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Transcriptional controls over neocortical projection neuron identity and connectivity

presented by Mollie Ann Woodworth

candidate for the degree of Doctor of Philosophy and hereby certify that it is worthy of acceptance.

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Transcriptional controls over neocortical projection neuron identity and connectivity

A dissertation presented

by

Mollie Ann Woodworth

to

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in partial fulfillment of the requirements

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Transcriptional controls over neocortical projection neuron identity and connectivity

Abstract

The complex and sophisticated circuitry of the neocortex is assembled from an extraordinarily diverse repertoire of neuronal subtypes that reside in distinct functional areas. In recent years, a number of key regulators over neocortical projection neuron subtype and area specification have been identified. It is becoming increasingly clear that these regulators function within a highly-interconnected network, acting in parallel, synergistically, and cross-repressively to orchestrate cortical development. Moreover, an emerging understanding of cortical development has revealed that subtype and area identity are intimately interrelated, and that specification occurs based on several sequential molecular decision points.

Although great strides have been made in recent years toward understanding molecular controls over neocortical projection neuron development, many important controls remain to be discovered, and mechanisms by which recently-identified regulators act to delineate subtype and area identity are not well understood.

In this dissertation, I characterize functions of two zinc finger transcription factors, *Ctip2* and *Ctip1*, in postmitotic projection neuron subtype and area identity acquisition, using *in vivo* gain- and loss-of-function approaches in the mouse. I find that *Ctip2*, known for several years as a central functional control over corticospinal motor neuron (CSMN) terminal differentiation and connectivity, is required both cell-

autonomously (within CSMN) and non-cell-autonomously (within striatal medium-sized spiny neurons that surround CSMN axons traveling in the internal capsule) for CSMN to achieve proper connectivity with the spinal cord. In addition, I find that *Ctip1*, a transcription factor not previously functionally investigated in neocortical development, is a novel control over 1) corticothalamic and callosal projection neuron development and projection neuron migration; and 2) postmitotic area identity acquisition and the formation of sensory maps.

Taken together, these results reveal previously unknown functions of *Ctip1* in neocortical development, and novel sites of action for *Ctip2* control over CSMN connectivity. *Ctip1* and *Ctip2* are transcriptional controls over the postmitotic specification of neocortical projection neuron subtype and area identity, and over projection neuron connectivity with distant targets.

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Figures and Tables

Abbreviations

I-VI	neocortical layers I-VI
CC	corpus callosum
CFuPN	corticofugal projection neurons
CGE	caudal ganglionic eminence
COUP-TF	chicken ovalbumin upstream promoter transcription factor
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
DL	deep-layer
dLG	dorsal lateral geniculate nucleus of thalamus
E	embryonic day
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

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To Sheri McClarren

Chapter 1

Introduction

Publication

This chapter is adapted from an invited review in preparation for publication in *Nature Reviews Neuroscience*, to be submitted in December 2012. Some figures are adapted from an invited short review for publication in *Cell*, currently under editorial review.

Custo Greig, L.F.*, Woodworth, M.B.*, Galazo, M.J., Padmanabhan, H., and Macklis, J.D. "Molecular logic of neocortical projection neuron specification and development." *Nat. Rev. Neurosci.*, in preparation.

Woodworth, M.B.*, Custo Greig, L.F.*, Kreigstein, A.R., and Macklis, J.D. "Snapshot: Cortical Development." *Cell*, under review.

*equally-contributing first authors

Author contributions

I planned the review in collaboration with M.D./Ph.D. student Luciano Custo Greig, and we wrote the majority of the text together. Postdoctoral fellows Dr. Maria Galazo and Dr. Hari Padmanabhan will contribute to the completed review, but the text assigned to them has not yet been completed, so I wrote those sections for this thesis chapter. Luciano and I designed and prepared the figures together, with the exception of Figure 1.1, which was prepared in collaboration with Dr. Arnold Kreigstein.

1.1 Overview

The mature neocortex is populated by an extraordinarily diverse complement of cells, including neurons, astroglia, and oligodendroglia. Even considering variety only among neurons, hundreds or thousands of different subtypes are known to exist. In recent years, tremendous progress has been made toward understanding the molecular events that control the development of several important and prototypical types of neocortical neurons.

Neocortical circuits are built from two major classes of neurons, interneurons and projection neurons (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, and then migrate to the neocortex (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 (Molyneaux et al., 2007).

The most biologically meaningful way to classify projection neurons is hodological, or based on the target of their axons (Molyneaux et al., 2007). Projection neurons are first distinguished according to whether they project across the midline to the contralateral cortical hemisphere (callosal projection neurons, CPN) or ipsilaterally, away from the cortex (corticofugal projection neurons, CFuPN). CFuPN are further distinguished by the particular subcortical target of their axons, the thalamus (corticothalamic projection neurons, CThPN) or subcerebral/sub-forebrain structures (subcerebral projection neurons, SCPN). Each subtype encompasses a rich variety of subpopulations, which differ from each other in gene expression, cell morphology, electrophysiological properties, or targets of axon collaterals. In addition, projection

neurons acquire areal identities such that their connectivity and gene expression are partially determined by where in cortex they reside.

Projection neurons of the same subtype residing in different neocortical areas extend axons to anatomically and functionally distinct targets. The murine neocortex is organized into four primary areas (motor, somatosensory, visual, and auditory) defined by cytoarchitecture, connectivity, and patterns of gene expression (Mallamaci and Stoykova, 2006). For example, mature SCPN located in motor cortex send axons to the spinal cord, and are called corticospinal motor neurons (CSMN), while those located in visual cortex send axons to optic tectum, and are called corticotectal projection neurons (CTPN). Subtype identity and area identity are interrelated aspects of the unitary identity of a given projection neuron, and both are specified progressively over time.

Cortical development unfolds along three axes: time, subtype, and area. Most work to date has addressed these aspects separately, providing a descriptive analysis of individual molecular controls acting either in progenitors or in postmitotic neurons. However, it is becoming increasingly clear that molecular controls do not function in isolation, and that specification of subtype and area identity, as well as the timing of specification decisions, are intimately interrelated. In this chapter, I address progressive specification of projection neuron subtype and area identity over time, first by examining molecular programs at work in cortical progenitors, and then those acting as neurons become postmitotic, send axons to distant targets, and refine initially promiscuous patterns of gene expression and connectivity. At each stage, I consider genetic programs that establish boundaries between different projection neuron subtypes, and between adjacent cortical areas. Finally, I review known functions of the

zinc finger transcription factors *Ctip2* and *Ctip1*, the primary subjects of this dissertation, in non-neocortical tissues.

1.2 Early subtype and area specification in progenitors

Neocortical neurons arise from several progenitor zones

Several distinct progenitor zones located in distant regions of the forebrain generate neurons that migrate to and populate the neocortex. The largest group of neurons in the neocortex, projection neurons, is generated by progenitors in the dorsal pallium between approximately E11.5 and E15.5 in the mouse (Molyneaux et al., 2007). Postmitotic neuroblasts migrate radially from the cortical ventricular and subventricular zones into the cortical plate, with the earliest-born projection neurons settling in the deepest layers, and later-born projection neurons migrating past them to occupy progressively more superficial layers (**Figure 1.1**) (Angevine and Sidman, 1961).

A second class, the GABAergic interneurons, is born in rodents from progenitors in the medial and caudal ganglionic eminences of the subpallium (MGE and CGE, respectively) between E12.5 and E15.5. Interestingly, in primates, at least some interneurons are born from dorsal VZ/SVZ progenitors (Jones, 2009), although this does not appear to be the case in rodents. MGE- and CGE-derived postmitotic interneurons migrate tangentially through the ventral forebrain toward the cortex, enter the cortex through the marginal or intermediate zone streams, and then migrate radially to reach their final positions within the cortex (Marín and Rubenstein, 2001). Although it has long been known that interneurons take up residence with projection neurons of approximately the same birthdate, recent evidence suggests that it is the subtype

Figure 1.1 Distinct progenitor populations generate neocortical projection neurons in an “inside-out” fashion

Around E10.5 and before, the telencephalic wall is composed of undifferentiated neuroepithelial (NE) cells, which give rise to later progenitor populations. Radial glia (RG) divide asymmetrically to self-renew and generate either intermediate progenitor (IP) cells or neurons. IP cells divide symmetrically in sequential waves to produce progressively more superficial populations of the six-layered neocortex. In the mouse, small numbers of neurons are produced by outer radial glia (oRG).

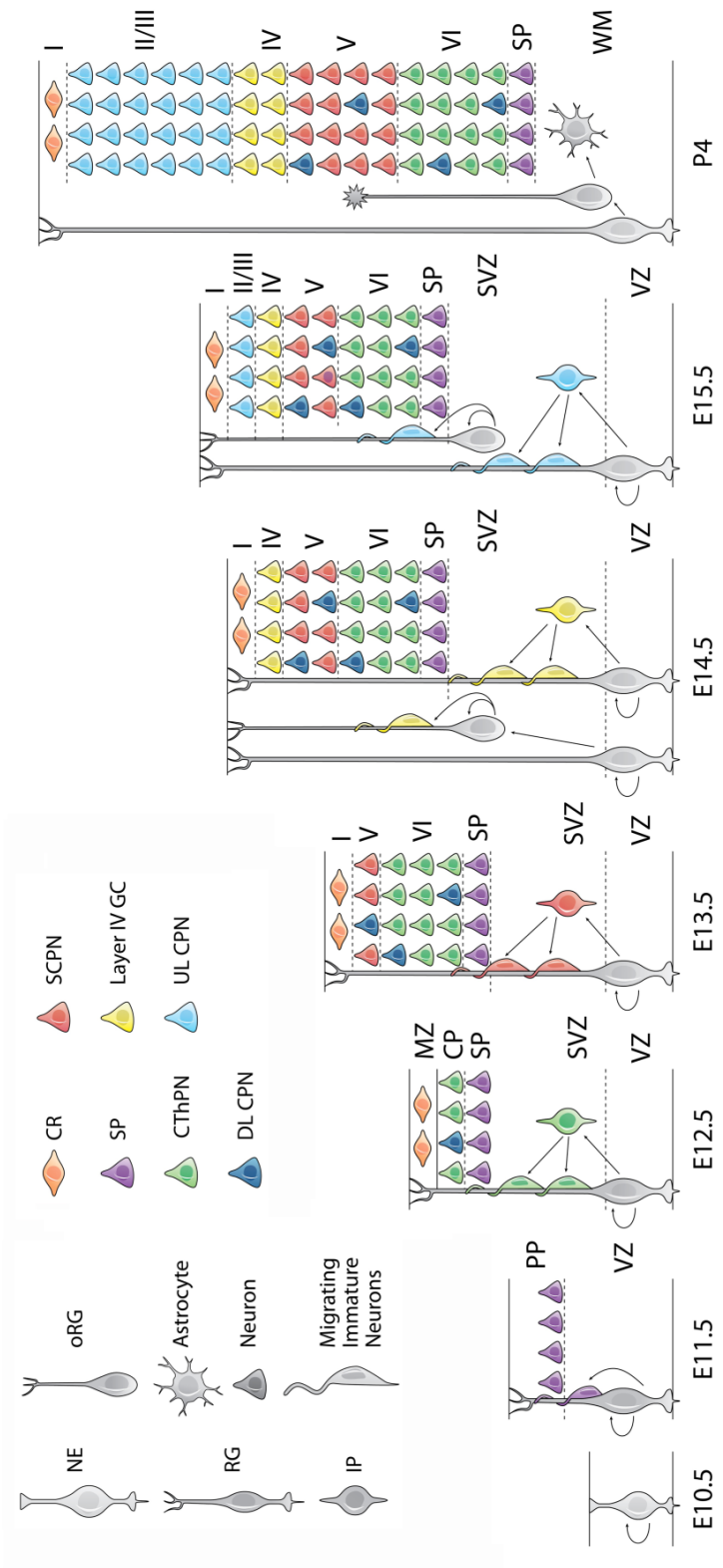

Figure 1.1 (Continued)

identity of projection neurons, rather than their birthdate, that attracts specific subtypes of interneurons to specific cortical layers (Lodato et al. 2011a).

A separate lineage of cortical interneurons, Cajal-Retzius neurons, is born from progenitors in multiple locations at the borders of the pallium: the cortical hem, the ventral pallium, and the septum (Bielle et al., 2005; Yoshida et al. 2006). Cajal-Retzius neurons then migrate into the preplate and compose the eventual layer I. This population has been extensively studied as a major source of the secreted glycoprotein *Reelin*, which is known to be critical for proper cortical lamination (Schiffmann et al., 1997). In addition to Cajal-Retzius neurons, small numbers of neocortical projection neurons may also be generated at the pallial/subpallial boundary (Morante-Oria et al., 2003; Teissier et al., 2010).

Progenitor populations over time

Within the pallium, several zones of progenitors emerge over developmental time (**Figure 1.1**). Early in development, the telencephalic wall is composed entirely of undifferentiated neuroepithelial cells, which give rise to later progenitor populations. Neuroepithelial cells primarily produce radial glia (RG), which establish contacts with both the apical and basal surfaces of the developing cortex, providing a physical scaffold for migration of newborn neurons (Lui et al., 2011). The proliferative zone of the neocortical domain is initially limited to a single layer of RG in the ventricular zone (VZ), but as neurogenesis proceeds, RG produce an additional progenitor class, the intermediate progenitor (IP) cells (Noctor et al., 2004). IP cells reside in the subventricular zone (SVZ), superficial to the VZ (Noctor et al., 2007). In humans, the outer SVZ (OSVZ), which is located superficial to the SVZ, houses the recently-identified outer radial glia (oRG), which make physical contact only with the basal surface of the

cortex (Hansen et al., 2010). A small population of oRG also exists in the rodent cortex, but rodent oRG are thought to be located in the superficial SVZ and intermediate zone, and the existence of a morphologically-identifiable rodent OSVZ is controversial (Wang et al., 2011; Martínez-Cerdeño et al., 2012).

The complex lineage relationships between these progenitor classes and their neuronal and glial progeny are beginning to become clear. As described above, early neuroepithelial cells divide to produce RG. RG then divide asymmetrically to self-renew and to generate transit-amplifying IP cells or neurons (Lui et al., 2011). IP cells, in turn, divide symmetrically to produce two neurons (Noctor et al., 2004). Although IP cells are initially outnumbered by RG, they generate the majority of projection neurons in the cortex throughout neurogenesis (Kowalczyk et al., 2009).

The evolution of additional progenitor populations in mammals has occurred concurrently with an increase in cortical neuron number and in diversity of projection neurons. The SVZ is common to all mammals, but is not present in the sauropsids from which mammals diverged (Cheung et al., 2010). This suggests that the emergence of the SVZ, and with it the ability to produce larger numbers and varieties of cortical neurons, may have enabled the elaboration of the six-layered mammalian neocortex (Martínez-Cerdeño et al., 2006).

Partially fate-restricted progenitors

The competence of progenitors to generate specific projection neuron subtypes becomes progressively limited over the course of development. Classic transplantation experiments demonstrate that early progenitors transplanted into later-stage cortex are capable of producing all subtypes of neurons, but later progenitors transplanted into earlier-stage cortex are only competent to produce upper-layer, *i.e.*, callosal, projection

Figure 1.2 Some transcriptional controls expressed with subtype specificity in postmitotic neurons are also present in progenitors

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).

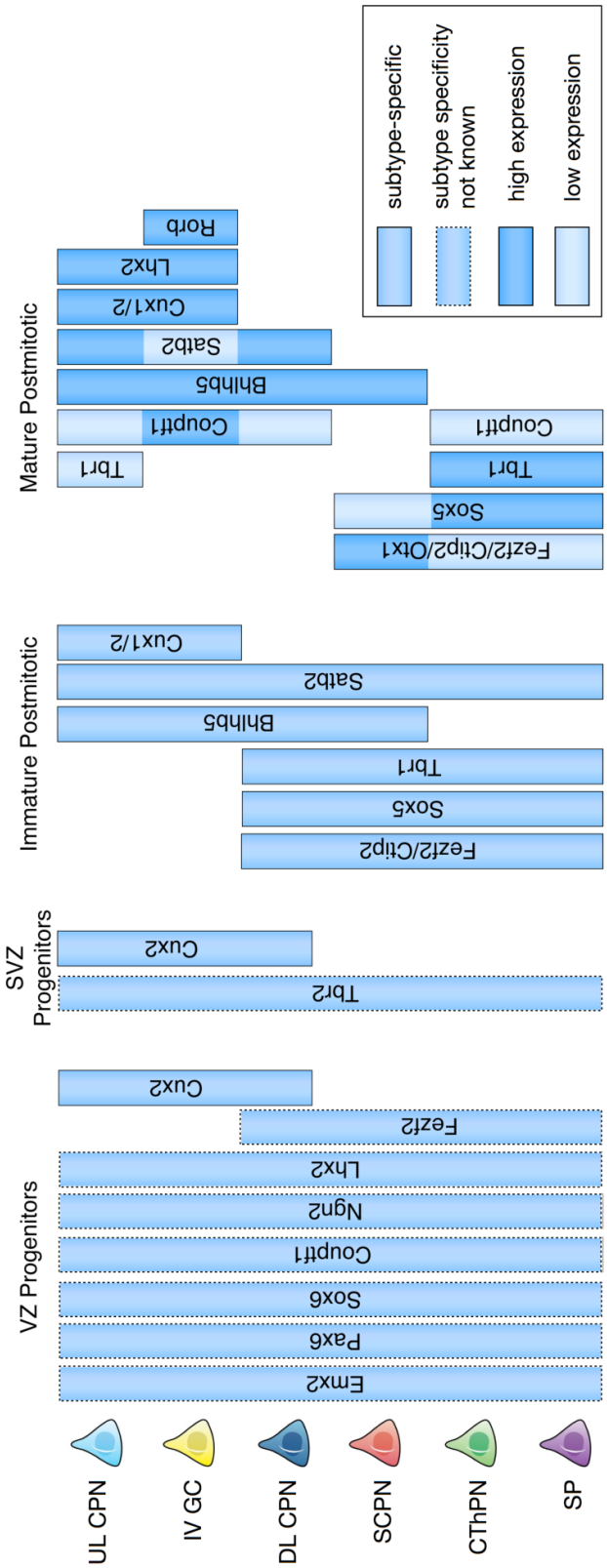

Figure 1.2 (Continued)

neurons (McConnell, 1988). In addition, retroviral lineage tracing experiments show that progeny of sparsely-labeled progenitors can be found in multiple layers after migration (Luskin et al., 1988; Walsh and Cepko, 1988), suggesting that early progenitors are competent to generate projection neurons that adopt all subtype and laminar fates, while late progenitors are competent to generate only upper-layer callosal projection neurons.

Interestingly, a number of genes expressed with postmitotic subtype specificity are also present in subsets of progenitors, suggesting that at least some progenitors may be committed to generating certain projection neuron subtypes (**Figure 1.2**). The transcription factor *Fezf2*, discussed in detail in Section 1.x, centrally controls the development of subcerebral projection neurons (Molyneaux et al., 2005; Chen et al., 2005a, 2005b). *Fezf2* is expressed at low levels in the VZ and SVZ (Molyneaux et al., 2005; Chen et al., 2005b), and its patchy expression in the proliferative zone suggests that *Fezf2* might be expressed in a subset of progenitors fated to produce SCPN. The homeodomain transcription factor *Lhx2* is expressed by neocortical progenitors, and also by upper-layer callosal projection neurons (Nakagawa et al, 1999; Bulchand et al., 2003; Molyneaux et al., 2009), suggesting that *Lhx2* marks progenitors restricted to producing callosal projection neurons. The transcription factors *Cux1* and *Cux2* are also expressed by SVZ progenitors and upper-layer CPN (Nieto et al., 2004; Molyneaux et al., 2009). Recent evidence from fate-mapping experiments suggests that *Cux2* might be a marker for a fate-restricted progenitor population that produces CPN for all layers of neocortex (Franco et al., 2012).

Positional information in progenitors

Neocortical arealization is initiated by expression of morphogens and signaling molecules from patterning centers at the borders of the neocortical primordium. Fibroblast growth factors *Fgf8* and *Fgf17* are secreted rostromedially by the commissural plate beginning at E9.5, and augmentation of either *Fgf* by *in utero* electroporation causes rostromedial areas of cortex to expand caudally (Bachler and Neubüser, 2001; Fukuchi-Shimogori and Grove, 2001); conversely, reduced *Fgf8* levels expressed from a hypomorphic allele cause caudal areas of cortex to expand rostrally (Garel et al., 2003). Caudomedially, *Wnt* and *Bmp* family members are secreted from the cortical hem, and, laterally, *Wnt* antagonist *Sfrp2* and several *Egf* family members are secreted from the antihem, although functions of hem- and antihem-secreted factors in neocortical arealization are not yet clear (Mallamaci and Stoykova, 2006). Hem-expressed genes, however, are critical for establishing boundaries between neocortex and the evolutionarily older paeleo- and archicortices (O'Leary et al., 2007; Molyneaux et al., 2007).

These gradients of diffusible factors induce graded expression of arealization-controlling transcription factors in VZ progenitors. The homeobox transcription factors *Pax6* and *Emx2* are expressed in reciprocal rostrolateral to caudomedial gradients, with *Pax6* expressed most highly rostrolaterally and *Emx2* expressed most highly caudomedially (Walther and Gruss, 1991; Gulisano et al., 1996). *Sp8* and *Couptf1*, in contrast, are expressed in reciprocal rostromedial to caudolateral gradients, with *Sp8* expressed most highly rostromedially and *Couptf1* expressed most highly caudolaterally (Sahara et al., 2007; Zhou et al., 2001). Because of the orthogonal orientation of these two pairs of gradients, expression levels of these four transcription factors can define any set of cortical coordinates (**Figure 1.3**). Therefore, while

Figure 1.3 Transcription factor expression gradients in the VZ and cortical plate evolve into discrete domains that demarcate cortical areas

Area identity is specified in a stepwise fashion, with early overlapping expression of critical controls resolving over the course of development to specific functional areas. Area identity begins to be imparted embryonically by smooth gradients of transcription factors *Pax6*, *Emx2*, *Sp8*, and *Couptf1* in progenitors. Postmitotic neurons inherit these gradients, and expression of critical controls, such as *Lmo4* and *Bhlhb5*, is initially also graded. During the first postnatal week, *Lmo4* and *Bhlhb5*, as well as downstream area-specific genes, become restricted to domains that sharply delineate cortical areas.

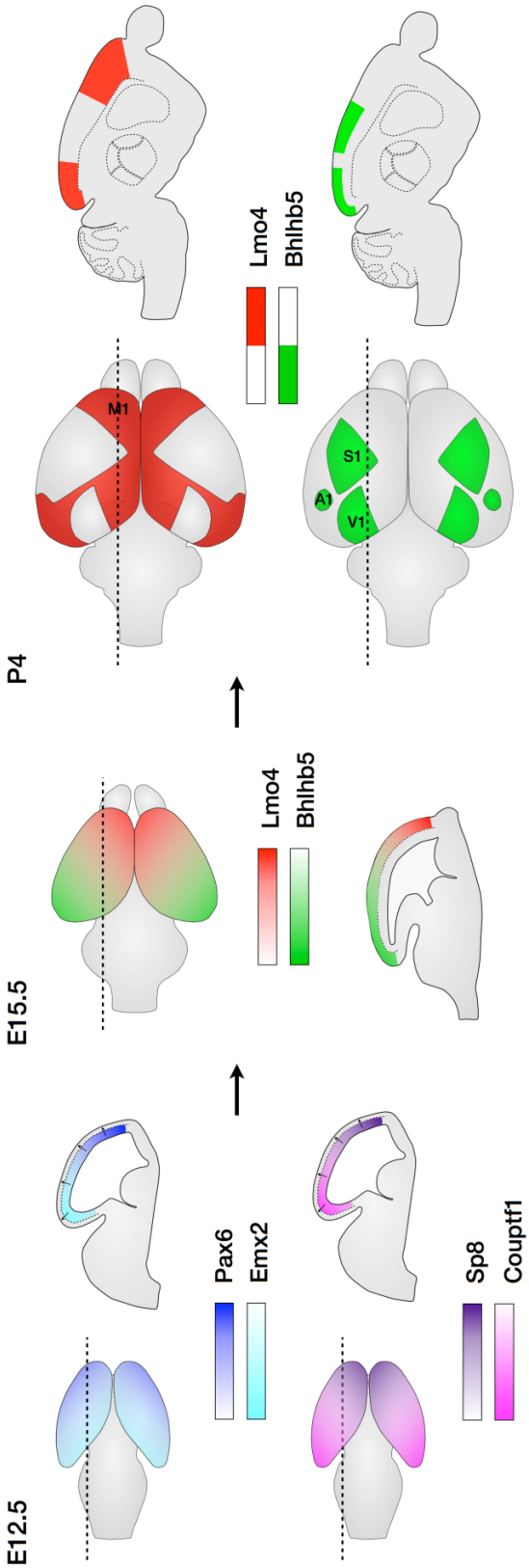

Figure 1.3 (Continued)

additional progenitor-level controls likely remain to be identified, it is clear that each postmitotic projection neuron emerges from the ventricular zone poised to acquire a specific area identity.

Transcription factor gradients in progenitors determine the final position and relative size of cortical areas. In concordance with their strong expression in caudal progenitors, *Emx2* and *Couptf1* promote specification of sensory areas. In *Nestin-Emx2* transgenic mice, in which expression of *Emx2* is driven in all cortical progenitors by the *Nestin* promoter, increased *Emx2* dosage in non-caudal progenitors leads to an increase in the size of visual cortex, and a concomitant decrease in the size of somatosensory and motor areas. In addition, somatosensory cortex is shifted rostrally. In the absence of one allele of *Emx2*, in contrast, motor areas are enlarged, and sensory areas are shifted caudally (Hamasaki et al., 2004). In *Couptf1* cortical conditional null mice, motor areas expand dramatically to occupy a large portion of cortex, while sensory areas are displaced to a narrow occipital band that contains compressed, but properly-configured, sensory representations (Armentano et al., 2007).

Pax6 and *Sp8* are expressed most highly in rostral parts of cortex, and, in accordance with this expression, function to promote specification of motor areas. Both *Sp8* conditional null and *Pax6*$^{sey/sey}$ ("small eye") mutant mice exhibit a drastic loss of motor areas, although interpretation of the *Pax6* findings is complicated by a concomitant decrease in the overall size of cortex (Sahara et al., 2007; Bishop et al., 2000). Gain- and loss-of-function *in utero* electroporation experiments independently support a direct role for *Sp8* in cortical area identity by cell-autonomous function in neocortical progenitors, as well as an indirect role by induction of *Fgf8* (Sahara et al., 2007).

Importantly, in mice misexpressing *Emx2*, *Couptf1*, *Pax6*, or *Sp8*, although the size and position of neocortical areas are shifted, area identity is otherwise established normally. Critically, thalamocortical input is redirected to properly innervate ectopically-located sensory areas, and downstream markers of molecular area identity are expressed appropriately in their new tangential location. Taken together, these results suggest that progenitor-based controls function to establish a broad framework for area identity, but that instructions from progenitors must be translated by independent postmitotic transcriptional controls that execute acquisition of area identity.

1.3 Postmitotic projection neuron subtype specification

Competition between cross-repressive genetic programs progressively establishes the subtype identity of postmitotic projection neurons, enabling the development of subtypes with strikingly different properties, including gene expression and projection patterns (and, not covered here, distinctive dendritic distributions and mature functional electrophysiological properties). These distinct subtypes can be visualized as separate points occupying coordinates in an n-dimensional "subtype space", with temporal and spatial boundaries separating them from each other. SCPN are generated contemporaneously with layer V CPN (Molyneaux et al., 2007), and changes in expression of several identified transcriptional controls can shift SCPN to become CPN, and *vice versa*. Similarly, CThPN and SCPN share a spatial boundary at the interface between layers V and VI, and this boundary is malleable with changes in transcription factor expression. CThPN are born concurrently with layer VI CPN, but genetic controls that delineate these two populations have not yet been reported. As

Figure 1.4 Competition between cross-repressive genetic programs progressively establishes projection neuron subtype identity

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.

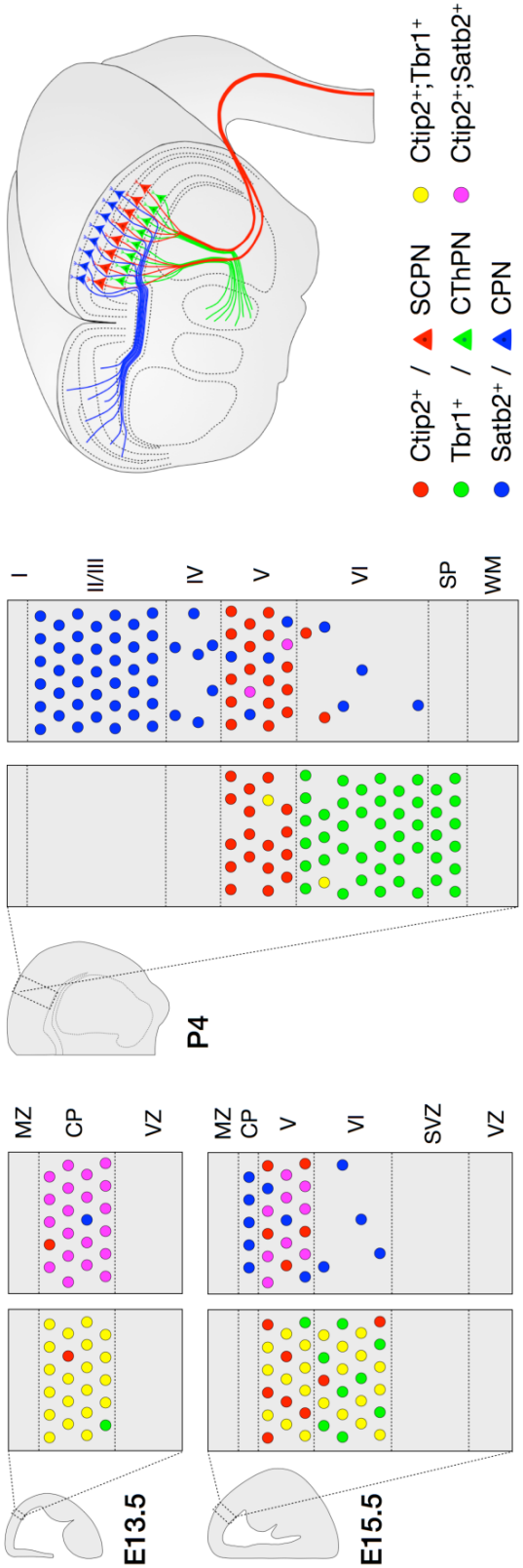

Figure 1.4 (Continued)

new regulators of subtype development are identified, the emerging picture is one of extensive transcriptional cross-repression between genetic programs driving the development of one subtype of projection neuron and those driving the development of alternate subtypes (**Figure 1.4**).

Boundary between SCPN and CThPN

Substantial plasticity exists in the specification of corticofugal projection neurons into either subcerebral projection neurons (SCPN) or corticothalamic projection neurons (CThPN). These populations are evolutionarily ancient and closely related, and are the only long-distance projection neurons in non-mammalian tetrapods (Cheung et al., 2007). Peak birth of CThPN occurs in mice at E12.5, and peak birth of SCPN occurs one day later, but generation of the two populations overlaps substantially; therefore, it is essential for SCPN and CThPN to distinguish themselves from each other. In the absence of critical controls that establish the boundary between the two subtypes, SCPN can expand as a population at the expense of CThPN, and *vice versa*.

The zinc finger transcription factor *Fezf2* (also known as *Fezl*/*Zfp312*) is a central regulator of SCPN identity. *Fezf2* is expressed by at least a subset of progenitors in the ventricular zone, and SCPN express high levels of *Fezf2* from embryonic development through adulthood (Arlotta et al., 2005; Özdinler et al., 2010). In the absence of *Fezf2* function, neurons in layer V fail to express SCPN-specific genes and to acquire pyramidal projection neuron morphology, and none project to the brainstem and spinal cord (Molyneaux et al., 2005; Chen et al., 2005a). Instead, more E13.5-born neurons in layer V express the corticothalamic control TBR1 and project to the thalamus (Molyneaux et al., 2005; McKenna et al., 2011), indicating that some SCPN are fate-

converted to CThPN in the absence of *Fezf2*. (Other SCPN in these mice are fate-converted to CPN, as discussed below.)

In CThPN, the T-box transcription factor *Tbr1* acts as a critical control. *Tbr1* was originally identified due to its strong layer VI-specific expression pattern (Bulfone et al., 1995), and, in the absence of *Tbr1*, neurons in layer VI fail to connect with the thalamus (Hevner et al., 2001). Instead, layer VI neurons express aberrantly high levels of *Fezf2* and CTIP2, and send axons to the brainstem and spinal cord. A major function of *Tbr1*, therefore, is to repress *Fezf2* expression in CThPN by directly binding to the *Fezf2* locus (McKenna et al., 2011; Han et al., 2011). Intriguingly, in $Tbr1^{-/-}$;$Fezf2^{-/-}$ double mutants, formation of the corticospinal tract is partially rescued, although projections to the thalamus are still completely absent (McKenna et al., 2011). A wide variety of genes are misregulated in $Tbr1^{-/-}$ cortex (Bedogni et al., 2010), and it is highly likely that *Tbr1* functions to control CThPN development through other means in addition to repressing *Fezf2*.

Timing of specification decisions in CFuPN

The SRY box transcription factor *Sox5* controls the emergence of corticofugal projection neuron subtypes by repressing expression of SCPN genes, including *Fezf2* and *Ctip2,* until generation of subplate neurons and CThPN is complete. In $Sox5^{-/-}$ mice, subplate neurons inappropriately project to the cerebral peduncle and take an abnormal laminar position in superficial layers of cortex, due to inappropriate activation of subcerebral molecular programs, including high expression of CTIP2 (Lai et al., 2008). In addition, CThPN and SCPN differentiate imprecisely, perhaps because *Sox5* normally directly represses *Fezf2* (Lai et al., 2008; Kwan et al., 2008; Shim et al., 2012). Expression of FOG2 and CTIP2, normally specific to CThPN and SCPN, respectively,

fails to resolve into distinct populations. Instead, FOG2 and CTIP2 are expressed by a single population of mixed SCPN/CThPN character (Kwan et al., 2008). Loss of *Sox5* results in a range of corticofugal projection abnormalities, including formation of an accessory subcerebral tract projecting through the external capsule, extensive defasciculation in the midbrain, and strikingly few axons reaching the pons and spinal cord (Lai et al., 2008). Projections to the thalamus are also compromised, especially as reported by the *Fezf2*-GFP and *Golli*-GFP transgenes (Kwan et al., 2008).

Couptf1 further controls the timing of CFuPN emergence. In the absence of *Couptf1*, somatosensory cortex neurons in layer VI express aberrantly high levels of CTIP2 and *Fezf2*, with extensive co-expression of CTIP2 and TBR1. More neurons in deep layers send axons subcerebrally, but, intriguingly, only the ectopic SCPN in layer VI are able to project to the spinal cord; axons of genuine SCPN located in layer V terminate in pons before entering the spinal cord (Tomassy et al., 2010). *Couptf1*, then, suppresses SCPN gene expression and projections in the latest-born, most superficially-located CThPN.

Boundary in "subtype space" between SCPN and CPN

Subcerebral projection neurons (SCPN) share a developmental "subtype space" boundary with callosal projection neurons (CPN), and especially with deep-layer CPN, which are generated during the same temporal window, and which reside intermingled with SCPN in layer V. From the time that axons of SCPN and CPN exit the cortical plate, they follow dramatically divergent trajectories, either away from cortex or toward the midline (Koester and O'Leary, 1993; Arlotta et al., 2005). Accordingly, high-level controls identified over SCPN and CPN development appear to function in large part by

repressing molecular programs that would instruct differentiation toward the alternate fate.

As described above, *Fezf2* functions centrally to specify SCPN identity, which requires suppression of deep-layer CPN fate. *Fezf2* overexpression *in vivo* is sufficient to redirect the axons of upper-layer CPN toward subcerebral targets (Molyneaux et al., 2005; Chen et al., 2008). In the absence of *Fezf2*, SCPN are never specified (Molyneaux et al., 2005; Chen et al., 2005a). Instead, neurons expressing an alkaline phosphatase reporter gene from the *Fezf2* locus extend axons across the corpus callosum, display electrophysiological characteristics typical of CPN, and express CPN-specific genes, suggesting that they have been fate-converted to CPN (Lodato et al., 2011a; Chen et al., 2008). Interestingly, these neurons appear to take on the identity of deep-layer CPN in particular, as they express broad CPN identity genes, such as *Satb2* and *Lpl*, but do not express genes specific to upper-layer CPN, such as *Inhba* and *Limch1* (Lodato et al., 2011a; Molyneaux et al., 2009).

The transcription factor *Satb2* is critical for CPN specification and concomitant repression of SCPN fate. *Satb2* is expressed at high levels by CPN (and likely also by associative neurons) in all layers of cortex (Alcamo et al., 2008; Britanova et al., 2008). In the absence of *Satb2*, although establishment of the midline is normal, almost no axons pass through the corpus callosum. Instead, neurons expressing a *LacZ* reporter allele from the *Satb2* locus project toward the brainstem and spinal cord. *Satb2* directly represses transcription of *Ctip2*, and, in the absence of *Satb2* function, upper-layer neurons ectopically express *Ctip2* as well as a number of other genes characteristic of SCPN, including *Clim1*, *Cdh13*, and *Grb14*. Conversely, expression of several genes characteristic of CPN, including *Cdh10*, *Dkk3*, and *Cux1*, is lost or severely reduced, suggesting partial fate conversion. These neurons do not appear to be completely fate-

converted, however, as they fail to express *Fezf2*. Recently, the transcriptional co-regulator *Ski* has been shown to be a critical component of the repressor complex recruited by *Satb2* to initiate HDAC1-dependent chromatin remodeling, and *Ski* loss-of-function mutants have abundant subcerebral projections at the expense of callosal projections, similar to *Satb2*$^{-/-}$ mice (Baranek et al., 2011).

Callosal projection neuron diversity

CPN are the largest class of projection neurons in the cortex, and the phylogenetic relationships between CPN of different subclasses are unclear. Most CPN reside in layer II/III, although smaller populations, born concurrently with neighboring deep-layer subtypes, are found in layers V and VI. CPN located in different layers, and even in different sublaminae, have remarkably distinctive patterns of gene expression from embryonic development onward (Molyneaux et al, 2009), consistent with the hypothesis that CPN in different layers might have been independently derived from existing projection neuron populations (Fame et al., 2010). However, recent fate-mapping evidence suggests that CPN in all layers might be born from lineage-restricted *Cux2*-positive progenitors (Franco et al., 2012). Further work is necessary to clarify evolutionary relationships between diverse types of CPN, and between CPN and other cortical projection neurons.

Progressive refinement of subtype identity

Mature deep-layer projection neurons are starkly different from each other in terms of projection patterns and gene expression, but these differences begin to emerge only after several days of postmitotic development. Newly-postmigratory projection neurons often co-express high levels of transcription factors that are later

restricted to only one subtype (**Figure 1.4**). For example, at E14.5, neurons in the cortical plate co-express CTIP2 and TBR1 or FOG2, which are later restricted to SCPN and CThPN, respectively (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, respectively, by E15.5 (Alcamo et al., 2008; Britanova et al., 2008). Some pairs of controls, such as *Clim1* in SCPN and *Lmo4* in CPN, do not become restricted to one subtype until after mice are born (Azim et al., 2009). This initially overlapping expression of transcriptional controls followed by later refinement is consistent with the hypothesis that postmitotic projection neurons are not finally fate-specified until one transcriptional program becomes more highly expressed than others, perhaps due to stochastic fluctuations in gene expression combined with exclusionary cross-repression of individual critical controls, and represses transcription of alternate programs. However, circumstances that favor the initial expression of one transcriptional program over another are not known.

1.4 Postmitotic projection neuron areal specialization

Little is known about the postmitotic regulators that transform continuous progenitor expression gradients into sharp areal boundaries and direct projection neurons to acquire areally-appropriate phenotypic characteristics. The postmitotic molecular controls over arealization that have been identified act by establishing boundaries that define primary sensory areas and non-primary sensory areas (including secondary sensory areas and motor areas).

Lmo4 is a Lim homeobox domain transcription factor expressed postmitotically in motor cortex and higher-order visual areas, but excluded from somatosensory and primary visual cortex. Loss of *Lmo4* function results in rostral expansion of gene expression patterns specific to somatosensory cortex genes, and a modest narrowing of vibrissal barrel fields (Huang et al., 2009), but no associated changes in projection patterns have yet been reported. Interestingly, *Lmo4* is expressed differentially between right and left hemispheres of human embryonic brains, and might be involved in left-right asymmetry between cortical hemispheres in humans (Sun et al., 2005).

The expression of the basic helix-loop-helix transcription factor *Bhlhb5* is essentially exactly complementary to that of *Lmo4*, with high expression restricted to somatosensory and primary visual cortex. *Bhlhb5* can first be detected in postmigratory cortical neurons in a gradient, from high expression caudomedially to low expression rostrolaterally, and it becomes refined to be specifically expressed in somatosensory and primary visual cortex by P4. In the absence of *Bhlhb5* function, molecular area identity is extensively disrupted in somatosensory and caudal motor cortex, and area-specific genes, including *Lmo4*, are aberrantly expressed. The position and configuration of the barrel field are unchanged, although thalamocortical input appears diffuse, and cytoarchitectural organization of vibrissal barrels is only faintly discernible (Joshi et al., 2008). *Bhlhb5*, therefore, is a transcription factor highly expressed in primary sensory cortex that centrally contributes to organization of hallmark somatosensory cortex-specific features, including vibrissal barrel fields and specific downstream gene expression.

Tbr1, discussed above as a critical control over corticothalamic projection neuron subtype identity, is expressed most highly in rostral areas of cortex, and regulates area identity in addition to its functions in subtype identity specification. In the

absence of *Tbr1*, genes typically expressed in caudal regions of cortex expand rostrally; *Bhlhb5* expression expands into motor cortex, and *Lmo4* expression expands into somatosensory cortex. Abnormalities in gene expression are not limited to CThPN or to layer VI, and can be observed as early as E14.5 (Bedogni et al., 2010). These data suggest that *Tbr1* is required for appropriate development of area identity before deep-layer projection neuron identity has been fully resolved (see Section 1.x). Thus, area identity and subtype identity are controlled, established, and refined in a parallel, but interdependent, manner.

Couptf1 is a critical transcriptional control over arealization of progenitors in the ventricular zone (see Section 1.x), and continues to be expressed in postmitotic neurons into adulthood (Tomassy et al., 2010; Lodato et al., 2011b). In concordance with its high-caudolateral to low-rostromedial expression in progenitors, *Couptf1* is expressed most highly in somatosensory and visual cortex in postnatal animals, and is largely excluded from motor cortex (Tomassy et al., 2010). Although *Couptf1* has been confirmed to act postmitotically in regulating migration of upper-layer callosal projection neurons (Alfano et al., 2011), it remains unclear whether *Couptf1* acts to regulate the development of area-specific gene expression and projection patterns by functions in progenitors only that are maintained postmitotically, or whether it has additional functions that emerge in postmitotic neurons.

A number of genes without known functions in area identity specification are nonetheless expressed in an area-specific fashion in postmitotic cortical neurons. Some are transcription factors, including *Rorb*, expressed highly in somatosensory and auditory cortex (Nakagawa and O'Leary, 2003); *Bcl6*, expressed in motor and somatosensory cortex, but excluded from visual cortex (Leamy et al., 2008); and *Id2*, expressed caudally in visual cortex and at lower levels rostrally (Neuman et al., 1993;

Rubenstein et al., 1999). Several members of the cadherin family are expressed in complex area-specific patterns. *Cdh8* is expressed at high levels in higher-order visual areas and motor cortex, and lower levels in primary visual cortex (Suzuki et al., 1997; Assimacopoulos et al., 2012), while *Cdh6* is expressed highly in somatosensory cortex and visual cortex (Suzuki et al., 1997). The transmembrane proteins *Mdga1* (Takeuchi et al., 2007) and *Efna5* (Dufour et al., 2003) are expressed specifically in somatosensory cortex. *Epha7* is specifically excluded from somatosensory cortex (Rubenstein et al., 1999), and *Odz3* is expressed in visual cortex (Leamy et al., 2008). Although these genes are likely downstream of major transcriptional controls over area identity acquisition, they are useful markers of specific areas of cortex.

In addition, serotonin (5-HT) immunolabeling in cortex is specific for thalamocortical afferents, which cluster in somatosensory, primary visual, and primary auditory cortex (Bennet-Clarke et al., 1993). In somatosensory cortex, 5-HT immunoreactivity delineates the rodent body map, and, in particular, the map of the vibrissae, discussed below.

The vibrissal barrel map in somatosensory cortex

One important and highly reproducible feature of somatosensory cortex in rodents is the vibrissal barrel map, a somatotopic representation of the individual vibrissae as arrayed on the snout (Woolsey and Van der Loos, 1970). Barrels form in cortex over the course of the first postnatal week, as input from thalamocortical afferents and layer IV cortical neurons organize to form cell-dense barrel walls surrounding the afferent-containing barrel hollows (López-Bendito and Molnár, 2003). The barrel map is plastic, and early ablation of one or more whiskers leads to the loss of their representations in cortex (Fox, 2008).

The barrel map is formed partly intrinsically to cortex, as a result of cortex-autonomous area identity specification programs, and partly extrinsically, as a result of interactions between cortical layer IV neurons and incoming thalamocortical afferent fibers. Just as changes in area induced by misexpression of progenitor area controls can change the location of cortical areas, they can also shift the size and position of the barrel map; in *Couptf1* conditional null mice, for example, motor cortex expands dramatically, and somatosensory cortex is confined to a small caudal region. This displaced somatosensory cortex is otherwise normally specified, and the barrel field, though shifted and reduced in size, is correctly patterned (Armentano et al., 2007). Further, overexpression of *Fgf8* at the caudal pole of cortex induces the development of an additional, duplicate barrel map that attracts thalamocortical input, and even responds to sensory deprivation (Assimacopoulos et al, 2012). Thalamocortical input is itself a major determinant of barrel map organization, and thalamocortical afferents begin to cluster in somatosensory cortex prior to reorganization of layer IV neurons into barrels (O'Leary et al., 1994). A number of mutants lacking barrels in somatosensory cortex have been described, including several related to synaptic function and plasticity, indicating a function for neuronal activity in the organization of barrels by thalamocortical afferents (Wu et al., 2011).

Area-specific regulation of projection neuron subtype distribution

Different areas of cortex are distinct from each other cytoarchitectonically, a feature identified histologically by early neuroanatomists (Brodmann, 1909). These cytoarchitectonic differences are related to the different proportions of projection neuron subtypes resident in different functional areas of cortex. For example, motor cortex contains an expanded layer V with many more subcerebral projection neurons

Figure 1.5 Projection neurons of the same subtype located in different cortical areas send axons to distinct targets

Projection neurons can be classified on the basis of their mature axonal projections. Corticothalamic projection neurons (CThPN) (A) are located in layer VI and send axons to thalamus; subcerebral projection neurons (SCPN) (B) are located in layer V and send axons to optic tectum, brainstem, or spinal cord; and callosal projection neurons (CPN) (C) are located in layers II/III, V, and VI and send axons through the corpus callosum (CC) to contralateral cortex. Importantly, neurons of each subtype are further specialized based on their positions in specific cortical areas. CThPN establish area-specific connections with thalamic nuclei (sensory cortex CThPN with VP; visual cortex CThPN with dLG) (A). SCPN in motor cortex project to the spinal cord (SC), while those in visual cortex project to the optic tectum (OT) (B). CPN establish mirror-image connections with CPN located in the same functional area in the contralateral hemisphere (C).

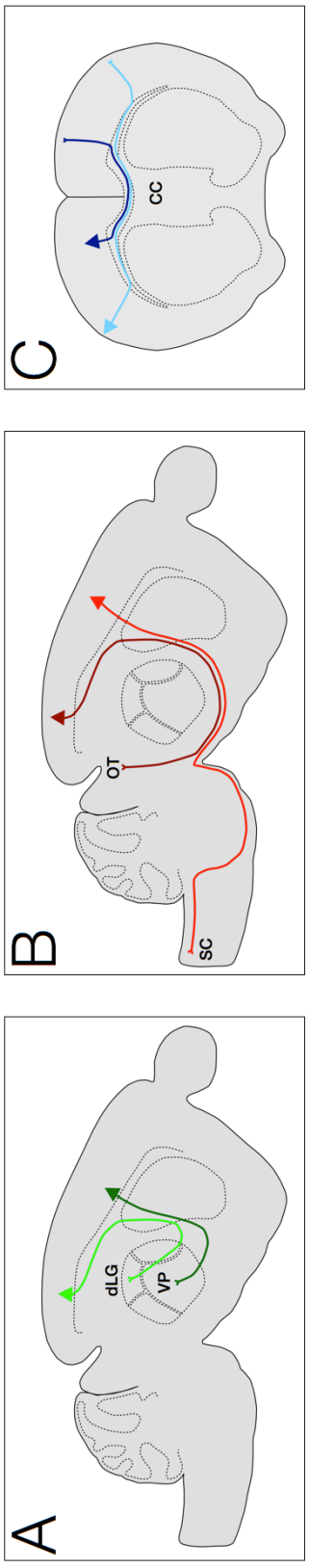

Figure 1.5 (Continued)

than are present in other areas, while layer IV is substantially reduced in size (Kandel et al., 2000). Conversely, in somatosensory and visual cortex, more callosal projection neurons are produced and maintained than in motor cortex (Aboitiz and Montiel, 2003). These differences allow the specialization of functional areas, enabling motor cortex to control output to the spinal cord, and sensory areas to process incoming modality-specific sensory information, and to connect with contralateral cortex. The mechanisms by which different areas produce varied complements of projection neuron subtypes are not well-understood.

Progressive refinement of area-specific projection patterns

Projection neurons of the same subtype that reside in different cortical areas send axons to distinct targets (**Figure 1.5**), and these area-specific projection patterns are refined over the first postnatal weeks.

Subcerebral projection neurons (SCPN) send axons from layer V of cortex to targets in the brainstem and spinal cord. In adult mice, corticospinal motor neurons (CSMN), the SCPN located in motor cortex, are the only projection neurons to maintain a connection with the spinal cord, while corticotectal projection neurons (CTPN), the SCPN located in visual cortex, are specialized to maintain a connection with the optic tectum. However, early in SCPN development, virtually all SCPN first project to the spinal cord, then extend collaterals to optic tectum and pons (O'Leary and Stanfield, 1985; O'Leary and Terashima, 1988). These projections are stabilized or eliminated according to the final area identity of the SCPN, and pruning is complete by approximately P21 (Bates and Killackey, 1984; O'Leary and Koester, 1993). Function of *Otx1*, a homeodomain transcription factor expressed by SCPN throughout cortex, is necessary for visual cortex SCPN to prune their spinal projection (Weimann et al.,

1999). In addition, *Ctip2* regulates SCPN pruning in a dose-dependent manner, as SCPN in somatosensory cortex fail to prune their spinal projections in *Ctip2*$^{+/-}$ mice (Arlotta et al., 2005).

Since callosal projection neurons (CPN) send axons to mirror-image locations on the contralateral hemisphere (and subsets of CPN send additional distributive projections ipsilaterally and/or contralaterally), callosal connectivity is necessarily area-specific. Like SCPN, CPN initially send promiscuous collaterals, then eliminate most collaterals during the first postnatal weeks. Early in development, retrograde labeling from the contralateral hemisphere reveals an even distribution of callosally-projecting neurons, but, by P21, only CPN located in certain patches of cortex, often at the borders of functional areas, can be labeled (Ivy et al., 1979). This final pattern is determined primarily through activity-dependent collateral elimination rather than by cell death, and can be perturbed by altering cortical activity (O'Leary et al., 1981; Luo and O'Leary, 2005).

Unlike SCPN or CPN, corticothalamic projection neurons (CThPN) do not initially project widely and then refine projections during the first postnatal weeks. CThPN make connections with thalamic nuclei in an area-specific manner, with motor cortex CThPN innervating motor nuclei (VL; ventrolateral nucleus), somatosensory cortex CThPN innervating sensory nuclei (VB and VP; ventrobasal nucleus and ventroposterior nucleus), and visual cortex CThPN innervating visual nuclei (LGN; lateral geniculate nucleus) (Grant et al., 2012). CThPN project with specificity very early in their development (O'Leary et al., 2007), potentially because CThPN make contact with modality-segregated thalamocortical projection neurons as they grow into the internal capsule, and might use thalamocortical projections to guide them to appropriate nuclei (Molnár and Cordery, 1999). Indeed, matching between *Eph* receptors on

thalamocortical axons and *ephrins* in cortex mediates the precision of thalamocortical innervation (Dufour et al., 2003), and might contribute to control over thalamocortical-corticothalamic axon matching. Early alterations in neocortical area identity are sufficient to change the topography of thalamocortical and corticothalamic projections; in *Couptf1* conditional null mice, for example, motor identity is dramatically expanded caudally, and somatosensory cortex CThPN adopt a motor CThPN identity and project to VL. In turn, thalamocortical neurons in VP project to ectopic areas that have adopted somatosensory identity (Armentano et al., 2007).

1.5 Functions of *Ctip1* and *Ctip2*

Ctip1 and *Ctip2* are highly-conserved C_2H_2 zinc finger transcription factors that function developmentally in many systems. While *Ctip2* is an important functional control over development of subcerebral projection neurons (Arlotta et al., 2005; Molyneaux et al., 2005; Lai et al., 2008) and striatal medium-sized spiny neurons (Arlotta et al., 2008), *Ctip1* has not previously been described to function in forebrain development.

Ctip1 and *Ctip2* were identified almost simultaneously by three groups investigating different biological systems, and using different methods. First, both genes were identified as interacting partners of COUP transcription factors in a yeast two-hybrid screen using a segment of *Couptf2* as bait (Avram et al., 2000). Soon after, *Ctip1* (called *Evi9*) was identified as a gene frequently targeted by activating retroviral insertion in murine leukemia (Nakamura et al., 2000). Later, *Ctip1* (called *Bcl11a*) was identified as the gene disrupted by a chromosomal translocation common in human B-

cell malignancies, and *Ctip2* (called *Bcl11b*) was discovered by its high conservation with *Ctip1* (Satterwhite et al., 2001). Both transcription factors were found to be sequence-specific DNA-binding proteins able to regulate gene expression even in the absence of *Couptf1* or *Couptf2* (Avram et al., 2002).

Ctip1 and *Ctip2* have been most fully investigated in the hematopoietic system, in which the two transcription factors have cross-repressive, as well as independent, functions. Consistent with overexpression of *Ctip1* being an activating mutation in leukemia, *Ctip1* is necessary for B cell development. In the absence of *Ctip1*, no B cells are produced (Liu et al., 2003). *Ctip1* is sharply and specifically down-regulated as pro-T cells become committed to a T cell fate (Tydell et al., 2007), and mice transplanted with *Ctip1*$^{-/-}$ fetal liver cells develop T cell leukemia (Liu et al., 2003). Intriguingly, *Ctip2* is required for appropriate T cell development (Wakabayashi et al., 2003), even from the earliest stages of T cell lineage commitment, and *Ctip2* upregulation is coincident with downregulation of *Ctip1* in T cell precursors (Li et al., 2010). These data suggest that T cell leukemia in *Ctip1*$^{-/-}$ might be caused by upregulation of *Ctip2*.

Ctip1 is also functionally important for the development of erythrocytes, and mediates switching between fetal and adult forms of hemoglobin. *Ctip1* was identified in genome-wide association studies as a locus linked to increased fetal hemoglobin levels in adults (Menzel et al., 2007). The single-nucleotide polymorphism identified at the *Ctip1* locus in human populations decreases expression of *Ctip1* in erythroblasts 3.5-fold, and these diminished levels of *Ctip1* permit the continued expression of fetal gamma-globin in adult humans and mice (Sankaran et al., 2008; Sankaran et al., 2009). To silence gamma-globin, *Ctip1* acts together with *Sox6*, a transcription factor also critical for neocortical development (Xu et al., 2010; Azim et al., 2009). Strikingly, reducing levels of *Ctip1* in a mouse model of sickle cell disease is sufficient to

ameliorate disease symptoms by re-activating transcription of fetal hemoglobin (Xu et al., 2011).

Ctip1 is known to be expressed in several regions of the developing nervous system, including cortex, hippocampus, striatum, cerebellum, and spinal cord (Leid et al., 2004; Kuo and Hsueh, 2007), but no investigation of its functions in cortical development has yet been published. *Ctip1* has been reported to interact with the serine kinase *Cask* and the neurogenesis regulator *Tlx* based on yeast two-hybrid screens (Kuo and Hsueh, 2010; Estruch et al., 2012), but these interactions are of questionable physiological relevance, since *Ctip1* appears to be primarily expressed in nuclei of postmitotic neurons (Chapter 3), not in the postsynaptic density, as would be required for interaction with *Cask*, nor the ventricular zone, as would be required for interaction with *Tlx*. In this dissertation, I demonstrate that *Ctip1* is a critical control over the development of both neocortical projection neuron subtype and area identity.

Ctip2 has been identified as a functional control over several discrete populations of neurons. In the absence of *Ctip2*, subcerebral projection neurons in neocortex migrate appropriately to layer V, but do not send axons to brainstem and spinal cord targets (Arlotta et al., 2005). Similarly, vomeronasal sensory neurons (VSN) are born in the absence of *Ctip2*, but fail to express mature VSN genes, or to send axons to the accessory olfactory bulb (Enomoto et al., 2011). *Ctip2* null hippocampal neurons do not integrate into dentate gyrus, leading to learning and memory defects (Simon et al., 2012). In the striatum and globus pallidus, *Ctip2* is expressed at high levels by medium-sized spiny neurons (MSN), and *Ctip2* null MSN do not develop characteristic patch-matrix striatal architecture or express MSN-characteristic genes (Arlotta et al., 2008). Intriguingly, reduced *Ctip2* expression in MSN has been implicated as a potential cause of Huntington's disease symptoms (Desplats et al., 2008). In

addition, *Ctip2* is expressed by and controls the development of other specific ectoderm-derived populations, including skin (Golonzhka et al., 2007; Golonzhka et al., 2009a; Zhang et al., 2012), teeth (Golonzhka et al., 2009b; Kyrylkova et al., 2012), and cochlear hair cells (Okumura et al., 2011).

1.6 Dissertation overview

In this dissertation, I characterize functions of the zinc finger transcription factors *Ctip2* and *Ctip1* in controlling the development of neocortical projection neuron identity and connectivity. I find that *Ctip2* controls corticospinal motor neuron axon extension, pathfinding, and fasciculation both CSMN-autonomously and non-CSMN-autonomously. I identify *Ctip1* as a novel transcriptional control over both cortical projection neuron subtype and area identity specification.

In Chapter 2, I report that *Ctip2*, known for several years as a marker of corticospinal motor neurons and a control over CSMN axon pathfinding and fasciculation (Arlotta et al., 2005), functions both within CSMN (CSMN-autonomously) and in striatal medium-sized spiny neurons (non-CSMN-autonomously) to guide CSMN axons to the spinal cord. Using cortical (*Emx1-Cre*) and striatal (*Gsx2-Cre*) conditional null mice, I show that *Ctip2* acts CSMN-autonomously to guide axons into the internal capsule, but non-CSMN-autonomously to cause CSMN axons to fasciculate and pathfind within the internal capsule. Caudal to striatum, *Ctip2* acts CSMN-autonomously to direct CSMN axon guidance and pathfinding to the spinal cord.

In Chapter 3, I describe functions of *Ctip1* in cortical projection neuron subtype development. *Ctip1*, a close paralog of *Ctip2*, has never been functionally investigated

in the brain. I find that *Ctip1* is expressed by corticothalamic and callosal projection neurons in all layers of cortex, but that *Ctip1* is progressively excluded over embryonic development from subcerebral projection neurons. In the absence of *Ctip1*, CThPN and deep-layer CPN differentiate aberrantly, and more deep-layer neurons adopt characteristics of SCPN. In addition, *Ctip1* controls the development of CThPN and CPN pioneer populations, and CThPN and CPN projections are consequently abnormal in *Ctip1* mutants. *Ctip1* overexpression *in vivo* suppresses SCPN gene expression and projection to the spinal cord, and causes electroporated neurons to project instead to the thalamus and across the corpus callosum.

In Chapter 4, I characterize *Ctip1* as a novel control over neocortical area identity acquisition and the development of sensory maps. *Ctip1* is highly expressed in all cortical areas during embryonic development, but expression later refines to sensory areas. In *Ctip1* conditional null mice, progenitors correctly specify area identity, but subsequent areal refinement fails to occur, and sharp gene expression boundaries between areas do not develop. Projection neurons lacking *Ctip1* do not acquire projection patterns dependent on appropriate area identity, including precise mirror-image targeting by CPN and pruning of spinal collaterals by SCPN in somatosensory and visual cortex. Further, in the absence of *Ctip1*, layer IV neurons in somatosensory cortex never aggregate into barrels, and thalamocortical input is disorganized and diffuse.

In Chapter 5, I conclude with a discussion of the impact of *Ctip1* and *Ctip2* on cortical projection neuron postmitotic development. I discuss the cross-repressive relationship between these two transcription factors, and how they act independently at different stages of projection neuron specification to direct the precision of cortical development. I describe how further knowledge of conserved controls over projection

neuron specification and differentiation can advance our understanding of the evolution of the brain, and inform future development of strategies to repair the damaged or degenerating nervous system.

Chapter 2

Cell-autonomous and non-cell-autonomous functions of *Ctip2* in corticospinal motor neuron development

Publication

This chapter is in the process of being prepared for manuscript submission in spring 2013. Experiments are ongoing.

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Author contributions

This project was initiated independently by me, and the experimental work was performed in collaboration with M.D./Ph.D. student Luciano Custo Greig. The majority of the experimental work and figure preparation was completed by Luciano and me together. Kevin Liu, an undergraduate working with me on his senior honors thesis, performed the analysis on *Ctip2* downstream targets in striatal medium-sized spiny neurons. Ryo Kominami of the Niigata University and his postdoctoral fellow Yoshinori Katsuragi made the *Ctip2*$^{fl/fl}$ mouse line.

2.1 Abstract

COUP-TF interacting protein 2 (*Ctip2*) has increasingly emerged as a central and critical control hub for neocortical projection neuron development, and, in particular, for the development of corticospinal motor neurons (CSMN). Little is known about the mechanisms by which it functions. In the absence of *Ctip2*, CSMN axons are misrouted and defasciculated, and never reach the spinal cord (Arlotta et al., 2005). Because medium-sized spiny neurons (MSN) in the striatum also require *Ctip2* function (Arlotta et al., 2008), and because CSMN axons are surrounded by MSN as they travel through striatum, we hypothesized that defective CSMN connectivity in *Ctip2*$^{-/-}$ mice might result from misregulation of axon growth and guidance controls normally provided by MSN. By examining mice conditionally null for *Ctip2* in either the cortex (*Ctip2*$^{fl/fl}$*;Emx1-Cre*) or the striatum (*Ctip2*$^{fl/fl}$*;Gsx2-Cre*), we find that *Ctip2* functions in CSMN development both within CSMN themselves, but also non-CSMN-autonomously, through its functions in MSN. *Ctip2* is required in CSMN for axon outgrowth from the cortex, for pathfinding in the midbrain and hindbrain, and for connectivity with the spinal cord, but not for fasciculation within the internal capsule. CSMN fasciculation within the internal capsule is achieved via *Ctip2* function in MSN, and is non-CSMN-autonomous. Together, these data indicate that *Ctip2* functions in multiple independent neuron populations to control CSMN axon guidance and connectivity with the spinal cord.

2.2 Introduction

Corticospinal motor neurons (CSMN) are a particularly important population of projection neurons, as they selectively degenerate in amyotrophic lateral sclerosis (ALS) and other “upper motor neuron” diseases, and are damaged in spinal cord injury, leading to devastating neurological deficits. In ALS, both CSMN and spinal motor neurons degenerate irreversibly, while in primary lateral sclerosis (PLS) and hereditary spastic paraplegia (HSP), CSMN uniquely degenerate (Fink, 2003; Özdinler et al., 2011). In spinal cord injury, loss of motor function results centrally from damage to the axons of the corticospinal tract. Because CSMN, once degenerated or axonally disrupted, do not regain function, ALS and other motor neuron diseases are currently incurable, and current treatments for spinal cord injury are critically limited.

Although CSMN morphology and function have been extensively studied, specific controls over CSMN specification and differentiation have been examined only relatively recently. One key transcription factor specifically expressed by CSMN beginning in progenitors, *Fezf2* (also known as *Fezl*), is necessary and sufficient for CSMN specification within developing cortex (Molyneaux et al., 2005; Chen et al., 2005a, 2005b). In the absence of *Fezf2*, CSMN are not specified, while overexpression of *Fezf2* generates neurons that extend CSMN-like axons toward subcerebral targets (Molyneaux et al., 2005; Chen et al., 2008). A number of CSMN-specific genes are not expressed in the absence of *Fezf2*, including COUP-TF interacting protein 2 (*Ctip2*) (Molyneaux et al., 2005; McKenna et al., 2010). *Ctip2* itself is a central regulator of CSMN differentiation, and multiple genetic controls over neocortical projection neuron differentiation operate at least in part by regulating *Ctip2* expression. *Sox5* controls

sequential generation of subcortical projection neuron types, and in *Sox5*$^{-/-}$ mice, temporally inappropriate expression of CTIP2 causes subplate and corticothalamic projection neuron (CThPN) axons to be aberrantly redirected toward subcerebral targets (Lai et al., 2008). In callosal projection neurons (CPN), the transcription factor SATB2 represses transcription at the *Ctip2* locus, and in *Satb2*$^{-/-}$ mice, upper-layer neurons express CTIP2 and extend axons subcerebrally, instead of across the corpus callosum (Alcamo et al., 2008; Britanova et al., 2008). *Ctip2* is positioned as a critical regulator of neocortical projection neuron differentiation and connectivity.

Ctip2 was first identified as an interacting partner of chicken ovalbumin upstream promoter transcription factors (COUP-TFs), which are themselves important regulators of nervous system development (Avram et al., 2000; Zhou et al., 1999; Tripodi et al., 2004; Tomassy et al., 2010). CTIP2, also known as B-cell leukemia/lymphoma 11b (BCL11B), is a C_2H_2 zinc finger transcription factor shown to act as both a repressor and an activator of transcription (Cismasiu et al., 2005; Cismasiu et al., 2006). Although CTIP2 was identified by its association with COUP-TFs, it can also regulate transcription independently as a sequence-specific DNA-binding protein (Avram et al., 2002). *Ctip2* functions centrally during development of several body systems, and has been investigated primarily for its role in the lymphoid system. *Ctip2* is a critical regulator of T cell development, and a known tumor suppressor (Wakabayashi et al., 2003a, 2003b). Pro-T cells sharply and specifically upregulate *Ctip2* when they commit to the T cell state, and *Ctip2*$^{-/-}$ mice are unable to produce mature T cells (Li et al., 2010; Tydell et al., 2007; Wakabayashi et al., 2003). *Ctip2* also controls differentiation of ameloblasts from oral ectoderm and of keratinocytes from basal cells of the epidermis (Golonzhka et al., 2007; Golonzhka et al., 2009a, 2009b). In the nervous system, *Ctip2* is critical for development of vomeronasal sensory neurons

and dentate gyrus granule neurons (Enomoto et al., 2011; Simon et al., 2012) in addition to its functions in corticospinal motor neurons and striatal medium-sized spiny neurons (Arlotta et al., 2005; Arlotta et al., 2008).

CTIP2 functions centrally in controlling appropriate differentiation of CSMN. In the absence of CTIP2, CSMN are born, express CSMN control genes such as *Fezf2*, and migrate to layer V, but they exhibit remarkable defects in axon pathfinding, fasciculation, and outgrowth (Arlotta et al., 2005). CSMN axons normally fasciculate in the internal capsule, the myelinated forebrain tract containing axons that project from cortex to subcortical targets. Strikingly, in *Ctip2*$^{-/-}$ mice, descending axons become misrouted and defasciculated as they pass through the forebrain. While wild-type axons efficiently project to the spinal cord, *Ctip2*$^{-/-}$ CSMN extend axons to ectopic targets in the forebrain and midbrain. These axons are clearly dysmorphic, and often possess bulbous structures suggestive of dysfunctional growth cones (Tom et al., 2004). Most critically, CSMN axons in *Ctip2*$^{-/-}$ mice do not successfully reach the spinal cord; those projections that exit the forebrain mostly terminate in the midbrain, with only rare axons reaching pons, and none reaching the pyramidal decussation. *Ctip2* is also expressed at high levels by medium-sized spiny neurons (MSN) in the striatum, and *Ctip2*$^{-/-}$ MSN lose their characteristic patch-matrix organization and mis-express key axon guidance molecules, attracting ectopic clusters of cells from outside the striatum (Arlotta et al., 2008). Because MSN surround CSMN axons as they penetrate through the internal capsule, and might provide important guidance cues to CSMN, and because the pallial-subpallial boundary (the embryonic border between cortex and striatum) is an important decision point for axons traveling through the internal capsule (Bloom et al., 2007), we reasoned that *Ctip2* might also contribute to CSMN development non-CSMN-autonomously via functions in MSN.

Using cortex-specific (*Ctip2$^{fl/fl}$;Emx1-Cre*) and striatum-specific (*Ctip2$^{fl/fl}$;Gsx2-Cre*) conditional mutant mice, we show here that *Ctip2* functions in CSMN development both CSMN-autonomously and CSMN-non-autonomously (via functions in MSN). *Ctip2* is necessary within CSMN themselves for axon outgrowth from the cortex, for pathfinding in the midbrain and hindbrain, and for connectivity with the spinal cord, but not for fasciculation within the internal capsule. CSMN fasciculation within the internal capsule is achieved via *Ctip2* function in MSN, and is non-CSMN-autonomous.

2.3 Results

Emx1-Cre efficiently excises *Ctip2* from neocortical neurons, but spares MSN

In order to delete *Ctip2* specifically from glutamatergic cortical projection neurons, we have crossed a loxP-flanked (floxed) *Ctip2* line (gift of Ryo Kominami, Niigata University) with an *Emx1-Cre* line (Gorski et al., 2002). These *Emx1-Cre;Ctip2$^{fl/fl}$* and *Emx1-Cre;Ctip2$^{fl/-}$* ("floxed over null") mice are born in expected Mendelian ratios, and survive until adulthood, unlike *Ctip2$^{-/-}$* mice, which invariably die at P0 (Wakabayashi et al., 2003a; Arlotta et al., 2005). Neither *Emx1-Cre;Ctip2$^{fl/fl}$* nor *Emx1-Cre;Ctip2$^{fl/-}$* tissue contains detectable levels of CTIP2 in layer V pyramidal neurons, even when subjected to citric acid antigen retrieval and examined at single-cell resolution on a confocal microscope; in the same sections, CTIP2 expression is preserved in MSN, and at low levels in cortical interneurons, both of which are derived from the subpallium (Figure 2.1).

Gsx2-Cre excises *Ctip2* from MSN

To directly examine MSN-specific effects on CSMN axon outgrowth, we have crossed the same *Ctip2*$^{fl/fl}$ line with *Gsx2-Cre* (gene formerly called *Gsh2*; gift of Nicoletta Kessaris, University College London) (Kessaris et al., 2006; Zhou et al., 2008). *Gsx2* is expressed by MSN and other neurons derived from progenitors in the lateral and medial ganglionic eminences (Hsieh-Li et al., 1995; Corbin et al., 2000). When *Gsx2-Cre* is crossed with a *Rosa26R-LacZ*$^{fl/fl}$ reporter line (Soriano, 1999), no CTIP2-positive pyramidal neurons express β-galactosidase. MGE-derived interneurons, identified by immunocytochemistry for parvalbumin, somatostatin, and neuropeptide Y (Wonders and Anderson, 1997), are the only β-galactosidase-positive neurons in cortex. *Gsx2-Cre;Ctip2*$^{fl/fl}$ tissue contains normal levels of CTIP2 in cortex, but expression of CTIP2 is abolished in striatum (Figure 2.2). *Gsx2-Cre;Ctip2*$^{fl/fl}$ striatum shows reduced expression of MSN marker DARPP-32, as reported for *Ctip2*$^{-/-}$ striatum (Arlotta et al., 2008).

Some CSMN axons conditionally null for *Ctip2* successfully fasciculate in the internal capsule

To compare abnormalities in axon tract morphology and cellular organization due to *Ctip2* status in CSMN and MSN, we performed Nissl stains on P0 tissue from wild-type, *Ctip2*$^{fl/fl}$*;Emx1-Cre*, *Ctip2*$^{fl/fl}$*;Gsx2-Cre*, *Ctip2*$^{fl/fl}$*;Emx1-Cre*;*Gsx2-Cre* ("double conditional"), and *Ctip2*$^{-/-}$ mice (Figure 2.3). Projections from CSMN lacking *Ctip2* appear disorganized within the cortex, evident from the lack of a well-defined border

Figure 2.1 *Emx1-Cre* excises *Ctip2* from cortex, and *Gsx2-Cre* excises *Ctip2* from striatum

Wild-type P0 layer V/VI CTIP2 expression (A-B'') is absent in *Ctip2$^{fl/fl}$;Emx1-Cre* forebrains (E-F''), but CTIP2 expression is maintained in MSN (C, G). Wild-type P0 MSN CTIP2 expression (A, C, C') is lost in *Ctip2$^{fl/fl}$;Gsx2-Cre* forebrains (I, K, K'), but cortical expression in maintained (J-J'). DARPP-32 is expressed normally in *Ctip2$^{fl/fl}$;Emx1-Cre* (H), but expression is lost in *Ctip2$^{fl/fl}$;Gsx2-Cre* (L).

Figure 2.1 (Continued)

Figure 2.2 *Gsx2-Cre* is expressed by neurons derived from the lateral and medial ganglionic eminiences

In P0 *Rosa26R-LacZ$^{fl/+}$;Gsx2-Cre* forebrains, β-galactosidase is detected in striatum by LacZ stain (A). In P14 *Rosa26R-LacZ$^{fl/+}$;Gsx2-Cre* cortex, β-galactosidase co-localizes with markers of subpallially-derived interneurons (arrowheads in B, C, D).

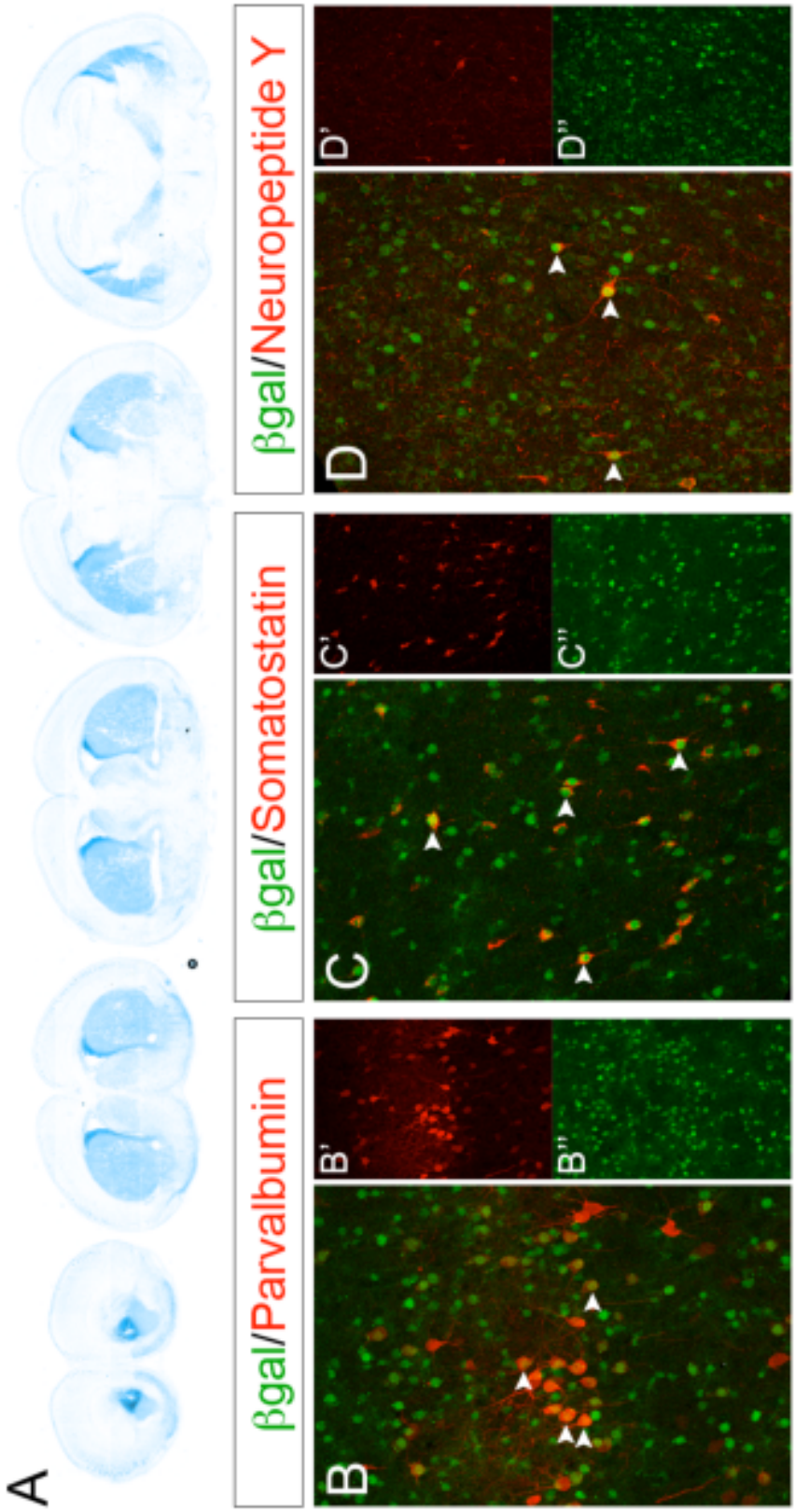

Figure 2.2 (Continued)

between subplate and cortical white matter in *Ctip2$^{fl/fl}$;Emx1-Cre*, *Ctip2$^{fl/fl}$;Emx1-Cre*;*Gsx2-Cre*, and *Ctip2$^{-/-}$* forebrains (arrows in Figure). Possibly in part as a result of this disorganization, axon fascicles passing through the dorsolateral part of the striatum, where lateral CSMN axons enter the internal capsule, are smaller and fewer in *Ctip2$^{fl/fl}$;Emx1-Cre* brains than in wild-type brains or *Ctip2$^{fl/fl}$;Gsx2-Cre* brains, and more axons pass through the external capsule (Figure 2.3).

However, in striking contrast with *Ctip2$^{-/-}$* CSMN axons, some axons from *Ctip2$^{fl/fl}$;Emx1-Cre* CSMN fasciculate successfully in the internal capsule; those fascicles passing through the ventromedial striatum, representing projections from medial CSMN, closely resemble wild-type fascicles (Figure 2.3). These projections are disturbed in *Ctip2$^{-/-}$* and *Ctip2$^{fl/fl}$;Gsx2-Cre* forebrains, but, in *Ctip2$^{fl/fl}$;Emx1-Cre* forebrain, those CSMN axons that successfully penetrate striatum are partially normalized by CTIP2 expression in MSN surrounding the internal capsule.

CSMN lacking *Ctip2* defasciculate in peduncle, and few enter pons

To analyze subcerebral projection neuron axon extension toward hindbrain and spinal cord, we placed crystals of DiI in sensorimotor cortex of P0 wild-type, *Ctip2$^{fl/fl}$;Emx1-Cre*, *Ctip2$^{fl/fl}$;Gsx2-Cre*, and *Ctip2$^{fl/fl}$;Emx1-Cre*;*Gsx2-Cre* fixed brain tissue, and we find that pathfinding and fasciculation at the midbrain-hindbrain junction is controlled by *Ctip2* expression in CSMN. Although those *Ctip2$^{fl/fl}$;Emx1-Cre* axons that penetrate the cortical white matter successfully fasciculate within the internal capsule (Figure 2.4), we find that in more caudal regions, where corticotectal axons turn away from the cerebral peduncle to enter the lenticular fascicle, axons from both

Ctip2$^{fl/fl}$*;Emx1-Cre* and *Ctip2*$^{fl/fl}$*;Emx1-Cre*;*Gsx2-Cre* cortex fail to turn properly. In contrast, the subset of *Ctip2*$^{fl/fl}$*;Gsx2-Cre* axons that have successfully passed the internal capsule do pathfind appropriately in the midbrain. Importantly, CSMN axons are dramatically defasciculated in the cerebral peduncle in *Ctip2*$^{fl/fl}$*;Emx1-Cre* tissue, and few axons, mostly defasciculated, enter the pons, in contrast to mostly normal-looking *Ctip2*$^{fl/fl}$*;Gsx2-Cre* axon trajectories through the cerebral peduncle and pons. *Ctip2*$^{fl/fl}$*;Emx1-Cre*;*Gsx2-Cre* pathfinding and fasciculation are more perturbed than in either *Ctip2*$^{fl/fl}$*;Emx1-Cre* or *Ctip2*$^{fl/fl}$;*Gsx2-Cre*, indicating that both sites of *Ctip2* function are important for CSMN axon extension to the hindbrain and spinal cord.

A subpopulation of CSMN conditionally null for *Ctip2* reach the spinal cord

Although ectopic expression of CTIP2 is sufficient to direct some callosal projection neuron axons into the internal capsule (Chen et al., 2008), and although several recently-identified molecular controls over neocortical projection neuron development act at least in part by suppressing CTIP2 expression by non-CSMN (Lai et al., 2008; Alcamo et al., 2008; Britanova et al., 2008; Tomassy et al., 2010), CTIP2 expression by CSMN is not necessary for a subset of CSMN axons to reach the spinal cord.

To investigate whether *Ctip2*$^{fl/fl}$*;Emx1-Cre* CSMN axons reach the spinal cord, we retrogradely labeled CSMN from cervical spinal cord at P2 using pressure-injected Alexa fluorophore-conjugated cholera toxin B (CTB). Some *Ctip2*$^{fl/fl}$*;Emx1-Cre* CSMN axons do indeed reach the spinal cord, but many fewer cell bodies are labeled in *Ctip2*$^{fl/fl}$*;Emx1-Cre* cortex than in wild-type (43±8%; Figure). *Ctip2*$^{fl/fl}$*;Emx1-Cre* are not merely delayed in reaching the spinal cord, as successful spinal cord connections by

Figure 2.3 *Ctip2*$^{fl/fl}$*;Emx1-Cre* CSMN axons fasciculate within the internal capsule at P0

CSMN axons lacking *Ctip2* form normal fascicles through ventromedial striatum (A-B, A'-B', F-G), but fasciculation is disturbed in brains lacking *Ctip2* in striatum (C-E, C'-E'). Brains lacking *Ctip2* in cortex show an abnormal border between subplate and cortical white matter (arrows in A-E), which will be investigated further with more precise methods.

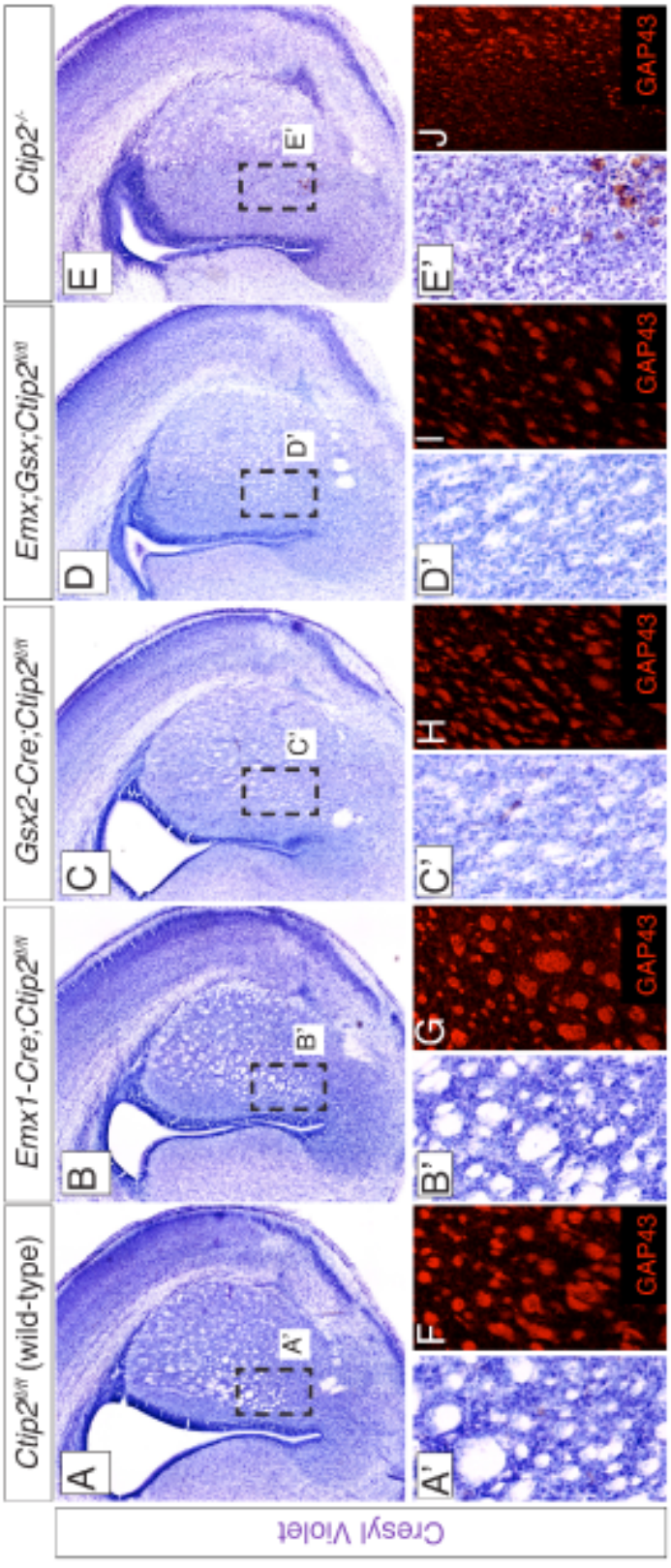

Figure 2.3 (Continued)

Figure 2.4 CSMN lacking *Ctip2* pathfind abnormally and defasciculate in cerebral peduncle

Ctip2$^{fl/fl}$*;Emx1-Cre* CSMN fail to travel in a tight fascicle in the midbrain (brackets in A-D), and corticotectal axons do not turn precisely to enter the lenticular fascicle (arrows in A-D). In contrast, *Ctip2*$^{fl/fl}$*;Gsx2-Cre* CSMN turn appropriately and are properly fasciculated. Few CSMN axons lacking *Ctip2* project successfully into pons (B, D), compared with axons from CSMN with intact Ctip2 expression (A, C). *Ctip2*$^{fl/fl}$*;Emx1-Cre;Gsx2-Cre* mutant projections are more severely affected than either single mutant.

Figure 2.4 (Continued)

CSMN are also reduced in number and areal distribution when retrograde labeling is performed at P21 (Figure 2.5). In agreement with the histological appearance of the internal capsule, a consistently larger percentage of *Ctip2$^{fl/fl}$;Emx1-Cre* CSMN located medially successfully project to the spinal cord, compared with those located laterally. This medio-lateral difference might be due to greater opportunity for medial CSMN axons to penetrate the internal capsule as they project laterally, their standard initial trajectory, or to spatially restricted compensation by other regulators

Taken together, these results indicate that, while CTIP2 expression is important in CSMN themselves for their axons to penetrate the internal capsule, those axons that successfully penetrate the internal capsule (whether stochastically or by expression of a functional substitute for CTIP2) can fasciculate and project to the spinal cord in the environment of a CTIP2-expressing striatum.

PlxnD1 **and** ***Efnb3*** **are potential** ***Ctip2*** **targets of in MSN that affect CSMN axon guidance**

Ctip2$^{-/-}$ MSN fail to form the characteristic patch-matrix cytoarchitecture of the striatum, and their expression of many genes typical to striatum is perturbed (Arlotta et al., 2008). We hypothesize that defasciculation of *Ctip2$^{-/-}$* and *Ctip2$^{fl/fl}$;Gsx2-Cre* CSMN axons might result in part from dysregulated expression of axon guidance and/or cytoarchitectural organizing molecules by MSN, which surround CSMN axons in the internal capsule.

To identify axon guidance molecules dysregulated in MSN lacking *Ctip2*, we utilized existing data generated from a microarray comparison between wild-type and *Ctip2$^{-/-}$*

striatum at P0 (Arlotta et al., 2008). Because 90–95% of the neurons within the striatum are CTIP2-expressing MSN, this microarray comparison was conducted using microdissected regions of striatum at matched locations in wild-type and *Ctip2*$^{-/-}$ littermates, without additional purification of MSN. To select candidates for further study, we identified genes with significant changes in expression between wild-type and *Ctip2*$^{-/-}$ striatum, and which are known or suspected to be located in the membrane or extracellular compartments, and therefore might function to guide CSMN axons passing through the internal capsule. We compiled a list of 29 gene candidates chosen for their potential function as axon guidance molecules based on their expression profiles and gene ontology (Table 1).

Using these criteria, we selected two top candidate genes, *Efnb3* and *PlxnD1*. These genes were selected due to their known functions in axon guidance (Bergemann et al., 1998; Chauvet et al., 2007), and abnormal expression of both genes in *Gsx2-Cre;Ctip2*$^{fl/fl}$ striatum was confirmed by qPCR and ISH (Figure). Expression of *Efnb3*, normally very low in MSN, is increased approximately two-fold in *Ctip2*$^{-/-}$ striatum, while normally robust expression of *PlxnD1* is decreased approximately two-fold. We hypothesize that aberrant expression of *Efnb3* and *PlxnD1* affects CSMN fasciculation and pathfinding in the internal capsule.

Efnb3 is known to be a strong negative guidance cue for CSMN, preventing them from re-crossing the midline once they have entered spinal cord (Kullander et al., 2001; Yokoyama et al., 2001). We hypothesize, therefore, that increased expression of *Efnb3* in *Gsx2-Cre;Ctip2*$^{fl/fl}$ MSN might cause CSMN axons to defasciculate or have otherwise aberrant pathfinding through the internal capsule. In contrast, *PlxnD1* is expressed highly in wild-type striatum, and plexin-semaphorin-neuropilin signaling is known to affect corticospinal tract development in the cerebral peduncle and spinal

Figure 2.5 A subpopulation of CSMN conditionally null for *Ctip2* reach the spinal cord

Fewer *Ctip2*$^{fl/fl}$*;Emx1-Cre* cortical neurons are retrogradely labeled from the spinal cord at either P2 (A, B) or P21 (C, D). This difference is more pronounced laterally (A'', B''; C', D')

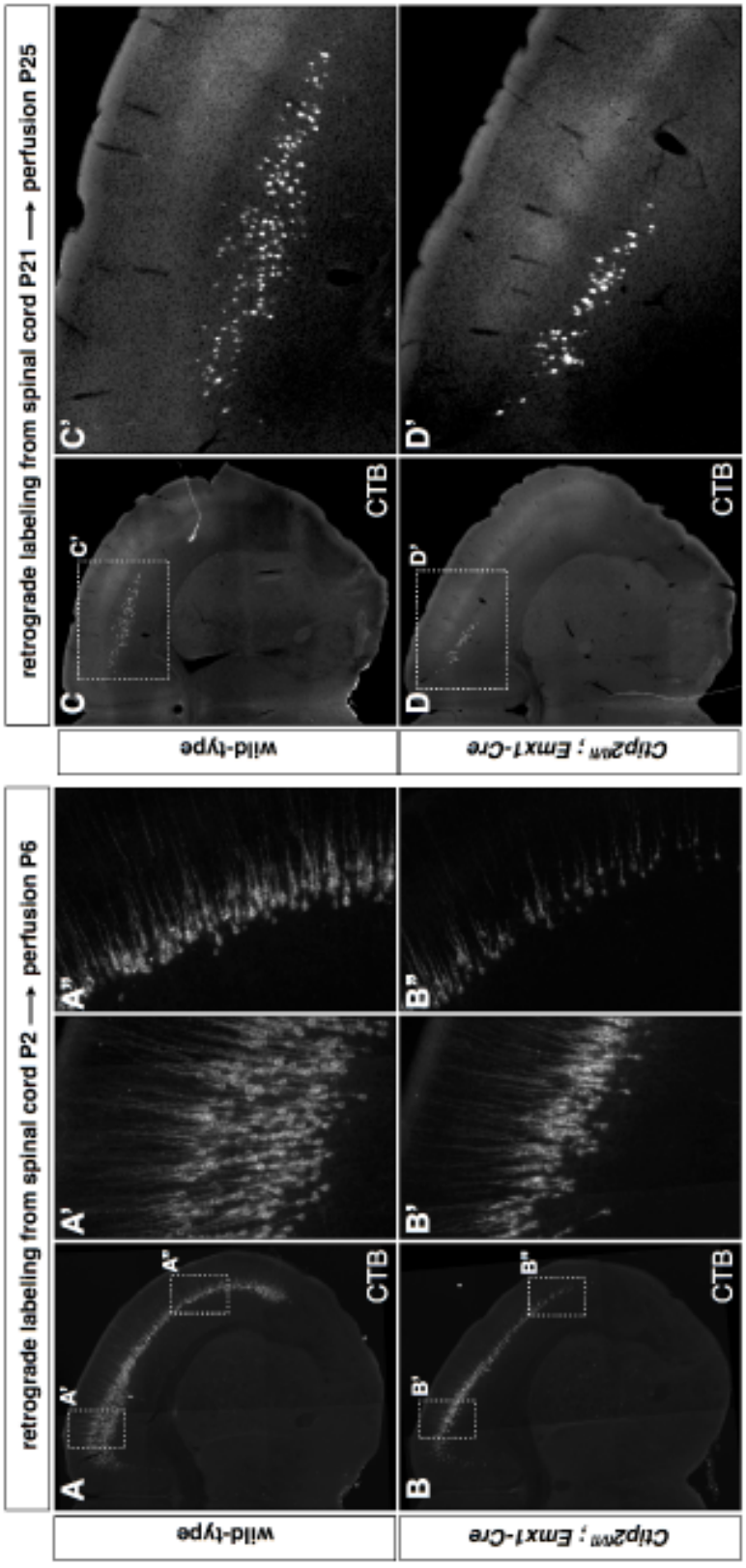

Figure 2.5 (Continued)

Gene Name	Accession	Fold Change	P-value	Full Name/Description	Gene Ontology			
					A	N	E	M
Adora2a	BB748999	-2.12	1.1×10^{-4}	Adenosine A2a receptor		✓	✓	✓
Cdh8	NM_007667	1.41	1.1×10^{-4}	Cadherin 8			✓	✓
Chrm1	BE947522	-3.07	9.9×10^{-8}	Cholinergic receptor, muscarinic 1				✓
Cxadr	BE824924	1.61	9.2×10^{-6}	Coxsackie virus and adenovirus receptor			✓	✓
Dcc	BE824778	2.33	1.5×10^{-6}	Deleted in colorectal carcinoma	✓	✓	✓	✓
Drd1a	BB282271	-2.89	3.5×10^{-6}	Dopamine receptor D1A				✓
Dscam	NM_031174	2.84	7.2×10^{-11}	Down syndrome cell adhesion molecule	✓			✓
Efnb3	BQ175608	2.53	2.0×10^{-39}	Ephrin B3	✓		✓	✓
Gabra1	BQ268470	1.90	3.2×10^{-3}	Gamma-aminobutyric acid (GABA) A receptor, subunit alpha 1			✓	✓
Gda	AW911807	-2.41	1.7×10^{-6}	Guanine deaminase			✓	
Gng7	BM114283	-4.45	1.5×10^{-16}	Guanine nucleotide binding protein (G protein), gamma 7				✓
Gpr88	BE947345	-1.95	1.1×10^{-3}	G-protein coupled receptor 88				✓
Kcnip2	AW490636	-6.80	1.7×10^{-11}	Kv channel-interacting protein 2				✓
Lpl	BC003305	-2.29	8.0×10^{-5}	Lipoprotein lipase			✓	✓
Neto1	AV346211	-2.46	7.6×10^{-30}	Neuropilin (NRP) and tolloid (TLL)-like 1				✓
Nexn	BM225804	-11.75	2.9×10^{-37}	Nexilin				✓
Olfm1	D78264	3.05	6.6×10^{-37}	Olfactomedin 1			✓	✓
Pde10a	AK014090	-4.38	8.8×10^{-10}	Phosphodiesterase 10A				✓
Plxnd1	BC019530	-7.78	2.6×10^{-16}	Plexin D1				✓
Rab3c	AY026947	-2.01	1.0×10^{-5}	RAB3C, member RAS oncogene family				✓
Rasgrp2	BE688720	-3.43	3.9×10^{-13}	RAS, guanyl releasing protein 2				✓
Reln	NM_011261	-4.10	5.4×10^{-14}	Reelin	✓		✓	
Resp18	NM_009049	3.72	1.0×10^{-5}	Regulated endocrine-specific protein 18			✓	
Serpine2	NM_009255	-2.34	1.1×10^{-23}	Serine (or cysteine) peptidase inhibitor, clade E, member 2		✓	✓	✓
Sez6	D29763	9.51	4.0×10^{-5}	Seizure related gene 6			✓	✓
Synpr	BC026512	-2.40	5.4×10^{-6}	Synaptoporin			✓	✓
Tgfa	M92420	-3.14	9.3×10^{-14}	Transforming growth factor alpha			✓	✓
Thbs3	NM_013691	-2.61	2.7×10^{-9}	Thrombospondin 3			✓	
Vldlr	BB127955	-3.11	2.0×10^{-5}	Very low density lipoprotein receptor			✓	✓

A: Biological processes including axon guidance or axon extension; **N**: Biological processes including neuron projection morphogenesis or neuron projection development; **E**: Cellular components including extracellular region, extracellular space, or extracellular matrix; **M**: Cellular components including membrane, integral to membrane, plasma membrane, or integral to plasma membrane

Table 2.1 Loss of *Ctip2* function in striatum causes misregulation of candidate genes involved in axon guidance

Using a combination of statistical, expression-based, and ontology-based criteria (see Experimental Procedures), we identified a selected group of high-priority candidate genes for further analysis as *Ctip2* downstream targets in MSN. Fold change indicates the magnitude of change between wild-type and *Ctip2*$^{-/-}$. Gene ontology categories are reproduced from the Gene Ontology Project.

cord (Chauvet et al., 2007; Faulkner et al., 2008). We hypothesize that reduced *PlxnD1* expression in MSN might remove a necessary guidance cue for CMSN axons, contributing to fasciculation and pathfinding defects observed in *Gsx2-Cre;Ctip2*$^{fl/fl}$ MSN.

Figure 2.6 *Efnb3* and *PlxnD1* are misregulated in *Ctip2*$^{-/-}$ and *Ctip2*$^{fl/fl}$*;Gsx2-Cre* striatum

Efnb3 is expressed at low levels in wild-type and *Ctip2*$^{fl/fl}$ striatum, but expression is dramatically increased in *Ctip2*$^{-/-}$ and *Ctip2*$^{fl/fl}$*;Gsx2-Cre* striatum (A-D). *PlxnD1*, expressed robustly in wild-type and *Ctip2*$^{fl/fl}$ striatum, is absent from *Ctip2*$^{-/-}$ and *Ctip2*$^{fl/fl}$*;Gsx2-Cre* striatum (E-H). Quantitative PCR confirms microarray and *in situ* findings (I).

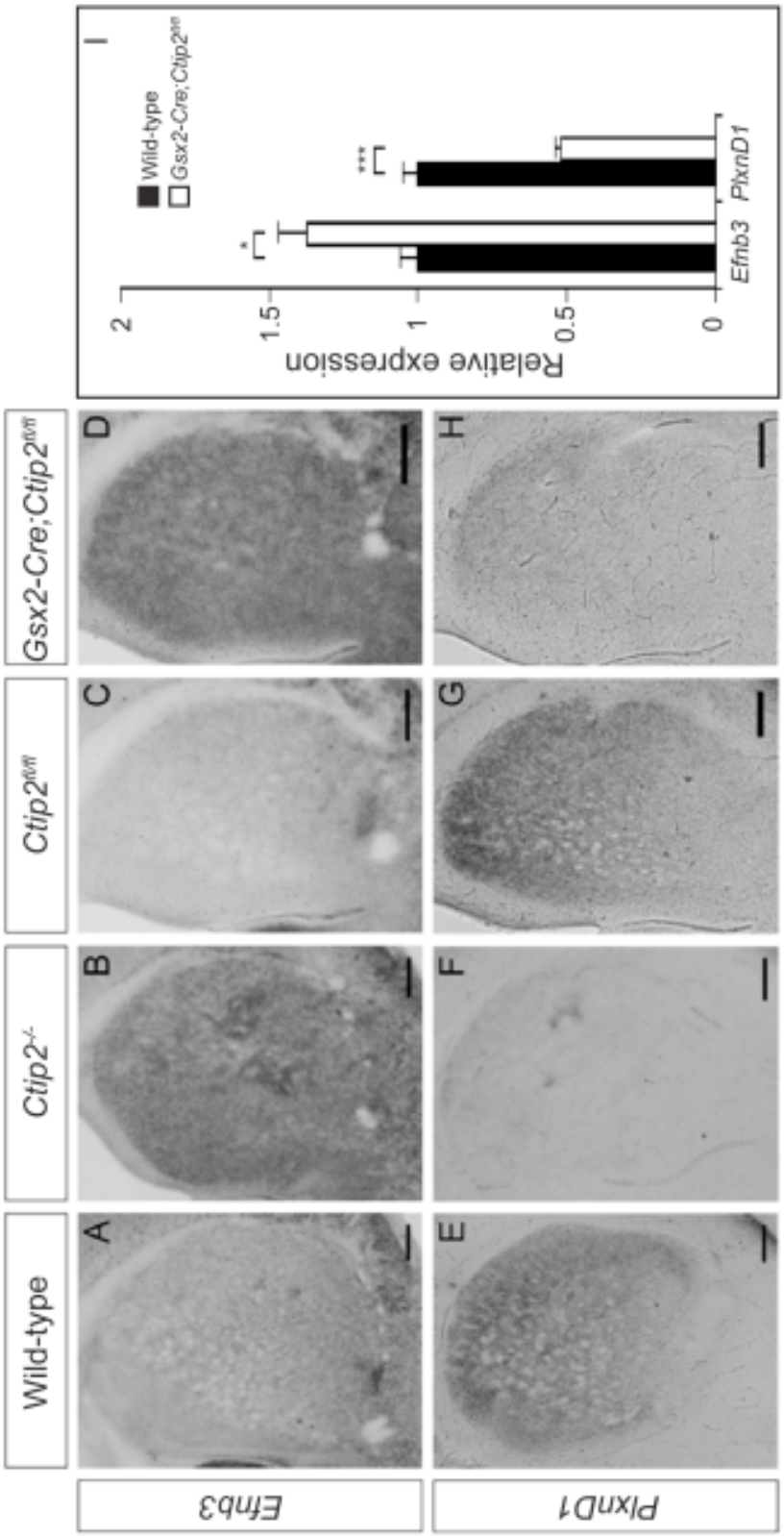

Figure 2.6 (Continued)

2.4 Discussion

The corticospinal tract is the longest axon tract in the mammalian central nervous system, and CSMN axons complete a particularly complex trajectory through multiple brain regions to reach their final targets in the spinal cord (Stanfield, 1992). CSMN and other corticofugal axons first must pierce the cortical white matter and enter into the internal capsule, a process mediated by semaphorin signaling that repels corticofugal axons from cortex (Polleux et al., 1998; Bagnard et al., 1998). The pallial-subpallial boundary is an early decision point for corticofugal axons, and disruption of guidance molecules at this boundary can cause axons to stall here (Bloom et al., 2007; Zhou et al., 2008). Once they have entered the internal capsule, CSMN axons must fasciculate with each other, which is thought to be an important element of further pathfinding (Stanfield 1992), then pass the telencephalic-diencephalic boundary (ten Donkelaar et al., 2004). CSMN axons continue to travel through the midbrain and hindbrain, reach the pyramidal decussation, and cross to the contralateral side to enter the spinal cord (Vulliemoz et al., 2005).The peak birthdate of CSMN is E13.5 in mouse, and CSMN axons spend the next week alternately growing and pausing until they reach the pyramidal decussation at P0 (Molyneaux et al., 2007; Schreyer and Jones, 1982). Because CSMN must interpret a multitude of cues to arrive at their spinal targets, exploring the interplay between CSMN-autonomous and CSMN-non-autonomous sources of guidance is particularly interesting.

In this work, we find that the transcription factor *Ctip2* is necessary within CSMN for entry into the internal capsule, pathfinding in the midbrain, and normal connectivity with the spinal cord. *Ctip2* is not necessary within CSMN for fasciculation

within the internal capsule, but is instead required by MSN surrounding CSMN axons in the internal capsule. In *Ctip2*$^{-/-}$ mice, no axons reach the spinal cord, but deleting *Ctip2* only from cortex, with normal *Ctip2* expression in MSN (*Ctip2*$^{fl/fl}$*;Emx1-Cre*), results in approximately 40% of CSMN axons reaching the spinal cord. This is a remarkable degree of rescue, especially since we find that the CSMN of mice lacking *Ctip2* only in MSN (*Ctip2*$^{fl/fl}$*;Gsx2-Cre*) are impaired only in the internal capsule, and that their later pathfinding is approximately normal.

Our data showing that *Ctip2* function in MSN controls CSMN axon fasciculation in the internal capsule led us to investigate axon guidance molecules regulated by *Ctip2* in MSN. We find that candidate controls *Plxnd1* and *Efnb3* are strikingly misregulated in the striatum in the absence of *Ctip2*. We will investigate the functional effect(s) of misexpressing these candidate controls in wild-type tissue, and determine whether reduction of *Plxnd1* or overexpression of *Efnb3* might cause defasciculation of wild-type CSMN axons.

Ctip2$^{-/-}$ mice invariably die within 8-12 hours of birth, and, therefore, all analyses conducted to date have been performed at P0 or earlier (Arlotta et al., 2005; Arlotta et al., 2008). In contrast, *Ctip2*$^{fl/fl}$*;Emx1-Cre* mice survive until adulthood, enabling more detailed analysis of CSMN projections using anterograde, retrograde, and genetic labeling. We plan to visualize CSMN axon projections in *Ctip2*$^{fl/fl}$*;Emx1-Cre* tissue by utilizing genetic CSMN labels including placental alkaline phosphatase (PLAP) inserted into the *Fezf2* locus (*Fezf2*^{hPLAP}; gift of Susan McConnell) (Chen et al., 2005a) and *6430573F11Rik*-GFP (Gong et al., 2003). Using these tools, we will determine whether CSMN lacking *Ctip2* project to ectopic structures, and whether they are abnormally distributed within the internal capsule.

Our preliminary data suggest that CSMN located medially in cortex are more successful establishing corticospinal projections than are lateral CSMN. We will retrogradely label CSMN from cervical spinal cord, and quantify cell bodies labeled in defined medio-lateral sectors of cortex. We will determine whether differential projection success is defined by 1) sharp areal boundaries (*e.g.*, medial CSMN in motor areas, lateral CSMN in somatosensory areas), potentially suggesting areally-expressed gene(s) capable of partially compensating for *Ctip2* in motor areas, or 2) gradual loss of projections from medial to lateral, suggesting increased probability of penetrating the internal capsule for medial *Ctip2$^{fl/fl}$;Emx1-Cre* CSMN, whose laterally-directed axons contact dorsal striatum longer than lateral CSMN.

In this work, we find that a single transcription factor expressed in two independent neuron populations, derived from separate progenitors, functions to control development of a single axonal projection. This effect could be mediated by homophilic adhesion molecules expressed by both CSMN axons and MSN surrounding the internal capsule, and, indeed, some well-known axon guidance molecules are expressed selectively by CSMN compared with their fellow corticofugals, corticothalamic projection neurons (B. J. Molyneaux, personal communication). These molecules might represent further candidates for MSN-expressed controls over CSMN axon guidance through the internal capsule.

The existence of a non-CSMN-autonomous transcriptional control over CSMN axon guidance has significant implications for potential future repair strategies for this clinically important neuron type. This work suggests that endogenous repair of diseased or injured circuitry might require considerable manipulation of non-CSMN-autonomous cues, some of which might not be present after development is complete. However, it is encouraging to note that appropriate guidance cues from MSN

surrounding CSMN axons in the internal capsule are able to fasciculate CSMN axons, even in the absence of important CSMN-intrinsic transcriptional programs; if induced CSMN can be directed to enter the internal capsule, striatal programs of fasciculation might be sufficient to guide axons a significant distance through the brain.

2.5 Experimental Procedures

Animals

All mouse studies were approved by the Massachusetts General Hospital and/or Harvard University IACUCs, 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. Unless noted otherwise, all experiments with *Ctip2$^{fl/fl}$;Emx1-Cre* or *Ctip2$^{fl/fl}$;Gsx2-Cre* were controlled with *Ctip2$^{fl/fl}$* (Cre wild-type), due to slightly lower expression of CTIP2 from the floxed locus than from the wild-type locus.

Ctip2$^{-/-}$ and *Ctip2$^{fl/fl}$* mice were generated by Kominami and colleagues (Wakabayashi et al., 2003), and were generously supplied by Ryo Kominami. *Emx1-Cre* (stock number 005628) and *Rosa26R-LacZ* (stock number 003474) mice were purchased from Jackson Laboratories. *Gsx2-Cre* mice were generated by Kessaris and colleagues (Kessaris et al., 2006), and were generously supplied by Nicoletta Kessaris and David Price.

Immunocytochemistry

Mice were transcardially perfused with 4% paraformaldehyde, and brains were dissected and post-fixed at 4°C overnight in 4% paraformaldehyde. 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. Primary antibodies and dilutions used: rat anti-CTIP2, 1:200 (Abcam); rabbit anti-DARPP32, 1:250 (Chemicon); mouse anti-GAP43, 1:500 (Millipore). Secondary antibodies were chosen from the Alexa series (Invitrogen), and used at a dilution of 1:500.

Nissl staining

Tissue was prepared as for immunocytochemistry above, then mounted on gelatin-coated slides and allowed to dry. Tissue was wetted, then stained in 0.25% cresyl violet for 4 minutes. Tissue was rinsed and dehydrated through a series of ethanol and xylene baths (Two minutes each: 50% EtOH, 70% EtOH, 95% EtOH, 100% EtOH; 45 minutes xylenes) and mounted in DPX mountant.

Anterograde labeling

Projection neurons were labeled by insertion of a small crystal of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) into sensorimotor cortex in P0 fixed tissue. Tissue was incubated in PBS/0.05% sodium azide at 37°C for four weeks to allow DiI transport to occur. DiI-labeled brains were sectioned sagittally at 70μm on a vibrating microtome. Images shown are a composite of multiple sagittal montages collapsed into a single image of the corticospinal tract.

Retrograde labeling

Projection neurons were labeled from cervical spinal cord under ultrasound guidance at P2 by pressure injection of Alexa fluorophore-conjugate cholera toxin B (Invitrogen). Tissue was collected at P6 and processed for immunocytochemistry. For retrograde labeling of adult CSMN, labeling was performed at P21 and tissue collected at P25.

Microarray data analysis

The microarray comparison discussed was performed by former laboratory members Bradley Molyneaux and Paola Arlotta (Arlotta et al., 2008).

We selected only gene candidates with absolute fold change greater than 2 and $p<0.05$ between wild-type and *Ctip2*$^{-/-}$ striatum, and excluded probes with very low expression intensity in order to select target genes expressed at high enough levels to have biologically-significant effects. These parameters identified 433 genes differentially expressed between wild-type and *Ctip2*$^{-/-}$ striatum. The candidate list was further winnowed by selecting genes with membrane or extracellular localization using gene ontology databases, including The Gene Ontology project (http://www.geneontology.org/), Nextbio (http://www.nextbio.com/), and the UCSC Genome Browser (http://genome.ucsc.edu). Of the 433 genes differentially expressed between wild-type and *Ctip2*$^{-/-}$ striatum, 28 are known to be exported to the extracellular space, 111 have membrane-associated domains, and 36 are included in both categories. Next, we narrowed the list of gene candidates by identifying those that

are normally specifically expressed in striatum, or normally excluded from the striatum, by comparing gene expression profiles using online expression databases such as Allen Brain Atlas (http://www.brain-map.org), Genepaint (http://www.genepaint.org/), and Brain Gene Expression Map (BGEM) (http://www.stjudebgem.org/). Gene candidates not typically expressed in the striatum were included only if expression was increased in *Ctip2*$^{-/-}$ striatum in the microarray data set.

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Chapter 3

Ctip1 regulates balance of projection neuron subtype specification in deep cortical layers

Publication

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*equally-contributing first authors

Author contributions

This project was initiated independently by me, and experimental work was performed in equal collaboration with M.D./Ph.D. student Luciano Custo Greig. All experimental work and figure preparation was completed by Luciano and me together, and we wrote the manuscript together. Postdoctoral fellow Maria Galazo performed corticothalamic retrograde labeling experiments. Philip Tucker of the University of Texas at Austin and his postdoctoral fellow Gregory Ippolito made the *Ctip1*$^{fl/fl}$ mouse line.

3.1 Abstract

Molecular events that regulate precise differentiation, migration, and connectivity of diverse neocortical projection neuron subtypes remain poorly understood. We report here that the transcription factor *Ctip1* controls precision of neocortical development by regulating subtype identity. *Ctip1* is expressed by callosal and corticothalamic projection neurons, but is excluded over time from subcerebral projection neurons. Loss of *Ctip1* function results in a striking bias in favor of subcerebral development at the expense of corticothalamics and deep-layer callosals. In addition, those corticothalamic and callosal projections that exist are remarkably abnormal, because *Ctip1* regulates development of their pioneer populations. Misexpression of *Ctip1 in vivo* represses subcerebral gene expression and projections. During corticogenesis, *Ctip1* regulates migration, but not subtype identity, of late-born neurons. These data indicate that *Ctip1* controls the balance between specification of different projection neuron subtypes in deep cortical layers, enabling cortex to populate specific functional areas with precise ratios of different subtypes.

3.2 Introduction

The remarkable complexity of the neocortex is precisely orchestrated during development, as millions of neurons are born from progenitors, migrate to birthdate-appropriate layers, and adopt subtype-specific gene expression and projection patterns. Projection neuron subtypes are generated in sequential waves between approximately embryonic day (E) 11.5 and E15.5 in mice. Subplate neurons are born first, around E11.5, migrate to occupy the deepest positions in the neocortex, and pioneer projections to the thalamus (Wang et al., 2010), while closely-related corticothalamic projection neurons (CThPN), born around E12.5, migrate to layer VI and establish connections with specific thalamic nuclei (Grant et al., 2012). Subcerebral projection neurons (SCPN), including corticospinal motor neurons, are generated around E13.5 and migrate to layer V before sending axons to targets in the brainstem and spinal cord (Stanfield, 1992). Callosal projection neurons (CPN) extend axons across the corpus callosum to the contralateral hemisphere. Most CPN are born around E15.5 and reside in layer II/III, but smaller populations in layers V and VI are born concurrently with other deep-layer subtypes (Molyneaux et al., 2007).

Although the diversity among neurons in the neocortex has long been appreciated, key transcription factor controls over subtype development have only been identified in recent years. One such transcription factor, *Fezf2*, is expressed at high levels by SCPN beginning in progenitors, and centrally regulates SCPN specification (Molyneaux et al., 2005; Chen et al., 2005a, 2005b). In the absence of *Fezf2*, layer V neurons do not express SCPN-specific genes or project subcerebrally (Molyneaux et al., 2005; Chen et al., 2005a), and, instead, are fate-converted to CPN

and CThPN (Chen et al., 2008; Lodato et al., 2011; McKenna et al., 2011). Repression of *Fezf2* is critical for appropriate development of non-SCPN subtypes. *Fezf2* is repressed by *Sox5* in CThPN and subplate neurons (Lai et al., 2008; Kwan et al., 2008; Shim et al., 2012) and by *Tbr1* in CThPN (Bedogni et al., 2010; McKenna et al., 2011; Han et al., 2011). Conversely, *Fezf2* acts in SCPN to prevent expression of transcription factors key for specification of alternate subtype fates, including *Tbr1* (Begdoni et al., 2010; McKenna et al., 2011) and *Satb2* (Alcamo et al., 2008; Britanova et al., 2008), and to promote expression of *Ctip2* (Molyneaux et al., 2005; Chen et al., 2008). Although these central controls begin to be expressed immediately after neurons exit the cell cycle, subtype identity continues to be refined as neurons mature, as, for example, initially promiscuous expression of *Ctip2* and *Satb2* in early postmitotic layer V neurons resolves to subtype-specific expression in SCPN and CPN by late embryogenesis (Alcamo et al., 2008; Britanova et al., 2008; Azim et al., 2009b).

Ctip2 is expressed at high levels by SCPN, and functions centrally in controlling SCPN terminal differentiation. In the absence of *Ctip2*, SCPN are born and migrate to layer V, but their axons are defective in outgrowth, pathfinding, and fasciculation, and never reach the spinal cord (Arlotta et al., 2005). Multiple pathways controlling projection neuron specification and postmitotic differentiation operate at least in part by regulating *Ctip2* expression (Molyneaux et al., 2005; Chen et al., 2005a, 2005b; Lai et al., 2008; Kwan et al., 2008; Alcamo et al., 2008; Britanova et al., 2008; Bedogni et al., 2010; McKenna et al., 2011; Tomassy et al., 2010). A paralogous zinc finger transcription factor, *Ctip1* (also known as *Bcl11a*/*Evi9*), is also expressed in the neocortex, as well as in the hippocampus, striatum, and cerebellum (Leid et al., 2004). *Ctip2* and *Ctip1* are closely related, with approximately 60% identity between their nucleotide sequences (Avram et al., 2000; Satterwhite et al., 2001).

Ctip1 was first identified as an interacting partner of COUP transcription factors (Avram et al., 2000), one of which, *Couptf1*, is itself an important regulator of neocortical projection neuron development (Zhou et al., 1999; Armentano et al., 2007; Tomassy et al., 2010; Alfano et al., 2011). *Ctip1* binds DNA in a sequence-specific manner (Avram et al., 2002; Xu et al., 2010), and its transcriptional functions are best-understood in the hematopoietic system, where it controls the development of B cells (Liu et al., 2003) and regulates the switch between fetal and adult forms of hemoglobin (Sankaran et al., 2008; Sankaran et al., 2009; Xu et al., 2011). In the nervous system, *Ctip1* is important for morphogenesis and projections of dorsal spinal cord neurons (John et al., 2012), and for axon branching and dendrite outgrowth in cultured hippocampal neurons (Kuo et al., 2009; Kuo et al., 2010). Although *Ctip1* is known to be expressed in the neocortex (Leid et al., 2004; Kuo and Hsueh, 2007), its functions in neocortical development have not previously been investigated.

Here, we report that *Ctip1* is a novel control over projection neuron subtype identity, as well as migration of late-born cortical neurons. *Ctip1* is expressed by CPN, CThPN, and subplate neurons, but is progressively excluded over embryonic development from *Ctip2*-expressing SCPN. In deep layers, *Ctip1* controls the balance between generation of SCPN and CThPN or CPN. In the absence of *Ctip1*, SCPN expand as a population at the expense of CThPN and deep-layer CPN, with higher expression of genes characteristic of SCPN, and more neurons projecting to the cerebral peduncle, and with a reduction in CThPN and CPN gene expression and projections. In contrast, *Ctip1* overexpression *in vivo* is sufficient to repress *Ctip2*, and reduce the number of neurons projecting subcerebrally, while increasing the number of neurons projecting through the corpus callosum and to thalamus. In addition, in the absence of neocortical *Ctip1*, upper-layer projection neurons migrate defectively,

resulting in imprecise layering of the neocortex mediated through the *Reelin* pathway. In an accompanying manuscript, we find that *Ctip1* is also a novel control over the development of neocortical area identity and the formation of sensory maps (Chapter 4).

Figure 3.1 *Ctip1* is expressed by postmitotic corticothalamic and callosal projection neurons, but is progressively excluded from subcerebral projection neurons

Postmitotic cortical plate neurons express CTIP1 at E12.5 (A) and E16.5 (B). At P4, projection neurons in layer II/III, V, VI, and subplate express CTIP1 (C). CTIP1 co-localizes with FOG2, as well as cholera toxin B (CTB) injected into thalamus (D). CPN express both CTIP1 and SATB2, and are retrogradely labeled by injection of CTB into corpus callosum (E). CSMN are retrogradely labeled by CTB injection into cervical spinal cord and express CTIP2, but not CTIP1 (F). At E14.5, CTIP2 and CTIP1 are extensively co-expressed by newly postmitotic projection neurons (G-I). At E17.5, developing CSMN express CTIP2 (K), while developing CPN express CTIP1 (J), and this expression is fully segregated at P4 (M-O). Some neurons with small nuclei, possibly interneurons, co-express *Ctip1* and *Ctip2* at high levels (arrows in M-O).

Figure 3.1 (Continued)

Figure 3.2 CTIP1 is not expressed by neocortical progenitors

Although *Ctip1* mRNA can be seen in the neocortical ventricular zone at E14.5 (arrow, A), protein expression is restricted to postmitotic neurons (arrow, B). CTIP1 expression does not co-localize with intermediate progenitor gene TBR2 (C).

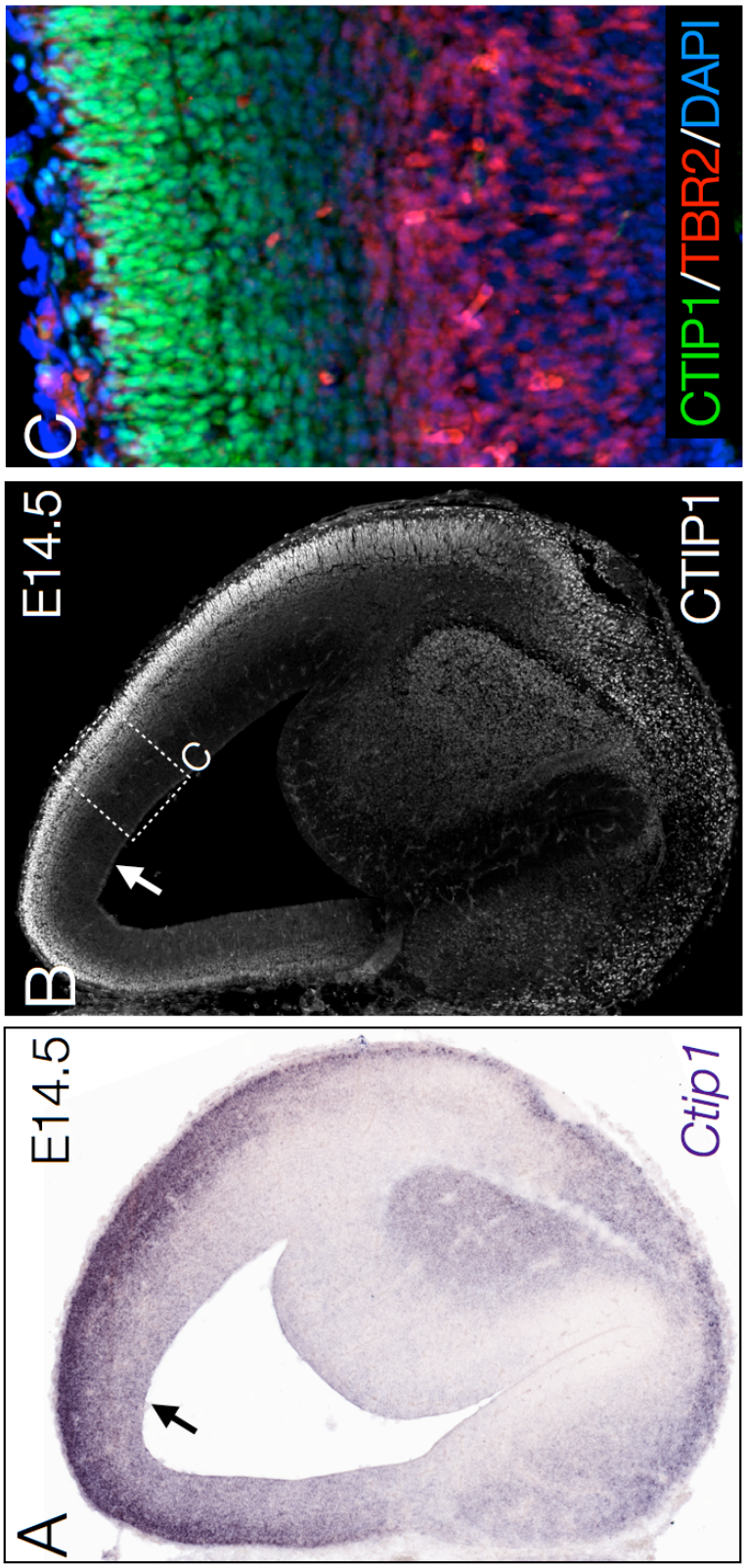

Figure 3.2 (Continued)

3.3 Results

CTIP1 is expressed by CPN, CThPN, and subplate neurons, but is largely excluded from SCPN

CTIP1 is first detected by immunocytochemistry at approximately E12.5 in young postmitotic glutamatergic projection neurons in the cortical plate (**Figure 3.1A**). Immunostaining is absent from progenitor zones (**Figure 3.2B**), although some expression of *Ctip1* mRNA can be observed by *in situ* hybridization (**Figure 3.2A**). This suggests that *Ctip1* begins to be transcribed as neurons exit the cell cycle, but detectable amounts of protein are not present until postmitotic neurons migrate to the cortical plate (**Figure 3.2B**). CTIP1 continues to be expressed by postmitotic cortical neurons throughout embryogenesis (**Figure 3.1B**), though expression begins to decrease after the first postnatal week. At postnatal day (P) 4, CTIP1 is highly expressed in subplate, layer V, and upper layer II/III in all areas of neocortex (**Figure 3.1C**). CTIP1 is also expressed in layer VI in somatosensory and visual cortex. Similarly, expression in deeper layer II/III is also somatosensory-specific (Chapter 4).

To investigate the subtype specificity of *Ctip1* expression, we retrogradely labeled CPN, SCPN, or CThPN with Alexa fluorophore-conjugated cholera toxin B (CTB) at early postnatal ages (P1-P3) and collected labeled brains at P4. In addition, we performed dual immunocytochemistry for CTIP1 with SATB2, CTIP2, and FOG2, well-characterized markers of CPN, SCPN, and CThPN, respectively (Arlotta et al., 2005; Alcamo et al., 2008; Britanova et al., 2008; Molyneaux et al., 2007). These results reveal

that CTIP1 is expressed by CPN and CThPN, but is essentially excluded from SCPN (**Figure 3.1D-F**). CTIP1 is expressed by retrogradely-labeled CPN in layer II/II, layer V, and layer VI, and largely co-localizes with SATB2 in CPN. Similarly, retrogradely-labeled CThPN express both CTIP1 and FOG2. Almost no neurons in motor cortex retrogradely labeled from the spinal cord express CTIP1, and there is little to no co-expression of CTIP1 and CTIP2 (**Figure 3.1F**). SCPN in somatosensory and visual cortex faintly express CTIP1, at much lower levels than neighboring CPN, CThPN, and subplate neurons (Chapter 4).

Having determined that CTIP1 is expressed at high levels by CPN and CThPN, but not by SCPN, we next examined the timecourse of CTIP1 and CTIP2 expression in embryonic cortex. We find that, at E14.5, all early postmitotic layer V neurons co-express CTIP1 and CTIP2 at high levels, but, by E17.5, CTIP1 and CTIP2 are expressed separately by two largely distinct populations, corresponding to CPN and SCPN, respectively (**Figure 3.1G-L**). A small population of neurons co-expresses CTIP1 and CTIP2 at P4, but nuclei of these neurons are smaller than layer V pyramidal neurons, and likely belong to interneurons (arrows in **Figure 3.1M-O**; Arlotta et al., 2005).

The early segregation of cortical CTIP1 and CTIP2 expression, together with their functions controlling differentiation of closely-related immune cell types (Wakabayashi et al., 2003; Liu et al., 2003), led us to hypothesize that *Ctip2* and *Ctip1* might interact cross-repressively to control differentiation of deep-layer projection neurons into distinct subtypes. We examined *Ctip2* expression in the neocortex of *Ctip1*$^{-/-}$ mice, and find increased levels of CTIP2 in layers V and VI by both immunocytochemistry and qPCR (1.5-fold, p=0.014). Conversely, there is increased CTIP1 expression in layers V and VI of *Ctip2*$^{-/-}$ neocortex (1.5-fold, p=0.049) (**Figure 3.3**).

Figure 3.3 *Ctip2*$^{-/-}$ cortex expresses higher levels of *Ctip1*, and *Ctip1*$^{-/-}$ cortex expresses higher levels of *Ctip2*

CTIP2 is increased in layers V and VI of *Ctip1*$^{-/-}$ cortex (A, B), and CTIP1 is increased in layers V and VI of *Ctip2*$^{-/-}$ cortex (D, E). These changes are confirmed by qPCR, $p<0.05$ (C, F).

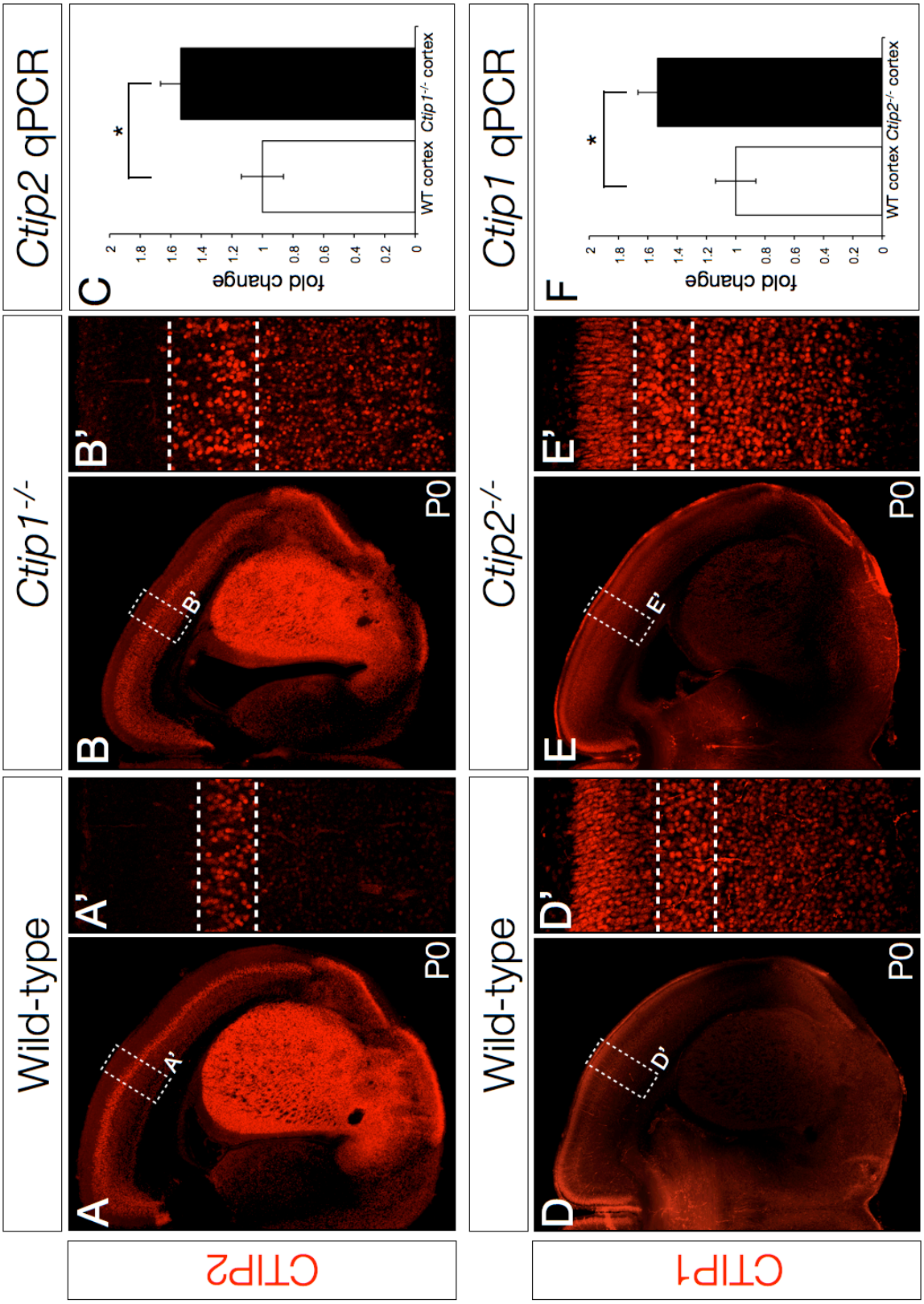

Figure 3.3 (Continued)

Figure 3.4 In the absence of neocortical *Ctip1*, superficial-layer projection neurons migrate defectively

Ctip1$^{fl/fl}$*;Emx1-Cre* cortex exhibits defective lamination at P4 by Nissl stain (A, B). When BrdU is administered to pregnant females at E14.5 and E15.5, BrdU-labeled neurons in *Ctip1*$^{fl/fl}$*;Emx1-Cre* cortex are significantly more likely to remain in deep layers than wild-type (C, D, F, G), and the overall laminar distribution of labeled neurons is strikingly abnormal (E, H).

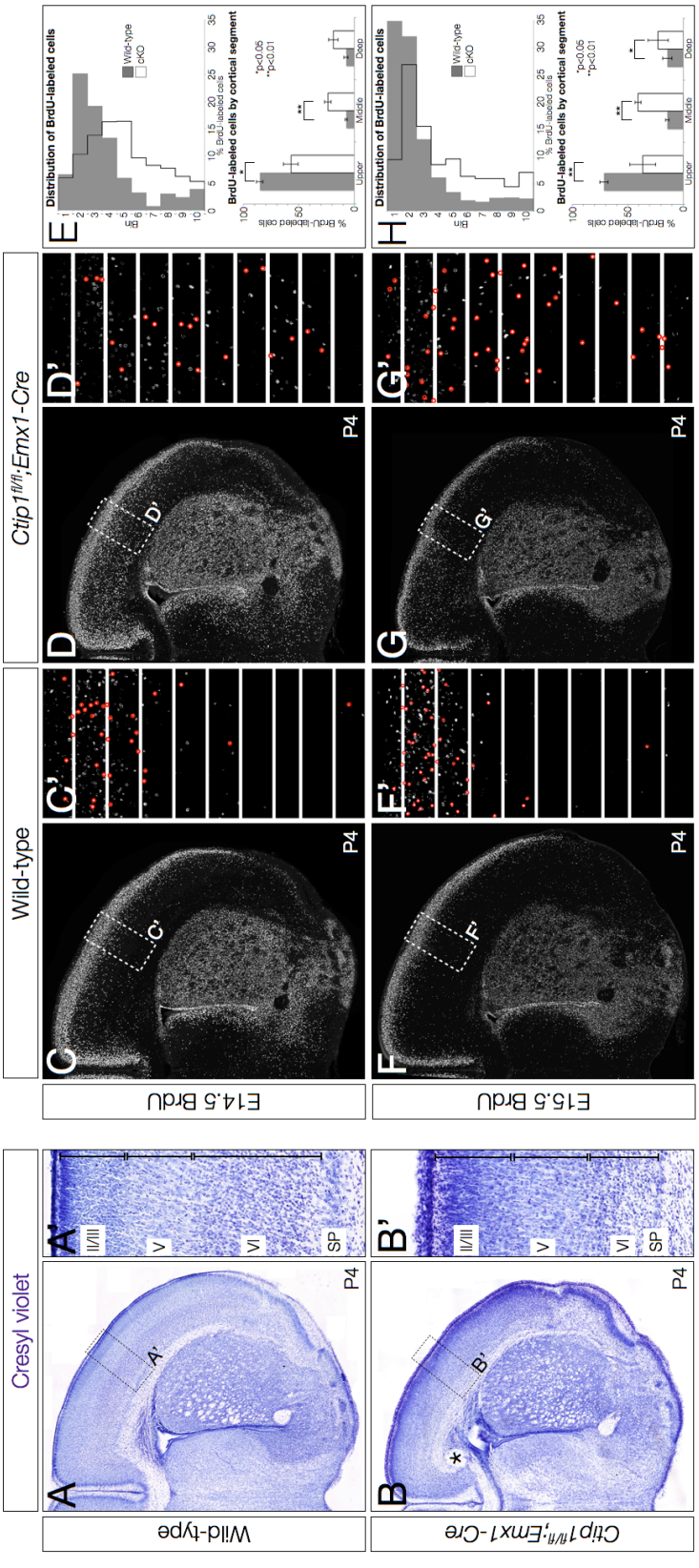

Figure 3.4 (Continued)

Projection neurons migrate abnormally in *Ctip1* mutants

Since *Ctip1*$^{-/-}$ mice die within hours of birth (Liu et al., 2003), we obtained *Ctip1*$^{fl/fl}$ mice, which survive to adulthood (Sankaran et al., 2009), to study *Ctip1* function as neocortical projections are established and refined. We deleted *Ctip1* specifically from cortical neurons by crossing these *Ctip1*$^{fl/fl}$ mice with mice expressing *Emx1-Cre*, which is expressed beginning in cortical progenitors (Gorski et al., 2002), and which spares *Ctip1* expression in non-cortical neuronal populations, including cortical interneurons, striatal medium-sized spiny neurons, and dorsal spinal neurons (Leid et al., 2004; Arlotta et al., 2008; John et al., 2012). We compared wild-type and *Ctip1*$^{fl/fl}$*;Emx1-Cre* (conditional null) tissue at P4 by Nissl stain, and find that conditional nulls exhibit defective cortical layering, with a lack of the clear distinction between adjacent layers visible in wild-type tissue (**Figure 3.4A-B**). These defects in lamination persist until at least P21, strongly suggesting that they are not resolved later in development (**Figure 3.5C-D**).

To investigate whether *Ctip1*$^{fl/fl}$*;Emx1-Cre* neurons form imprecise layers as a result of abnormal migration, we birthdated cortical neurons in conditional null and wild-type cortex by injecting BrdU at E11.5, E12.5, E13.5, E14.5, and E15.5. We analyzed the laminar position of labeled neurons at P4, after migration is normally complete. Significant numbers of E14.5- and E15.5-born neurons are ectopically located in deep layers in *Ctip1*$^{fl/fl}$*;Emx1-Cre* mutants (**Figure 3.4C-H**; $p<0.05$ for neurons located in bins 5, 6, and 7 at both E14.5 and E15.5), indicating that late-born projection neurons fail to migrate appropriately to upper layers in the absence of *Ctip1*. In contrast, migration of conditional null neurons born at E11.5, E12.5, and E13.5 is not significantly affected (**Figure 3.6**).

Migration defects in *Ctip1*$^{fl/fl}$*;Emx1-Cre* mice might be due to defective signaling from post-migratory neurons in the cortical plate to migrating neurons, or they might be due to deficient interpretation of cues by migrating neurons themselves. To distinguish between these possibilities, we electroporated E14.5 wild-type or *Ctip1*$^{fl/fl}$ embryos with a CMV/β-actin promoter-driven *Cre*-IRES-*Egfp* construct, and examined electroporated tissue at E17.5. This experiment deletes *Ctip1* from only a small fraction of cortical neurons, allowing deletion of *Ctip1* in an otherwise wild-type context. *Ctip1*$^{fl/fl}$ neurons electroporated with *Cre* at E14.5 are four times less likely than wild-type neurons to have entered the cortical plate at E17.5 (**Figure 3.7C-D**; 13% *Ctip1*$^{fl/fl}$ vs. 51% wild-type; p=0.004). Neurons in *Ctip1*$^{fl/fl}$ brains electroporated only with a CMV/β-actin promoter-driven *tdTomato* construct migrate normally into the cortical plate (**Figure 3.7A-B**), indicating that defects in migration in neurons lacking *Ctip1* are cell-autonomous.

The defects in late-born projection neurons in *Ctip1*$^{fl/fl}$*;Emx1-Cre* cortex are similar to previously-reported phenotypes for *Reelin* pathway mutants (Trommsdorff et al., 1999; Hack et al., 2007), so we next investigated whether expression of known components of the *Reelin* pathway are disrupted by the loss of *Ctip1*. We find that expression of *Reelin* is dramatically decreased at P0 in *Ctip1*$^{fl/fl}$*;Emx1-Cre* cortex, both within individual Cajal-Retzius cells and as a result of fewer Cajal-Retzius cells being present in layer I (**Figure 3.7F-G**). Because it is known that loss of Cajal-Retzius cells alone does not lead to significant cortical migration abnormalities (Yoshida et al., 2006), and because our electroporation experiments described above suggest that migration defects in the absence of *Ctip1* are cell-autonomous, we investigated receptors and downstream effectors of *Reelin* by RNA sequencing (RNA-seq). Indeed, expression of *Reelin* receptor *Vldlr* is significantly reduced in *Ctip1*$^{fl/fl}$*;Emx1-Cre* cortex (p=0.001),

Figure 3.5 *Couptf1* downstream target *Rnd2* is not misregulated in the absence of *Ctip1*, and migration defects persist until adulthood

Expression of cortical *Rnd2* is not increased in *Ctip1$^{fl/fl}$;Emx1-Cre* brains by *in situ* hybridization at E14.5 (compare levels in cortex at arrows in A, B with levels in thalamus at asterisk in A, B). Migration defects persist until at least early adulthood, as P21 Nissl staining reveals that cortical tissue is still defectively laminated (C, D).

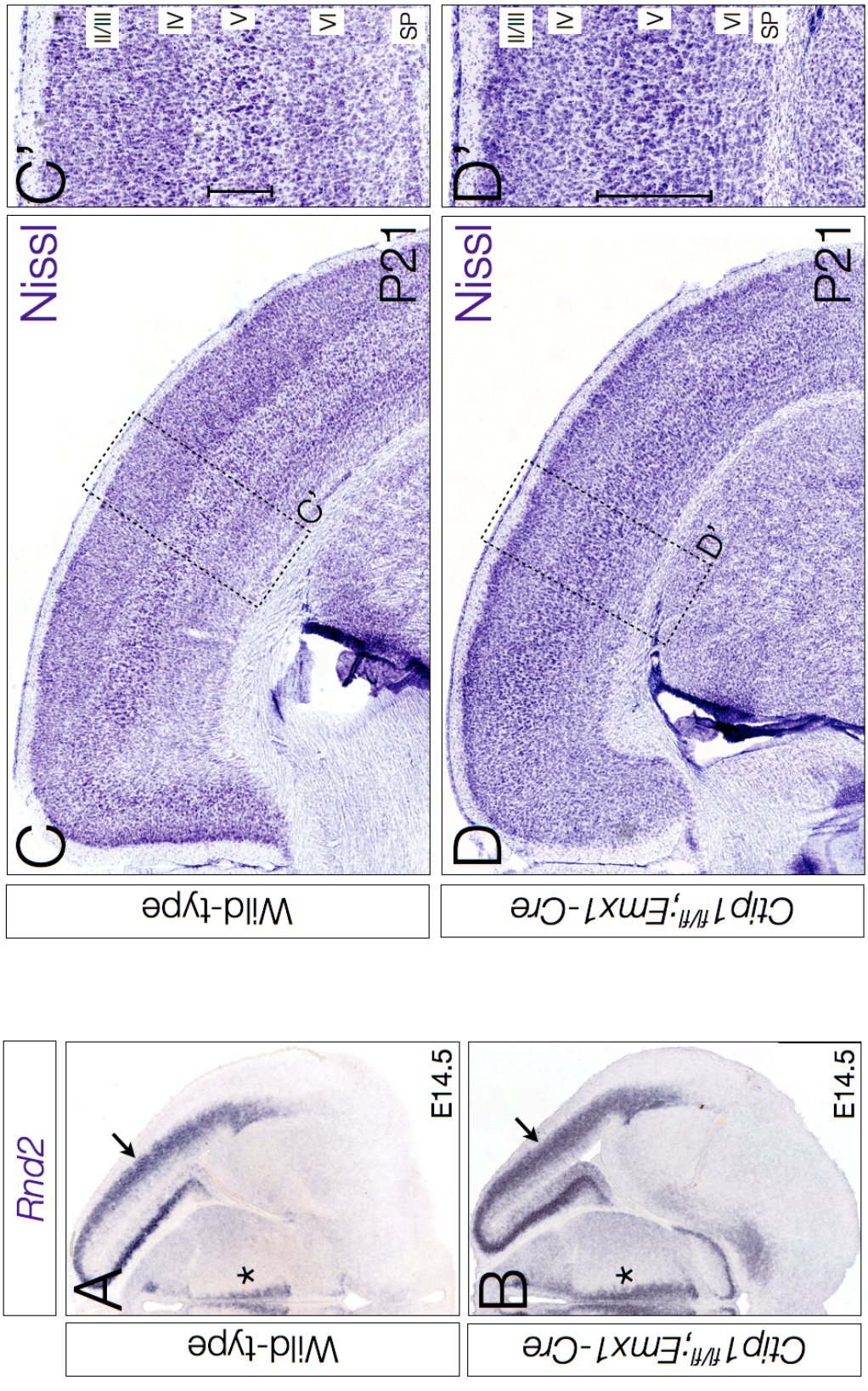

Figure 3.5 (Continued)

Figure 3.6 Migration of deep-layer neurons is not substantially affected in the absence of *Ctip1*

When BrdU is administered to pregnant females at E11.5, E12.5 and E13.5, BrdU-labeled neurons in *Ctip1*$^{fl/fl}$*;Emx1-Cre* cortex are equally likely to remain in deep layers compared with wild-type (A, B, D, E, G, H), and the overall laminar distribution of labeled neurons is normal (C, F, I).

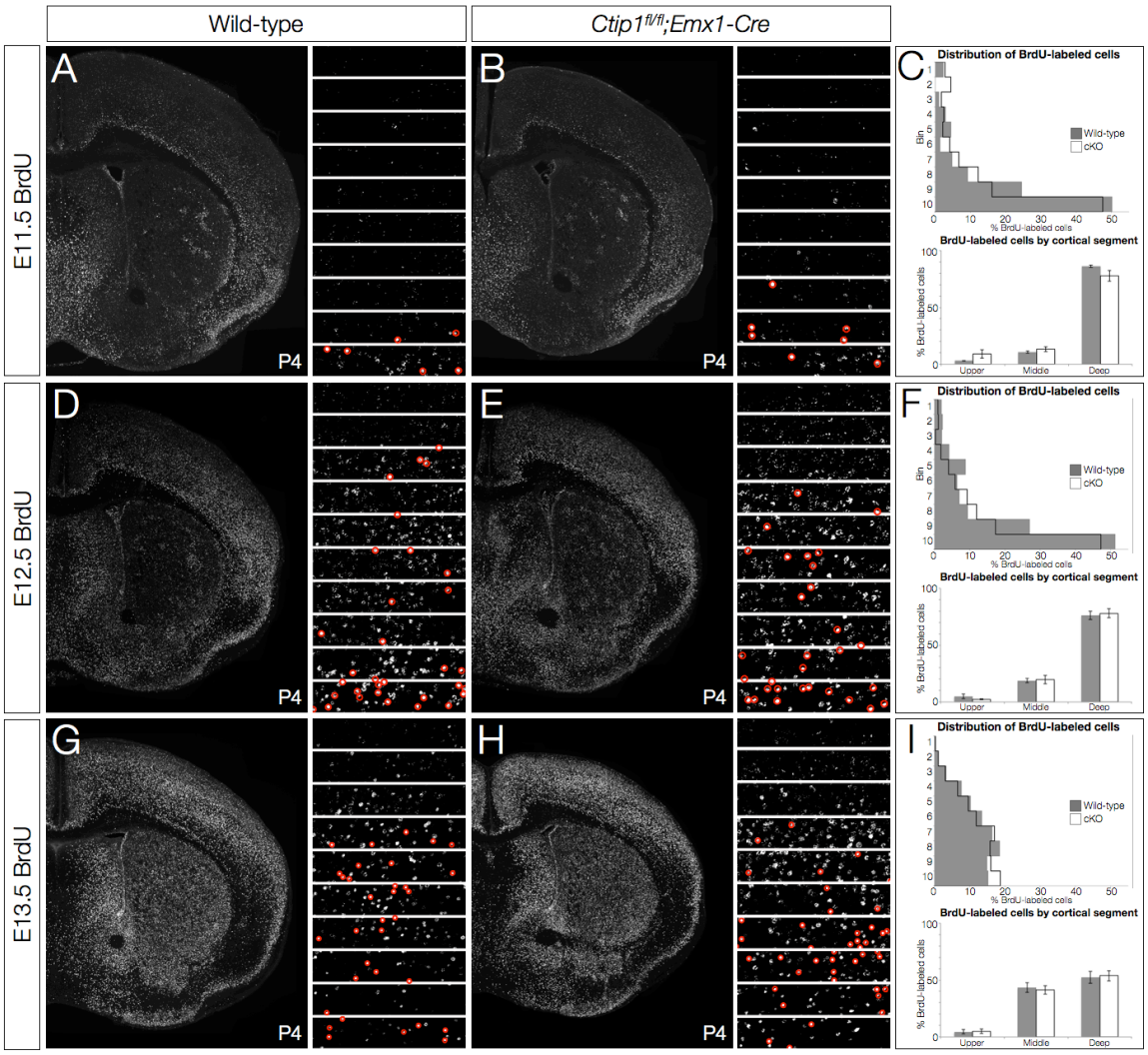

Figure 3.6 (Continued)

Figure 3.7 Migration defects in the absence of *Ctip1* are cell-autonomous

Although cortical neurons migrate normally in both wild-type and *Ctip1*$^{fl/fl}$ cortex with electroporation of *tdTomato* into E14.5 cortical progenitors (A, B), electroporation of *Cre*-IRES-*Egfp* causes *Ctip1*$^{fl/fl}$ neurons to stall in the intermediate zone (C, D, E). In addition, expression of migration guidance molecule *Reelin* is strikingly decreased in *Ctip1*$^{fl/fl}$*;Emx1-Cre* cortex at P0 (F, G).

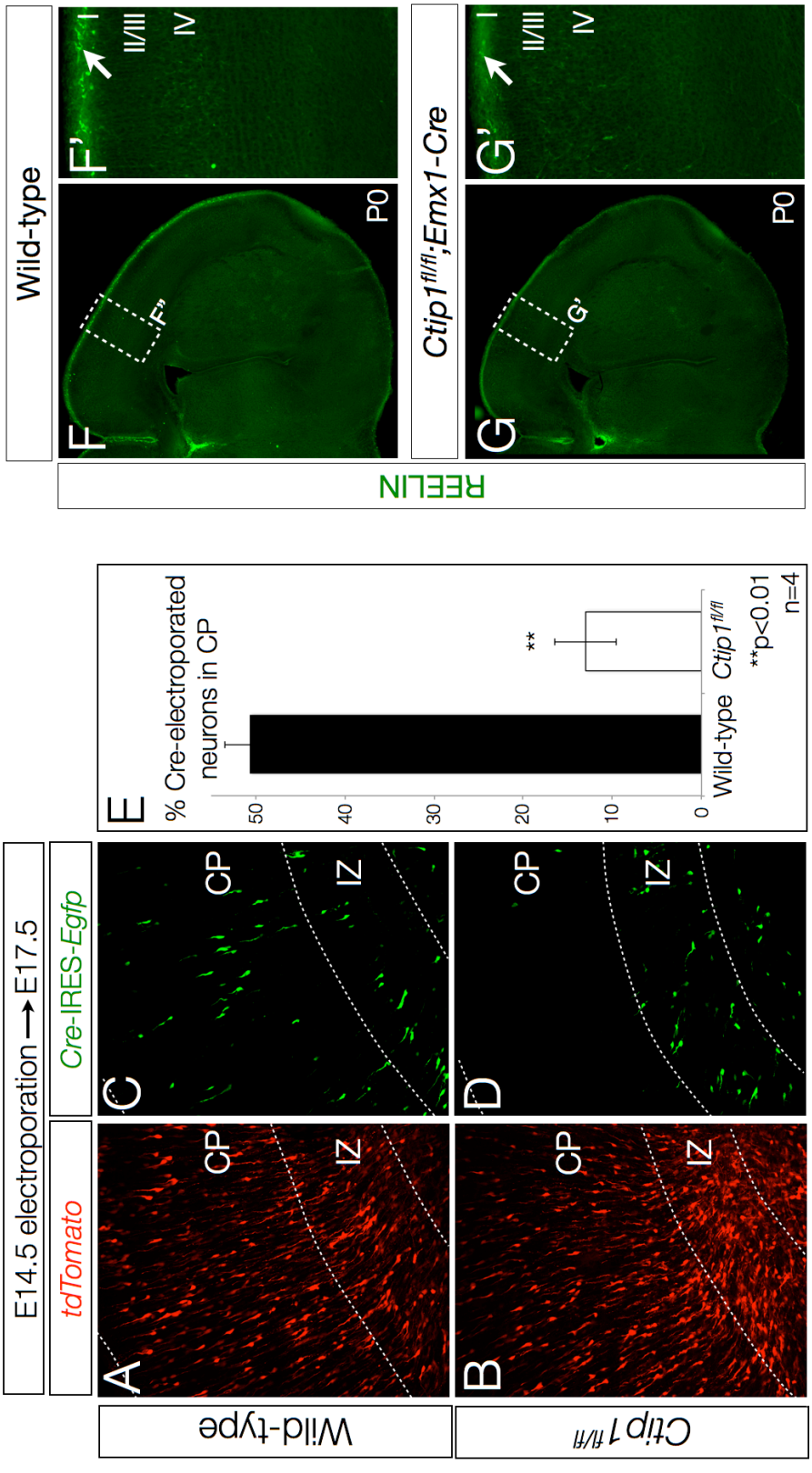

Figure 3.7 (Continued)

although expression of *Reelin* receptor *Apoer2* is normal (p=0.13). We also find decreased expression of the *Reelin* downstream effector *Dab1* in *Ctip1$^{fl/fl}$;Emx1-Cre* cortex (p=0.013). We are working to supplement these results with immunocytochemistry and/or *in situ* hybridization, and are also pursuing *in utero* electroporation experiments to determine whether restoring expression of *Vldlr* or *Dab1* is sufficient to rescue deficient migration caused by loss of *Ctip1* function. In the absence of *Ctip1*, multiple genes involved in *Reelin* signaling are misregulated, suggesting a possible pathway underlying *Ctip1*-related projection neuron migration defects.

More projection neurons develop an SCPN identity in the absence of *Ctip1* function

Nissl staining of *Ctip1$^{fl/fl}$;Emx1-Cre* tissue reveals an expansion of layer V, particularly of large pyramidal cells with characteristic SCPN morphology, with a concomitant decrease in the size of layer VI (**Figure 3.4A-B**). To investigate whether more neurons in *Ctip1$^{fl/fl}$;Emx1-Cre* mice differentiate into SCPN, we performed immunocytochemistry and *in situ* hybridization at P0 for several genes expressed specifically by SCPN, including CTIP2, *Fezf2*, *Clim1*, and *S100a10* (Arlotta et al., 2005; Molyneaux et al., 2005; Azim et al., 2009; Molyneaux et al., 2007). We find that high-level expression of these genes is expanded radially in *Ctip1$^{fl/fl}$;Emx1-Cre* cortex, especially in somatosensory cortex, where layer V is typically thinner than in motor cortex (**Figure 3.8A-H**). This expansion appears to be primarily from layer V into layer

VI, suggesting that layer VIa neurons are converted to SCPN. Indeed, when we perform immunocytochemistry at P0 for genes specific to CThPN, including FOG2, TBR1, TLE4, and DARPP32 (Molyneaux et al., 2007; Hevner et al., 2001; Ouimet et al., 1984), we find a striking reduction in the radial thickness of layer VI (**Figure 3.8I-P**). Even within this thinner layer VI, neurons expressing CThPN controls also express inappropriately high levels of CTIP2 and *Fezf2* (asterisks in **Figure 3.8B, D**), suggesting a potentially mixed SCPN/CThPN identity. These results indicate that, in the absence of *Ctip1* function, more projection neurons adopt an SCPN identity, and fewer adopt a CThPN identity.

More neurons project subcerebrally in *Ctip1$^{fl/fl}$;Emx1-Cre* mice, and fewer project to thalamus

We next investigated whether, beyond dysregulation of characteristic subtype-specific genes, cortical projection patterns change in the absence of *Ctip1*. We retrogradely labeled SCPN from the cerebral peduncle at P1 revealing that significantly more axons project to subcerebral targets in *Ctip1$^{fl/fl}$;Emx1-Cre* than in wild-type brains (**Figure 3.9A-B**). In particular, the number of SCPN in somatosensory cortex increases 1.4-fold ($p=0.003$), and the number of SCPN in visual cortex increases 1.2-fold ($p=0.014$). The number of SCPN in motor cortex does not change, suggesting that already low expression of CTIP1 in wild-type motor cortex enables development of the maximum number of SCPN that motor cortex can generate, via elimination of *Ctip2* repression by *Ctip1*. In concordance with expression of SCPN-specific genes expanding deeper into layer VI, we observe that most additional SCPN are located in the upper segment of layer VI, and we hypothesize that these neurons are born at a

Figure 3.8 More neurons in *Ctip1*$^{fl/fl}$*;Emx1-Cre* cortex adopt a subcerebral identity, and fewer adopt a corticothalamic or subplate identity

In the absence of *Ctip1*, expression of SCPN identity genes CTIP2, *Fezf2*, *Clim1*, and *S100a10* increases in cortex (A-H), especially in somatosensory cortex (arrows in A-H). In tandem, expression of CThPN identity genes FOG2, TBR1, TLE4, and DARPP32 is reduced (I-P; radial bar is the same size between images). Even layer VI neurons that continue to express CThPN identity genes express aberrantly high levels of CTIP2 and *Fezf2* (asterisks in B, D), suggesting a mixed CThPN/SCPN identity.

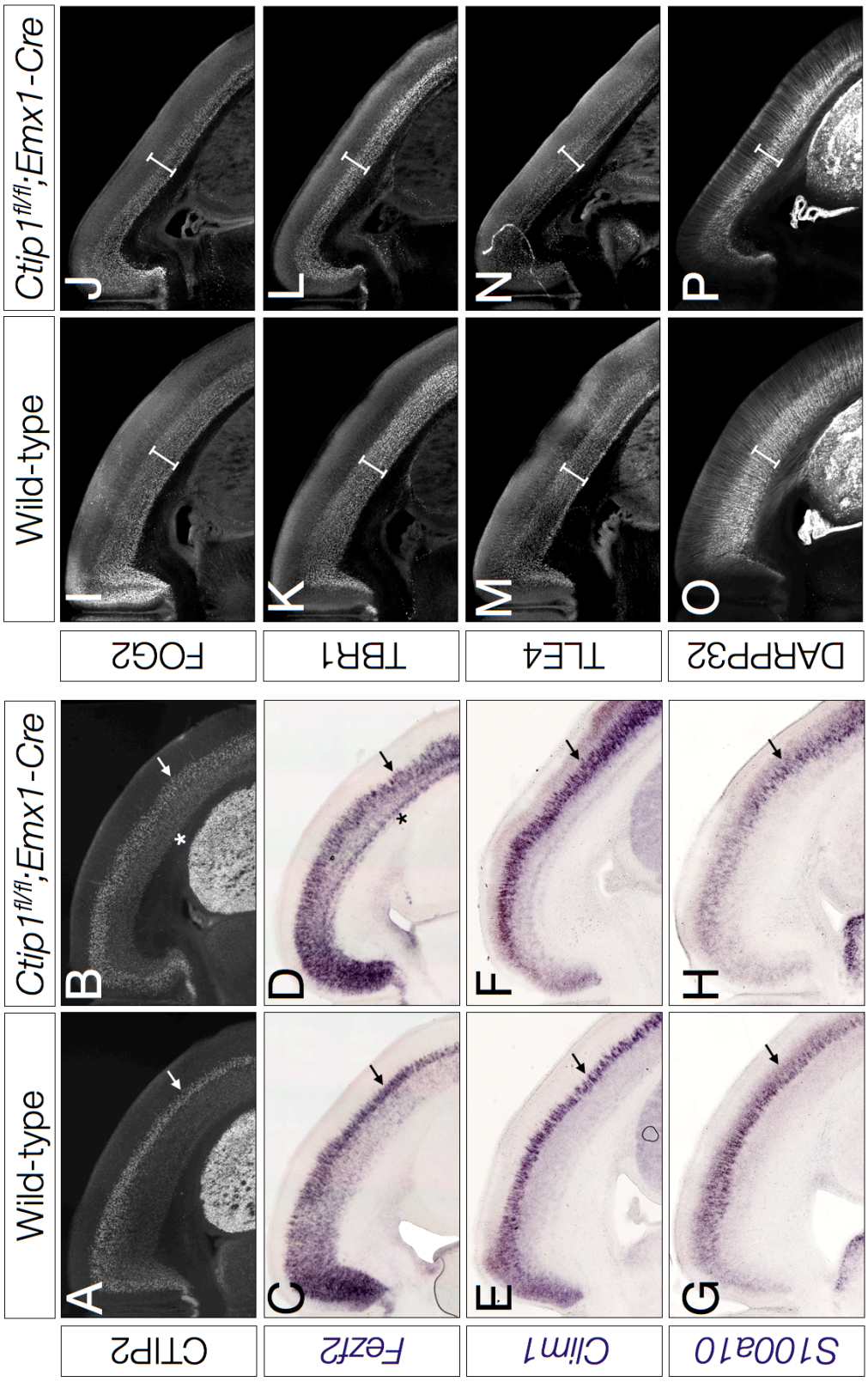

Figure 3.8 (Continued)

Figure 3.9 More neurons in *Ctip1$^{fl/fl}$;Emx1-Cre* somatosensory and visual cortex project toward subcerebral targets, and fewer project toward thalamic targets

More neurons in *Ctip1$^{fl/fl}$;Emx1-Cre* cortex are retrogradely labeled by injection of CTB into cerebral peduncle at P1 (A, B), and the distribution of retrogradely-labeled SCPN is uniform rather than highly area-specific (inset; traced from images in A, B). The increase in SCPN is exclusive to somatosensory and visual cortex. Further, crossing wild-type and conditional null mice with a *Rosa26R-tdTomato* reporter reveals fewer projections entering thalamus in conditional null brains (arrows and brackets in G, H), and reduced labeling in thalamus itself (arrows in D, E). DAPI-stained wild-type images of the levels shown are provided for reference (C, F).

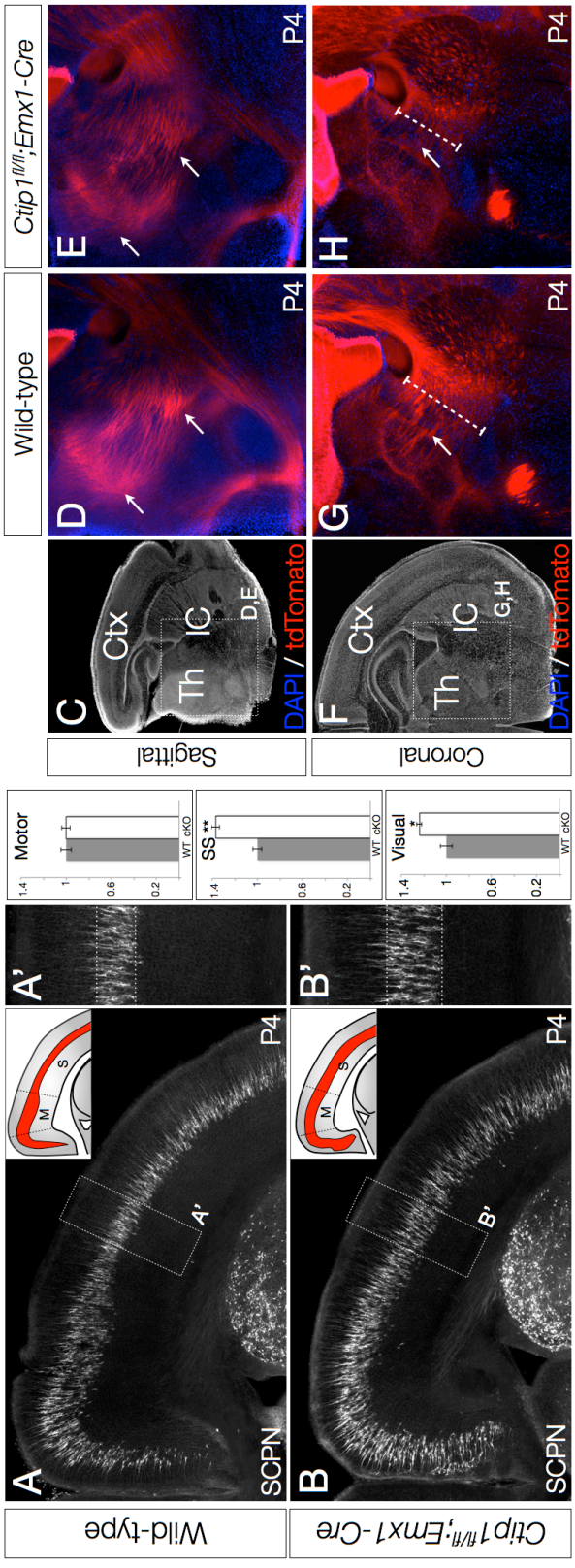

Figure 3.9 (Continued)

time when CThPN are normally generated. We have combined saturating BrdU birthdating at E13.5 and E14.5 with retrograde labeling from the cerebral peduncle to determine whether more retrogradely-labeled neurons in *Ctip1$^{fl/fl}$;Emx1-Cre* brains are born prematurely, and analysis of these data is in progress.

Because expression of genes characteristic of CThPN is reduced in *Ctip1$^{fl/fl}$;Emx1-Cre* cortex, we investigated CThPN projections in the absence of *Ctip1* function. We visualized cortical projections by examining P4 wild-type and *Ctip1$^{fl/fl}$;Emx1-Cre* mice carrying a *Rosa26R-tdTomato* allele (Madisen et al., 2010), which strongly labels all descending projections from cortex. We find that fewer CThPN turn into the thalamus from internal capsule in the absence of *Ctip1* (**Figure 3.9C-H**). We are currently pursuing retrograde labeling experiments with CTB to quantify the degree of reduction in CThPN projections. Taken together, these results indicate that *Ctip1* controls the development of corticothalamic projection neurons by suppressing their alternative differentiation into subcerebral projection neurons.

In contrast to these striking abnormalities of deep-layer CThPN and SCPN allocation, superficial-layer neurons neither express SCPN-specific controls nor project through the cerebral peduncle, except in cingulate cortex (**Figure 3.8, Figure 3.9**). Superficial-layer CPN are normally specified (**Figure 3.10**), and appropriately project to the contralateral hemisphere. Loss of *Ctip1* function contrasts with the loss-of-function phenotype observed for another important negative regulator of SCPN development, *Satb2*, in which large numbers of superficial-layer neurons express CTIP2 and project through the cerebral peduncle (Alcamo et al., 2008; Britanova et al., 2008).

Development of CThPN and CPN pioneer populations is perturbed in the absence of *Ctip1* function

Ctip1 is expressed at high levels by subplate neurons and by superficial-layer neurons in cingulate cortex (**Figure 3.1C**), which pioneer projections to the thalamus and through the corpus callosum, respectively (Koester and O'Leary, 1994; McConnell et al., 1989). Since both corticothalamic and callosal projections are defective in *Ctip1$^{fl/fl}$;Emx1-Cre* brains, we investigated whether development of subplate and cingulate callosal pioneer populations is also abnormal.

Although subplate is still morphologically distinguishable in the absence of *Ctip1* (**Figure 3.4B**), the subplate markers *Ctgf*, *Pcp4*, and NURR1 (Heuer et al., 2003; Molyneaux et al., 2007; Lai et al., 2008; Hoerder-Suabedissen et al., 2009) are nearly absent in P0 *Ctip1$^{-/-}$* cortex (**Figure 3.11A-F**), and, instead, neurons in subplate express aberrantly high levels of *Fezf2* (asterisk in **Figure 3.8D**). Further, subplate axons in *Ctip1$^{-/-}$* mice do not extend as far into the internal capsule at E14.5 as subplate axons in wild-type mice (**Figure 3.11G-I**), suggesting that, with high expression of *Fezf2* and low expression of typical subplate markers, their axons behave like subcerebral projection neurons instead of subplate pioneer axons.

Ctip1$^{fl/fl}$;Emx1-Cre brains have partial agenesis of the corpus callosum, with prominent Probst bundles representing axons that have failed to cross the midline (Richards et al., 2004), and the corpus callosum is thinner in *Ctip1$^{fl/fl}$;Emx1-Cre* mice than in wild-type (**Figure 3.4A-B**). These data suggest that development of callosal projections is disrupted in the absence of *Ctip1*. In addition, many upper-layer neurons in cingulate cortex, the earliest-crossing callosal population, are abnormally

Figure 3.10 Superficial-layer CPN are normally specified in *Ctip1$^{fl/fl}$;Emx1-Cre* mice

Superficial-layer CPN express control genes SATB2 (A-B), CUX1 (C-D), and LHX2 (E-F) in *Ctip1$^{fl/fl}$;Emx1-Cre* brains at P0 as expected.

Figure 3.10 (Continued)

Figure 3.11 Subplate neurons express diminished levels of subplate genes, and defectively pioneer the corticothalamic projection in the absence of *Ctip1*

Subplate neurons normally express *Ctgf*, *Pcp4*, and NURR1 (arrows in A, C, E); all three are strikingly reduced in *Ctip1* null subplate (arrows in B, D, F) at P0, but expression in other populations is unaffected (*e.g.*, arrowheads in E, F). Subplate projections have not pioneered into the internal capsule as far at E14.5 in *Ctip1* null mice as in wild-type (preliminary data G-I; 15 sections counted of n=1 pair genetically labeled with *Rosa26R-tdTomato*). Dotted line in G-H represents the axon front.

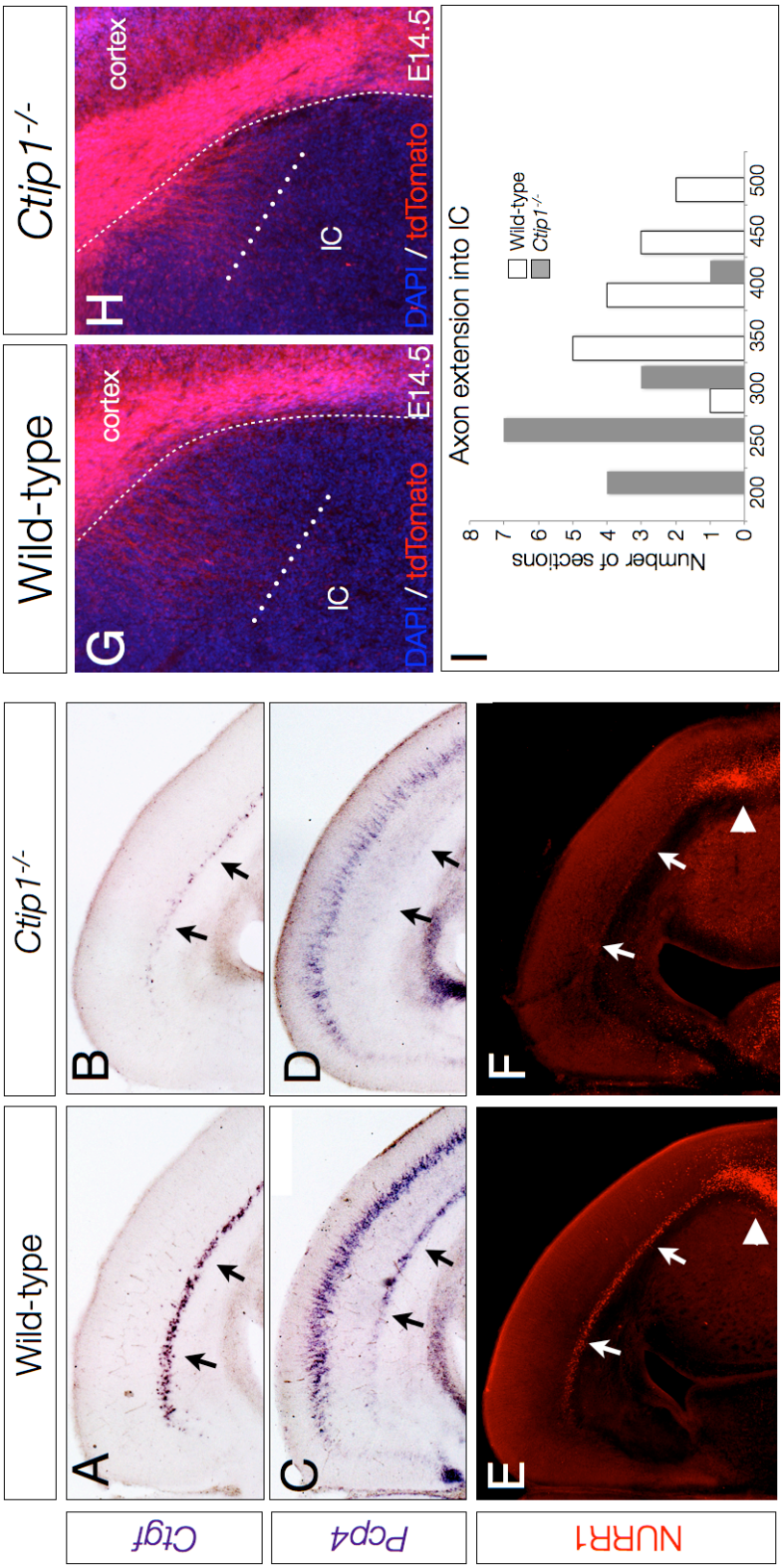

Figure 3.11 (Continued)

retrogradely labeled with injection of CTB from the cerebral peduncle (**Figure 3.9A-B;** close-up in **Figure 3.12G-H**), suggesting that cingulate cortex CPN in *Ctip1*$^{fl/fl}$*;Emx1-Cre* brains are fate-converted to SCPN. Consistent with this interpretation, superficial-layer neurons in cingulate cortex fail to express genes typical of cingulate cortex CPN, such as *Lpl* and *Dkk3* (Molyneaux et al., 2009), and instead aberrantly express CTIP2 (**Figure 3.12A-F**).

To further investigate cingulate pioneer projections in the absence of *Ctip1*, we electroporated CMV/β-actin promoter-driven *Cre*-IRES-*Egfp* into the medial VZ at E13.5, and examined projection patterns at E16.5. These experiments reveal that axons of *Ctip1*$^{fl/fl}$ neurons electroporated with *Cre*-IRES-*Egfp* trail behind those of neurons electroporated only with CMV/β-actin promoter-driven *tdTomato*, and that fewer *Cre*-electroporated neurons have crossed the midline at E16.5 than *tdTomato*-electroporated neurons (**Figure 3.12I-K**). Potentially because cingulate CPN fail to pioneer the callosum, fewer deep-layer neurons cross the callosum in *Ctip1*$^{fl/fl}$*;Emx1-Cre* cortex compared with wild-type (**Figure 3.12L-N**; p=0.005). Strikingly, layer VI CPN (56% fewer cross; p=0.003) are more severely affected than layer V CPN (19% fewer cross; p=0.03), suggesting that later-born CPN are able to follow the few early-born CPN that manage to cross the midline, even in the absence of a fully-functional cingulate pioneer population.

Ctip1 overexpression represses CTIP2 in layer V neurons, preventing them from extending axons subcerebrally

To further investigate genetic cross-repressive interactions between *Ctip1* and *Ctip2*, we tested whether overexpression of *Ctip1* is sufficient to repress endogenous expression of *Ctip2* in wild-type SCPN. We electroporated CMV/β-actin promoter constructs driving expression either of control IRES-*nEgfp* (nuclear EGFP) or of *Ctip1*-IRES-*nEgfp* into the ventricular zone of E12.5 wild-type embryos, and examined CTIP2 expression at P4 by immunocytochemistry (**Figure 3.13A-F**). Strikingly, while many *Egfp*-electroporated layer V neurons are CTIP2-positive, almost no *Ctip1*-electroporated layer V neurons are CTIP2-positive. Conversely, overexpression of *Ctip2* is sufficient to repress endogenous *Ctip1* in upper-layer neurons. When we electroporate CMV/β-actin promoter constructs driving expression of *Ctip2*-IRES-*Egfp* at E14.5, we find that almost no upper-layer neurons express *Ctip1* at P4 compared with upper-layer neurons electroporated with control IRES-*nEgfp* (**Figure 3.14**). High-level expression of *Ctip1*, therefore, is sufficient to repress expression of *Ctip2*, and high-level expression of *Ctip2* is sufficient to repress expression of *Ctip1*. We are pursuing electroporation experiments at E12.5 with *Ctip2*-IRES-*nEgfp* and *Fezf2*-IRES-*nEgfp* to determine whether expression of *Ctip2* or *Fezf2* is sufficient to repress *Ctip1* expression in layer V neurons.

This cross-repression between *Ctip1* and *Ctip2*, a central transcriptional control over SCPN development, motivated us to investigate whether *Ctip1* is also sufficient to prevent layer V neurons from projecting subcerebrally. We electroporated CMV/β-actin promoter-driven IRES-*Egfp* or *Ctip1*-IRES-*Egfp* constructs into wild-type E12.5 embryos, and examined axon projection patterns of electroporated neurons at P4. In

Figure 3.12 Cingulate CPN fail to pioneer the callosum in the absence of *Ctip1* function, impairing projections of deep-layer CPN

Cingulate cortex CPN in *Ctip1*$^{fl/fl}$*;Emx1-Cre* cortex express low levels of characteristic cingulate CPN genes *Dkk3* and *Lpl* (A-D), but express aberrantly high levels of CTIP2 (arrows in E, F) and project to subcerebral targets (arrows in G, H). Cingulate CPN in *Ctip1*$^{fl/fl}$ mice electroporated with *tdTomato* appropriately pioneer the corpus callosum at E16.5 (J), while those electroporated with *Cre* lag behind (K). Failure of cingulate CPN to pioneer the corpus callosum results in a reduction of retrogradely-labeled CPN in layers V and VI (L-N).

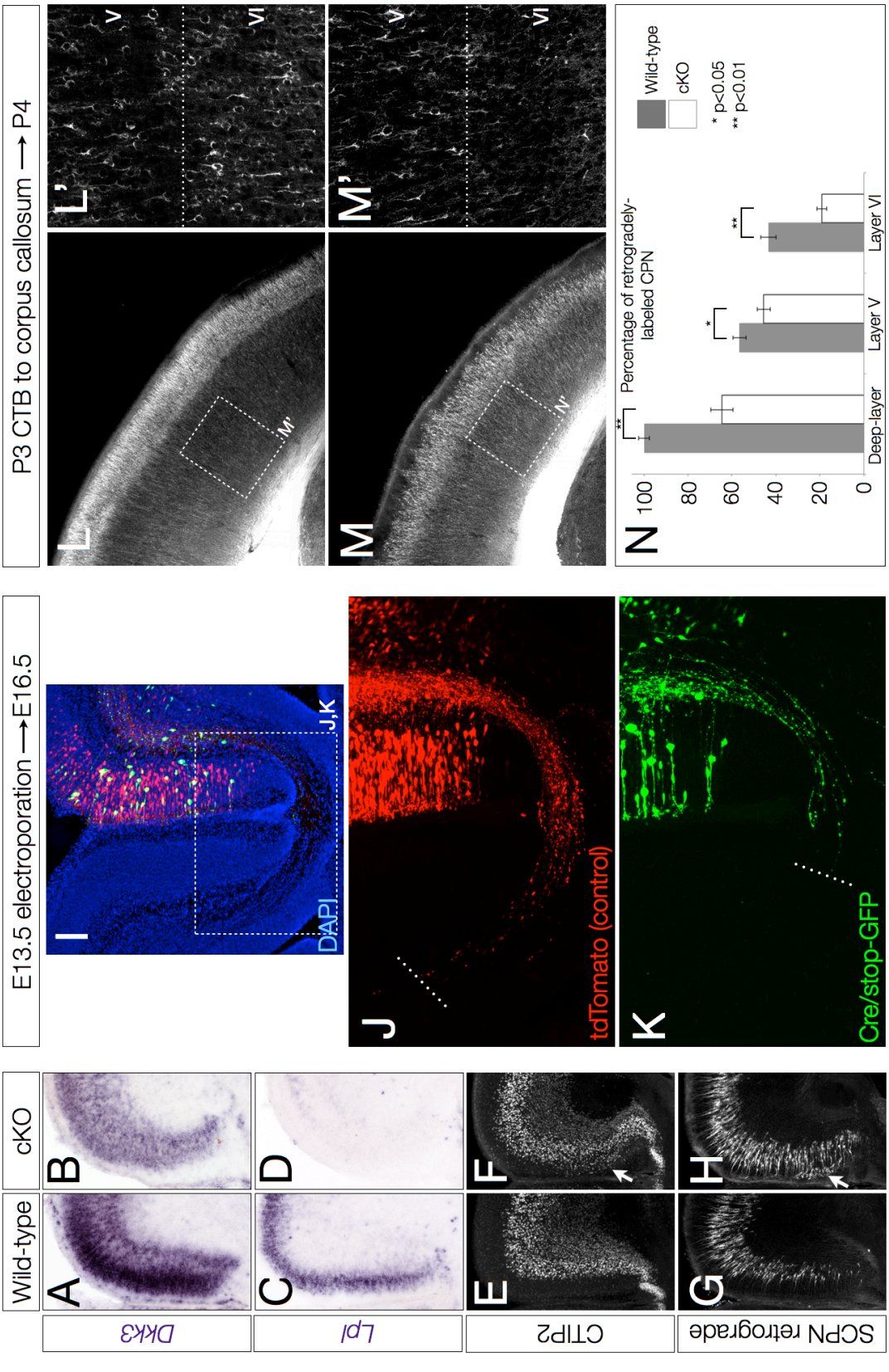

Figure 3.12 (Continued)

Figure 3.13 *Ctip1* misexpression *in vivo* at E12.5 represses SCPN identity and projection to the spinal cord

Many neurons electroporated with nuclear *Egfp* (*nEgfp*) at E12.5 express CTIP2 (A-C), while almost no neurons electroporated with *Ctip1*-IRES-*nEgfp* do so (D-F). Neurons electroporated with *Ctip1*-IRES-*Egfp* send few projections to brainstem (G-H). Neurons electroporated at E12.5 with *Ctip1*-IRES-*nEgfp* send fewer projections to spinal cord by P2 than those electroporated with *nEgfp* (I-N, U), while sending more projections across the corpus callosum (O-T, V).

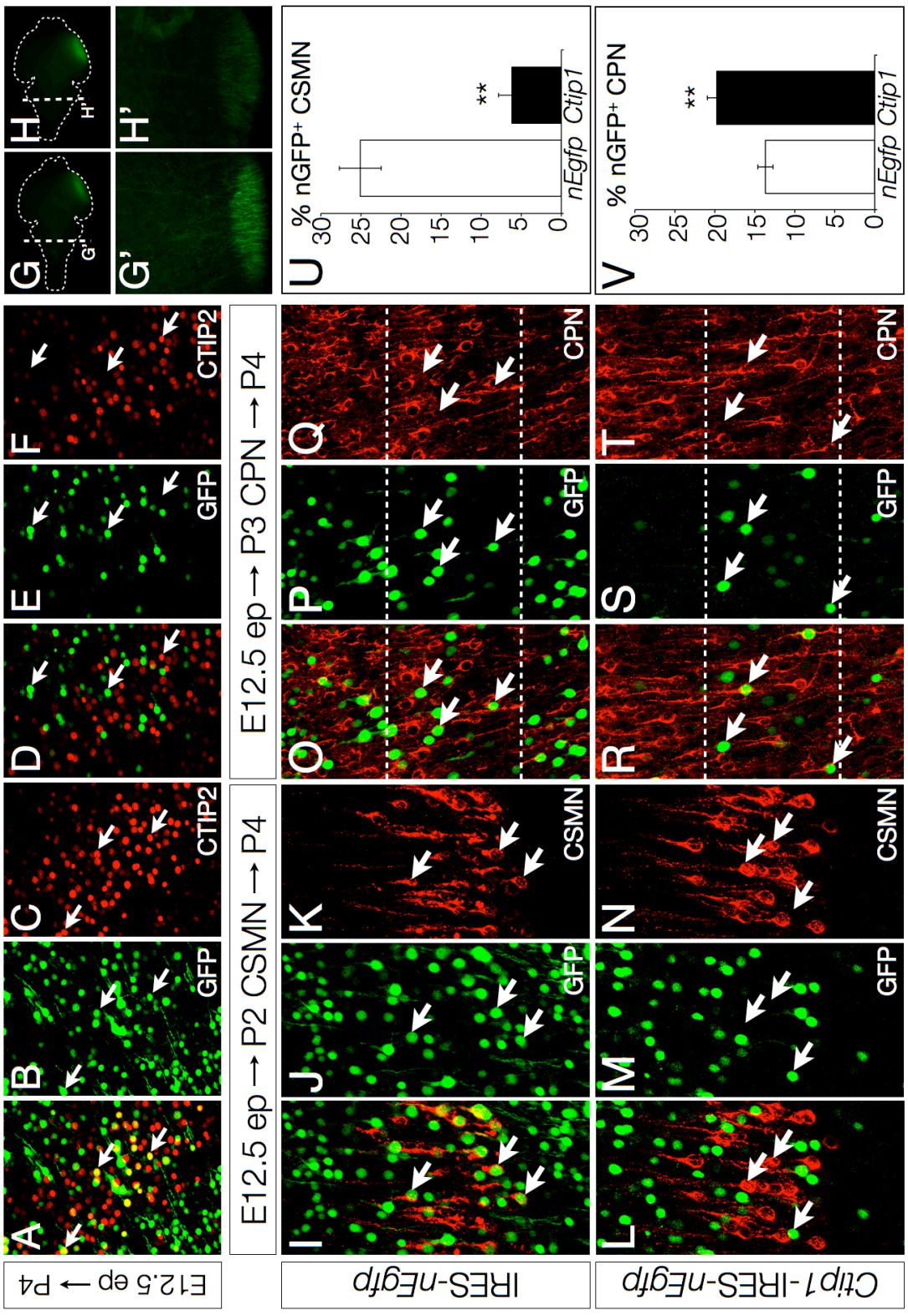

Figure 3.13 (Continued)

agreement with the results above, there are strikingly fewer axons from *Ctip1*-overexpressing neurons in cerebral peduncle at P4 (**Figure 3.13G-H**), while many axons are visible terminating in the thalamus, or passing through the corpus callosum. This indicates that *Ctip1* specifically represses the development of subcerebral projections, while not impairing callosal or corticothalamic projections.

Because large numbers of neurons project to thalamus and across the corpus callosum even when electroporated with IRES-*Egfp* alone, any axons added due to rerouting of comparatively few SCPN by overexpression of *Ctip1* might be difficult to identify. Therefore, we pursued further experiments combining electroporation of nuclear-localized EGFP (*nEgfp*) with retrograde labeling, which enables quantitative assessment of the projection patterns of deep-layer neurons in response to *Ctip1* overexpression. We electroporated CMV/β-actin promoter-driven IRES-*nEgfp* or *Ctip1*-IRES-*nEgfp* constructs into wild-type embryos at E12.5, then retrogradely labeled SCPN or CPN with CTB. Strikingly, we find a five-fold reduction in the percentage of *Ctip1*-electroporated neurons projecting to spinal cord, compared with *nEgfp*-electroporated neurons (25% vs. 6%; p=0.008; **Figure 3.13I-N**). Further, we find a small, but significant increase in the number of neurons projecting across the corpus callosum (20% vs. 13%; p=0.017; **Figure 3.13O-T**), indicating that axons of *Ctip1*-electroporated neurons are redirected from cerebral peduncle toward contralateral cortical targets. We are currently pursuing the same electroporation experiments, combined with retrograde labeling from thalamus, to determine whether some *Ctip1*-electroporated neurons are also redirect to thalamic targets. Taken together, these data demonstrate that *Ctip1* overexpression is sufficient to respecify SCPN to adopt gene expression and projection patterns characteristic of CPN, and potentially also of CThPN.

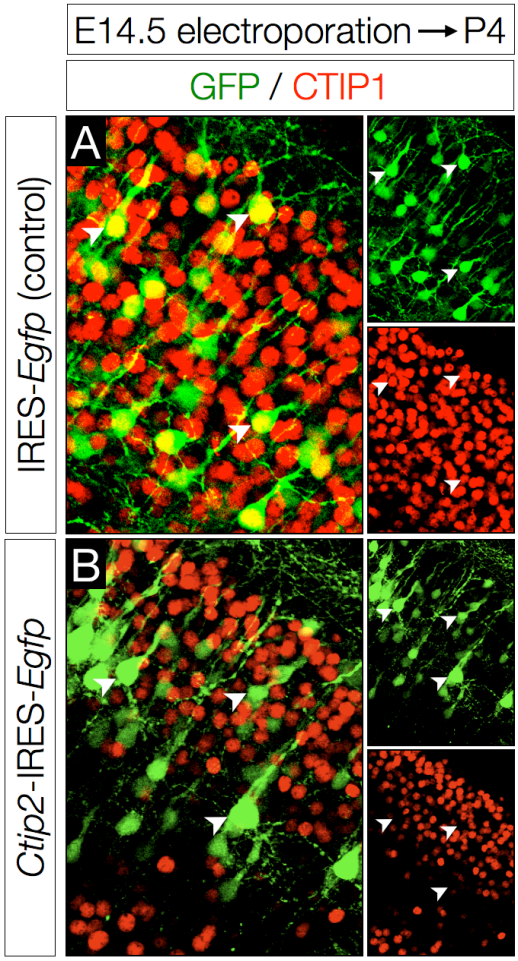

Figure 3.14 *Ctip2* overexpression in superficial-layer CPN reduces expression of CTIP1

Many neurons electroporated with *Egfp* at E14.5 express CTIP1 (A), but no neurons electroporated with *Ctip2*-IRES-*Egfp* co-express CTIP1 (B).

3.4 Discussion

Although several key controls over projection neuron subtype development have been identified in recent years, it is clear that additional transcriptional regulators remain to be discovered, particularly those that exert fine control over the final distribution of subtypes present in specific areas of cortex. In this work, we show that the transcription factor *Ctip1* directs the allocation of corticothalamic, subcerebral, and callosal projection neurons in deep cortical layers.

Ctip1 is expressed by maturing corticothalamic and callosal projection neurons, and is excluded early in subtype development from subcerebral projection neurons. In the absence of cortical *Ctip1*, more deep-layer neurons adopt gene expression and projection patterns characteristic of SCPN, at the expense of CThPN and deep-layer CPN. Overexpression of *Ctip1 in vivo* is sufficient to induce layer V neurons to project to contralateral cortex rather than to spinal cord. *Ctip1* also directs the development of callosal and corticothalamic pioneer populations, and in the absence of *Ctip1*, pioneer projections from cingulate cortex and subplate are defective. Independent of its functions in specifying deep-layer CPN, *Ctip1* controls migration of upper-layer CPN, and in the absence of *Ctip1*, late-born neurons are cell-autonomously delayed in entering the cortical plate. We conclude that *Ctip1* directs the precision of cortical development by controlling the subtype-specific differentiation of deep-layer projection neurons, and the migration of superficial-layer projection neurons.

Relationship between *Ctip1* and *Couptf1*

Ctip1 was originally identified as a *Couptf*-interacting protein (Avram et al., 2000), and *Couptf1* regulates migration of upper-layer callosal projection neurons by repressing expression of the small GTP-binding protein *Rnd2* (Heng et al., 2008; Alfano et al., 2011). Therefore, we first hypothesized that *Ctip1* might interact with *Couptf1* to regulate migration through *Rnd2*. However, in the absence of *Ctip1* function, expression of *Rnd2* is normal (**Figure 3.5A-B**), and that *Ctip1* instead directs upper-layer neuron migration through the *Reelin* pathway.

Furthermore, we do not observe the same abnormalities in deep-layer neuron specification in the absence of *Ctip1* that are seen in the absence of *Couptf1*, including a "motorized" layer VI and extensive co-expression of CTIP2 and TBR1 in layer VI (Tomassy et al., 2010). In *Ctip1$^{fl/fl}$;Emx1-Cre* mice, more large pyramidal neurons in layer V express CTIP2, and these neurons project in expected proportions to the spinal cord and other subcerebral targets (**Figure 3.8**, **Figure 3.9**). In contrast, in *Couptf1* mutants, layer V neurons project subcerebrally, but not to the spinal cord; only layer VI neurons project to the spinal cord (Tomassy et al., 2010). Loss of *Couptf1* causes a temporal shift in deep-layer subtype identity, with superficial layer VI neurons taking on characteristics of CSMN, and genuine layer V CSMN failing to connect with the spinal cord, while loss of *Ctip1* expands SCPN (and CSMN) as a population at the expense of CThPN.

Although *Ctip1* and *Couptf1* are capable of interacting *in vitro* (Avram et al., 2000), we find no evidence to suggest that they interact to control either subtype specification or migration in the developing cortex.

Migration control over superficial-layer neurons

Expression of *Ctip1* mRNA can be seen at E14.5 in the pallial ventricular zone (**Figure 3.2A**), suggesting that *Ctip1* begins to be transcribed by postmitotic superficial-layer projection neurons as they exit the cell cycle and begin to migrate into the cortical plate. In the absence of *Ctip1*, these superficial-layer CPN are specified normally, but they are delayed in entering the cortical plate and lag behind wild-type neurons even at P4, when migration is largely finished. We do not find significant abnormalities in the migration of projection neurons born at E11.5, E12.5, or E13.5 by BrdU birthdating, although all cortical layers are indistinct by Nissl stain (**Figure 3.4B**). It is possible that deep-layer neurons also become positioned in final laminar locations with less precision in the absence of *Ctip1*, but that this is not discernible because deep-layer neurons do not need to migrate as far to reach their final locations in the cortical plate. Disturbances in deep-layer neuron migration, therefore, might be more difficult to identify by BrdU birthdating.

Reelin, *Dab1*, and *Vldlr*, components of the *Reelin* signaling pathway, are strikingly misregulated in *Ctip1* conditional null cortex. Although migration of all cortical projection neurons is defective in *Reelin* null mice (Caviness 1982; Boyle et al., 2011), loss of other components of the *Reelin* pathway appear to affect upper-layer and deep-layer neurons differentially (Herrick and Cooper, 2004; Hack et al., 2007; Franco et al., 2011). This is perhaps because deep-layer neurons primarily migrate via somal translocation, while upper-layer neurons are guided by radial glial processes (Nadarajah and Parnavelas, 2002), and, therefore, different molecular signals are necessary for upper-layer neurons to start and stop migration, compared with deep-layer neurons.

Other migration-related genes downstream of *Ctip1* might regulate interaction of migrating neurons with radial glia, causing *Ctip1*-deficient superficial-layer neurons to migrate poorly.

Subtype control over deep-layer neurons

Ctip1 controls the balance between specification of different subtypes of deep-layer cortical projection neurons, and in the absence of *Ctip1*, more SCPN are generated at the expense of CThPN and deep-layer CPN. Although *Ctip1* is also expressed at high levels by superficial-layer CPN, these neurons are specified normally in the absence of *Ctip1*, and successfully cross the corpus callosum (see superficial layers in **Figure 3.12L-M**). These data are in agreement with the hypothesis that superficial-layer and deep-layer CPN are substantially different from each other, perhaps reflecting distinct evolutionary events of projection neuron diversification (Molyneaux et al., 2009; Azim et al., 2009b; Fame et al., 2011). *Ctip1* is expressed both by superficial-layer and deep-layer CPN, but is necessary for subtype specification only for deep-layer CPN.

In *Ctip1*$^{fl/fl}$*;Emx1-Cre* mice, neurons with molecular and anatomical characteristics of SCPN expand radially into what would normally be layer VI, indicating that CThPN located in the more superficial segment of layer VI are fate-converted to SCPN. This population of CThPN, located at the interface between layer V and layer VI, is particularly vulnerable to switching fates due to ectopic expression of SCPN genetic controls (Tomassy et al., 2010; M. J. Galazo and J. D. Macklis, unpublished data). In the absence of *Ctip1*, all layer VI neurons express higher-than-normal levels of *Fezf2* and

Ctip2 (asterisks in **Figure 3.8B, D**); however, only those neurons located in the more superficial segment of layer VI cease to express CThPN controls (**Figure 3.8I-N**) and consequently project to the cerebral peduncle (**Figure 3.8A-B**). The existence of this subpopulation of CThPN, which is generated immediately before SCPN specification begins, and which resides immediately adjacent to layer V, suggests that these closely-related corticofugal neurons require precise transcriptional control by *Ctip1* and other genes for correct specification into distinct projection neuron subtypes, correctly allocating CThPN and SCPN depending on cortical area.

Intriguingly, *Ctip1* controls subtype specification, but is itself non-subtype-specific, as it is expressed at high levels by superficial-layer CPN, deep-layer CPN, and CThPN (**Figure 3.1D-F**). Although some previously-identified controls are expressed by multiple subtypes, they are generally expressed at high levels by one subtype and low levels by another: *Ctip2* and *Fezf2* by SCPN (high) and CThPN (low), *Tbr1* by CThPN (high) and upper-layer CPN (low), and *Sox5* by CThPN (high) and SCPN (low) (Arlotta et al., 2005; Molyneaux et al., 2005; Hevner et al., 2001; Lai et al., 2008). *Ctip1*, in contrast, is specific only in its exclusion from SCPN. This widespread expression pattern explains why *Ctip1* was not previously identified as a candidate in microarray-based screens for genes involved in subtype specification (Arlotta et al., 2005; Chen et al., 2005a, 2005b). These data suggest that further unidentified controls over subtype development might expand or contract one population of neurons by action in multiple physically or temporally adjacent populations.

Ctip1* and *Ctip2

Ctip1 and *Ctip2*, paralogous and highly similar transcription factors, are initially co-expressed in developing cortex, but later are expressed in a complementary fashion, with CTIP1 expressed by CPN and CThPN, and CTIP2 expressed by SCPN (**Figure 3.1**). Expression of CTIP2 increases in the absence of *Ctip1*, and expression of CTIP1 increases in the absence of *Ctip2* (**Figure 3.3**). Furthermore, overexpression of either *Ctip1* or *Ctip2 in vivo* is sufficient to repress expression of the other in both deep-layer and superficial-layer neurons (**Figure 3.13**, **Figure 3.14**). From these data, we conclude that *Ctip1* and *Ctip2* cross-repressively interact to control the development of cortical projection neurons. *Ctip1* and *Ctip2* are thought to repress each other in development of other cell types, as well; *Ctip1* expression is sharply downregulated as *Ctip2* begins to be expressed in maturing T cells (Tydell et al., 2007), and expression of *Ctip1* is significantly increased in P0 *Ctip2*$^{-/-}$ striatal medium-sized spiny neurons (Arlotta et al., 2008).

In conclusion, our results indicate that *Ctip1* is a central functional control over the precision of neocortical development. *Ctip1* directs the development of corticothalamic and callosal projection neurons at multiple stages. First, *Ctip1* specifies subtype identity in deep-layer neocortical projection neurons, preventing corticothalamic and callosal projection neurons from acquiring characteristics of subcerebral projection neurons. Second, *Ctip1* directs the development of subplate neurons and cingulate cortex neurons, allowing these populations to pioneer corticothalamic and callosal projections, respectively. Finally, *Ctip1* controls the migration of superficial-layer callosal projection neurons, allowing these neurons to

settle in a precise laminar location. Identification of further fine transcriptional controls over the precise differentiation, migration, and connectivity of neocortical projection neurons will allow a better understanding of the staggering complexity of the mature neocortex.

3.5 Experimental Procedures

Animals

All mouse studies were approved by the Massachusetts General Hospital and/or 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. Unless noted otherwise, all experiments with *Ctip1*$^{fl/fl}$*;Emx1-Cre* were controlled with *Ctip1*$^{wt/wt}$*;Emx1-Cre*, and *Ctip1*$^{-/-}$ with *Ctip1*$^{wt/wt}$.

Ctip1$^{fl/fl}$ mice were generated by Tucker and colleagues (Sankaran et al., 2009). *Ctip1*$^{-/-}$ mice were generated by Copeland and colleagues (Liu et al., 2003), and were obtained from the RIKEN BioResource Center. *Emx1-Cre* (stock number 005628) and *Rosa26-tdTomato-Ai9* (stock number 007909) mice were purchased from Jackson Laboratories.

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. Primary antibodies and dilutions used: rat anti-BrdU, 1:500 (Accurate); mouse anti-CTIP1 clone 14B5, 1:500 (Abcam), rabbit anti-CTIP2, 1:200 (Abcam); rat anti-CTIP2,

1:200 (Abcam); rabbit anti-DARPP32, 1:250 (Chemicon); rabbit anti-FOG2, 1:250 (Santa Cruz); chicken anti-GFP, 1:200 (Aves); rabbit anti-GFP, 1:500 (Invitrogen); rabbit anti-NFIB, 1:200 (Active Motif); goat anti-NURR1, 1:100 (R&D Systems); mouse anti-SATB2, 1:200 (Abcam); rabbit anti-TLE4, 1:200 (Santa Cruz); rabbit anti-TBR1, 1:200 (Santa Cruz). With the exception of rabbit/chicken anti-GFP, staining for all antibodies was optimized by a 10-minute antigen retrieval at 95°C in 0.01M citric acid, pH 6.0; rat anti-BrdU requires a 90-minute antigen retrieval at room temperature in 2N HCl. Secondary antibodies were chosen from the Alexa series (Invitrogen), and used at a dilution of 1:500.

Non-radioactive *in situ* hybridization was performed as previously described (Arlotta et al., 2005). Probes for *Clim1*, *Ctgf*, *Fezf2*, *Pcp4*, and *S100a10* were previously described (Arlotta et al., 2005, Lai et al., 2008). RT-PCR was used to generate GenePaint-validated riboprobes for *Apoer2* (riboprobe 4194), *Dab1* (riboprobe 305), *Rnd2* (riboprobe 1717), and *Vldlr* (riboprobe 993).

BrdU birthdating

Timed pregnant females were intraperitoneally injected with bromodeoxyuridine (50 mg/kg) at E11.5, E12.5, E13.5, E14.5, or E15.5. Littermate pairs of *Ctip1*$^{fl/fl}$*;Emx1-Cre* and *Ctip1*$^{wt/wt}$*;Emx1-Cre* pups were collected at P4 and processed for BrdU immunocytochemistry (Magavi 2000). Six anatomically-matched sections from each mouse (n=4 of each genotype at each age) were selected, and single confocal slices of somatosensory cortex were imaged. Images were counted by investigators blinded to genotype (Molyneaux et al., 2005), dividing cortex into ten bins of equal size.

In utero electroporation

For overexpression experiments, a CMV/β-actin promoter plasmid (derived from CBIG; gift of C. Lois) was used to drive expression of IRES-*Egfp* (control) or *Ctip1 XL*-IRES-*Egfp* (experimental); or IRES-*nEgfp* (nuclear EGFP) (control) or *Ctip1 XL*-IRES-*nEgfp* (experimental) in CD1 timed pregnant females at E12.5. For some experiments, pups were screened for *Egfp* expression on a fluorescent dissecting microscope at birth, and electroporated pups were retrogradely labeled (as described below). Brains were collected at P4.

For loss-of-function experiments, two CMV/β-actin promoter plasmids were co-electroporated, driving expression of *tdTomato* and *Cre*-IRES-*Egfp* in $Ctip1^{wt/wt}$ (control) or $Ctip1^{fl/fl}$ (experimental) embryos. For migration experiments, plasmids were electroporated at E14.5, and brains were collected at E17.5. For cingulate CPN experiments, plasmids were electroporated at E13.5, and brains were collected at E16.5. Electroporation conditions were described previously (Molyneaux 2005).

Retrograde labeling

Projection neurons were labeled from their axon termini (spinal cord, cerebral peduncle, thalamus, or contralateral cortex) under ultrasound guidance between P1 and P3 by pressure injection of Alexa fluorophore-conjugated cholera toxin B (Invitrogen). Tissue was collected at P4, and processed for immunocytochemistry.

Six anatomically-matched sections from each mouse (n=4 for each genotype) were selected, and single confocal slices were imaged, then images were counted by investigators blinded to genotype.

RNA sequencing (RNA-seq)

Whole somatosensory cortex was dissected from P4 wild-type and *Ctip1*$^{fl/fl}$*;Emx1-Cre* mice. RNA libraries were prepared with the NEBNext RNA Library Preparation Kit (New England Biolabs), and sequenced on an Illumina HiSeq 2000. Data analysis was performed using the TopHat and Cufflinks software tools (Trapnell et al., 2012).

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Chapter 4

Ctip1 controls refinement of area identity and organization of sensory maps in the developing neocortex

Publication

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*equally-contributing first authors

Author contributions

This project was initiated independently by M.D./Ph.D. student Luciano Custo Greig, and we performed experimental work in equal collaboration. All experimental work and figure preparation was completed by Luciano and me together, and we wrote the manuscript together. Former undergraduate Kevin Liu contributed quantitative PCR. Technician Chloé Greppi performed *in situ* hybridization.

4.1 Abstract

While great progress has been made toward identifying transcription factor controls over arealization of neocortical progenitors, much less is known about the postmitotic regulators that transform continuous expression gradients into sharp areal boundaries and direct neocortical projection neurons to acquire areally-appropriate phenotypic characteristics. In this chapter I show that *Ctip1* is critical for developmental refinement of area identity. I find that *Ctip1* is highly expressed in primary sensory areas during early postnatal development, and that it is a critical control over refinement of molecular area identity, areal precision of CPN and SCPN connectivity, organization of thalamocortical axons into sensory maps, and aggregation of layer IV neurons into barrels. These experiments represent the first investigation of transcriptional mechanisms underlying homotypic CPN connectivity and provide the first demonstration that cortical transcription factors not only dictate the size and position of primary sensory areas, but are also required for the organization of thalamocortical input into sharply-defined and appropriately-shaped maps.

4.2 Introduction

The neocortex is responsible for processing different modalities of sensory information, generating precise motor output, and conducting higher-order cognitive tasks. This broad range of functions is made possible by its tangential organization into specialized areas defined by distinct cytoarchitecture, connectivity, and patterns of gene expression (Rakic et al., 1988). In rodents, the neocortex is organized into four primary areas: motor (M1), somatosensory (S1), visual (V1), and auditory (A1). Area identity begins to be specified early in development by morphogens and signaling molecules secreted from early patterning centers, which induce graded ventricular zone expression of several key transcription factors, including *Emx2*, *Couptf1*, *Pax6*, and *Sp8* (Rash et al., 2006). These progenitor-level controls have been extensively studied, and appear to determine the final size and position of cortical areas (O' Leary et al., 2007).

Substantially less is known about the postmitotic regulators that transform these continuous expression gradients into sharp areal boundaries, direct neurons to acquire areally appropriate projections, and organize local cortical cytoarchitecture to receive modality-specific input. Only two controls, the basic-helix-loop-helix transcription factor *Bhlhb5* and the LIM-homeodomain transcription factor *Lmo4*, have been reported to function in postmitotic acquisition of area identity (Joshi et al., 2008; Ross et al., 2012; Sun et al., 2005; Huang et al., 2009). *Bhlhb5* is highly expressed in somatosensory and primary visual cortex, while *Lmo4* is expressed in a complementary pattern, strongest in motor cortex and higher-order visual areas (Joshi et al., 2008; Sun et al., 2005). In the absence of *Bhlhb5*, molecular area identity is strikingly disrupted in somatosensory and caudal motor cortex (Joshi et al., 2008). Loss of *Lmo4* function also causes shifts in

area-specific gene expression, but these changes are less extensive (Huang et al., 2009). While these studies have begun to define molecular mechanisms directing area-specific differentiation, many important questions remain to be addressed.

In particular, few controls over area-specific projection patterns have been identified, even though it has long been appreciated that projection neurons of the same subtype residing in different cortical areas send axons to distinct targets. Corticothalamic projection neurons (CThPN) establish reciprocal connections with specific thalamic nuclei in an area-dependent manner (Jones, 1985). Subcerebral projection neurons (SCPN) send axons to the spinal cord and establish collaterals to pontine, midbrain, and cerebellar nuclei, but their final connectivity is determined by developmental pruning, such that SCPN in motor cortex maintain projections to the spinal cord (corticospinal motor neurons; CSMN), and SCPN in visual cortex maintain projections to the optic tectum (corticotectal projection neurons; CTPN) (Stanfield et al., 1982; Thong and Dreher, 1986). Lastly, callosal projection neurons (CPN) extend axons to mirror-image locations on the contralateral hemisphere, enabling highly-organized inter-hemispheric transfer and integration of motor, somatosensory, visual, and auditory information (Yorke and Caviness, 1975). Little is known about the postmitotic controls that bring about these areal differences in projection patterns. Although areal refinement of SCPN connectivity requires *Otx1* function (Weimann et al., 1999), it remains unclear how this transcription factor, which is broadly expressed in all SCPN, selectively instructs neurons in visual cortex to eliminate their projections to the spinal cord.

Another important aspect of area identity is the ability of defined regions of cortex to attract thalamocortical axons, and organize sensory input into topographic maps. Several lines of investigation indicate that cortex-autonomous programs

determine the size, shape, and position of sensory maps (O'Leary et al., 2007). Perhaps the most definitive evidence in favor of this model is that cortex-specific deletion of *Couptf1* causes a striking caudal shift of sensory maps, which become substantially smaller as motor cortex expands into sensory territory (Armentano et al., 2007). Importantly, other aspects of sensory map formation appear to be autonomously controlled by thalamic afferents. For instance, when a slab of V1 cortex is transplanted into S1 territory, thalamocortical axons are able to cluster normally and induce barrel cytoarchitecture in the transplanted tissue (Schlaggar and O'Leary, 1991). Activity-dependent mechanisms are also critical for normal differentiation of sensory areas, and a number of pre- and post-synaptic neurotransmitter-receptor pathways have been implicated in this process (López-Bendito and Molnár, 2003; Wu et al., 2011). However, the molecular controls that direct the cytoarchitectural reorganization of cortical neurons are not well-understood (Li and Crair, 2011). Ectopic overexpression of the transcription factor *Rorb* in non-sensory areas induces formation of barrel-like clusters that attract thalamocortical input, but it is not known whether *Rorb* is necessary for barrel development (Jabaudon et al., 2011). Here, we identify *Ctip1* as a critical control over cytoarchitectural organization of layer IV and formation of sensory maps, as well as postnatal refinement of molecular area identity and area-specific connectivity of SCPN and CPN.

Ctip1 was first described as an interacting partner of COUP transcription factors (COUP-TFs), which recruit *Ctip1* to potentiate transcriptional repression (Avram et al., 2000). However, *Ctip1* is itself a transcription factor, and can regulate gene expression independently of COUP-TFs by binding specific DNA sequence motifs through its zinc finger domains (Avram et al., 2002; Xu et al., 2010). *Ctip1* has been most extensively studied in the hematopoietic system, in which it controls specification of B-cells (Liu et

al., 2003), and regulates the developmental switch from γ-globin to β-globin in red blood cells (Sankaran et al., 2008; Sankaran et al., 2009; Xu et al., 2011), but it is also important in the nervous system. *In vitro* studies support a role for *Ctip1* in hippocampal neuron axon branching and dendrite outgrowth (Kuo et al., 2009; Kuo et al., 2010). Most recently, *Ctip1* was reported to regulate dorsal interneuron morphogenesis and sensory circuit formation in the spinal cord (John et al., 2012). However, beyond broad descriptions of expression in cortex (Leid et al., 2004; Kuo and Hsueh, 2007), *Ctip1* has not been functionally investigated in cortical development.

Although *Ctip1* is uniformly expressed throughout the cortical plate early in development, our studies reveal that this expression pattern is later refined, such that *Ctip1* expression remains high in primary sensory areas, and becomes very sparse in motor cortex. This observation led us to hypothesize that *Ctip1* might regulate acquisition of projection neuron area identity. We investigated this possibility by examining area-specific gene expression, projection patterns, and sensory input maps in cortex-specific *Ctip1* conditional null mutant mice. While there are only subtle defects in molecular area identity at birth, there is a striking failure of subsequent delineation of sensory areas, as indicated by absent, reduced, or ectopic expression of multiple area-specific genes, including the area identity controls *Bhlhb5* and *Lmo4*. In addition, SCPN in visual cortex aberrantly maintain spinal axons, and CPN project inappropriately to non-mirror image coordinates on the contralateral hemisphere. The topographic organization of thalamocortical input is severely disrupted, and barrel cytoarchitecture completely fails to emerge in somatosensory cortex. Mosaic analysis indicates that, in the context of otherwise wild-type cortex, *Ctip1* null neurons are excluded from layer IV,

and their dendrites avoid thalamocortical input. In an accompanying manuscript, we find that *Ctip1* also critically controls subtype specification in deep cortical layers, and directs migration of superficial-layer projection neurons (Chapter 3).

4.3 Results

Ctip1 is highly expressed in primary sensory areas

CTIP1 can first be detected in the cortical plate at E12.5, as it begins to be expressed by newly-born postmitotic glutamatergic neurons (Chapter 3). During these early stages of forebrain development, CTIP1 is present at approximately equivalent levels throughout the mediolateral and rostrocaudal extents of cortex (**Figure 4.1A and 4.1B**). However, over the first week of postnatal development, *Ctip1* expression undergoes progressive areal refinement. By P4, a clear difference already exists between motor cortex rostrally, where expression of *Ctip1* is lower, and sensory areas caudally, where expression of *Ctip1* is higher (**Figure 4.1C**). By P7, after further refinement, it is possible to distinctly recognize primary somatosensory, visual, and auditory cortex by CTIP1 immunocytochemistry (**Figure 4.1D**). Notably, expression of *Ctip1* is not uniformly patterned in all layers of the neocortex. Areal differences are most striking in layers VI, IV, and deeper II/III, although expression is sparser and levels are lower in motor cortex across all layers. Only subplate exhibits uniform expression of *Ctip1* across all cortical areas.

Figure 4.1 Neocortical expression of *Ctip1* becomes areally patterned as the neocortex matures

CTIP1 is uniformly expressed by newly postmitotic cortical plate neurons at E12.5 (A) and E16.5 (B). By P4, CTIP1 expression is higher in sensory cortex (C'') than in motor cortex (C'), and, by P7, CTIP1 is highly expressed in primary somatosensory, visual, and auditory cortex (D).

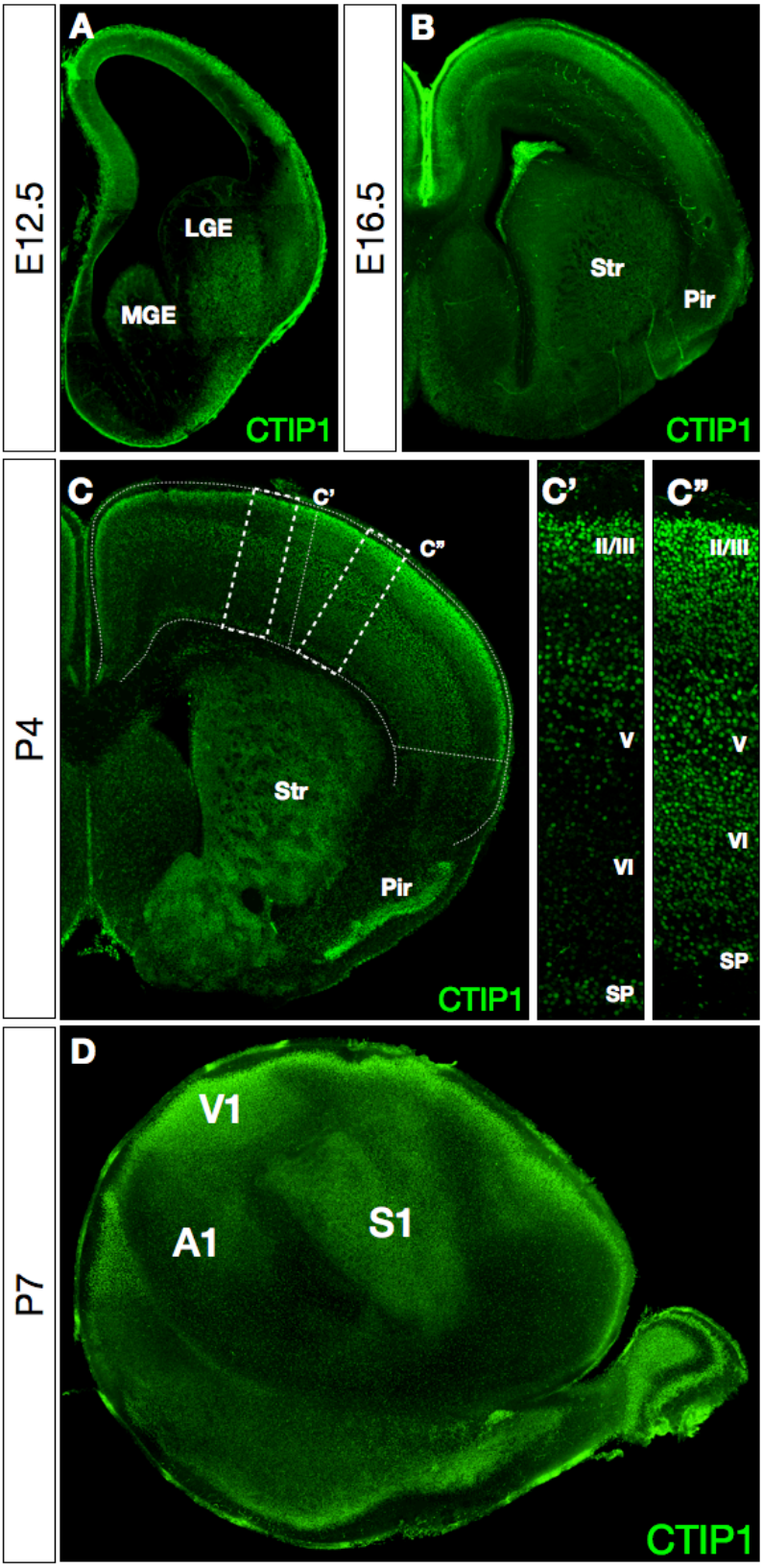

Figure 4.1 (Continued)

Figure 4.2 *Ctip1* is homogeneously deleted by *Emx1-Cre*

Emx1-Cre deletes *Ctip1* across all areas of cortex in *Ctip1*$^{fl/fl}$*;Emx1-Cre* mutants (A-B)**.**

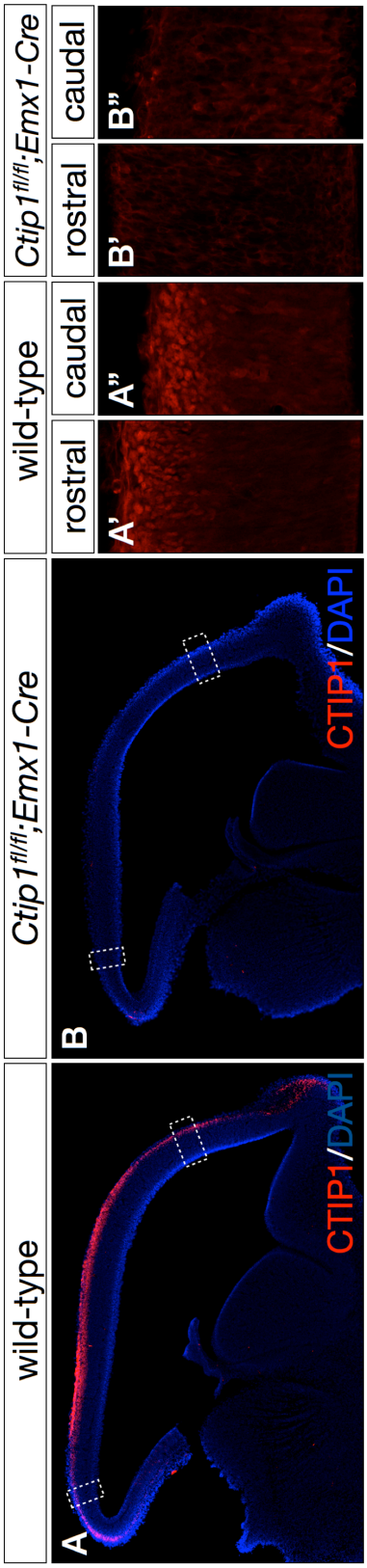

Figure 4.2 (Continued)

Acquisition of molecular area identity is extensively disrupted in the absence of *Ctip1*

This tangential refinement of *Ctip1* expression motivated us to investigate potential roles of *Ctip1* in postmitotic acquisition of area identity. Because *Ctip1*$^{-/-}$ mice die at birth (Liu et al., 2003), and cortical arealization continues to develop over the first week of postnatal development, we pursued loss-of-function studies using *Ctip1*$^{fl/fl}$*;Emx1-Cre* mice, which survive to adulthood. *Emx1-Cre* is known to mediate recombination in pallial progenitors beginning as early as E10.5 (Gorski et al., 2002), two days before the onset of CTIP1 expression. Accordingly, there is no detectable expression of CTIP1 in the cortical plate of these mutants. Although *Emx1* is expressed in a high-posteromedial to low-anterolateral gradient (Gulisano et al., 1996), even low levels of *Cre* expression appear to be sufficient to mediate recombination, as *Emx1-Cre* uniformly deletes *Ctip1* along the entire rostrocaudal and mediolateral extents of cortex (**Figure 4.2**). Importantly, these data rule out the possibility that areal phenotypes result from incomplete recombination in some regions of *Ctip1*$^{fl/fl}$*;Emx1-Cre* cortex.

As a first step toward interrogating area development in the absence of *Ctip1* function, we examined expression of *Lmo4* and *Bhlhb5*. Whole-mount β-galactosidase staining was performed at P4 on brains from *Ctip1*$^{fl/fl}$*;Emx1-Cre* or wild-type mice carrying either an *Lmo4-LacZ* or a *Bhlhb5-LacZ* reporter allele (Deng et al., 2010; Feng et al., 2006). In wild-type mice, *Lmo4* is highly expressed rostrally in motor cortex, and caudally in higher-order visual areas, but is excluded from somatosensory cortex, with sharp boundaries between these regions (**Figure 4.5A**), while *Bhlhb5* is expressed only in primary sensory areas (**Figure 4.5C**). Strikingly, we find that, in the absence of *Ctip1* function, expression of *Lmo4* in motor and cingulate cortex expands into

somatosensory territory, and occipital expression becomes compressed to a narrow band, such that visual cortex (V1) is no longer discernible (**Figure 4.5B**). *Bhlhb5* expression in sensory areas becomes diffuse, such that primary somatosensory, visual, and auditory cortex can no longer be discerned (**Figure 4.5D**). Notably, while the boundaries between areas become blurred, expression of *Bhlhb5* and *Lmo4* remains complementary, indicating that the cross-repressive genetic interactions directing areal segregation of *Bhlhb5* and *Lmo4* expression are maintained even in the absence of *Ctip1* function.

Because *Bhlhb5* and *Lmo4* are themselves important regulators of area identity acquisition, we reasoned that changes in their expression would be likely to affect molecular area identity more broadly. To rigorously investigate this possibility, we performed ISH for several genes with areally-restricted expression patterns at P7. *Cdh8* and *Epha7* are normally expressed in rostral and occipital cortex, but excluded from somatosensory cortex (**Figures 4.3A and 4.3C**). We find that, as with *Lmo4*, expression of both *Cdh8* and *Epha7* expands in the absence of *Ctip1* function, filling in the "gap" in each of their expression domains normally present in somatosensory cortex (**Figures 4.3B and 4.3D**). Conversely, as with *Bhlhb5*, *Efna5* in somatosensory cortex is strikingly reduced, and sharp boundaries are no longer present with motor cortex rostrally, or with visual cortex caudally (**Figures 4.3E and 4.3F**). In addition, *Id2* expression is lost in layer II/III of higher-order visual areas, and this is accompanied by an expansion of *Cdh6* expression into this same territory (**Figures 4.3G-4.3J**). Importantly, while the boundary between motor and sensory cortex becomes blurred in *Ctip1$^{fl/fl}$;Emx1-Cre* mice, expression of all of these genes within the motor domain remains normal (**Figures 4.3A-4.3J**). Taken together, these results indicate that the molecular boundaries

Figure 4.3 Establishment of precise molecular boundaries delineating motor and sensory areas is severely disrupted in *Ctip1$^{fl/fl}$;Emx1-Cre* cortex

In the absence of *Ctip1* function, molecular areal identity in somatosensory cortex is severely disrupted. *Cdh8* and *EphA7*, excluded from upper layers in wild-type somatosensory cortex, are expressed in *Ctip1$^{fl/fl}$;Emx1-Cre* somatosensory cortex (A-D). Sharp caudal boundaries of *Efna5* and *Cdh6* expression are absent in *Ctip1$^{fl/fl}$;Emx1-Cre* (E-I).

Figure 4.3 (Continued)

Figure 4.4. Graded expression of *Pax6*, *Emx1*, and *Couptf1* in progenitors is not affected by loss of *Ctip1* function

Progenitor-level controls over area identity are normally expressed in the absence of *Ctip1*. *Couptf1* (A-B), *Emx1* (C-D), and *Pax6* (E-F), important regulators of early area development in the ventricular zone, are present in expected expression gradients in both wild-type and *Ctip1*$^{fl/fl}$*;Emx1-Cre* cortex at E13.5.

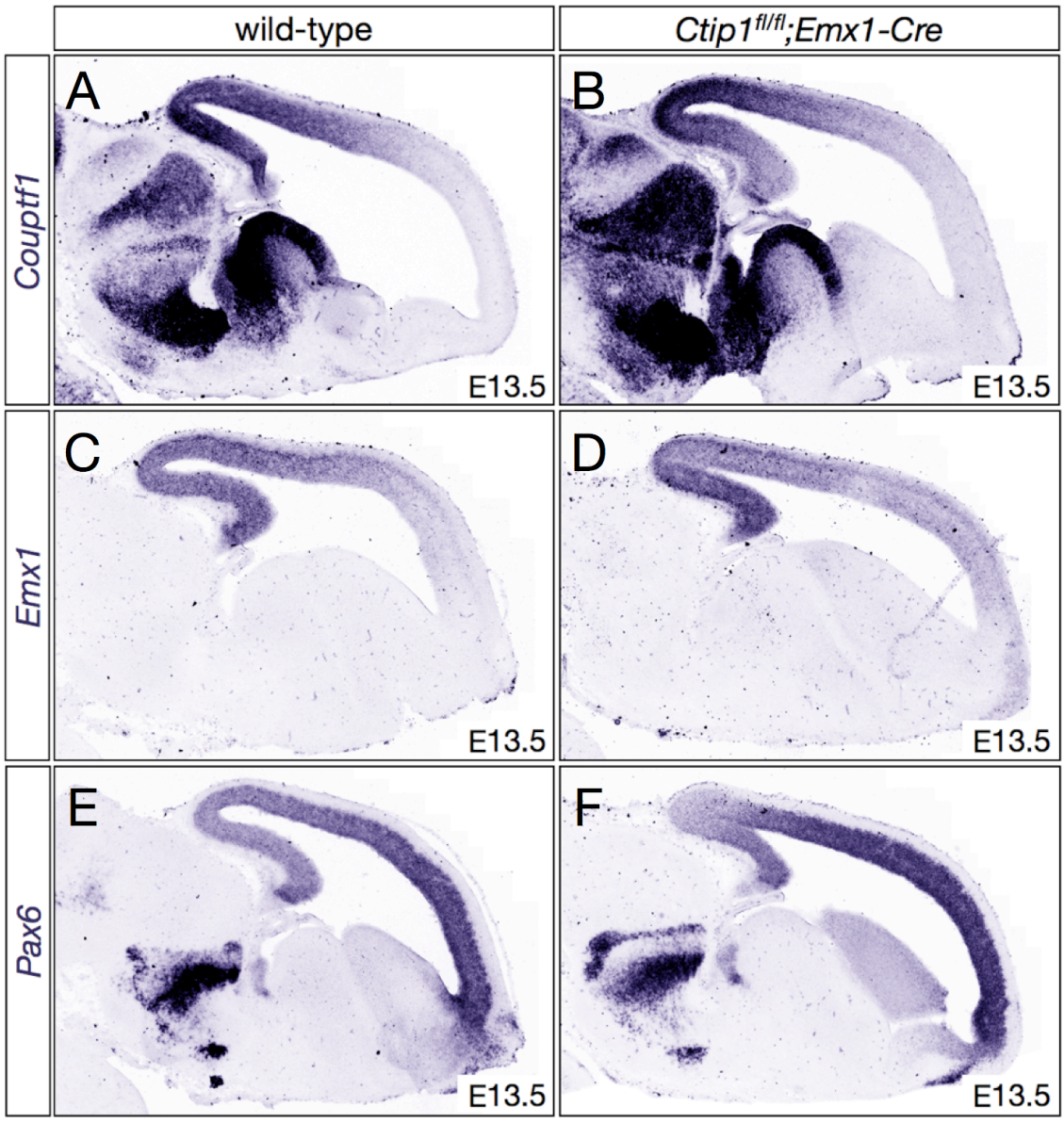

Figure 4.4 (Continued)

Figure 4.5 *Ctip1* controls refinement of broad expression gradients into precise areal boundaries

Expression domains of critical transcriptional controls over area identity are strikingly abnormal in the absence of *Ctip1*. In wild-type P4 cortex, *Lmo4* is excluded from somatosensory cortex (A), while *Bhlhb5* is expressed highly in somatosensory and visual cortex (C). In *Ctip1*$^{fl/fl}$*;Emx1-Cre* P4 cortex, both *Lmo4* and *Bhlhb5* fail to respect these areal boundaries, which become blurred rather than sharp. *Bhlhb5* expression becomes progressively disrupted over time in the absence of *Ctip1*. At P0, shortly after postmitotic refinement area identity has begun, *Bhlhb5* expression is broadly normal in *Ctip1*$^{fl/fl}$*;Emx1-Cre* cortex, although rostromedial and caudomedial expression boundaries lack precision (E, F). However, at P7, *Bhlhb5* expression is strikingly reduced, and primary sensory areas cannot be discerned (G, H).

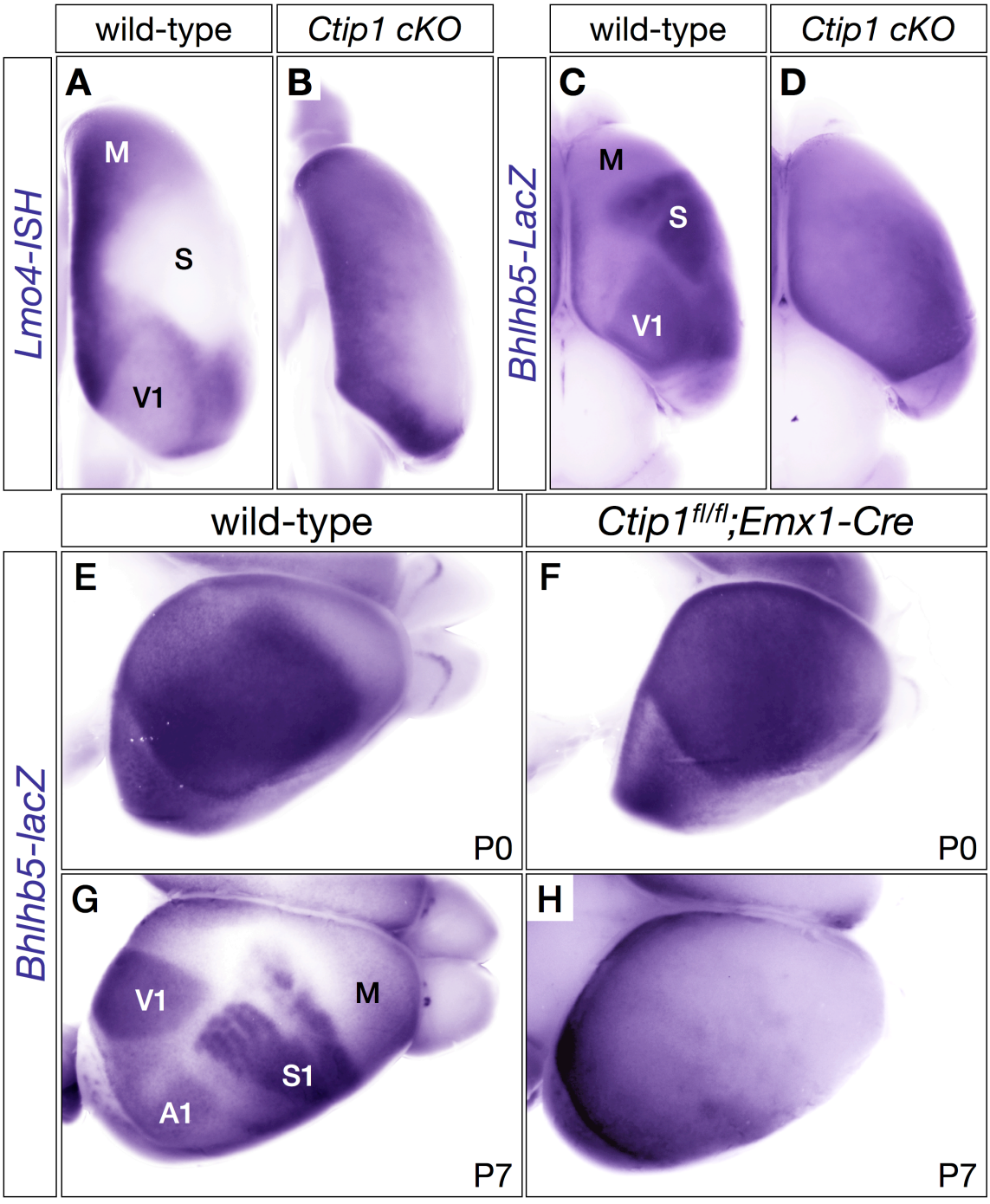

Figure 4.5 (Continued)

dividing sensory areas from motor areas, as well as those separating primary sensory areas from higher-order sensory areas, are not properly established in the absence of *Ctip1* function.

Ctip1 controls postmitotic refinement of broad expression gradients into sharply-delineated areal domains

In order to determine the specific stage(s) at which *Ctip1* acts to control area development, we carefully examined the full course of area identity specification and subsequent refinement in *Ctip1$^{fl/fl}$;Emx1-Cre* mice. Because CTIP1 immunostaining is present only in the cortical plate, and not in proliferative zones (**Figure 4.1**), we hypothesized that *Ctip1* acts postmitotically to control area identity acquisition. However, because *Ctip1* expression can be observed in the ventricular and subventricular zones by *in situ* hybridization (Chapter 3), very low levels of CTIP1 protein, not detected by immunocytochemistry, might be present in progenitors. To investigate the theoretical possibility that *Ctip1* might be necessary for early progenitor arealization, we performed *in situ* hybridization for *Pax6*, *Emx1*, and *Couptf1* in *Ctip1$^{fl/fl}$;Emx1-Cre* mice at E13.5. This analysis reveals that ventricular zone expression gradients of these critical transcription factors, which are the earliest manifestation of neocortical arealization, are identical in wild-type and *Ctip1$^{fl/fl}$;Emx1-Cre* brains (**Figures 4.4A-4.4F**), thus progenitor areal organization is independent of *Ctip1* function. Once transcription factor expression gradients are established in the ventricular zone, progenitors convey this positional information to their neuronal progeny, such that early gradients of *Lmo4* and *Bhlhb5* expression in the cortical plate closely parallel those of

Pax6 and *Emx1* in the ventricular zone. We examined *Ctip1*$^{fl/fl}$*;Emx1-Cre* and wild-type brains carrying *LacZ* reporter alleles for either *Lmo4* or *Bhlhb5*, and find that initially their expression gradients are largely preserved, even in the absence of *Ctip1* function, but refinement of this rudimentary area identity over the first week of postnatal development completely fails to take place. At P0, expression of *Bhlhb5* is still grossly normal in *Ctip1*$^{fl/fl}$*;Emx1-Cre* brains, but the boundaries between areas are not as sharply defined as they are in wild-type controls (**Figures 4.5E and 4.5F**). However, extensive abnormalities emerge by P4, as expression of *Lmo4* persists in somatosensory territory rostrally, and becomes compressed to a narrow band caudally (**Figures 4.5A and 4.5B**). In parallel, expression of *Bhlhb5* remains diffuse, rather than becoming restricted to primary somatosensory, visual, and auditory cortex (**Figures 4.5C and 4.5D**). This cumulative failure of refinement is even more striking at P7, when area identity has normally reached almost full maturity. At this age, *Bhlhb5* delineates primary sensory areas in exquisite detail, such that even individual barrels can be distinguished on the surface of cortex (**Figure 4.5G**). In contrast, in *Ctip1*$^{fl/fl}$*;Emx1-Cre* brains, expression of *Bhlhb5* is diffuse and weak, with no clear boundaries between areas (**Figure 4.5H**). Taken together, these data indicate that positional information is successfully transmitted from progenitors to early postmitotic neurons, in the absence of *Ctip1* function, but subsequent postmitotic refinement of these gradients into crisply-defined areal domains is severely impaired.

Figure 4.6 Imprecise topography of CPN connections in *Ctip1*$^{fl/fl}$*;Emx1-Cre* cortex

Ctip1 function is necessary for CPN to project homotypic coordinates on the contralateral hemisphere. In wild-type embryos, CPN electroporated with a CAG-*Cre*-IRES-*Egfp* expression construct at E14.5 project to homotypic coordinates on the contralateral hemisphere ("x", B-C). *Ctip1*$^{fl/fl}$ CPN electroporated with a CAG-*Cre*-IRES-*Egfp* expression construct project more to more rostrolateral or caudolateral coordinates on the contralateral hemisphere ("x", E-F).

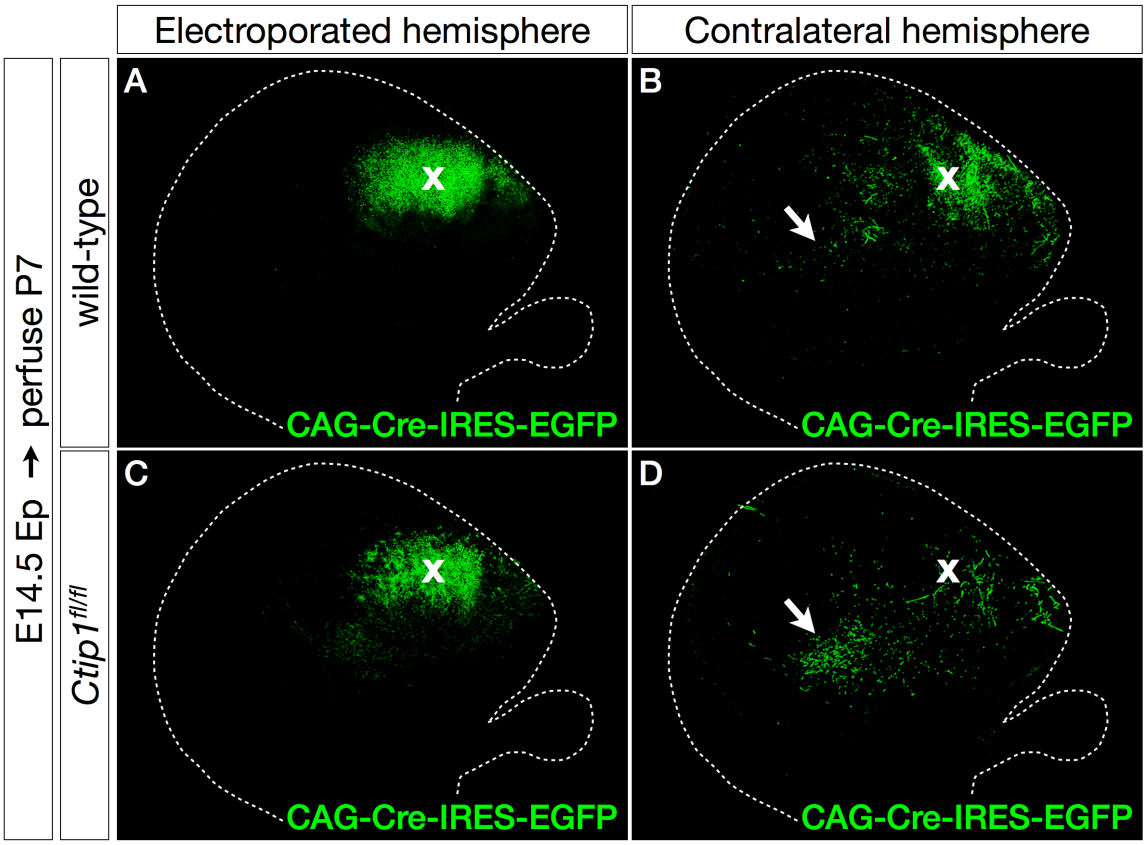

Figure 4.6 (Continued)

Figure 4.7 In the absence of *Ctip1* function, CPN aberrantly project to non-homotypic locations on the contralateral hemisphere

In the absence of *Ctip1* function, homotypic targeting of callosal projections loses precision. In wild-type mice, injection of AAV-EGFP into somatosensory cortex (A) anterogradely labels spatially-restricted homotypic projections in the contralateral hemisphere (B). However, injection of AAV-EGFP into *Ctip1*$^{fl/fl}$*;Emx1-Cre* somatosensory cortex (C) labels projections that cover a wide area of the contralateral hemisphere (D).

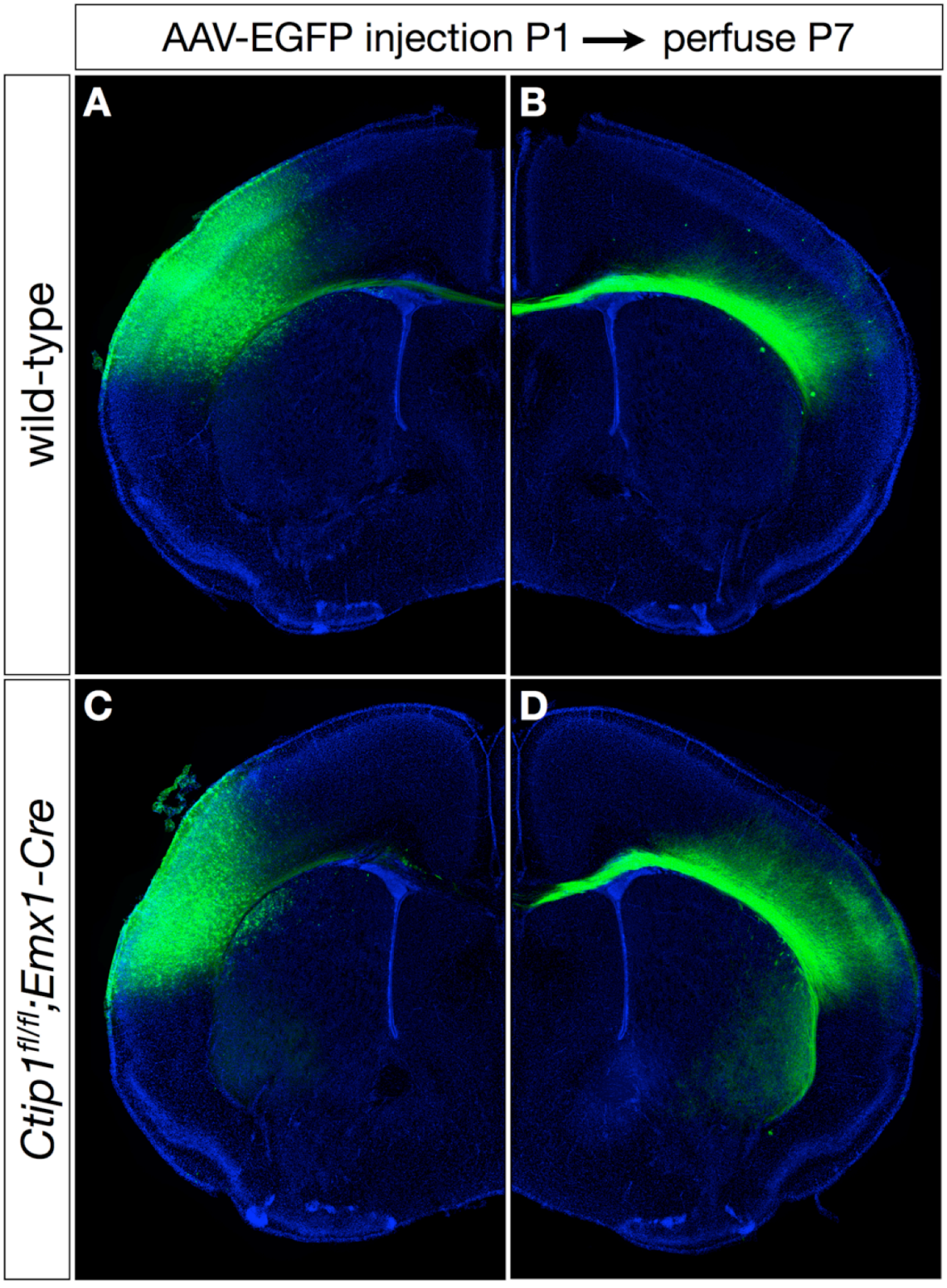

Figure 4.7 (Continued)

Ctip1 is required cell-autonomously by callosal projection neurons for pathfinding to homotypic contralateral coordinates

Callosal projection neurons (CPN) connect mirror image locations in corresponding cortical areas of either cerebral hemisphere to enable highly-organized inter-hemispheric transfer of information (Richards et. al., 2004). The cues that guide CPN axons to precise homotypic coordinates during development are not known (Fame et al., 2010), and whether this precise connectivity reflects the area identity of CPN in different regions of the cortex has not been previously investigated. However, in many other systems, topographic organization depends on graded expression of guidance receptors (Luo and Flanagan, 2007), and, in cortex, such gradients are generally regulated by transcription factors that control area identity acquisition. It is therefore possible that areally-specified expression of axon guidance cues, axon guidance receptors, and cell adhesion molecules underlies this very precise connectivity. Given that *Ctip1* is highly expressed by CPN, and given the severe disruption of molecular area identity in *Ctip1* mutants, we hypothesized that *Ctip1* is important for the establishment of homotypic connectivity of CPN.

To address this question, we focally injected adeno-associated virus expressing EGFP under a CMV/β-actin promoter (AAV-EGFP) into the cortex of *Ctip1*$^{fl/fl}$*;Emx1-Cre* and control mice, and examined whether CPN project to appropriate contralateral coordinates. Normally, CPN axons innervate a narrow mediolateral target area on the contralateral hemisphere (**Figures 4.6A and 4.6B**). While many CPN axons continue to project appropriately in *Ctip1*$^{fl/fl}$*;Emx1-Cre* brains, a substantial number invade the region of cortex immediately adjacent to the predicted target area (**Figures 4.6C and 4.6D**). This lack of precision in targeting might result from a corresponding lack of

definition in the expression gradients of axon guidance molecules, similar to those we observe for *Epha7*, *Efna5*, *Cdh8*, and *Cdh6* (**Figures 4.3A-4.3J**).

If the gradients of molecules controlling homotypic connectivity are areally specified, they would be predicted to change symmetrically in the absence of *Ctip1* function, affecting both CPN and their contralateral projection targets, and leaving CPN targeting relatively intact, albeit less sharply defined. We therefore hypothesized that disrupting Ctip1 expression unilaterally might result in a more dramatic re-routing of CPN axons, by creating an areal mismatch between the two hemispheres. To investigate this possibility, we electroporated a CMV/β-actin promoter-driven *Cre*-IRES-*Egfp* overexpression construct into the ventricular zone of E14.5 *Ctip1*$^{fl/fl}$ and wild-type embryos. Brains were collected at P7, divided along the midline, and each hemisphere was flattened and sectioned tangentially. In wild-type mice, electroporated neurons project almost exclusively homotypically, as expected (**Figures 4.7A and 4.7B**). In striking contrast, in *Ctip1*$^{fl/fl}$ mice, electroporated neurons projected few axons homotypically, with the majority of axons shifting to more caudolateral positions (**Figures 4.7C and 4.7D**). Taken together, these results indicate that CPN growth cones translate molecular areal identity, combinatorially imparted by transcription factors in the nucleus, into a precise axonal projection path leading to homotypic locations in the contralateral hemisphere.

Figure 4.8 SCPN in somatosensory and visual cortex, but not in motor cortex, express *Ctip1*, and, in the absence of *Ctip1* function, maintain aberrant spinal projections at P21

Subcerebral projection neurons (SCPN) in visual cortex maintain spinal projections in the absence of neocortical *Ctip1*. SCPN in motor cortex do not express *Ctip1* (A), while SCPN in somatosensory cortex express low levels (B). Subcerebral projection neurons (SCPN) in visual cortex maintain spinal projections in the absence of neocortical *Ctip1*. In wild-type animals, SCPN in motor cortex (red) maintain their projections to the spinal cord at P21, while SCPN in visual cortex (green) do not project beyond the pons. In P21 *Ctip1*$^{fl/fl}$*;Emx1-Cre* mice, SCPN in motor cortex project normally, but SCPN in visual cortex project beyond the cerebral peduncle and into the spinal cord.

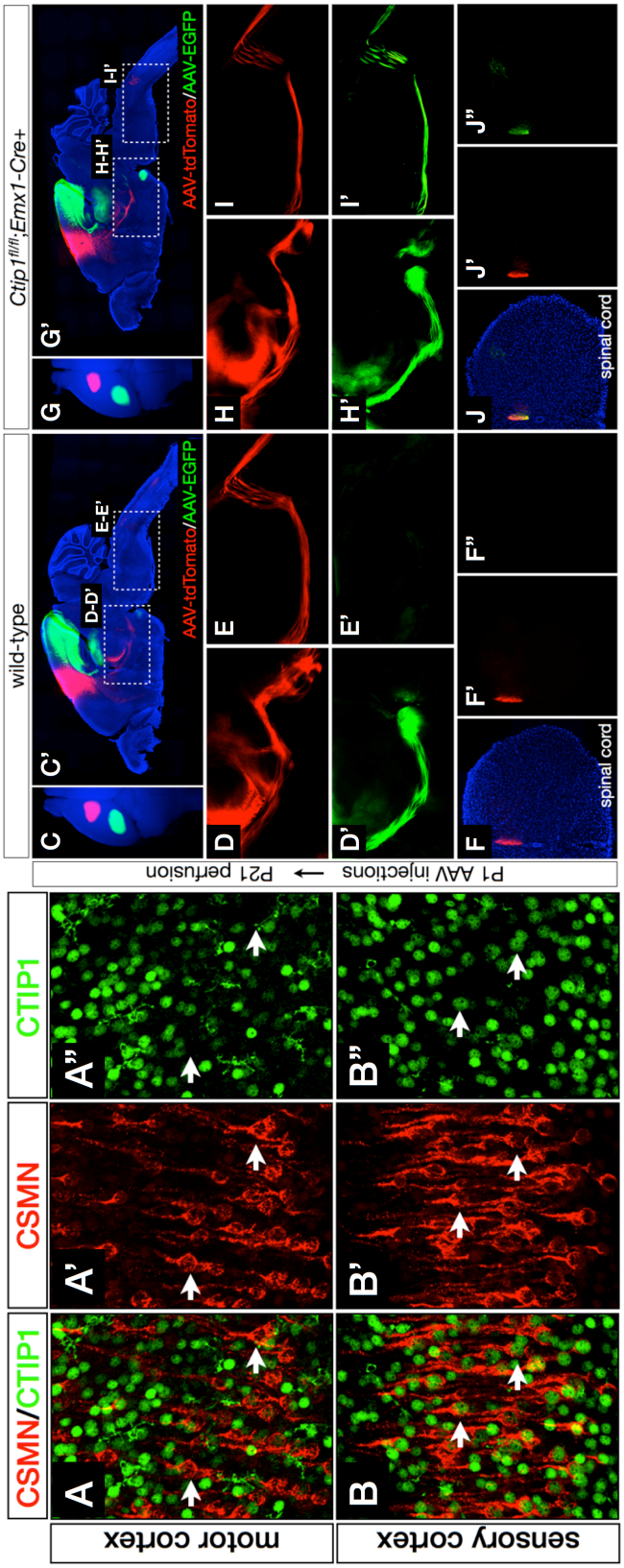

Figure 4.8 (Continued)

Figure 4.9 *Ctip1* is required for repression of CSMN-specific genes in sensory cortex SCPN

Subcerebral projection neurons in somatosensory cortex require *Ctip1* function to acquire sensory identity. In the absence of *Ctip1* function, genes normally restricted to motor cortex expand laterally into somatosensory areas (C-H), and SCPN in somatosensory cortex fail to prune spinal projections by P21 (I-P).

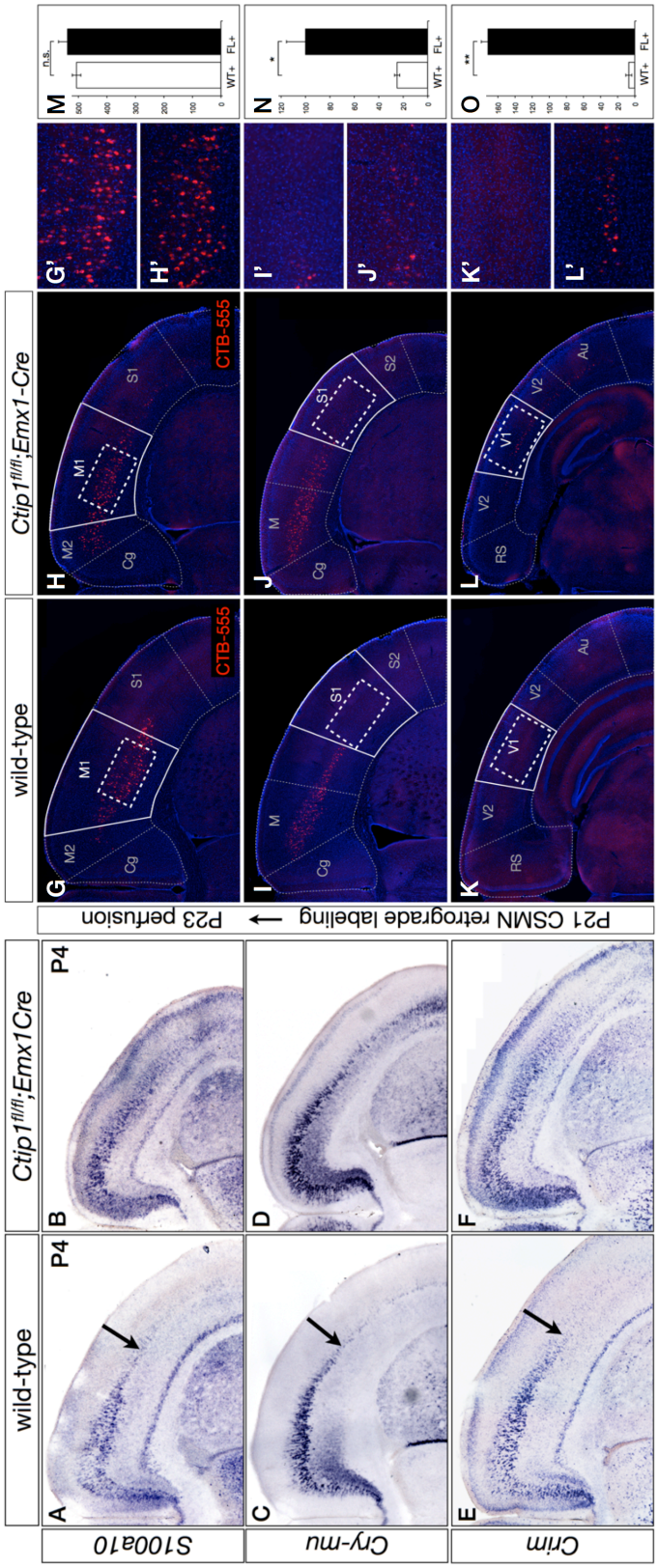

Figure 4.9 (Continued)

Subcerebral projection neurons in sensory areas acquire motor identity in the absence of *Ctip1* function

While CTIP1 is progressively excluded from SCPN in motor cortex, it continues to be expressed at low levels by SCPN in somatosensory and visual cortex through early postnatal development (**Figures 4.8A and 4.8B**), suggesting that *Ctip1* function might be necessary to prevent SCPN in sensory areas from acquiring motor identity. To investigate this hypothesis, we injected AAV-tdTomato into motor cortex, and AAV-EGFP into visual cortex of *Ctip1*$^{fl/fl}$*;Emx1-Cre* and wild-type mice. Initially, SCPN in both motor and visual cortex project to the spinal cord and extend collaterals to multiple brainstem nuclei. From P7 to P21, this connectivity undergoes substantial refinement, with SCPN in motor cortex (CSMN) pruning their collaterals to the optic tectum, and SCPN in visual cortex (CTPN) pruning their spinal axons. We find that almost no neurons from visual cortex project beyond pons in wild-type mice (**Figures 4.8C-4.8F**). In striking contrast, in *Ctip1*$^{fl/fl}$*;Emx1-Cre* mice, neurons from visual cortex project robustly past the pons, through the medulla, and into the dorsal corticospinal tract (**Figures 4.8G-4.8J**). To further investigate this phenotype, we retrogradely labeled spinal cord-projecting neurons at P21 by injection of Alexa fluorophore-conjugated cholera toxin B (CTB) into the corticospinal tract, and quantified retrogradely labeled neurons in different cortical areas. In the absence of *Ctip1* function, the number of neurons from motor cortex that maintain spinal projections does not change (**Figures 4.9G, 4.9H and 4.9M**). In contrast, the number of neurons maintaining spinal projections increases five-fold in somatosensory cortex and 20-fold in visual cortex, and the distribution of these neurons expands beyond the normal boundaries at the medial limit of the barrel field and the rostral limit of V1 (**Figures 4.9I-4.9L and 4.9N-**

4.9O). These findings indicate that *Ctip1* is necessary for correct refinement of SCPN connectivity.

At least two different mechanisms of action might explain the pruning abnormalities present in the absence of *Ctip1* function. First, *Ctip1* might be acting early in development to specify the area identity of SCPN, thereby shaping their subsequent pruning decisions. Alternatively, because more SCPN are generated in sensory areas in *Ctip1$^{fl/fl}$;Emx1-Cre* mice (Chapter 3), a subset of this larger population might stochastically maintain spinal projections. We favor the former interpretation, since the two phenotypes are not proportional: the number of subcerebral projection neurons in sensory areas increases only 1.5-fold, while there is a five- to 20-fold increase in the number of these neurons maintaining spinal projections. To more directly address whether SCPN in sensory cortex acquire a motor identity, we performed ISH for genes specifically expressed by CSMN. In wild-type brains at P4, *S100a10*, *Crym*, and *Crim1* are strongly expressed in layer V of motor areas, but virtually absent in sensory areas (**Figures 4.9A, 4.9C and 4.9E**). In *Ctip1$^{fl/fl}$;Emx1-Cre* brains, however, there is a dramatic expansion of the expression domain of these genes into sensory areas (**Figures 4.9B, 4.9D and 4.9F**). These data indicate that SCPN in sensory cortex aberrantly acquire motor identity well before pruning begins, and that motorized gene expression, rather than being secondary to changes in connectivity, reflects a transcriptionally-specified change in area identity.

Thalamocortical input fails to organize into precise sensory maps in the absence of *Ctip1* function

Given the striking abnormalities in the refinement of area-specific gene expression and output connectivity in *Ctip1* conditional mutants, we next examined sensory input. Although several lines of investigation indicate that cortex-autonomous programs initiated in progenitors determine the final size and position of sensory maps, postmitotic transcriptional controls identified to-date have only subtle effects on sensory map formation. To investigate whether *Ctip1* is important for this process, we visualized thalamocortical afferents at P7 by serotonin (5-HT) immunohistochemistry on tangential sections through layer IV of flattened cortices. In wild-type controls, sensory maps can be discerned in sharp and unambiguous detail. These include the triangular visual map in occipital cortex, and the somatosensory "rodunculus" in parietal cortex, with its somatotopic representations of the hindlimb, forelimb, and lower jaw, and its field of vibrissal barrels, precisely arranged to reflect the positions of the whiskers on the snout (**Figure 4.10A**). In *Ctip1*$^{fl/fl}$*;Emx1-Cre* mice, the anatomical regions that would normally correspond to the visual map and the barrel field appear to receive completely disorganized innervation (**Figure 4.10B**), while representations of the hindlimb, forelimb, and lower jaw are still somewhat recognizable, albeit aberrantly indistinct.

To more deeply investigate whether thalamocortical axons broadly target the correct cortical areas, we injected cholera toxin B conjugated to Alexa-555 and Alexa-488 (CTB-555 and CTB-488), respectively, into motor and somatosensory cortex of P0 wild-type and *Ctip1*$^{fl/fl}$*;Emx1-Cre* mice. We then examined thalamic sections to determine whether thalamocortical axons projecting to motor and somatosensory cortex originate from appropriate thalamic nuclei. Although the boundaries between

thalamic nuclei are less sharply defined in *Ctip1*$^{fl/fl}$*;Emx1-Cre* brains than in wild-type brains, the overall topography of connections remains remarkably normal (**Figure 4.11A-4.11F**). Similarly, there are no significant perturbations observed following CTB-555 and CTB-488 injection into visual and somatosensory cortex (**Figure 4.11G-4.11J**). Taken together, these data indicate that thalamocortical axons target appropriate areas of cortex in the absence of *Ctip1* function, but are unable to form topographically-organized sensory maps.

Ctip1 directs cytoarchitectural organization of layer IV neurons

In parallel to the establishment of sensory maps by thalamocortical axons, cortical cytoarchitecture must be reorganized in order to receive and process sensory input (López-Bendito and Molnár, 2003). The most striking example of cytoarchitectural organization of layer IV neurons can be found in barrel cortex, where information from each whisker is relayed to an individual barrel. Barrels consist of a central cell-sparse "hollow" occupied by thalamocortical axons, and a cell dense wall made-up of layer IV neurons, which orient their dendrites centrally to receive sensory information. Neighboring barrels are separated by cell-sparse septae.

We hypothesized that sensory maps might fail to form in *Ctip1*$^{fl/fl}$*;Emx1-Cre* mice, because cortical cues normally provided to thalamocortical axons are lost. Supporting this interpretation, cytoarchitectural organization of barrels is entirely absent in *Ctip1*$^{fl/fl}$*;Emx1-Cre* cortex. This is the case whether barrel cytoarchitecture is examined directly by DAPI staining (**Figures 4.12A and 4.12B**), or indirectly by expression of barrel-specific genes, such as *Rorb* (**Figures 4.12C and 4.12D**). Instead of the normal

pattern of cell-dense walls and cell-sparse hollows (**Figure 4.12A**), layer IV cytoarchitecture becomes uniform, without even minimal organization, in the corresponding region of *Ctip1*$^{fl/fl}$*;Emx1-Cre* cortex (**Figure 4.12B**). Similarly, *Rorb* and *Cux1*, which are strongly expressed by layer IV neurons of the barrel walls and hollows, but completely excluded from septae (**Figure 4.12C**), are present at reduced levels and evenly distributed in the absence of *Ctip1* function (**Figure 4.12D**). Recent work from our laboratory shows that *Rorb* overexpression is sufficient to induce formation of barrel-like aggregates, and to attract thalamocortical axons (Jabaudon et al., 2011), suggesting that dysregulated *Rorb* expression might be a potential mechanism for the abnormal organization of both sensory input and layer IV neurons in *Ctip1*$^{fl/fl}$*;Emx1-Cre* mice.

In the absence of *Ctip1*, layer IV neurons are excluded from barrels and their dendrites avoid barrel hollows

To gain further insight into this failure of barrel cytoarchitecture to emerge in the complete absence of cortical *Ctip1* function, we investigated the effects of cell-autonomous loss of *Ctip1* function by electroporating a *Cre*-IRES-*Egfp* expression construct into somatosensory cortex of *Ctip1*$^{fl/fl}$ embryos or wild-type controls at E14.5. On the tangential axis, electroporated *Ctip1* null neurons located in layer IV completely avoid barrels, and instead position themselves exclusively within septae (**Figure 4.12E**). In contrast, electroporated wild-type neurons adopt an unbiased distribution (**Figure 4.12G**), with more electroporated neurons are present in barrel walls, where cell density

Figure 4.10 Thalamocortical input to primary sensory areas is strikingly disorganized in the absence of *Ctip1* function

Neocortical *Ctip1* function is necessary for organization of sensory maps. Thalamic input, visualized by serotonin IHC, aggregates into a precise array of vibrissal barrels in wild-type somatosensory cortex (A), but is extremely disorganized in *Ctip1$^{fl/fl}$;Emx1-Cre* cortex (B).

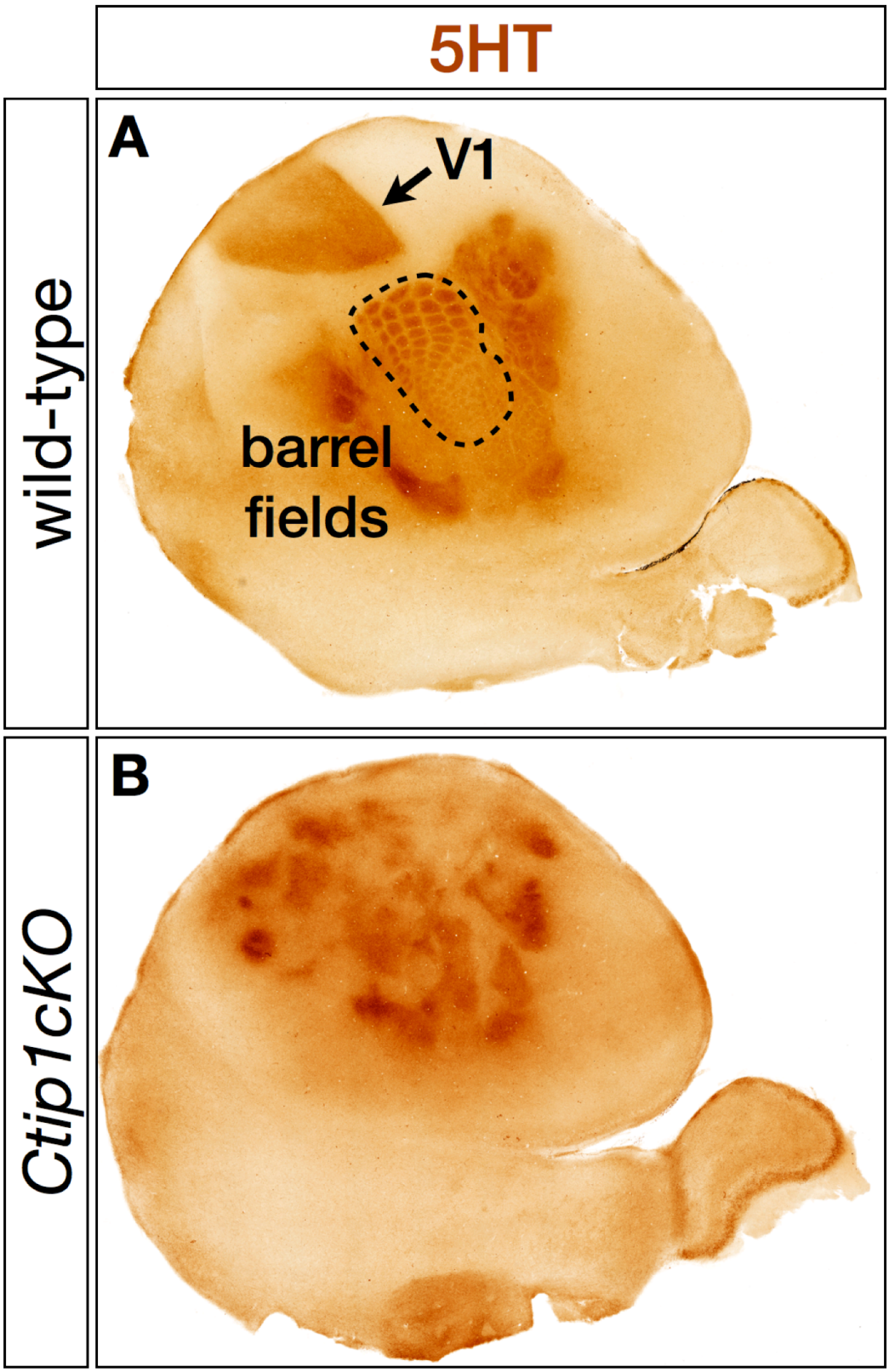

Figure 4.10 (Continued)

Figure 4.11 Overall topography of thalamocortical projections is preserved

Although thalamocortical input fails to organize in functional areas in the absence of *Ctip1* function, overall topography of thalamocortical projections is maintained. Injection of CTB into motor and somatosensory areas of wild-type cortex at P0 retrogradely labels motor and sensory nuclei, respectively, in thalamus (A-F). Similarly, injection of CTB into somatosensory and visual areas of *Ctip1*$^{fl/fl}$*;Emx1-Cre* cortex labels sensory and visual nuclei, respectively (G-L).

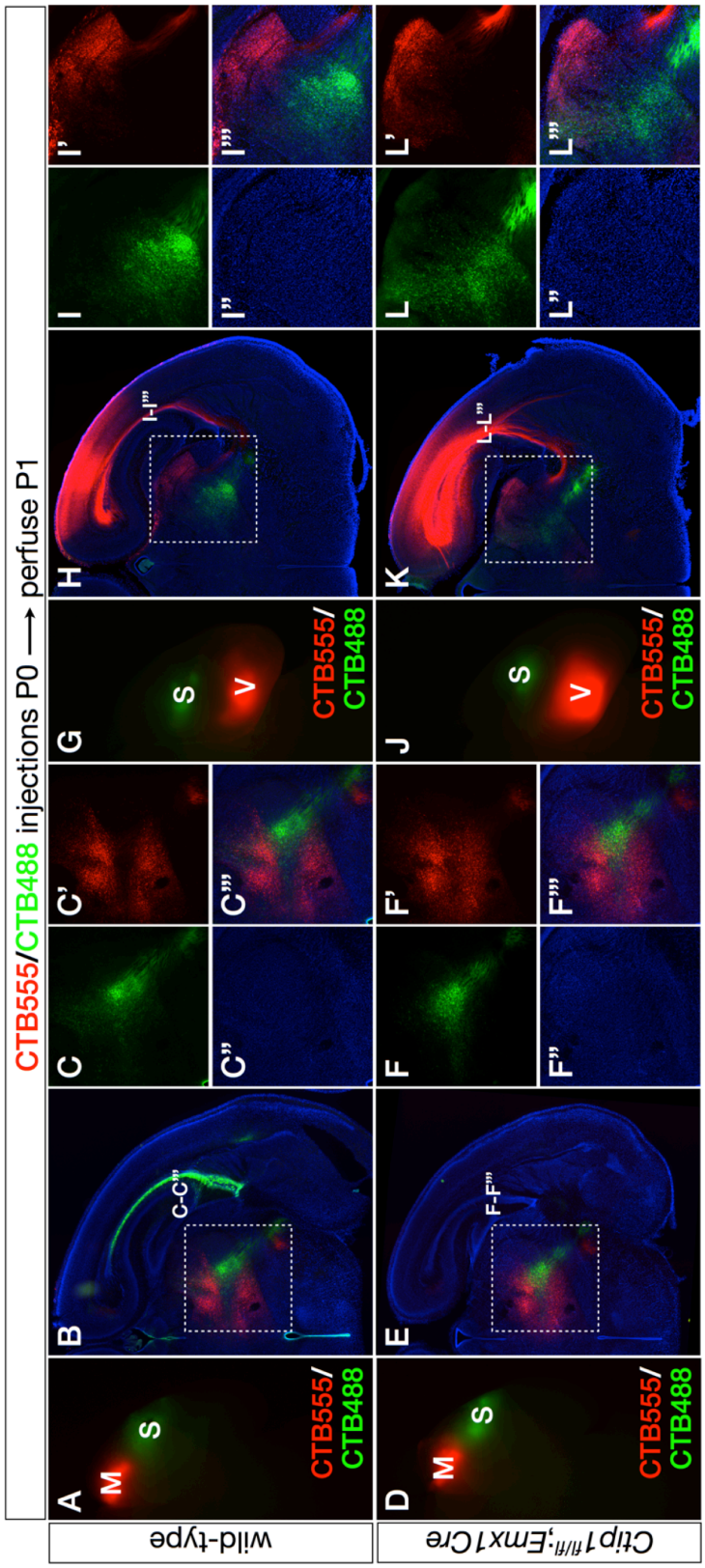

Figure 4.11 (Continued)

Figure 4.12 *Ctip1* function is required for organization of layer IV neurons into cytoarchitecturally distinct barrels

Ctip1 is required for cytoarchitectural organization of layer IV neurons and mosaic loss of *Ctip1* function causes layer IV neurons to be excluded from barrels. Wild-type neurons electroporated with *Cre* at E14.5 are evenly distributed in somatosensory cortex (A) and take up positions in both barrel walls and barrel hollows (B-B'''). In contrast, $Ctip1^{fl/fl}$ neurons electroporated with *Cre* at E14.5 are positioned exclusively in barrel septae (D-D'''), with no electroporated neurons in walls or hollows, leading to a reticular appearance in barrel cortex (C).

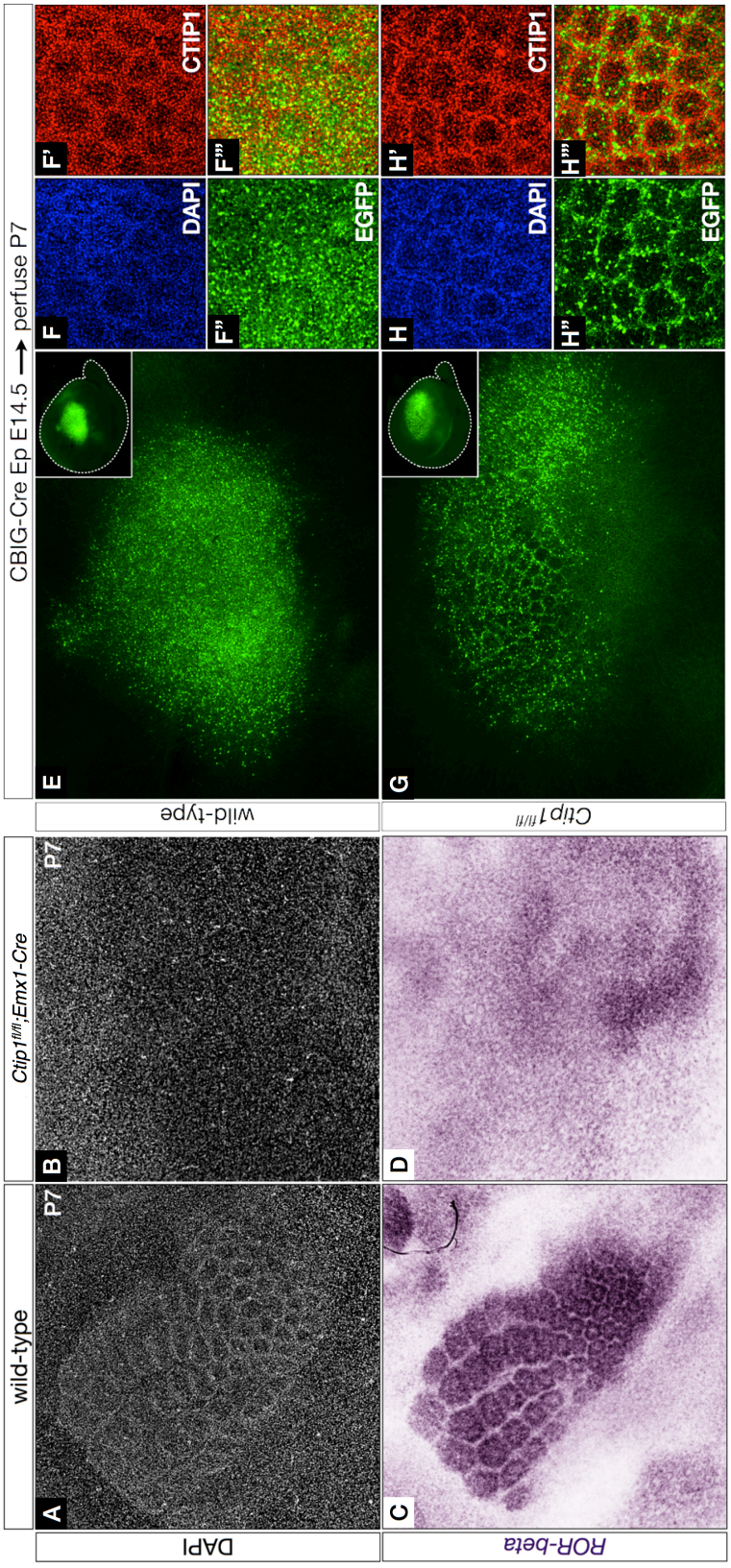

Figure 4.12 (Continued)

is higher. In addition, while most wild-type layer IV neurons are located in the barrel walls and orient their dendrites toward the center of each barrel to synapse with thalamocortical axons (**Figure 4.12F**), the dendritic trees of *Ctip1* null neurons accumulate in septae, completely avoiding barrels (**Figure 4.12H**). These experiments indicate that *Ctip1* function is necessary for the aggregation of layer IV neurons into barrels, and for their assembly into circuits that receive sensory information from thalamocortical afferents. Therefore, it seems likely that abnormal expression of *Rorb* and other coordinately-regulated genes in *Ctip1* mutants results in layer IV neurons being unable to aggregate with each other, or establish synapses with thalamocortical axons, which, in turn, precludes activity-dependent interactions known to be necessary for emergence of cortical cytoarchitecture and sensory maps.

Integrating data on gene expression, output connectivity, and organization of sensory input, these experiments indicate that *Ctip1* is critical for developmental refinement of area identity in the neocortex. Loss of *Ctip1* function results in a striking failure of postmitotic refinement, as broad expression gradients inherited from progenitors are not transformed into sharply-defined domains corresponding to functional areas. Importantly, expression of *Lmo4* and *Bhlhb5*, two postmitotic transcriptional regulators of area identity, expands beyond normal boundaries. This blurring of boundaries is also reflected in the abnormal point-to-point connectivity of CPN, which project with less precision to mirror-image targets. SCPN with motor identity expand into somatosensory and visual cortex, indicating that *Ctip1* normally represses motor identity in these neurons. Organization of sensory input is also severely disrupted, as thalamocortical axons fail to cluster into clear topographic maps, and layer IV neurons fail to organize into characteristic cytoarchitectural patterns. Therefore, *Ctip1* is a centrally important high-level control over refinement of area identity.

4.4 Discussion

Elucidating the transcriptional programs that direct specialization of cortical areas is of great interest toward understanding how area-specific connectivity, cytoarchitecture, and function emerge during development of the cerebral cortex, and also toward understanding how this complexity arose during evolution. We report that *Ctip1* is highly expressed in primary sensory areas during early postnatal development, and that it is a critical control over refinement of molecular area identity, areal precision of CPN and SCPN connectivity, organization of thalamocortical axons into sensory maps, and aggregation of layer IV neurons into barrels. These experiments represent the first investigation of transcriptional mechanisms underlying homotypic CPN connectivity and provide the first demonstration that cortical transcription factors not only dictate the size and position of primary sensory areas, but are also required for the organization of thalamocortical input into sharply-defined and appropriately-shaped maps.

Postmitotic refinement of molecular area identity

Genes belonging to diverse functional categories, including cell adhesion molecules (*e.g.*, *Cdh6* and *Cdh8*; Suzuki et al., 1997), intracellular signaling molecules (*e.g.*, *Plcb1*; Hannan et al., 2001), transcriptional regulators (*e.g.*, *Id2* and *Rorb*; Neuman et al., 1993; Nakagawa and O'Leary, 2003), and axon guidance receptors and ligands (*e.g.*, *Epha7* and *Efna5*; Rubenstein et al., 1999; Dufour et al., 2003), have been

identified to be expressed in an area-specific fashion. Although these genes are useful readouts of area identity, their complex expression patterns do not directly inform our understanding of the basic organizational principles that govern cortical arealization, since they represent the final outcome of intersectional regulation by multiple transcriptional programs acting in specific cortical layers and areas. The recent identification of *Bhlhb5* and *Lmo4* as important transcriptional controls over postmitotic acquisition of area identity has made it possible to begin deconstructing this complex regulatory network into individual “developmental vectors”.

We report here that *Ctip1* function is necessary for developmental refinement of *Bhlhb5* and *Lmo4* expression, and for the establishment of sharp gene expression boundaries between their respective domains (**Figure 4.5**). Notably, in the absence of *Ctip1* function, while motor and cingulate *Lmo4* expression expand into somatosensory territory, expression in higher-order visual areas recedes caudally into a narrow band of abnormally high expression. These symmetrically opposite changes suggest that *Lmo4* expression in motor and cingulate cortex might be established by mechanisms distinct from those acting in higher-order-visual areas. It is possible that the transcriptional networks responsible for setting up boundaries between sensory cortex and either motor or cingulate cortex are more robust than those responsible for setting up boundaries between primary and secondary sensory areas, and, therefore, the former set of boundaries is largely maintained, even in the absence of *Ctip1* function, while the latter set of boundaries is entirely lost. This model agrees well with our data that, in *Ctip1* mutants, *Bhlhb5* expression no longer distinguishes primary sensory areas from higher-order sensory areas, and thalamocortical input fails to organize into a discernible barrel field or visual map. Therefore, while *Ctip1* contributes to the establishment of the broad molecular boundaries that define the sensory domain, it is most critical for the

delineation of primary sensory areas and higher-order sensory areas within the sensory domain.

Homotypic CPN connectivity is areally specified

The corpus callosum is the largest commissural tract in the forebrain of placental mammals, and allows for point-to-point interhemispheric transfer and integration of information. Although others have investigated homotypic targeting of CPN axons in the context of area identity acquisition (Weimann et al., 1999), these studies did not identify any abnormalities. Here, we provide direct experimental evidence that mirror-image connectivity of CPN is, in fact, areally specified. Anterograde labeling reveals that homotypic targeting of CPN axons is strikingly imprecise in *Ctip1*$^{fl/fl}$*;Emx1-Cre* mice (**Figure 4.7**), likely as a result of a corresponding loss of precision in the gradients and counter-gradients of guidance molecules that direct CPN axons to precise contralateral coordinates (as we observe for *Cdh6*, *Cdh8*, *Epha7*, and *Efna5*; **Figure 4.3**). In further support of this conclusion, our *in utero* electroporation experiments show that cell-autonomous loss of *Ctip1* function causes a directional shift in CPN projections (**Figure 4.6**). We propose that this phenotype results from a mismatch between the abnormal area identity of electroporated (*Ctip1* null) neurons and their position in an otherwise wild-type brain. Prior work in the visual and olfactory systems indicates that two broad mechanisms generally underlie precise topographic connectivity: 1) adhesive and repulsive axon-axon interactions that help maintain the ordered arrangement of axons as they travel to their targets; and 2) gradients of axon guidance cues and/or receptors that direct axons to specific

coordinates (Luo and Flanagan, 2003). Both mechanisms are likely to be involved in homotypic targeting of CPN axons, and it will be important both to identify individual guidance molecules, and to determine how their expression gradients are regulated by transcriptional controls over area identity acquisition.

Acquisition of area identity by SCPN

Area-specific connectivity of SCPN arises by stereotyped pruning of a common set of branches during the first three weeks of postnatal development (O'Leary, 1992). SCPN from all areas of cortex first project axons to the spinal cord, and extend collaterals to pontine, midbrain, and cerebellar nuclei. Some of these exuberant projections are then eliminated, such that SCPN in motor cortex (CSMN) maintain their spinal axons and prune their tectal collaterals, and SCPN in sensory cortex (CTPN) maintain their tectal collaterals and prune their spinal axons (Stanfield et al., 1982; Thong and Dreher, 1986). The mechanisms underlying this process of refinement are still not well understood, and it is unclear what combination of genetic programs and/or neuronal activity orchestrates axon pruning (Vanderhaeghen and Cheng, 2010). The homeodomain transcription factor *Otx1* is expressed broadly by SCPN in all areas of cortex, and in *Otx1* null mice, SCPN in visual cortex fail to prune their spinal axons (Weimann et al., 1999). However, it remains unclear whether Otx1 is instructive for this process, resulting in visual cortex SCPN taking on a motor identity in *Otx1* null mice, or permissive, resulting in visual cortex SCPN simply lacking the necessary cellular machinery to prune spinal axons in *Otx1* null mice. Abnormal pruning has also been noted in secondary somatosensory cortex of *Ctip2*$^{+/-}$ mice (Arlotta et al., 2005), but it is

similarly not known how decreased dose of *Ctip2* causes this phenotype. Interestingly, transplantation experiments suggest that, even if genetic programs dictate pruning, extrinsic influences can change pruning properties of occipital neurons transplanted into motor cortex (Stanfield and O'Leary, 1985; O'Leary and Stanfield 1989).

We find that expression of *Ctip1* becomes specific to SCPN in sensory areas by P4, before the initial extension of corticospinal axons and brainstem collaterals is complete. In the absence of *Ctip1* function, SCPN in visual and somatosensory cortex aberrantly maintain spinal projections, and express genes characteristic of motor SCPN (**Figure 4.8**). These changes in area-specific gene expression indicate that *Ctip1* is not simply necessary for pruning in a general sense, but, rather, specifically represses motor SCPN identity. Based on these findings, one possible model might be that *Ctip1* itself, or one of its downstream targets, modifies *Otx1* function in an area-specific fashion, enabling activation of genes required for pruning of spinal axons, and/or repression of genes required for maintaining spinal axons. Notably, however, not all SCPN in sensory areas maintain spinal projections, even in the absence of *Ctip1* or Otx1 function (Weimann et al., 1999), indicating that additional molecular controls are able to independently repress motor identity in sensory SCPN.

Organization of thalamocortical axons into sensory maps

The establishment of sensory maps by thalamocortical axons, and in particular the vibrissal barrel field in somatosensory cortex, has been intensively investigated, because it provides an excellent system to study topographic organization and activity-dependent plasticity (López-Bendito and Molnár, 2003). In mice, thalamic axons arrive

at the appropriate cortical regions on ~E16.5, and pause at the subplate before beginning their invasion of the cortical plate at ~P0 (Lund and Mustari, 1977). Previous studies have demonstrated that thalamocortical axons are attracted to specific regions of cortex by areally-specified cortical cues, and, under permissive conditions, form sensory maps at these locations (O'Leary et al., 2007; Armentano et al., 2007). In *Ctip1$^{fl/fl}$;Emx1-Cre* mice, thalamocortical axons seem to target appropriate areas, indicating that these early cues are largely normal (**Figure 4.11**). Over the first week of postnatal development, thalamocortical axons in primary somatosensory, auditory, and visual cortex organize into precisely-defined maps, with parallel changes unfolding in the cytoarchitectural organization of layer IV neurons. These changes are particularly striking in the barrel field, where thalamocortical axons relaying information from a single whisker cluster together, and layer IV neurons aggregate around them.

Multiple lines of investigation suggest that aggregation of cortical neurons into barrels requires signals from thalamocortical axons (Wu et al., 2011). In adenylyl cyclase 1 (*Adcy1*) null mice, long-studied as the spontaneous mutation known as *barrelless* (*Brl*), thalamocortical axons fail to segregate into individual barrels (Van der Loos et. al., 1986; Abdel-Majid et al., 1998). However, it has recently been reported that cortex-specific conditional deletion of *Adcy1* does not disrupt proper arrangement of either thalamocortical axons or layer IV neurons, indicating that abnormalities in *Brl* mutants are secondary to a loss of signals normally conveyed by thalamocortical axons (Iwasato et al., 2008). In addition, it has been shown that mutation of the G-protein-coupled phosphodiesterase *Plc*β*1*, which is thought to be mainly postsynaptic, results in a complete lack of cytoarchitectural organization of layer IV, while thalamocortical axons segregate normally into barrels (Hannan et al., 2001). These data suggest that thalamocortical axons are able to organize into sensory maps, even if cortex fails to

establish a proper cytoarchitectural framework. In this manuscript, we report a striking failure in the formation of precise maps by thalamocortical axons (**Figure 4.10**), and in the concurrent cytoarchitectural reorganization of layer IV neurons in cortex-specific *Ctip1* mutants (**Figure 4.12**). Therefore, proper interactions with and instructive cues from cortex are critical for thalamocortical axons, not only as they pathfind to appropriate areas, but also later as they organize into precise maps. Overall, integrating our findings with prior work in the field favors a model of interdependent barrel field development, in which bidirectional communication between thalamocortical axons and cortical neurons is absolutely required.

Dual control of subtype and area development

Over the course of cortical evolution, radial expansion and neuronal diversification have been accompanied by regional specialization, allowing for increased sophistication of cortical circuitry, and broadening the repertoire of functionally-specialized areas (Rakic, 2009; Molnár, 2011). Although most transcription factors important for cortical development have been described to control either subtype or area specification, recent reports suggest that *Tbr1* and *Couptf1*, which had previously been thought to regulate subtype and area identity, respectively, actually regulate both. Here, and in an accompanying manuscript (Chapter 3), we present evidence that *Ctip1* also has dual roles controlling both subtype and area specification. This is not entirely surprising, as the neocortex was first divided into areas by early neuroanatomists on the basis of regional differences in laminar morphology, cell density, and thickness (Brodmann, 1909). Cytoarchitectural differences reflect whether an area

is specialized for input, output, or integration, and arise from areal adjustments in the relative proportion of neurons instructed to differentiate into CThPN, SCPN, layer IV granule neurons, or CPN. Therefore, in addition to specific input and output connectivity, areal specialization requires production of specific ratios of projection neurons, so it is parsimonious for some transcription factors to be involved both in arealization and subtype specification. These results support a model in which, over the course of evolution, additional transcription factors were recruited to control cortical development, progressively adding new layers of complexity, in terms of both neuronal diversity and areal specialization, to an existing evolutionarily ancestral framework.

4.5 Experimental Procedures

Animals

All mouse studies were approved by the Massachusetts General Hospital and/or 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. Unless noted otherwise, all experiments were performed using mice maintained on a C57BL6/J background, with *Ctip1*$^{fl/fl}$*;Emx1-Cre* mice as experimentals and *Ctip1*$^{wt/wt}$*;Emx1-Cre* mice as controls.

Ctip1$^{fl/fl}$ mice were generated by Tucker and colleagues (Sankaran et al., 2009). *Bhlhb5-LacZ* and *Lmo4-LacZ* mice were generated and generously shared by Gan and colleagues (Deng et al., 2010; Feng et al., 2006). *Emx1-Cre* mice were generated by Jones and colleagues (Gorski et al., 2002), and purchased from Jackson Laboratories (stock number 005628), while *Ntsr1-Cre* mice were generated by Gerfen and colleagues (Gong et al., 2007), and purchased from the MMRRC (stock number 030648-UCD).

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). Embryonic brains were fixed at 4°C overnight, dissected, cryoprotected in 30% sucrose and sectioned at 20μm on a cryostat (Leica). The

following primary antibodies and dilutions used: rabbit anti-5HT, 1:20,000 (Immunostar); goat anti-BHLHB5, 1:300 (Santa Cruz); rabbit anti-COUPTFI, 1:500 (gift from M. Studer), mouse anti-CTIP1 clone 14B5, 1:500 (Abcam); rabbit anti-CUX1, 1:200 (Santa Cruz) chicken anti-GFP, 1:200 (Aves); rabbit anti-GFP, 1:500 (Invitrogen); goat anti-LMO4, 1:200 (Santa Cruz); rabbit anti-RORβ, 1:1000 (Diagenode). Alexa fluorophore-conjugated secondary antibodies (Invitrogen) were used at a dilution of 1:500. For 5HT immunoshistochemistry, sections were developed using the Vectastain ABC kit.

Riboprobes synthesis and nonradioactive *in situ* hybridization were performed using standard methods (Tiveron et al., 1996). Probes for *Crim1*, *Cry-mu*, *Diap3*, and *Igfbp4* were previously described (Arlotta et al., 2005). Probes for *Cdh6*, *Cdh8*, *EphA7*, *EphrinA5*, *Id2* and *Rorb* were generated by RT-PCR.

In utero **electroporation**

For mosaic loss of function experiments, a CMV/β-actin promoter plasmid (derived from CBIG; gift of C. Lois) was used to drive expression of IRES-*Egfp* (control) or *Cre*-IRES-*Egfp* (experimental). Surgeries were performed as previously described (Molyneaux et al., 2005). Briefly, plasmids were microinjected into the lateral ventricle of developing embryos under ultrasound guidance, and electroporated into cortical progenitors using a square wave electroporator (CUY21EDIT, Nepa Gene, Japan), set to deliver five 30V pulses of 50ms, separated by 950ms intervals.

Retrograde labeling

CSMN were labeled from their axonal projections in the spinal cord at P21 by pressure injection of ~240nl of Alexa fluorophore-conjugated cholera toxin B (CTB) (Invitrogen) into the dorsal corticospinal tract between C1 and C2. Brains were collected three days later. For quantification, four adjacent sections from anatomically-matched rostrocaudal positions were counted from each mouse (n=3 for each genotype) by investigators blinded to genotype. Thalamocortical neurons were labeled from their axonal projections in the cortex at P0 by pressure injection of ~80nl of Alexa fluorophore-conjugated CTB into appropriate cortical areas. Brains were collected on the following day.

Anterograde labeling with AAV

All virus work was approved by the Harvard Committee on Microbiological Safety, and conducted according to institutional guidelines. The pAAV-EGFP construct was obtained from the MGH Virus Core and contains the following elements flanked by AAV2 ITRs: a CMV/β-actin promoter, the coding sequence for *Egfp*, the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), a bovine GH pA signal, and an SV40 pA signal (full sequence available upon request). The pAAV-*tdTomato* construct was generated by replacing the *Egfp* coding sequence in pAAV-*Egfp* with the coding sequence for tdTomato obtained from pTdTomato-N1 (Clonetech). Both constructs were packaged and serotyped with the AAV2/1 capsid protein by the MGH Virus Core.

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Chapter 5

Discussion

5.1 Transcriptional controls over neocortical projection neuron development

During the development of the mammalian telencephalon, spatially and temporally heterogeneous progenitors generate a rich variety of projection neuron subtypes. Recent work has identified combinatorial programs of transcription factor controls over the specification and differentiation of corticospinal motor neurons (CSMN) and other neocortical projection neurons (Molyneaux et al., 2007). One of these controls, *Ctip2*, has increasingly emerged as a critical regulator of CSMN development and connectivity, as well as a common target for regulation by multiple projection neuron subtype differentiation pathways (Arlotta et al., 2005; Molyneaux et al., 2005; Alcamo et al., 2008; Britanova et al., 2008; Chen et al., 2008; Lai et al., 2008; Tomassy et al., 2010; McKenna et al., 2011).

In this dissertation, I have characterized functions of *Ctip2* and its paralog *Ctip1* in the control of neocortical projection neuron subtype and area identity acquisition. *Ctip2*, a central control over CSMN terminal differentiation and connectivity, is necessary autonomously within CSMN for axon extension, pathfinding in the midbrain and brainstem, and projection to the spinal cord. However, *Ctip2* is also required non-CSMN-autonomously, within striatal medium-sized spiny neurons, for CSMN fasciculation and pathfinding within the internal capsule. A highly-related zinc finger transcription factor, *Ctip1*, controls the balance between specification of different subtypes of deep-layer projection neurons, favoring the development of corticothalamic projection neurons (CThPN) and deep-layer callosal projection neurons (CPN) at the expense of subcerebral projection neurons (SCPN). In addition, *Ctip1* controls postmitotic refinement of neocortical projection neuron area identity, directing precise area-specific gene expression and projection patterns. In the absence of *Ctip1*, area-

specific genes are diffuse and not restricted to sharply-delineated functional areas, and cardinal areal features, such as the somatosensory barrel field, do not develop. Taken together, I find that *Ctip1* and *Ctip2* independently and cross-repressively function to control projection neuron development.

5.2 Cross-repression between *Ctip1* and *Ctip2*

Cross-repression between opposing transcriptional controls is a common mechanism for boundary establishment in the central nervous system. In the forebrain, pallial and subpallial progenitor domains are delineated by cross-repressive transcriptional interactions between *Pax6* dorsally and *Gsx2* ventrally (Toresson et al., 2000; Stoykova et al., 2000; Schuurmans and Guillemot, 2002), and between *Sox6* dorsally and *Sox5* ventrally (Azim et al., 2009). Next, in the pallium, opposing transcription factor gradients direct progenitors to acquire an initial positional identity. *Emx2*, expressed most highly in caudal areas, and *Pax6*, expressed most highly in rostral areas, are directly cross-repressive, and further specificity is added to the system by repression of *Emx2* by *Couptf1* and repression of *Pax6* by *Sp8* (Bishop et al., 2002; Hamasaki et al., 2004; O'Leary et al., 2007). As projection neurons become postmitotic and begin to acquire a specific subtype identity, cross-repression between *Ctip2* and *Satb2* (Alcamo et al., 2008; Chen et al., 2008) drives a neuron to project either subcerebrally or callosally. In this dissertation, I find that *Ctip1* and *Ctip2*, like many other sets of transcriptional controls across all stages of cortical development, act cross-repressively to specify projection neuron identity.

In Chapter 3, I describe a genetic cross-repressive interaction between *Ctip1* and *Ctip2*. *Ctip1* expression increases in layer V in *Ctip2*$^{-/-}$ cortex, and *Ctip2* expression increases in *Ctip1*$^{-/-}$ cortex (**Figure 3.3**). Furthermore, overexpression of either *Ctip1* or *Ctip2* in wild-type embryos is sufficient to repress transcription of the other protein (**Figure 3.13**). From these data, I conclude that *Ctip1* and *Ctip2* cross-repressively interact to control the development of cortical projection neuron subtype identity. Intriguingly, *Ctip1* expression also increases in *Ctip2*$^{-/-}$ striatal medium-sized spiny neurons (Arlotta et al., 2008), and I find that *Ctip2* expression increases in *Ctip1*$^{-/-}$, but not *Ctip1*$^{fl/fl}$*;Emx1-Cre*, striatum (data not shown); *Ctip1* is sharply and specifically downregulated in the immune system as pro-T cells become committed to a T-cell fate and increase expression of *Ctip2* (Tydell et al., 2007; Li et al., 2010). These data suggest that *Ctip1* and *Ctip2* might have previously-unrecognized cross-repressive functions in cell-type specification decisions across a range of biological systems. Since *Ctip1* and *Ctip2* are both expressed in hippocampus, but in spatially-segregated neuron populations (data not shown), further cross-repressive interactions might also be important for development of hippocampus.

Since *Ctip1* and *Ctip2* are sequence-specific DNA-binding proteins, transcriptional regulation is a likely mechanism for the genetic cross-repression I observe in cortex. Recent work has established CTIP1 binding sites within the human beta-globin locus (Xu et al., 2010) using chromatin immunoprecipitation (ChIP) with the same CTIP1 antibody that we use in tissue (Abcam clone 14B5); the CTIP2 antibody that we use in tissue is also reportedly ChIP-grade (Abcam clone 25B6). It would be interesting to perform ChIP with our CTIP1 and CTIP2 antibodies, followed either by quantitative PCR or by deep sequencing (ChIP-seq), to determine whether CTIP1 and/or CTIP2 binds the genomic locus of the other. I have processed samples from

wild-type cortex at P0, when both CTIP2 and CTIP1 are highly expressed, for this purpose, and intend to perform ChIP-seq to address this question.

I find that increased CTIP2 expression in *Ctip1*$^{fl/fl}$*;Emx1-Cre* cortex causes the production of more neurons with characteristics of SCPN (Chapter 3). Intriguingly, in *Ctip2*$^{fl/fl}$*;Emx1-Cre* cortex, where expression of CTIP1 is increased in layer V, fewer SCPN reach the spinal cord at early postnatal ages, and motor cortex SCPN are more likely to have reached the spinal cord at P2 than SCPN located in somatosensory cortex (**Figure 2.5A-B**). Further, in young adult mice, CSMN located laterally within motor cortex are more likely to prune their spinal projection than CSMN located medially (**Figure 2.5C-D**). I hypothesize that, just as loss of *Ctip1* function shifts motor area identity laterally, such that SCPN in somatosensory cortex maintain motor area identity (**Figure 4.8**), overexpression of CTIP1 in *Ctip2*$^{fl/fl}$*;Emx1-Cre* mice might shift somatosensory area identity medially. These results might also suggest a previously unknown function of *Ctip2* in area identity acquisition and maintenance.

5.3 Integrating functions of *Ctip1* and *Ctip2* in cortical projection neuron development

Ctip1 and *Ctip2* are postmitotic transcription factors that function in acquisition of specific subtype and area identity, and in the development of subtype- and area-specific projection neuron connectivity. Although I observe expression of CTIP1 and CTIP2 protein only in postmitotic neurons (**Figure 3.2, Figure 2.1**), even during embryonic development, I observe expression of both *Ctip1* and *Ctip2* mRNA in cortical

proliferative zones (**Figure 3.2** and data not shown). These data suggest that *Ctip1* and *Ctip2* expression is tightly controlled, and that, like many other developmentally-regulated genes, their genomic loci might be poised for active transcription, which begins as soon as neurons become postmitotic (Zeitlinger et al., 2007).

Early subtype identity specification

Ctip1 and *Ctip2* act first in cortical development to specify subtype identity. Projection neuron subtype specification is a progressive process that begins as postmitotic neurons migrate to and settle in the cortical plate. Newly-postmigratory E14.5 deep-layer projection neurons express high levels of both CTIP1 and CTIP2 (**Figure 3.1**), but most layer V neurons express either CTIP1 or CTIP2 exclusively by E17.5. The mechanisms by which this transition occurs are not known, but it is plausible that small stochastic variations in expression of the two transcription factors might lead to one transcription factor becoming slightly more highly-expressed, then binding to the genomic locus of the other transcription factor and preventing further transcription. This negative feedback loop could result in stark gene expression differences following an initial period of approximately equal expression.

As deep-layer projection neurons begin to express either CTIP1 or CTIP2 by E16.5-E17.5, their axons are traveling through the internal capsule (CThPN and SCPN) or approaching the midline (CPN) (Grant et al., 2012; Lindwall et al., 2007). In fact, corticofugal projection neuron fibers pause between E16 and E17.5 at the edge of the thalamic reticular nucleus, and only after E17.5 do CThPN axons resume growing into the thalamus and SCPN axons toward more caudal regions of the brain (Molnár and Cordery, 1999). Whether a neuron expresses CTIP1 or CTIP2 at E17.5, and at what dose, might influence its axon to enter thalamus or to continue projecting toward the

spinal cord. At this stage, CTIP2 also functions in striatal medium-sized spiny neurons to enable fasciculation of corticofugal axons in the internal capsule (**Figure 2.3**).

Area identity acquisition during the first postnatal week

As neocortical projection neurons establish initial patterns of subtype-specific connectivity during the first days of postnatal life, functional areas begin to acquire distinctive patterns of gene expression and innervation in a process that requires *Ctip1* function. While gradients of gene expression in progenitors and postmitotic expression of area controls such as *Bhlhb5* are both normal embryonically in *Ctip1*$^{fl/fl}$*;Emx1-Cre* mutants (**Figure 4.4**), area-specific gene expression patterns fail to coalesce between P4 and P7 into sharply-demarcated zones (**Figure 4.5**). These data indicate that initial area identity specification occurs appropriately in these mutants, but that subsequent postmitotic refinement of these gradients into crisply-defined areal domains is severely impaired.

Later area-specific pruning of collaterals

Finally, some projection neuron subtypes project promiscuously during development, and *Ctip1* is required for these supernumerary collaterals and axons to be pruned between P7 and P21. In the absence of *Ctip1*, many CPN are unable to project to the contralateral hemisphere (**Figure 3.12**), and those CPN able to cross the callosum project to mirror-image targets with less precision than their wild-type counterparts (**Figure 4.6**). SCPN located in motor cortex normally prune collaterals to tectal and pontine targets, while SCPN located in visual cortex normally maintain connections with the tectum and prune their spinal axons. We are currently investigating whether ectopic expression of *Ctip1* postnatally in motor cortex is

sufficient to induce CSMN to maintain their tectal collaterals, given that *Ctip1* loss-of-function causes corticotectals and other sensory area SCPN to maintain their spinal collaterals (**Figure 4.8**).

5.4 Downstream targets of *Ctip1* and *Ctip2*

Since *Ctip1* and *Ctip2* are transcription factors and likely regulate suites of genes in concert, future work to identify programs of gene expression downstream of *Ctip1* and *Ctip2* will provide insight into mechanisms of projection neuron subtype and area identity development. As discussed above, I have prepared samples of P0 wild-type cortical tissue to perform ChIP-seq, which will identify sites of increased CTIP1 or CTIP2 binding in an unbiased fashion. As deep sequencing capacities evolve, it will be more biologically relevant to perform ChIP-seq on retrogradely-labeled and FACS-purified projection neuron subtypes. The cellular yield on these experiments is very low, since relatively few projection neurons are produced compared with more abundant cell types such as hematopoietic cells, and because some neurons are lost at each stage of the experiment. I have attempted to perform retrograde labeling and FACS purification followed by deep sequencing, and I have determined that, given current limitations of deep-sequencing technology, directly dissecting tissue from defined areas of cortex is a more efficient way to perform this experiment.

Genes downstream of Ctip2

I find that *Ctip2* is cell-autonomously necessary in CSMN for axon outgrowth from the cortical plate, for pathfinding in the midbrain and pons, and for connection

with the spinal cord (Chapter 2). Since *Ctip2* functions at several spatially- and temporally-distinct stages of CSMN axon development, I might find molecules in a range of functional categories that are regulated by *Ctip2* expression in CSMN. Receptors for axon guidance cues would likely be critical downstream targets of *Ctip2*, although as the CSMN growth cone becomes progressively more distant from the nucleus, *Ctip2* might also regulate intermediates that control local protein synthesis in the growth cone. In addition to CSMN-autonomous functions of *Ctip2*, I have preliminary data to suggest that axon guidance molecules and growth factors, including *Plxnd1* and *Efnb3*, are regulated by *Ctip2* in striatal medium-sized spiny neurons (**Figure 2.6**).

Genes downstream of Ctip1

Ctip1, like *Ctip2*, functions at temporally-distinct stages of projection neuron development, and might regulate different suites of downstream genes at different stages. In addition, *Ctip1* might control distinct sets of targets in CThPN, deep-layer CPN, and upper-layer CPN, or in somatosensory and primary visual cortex. Relative to *Ctip2*, *Ctip1* appears to be a higher-level control over neocortical development, as *Ctip1* directly or indirectly regulates a broad range of other transcriptional controls, including *Fezf2*, *Tbr1*, *Fog2*, and *Tle4* in subtype identity (**Figure 3.8**), and *Bhlhb5* and *Lmo4* in area identity (**Figure 4.5**). I might find that *Ctip1* functions primarily by regulating expression of other transcription factors, while *Ctip2* more directly controls CSMN axon development by initiating or repressing expression of functional molecules.

Regulation of Ctip1 *isoforms*

Ctip1 has several known isoforms, and differential isoform expression or regulation might influence transcriptional functions of *Ctip1*. The longest *Ctip1* isoform, known as *Ctip1-XL*, is most structurally similar to *Ctip2*, with six zinc fingers. Other identified isoforms include *Ctip1-L*, with three zinc fingers, *Ctip1-S*, with two zinc fingers, and *Ctip1-XS*, with only one zinc finger; these four isoforms share the same N terminus, but truncate at different points, with *Ctip1-L* and *Ctip1-S* sharing a 3' exon not transcribed in *Ctip1-XL* or *Ctip1-XS* (Nakamura et al., 2000; Satterwhite et al., 2001; Liu et al., 2006).

We have elected to focus on *Ctip1-XL* in our work, since it is most similar to *Ctip2*, since it has been reported by Western blot analysis to be highly expressed in fetal brain tissue (Satterwhite et al., 2001), and since it appears to be the primary functional isoform in the hematopoietic system (Liu et al., 2006; Xu et al., 2010). I have performed immunostaining with CTIP1 antibodies that recognize different isoforms: one specific to the *Ctip1-XL* isoform (Novus Biologicals), one that recognizes both *Ctip1-L* and *Ctip1-XL* (Abcam clone 14B5), and one that recognizes all known isoforms (Abcam clone 15E3C11), and I find identical expression patterns for all three antibodies (data not shown). These data suggest that *Ctip1-XL* is the primary functional isoform in the brain, but other isoforms could bind different genomic loci or contribute to regulation of *Ctip1-XL* expression.

5.5 Lessons from development provide insight into evolution and disease

Understanding programs of molecular controls over specification and precise differentiation of neocortical projection neurons is of great interest toward understanding the development, organization, evolution, and function of the cerebral cortex, and toward informing strategies for nervous system repair, or against degenerative disease and acquired injuries.

Evolution of neocortical projection neuron populations

In this dissertation, I investigate functions of the highly-conserved paralogous transcription factors *Ctip1* and *Ctip2* in neocortical projection neuron development. Although these two genes are highly similar in sequence and structure, they mediate very different functions in projection neuron subtype specification, highlighting the utility of gene duplication for enabling diversity to develop within a biological system. Further, the subtype-specific expression and function of *Ctip1* (**Figure 3.1**) imply an evolutionary lineage relationship between CThPN and deep-layer CPN (Lai et al., 2008; Azim et al., 2009b). These data join other studies suggesting that a major function of transcriptional controls expressed by CThPN and CPN is to suppress expression of *Ctip2* and development of SCPN (Lai et al., 2008; Alcamo et al., 2008; Britanova et al., 2008; Tomassy et al., 2010; Bedogni et al., 2010; Han et al., 2011; McKenna et al., 2011). It is particularly noteworthy that *Ctip2* expression and pyramidal tract formation are abolished in the absence of *Fezf2*, but that both are partially restored in compound *Fezf2*$^{-/-}$*;Tbr1*$^{-/-}$ mutants (McKenna et al., 2011), implying that expression of *Ctip2* and subsequent subcerebral projection might represent a default state for deep-layer projection neurons.

Implications for CSMN disease and circuit repair

The long-term goals of these investigations into development of neocortical projection neurons are both to elucidate controls over the neuron subtype-specific development of CSMN circuitry, and to potentially enable repair of degenerating or injured CSMN. Since CSMN are the brain neurons that degenerate in amyotrophic lateral sclerosis (ALS), and are a central population damaged in spinal cord injury, a detailed understanding of molecular controls regulating the generation and maturation of CSMN, centrally including *Ctip2*, is important for the potential future development of cellular repair strategies for ALS, spinal cord injury, and other diseases affecting CSMN. These experiments suggest (Chapter 2) that endogenous repair of diseased or injured circuitry might require considerable manipulation of guidance cues derived from sources in the midbrain and hindbrain, some of which may not exist after development is complete. However, these experiments also imply that appropriate guidance cues from MSN surrounding the internal capsule are able to fasciculate CSMN axons, even in the absence of important CSMN-intrinsic transcriptional programs. If induced or transplanted CSMN can be directed to enter the internal capsule, striatal programs of fasciculation might be sufficient to guide axons a significant distance through the brain.

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