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Abstract

An extraordinary variety of neuronal subtypes, each with distinct morphologies, patterns of connectivity, and electrophysiological properties, is generated during neocortical development. Elucidating programs of molecular controls that govern progressive specification of neuronal subtype identity in the cerebral cortex contributes toward our understanding its development, organization, evolution, and function. Establishing a basic framework for how and when cell fate specification decisions are made will enable more efficient manipulation of these transitions *in vitro*, and therefore has important practical implications for biomedical research, as it will help improve protocols for generation of specific cortical neuron subtypes for use in disease modeling, drug screening, and therapeutic transplantation.

Previous work has shown that a subset of cortical progenitors that express the transcription factor *Cux2* is committed to generating interhemispheric projection neurons. However, it remains unclear whether other fate-restricted progenitor populations establish other distinct lineages. Current evidence indicates that at least two distinct lineages exist, but it is not known whether they are entirely mutually exclusive or whether some progenitors join the *Cux2*-positive lineage after generating deep-layer neuronal subtypes, therefore changing their competence state. *Fezf2* is a particularly attractive candidate gene that might be specifically expressed by *Cux2*-negative progenitors, as it is sparsely expressed in the proliferative zones and, later in development, is specifically expressed by postmitotic subcortical projection neurons, and excluded from interhemispheric projection neurons.

Here we investigate whether the zinc finger transcription *Fezf2* is specifically expressed by fate-restricted progenitors committed to generating subcortical projection neurons and whether its critical function in subtype specification takes place primarily in progenitors or in postmitotic neurons. First, we generated *Fezf2-CreERT2* mice, using CRISPR-Cas9 technology to introduce an *IRES-CreERT2* expression cassette into the 5' UTR of the *Fezf2* gene. We show that postnatal induction of recombination in these mice faithfully recapitulates the endogenous expression pattern of *Fezf2*, which is largely restricted to subcortical projection neurons in deep layers of cortex. Interestingly, induction of recombination at E10.5 demonstrates that *Fezf2*-

positive progenitors have a broad fate potential and are able to generate both subcortical and interhemispheric projection neurons, as well as glial cells, which can be found distributed across all layers of cortex. Over the course of corticogenesis, however, as *Cux2*-positive progenitors become more prevalent, expression of *Fezf2* in progenitors appears to decrease substantially, and the bulk of recombination following tamoxifen induction occurs in postmitotic subcortical projection neurons. Interestingly, misexpression of *Fezf2* restricted to the progenitor stage directs fate specification of neuronal progeny toward a subcortical fate and is sufficient to repress transition by progenitors into a *Cux2*-postive molecular program. Taken together, these data support a model in which *Fezf2*-positive progenitors are multipotential, but can downregulate *Fezf2*, thereby transitioning to a fate-restricted *Cux2*-positive state.

Table of Contents

Abstract ..... **ii**
Table of Contents ..... **vi**
List of Abbreviations ..... **vii**
Introduction **1**
Overview 2
Progenitor diversity and corticogenesis 3
Projection neuron diversity in the cerebral cortex 4
Projection neuron subtype specification 5
Delineation of SCPN and CThPN subtype identity 5
Temporal control over generation of CFuPN subtypes 7
Delineation of CFuPN and CPN subtype identity 8
Epistasic analysis of subtype specification 9
Early specification of subtype identity in progenitors 10
Toward a molecular logic of neocortical development 12
Thesis overview 13
Experimental Procedures **14**
Animals 15
Immunocytochemistry and *in situ* hybridization 15
In utero electroporation 16
Retrograde labeling 16
Statistical analyses 16
Results **18**
Generation of *Fezf2*-CreERT2 knock-in mice 19
The *Fezf2-CreERT2* allele mediates corticofugal-specific recombination 19
Fezf2-expressing cortical progenitors are not lineage-restricted 20
Fewer progenitors express *Fezf2* after deep-layer neurons are generated 22
Fezf2 functions in progenitors to repress expression of *Cux2* 23
Fezf2 expression in progenitors biases subtype fate-specification 24
Discussion **26**
Competing models: progenitor fate restriction vs. progenitor diversification 27
Progressive acquisition of subtype identity 27
Cell-autonomous and non-cell-autonomous mechanisms of cortical development 28
Future directions 29
Summary **30**
References **32**
Figures **48**

List of abbreviations

I-VI	neocortical layers I-VI
CC	corpus callosum
CFuPN	corticofugal projection neurons
CGE	caudal ganglionic eminence
CP	cortical plate
CPN	callosal projection neurons
CR	Cajal-Retzius neurons
CSMN	corticospinal motor neurons
CTPN	corticotectal projection neurons
CThPN	corticothalamic projection neurons
CTIP	COUP-TF interacting protein
Ctx	cortex
Cux2	cut-like homeobox 2
DL	deep-layer
dLG	dorsal lateral geniculate nucleus of thalamus
E	embryonic day
Fezf2	forebrain embryonic zinc-finger 2
GC	granule cells
IC	internal capsule
IP	intermediate progenitor
IZ	intermediate zone
LGE	lateral ganglionic eminence
M	motor (cortex)
MGE	medial ganglionic eminence
NE	neuroepithelial
oRG	outer radial glia
OSVZ	outer subventricular zone
OT	optic tectum
P	postnatal day
PP	preplate
RG	radial glia
S/S1	somatosensory/primary somatosensory (cortex)
SC	spinal cord
SCPN	subcerebral projection neurons
SP	subplate
Str	striatum
SVZ	subventricular zone
Th	thalamus
UL	upper-layer
V/V1	visual/primary visual (cortex)
VB	ventrobasal nucleus of thalamus
VL	ventrolateral nucleus of thalamus
VP	ventroposterior nucleus of thalamus
VZ	ventricular zone

Introduction

Overview

The mammalian neocortex is responsible for processing multiple modalities of sensory information, controlling motor output, and mediating higher-order cognitive functions. Its organization into only six histologically distinct layers belies an extraordinary diversity of neuronal subtypes, which serve as building blocks for its computationally powerful neural circuitry. In recent years, tremendous progress has been made toward understanding the molecular events that control the development of several important types of neocortical neurons (Greig et al., 2013).

Two major classes of neurons, interneurons and projection neurons, populate the neocortex (Parnavelas, 2000). Interneurons connect locally within the neocortex, are largely inhibitory, and are generated by progenitors in the subpallial (ventral) proliferative zone of the telencephalon before migrating to the neocortex (Cobos et al., 2001; Wichterle et al., 2001; Wonders and Anderson, 2006). In contrast, projection neurons send axons to distant brain targets, are excitatory, and are generated by progenitors in the pallial (dorsal) proliferative zone (Gorski et al., 2002; Molyneaux et al., 2007). Interneuron diversity and development have been reviewed elsewhere (Batista-Brito and Fishell, 2009; Corbin and Butt, 2011; Fishell and Rudy, 2011); here, we will focus exclusively on projection neurons.

Individual phenotypic characteristics, such as dendritic morphology, electrophysiological properties, or projection patterns have been used in the past to systematically classify projection neurons (Migliore and Shepherd, 2005; Molyneaux et al., 2007; Oberlaender et al., 2012; Spruston, 2008). While these classification schemes have facilitated investigation of projection neuron development and function, a more comprehensive understanding of neuronal diversity will require integration of these and other phenotypic data points, including transcriptomic and epigenomic profiles (Defelipe et al., 2013). Here, we group neurons primarily by the target of their axons, both because hodology is centrally related to function, and because establishment of appropriate projections indicates successful stepwise execution of elaborate developmental molecular programs (**Figure 1**).

Progenitor diversity and corticogenesis

Early in development, the telencephalic wall consists exclusively of undifferentiated neuroepithelial cells (**Figure 2A**). As these progenitors proliferate and expand in number, some begin to differentiate into radial glia (RG), establishing the ventricular zone (VZ) (Haubensak et al., 2004). RG, in turn, give rise to additional progenitor classes, including outer radial glia (oRG) and intermediate progenitors (IP), which together form the subventricular zone (SVZ) (Noctor et al., 2007; 2004).

These progenitor populations each have distinct morphological properties and follow specific patterns of cell division. RG span the thickness of cortex from the ventricular (apical) surface to the pial (basal) surface, and are used as a scaffold by newly-born neurons as they migrate into cortex (Rakic, 1971). They primarily divide asymmetrically to self-renew, while also giving rise to oRG, IP, or neurons (Miyata et al., 2001; Noctor et al., 2001). oRG are also unipolar and undergo asymmetric divisions to self-renew and generate neurons, but can be distinguished from RG by their lack of an apical process (Fietz et al., 2010; Hansen et al., 2010; Wang et al., 2011). First characterized in the outer SVZ of developing human cortex (Hansen et al., 2010), oRG were initially thought to be present only in gyrencephalic animals (Fietz et al., 2010), but a small population also exists in the SVZ of rodents (Martínez-Cerdeño et al., 2012; Wang et al., 2011). Unlike RG and oRG, IP have a multipolar morphology and undergo limited proliferative divisions, more often dividing symmetrically to produce two neurons (Haubensak et al., 2004; Kowalczyk et al., 2009; Noctor et al., 2004; Sessa et al., 2008; Wu et al., 2005). A fourth class of progenitors, the short neural precursors (SNP), reside in the VZ and have a basal process that does not reach the pia, but in other respects appear similar to IP, suggesting that they might represent RG in the process of becoming IP (Gal et al., 2006; Kowalczyk et al., 2009; Stancik et al., 2010).

Neocortical progenitors begin to produce excitatory cortical projection neurons around embryonic day 10.5 (E10.5) (Angevine and Sidman, 1961; Rakic, 1974). The earliest-born neurons migrate away from the ventricular surface to segregate from progenitors and form the preplate (Marin-Padilla, 1978; Raedler and Raedler, 1978).

Later-born neurons migrate into the preplate, splitting it into the marginal zone and subplate, and establishing the cortical plate between the two (Luskin and Shatz, 1985). Throughout the rest of corticogenesis, newly born neurons migrate into the cortical plate organizing themselves in an "inside-out" fashion (**Figure 2B**), such that early-born neurons populate deeper neocortical layers (VI, then V), and late-born neurons migrate past them to progressively populate more superficial layers (IV, then II/III).

Projection neuron diversity in the cerebral cortex

Projection neurons are first classified according to whether they extend axons within one hemisphere (associative projection neurons), across the midline (commissural projection neurons), or away from cortex (corticofugal projection neurons, CFuPN). Associative projection neurons include intrahemispheric short-distance projection neurons, which project within a single cortical column or to nearby cortical columns (*e.g.*, layer IV granule cells), as well as intrahemispheric long-distance projection neurons, which project to adjacent or distant cortical areas (*e.g.*, frontal and backward projection neurons, FPN and BPN). Most neocortical commissural neurons project axons across the corpus callosum to the contralateral cortical hemisphere (callosal projection neurons, CPN), while CFuPN extend axons subcortically, either to the thalamus (corticothalamic projection neurons, CThPN) or to subcerebral targets (subcerebral projection neurons, SCPN).

Projection neurons of each subtype are further specialized according to their rostrocaudal and mediolateral positions, reflecting the tangential organization of the neocortex into functional areas (motor, somatosensory, visual, and auditory). Projection neurons of the same subtype residing in different areas extend axons to anatomically and functionally distinct targets. CThPN establish connections with modality-specific thalamic nuclei in an area-dependent manner, while SCPN maintain projections to the spinal cord (corticospinal motor neurons; CSMN), the pons (corticopontine projection neurons), or the optic tectum (corticotectal projection neurons; CTPN) depending on their areal location. Similarly, CPN extend axons to mirror-image locations in the same

functional area of the contralateral hemisphere, enabling bilateral integration of motor, somatosensory, visual and auditory information.

Projection neuron subtype specification

Postmitotic controls are critical to specify the precise subtype identities and to direct the differentiation of newly born neurons. High-throughput efforts to define laminar- and subtype-specific gene expression patterns in the neocortex(Arlotta et al., 2005; Gray et al., 2004; Lein et al., 2007; Magdaleno et al., 2006; Sugino et al., 2006; Visel et al., 2013; 2004) have led to the identification of an increasing number of controls over subtype development. One recurring theme that has emerged from these recent studies is that extensive transcriptional cross-repression between competing genetic programs drives specification of alternate subtype identities, and this section is organized to reflect these developmental decision points.

Delineation of SCPN and CThPN subtype identity

SCPN and CThPN are closely related projection neuron subtypes that reside in the deep layers of the neocortex and are sequentially generated early in corticogenesis. Substantial plasticity exists in the specification of corticofugal projection neurons (CFuPN) into either SCPN or CThPN, and each population can expand at the expense of the other in the absence of critical controls (**Figure 3** and **Figure 4**).

The zinc finger transcription factor *Fezf2* is critical for specification of SCPN. It is expressed by a subset of VZ progenitors while deep cortical layers are being generated, and later expressed by postmitotic CFuPN, at high levels by SCPN, and at lower levels by CThPN and SP neurons (Arlotta et al., 2005; Chen et al., 2005a; 2005b; Hirata et al., 2004; Inoue et al., 2004; Molyneaux et al., 2005). In *Fezf2* null mice, the large pyramidal neurons that normally define layer V are entirely absent. Even more strikingly, expression of SCPN-specific genes is lost, and no cortical neurons project to the brainstem and spinal cord (Chen et al., 2005a; Molyneaux et al., 2005). Instead, expression of *Tbr1*, a transcription factor critical for CThPN development(Bedogni et al., 2010; McKenna et al., 2011), expands into presumptive layer V (Molyneaux et al.,

2005), and many of these *Tbr1*-expressing neurons project to thalamus (McKenna et al., 2011), indicating that some SCPN are fate-converted to CThPN (while other SCPN are fate-converted to CPN, as discussed below). Thus, *Fezf2* specifies SCPN identity, at least in part by repressing CThPN identity. In addition to being a "master" regulator of SCPN development, *Fezf2* also functions in the specification of CFuPN identity more broadly. CThPN and SP neurons appear disorganized in *Fezf2* null mice, and a number of CThPN-specific genes, including DARPP-32, *Grg4*, and *Foxp2*, fail to be expressed (Chen et al., 2005a; Molyneaux et al., 2005). These findings suggest that low-level *Fezf2* expression by CThPN and SP neurons is necessary for precise differentiation of these populations. Furthermore, misexpression of *Fezf2* by *in utero* electroporation causes layer II/III CPN to redirect their axons toward subcortical targets, including the thalamus, brainstem, and spinal cord (Chen et al., 2008; 2005b; Molyneaux et al., 2005; Rouaux and Arlotta, 2013). Taken together, these data indicate that *Fezf2* instructs CFuPN identity, and not SCPN identity alone.

A second transcription factor, *Ctip2* (*COUPTF-interacting protein 2*), functions downstream of *Fezf2* to control appropriate differentiation of SCPN. Although SCPN are still born and migrate normally to layer V in the absence of *Ctip2*, they exhibit striking defects in axon outgrowth, fasciculation, and pathfinding. Most critically, SCPN axons fail to reach the spinal cord, as they become misrouted and defasciculated in the midbrain, only rarely reaching pons, and never reaching the pyramidal decussation (Arlotta et al., 2005). While activation of *Ctip2* by *Fezf2* is critical for SCPN development, several controls over CPN, CThPN, and SP development operate at least in part by repressing *Ctip2* expression (*e.g.*, *Satb2* (Britanova et al., 2008; Chirivella et al., 2008), *Sox5* (Lai et al., 2008), and *Couptf1* (Tomassy et al., 2010)), indicating that *Ctip2* is a critical target for transcriptional regulation during neocortical projection neuron development.

Tbr1 (*T-box brain 1*) acts in opposition to *Fezf2* and *Ctip2* to specify CThPN identity. *Tbr1* is expressed postmitotically by CThPN and SP neurons, and at lower levels by Cajal-Retzius cells and CPN (Bedogni et al., 2010; McKenna et al., 2011). In

the absence of *Tbr1*, subplate is not morphologically discernible, and subplate-specific genes fail to be expressed (Bedogni et al., 2010). Similarly, early-born neurons that would normally develop into CThPN express aberrantly high levels of *Fezf2* and *Ctip2*, as well as several other SCPN-specific genes, and extend axons toward subcerebral targets instead of the thalamus (Bedogni et al., 2010; Han et al., 2011; McKenna et al., 2011). *Tbr1* directly binds to highly conserved regulatory regions to repress expression of *Fezf2*, therefore functioning, at least in part, by preventing SCPN specification (Han et al., 2011; McKenna et al., 2011).

Temporal control over generation of CFuPN subtypes

CFuPN are generated in temporally-overlapping waves and share the same core developmental program; however, specific controls direct the sequential generation of SP neurons, CThPN, and SCPN, ensuring precise acquisition of molecular identity by each subtype.

The transcription factor *Sox5* (*SRY-box containing 5)* controls the orderly emergence of CFuPN subtypes by repressing high-level expression of SCPN genes, including *Fezf2* and *Ctip2,* until generation of subplate neurons and CThPN is complete (Kwan et al., 2008; Lai et al., 2008; Shim et al., 2012). *Sox5* directly represses *Fezf2* by binding an enhancer element required for *Fezf2* expression in the forebrain (Shim et al., 2012). In *Sox5*$^{-/-}$ mice, subplate neurons express inappropriately high levels of CTIP2, take an abnormal laminar position in superficial cortical layers, and project to the cerebral peduncle (Lai et al., 2008). In addition, FOG2 and CTIP2, normally specific to CThPN and SCPN, respectively, are co-expressed by a single population of mixed SCPN/CThPN character, indicating imprecise differentiation (Kwan et al., 2008). Loss of *Sox5* results in widespread CFuPN pathfinding defects, including the formation of an accessory subcerebral tract projecting through the external capsule and extensive defasciculation of SCPN axons in the midbrain(Lai et al., 2008). Corticothalamic projections are also severely compromised, as reported by the *Golli*-GFP and *Fezf2*-GFP transgenes, as well as pancortical *Emx1-Cre;CAG-Cat-GFP* labeling (Kwan et al., 2008).

Couptf1 suppresses SCPN identity in the latest-born, most superficially located CThPN. In the absence of *Couptf1*, layer VIa neurons in somatosensory cortex express aberrantly high levels of CTIP2 and *Fezf2*, while also maintaining expression of TBR1. Although more deep-layer neurons project subcerebrally, only the axons of SCPN prematurely generated at E12.5 and located in layer VIa are able to reach the spinal cord. Axons of SCPN generated at E13.5 and located in layer V, in contrast, aberrantly terminate in pons before entering the spinal cord (Tomassy et al., 2010).

Delineation of CFuPN and CPN subtype identity

CFuPN share a developmental boundary with CPN, and especially with deep-layer CPN, which are generated during the same temporal window, and reside intermingled with CFuPN in layers V and VI. From the time CFuPN and CPN axons exit the cortical plate, they follow dramatically divergent trajectories, either away from cortex or toward the midline (Koester and O'Leary, 1993). Accordingly, some critical controls over CFuPN and CPN development function, in large part, by repressing molecular programs that would instruct differentiation toward the alternate fate.

As described above, *Fezf2* functions centrally to specify CFuPN identity, which requires suppression of CPN fate. *Fezf2* overexpression *in vivo* is sufficient to redirect the axons of superficial-layer CPN toward subcortical targets (Chen et al., 2008; Molyneaux et al., 2005; Rouaux and Arlotta, 2013). In the absence of *Fezf2*, neurons expressing alkaline phosphatase from the *Fezf2* locus extend axons across the corpus callosum. In addition, more neurons in layer V display electrophysiological characteristics typical of CPN and express CPN-specific genes, suggesting that many SCPN are fate-converted to CPN (Chen et al., 2008). Interestingly, these neurons appear to take on a deep-layer CPN identity, expressing broad CPN identity genes, such as *Satb2* and *Lpl*, but not expressing genes specific to superficial-layer CPN, such as *Inhba* and *Limch1* (Lodato et al., 2011; Molyneaux et al., 2009).

The transcription factor *Satb2* (*special AT-rich sequence binding protein 2*) is critical for CPN specification and concomitant repression of CFuPN fate. *Satb2* is expressed at high levels by CPN (and likely also by associative neurons) in all layers of

cortex (Britanova et al., 2008; Chirivella et al., 2008). In the absence of *Satb2*, almost no axons cross through the corpus callosum, even though establishment of the midline appears normal. Instead, neurons expressing *LacZ* from the *Satb2* locus project toward the brainstem and spinal cord (Chirivella et al., 2008; Srinivasan et al., 2012). Expression of several genes characteristic of CPN, including *Cdh10*, *Dkk3*, *Sip1*, and *Cux1*, is lost or severely reduced in the *Satb2* null. Conversely, superficial-layer neurons express high levels of *Ctip2*, as well as a number of other genes characteristic of SCPN, including *Clim1*, *Cdh13*, and *Grb14*. *Satb2* operates by directly repressing *Ctip2*, rather than by upstream control of *Fezf2*, and, consequently, *Satb2* null CPN are not fully fate-converted to SCPN (Britanova et al., 2008; Chirivella et al., 2008). Recently, the transcriptional co-regulator *Ski* (*ski sarcoma viral oncogene homolog)* has been shown to be a critical component of the repressor complex recruited by *Satb2* to initiate HDAC1-dependent chromatin remodeling, and *Ski*$^{-/-}$ mice largely phenocopy *Satb2*$^{-/-}$ mice (Baranek et al., 2012).

Epistasic analysis of subtype specification

Transcription factors that specify subtype identity have been shown in several instances to directly repress each other, raising the possibility that suppressing differentiation programs for alternate fates, rather than actively specifying a particular fate, might be their primary function in some cases. Under this model, simultaneous deletion of two competing transcription factors, such as *Tbr1* and *Fezf2* or *Satb2* and *Ctip2*, might partially restore proper subtype specification. Indeed, formation of the corticospinal tract (lost in *Fezf2*$^{-/-}$) is partially rescued in *Tbr1*$^{-/-}$;*Fezf2*$^{-/-}$ mice, although projections to the thalamus (lost in *Tbr1*$^{-/-}$) are still completely absent(McKenna et al., 2011). Similarly, formation of the corpus callosum (lost in *Satb2*$^{-/-}$) is partially rescued in *Satb2*$^{-/-}$;*Ctip2*$^{-/-}$ mice (Srinivasan et al., 2012). These results suggest that downstream programs are able to direct some neurons to differentiate appropriately, even in the absence of important specification controls, as long as competing specification controls are not active.

Early specification of subtype identity in progenitors

In aggregate, neocortical progenitors generate different projection neuron subtypes in sequential waves; however, the lineage relationships leading from progenitor cells to specific neuronal subtypes, and the molecular mechanisms that determine the fixed order in which neuronal subtypes are generated, remain largely unknown.

The classical model of progenitor fate restriction proposes that a single lineage of progenitors generates all subtypes of projection neurons, and that the competence of a given progenitor to generate specific subtypes becomes progressively limited over the course of development. In support of this model, early-stage progenitors transplanted into late-stage cortex are capable of producing all subtypes, but late-stage progenitors transplanted into early-stage cortex are competent only to produce superficial-layer subtypes (Frantz and McConnell, 1996; McConnell and Kaznowski, 1991; McConnell, 1988). In addition, retroviral lineage tracing experiments show that single progenitors labeled early in corticogenesis are competent to produce neurons of all layers (Luskin et al., 1988; Price and Thurlow, 1988; Walsh and Cepko, 1988). Later in corticogenesis, however, labeled progenitors primarily give rise to progeny residing in superficial layers (Reid et al., 1995). *In vitro* studies of both dissociated and embryonic stem (ES) cell-derived cortical progenitors indicate that these progenitors are capable of autonomously recapitulating the sequential generation of neuronal subtypes characteristic of corticogenesis *in vivo* (Eiraku et al., 2008; Gaspard et al., 2008; Nasu et al., 2012; Shen et al., 2006). Together, these various approaches demonstrate a narrowing of competence in the overall progenitor population over time, but do not establish that every progenitor is capable of producing all subtype fates.

An alternative model, one of progenitor diversification, proposes that independent, fate-restricted lineages of progenitors generate specific neuronal subtypes. Early evidence for this model came from the expression patterns of a number of subtype-specific transcription factors that are also present in progenitors, suggesting that different subsets of progenitors may be committed to generating

particular classes of projection neurons. *Fezf2* (*Fez family zinc finger 2*) is sparsely expressed in the proliferative zones during deep-layer neurogenesis, and is specific postmitotically to CFuPN (Arlotta et al., 2005; Chen et al., 2005b; Hirata et al., 2004; Inoue et al., 2004; Molyneaux et al., 2005). Conversely, *Cux1* and *Cux2* (*cut-like homeobox 1 and 2*) are expressed in the VZ and SVZ primarily during superficial-layer neurogenesis, and are specific postmitotically to CPN and other superficial-layer neurons (Molyneaux et al., 2009; Nieto et al., 2004; Zimmer et al., 2004). Direct evidence for a partially fate-restricted progenitor in the neocortex derives from recent genetic fate mapping using a *Cux2-CreERT2* line, which demonstrates that a subset of progenitors present froms the earliest stages of corticogenesis exclusively produces CPN and other superficial-layer neuron subtypes (**Figure 5A**). While deep-layer neurons are being generated, *Cux2*$^{+}$ progenitors mainly undergo proliferative divisions, expanding as a population while producing only a limited number of neurons, which likely become deep-layer CPN. Later, they switch to a neurogenic mode of division to generate superficial-layer neurons (Franco et al., 2012).

Subsequently, others have investigated whether the zinc finger transcription *Fezf2* is specifically expressed by a complementary population of *Cux2*$^{-}$ fate-restricted progenitors committed to generating subcortical projection neurons (Guo et al., 2013). These authors generate a transgenic mouse line using 36 kb of 5′ and 160 kb of 3′ sequence flanking the *Fezf2* gene and replacing the coding sequence for *Fezf2* with that of *CreERT2*. Using one of the transgenic lines generated by this approach, the authors conclude that *Fezf2*$^{+}$ progenitors are multipotential. However, it is widely known that transgenic mouse lines do not always recapitulate endogenous gene expression, a phenomenon that has been extensively demonstrated in the nervous system by the GENSAT project (Gong et al., 2003). Additional methodological flaws limit the interpretation of these results. First, the authors validate expression of *CreERT2* in progenitors by comparing it to the signal obtained using a *Fezf2 in situ* hybridization probe that can detect transcript generated by the *Fezf2-CreERT2* transgene, which includes most of the *Fezf2* open reading frame. Second, using a *CreERT2 in situ* hybridization probe, the authors demonstrate that their transgenic line

shows no subtype specificity postmitotically, with extensive expression in superficial layers, which is never seen from the native *Fezf2* locus. Therefore, it is not possible to draw any conclusions about the fate potential of *Fezf2*-positive progenitors based on these data, only about the specificity of the promoter elements included in transgene as they function in the unknown genomic context of random integration for this particular founder line.

Importantly, a number of different models of cortical subtype generation, ranging from strict sequential progression through competence states to immediate single-lineage commitment, can be entertained on the basis of current evidence. Of these, only the classical sequential competence model (**Figure 5B**) seems inconsistent with the experimental data, because this model predicts that fate-restricted *Cux2*$^{+}$ progenitors should not be present early in corticogenesis. Although two distinct lineages appear to exist (Franco et al., 2012), it is not clear whether they are entirely mutually exclusive (**Figure 5C**), or whether some subset of *Cux2*$^{-}$ progenitors joins the *Cux2*$^{+}$ lineage after generating deep-layer neuronal subtypes (**Figure 5D**). Importantly, the *Cux2*$^{+}$ and the *Cux2*$^{-}$ lineages each include multiple projection neuron subtypes, and it remains to be determined whether further fate-restricted sub-lineages emerge (**Figure 5E**) or fate specification is resolved postmitotically.

Toward a molecular logic of neocortical development

A deeper understanding of the molecular programs that govern progressive specification of neuronal subtype identity will necessitate establishing a basic framework for how and when cell fate specification decisions are made. These developmental pathways can be represented using first-order Boolean logic, with decision points represented by “molecular logic gates” (**Figure 6**). Each of these transitions is regulated by the coordinated activity of multiple transcriptional regulators and chromatin-modifying proteins, which direct extensive changes in the transcriptional and epigenetic state of a cell. Although this introduction has focused exclusively on regulation of subtype specification, other aspects of neuronal

development, such as area specification and migration, proceed in parallel, orchestrated by partially intersecting molecular programs.

Thesis overview

In this thesis, we investigate whether the zinc finger transcription *Fezf2* is specifically expressed by fate-restricted progenitors committed to generating subcortical projection neurons. Further, we investigate whether the critical function of *Fezf2* in subtype specification takes place primarily in progenitors or in postmitotic neurons. To address these questions it was necessary to generate tamoxifen-inducible *Fezf2*-CreERT2 mice, using CRISPR-Cas9 technology to introduce an *IRES-CreERT2* expression cassette into the 5' UTR of the *Fezf2* gene. Unlike in the *Fezf2*-CreERT2 mouse line previously reported (see previous section), we find that postnatal induction of recombination in our mice faithfully recapitulates the endogenous expression pattern of *Fezf2*, which is largely restricted to subcortical projection neurons in deep layers of cortex. Intriguingly, induction of recombination during early cortical neurogenesis, at E10.5, demonstrates that *Fezf2*-positive progenitors have a broad fate potential, as they are able to generate both projection neurons of multiple classes, as well as glial cells, distributed across all layers of cortex. Over the course of corticogenesis, however, at the time *Cux2*-positive progenitors are becoming more productive, expression of *Fezf2* in progenitors appears to decrease substantially, and the bulk of recombination following mid-corticogenesis tamoxifen induction occurs in postmitotic subcortical projection neurons. Interestingly, progenitor-stage-restricted misexpression of *Fezf2* directs fate specification of their neuronal progeny toward a subcortical fate, sufficient to repress transition by progenitors into a *Cux2*-postive molecular program. Taken together, these data support a model in which *Fezf2*-positive progenitors are multipotential, but can downregulate *Fezf2*, thereby transitioning to a fate-restricted *Cux2*-positive state.

Experimental Procedures

Animals

All mouse studies were approved by the Harvard University IACUC, and were performed in accordance with institutional and federal guidelines. The date of vaginal plug detection was designated E0.5, and the day of birth as P0. *Rosa26R-tdTomato-Ai9* (stock number 007909) and *Rosa26R-NZG* (stock number 012429) mice were purchased from Jackson Laboratories. *Nex1-Cre* mice were generated by Nave and colleagues (Goebbels et al., 2006). *Fezf2-CreERT2* mice were generated at the Harvard Genome Modification Facility using the targeting constructs described above. Briefly, for generation of the *Fezf2-IRES-CreERT2* targeting construct, the 5' and 3' homology arms were obtained by PCR from C57BL/6 mouse genomic DNA. Gibson molecular assembly was used to clone the homology arms were into a pUC19 backbone, along with an internal ribosomal entry site (IRES) and the coding sequence for CreERT2. The targeting construct and *in vitro* transcribed sgRNA were microinjected into B6D2F1 embryos, which were then transferred into pseudo-pregnant surrogates (Wang et al., 2013). Correctly targeted founder mice were identified using a PCR-based screening strategy using the following primers: *Fezf2*-F (5'-CCCATAAGGAGCGCTTGCAT-3') and *CreERT2*-R (5'-CCCTAGGAAT GCTCGTCAAGA-3').

Tamoxifen induction was accomplished by intraperitoneal injection of tamoxifen dissolved in corn oil (Sigma-Aldrich T5648 and C8267; 4mg/ml) into pregnant dams. For injections at E14.5 or later, tamoxifen was supplemented with progesterone to prevent abortions (Sigma-Aldrich; 2mg/ml). For fate-mapping and acute labeling experiments 1mg and 10mg of tamoxifen were used, respectively. Given the high incidence of dystocia following administration of high doses of tamoxifen, surviving pups were delivered by Caesarean section at E19.5 and cross-fostered by lactating CD1 surrogates from Charles River Labs. For postnatal tamoxifen inductions, 2mg tamoxifen was injected intraperitoneally at P4.

Immunocytochemistry and *in situ* hybridization

Mice were transcardially perfused with 4% paraformaldehyde, and brains were dissected and post-fixed at 4°C overnight. Tissue was sectioned at 50μm on a

vibrating microtome (Leica). Non-specific binding was blocked by incubating tissue and antibodies in 8% goat serum/0.3% bovine serum albumin in phosphate-buffered saline. For DAPI staining, tissue was mounted in DAPI-Fluoromount-G (SouthernBiotech).

Primary antibodies and dilutions used: rat anti-CTIP2, 1:200 (Abcam); chicken anti-GFP, 1:200 (Abcam); rabbit anti-GFP, 1:500 (Invitrogen); rabbit anti-Pax6, 1:500 (Millipore); rabbit anti-RFP 1:500 (Rockland); and mouse anti-SATB2, 1:200 (Abcam). Tissue was incubated with primary antibody at 4°C overnight. Secondary antibodies were chosen from the Alexa series (Invitrogen), and used at a dilution of 1:500 for 4 hours at room temperature.

***In utero* electroporation**

Overexpression constructs were generated using a *CMV/β-actin* promoter plasmid (derived from CBIG; gift of C. Lois), with or without a *loxP-3xSV40-loxP* transcriptional STOP cassette, and either *EGFP*, *myc-zsGreen*, *tdTomato* or *EGFP-shRNA*. Progenitor-specific expression constructs were generated by modifying a previously described *Nestin* promoter plasmid by introducing a *loxP-EGFP-2A-Fezf2-loxP* expression cassette (Wang et al., 2007). Electroporation conditions were described previously (Greig et al., 2016).

Retrograde labeling

Projection neurons were labeled from their axon termini under ultrasound guidance (Vevo 770) between P1 and P3 by pressure injection of Alexa fluorophore-conjugated cholera toxin B (Invitrogen). SCPN were labeled by injection into cerebral peduncle at P2, and CPN were labeled by injection into contralateral corpus callosum at P3. Tissue for all injections was collected at P7, and processed as for immunocytochemistry, above.

Statistical analyses

All experiments were performed with at least three independent replicates (n=3). For quantification, anatomically-matched sections from each mouse (n=3 for each

condition) were selected, and single confocal slices of somatosensory cortex were imaged. Cells were counted within a box of pre-defined size spanning the radial thickness of cortex. All error bars represent mean ± standard error.

Results

Generation of *Fezf2*-CreERT2 knock-in mice

In order to genetically label *Fezf2*-expressing cortical progenitors and to characterize the fate potential of this progenitor subset, we generated *Fezf2*-CreERT2 mice using CRISPR/Cas9 technology. The targeting construct was designed to introduce an *IRES*-CreERT2 cassette into the 5' untranslated region (UTR) of the endogenous *Fezf2* locus without disrupting the coding sequence. Guide RNA (sgRNA) sequences targeting the 3' UTR of *Fezf2* were designed *in silico* using an online CRISPR algorithm maintained by the Zhang lab at MIT. We selected those sgRNAs with the fewest potential off-target sites, cloned them into a dual promoter construct for expression of sgRNAs and Cas9. Candidate sgRNAs were tested for cleavage efficiency at the *Fezf2* locus by transfecting NIH-3T3 cells and performing surveyor assays (data not shown). Following microinjection of Cas9 mRNA, sgRNAs, and the targeting construct into B6D2F1 embryos and transfer into pseudopregnant surrogates, a total of 63 mice were born and screened for site-specific genomic insertion by PCR using two independent primer sets binding on either side of the 5' homology arm (**Figure 7A-B**). On the basis of these screening PCRs, seven founders were identified as candidates that potentially carried the knock-in allele (#40, 41, 42, 44, 61, 70 and 72).

The *Fezf2-CreERT2* allele mediates corticofugal-specific recombination

To conclusively determine whether each newly generated mouse line was correctly targeted, *Fezf2-CreERT2* founder mice were cross-bred with mice carrying a *Rosa26-CAG-floxed(Neo-STOP)-EGFP* reporter allele (*R26-NG*). The recombination patterns obtained were then compared with the spatial and cellular expression pattern of endogenous *Fezf2* expression (**Figure 7C-F**). For these experiments, recombination was induced by tamoxifen administration at postnatal day 4 (P4), after corticogenesis is complete. At this age, expression of *Fezf2* is limited to subcortical projection neurons, with higher levels of expression by subcerebral projection neurons in layer V, and lower levels of expression by corticothalamic projection neurons in layer VI. The distribution of EGFP-labeled cells was analyzed at P7. Although multiple founder lines

were found to faithfully recapitulate the spatial and cellular specificity of endogenous *Fezf2* expression, we selected line #41 for further analysis. Importantly, most recombined cells were found to reside in layer V, with a smaller number in layer VI, indicating that the distinguishable levels of *Fezf2* expression by subcerebral projection neurons (SCPN) and corticothalamic projection neurons (CThPN) are paralleled by differential expression of CreERT2, resulting in higher and lower recombination probabilities, respectively. No recombined neurons were identified in superficial layers, consistent with the very well-established absolute lack of *Fezf2* expression in postmitotic superficial layer neurons (Molyneaux et al., 2005; Chen, et al. 2005a; Chen et al., 2005b). As expected from this laminar distribution, there is robust labeling of SCPN descending through the diencephalon and entering the cerebral peduncle, with comparatively few axons entering the thalamus or crossing the corpus callosum.

Within layers V and VI, *Fezf2* expression is further restricted to subcortical projection neurons, and is largely excluded from interhemispheric projections neurons. We therefore sought to further characterize the subtype specificity of *Fezf2*-CreERT2 mediated recombination by combining postnatal tamoxifen induction with retrograde labeling from the cerebral peduncle and corpus callosum (**Figure 8**). As expected, we find that a large number of layer V recombined neurons project to the cerebral peduncle and very few project across the corpus callosum. Despite nearly saturating retrograde labeling of SCPN, a substantial proportion of layer V neurons were not labeled from either the cerebral peduncle or corpus callosum. This population of layer Va neurons likely consists primarily of corticostriatal projection neurons (CStrPN). Because recombination efficiency is dependent on expression levels, as illustrated by the different recombination efficiencies in layers V and VI and by additional findings discussed in later sections, these data suggest that SCPN express significantly lower levels of *Fezf2* than CStrPN at early postnatal stages.

***Fezf2*-expressing cortical progenitors are not lineage-restricted**

To investigate whether *Fezf2*-positive progenitors comprise an independent lineage that gives rise to subcortical, and not interhemispheric, projection neurons, we

performed a series of fate-mapping experiments. *Fezf2-CreERT2* mice were cross-bred with *R26-NG* reporter mice, and timed-pregnant mice were injected with tamoxifen at E10.5, E14.5 or E16.5. Analysis of labeled projection neurons was performed at P7 on the basis of: 1) laminar position, 2) axonal projections, and 3) molecular identity.

First, we examined the fate potential of *Fezf2*-positive progenitors at E10.5, before the proliferative zones (ventricular and subventricular zones- VZ and SVZ) of the telencephalon have generated any projection neurons, thereby guaranteeing that only progenitors, and no postmitotic neurons, are labeled upon tamoxifen induction. As expected, fate mapping of *Fezf2*-positive progenitors at this early developmental stage results in clonal labeling, with clear clustering of cells into characteristic ontogenetic cortical columns (**Figure 9**). Recombined neurons are present in all layers, with no bias toward superficial-layer or deep-layer fates. Robust axonal labeling is present in the corpus callosum, internal capsule, and thalamus. Most recombined cells are SATB2-expressing CPN, rather than CTIP2-expressing SCPN or CThPn, reflecting the larger proportion of CPN relative to subcortical projection neurons in cortex. Labeled glial cells, most of which have the morphologic appearance typical of astrocytes, are also scattered across all cortical layers.

In contrast, induction of recombination at E14.5, when deep-layer neurons have already been produced, results in very robust labeling in layers V and VI, without any obvious clonal distribution (**Figure 10**). Scattered neurons, likely derived from *Fezf2*-positive progenitors, are labeled in superficial layers. The presence of astrocytes further supports this interpretation, as these glial cells are born later in corticogenesis that this tamoxifen induction, starting on E17.5. Most recombined cells in deep layers are CTIP2-expressing SCPN or CThPN, rather than SATB2-expressing CPN, reflecting the subtype specificity of postmitotic *Fezf2* expression, in distinct contrast to the lack of subtype specificity of *Fezf2*-positive progenitors labeled at E10.5. Axons are abundantly labeled in the internal capsule and thalamus, while few axons are labeled in the corpus callosum. Importantly, fewer superficial-layer neurons are fate-mapped at E14.5 compared to E10.5, suggesting that a smaller fraction of all cortical progenitors

express *Fezf2* at E14.5, or that they do so at lower levels. This discrepancy would be consistent with progenitors downregulating expression of *Fezf2* as they become *Cux2*-positive and begin to generate superficial layer neurons, particularly given that the number of *Cux2*-positive progenitors increases over this same interval.

Next, we fate-mapped *Fezf2*-positive progenitors at E16.5, a transitional period during which neurogenesis is coming to a close and gliogenesis is beginning. Labeled cells are found almost exclusively in deep cortical layers (98.7±1.3%), and nearly all recombined cells are CTIP2-expressing SCPN or CThPN, rather than SATB2-expressing CPN (**Figure 10**). The near absence of superficial-layer neurons and astrocytes suggests a further reduction in the population of progenitors that express *Fezf2*, relative to E14.5. Given the lack of lineage restriction of *Fezf2*-positive progenitors and the lack of postmitotic *Fezf2* expression in superficial layers, it is possible to infer how much recombination takes place in progenitors at any given developmental stage by focusing on the absolute number of superficial-layer neurons labeled. Interestingly, there is a very clear reduction of progenitor recombination over the course of corticogenesis, with 329.3±59.6 cells/mm^2 with tamoxifen induction at E10.5, 84.0±20.4 cells/mm^2 at E14.5 and 1.3±1.6 cells/mm^2 at E16.5. We cannot discern whether this represents a reduction in the numbers of *Fezf2*-positive progenitors or, instead, in the levels of *Fezf2* expression by each individual progenitor. Moreover, even if progenitors have completely turned off *Fezf2* expression by E14.5, there might be residual recombination as a result of residual Cre-ERT2 protein and mRNA, although this would be expected to decrease over time both a result of protein/mRNA turnover and of dilutional effects from continued cell division.

Fewer progenitors express *Fezf2* after deep-layer neurons are generated

Subcortical projection neurons are born between E11.5 and E13.5, while the majority of interhemispheric projection neurons are born later in corticogenesis, between E14.5 and E16.5 (Greig et al., 2013). Interestingly, *Cux2*-positive progenitors expand as a population over the course of development. Because the vast majority of superficial layer neurons are derived from the *Cux2* lineage, and our experiments

demonstrate robust superficial-layer labeling in the *Fezf2* lineage, it stands to reason that the majority of *Cux2*-positive progenitors, in fact, arise from originally *Fezf2*-positive progenitors. If this transition also involves downregulation of *Fezf2* expression by *Cux2*-positive progenitors, a smaller proportion of cortical progenitors should express *Fezf2* as *Cux2*-positive progenitors expand as a population.

To investigate whether a smaller proportion of cells in the VZ and SVZ express *Fezf2* as progenitors transition from production of primarily subcortical to primarily interhemispheric projection neurons, we induced recombination in *Fezf2-CreERT2;R26-floxed(STOP)tdTomato* animals at either E12.5 or E14.5 with a high dose of tamoxifen and analyzed progenitor labeling 24h later. At both developmental stages, recombination is present both in the ventricular and subventricular zones (VZ and SV; progenitors) and in the cortical plate (CP; postmitotic neurons). Interestingly, the density of labeling in the VZ and SVZ is much higher at E12.5 than it is at E14.5, consistent with the substantial reduction in superficial-layer neuron labeling we observed with E14.5 tamoxifen induction relative to E10.5.

Fezf2* functions in progenitors to repress expression of *Cux2

Previous work in the field has demonstrated that *in utero* electroporation at E14.5 of a CMV/β-actin-promoter plasmid to drive ectopic expression of *Fezf2* starting at the progenitor stage, and continuing into the postmitotic period, causes layer II/III interhemispheric projection neurons to express genes characteristic of subcortical projection neurons and to redirect their axons towards the thalamus, brainstem, and spinal cord (Molyneaux et al., 2005; Chen et al., 2008). Postmitotic overexpression of *Fezf2* has similar effects, indicating that *Fezf2* is sufficient to reprogram projection neuron subtype identity in the cerebral cortex (Rouaux et al., 2013; De la Rossa et al., 2013). However, there is little evidence that *Fezf2* regulates gene expression in progenitors, and no evidence that it affects their fate potential.

To investigate whether *Fezf2* expression by progenitors influences their ability to transition into the *Cux2*-positive lineage, we generated a *Nestin*-promoter plasmid to drive ectopic expression of *EGFP*-2A-*Fezf2* (or an *EGFP*-only control) in progenitors

and electroporated them into *Cux2-CreERT2* embryos (Wang et al., 2007; Franco et al., 2012). These constructs were electroporated together with a constitutively active *CAG-EGFP* plasmid to identify all transfected neurons, and a reporter *CAG-floxed(STOP)-tdTomato* plasmid to identify cells that undergo recombination. Electroporations were performed at E12.5, tamoxifen was injected 36h later, and brains were collected for analysis 36h after induction of recombination. The proportion of electroporated cells in the *Cux2* lineage (tdTomato-positive/EGFP-positive) was then quantified. In control experiments, 35.1±4.6% of electroporated cells were in the *Cux2* lineage. Importantly, some of these tdTomato-labeled cells in control experiments were, in fact, *Cux2*-positive progenitors, as confirmed by PAX6 co-immunostaining. Strikingly, ectopic expression of *Fezf2* resulted in a dramatic reduction of recombination, with only 4.4±1.3% of electroporated cells expressing *Cux2*. These findings suggest that *Fezf2* maintains the multipotential state of progenitors that generate subcortical projection neurons, and actively represses molecular programs present in progenitors that become fate-restricted and commit to generating interhemispheric projection neurons.

Fezf2 **expression in progenitors biases subtype fate-specification**

We next investigated whether *Fezf2* expression by progenitors affects their fate-specification decisions. We also used a *Nestin* promoter to drive ectopic *Fezf2* expression in these experiments, since it is active only in progenitors, and would not continue to drive expression of ectopic *Fezf2* in postmitotic neurons as they acquire their subtype identity. To ensure that ectopic *Fezf2* expression does not bias differentiation of postmitotic neurons toward a subcortical subtype phenotype, we performed experiments in *Nex1-Cre* mice, in which expression of *Cre* begins immediately as neurons become postmitotic, but excludes progenitors (Goebbels et al., 2006; Woodworth et al., 2016). By flanking the *EGFP*-2A-*Fezf2* expression cassette with loxP sites, we were able to delete *Fezf2* from the *Nestin* promoter plasmid, preventing any leaky expression in postmitotic neurons. In addition, we further accelerated degradation of any residual *Fezf2* mRNA carried over from progenitors by co-electroporating a *CAG-floxed(STOP)-EGFPshRNA* plasmid to target any *Fezf2-2A-*

EGFP transcript remaining in postmitotic neurons, but not endogenous *Fezf2*, for degradation. Finally, a *CAG-floxed(STOP)-tdTomato* plasmid was co-electroporated to identify transfected cells.

Nex1-Cre-positive embryos were electroporated at E12.5 and analysis of resulting neuronal progeny for subtype identity was performed at P7. Progenitors electroporated with a control *Nestin*-floxed(EGFP) plasmid produced both deep- and superficial-layer neurons, including many CTIP2-expressing sucortical projection neurons. Interestingly, progenitors electroporated with *Nestin*-floxed(EGFP-2A-*Fezf2)* produce more than three times as many CTIP2-expressing subcortical projection neurons ($p<0.01$). The number of neurons derived from progenitors electroporated with *Nestin*-floxed(EGFP-2A-*Fezf2)* that extend axons into the internal capsule is also strikingly increased. However, we do not find a significant shift in the proportion of neurons residing in deep versus superficial layers of cortex. Taken together, our results suggest that *Fezf2* can function in progenitors to bias subtype identity acquisition, but the low levels of ectopic expression driven by the *Nestin* promoter are not sufficient to overcome the regulatory mechanisms driving fate restriction of multipotential progenitors toward the generation of superficial-layer neurons.

Discussion

Competing models: progenitor fate restriction vs. progenitor diversification

The identification of a lineage of cortical progenitors fate-restricted to generate interhemispheric projection neurons has challenged a longstanding view that cortical progenitors comprise a homogenous population that progresses through sequential competence states to generate different projection neuron types (Franco et al., 2012). More recent reports have suggested that these results could not be replicated (Guo et al., 2013), but the lack of subtype specificity was later found to result from epigenetic changes in the *Cux2*-CreERT2 locus that were triggered when these mice were bred to homozygosity, effectively producing *Cux2* nulls (Gil-Sanz et al., 2015). This debate in the field has been further complicated by the flawed assumption that transgenic mice can be used to define the endogenous expression pattern of tissue- and cell-type-specific genes (Guo et al., 2013).

Our results indicate that a hybrid model of progenitor fate restriction and diversification is necessary to explain the lineage relationships that connect progenitor cells with specific neuronal subtypes. Based on our fate-mapping experiments, *Fezf2*-positive progenitors are multipotential. However, *Fezf2* appears to repress the transition of cortical progenitors into the *Cux2*-lineage, and, after generation of subcortical projection neurons is complete at E14.5, few superficial-layer neurons are labeled by fate-mapping. These data suggest that although *Cux2*-positive progenitors are in the *Fezf2* lineage, they do not express *Fezf2* at mid-corticogenesis, and that downregulation of *Fezf2* might, in fact, be critical for interhemispheric progenitor fate-restriction. Therefore, progenitors do, in fact, change their competence over development, but diverse subsets coexist at most developmental stages and generate distinct classes of projection neurons.

Progressive acquisition of subtype identity

Interestingly, newly postmitotic projection neurons co-express high levels of transcription factors that are later restricted to only one subtype. For example, at E14.5, neurons in the cortical plate co-express CTIP2 and TBR1/FOG2, which are later restricted to SCPN and CThPN, respectively (Deck et al., 2013; Kwan et al., 2008).

Similarly, at E13.5, presumptive layer V neurons co-express CTIP2 and SATB2, which begin to become restricted to SCPN and CPN by E15.5 (Chirivella et al., 2008; Srinivasan et al., 2012). Some genes, such as *Clim1* in SCPN and *Lmo4* in CPN, do not become restricted to one subtype until after mice are born (Azim, 2009). The brief period during which immature postmitotic neurons co-express multiple subtype controls might correspond to a particularly plastic state, when decisions regarding subtype identity are being crystallized. However, this initially widespread expression of subtype controls followed by later refinement is intriguing, given that some progenitors are already committed to generating specific neuronal subtypes (Franco et al., 2012).

The timing of different fate specification decisions might be linked to biologically meaningful decision points, which might necessitate commitment of progenitors or favor later resolution postmitotically. Because CFuPN and deep-layer CPN begin to extend axons in different directions even as they migrate through the intermediate zone (Lickiss et al., 2012), specification into one of these two broad fates might need to occur in progenitors. In contrast, CThPN and SCPN axons travel through the internal capsule together for several days before their trajectories diverge (Molnár and Cordery, 1999). This coincides with a period during which newly-postmigratory CFuPN transition from co-expressing high levels of TBR1 and CTIP2 to expressing one or the other, potentially reflecting postmitotic commitment (Deck et al., 2013).

Cell-autonomous and non-cell-autonomous mechanisms of cortical development

A complex interplay of cell-intrinsic programs and cell-extrinsic cues guides the progressive differentiation of cortical projection neurons throughout development. Positional information is first imparted on the neural tube by rostro-caudal and dorso-ventral gradients of morphogens, including FGFs, BMPs, Wnts, and SHH (Rallu et al., 2002). These extrinsic cues induce expression of cross-repressive transcriptional programs that specify and maintain dorsal telencephalic identity, including *Pax6*, *Ngn2*, *Sox6*, and *Emx2* (Schuurmans and Guillemot, 2002). Much less is known about how extrinsic cues influence subtype specification, although it has been proposed that NTF3 and FGF9 secreted by the cortical plate regulate the transition from deep-layer to

superficial-layer neurogenesis (Seuntjens et al., 2013). Interestingly, the onset of NTF3 and FGF9 production by the cortical plate coincides with a progressive expansion of the *Cux2*-positive population of cortical progenitors, and presumably a corresponding reduction in the number of *Fezf2*-positive progenitors (Franco et al., 2012).

Future directions

Although our experiments strongly suggest that *Cux2*-positive progenitors are derived from *Fezf2*-positive progenitors, they certainly do not preclude the existence of a subcortical-fate-restricted progenitor lineage. It is possible that before progenitors begin to generate projection neurons, they must first commit to either the subcortical or interhemispheric lineage, and that *Fezf2* is expressed by both progenitors committed to the subcortical lineage and by a primitive uncommitted population that undergoes proliferative divisions only, becoming fate-restricted before commencing neurogenic divisions. Alternatively, *Fezf2*-positive progenitors might join the *Cux2*-positive lineage after generating subcortical projection neurons, therefore changing their competence state. This question could be experimentally addressed by generating a *Fezf2*-Flpo knock-in line in order to simultaneously fate-map both populations of progenitors. The behavior of each population could be observed by performing live imaging in cortical slice cultures.

While our electroporation experiments, in which we drive ectopic *Fezf2* expression in cortical progenitors, are highly suggestive for a role of *Fezf2* in regulating the transition of progenitors into the *Cux2* lineage, it would be important to further delineate possible functions of *Fezf2* in cortical progenitor lineage commitment. One possibility might be to cross-breed *Fezf2* null and *Cux2-CreERT2* animals in order to investigate whether *Cux2*-positive progenitors expand as a population in absence of *Fezf2* function. This would provide evidence that *Fezf2* does, in fact, maintain competence of progenitors to generate subcortical projection neurons, and that abnormal fate specification in *Fezf2* null mice has its roots in molecular abnormalities that arise at the progenitor stage.

Summary

Taken together, the results presented in this thesis demonstrate that *Fezf2* is expressed by multipotential cortical progenitors. However, *Fezf2*-positive progenitors do not necessarily give rise directly to both subcortical and interhemispheric projection neurons. In fact, we find that the number of *Fezf2*-positive progenitors decreases over the course of development, and that very few, if any, superficial-layer neurons are fate-mapped in the *Fezf2*-lineage at E14.5 or later. Therefore, our data support a model in which *Fezf2* is expressed by multipotential progenitors, a subset of which transitions into the *Cux2*-positive lineage. These progenitors subsequently downregulate expression of *Fezf2* and become fate-restricted, so that they are able to generate only interhemispheric projection neurons. Importantly, we provide evidence that *Fezf2* functions in progenitors to repress the transition into the *Cux2* lineage and that it can therefore bias progenitors toward generating neurons that acquire subcortical fates.

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Figures

Figure 1. Projection neuron diversity in the cerebral cortex is organized along the radial (subtype identity) and tangential (area identity) axes.

(a) Commissural neurons, such as callosal projection neurons, reside in layers II/III, V, and VI and project axons across the corpus callosum to the contralateral cortical hemisphere.

(b) Associative projection neurons include intrahemispheric short-distance projection neurons, such as layer IV granule cells, as well as intrahemispheric long-distance projection neurons, which project to adjacent or distant cortical areas, such as frontal and backward projection neurons, FPN and BPN.

(c) Corticofugal projection neurons reside in layer VI (corticothalamic projection neurons, left) or layer V (subcerebral projection neurons, right) and send axons to targets outside the cortex.

(d) Some neurons project to multiple targets, falling into more than one of the categories above, such as intratelencephalic corticostriatal projection neurons, which send axons to both the ipsilateral and contralateral striatum, and a collateral into contralateral cortex.

Projection neurons of each subtype are further specialized according to their rostrocaudal and mediolateral positions, reflecting the tangential organization of the neocortex into functional areas (motor, somatosensory, visual, and auditory).

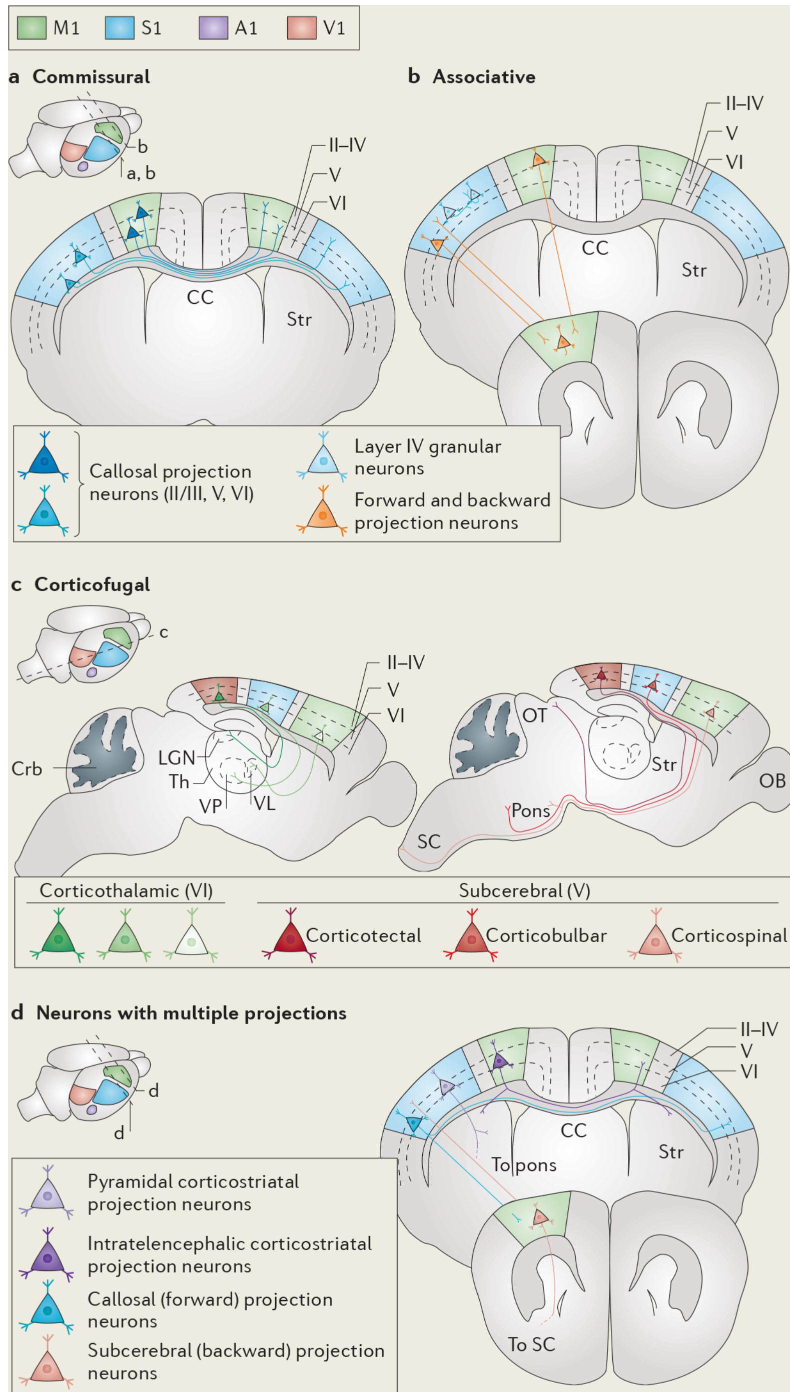


Figure 1

Figure 2. Neocortical projection neurons are generated in an "inside-out" fashion by diverse progenitor types in the VZ and SVZ.

(a) Projection neurons begin to be generated by radial glia (RG) in the ventricular zone (VZ) around E11.5, and by intermediate progenitors (IP) and outer radial glia (oRG) soon thereafter. Newly-born neurons migrate along radial glia to reach their final laminar destinations.

(b) Distinct projection neuron subtypes are born in sequential waves over the course of neurogenesis. The peak birth of subplate (SP) neurons occurs around embryonic day (E) 11.5, with the peak birth of corticothalamic projection neurons (CThPN) and subcerebral projection neurons (SCPN) occuring at E12.5 and E13.5, respectively. Layer IV granule cells (GC) are born around E14.5. Some callosal projection neurons (CPN) are born starting at E12.5, and those CPN born concurrently with CThPN and SCPN also migrate to deep layers. Most CPN are born between E14.5 and E16.5, and these late-born CPN migrate to superficial cortical layers.

NE, neuroepithelial cell; CR, Cajal-Retzius cells; WM, white matter

Figure 2

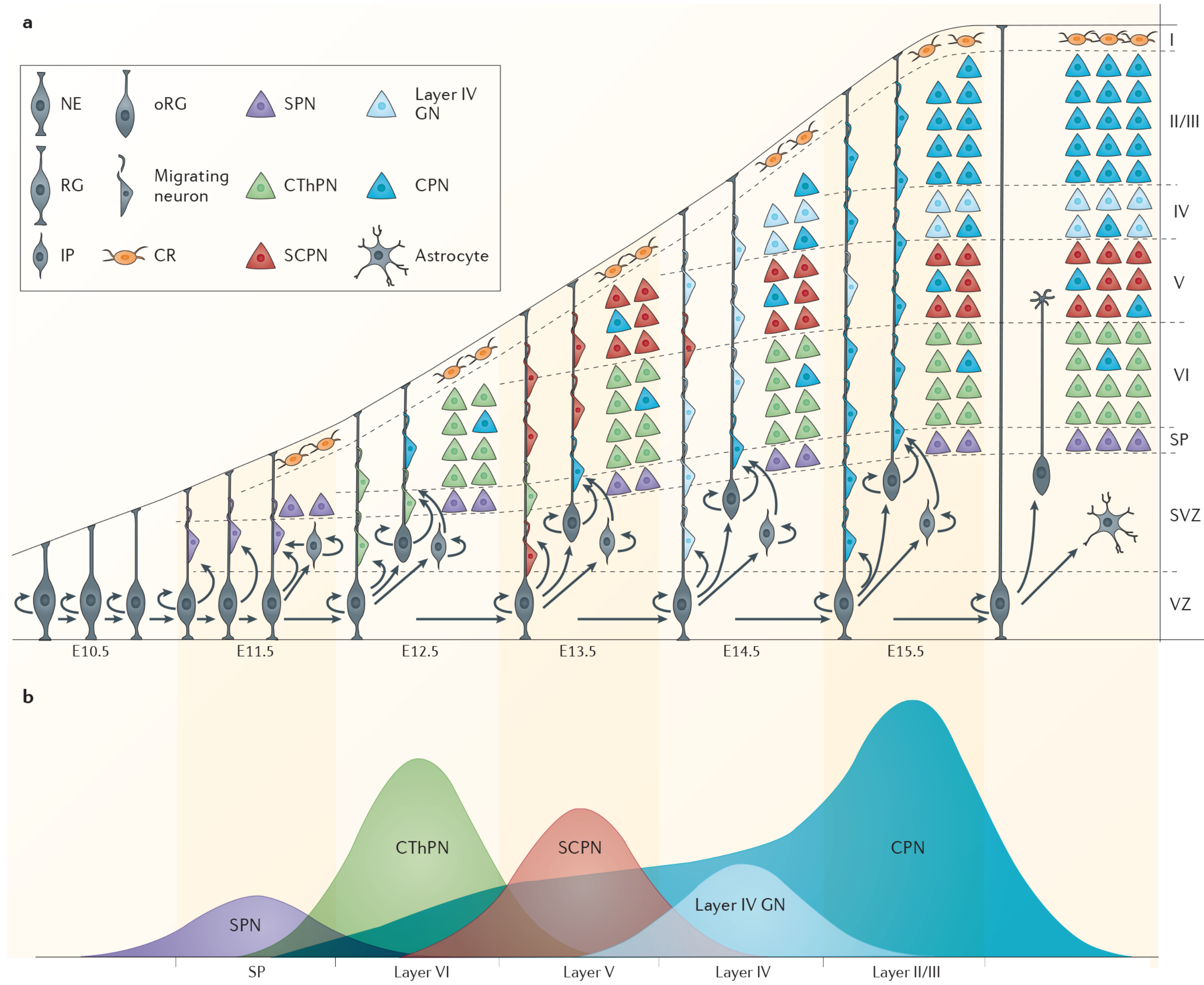


Figure 3. Competing molecular programs direct differentiation of newly postmitotic projection neurons into one of three broad subtype identities.

Boundaries between subtype identities are established in "subtype space" by the action of cross-repressive molecular controls. One boundary exists between neurons specified as SCPN and those specified as CThPN, with another between SCPN/CThPN (CFuPN) and CPN. The boundaries between CFuPN and deep-layer or superficial-layer CPN may shift independently. Interaction arrows represent known cases of genetic or transcriptional activation or repression, and further interactions and molecular controls likely remain to be identified. Changes in expression of a number of key regulators can cause boundaries between subtypes to shift, as neurons partially or completely acquire gene expression, cellular morphology, and projection patterns characteristic of other subtypes.

Figure 3

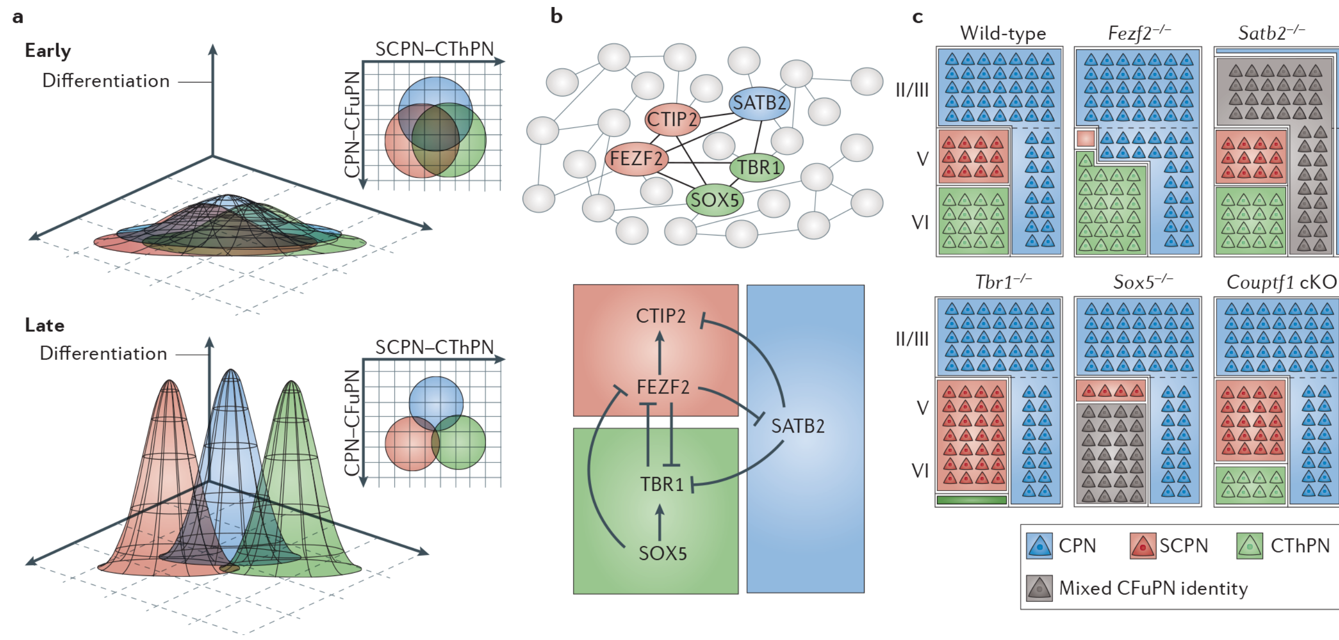
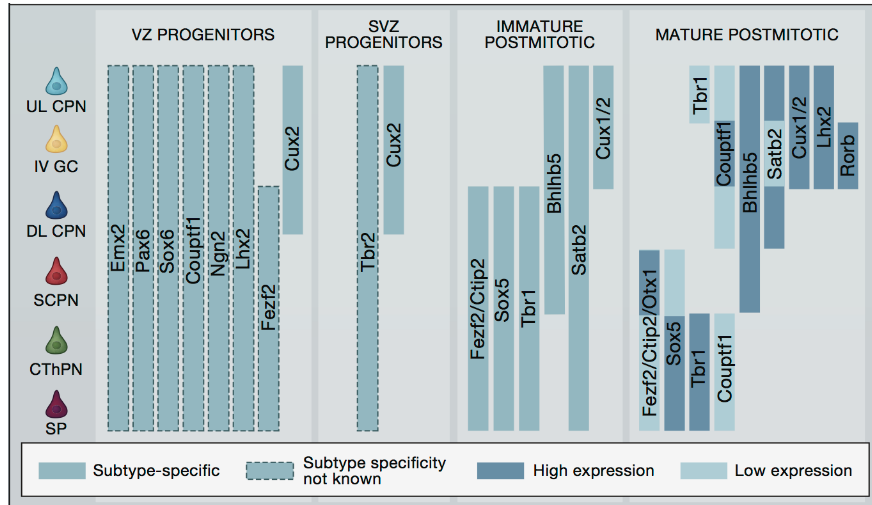


Figure 4. Competition between cross-repressive genetic programs progressively establishes projection neuron subtype identity.

(Top panel) Projection neuron subtype identity is progressively specified by combinatorial transcription factor programs. Some transcription factors expressed by specific subtypes of postmitotic projection neurons are also expressed by subsets of progenitors, implying that some progenitors might be partially or wholly fate-restricted. For example, *Cux2* is expressed by VZ and SVZ progenitors, as well as layer IV granule cells and upper-layer callosal projection neurons; *Fezf2* is expressed by VZ progenitors, as well as subcerebral projection neurons (and, at low levels, corticothalamic projection neurons).

(Bottom panel) Postmitotic projection neurons initially co-express high levels of controls that are later mutually exclusive, including *Ctip2*, *Tbr1*, and *Satb2*. By E15.5, many neurons exclusively express one of these three controls. By P4, segregation of these programs is complete, and each subtype sends axons to spatially distinct targets.



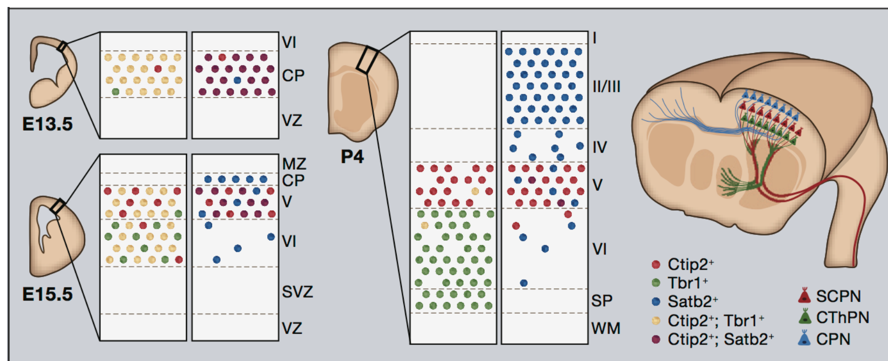


Figure 4

Figure 5. Distinct progenitor lineages produce deep-layer (corticofugal) and superficial-layer (commissural and associative) projection neurons.

(a) Fate-mapping experiments have established that superficial-layer projection neurons derive from *Cux2*$^{+}$ progenitors, while deep-layer neurons derive from *Cux2*$^{-}$ progenitors. Although the previously-accepted model of "sequential competence states" (b) has been refuted, the precise structure of lineage trees during corticogenesis remains unknown. It is possible that progenitors commit to independent lineages before the onset of neurogenesis (c), or that some progenitors first give rise to neurons of one lineage and later commit to a different lineage (d). Similarly, progenitors might be multipotential, giving rise to more than one type of neuron, or become progressively fate restricted until they are unipotential (e).

Figure 5

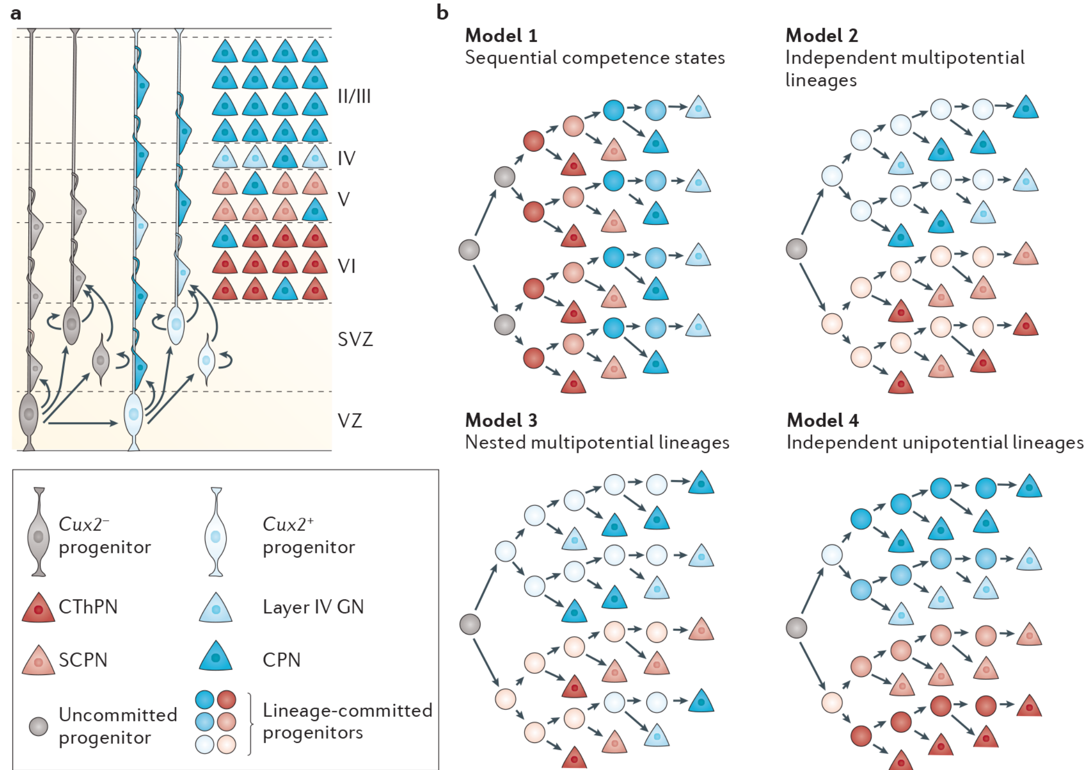


Figure 6. Molecular logic of subcerebral projection neuron development.

The neocortical domain is established by transcription factors that act combinatorially to repress subpallial programs (*e.g.*, *Pax6*, *Emx2*, and *Sox6*) and cortical hem programs (*e.g.*, *Lhx2* and *Foxg1*). Subsequently, neocortical progenitors are further specified into at least two partially fate-restricted lineages by yet unidentified molecular controls. Progenitors that are *Cux2*$^{-}$, and possibly *Fezf2*$^{+}$, generate CFuPN, while progenitors that are *Cux2*$^{+}$ generate CPN and other neurons in superficial layers. CFuPN become committed to a specific subtype at a decision point gated by cross-repression between *Fezf2*, which directs SCPN specification, and *Tbr1* and *Sox5*, which direct CThPN specification. Once SCPN are specified, *Ctip2* promotes subsequent differentiation steps, including axon outgrowth, fasciculation, and targeting. Additional controls instruct further specialization of SCPN subpopulations, including collateralization and pruning decisions.

Figure 6

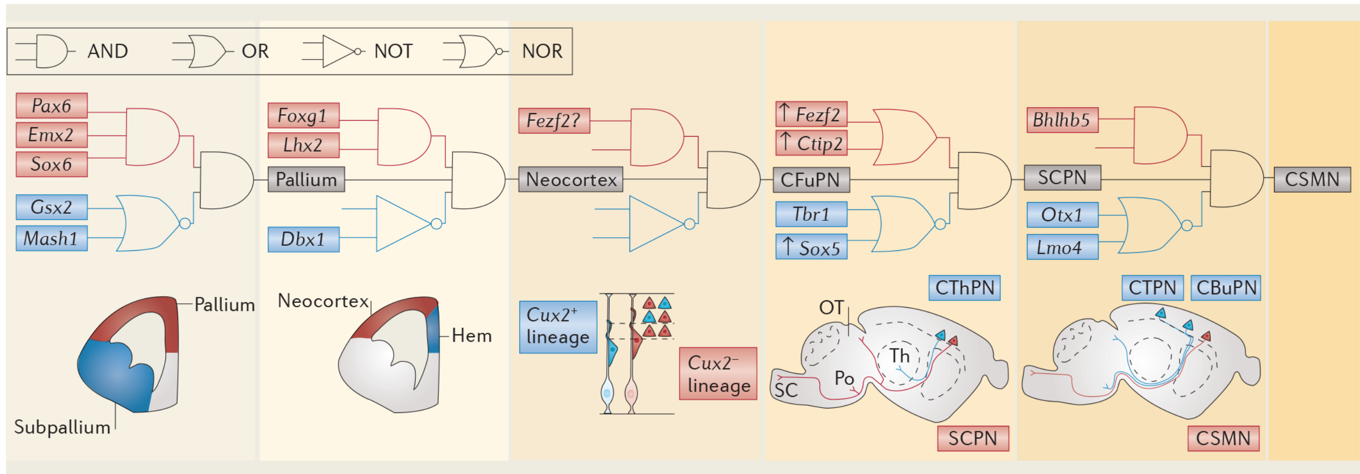


Figure 7. Fezf2-CreERT2 mice express tamoxifen-inducible Cre recombinase under the control of the Fezf2 genomic locus.

The *Fezf2* genomic locus is edited to insert IRES-CreERT2 into exon 4 of *Fezf2* (A). Founder screening PCR indicates establishment of multiple successful founder lines (B). *Fezf2*-CreERT2 is expressed in the same pattern as *Fezf2* mRNA in early postnatal brain (C-F), and reporter GFP marks large pyramidal projection neurons in layer V of cortex that send axons to subcerebral targets (arrow in F).

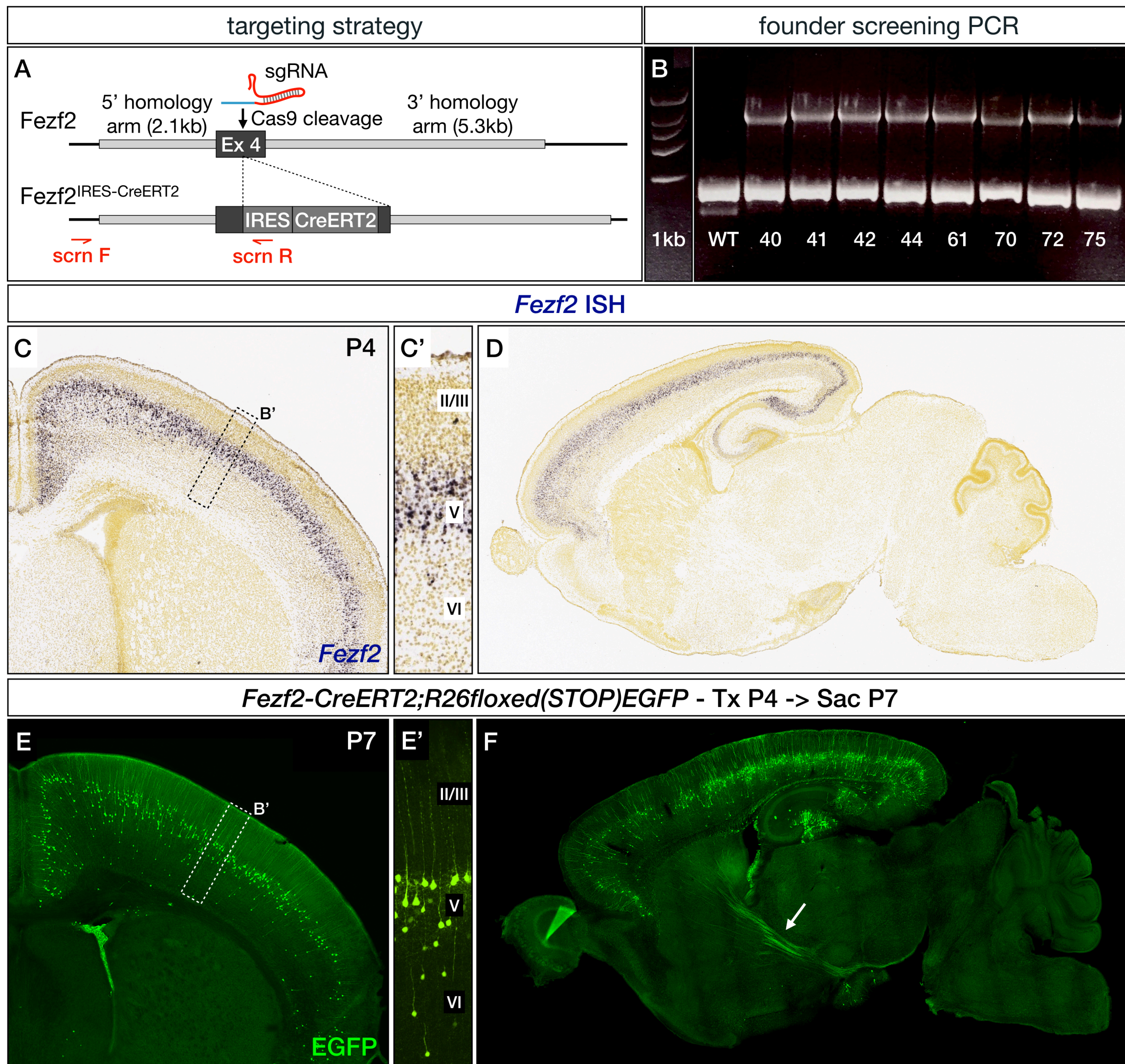


Figure 7

Figure 8. *Fezf2*-CreERT2 is specific to subcerebral projection neurons in postnatal brain.

Fezf2-CreERT2 recombination (A) occurs in subcerebral projection neurons, labeled by retrograde injection from the cerebral peduncle (B), but not callosal projection neurons, labeled by retrograde injection from the contralateral cortical hemisphere (C). Schematic of labeling approach (D). Confocal images of a representative *Fezf2*-CreERT2-recombined neuron co-labeled with retrograde tracing from the cerebral peduncle, but not from the contralateral cortical hemisphere (E-K).

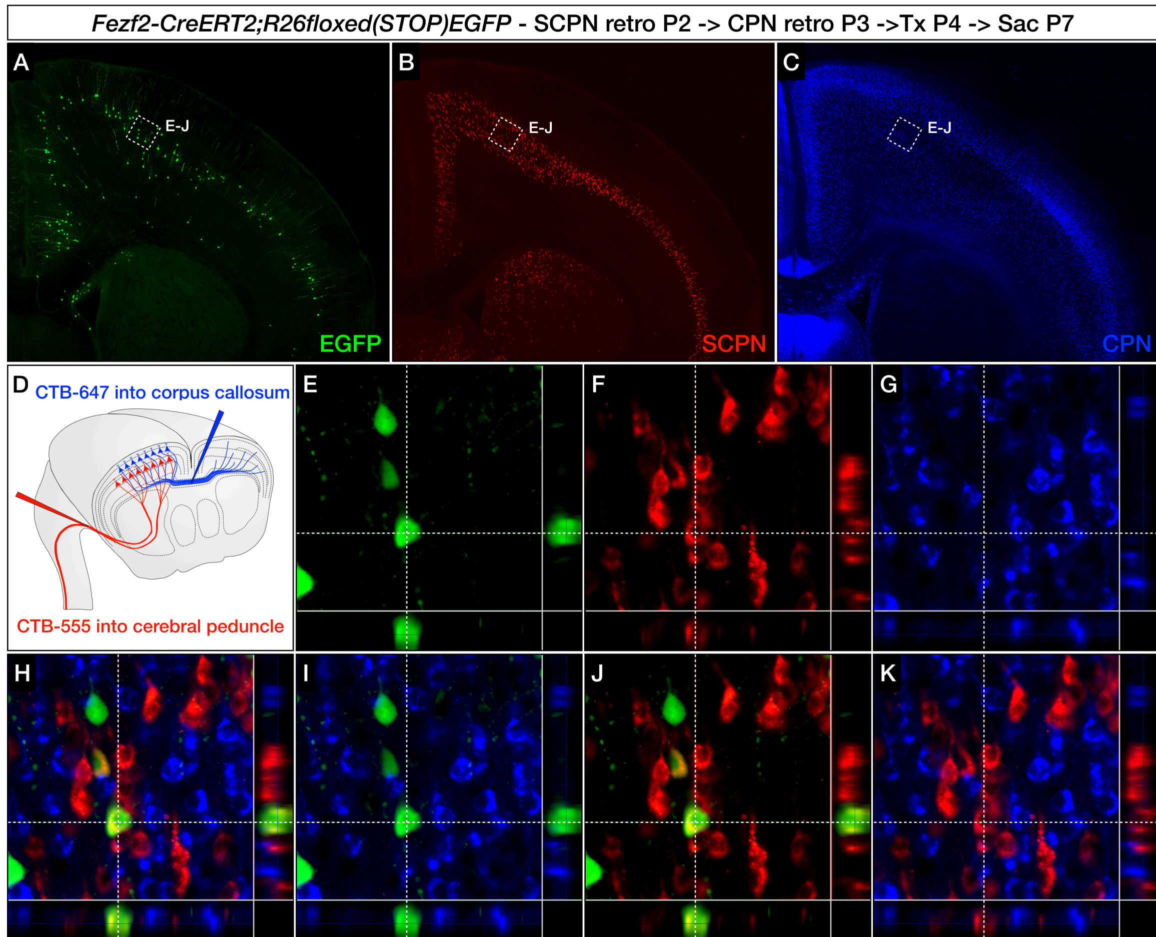


Figure 8

Figure 9. Fate mapping *Fezf2*-positive progenitors in early cortical development (E10.5) results in widespread clonal labeling.

Tamoxifen administration at E10.5 in *Fezf2*-CreERT2-positive embryos labels widespread cortical neurons at P7 (A-C). Recombined cells are present in all layers, with no bias toward superficial-layer or deep-layer fates (D-E). Most recombined cells are SATB2-expressing callosal projection neurons (G, I), rather than CTIP2-expressing subcerebral projection neurons (F, H), reflecting the larger percentage of callosal projection neurons in P7 cortex. Quantification (J).

Figure 9

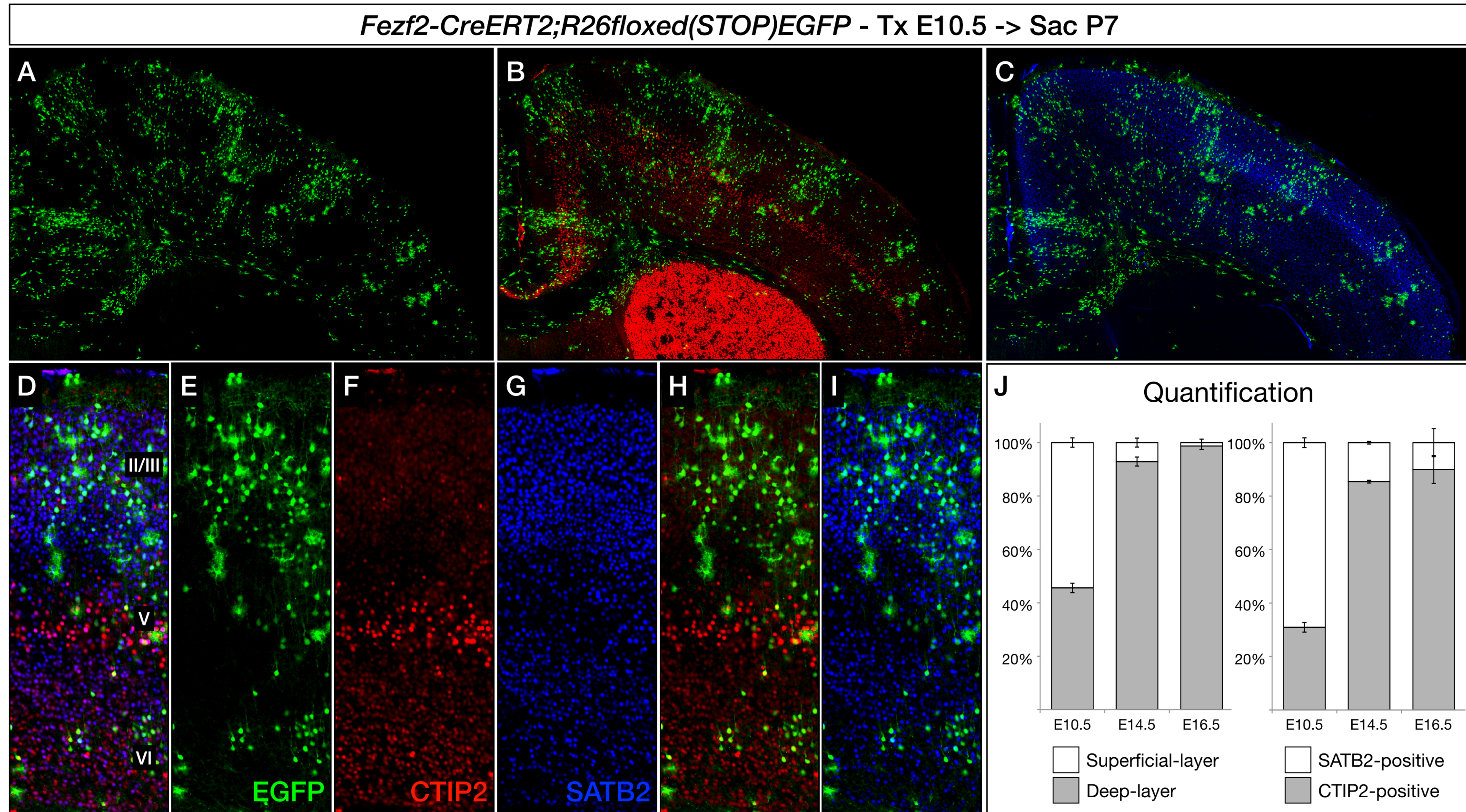


Figure 10. Fate mapping *Fezf2*-positive progenitors in mid-cortical development (E14.5) results in pronounced bias toward neurons that take up deep-layer fates.

Tamoxifen administration at E14.5 in *Fezf2*-CreERT2-positive embryos labels primarily deep-layer cortical neurons at P7 (A-C). Recombined cells are present primarily in layers V and VI, with few cells adopting superficial-layer fates (D-E). Most recombined cells are CTIP2-expressing subcerebral projection neurons (F, H), rather than SATB2-expressing callosal projection neurons (G, I), reflecting a substantial bias toward adoption of subcerebral and corticothalamic fates in P7 cortex. Quantification (J).

Figure 10

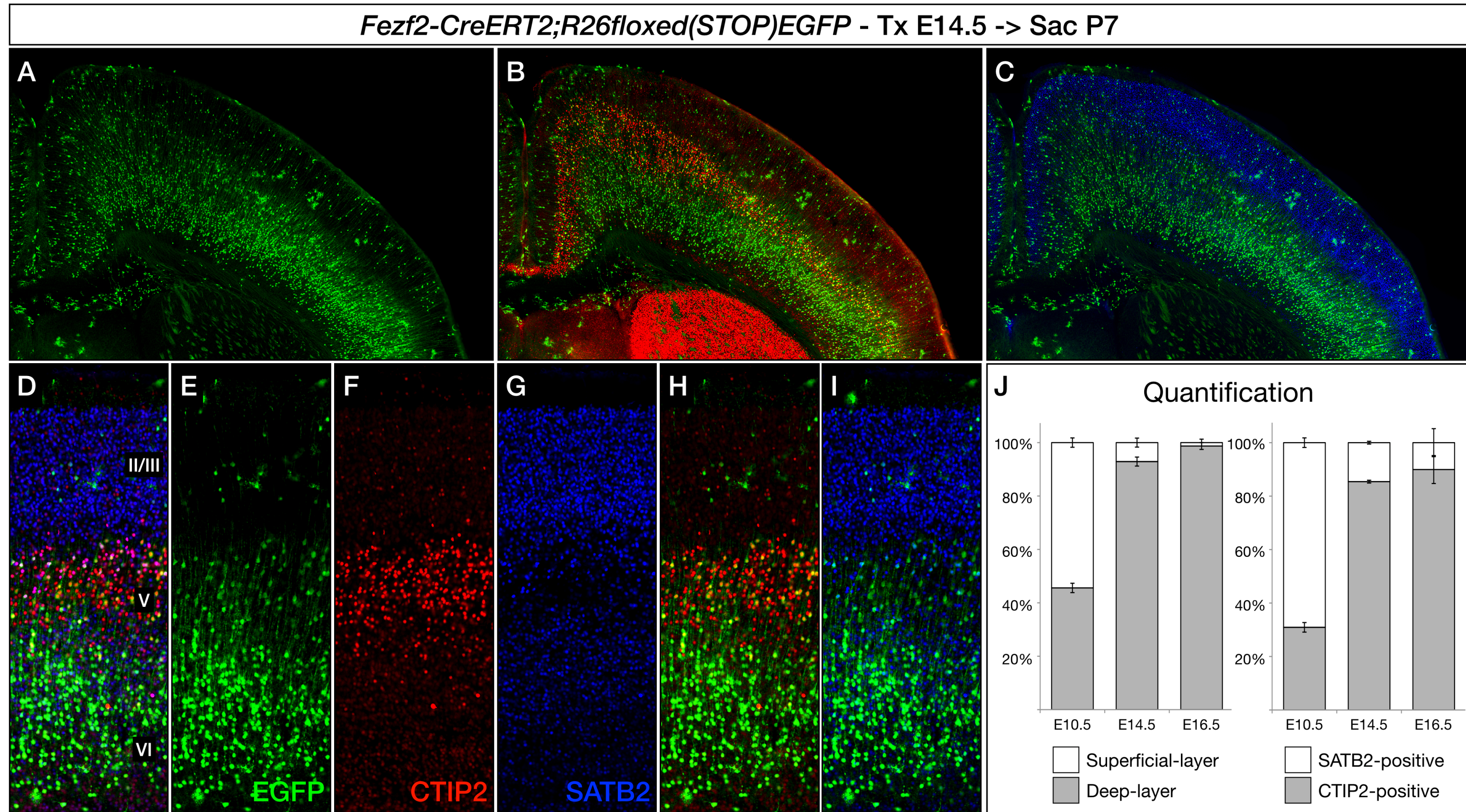


Figure 11. Fate mapping *Fezf2*-positive progenitors in late cortical development (E16.5) results in pronounced bias toward neurons that take up deep-layer fates.

Tamoxifen administration at E16.5 in *Fezf2*-CreERT2-positive embryos labels primarily deep-layer cortical neurons at P7 (A-C). Recombined cells are present almost exclusively in layers V and VI, with very few cells adopting superficial-layer fates (D-E). Nearly all recombined cells are CTIP2-expressing subcerebral projection neurons (F, H), rather than SATB2-expressing callosal projection neurons (G, I), reflecting a profound bias toward adoption of subcerebral and corticothalamic fates in P7 cortex. Quantification (J).

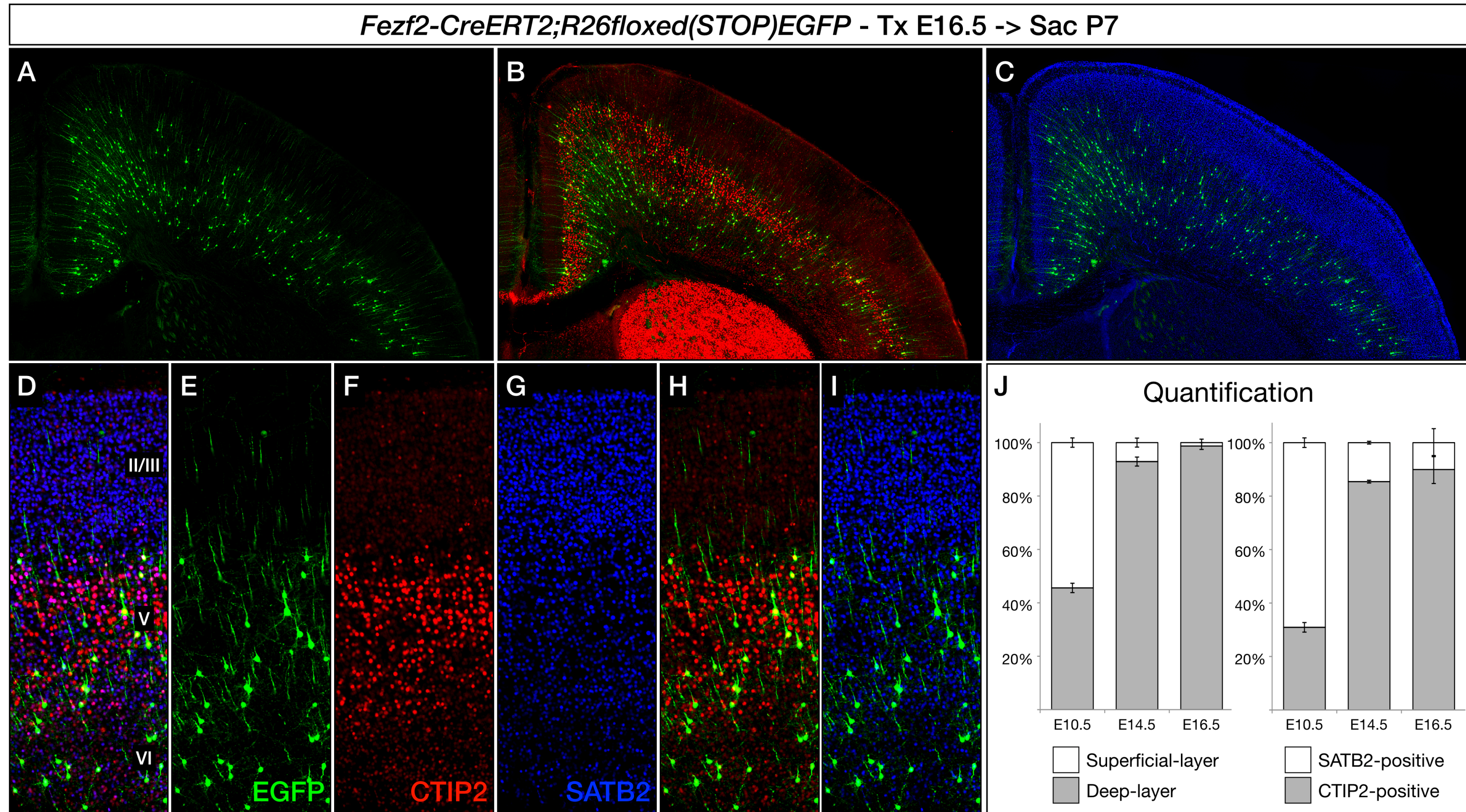


Figure 11

Figure 12. *Fezf2*-CreERT2 marks both progenitors and postmitotic neurons 24 hours after mid-embryonic tamoxifen administration.

Tamoxifen administration at E12.5 in *Fezf2*-CreERT2-positive embryos (A) marks both ventricular zone progenitors and newborn neurons in the cortical plate (B). Tamoxifen administration at E14.5 in *Fezf2*-CreERT2-positive embryos (C) marks ventricular zone and subventricular zone progenitors, as well as newborn neurons in the cortical plate (D).

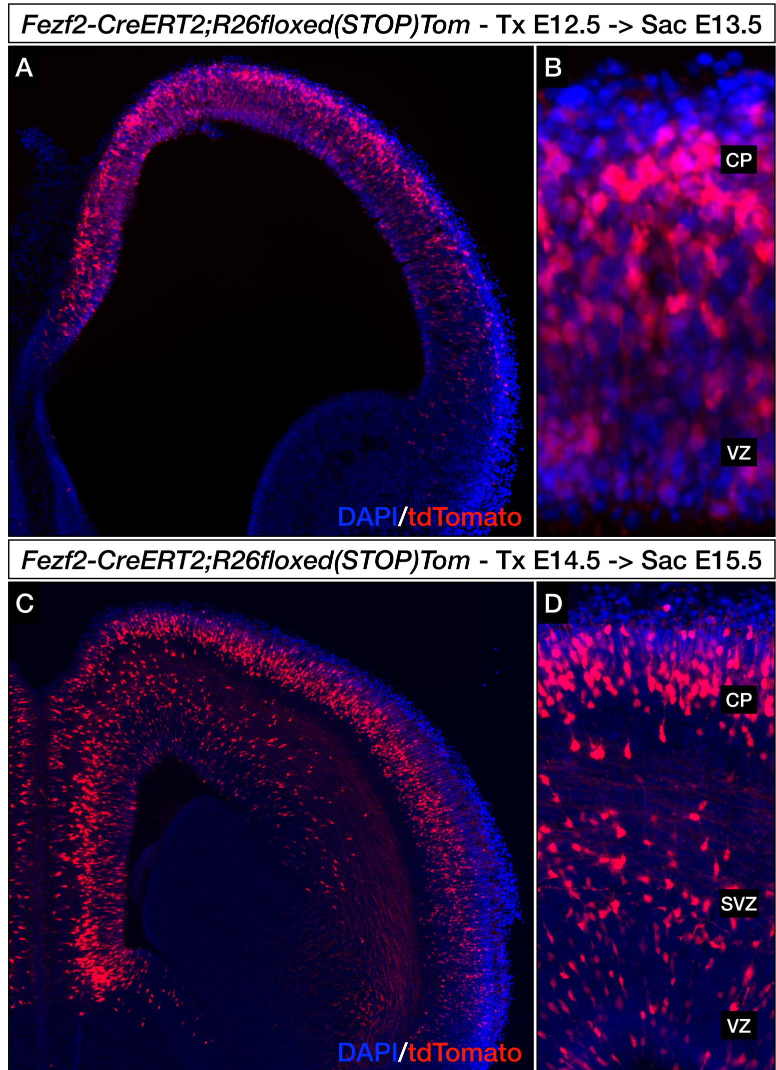


Figure 12

Figure 13. At E13.5, *Cux2* is expressed by progenitors and their neuron progeny, but *Cux2* expression is abolished in progenitors that overexpress *Fezf2* from E12.5.

At E12.5, cortical progenitors in *Cux2*-CreERT2-positive embryos are electroporated with plasmids encoding lox-STOP-lox tdTomato (CreERT2 reporter), constituitively active (CAG) EGFP, and Nestin-promoter-driven EGFP (control, A) or EGFP-2A-Fezf2 (experimental, E). Tamoxifen is administered at E13.5, and embryos are collected at E15.5. In control electroporations, PAX6-positive progenitors and PAX6-negative neurons are both fate-mapped by *Cux2*-CreERT2 (C-D''). In EGFP-2A-*Fezf2* electroporations, neither progenitors nor postmitotic neurons are fate-mapped by *Cux2*-CreERT2.

Figure 13

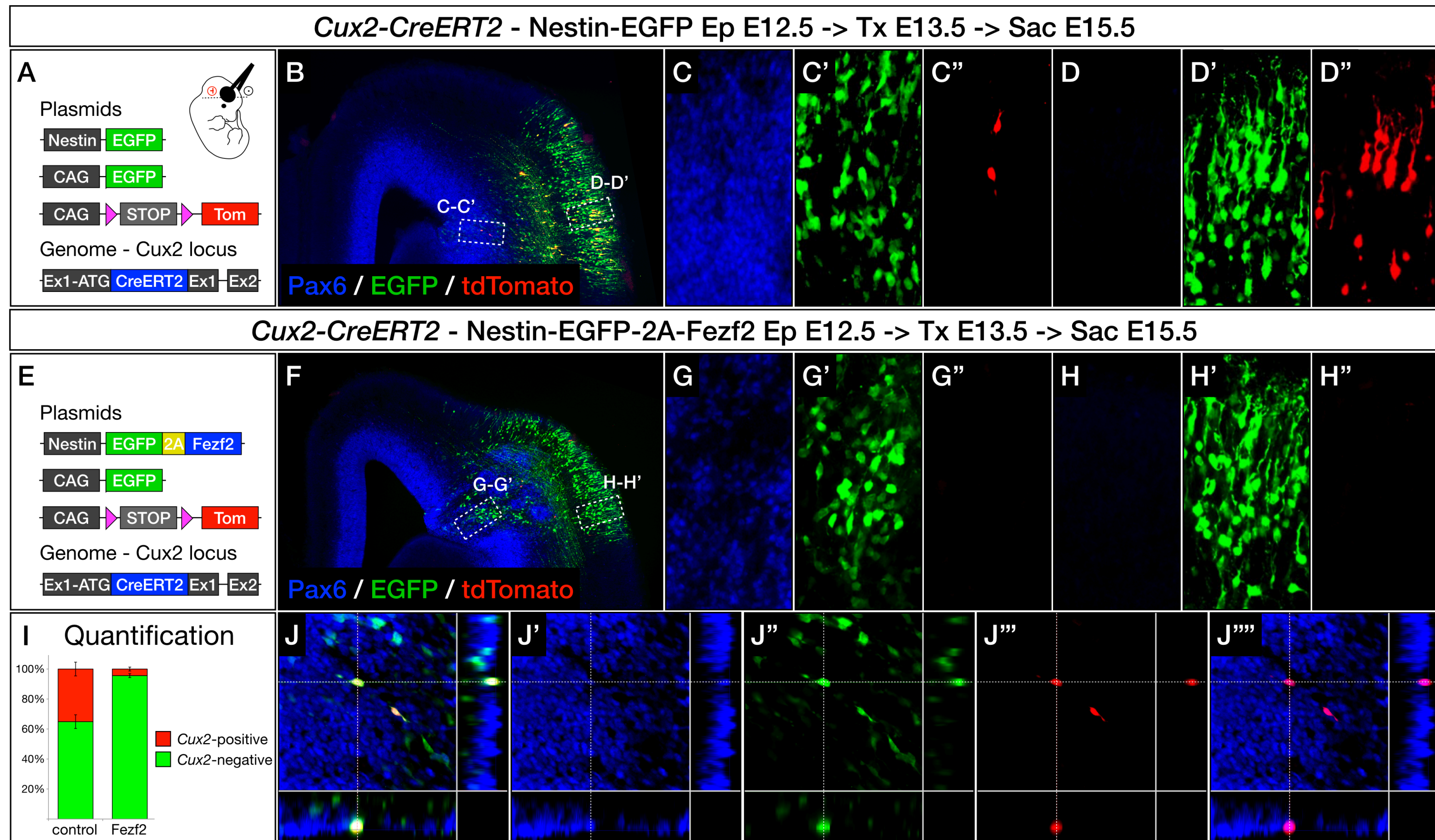


Figure 14. Progenitor-specific overexpression of *Fezf2* produces postmitotic subcerebral projection neurons.

Schematic of approach (A). *Nex1*-Cre-positive embryos are electroporated at E12.5, leading to Cre recombination only in postmitotic neurons, but no recombination in progenitors (left panel). Electroporated progenitors express *Nestin*-promoter-driven EGFP-2A-*Fezf2* (middle panel), while postmitotic neurons express zsGreen and GFP shRNA, silencing expression of EGFP-2A-*Fezf2* (right panel). Progenitors electroporated with *Nestin*-floxed-EGFP plasmids (control, B-C) produce some CTIP2-expressing postmitotic subcerebral projection neurons (arrows, D-D''), and more neurons that do not express CTIP2 (arrowheads, D-D''). Progenitors electroporated with *Nestin*-floxed-EGFP-2A-*Fezf2* (E-F) produce significantly more CTIP2-expressing subcerebral projection neurons than control (arrows in G-G''). Quantification (H). Progenitors electroporated with *Nestin*-floxed-EGFP-2A-*Fezf2* produce more postmitotic neurons that send projections through the internal capsule and fewer that send projections through the corpus callosum (K, M) than progenitors electroporated with control constructs (J, L).

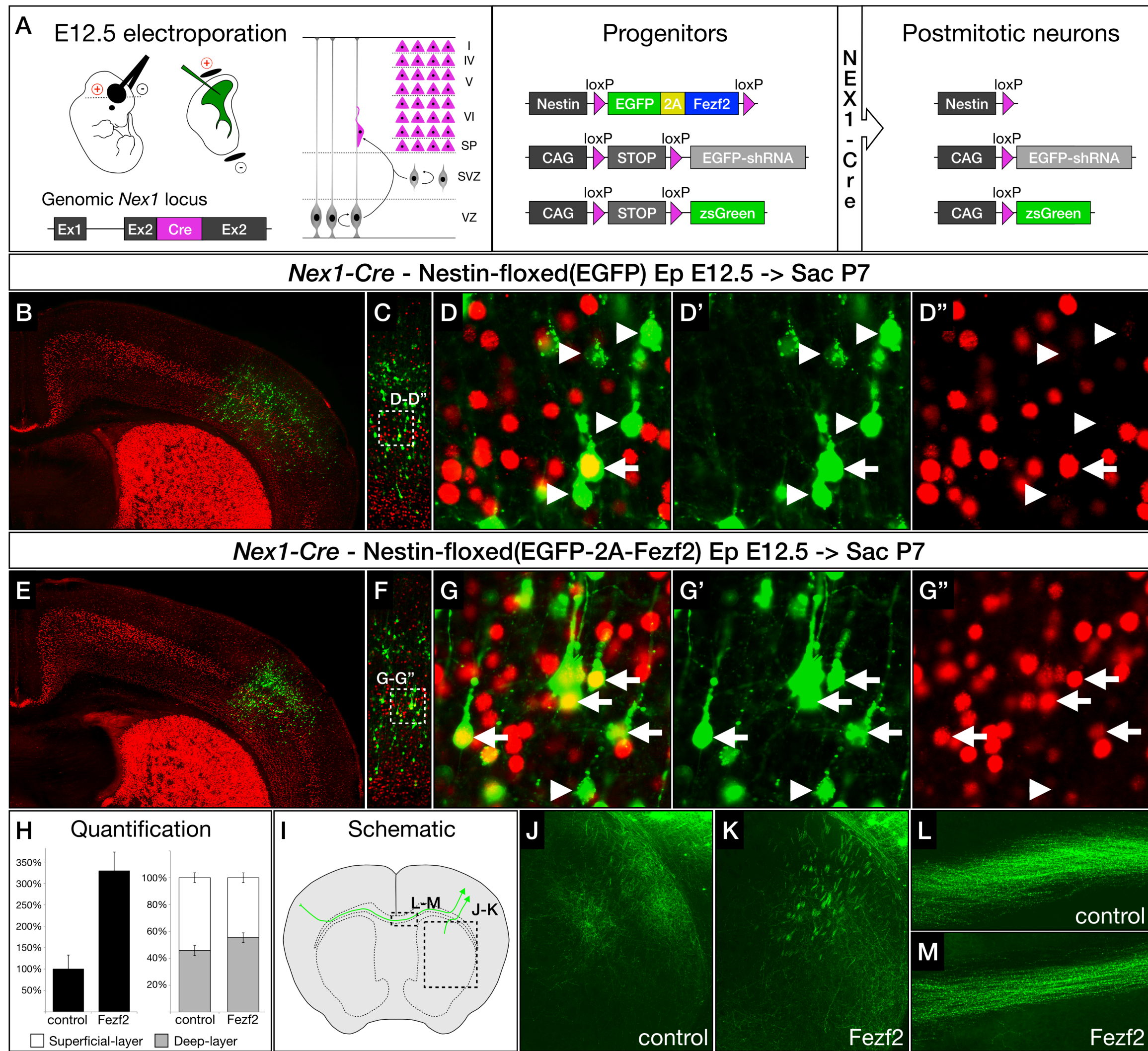


Figure 14