentially affected by the absence of SOX5 function. Consistent with the interpretation that SOX5 does not control the development of layer V callosal neurons and superficial layer projection neurons, callosal projections in Sox5−/− mice are largely unaffected, although a small subset of aberrantly located CTIP2-positive neurons extend anomalous callosal projections (Figure S3).

Taken together, these data indicate that SOX5 functions to regulate early differentiation of CFu neurons, including proper laminar positioning and subcerebral connectivity.

**Sox5−/− Subplate Neurons Aberrantly Differentiate into Normally Later-Born Subcerebral Projection Neurons**

As a first step in assessing the origin of the abnormally located subcerebrally projecting neurons in Sox5−/− cortex, we performed migrational analysis via BrdU birthdating. We compared the laminar positions of pulse-labeled neurons in WT and Sox5−/− neocortex at P0. BrdU was injected in utero at four stages of cortical development: E12.5 (SP and CTh neuron production), E13.5 (peak subcerebral neuron production), E14.5 (peak layer IV neuron production), and E15.5 (peak layer II/III neuron production). Proportionally more E12.5- and E13.5-labeled birthdated cells in Sox5−/− cortex localize to superficial layers than in WT cortex (Figures 4A, 4B, 4E, 4F, and 4I). This abnormal migration is limited to neurons born early during corticogenesis: the laminar distributions of E14.5- and E15.5-labeled birthdated cells are similar in WT and Sox5−/− cortex (Figure 4C, 4D, 4G, 4H, and 4I). These data are in keeping with the specific expression of SOX5 in CFu neuron populations and reinforce that SOX5 functions selectively during early stages of corticogenesis, at the time when the sequential birth of CFu neurons normally occurs.

The presence of early-born (BrdU-labeled at E12.5) and Ctgf-positive neurons in superficial layers of Sox5−/− cortex suggests that these cells are abnormally located and/or aberrantly differentiated SP neurons. We thus specifically investigated the fate of SP neurons in Sox5−/− cortex by BrdU labeling at their peak time of birth, E11.5. Cells born at E11.5 are almost exclusively confined to the SP in WT mice (Figure 5A). In striking contrast, in Sox5−/− mice, E11.5 BrdU-labeled cells are not appropriately located in superficial laminae (Figure 5B). Importantly, these E11.5 BrdU-labeled cells strongly express CTIP2 (Figures 5C–5C†), a protein normally expressed at high level in subcerebral projection neurons only, and not significantly in WT SP neurons (Arlotta et al., 2005). Together, these data indicate that SP neurons differentiate abnormally in the absence of SOX5: they adopt molecular and projection characteristics of subcerebrally projecting neurons.

The abnormal location of early-born neurons in Sox5−/− cortex, therefore, appears to reflect primarily a fate switch with abnormal differentiation and secondary migrational abnormalities. Our analyses identify substantial further evidence for this interpretation. Superficially located neurons in Sox5−/− cortex express Cry-mu, a gene normally expressed by subcerebral projection neurons, but not by SP neurons (Figures 5D and 5E; Arlotta et al., 2005). Calretinin (calret)-positive neurons, which are largely restricted to the SP during corticogenesis ( Fonseca et al., 1995; Schierle et al., 1997), are present in superficial layers of Sox5−/− cortex, where they strongly and aberrantly express CTIP2 (Figures 5F–5I†). In contrast to other SP neuron markers, calret has the advantage of being present in the cytoplasm, allowing analysis of cell morphology. While calret-positive SP neurons in WT cortex typically have an elongated horizontal shape (Figures 5F and 5H), the superficially positioned calret-CTIP2 double-positive neurons in Sox5−/− cortex are distinctly pyramidal shaped (Figures 5G and 5I), resembling that of normal layer V subcerebral projection neurons (Figure 1O). Of note, many neurons also express calret in deep layers of Sox5−/− cortex, suggesting that calret is not suppressed in later-born CFu neuron populations.

Finally, we directly assessed the date of birth of the aberrantly located CTIP2-expressing neurons in Sox5−/− cortex using BrdU labeling, which confirmed that these neurons in Sox5−/− mice are born earlier than WT CTIP2-positive subcerebral projection neurons of layer V (Figure S4).

Taken together, these molecular, morphological, and connectivity data indicate that SOX5 functions to regulate and coordinate the timing of emergence of the sequentially generated populations of CFu neurons during early corticogenesis.

**Combined Loss of CTIP2 and SOX5 Rescues the Preplate Splitting Defect Caused by Aberrant Differentiation of Subplate Neurons in Sox5−/− Mice**

We next investigated whether the abnormal differentiation of SP neurons in Sox5−/− mice disrupts their normal function. Normally at E12.5, neurons that will form layer VI migrate into the preplate, dividing this structure into marginal zone (MZ) and SP in a process called “preplate splitting.” We examined whether the
Figure 3. Loss of SOX5 Function Causes Aberrant Subcerebral Projections

(A) Sagittal schematic view of the mouse brain, showing FG injection site in the cerebral peduncle (CeP) for retrograde labeling of subcerebral projection neurons in the neocortex (Cx).

(B) FG injection into the CeP of WT mice at E17.5 exclusively labels CTIP2-positive neurons in layer V.

(C–F) In Sox5+/− littermates, retrogradely labeled neurons (green) are CTIP2 positive (red) but are abnormally located in superficial (D–D′) and deep (F–F′) cortical layers (arrows).

(G–H) Dil anterograde labeling of the corticospinal tract (CST) in P0 WT (G–G′) and Sox5−/− (H–H′) mice. (G and H) Ventral views of whole-mount brains showing the CST (arrowheads) and an aberrant fiber tract in Sox5−/− brain (arrow). (G′ and H′) Sagittal composite views of the area outlined by the red box (inset in G′); same brain as in (G) and (H), respectively. (G′′ and H′′) Rostral coronal sections of brains in (G) and (H), at the positions indicated by the left dotted lines. (G′′′ and H′′′) High-magnification views of the area in the red box (inset in G′′′) at caudal locations, as indicated by the right dotted lines in (G) and (H), respectively. The CST in Sox5−/− brains exhibits fasciculation defects and a substantially limited caudal extension, but its general course is preserved, and many axons reach the pons. An additional component of the CST inappropriately courses through the Sox5−/− external capsule, extending ventrocaudally toward the cerebral peduncle (arrows in H′′′–H′′′). Scale bars: 0.5 mm (G–G′′ and H–H′′′), 100 μm (G′′ and H′′′), 50 μm (B and O), 10 μm (D–F′). AC, anterior commissure; CeP, cerebral peduncle; Cx, cortex; IC, internal capsule; Mid, midbrain; OB, olfactory bulb; Th, thalamus.
aberrant differentiation of SP neurons in Sox5<sup>−/−</sup> cortex affects their segregation away from the MZ and impairs preplate splitting.

We find that defects in SP neurons in Sox5<sup>−/−</sup> cortex result in incomplete preplate splitting. Cajal Retzius cells of the MZ normally express reelin and calret, while SP neurons express calret but not reelin (Meyer et al., 2000; data not shown). At E13.5, when preplate splitting is normally complete (Figure 6A), calret-positive reelin-negative neurons, i.e., SP neurons, in Sox5<sup>−/−</sup> cortex are abnormally dispersed throughout the developing cortical plate and remain apposed to the MZ throughout corticogenesis (Figure 6B). These findings indicate that preplate splitting is disrupted in Sox5<sup>−/−</sup> mice, likely a consequence of abnormal differentiation of SP neurons.

A preplate splitting defect might theoretically affect the development and migration of later-born neurons and account, at least in part, for the aberrant differentiation of CFu neurons in Sox5<sup>−/−</sup> cortex. We tested this possibility by examining whether re-expression of SOX5 in Sox5<sup>−/−</sup> cortical neurons, after abnormal preplate splitting has already occurred, is sufficient to restore proper differentiation of subcerebral projection neurons.

We find that in utero retroviral transfer of the Sox5 gene in Sox5<sup>−/−</sup> E12.5 embryos enabled CTIP2-positive (i.e., subcerebral projection) neurons to reach layer V, their correct laminar position, whereas the laminar location of CTIP2-positive neurons infected with a control virus (expressing solely GFP), in the same embryo, remained abnormal. Only 23% of CTIP2-positive neurons infected with the control virus were located in layer V (7/30) versus 66% of CTIP2-positive neurons infected with the SOX5-expressing virus (37/56, n = 2 mice, p = 0.0002) (Figure S5). Re-expression of SOX5 at a time when subcerebral projection neurons are being born is thus sufficient to rescue the laminar positioning of CTIP2-positive subcerebral projection neurons in Sox5<sup>−/−</sup> mice, indicating that the abnormal differentiation of these neurons is at least predominantly cell-autonomous.

In the absence of SOX5, CTIP2 is abnormally expressed at high levels in mutant SP neurons, while it is minimally expressed in WT SP neurons (Figures 5H–5I′ and 5I–5I′). We therefore hypothesized that SOX5 normally regulates the emergence of CTIP2 expression (and potentially of other coordinately regulated genes) in CFu neurons and that loss of SOX5 function
would result in inappropriate and premature expression of CTIP2. This hypothesis is consistent with the nominally accepted role of SOX5 as a transcriptional repressor. Given the critical role of CTIP2 in normal differentiation of subcerebral projection neurons (Arlotta et al., 2005), inappropriate expression of CTIP2 might be causative in the aberrant differentiation of SP neurons.

Figure 5. Sox5−/− Subplate Neurons Abnormally Differentiate into Normally Later-Born Subcerebral Projection Neurons
(A and B) Distribution of E11.5 BrdU birthdate-labeled cells (green, circled in red) in P0 neocortex of WT and Sox5−/− mice.
(C–C′) Magnification of the boxed region in (B). Cells born at E11.5 are found throughout Sox5−/− cortex and express the subcerebral projection neuron marker CTIP2.
(D and E) In situ hybridization for the normally layer V subcerebral projection neuron marker Crystallin-mu (Cry-mu) shows abnormally located Cry-mu-expressing neurons in the superficial (arrows) and deepest layers of Sox5−/− cortex.
(F–I′) ICC for CTIP2 (red) and the SP neuron marker calretinin (calret, green). (F and H–H′) In WT cortex, calret-positive neurons are found mostly in the SP and have low or absent CTIP2 expression (empty arrows in H–H′), magnified from boxed region in (F). (G and I–I′) In Sox5−/− cortex, calret-positive neurons are aberrantly located throughout the cortex, quite notably in the superficial cortical plate, and both have a pyramidal morphology and strongly express the subcerebral projection neuron marker CTIP2 (solid arrows in I–I′), magnified from boxed region in (G).
Scale bars: 50 µm (A, B, and D–G), 20 µm (C–C′, H–H″, and I–I′).
into mixed-phenotype subcerebrally projecting neurons and secondarily disrupt preplate splitting.

To test this hypothesis, we generated Sox5;Ctip2 double null mutant mice. We reasoned that, if inappropriate expression of Ctip2 causes abnormal differentiation of SP neurons, then the preplate splitting defect should be less severe in Sox5;Ctip2 double null cortex than in Sox5 single null cortex. In agreement with this hypothesis, we find that genetic ablation of Ctip2 partially rescues preplate splitting; twice as many SP neurons are apposed to the MZ in Sox5−/− than in Sox5−/−;Ctip2−/− mice (8.5% versus 4.0%, n = 2 for Sox5−/−, n = 3 for Sox5−/−; Ctip2−/−; Figures 6C–6G). Interestingly, this rescue shows a dose dependence with Ctip2 levels, since in Sox5−/−;Ctip2+/− mice, the proportion of MZ-apposed SP neurons is intermediate between Sox5−/− and Sox5−/−;Ctip2−/− mice (5.5%, p < 0.05; Figure 6G). Taken together, these findings strongly support the interpretation that, during early corticogenesis, SOX5 function leads to Ctip2 repression in SP neurons and that absence of this repression results in inappropriate adoption of mixed and aberrant characteristics of normally later-born subcerebral projection neurons.

Gain-of-Function Analysis: SOX5 Misexpression Disrupts Normal Differentiation of Callosal Projection Neurons and Shifts Their Differentiation toward Corticofugal Neuron Fate

Our loss-of-function results indicate that lack of SOX5 function leads early-born CFu neurons to inappropriately and prematurely develop characteristics of later-born CFu neurons. We next investigated whether overexpression of SOX5 past the normal period of generation of CFu neurons (here referred to as misexpression) might induce the converse in later-born, non-CFu neuron populations, i.e., abnormal acquisition of characteristics of an earlier-born neuron subtype. Specifically, we examined whether misexpression of SOX5 in the developing cortex after E13.5, past the normal period of generation of CFu neurons, would cause differentiating CPN to develop characteristics of (previously born) CFu neurons.

We misexpressed SOX5 at multiple developmental stages. First, we misexpressed SOX5 from E12.5 onward via in utero electroporation in WT mice using a plasmid construct in which expression of the Sox5 coding sequence is under the control of a constitutively active CMV/β actin promoter with an IRES-GFP sequence.
element for identification of electroporated cells (Molyneaux et al., 2005). A control vector containing only GFP was used in parallel control experiments.

Misexpression of SOX5 dramatically affects the differentiation of CPN (Figure 7), which normally do not express this transcription factor. When the control vector was used, axons of GFP-positive CPN always crossed the midline (5/5 embryos) and had typically reached lateral regions in the opposite hemisphere by E18.5 (3/5 embryos; Figures 7C and 7'C). In striking contrast, when the SOX5 expression vector was used, axons of SOX5-GFP double-positive CPN never extended laterally into the opposite hemisphere (0/5 embryos) and only occasionally even reached the midline (2/5 embryos; Figures 7E and 7'E). CFu neurons in layers V and VI were not significantly affected by overexpression of SOX5, as demonstrated by the similar numbers of subcortical and subcerebral GFP-positive axons in the internal

Figure 7. SOX5 Misexpression Impairs Callosal Axonal Projections and Generates De Novo Corticofugal Axons

(A) Schematic view of a coronal brain section indicating regions imaged in (B–E). (C') and (E) are enlarged from boxed regions in (C) and (E), respectively. (B–E) Images from coronal brain sections after electroporation at E12.5, collected at E18.5. (B and D) The laminar locations of neurons labeled by electroporation with the Sox5GRFP construct at E12.5 (green cells in [D]) do not differ detectably from those electroporated with the ControlGRFP construct (green cells in [B]). (B, C, and C') Callosal axons are unaffected (solid arrowheads in [B] and project to the opposite hemisphere in control electroporations (solid arrowheads in [C']). (D, E, and E') Callosal projections are dramatically impaired by SOX5 misexpression, not extending for more than a few hundred microns (empty arrowheads in [D]). (F) Schematic view of a coronal brain section indicating regions imaged in (G–J). (G–J) Images from coronal brain sections after electroporation at E14.5, collected at E18.5. (G and I) Neurons labeled by electroporation with either the ControlGRFP (G) or Sox5GRFP (I) construct at E14.5 are equivalently localized to the most superficial portion of the cortical plate. (J and J') SOX5 misexpression generates neurons that send axons through the striatum (Str) (arrowheads), whereas subcortical projections are never present in control electroporated mice (H). The dotted lines in (H) and (J) outline the striatum, where CTIP2 is expressed (Arlotta et al., 2005, 2008). The subcortical fibers in (J) are shown in magnified view in (J'). Scale bars: 200 μm (B–C', D–E', and G–J), 100 μm (J').
capsule and cerebral peduncle when either plasmid was used (data not shown). Taken together, these data indicate that misexpression of SOX5 specifically disrupts the proper differentiation of CPN.

We investigated the possibility that this reduced callosal axon extension might reflect aberrant development of CFu neuron characteristics in callosal neurons when SOX5 is misexpressed. This hypothesis predicts that at least a subset of callosal neurons misexpressing SOX5 might develop anomalous corticofugal axons. Therefore, we examined whether corticofugal axons might originate from normally callosal-projecting neurons in Sox5-electroporated brains. In order to avoid the confounding presence of normal corticofugal projections, we expressed SOX5 from E14.5 on, a time after which CFu neurons are no longer normally generated. We find that, whereas CPN never extended corticofugal axons in brains electroporated with the control vector (0/4 mice; Figure 7H), corticofugal axons were always visible in the internal capsule of Sox5-electroporated mice (3/3 mice; Figures 7J and 7J'). Taken together, these results strongly indicate that misexpression of SOX5 beyond the normal period of generation of CFu neurons is sufficient to cause a partial fate-switch of callosal neurons and the abnormal development of neurons with corticofugal features at late stages of corticogenesis.

Integrating the loss-of-function and gain-of-function data, these experiments indicate a critical role for SOX5 in regulating the temporal precision of development of sequentially born populations of CFu neurons. SOX5 thus regulates the complex emergence of neuronal diversity of CFu neurons. This regulation defines and controls the proper organization, output circuit formation, and function of the neocortex.

**DISCUSSION**

The identification and characterization of molecular controls over the differentiation of newborn neocortical neurons into distinct projection neuron subtypes are important for understanding how cortical architecture, connectivity, and function emerge during development. We show here that the transcription factor SOX5 is specifically expressed in the three central and closely related corticofugal neuron subtypes and that it controls the timing of critical fate decisions during corticogenesis that are central to the generation of the cellular, anatomical, and functional diversity of neocortical output neurons.

By combining anatomical, molecular, and birthdating approaches in null mutant mice, we show that SOX5 is required for the normal sequential and coordinated generation of the three principal CFu neuron subtypes, during which SP neurons are born first, CTh neurons second, and subcerebral projection neurons last. Loss of SOX5 function specifically disrupts the subtype-specific differentiation programs of CFu neurons, causing the premature and inappropriate acquisition of subcerebral projection neuron features by early-born SP neurons, leading to a blurring of the identities of these normally precisely defined neuron subtypes.

Remarkably, while early-born neurons incorrectly develop characteristics of later-born neurons in the absence of SOX5, misexpression of this transcription factor after the normal period of generation of CFu neurons has a reciprocal effect: the abnormal development of neurons with features of (normally earlier-born) CFu neurons at late stages of corticogenesis. We conclude from these developmental expression, loss-of-function, and gain-of-function studies that SOX5 is a critical regulator of CFu neuron differentiation programs, acting to precisely control the temporal sequence of differentiation and the identity of the closely related, yet distinct, subtypes of neurons.

**Role of SOX5 in the Delineation of the Sequentially Born Corticofugal Neuron Subtypes**

In contrast to WT mice, in which the sequentially born CFu neuronal subtypes express characteristic levels and combinations of genes and have a defining connectivity, several idiosyncratic features that normally distinguish these distinct populations are found across CFu neuron subtypes in Sox5<sup>−/−</sup> brain. This results in an overlap, or “confusion,” in the molecular, laminar, and connectivity identities of these neurons. Interestingly, even these abnormal and partially “confused” neurons can still be distinguished somewhat by their relative expression of a core set of subcerebral projection neuron (including CSMN) specific genes and SP markers, and by their dates of birth (Figures 2 and 5). A first subset of neurons in Sox5<sup>−/−</sup> cortex, located in the subcortical white matter and deep layer VI, exclusively expresses CSMN-specific genes (in particular Ctip2 and Fezf2) and not SP neuron markers (Ctgf, cafret) or TBR1. This strongly suggests that these neurons are the “genuine” subcerebral projection neurons that have failed to complete their migration into layer V. A second subset of abnormal neurons, located mostly within layer VI of Sox5<sup>−/−</sup> cortex, expresses a “confused” combination of CSMN-specific genes, layer VI genes, and cafret, thus displaying mixed molecular features of all CFu neurons. Finally, abnormal neurons located in the superficial layers of Sox5<sup>−/−</sup> cortex are earliest born and express only a subset of CSMN-specific genes (e.g., Ctip2, Cry-mu, but not Fezf2), while expressing both SP markers cafret and Ctgf; these are therefore likely to be “genuine” SP neurons with aberrant differentiation. Taken together, these findings demonstrate that loss of SOX5 function causes a striking lack of precision of CFu neuron differentiation and dramatically reduces neuronal diversity, eliminating the accurate molecular and connection features of CFu populations that underlie the precision of cortical output function.

SOX5 is expressed in CFu neurons, but not in CPN of layers II/III and V, suggesting that the sequentially born CFu neuron subtypes share a common lineage distinct from that of intracortical projection neurons. Loss of SOX5 function, however, does not affect the three CFu neuron subtypes equally; this might be informative regarding their relative lineage relationships. For example, differentiating SP neurons are exquisitely sensitive to the absence of SOX5 function and undergo a dramatically aberrant differentiation in its absence, while next-born CTh neurons mostly remain within layer VI and establish relatively normal projections to the thalamus (Figure S2). The contrast between the relative sparing of layer VI CTh neurons and the clearly abnormal differentiation of SP neurons in Sox5<sup>−/−</sup> mice is somewhat surprising given the laminar proximity and the functional and molecular similarities of these neuronal subtypes. The fact that subcerebral projection neurons and SP neurons are both severely...
affected by loss of SOX5 function suggests that they might be more closely related in lineage to each other than to layer VI CTh neurons. In support of this hypothesis, we find that a significant proportion of CSMN-specific genes (9/18 examined) are also expressed in SP neurons during cortical development, but not in layer VI neurons. In contrast, callosal projection neuron specific genes in layer V are only rarely expressed by SP neurons (Arlotta et al., 2005; P.A. and B.J.M., unpublished data), demonstrating that the correlated gene expression pattern of subcerebral projection neurons with SP neurons is not simply due to subcerebral projection neurons’ layer V location. These data regarding multiple molecular linkages between neuron subtypes therefore suggest a previously unsuspected level of ontogenetic connection between SP neurons and subcerebral projection neurons.

It is interesting to note that the period of corticogenesis during which SP neurons are born progressively lengthens with mammalian evolution, resulting in a considerably expanded SP in primates (Kostovic and Rakic, 1990). The anatomical and temporal expansion of the SP during evolution is thought to have played a critical role in enabling afferent fibers to invade the cortex from below, instead of from the cortical surface, as is the case in sauropods (reptiles and birds). This, in turn, is thought to have allowed the cortex to expand from three to six layers in an inside-out fashion (Marin-Padilla, 1992; Molnar et al., 2006; Super and Uylings, 2001). It is interesting to speculate that SOX5 function might have facilitated SP expansion by preventing the emergence of normally later-born neuron features in SP neurons, enabling this critical neuronal population to be generated over a prolonged period of corticogenesis, simultaneously with other subtypes of CFu neurons.

Role of SOX5 in the Differentiation of Projection Neurons

The subcortical location and gene expression profile of the “genuine” subcerebral projection neurons in Sox5−/− cortex are quite similar to those of heterotopic Ctip2-positive subcerebrally projecting neurons generated after acute overexpression of the transcription factor FEZF2, a gene that is critical for subcerebral projection neuron specification (Molyneaux et al., 2005). In these previous experiments, overexpression of FEZF2 induced a fate switch of progenitors for superficial layer cortical neurons and their differentiation into subcerebral projection neurons, resulting in secondary migratory arrest and white matter heterotopias (Molyneaux et al., 2005). The similar presence of heterotopic white matter subcerebral projection neurons in Sox5−/− mice suggests that premature expression of later differentiation genes like Ctip2, a critical control over CSMN axon outgrowth and fasciculation (Arlotta et al., 2005), may lead to premature migratory arrest. SOX5 therefore might act in migrating prospective subcerebral projection neurons, to control the proper timing and emergence of their early differentiation program. In support of this interpretation, SOX5 has been reported to set the proper pace and sequence of gene expression in chondroblasts during cartilage development and in oligodendrocyte precursors during spinal cord development, as shown by the premature differentiation of these cell types in the absence of SOX5 function (Smits et al., 2001; Stolt et al., 2006).

Consistent with an intrinsic role of SOX5 in regulating the sequential emergence of distinct CFu neuron subtypes, misexpression of SOX5 in late-born CPN is sufficient to induce a partial CFu neuron differentiation program and cause the acquisition of corticofugal features. SOX5 misexpression from E12.5 onward resulted in impaired callosal axonal extension, possibly reflecting the partial adoption of a CFu neuron differentiation program by the layer V CPN and the subsequent interference of normal callosal projection neuron development.

The specificity of SOX5’s effects in callosal neurons, which do not normally express SOX5 (Figures 1P–1Q), likely reflects the lineage proximity of neocortical projection neurons. Despite some distinctive and fundamental molecular and functional differences, all cortical projection neurons share highly similar general features and transcriptional profiles and are therefore likely to include SOX5 “downstream” effector genes. As also recognized in other systems, the cell-subtype-specific differentiation of individual subpopulations of neocortical projection neurons therefore likely relies on differences in the combinatorial interactions of a relatively small number of genes (~2% of genes; Arlotta et al., 2005, and unpublished observations) rather than on fundamental differences in the overall components of differentiation pathways.

Neocortical Efferent and Afferent Tracts in Sox5−/− Brain

The aberrant differentiation and redistribution of CFu neurons in Sox5−/− cortex have dramatic consequences on cortical connectivity. In Sox5−/− cortex, CFu neurons that do not normally send subcerebral projections extend their axons to subcerebral targets, including via an exuberant ectopical tract, presumably a consequence of the aberrant emergence of neurons with subcerebral projection neuron features from the earliest stages of corticogenesis. As demonstrated by retrograde labeling from a subcerebral target, both nominal SP neurons and “genuine” subcerebral projection neurons send subcerebral projections in Sox5−/− brains. The presence of layer VI subcerebrally projecting Ctip2-negative neurons in lateral cortical areas suggests that at least some CTh neurons also send subcerebral projections. Therefore, the lack of precision of neuronal subtype-specific expression of subcerebral projection neuron genes across CFu neuron subtypes in Sox5−/− cortex is accompanied by a corresponding lack of precision and diversity in the connectivity of these neurons, leading to an increased propensity to project subcerebrally.

A surprising axonal pathfinding feature of Sox5−/− mice is the seemingly preserved ability of thalamocortical neurons to find their target despite the lack of a morphological SP (Figures S1B and S2). The SP has been proposed to guide the development of thalamocortical axons, which is perturbed to varying degrees when preplate splitting is abnormal or when the SP is injured (Allenberger and Shatz, 1994; Hevner et al., 2001; McConnell et al., 1989, 1994; Rakic et al., 2006). In turn, thalamocortical and corticothalamic interactions have been thought to contribute to each other’s axonal pathfinding and connectivity (Molnar and Blakemore, 1995). Thus, the globally preserved anatomy of afferent and efferent thalamic pathways in Sox5−/− mice suggests that, despite aberrant differentiation, at least
a subset of SP neurons retains the ability to enable appropriate thalamocortical and corticothalamic connectivity.

**Proposed Mechanisms of SOX5 Action**

The nominal SP neurons of Sox5−/− cortex are an extremely interesting population, since they can shed light on the functional consequences and mechanisms of the inappropriate initiation of a subcerebral projection neuron differentiation program. Aberrant differentiation of Sox5−/− SP neurons appears to dramatically affect their normal function during preplate splitting, by impeding their proper segregation away from the MZ. This impediment results at least in part from increased CTIP2 expression in these earliest-born neurons, since genetic elimination of CTIP2 function in Sox5;CTip2 double null mice partially rescues this phenotype.

Consistent with the nominal role of SOX5 as a transcriptional repressor, we propose that SOX5 normally inhibits the expression of coordinately regulated genes, including CTIP2, whose levels normally progressively increase as the distinct subtypes of CFu neurons are sequentially generated. Interestingly, loss of SOX5 function has strikingly different consequences in medial motor versus lateral nonmotor regions, even though this transcription factor is normally expressed in both areas evenly, suggesting that areal-specific interactions occur with other proteins expressed along gradients. This inhibitory role of SOX5 likely requires interactions with additional temporally regulated and cell-type-specific gene products, since elimination of CTIP2 function only partially corrects preplate splitting.

Repression of the inappropriate expression of CTIP2 (and other genes) by SOX5 during early corticogenesis offers interesting parallels with the “derepression model” proposed for spinal cord development, according to which cell fate is progressively specified by the sequential disinhibition of effector genes (Lee and Pfaff, 2001). Similarly, other genes have been shown to act by repressing differentiation of one cell subtype into another related subtype. One example is Foxg1, which is required to constitutively repress Cajal–Retzius differentiation in deep layer neurons (Hanashima et al., 2004). Another example is Notch1, which has been shown to inhibit photoreceptor fate in the mammalian retina (Jadhav et al., 2006).

In conclusion, our results indicate that SOX5 exerts critical control over the sequential and precise differentiation of the distinct CFu neuron subtypes. The data indicate that SOX5 coordinates the temporal generation of these sequentially born neuron subtypes by repressing coordinately regulated genes and by preventing inappropriate and premature emergence of subcerebral projection neuron features early in corticogenesis. Because in the neocortex SOX5 is not detectably expressed in dividing neural precursors, but is exclusively expressed in postmitotic neurons, it is likely that it regulates the expression of genes poised for activation in each of the CFu neuron populations. Further elucidation of the combinatorial molecular programs by which SOX5 functions to regulate fate specification should offer important new insights into the evolutionary and functional relationships between distinct neuronal populations, the generation of neuronal diversity, the precision of neuronal differentiation, and potential plasticity in the mammalian neocortex.

**EXPERIMENTAL PROCEDURES**

Sox5+/− mice were the generous gift of V. Lefebvre (Smits et al., 2001) (Sox5 GenBank accession number 20678). Ctip2−/− mice were the generous gift of R. Kominami and colleagues (Wakabayashi et al., 2003). The day of the vaginal plug detection was designated as embryonic day 0.5 (E0.5). The day of birth was designated postnatal day 0 (P0). All mouse studies were approved by the Massachusetts General Hospital IACUC and were performed in accordance with institutional and federal guidelines.

**Immunocytochemistry and In Situ Hybridization**

Brains were fixed and stained using standard methods (Frickert-Gates et al., 2002). Primary antibodies and dilutions were used as follows: rat anti-CTIP2, 1:1000 (Abcam); rabbit anti-TBR1 antibody, 1:8000, gift of R. Hevner; rabbit anti-ROXl, 1:2000, gift of H. Stunnenberg (Gawlas and Stunnenberg, 2000); rat anti-BrdU, 1:750 (Accurate); mouse anti-BrdU, 1:750 (Chemicon); rabbit anti-GFP, 1:1000 (Molecular Probes); goat anti-SOX5, 1:250 (Santa Cruz Biotech); rabbit anti-β-gal, 1:5000 (MP Biomedical); mouse anti-reelin, 1:500 (Chemicon); rabbit anti-FG, 1:500 (Chemicon); rabbit anti-calretinin, 1:1000 (Chemicon). Appropriate secondary antibodies were from the Molecular Probes Alexa series.

Nonradioactive in situ hybridization was performed using reported methods (Berger and Hediger, 2001). Riboprobes were generated as previously described (Arlotta et al., 2005).

**BrdU Birthdating**

Timed pregnant females received a single intrauterine injection of BrdU (100 mg/kg) at E11.5, E12.5, E13.5, E14.5, or E15.5. Pups were collected at birth and processed for BrdU immunocytochemistry (ICC) (Magavi et al., 2000). Quantification of first-generation BrdU-labeled cells and distribution within cortical layers were analyzed using established methods (Molynieux et al., 2005). Four anatomically matched sections from each mouse were selected, BrdU ICC and DAPI staining were performed, and fluorescence micrographs were obtained spanning motor cortex of both hemispheres. Two or three sets of WT and Sox5−/− littersmates were examined for each time point. The full cortical thickness was divided evenly into ten bins, and the distribution of first-generation BrdU-positive cells (defined a priori as having strong and homogeneous nuclear labeling) was determined for all bins. A priori criteria were defined for blinded analysis by two independent investigators; interobserver assessments for distribution of labeled cells were nearly indistinguishable. One investigator was not involved in the study, and both were blinded to the genotype and time of BrdU injection. The unpaired t test was used for statistical analysis.

**Anterograde and Retrograde Labeling**

Anterograde and retrograde Di tracing in postfixed brains were performed as previously described (Arlotta et al., 2005; O’Leary and Terasima, 1998). Subcerebrally, subcortically, and callosally projecting neurons in the motor cortex were retrogradely labeled via FG injections into the peduncle, thalamus, and contralateral hemisphere, respectively, at E17.5, P0, or P1 under ultrasound guidance (Vevo 660, VisualSonics; Arlotta et al., 2005). E17.5 pregnant mice were deeply anesthetized with Avertin, and each embryo was injected through the uterine wall (Arlotta et al., 2005). Injected mice were collected at P0, P3, or P4 and processed for ICC.

**SOX5 Electroporation**

For control experiments, a vector containing IRES-GFP under the control of a constitutively active CMV/I actin promoter was used (controlGFP, a generous gift of C. Lois, MIT; Molynieux et al., 2005). Sox5 was cloned into this vector to create the construct Sox5GFP for misexpression. 750 nl of purified DNA (0.5–1.0 μg/μl) mixed with 0.005% Fast Green was injected in utero into the lateral ventricle of CD1 embryos at E12.5 or E14.5 under ultrasound guidance and electroporated into the neocortical ventricular zone essentially according to Saito and Nakatsuji (2001) and Molynieux et al. (2005). For E12.5 electroporation, five controlGFP-electroporated pups from four different litters and five Sox5GFP-electroporated pups from five different litters were analyzed. For E14.5 electroporation, four controlGFP-electroporated pups from three litters and five Sox5GFP-electroporated pups were analyzed.
different litters and three Sox5/GFP-electroporated pups from two different litters were analyzed.

Supplemental Data
The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/57/2/232/DC1.

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SOX5 Controls Development of Corticofugal Neurons


Supplemental Data

SOX5 Controls the Sequential Generation of Distinct Corticofugal Neuron Subtypes

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Supplemental Experimental Procedures

Viral Gene Transfer. One μl of a solution containing both a retrovirus expressing SOX5 and GFP (1.49 x10^8 infectious particles/ml) and a control retrovirus expressing only GFP (1.7 x10^8 infectious particles/ml) was injected into the lateral ventricle of embryos of E12.5 timed-pregnant Sox5+/− females. Infected Sox5−/− embryos were collected at E18.5 (n=2 embryos from two different litters). Within each Sox5−/− embryo, the laminar locations of CTIP2-positive neurons infected with SOX5-expressing virus were compared to the laminar locations of CTIP2-positive neurons infected with control GFP-expressing virus. For this purpose, the cortex was divided into ten equal bins and layer V was defined as bins number 4 and 5. The proportion of infected CTIP2-positive neurons within layer V was determined for mice infected with each virus. Fisher's exact test was used for statistical analysis.
Figure S1. Laminar and Areal Distribution of Neuronal Subtype-Specific Markers in Sox5<sup>−/−</sup> Cortex

(A, B) Nissl staining reveals the absence of a morphological subplate (outlined by dotted lines) in Sox5<sup>−/−</sup> cortex. (C, D) Immunocytochemistry for the layer VI marker TBR1 shows little difference in cell distribution medio-laterally between WT and Sox5<sup>−/−</sup> cortex. (E, F) However, the layer V/VI/subplate maker TLE4 shows TLE4-positive neurons located deeper within layer VI in the medial region of the Sox5<sup>−/−</sup> cortex. (G, H) Higher magnification images of boxed regions in E and F, highlighting the absence of TLE4-positive CFu neurons from layer V in Sox5<sup>−/−</sup> cortex. (I-P) In situ hybridization with layer-specific markers in WT (I, K, O) and Sox5<sup>−/−</sup> cortex (J, L, P). (I, J) Fez2-positive neurons are aberrantly located in layer VI in Sox5<sup>−/−</sup> cortex, but found only in medial cortex. (K, L) Crim1 strongly labels layer V and subplate neurons in WT cortex (K), but, in Sox5<sup>−/−</sup> cortex (L), Crim1-positive neurons are aberrantly distributed from layer VI to the most superficial parts of CP. (M, N) Higher magnification images of boxed regions in K and L, highlighting the aberrantly-located Crim1-positive neurons in superficial CP (arrowheads). (O, P) Cux2, a layer II/III marker, shows identical laminar and areal distribution in WT and Sox5<sup>−/−</sup> cortex. Scale bars, 0.5 mm (C-F, I-L, O, P), 50 μm (A, B, G, H, M, N).
Figure S2. Corticothalamic and Thalamocortical Projections Are Not Substantially Affected by Loss of SOX5

(A, B) DiI anterograde labeling of corticothalamic projections shown in sagittal view at P0. Arrows indicate corticothalamic axons projecting medially from the internal capsule (IC) toward the thalamus (Th). The course of corticothalamic fibers is similar between WT and Sox5−/− brains. (C, D) DiI anterograde labeling from the thalamus shown in coronal view at P0. The trajectory of thalamocortical axons is essentially identical between WT and Sox5−/− brains. Thalamocortical axons course through the internal capsule (IC; solid arrows), turn medially at the pallial-subpallial junction (empty arrows), and extend radial processes into cortex (arrowheads). Scale bars, 100 μm.
Figure S3. A Subset of CTIP2-Positive Neurons Aberrantly Send Callosal Projections in Sox5-/- Cortex

(A-E'') Injection of FG in one hemisphere labels callosal projection neurons in contralateral cortex. (A-B'') In Sox5-/- cortex, as in WT, FG-labeled callosal projection neurons (green, empty arrows) do not express CTIP2 (red, solid arrows). Boxed region in A shown at higher magnification in B-B''. (C-E'') In Sox5-/- cortex, some FG-labeled callosal projection neurons aberrantly express CTIP2 (arrows), both in the superficial cortical plate (D-D'') and in layer V (E-E''). Boxed regions in C shown at higher magnification in D-D'' and E-E''. Scale bars, 50 µm (A, C), 10 µm (B-B'', E-E'').
Figure S4. Heterotopic CTIP2-Positive Neurons in Sox5\(^{+/−}\) Cortex Are Born Earlier than WT CTIP2-Positive Subcerebral Projection Neurons

(A-B', D-E') Representative photomicrographs of P0 WT and Sox5\(^{+/−}\) cortical sections after E12.5 BrdU injection, showing immunocytochemistry for CTIP2 (highlighted with red dots in A, A'', D, D'') and BrdU (highlighted with green dots in A', A'', D', D''). Boxed regions in A, A'', D, D'' shown at higher magnification in B, B', E, and E', respectively. While only \(~1\%\) of CTIP2-positive neurons in layer V of WT cortex are labeled by \textit{in utero} BrdU injection at E12.5 (B, B', C, F), \(~9\%\) of CTIP2-positive neurons in layer V of Sox5\(^{+/−}\) littermates are co-labeled by BrdU (arrows; E, E', F). (C) Graphical representation of the results for WT cortex: percentages of CTIP2-positive neurons in each layer that are also BrdU-positive; shown for each of 10 bins. Open circles and squares denote values from two separate experiments. In WT cortex, neurons born at E12.5 overwhelmingly migrate to the subplate and layer VI. As a result, CTIP2-positive neurons in the deepest bins have the highest rates of BrdU labeling. In contrast, very few CTIP2-positive neurons in layer V (subcerebral projection neurons) are born at E12.5 in WT mice, as indicated by the very low percentage of BrdU-positive neurons among CTIP2-positive neurons in layer V. (F) In striking contrast, in Sox5\(^{+/−}\) cortex, CTIP2-positive neurons in layer V are labeled at a much higher rate, indicating an abnormally early birthdate. The rate of labeling of the CTIP2-positive neurons in layer V is comparable to the rate of labeling of CTIP2-positive neurons in the deepest regions of WT cortex (deepest bins in C), indicating similar birthdates. Dotted lines connect data from littermate WT and Sox5\(^{+/−}\) brains. Asterisks indicate significant differences. Abbreviations: n.a., not applicable, as no CTIP2-positive neurons are present in these bins. Scale bars, 50 \(\mu\text{m}\) (A-A'', D-D''), 20 \(\mu\text{m}\) (B, B', E, E').
Figure S5. Re-expression of SOX5 Rescues the Laminar Location of CTIP2-Positive Subcerebral Projection Neurons in Sox5<sup>−/−</sup> cortex

(A) E18.5 Sox5<sup>−/−</sup> cortex after E12.5 infection with both a Sox5-expressing retrovirus and control GFP retrovirus. Summary camera lucida drawing (from B-B′′ and C-C′′) of the distribution of SOX5-positive CTIP2-positive neurons (red outline) and control (Ctrl) GFP-positive CTIP2-positive neurons (green outline). Analysis of CTIP2-positive neurons enabled focus on the subcerebral projection neuron population. A greater proportion of SOX5-positive CTIP2-positive neurons are located in layer V, indicating that re-expression of SOX5 rescues the laminar location of subcerebral projection neurons (i.e. CTIP2-positive neurons) in Sox5<sup>−/−</sup> cortex. (B-C′′) E18.5 Sox5<sup>−/−</sup> cortex infected with both Sox5-expressing retrovirus (also contains IRES-GFP) and control GFP-only retrovirus. (B-B′′) Solid arrows indicate CTIP2-positive neurons infected with Sox5-expressing retrovirus. The empty arrow shows one of several CTIP2-negative Sox5-infected neurons. (B) Overlay. (B′) SOX5 only. (B′′) CTIP2 only. (C-C′′) Same area as shown in B-B′′, highlighting both Sox5-infected (yellow; asterisk marks one example) and control GFP-infected neurons. Solid arrows indicate CTIP2-positive neurons infected with control GFP retrovirus. The empty arrow shows one of several CTIP2-negative GFP-infected neurons. Since the Sox5-expressing virus also expresses GFP, SOX5-positive GFP-positive neurons are visible as yellow in this section (e.g. asterisk in C-C′′; also seen in B-B′′). (C) Overlay. (C′) GFP and SOX5 only. (C′′) CTIP2 only. Scale bars, 100 µm (A, B-C′′).