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Ctip1 regulates the balance between specification of distinct projection neuron subtypes in deep cortical layers

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SUMMARY

The molecular linkage between neocortical projection neuron subtype and area development, which enables the establishment of functional areas by projection neuron populations appropriate for specific sensory and motor functions, is poorly understood. Here, we report that *Ctip1* controls precision of neocortical development by regulating subtype identity in deep-layer projection neurons. *Ctip1* is expressed by postmitotic callosal and corticothalamic projection neurons, but is excluded over embryonic development from corticospinal motor neurons, which instead express its close relative, *Ctip2*. Loss of *Ctip1* function results in a striking bias in favor of subcerebral projection neuron development in sensory cortex at the expense of corticothalamic and deep-layer callosal development, while misexpression of *Ctip1 in vivo* represses subcerebral gene expression and projections. As we report in a paired paper, *Ctip1* also controls acquisition of sensory area identity. Therefore, *Ctip1* couples subtype and area specification, enabling specific functional areas to organize precise ratios of appropriate output projections.

INTRODUCTION

The remarkable complexity of the neocortex is precisely orchestrated during development, as many millions of neurons are born from progenitors, migrate to birthdate-appropriate layers, adopt subtype-specific gene expression and circuit connectivity, and organize into

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AUTHOR CONTRIBUTIONS

M.B.W. and J.D.M. conceived the project; M.B.W. and L.C.G. designed and performed all experiments; M.B.W., L.C.G., and J.D.M. analyzed and interpreted the data; and M.B.W. and L.C.G. made figures. K.X.L. performed qPCR experiments. G.C.I. and H.O.T. contributed a critical mouse line. M.B.W., L.C.G., and J.D.M. synthesized and integrated the findings, and wrote and revised the paper. All authors read and approved the final manuscript.

modality-specific functional areas. Subtype identity is tightly correlated with birthdate, as corticothalamic projection neurons (CThPN) are born around E12.5 in mice, subcerebral projection neurons (SCPN), including corticospinal motor neurons (CSMN), around E13.5, and most callosal projection neurons (CPN) between E14.5-E15.5, with a minority generated and resident with deep-layer subtypes (Woodworth et al., 2012). Nonetheless, functional areas of the cortex vary considerably in their subtype composition; for example, many more SCPN reside in mature motor cortex than in sensory cortex (Greig et al., 2013). This striking reciprocal linkage between subtype and area identity, with functional areas defined in large part by distinctive differences in subtype composition and, therefore, connectivity, has long been appreciated (Brodmann, 1909; Cajal, 1899), but its molecular control remains elusive.

One key transcription factor control over subtype development, *Ctip2*, is expressed at high levels by SCPN and functions centrally in SCPN terminal differentiation and connectivity (Arlotta et al., 2005). Multiple transcription factors controlling projection neuron specification and postmitotic differentiation operate at least in part by regulating *Ctip2* expression. For example, *Fezf2* regulates SCPN differentiation partly by promoting expression of *Ctip2* (Arlotta et al., 2005; Chen et al., 2008; Molyneaux et al., 2005), and by preventing expression of transcription factors key for specification of alternate subtype fates, including *Tbr1* (Bedogni et al., 2010; McKenna et al., 2011) and *Satb2* (Alcamo et al., 2008; Britanova et al., 2008). Conversely, repression of *Fezf2* and *Ctip2* by *Sox5* (Kwan et al., 2008; Lai et al., 2008; Shim et al., 2012) and *Tbr1* (Bedogni et al., 2010; Han et al., 2011; McKenna et al., 2011) in CThPN, and by *Satb2* in CPN (Alcamo et al., 2008; Britanova et al., 2008), is critical for appropriate development of these subtypes. Although these central controls begin to be expressed immediately after neurons exit the cell cycle, subtype identity continues to be refined as neurons mature (Azim et al., 2009), as, for example, initially promiscuous expression of *Ctip2* and *Satb2* in newly postmitotic layer V neurons resolves to subtype-specific expression in SCPN and CPN by late embryogenesis (Alcamo et al., 2008; Britanova et al., 2008).

A paralogous zinc finger transcription factor, *Ctip1* (also known as *Bcl11a*/*Evi9*), is also expressed during development. *Ctip2* and *Ctip1* are closely related, with approximately 60% identity between their nucleotide sequences (Avram et al., 2000; Satterwhite et al., 2001). In the hematopoietic system, *Ctip1* controls the development of B cells (Lee et al., 2013; Liu et al., 2006; 2003) and regulates switching between fetal and adult forms of hemoglobin (Bauer et al., 2013; Canver et al., 2015; Sankaran et al., 2008; 2009; Xu et al., 2011). *Ctip1* is expressed during development of many areas of the nervous system, including neocortex, hippocampus, striatum, and cerebellum (Arlotta et al., 2008; John et al., 2012; Kuo and Hsueh, 2007; Leid et al., 2004). Although *Ctip1* is known to regulate neuronal migration (Wiegreffe et al., 2015), its functions in neocortical subtype development are not known.

Here, we report that *Ctip1* controls projection neuron subtype identity and development of proper proportions of subtypes within a given functional area. *Ctip1* is expressed by CPN, CThPN, and subplate neurons, but is progressively excluded from *Ctip2*-expressing CSMN. 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, more neurons projecting

to the cerebral peduncle, and a reduction in CThPN and CPN gene expression and projections. In contrast, *Ctip1* overexpression *in vivo* represses *Ctip2* expression and reduces the number of neurons projecting subcerebrally, while increasing the number of neurons projecting through the corpus callosum. In accompanying work, we report that *Ctip1* controls the acquisition of sensory area identity and area-specific input/output connectivity (Greig et al., 2016), and thus serves to integrate the dual processes of subtype and area development in neocortex.

RESULTS

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

CTIP1 is first detected by immunocytochemistry at approximately E12.5 in young postmitotic glutamatergic projection neurons in the cortical plate (Figure 1A, Figure S1A). Immunostaining is absent from progenitor zones at mid-gestation (Figure S1D–E), although some expression of *Ctip1* mRNA can be observed by *in situ* hybridization at the same age (Figure S1B–C), suggesting 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. CTIP1 continues to be expressed in postmitotic cortical neurons throughout embryogenesis (Figure 1B), though expression begins to decrease after the first postnatal week. At postnatal day (P) 4, CTIP1 is highly expressed in all six layers of somatosensory cortex (Figure 1C–C′), while expression in motor cortex is largely restricted to subplate, layer V, and the most superficial aspect of layer II/III.

To investigate the subtype specificity of *Ctip1* expression, we retrogradely labeled CThPN, CPN, or CSMN with Alexa fluorophore-conjugated cholera toxin B (CTB) at early postnatal ages (P1-P3) and collected labeled brains at P4. In addition, we performed immunocytochemistry for CTIP1 together with FOG2, SATB2, and CTIP2, markers of CThPN, CPN, and SCPN, respectively (Woodworth et al., 2012). We find that CTIP1 is expressed by CPN and CThPN in sensorimotor cortex, but is essentially excluded from CSMN (Figure 1D–I). CTIP1 is expressed by 90% (±0.9%) of retrogradely labeled CThPN in layer VI, and co-localizes with FOG2 in 62% (±2.7%) of CThPN (Figure 1D, G). Similarly, 68% (±9.1%) of retrogradely labeled CPN express CTIP1, and CTIP1 and SATB2 are co-expressed by 59% (±7.9%) of CPN (Figure 1E, H), with no differences between superficial- and deep-layer CPN (61±7.3% superficial-layer, 57±9.0% deep-layer, not significantly different). Very few neurons labeled retrogradely from the spinal cord express CTIP1 (4.9%±1.1%), and there is very little co-expression of CTIP1 and CTIP2 (4.6% ±1.4%) in motor cortex (Figure 1F, I).

Having determined that CTIP1 is expressed at high levels by CThPN and CPN, but not by CSMN, we next investigated the temporal course of CTIP1 and CTIP2 expression in embryonic cortex. We find that, at E14.5, nearly all early postmitotic layer V neurons co-express CTIP1 and CTIP2 at high levels, but, by E17.5, CTIP1 and CTIP2 are expressed by two largely distinct populations within layer V (Figure S3A–D). Given the retrograde labeling data above (Figure 1E–I), we reasoned that these populations are likely immature CTIP1-expressing CPN and CTIP2-expressing CSMN.

The early segregation of cortical CTIP1 and CTIP2 expression, together with their functions controlling differentiation of closely-related immune cell types (Liu et al., 2003; Tydell et al., 2007; Wakabayashi et al., 2003), led us to hypothesize that *Ctip1* and *Ctip2* 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 qPCR (1.5-fold, $p<0.05$). Conversely, we find increased CTIP1 expression in layers V and VI of *Ctip2*$^{-/-}$ neocortex (1.5-fold, $p<0.05$) (Figure S3E–J). These data indicate that *Ctip1* and *Ctip2* are genetically cross-repressive, and suggest that these transcription factors might function to sharpen differentiation of distinct neuronal subtypes.

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

Since *Ctip1*$^{-/-}$ mice die almost immediately after birth (Liu et al., 2003), we obtained *Ctip1*$^{fl/fl}$ mice, which survive to adulthood (Lee et al., 2013; Sankaran et al., 2008), to study *Ctip1* function as neocortical projections are established and refined. We deleted *Ctip1* specifically from cortical neurons using *Emx1-Cre*, which is expressed beginning in cortical progenitors (Gorski et al., 2002).

Because *Ctip1* is expressed by CThPN and CPN, but not by CSMN, we investigated whether, in the absence of *Ctip1* function, more neocortical neurons differentiate into CSMN, and into SCPN more broadly. We compared wild-type and *Ctip1*$^{fl/fl}$*;Emx1-Cre* tissue at P4 by Nissl and DAPI stain, and find that conditional nulls exhibit defective cortical layering, with a lack of clearly distinct boundaries between adjacent layers, but that layer V, which contains SCPN, is expanded relative to other cortical layers ($p<0.01$, Figure 2A–D, Figure S2). To investigate further, we performed immunocytochemistry and *in situ* hybridization at P0 for several genes expressed by SCPN, including CTIP2, *Fezf2*, and *Clim1* (Arlotta et al., 2005; Azim et al., 2009; Molyneaux et al., 2005). We find that high-level expression of these genes is expanded radially in *Ctip1*$^{fl/fl}$*;Emx1-Cre* cortex, indicating increased layer V thickness (Figure 3A–F). This expansion is especially striking in somatosensory cortex, where layer V is normally thinner than in motor cortex, and where there are normally fewer SCPN than in motor cortex. In agreement with our Nissl results, this expansion appears to be primarily in the direction of deeper layers, suggesting that layer VIa CThPN are converted to SCPN.

Indeed, expression of genes specific to CThPN, including TBR1, FOG2, and DARPP-32 (Molyneaux et al., 2007), is markedly reduced in P0 *Ctip1*$^{fl/fl}$*;Emx1-Cre* cortex, and layer VI is radially thinner (Figure 3G–L). Even within the reduced domain of layer VI, neurons expressing CThPN molecular markers and developmental controls also express inappropriately high levels of CTIP2 and *Fezf2* (asterisks in Figure 3B, 3D), suggesting a mixed SCPN/CThPN identity. TBR1 is expressed by 31% fewer neurons in *Ctip1*$^{fl/fl}$*;Emx1-Cre* cortex ($p<0.05$; Figure 3T), while 32% more neurons express CTIP2 than in wild-type cortex ($p<0.01$; Figure 3S). These data strongly suggest that, in the absence of *Ctip1* function, many neurons that would normally have differentiated into CThPN have instead become SCPN. Expression of CPN genes SATB2, LHX2, and CUX1 (Molyneaux et al., 2009) is approximately normal in deep layers and superficial layers (Figure 3M–R, U).

Similarly, in conditional mutants in which *Ctip1* is deleted postmitotically (*Ctip1$^{fl/fl}$;Nex1-Cre*) (Goebbels et al., 2006), TBR1 expression is reduced relative to wild-type, CTIP2 expression is increased, and SATB2 expression is unchanged in both deep and superficial layers (Figure S3K–N), indicating that *Ctip1* functions postmitotically to specify neuronal subtype. These results demonstrate that, in the absence of *Ctip1* function, more projection neurons postmitotically adopt an SCPN molecular identity, and fewer adopt a CThPN identity.

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

We next investigated whether, beyond dysregulation of characteristic subtype-specific genes, cortical projection targeting is altered in the absence of *Ctip1*. We retrogradely labeled SCPN from the cerebral peduncle at P1 (labeling all neurons projecting to targets caudal to cerebrum), revealing that significantly more axons project to subcerebral targets in *Ctip1$^{fl/fl}$;Emx1-Cre* than in wild-type brains (Figure 4A–C). In particular, the number of SCPN in somatosensory cortex increases 1.4-fold ($p<0.01$), and the number of SCPN in visual cortex increases 1.2-fold ($p<0.05$). The number of SCPN in motor cortex, where CTIP1 expression is normally low (Figure 1C), does not change.

In concordance with expression of SCPN-specific genes expanding deeper into layer VI (Figure 3A–F), most additional SCPN in *Ctip1$^{fl/fl}$;Emx1-Cre* brains are located in the upper portion of layer VI. By combining BrdU birthdating at E12.5 with retrograde labeling from the cerebral peduncle, we find that 23% of *Ctip1* conditional null SCPN have incorporated BrdU at E12.5, when CThPN are usually generated, compared with 9% of wild-type SCPN (Figure 4D–F; $p<0.01$). In the absence of *Ctip1* function, newly born layer VI corticofugal neurons, normally destined to differentiate into CThPN, aberrantly differentiate into SCPN.

Because expression of genes characteristic of CThPN is reduced in *Ctip1$^{fl/fl}$;Emx1-Cre* cortex, and because additional neurons projecting through the cerebral peduncle in *Ctip1$^{fl/fl}$;Emx1-Cre* cortex are aberrantly differentiated E12.5-born neurons, we investigated CThPN projections in the absence of *Ctip1* function. We find that fewer CThPN in *Ctip1$^{fl/fl}$;Emx1-Cre* somatosensory cortex are retrogradely labeled by injection of CTB into thalamus than in wild-type cortex (0.5-fold, $p<0.05$)(Figure 4G–I). Further, we analyzed P0 *Ctip1$^{-/-}$* mice carrying a *Rosa26R-tdTomato* reporter allele (Madisen et al., 2010) and either an *Rbp4-Cre* or an *Ntsr1-Cre* allele (Gong et al., 2007), which are specific to SCPN and CThPN, respectively. Fewer CThPN in *Ctip1$^{-/-}$* cortex are labeled by *Ntsr1-Cre* than in wild-type cortex (0.5-fold, $p<0.01$), and more SCPN are labeled by *Rbp4-Cre* (1.6-fold, $p<0.01$) (Figure 4J–O), indicating that fewer neurons differentiate into mature CThPN in the absence of *Ctip1*. Taken together, these results indicate that *Ctip1* controls the development of corticothalamic projection neurons by suppressing their alternative differentiation into subcerebral projection neurons.

CPN pathfinding is disrupted in the absence of *Ctip1* function

Ctip1$^{fl/fl}$;Emx1-Cre mice exhibit partial agenesis of the corpus callosum, with prominent Probst bundles containing axons that have failed to cross the midline (asterisk in Figure 2B,

D; Richards et al., 2004), and the corpus callosum is thinner in *Ctip1*$^{fl/fl}$*;Emx1-Cre* than in wild-type mice (Figure 2A–D). These data indicate that development of the callosal projection is disrupted in the absence of *Ctip1*. In addition, many superficial-layer neurons in cingulate cortex, normally the earliest-crossing callosal population (Koester and O'Leary, 1994), are instead retrogradely labeled by injection of CTB from the cerebral peduncle (Figure 5A–B), suggesting that pioneering 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 *Dkk3* and *Lpl* (Figure 5C–F; (Molyneaux et al., 2009)), and instead aberrantly express CTIP2 (Figure 5G–H).

Potentially because cingulate CPN fail to pioneer the callosum, fewer deep-layer neurons in somatosensory cortex cross the callosum in *Ctip1*$^{fl/fl}$*;Emx1-Cre* cortex than in wild-type mice (Figure 5I–K; $p<0.01$). Strikingly, layer VI CPN are more severely affected (56% fewer cross; $p<0.01$) than layer V CPN (19% fewer cross; $p<0.05$), suggesting that many 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. However, even projections of later-born CPN are cell-autonomously disrupted in the absence of *Ctip1*, as *Cre* electroporation into *Ctip1*$^{fl/fl}$ cortex at E15.5 results in 52% fewer axons crossing the callosum (Figure S5I–K; $p<0.01$). These data indicate that axon pathfinding is disrupted in CPN across all layers of cortex in the absence of *Ctip1*.

Although the corpus callosum is thinner in *Ctip1*$^{fl/fl}$*;Emx1-Cre* mice, the anterior commissure is notably enlarged (arrows in Figure 5L–M). Indeed, many neurons in *Ctip1*$^{fl/fl}$*;Emx1-Cre* somatosensory cortex are retrogradely labeled by injection of CTB into the anterior commissure, while retrogradely-labeled neurons are not present in this location in wild-type somatosensory cortex (Figure 5N–R). These data indicate that, in the absence of *Ctip1* function, many commissural neurons aberrantly project to the contralateral hemisphere via this abnormal anterior commissure route instead of via the corpus callosum.

Late-born projection neurons migrate abnormally in *Ctip1* mutants

Although superficial-layer CPN outside cingulate cortex are not fate-converted to SCPN in *Ctip1*$^{fl/fl}$*;Emx1-Cre* mice (Figure 4A–B), superficial-layer neurons are improperly laminated (Figure 2A–D, Figure 5I–J) and impaired in their projections (Figure S5I–K). 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 into pregnant females 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. We find significant numbers of E14.5- and E15.5-born neurons ectopically located in deep layers in *Ctip1*$^{fl/fl}$*;Emx1-Cre* mutants (Figure 6A–G; $p<0.05$ 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 superficial layers. These neurons express markers of superficial-layer neurons, such as CUX1, appropriate to their birthdate, but not their laminar position (Figure S5F–H). In contrast, migration of neurons born at E11.5, E12.5, or E13.5 is not significantly affected (Figure S6A–I). These results indicate that many late-born *Ctip1*$^{fl/fl}$*;Emx1-Cre*

neurons migrate and become positioned in deep-layer cortex, leading to abnormal lamination of the cortex as a whole.

Migration defects in *Ctip1*$^{fl/fl}$*;Emx1-Cre* animals might be due to defective signaling to migrating neurons from post-migratory neurons in the cortical plate, or they might be due to aberrant interpretation of cues by migrating neurons themselves. To distinguish between these possibilities, we electroporated E14.5 wild-type or *Ctip1*$^{fl/fl}$ embryos with CMV/β-actin promoter-driven *Cre* and lox-STOP-lox-*Egfp* constructs, and examined electroporated tissue at E17.5 (Figure 6H). This experiment deletes *Ctip1* from only a small fraction of cortical neurons, allowing sparse 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 electroporated neurons to have entered the cortical plate at E17.5 (Figure 6I–K; 13% *Ctip1*$^{fl/fl}$ vs. 51% wild-type; $p<0.01$), indicating that migration abnormalities of superficial-layer neurons lacking *Ctip1* are cell-autonomous. Further, these defects are specific to neurons migrating to the superficial layers, because migration of *Ctip1*$^{fl/fl}$ neurons electroporated with *Cre* at E12.5 and examined at E15.5 is unaffected (not significant; Figure S6J–M).

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

To further investigate potential functions of *Ctip1* in repression of CSMN and SCPN specification, we tested whether overexpression of *Ctip1 in vivo* can repress endogenous expression of *Ctip2* by wild-type CSMN. We electroporated CMV/β-actin promoter constructs driving expression of either control IRES-*nEgfp* (nuclear EGFP) or *Ctip1*-IRES-*nEgfp* into the ventricular zone of E12.5 wild-type embryos, and examined CTIP2 expression at P4 by immunocytochemistry (Figure 7B–C). Strikingly, while many control E12.5 *Egfp*-electroporated layer V neurons are CTIP2-positive (39%), as expected, significantly fewer *Ctip1*-electroporated layer V neurons are CTIP2-positive (7%; $p<0.01$). These data demonstrate that high levels of CTIP1 are sufficient to repress expression of *Ctip2*.

Conversely, overexpression of *Ctip2* or *Fezf2* can repress endogenous *Ctip1* in layer V neurons. When we electroporate *Ctip2*-IRES-*nEgfp* or *Fezf2*-IRES-*nEgfp* at E12.5, we find that fewer layer V neurons express *Ctip1* at P4 compared with layer V neurons electroporated with control IRES-*nEgfp* (*nEgfp*, 64%; *Fezf2*, 37%, $p<0.01$; *Ctip2*, 8%, $p<0.01$; Figure S7). High-level expression either of *Ctip2* or *Fezf2* is therefore sufficient to repress expression of *Ctip1,* although *Ctip2* is more efficient than *Fezf2*.

This cross-repression between *Ctip1* and *Ctip2/Fezf2*, central transcriptional controls over SCPN development, motivated us to investigate whether *Ctip1* is also sufficient to prevent layer V neurons from projecting subcerebrally. We electroporated control IRES-*nEgfp* or *Ctip1*-IRES-*nEgfp* constructs into wild-type embryos at E12.5, then retrogradely labeled CSMN or CPN with CTB (Figure 7A). Strikingly, we find a five-fold reduction in the percentage of *Ctip1*-electroporated neurons projecting to the spinal cord, compared with control *nEgfp*-electroporated neurons (Figure 7F–H; 25% vs. 6%; $p<0.01$). Further, we find a significant increase in the number of *Ctip1*-electroporated layer V neurons projecting

across the corpus callosum (Figure 7I–K; 13% *nEgfp* vs. 20% *Ctip1*; $p<0.05$), indicating that the axons of some *Ctip1*-electroporated neurons are redirected from subcerebral targets and toward contralateral cortical targets. Taken together, these data demonstrate that *Ctip1* overexpression alters the balance of deep-layer projection neuron fate specification against SCPN and in favor of CPN, in both gene expression and axonal projections.

DISCUSSION

Although critical molecular 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, proportions, and balance of subtypes present in specific functionally specialized areas of the cortex. In this work, we identify that the transcription factor *Ctip1* directs the proportional allocation of corticothalamic, subcerebral, and callosal projection neurons in deep cortical layers.

Subtype control over deep-layer neurons

Ctip1 controls the balance between specification of distinct, functionally specialized subtypes of deep-layer cortical projection neurons, and, in the absence of *Ctip1* function, more SCPN are generated at the expense of CThPN and deep-layer CPN. In $Ctip1^{fl/fl}$;*Emx1-Cre* mice, an aberrantly expanded population of neurons with molecular and anatomic characteristics of SCPN occupies an expanded layer V (Figure 3), and more neurons born at E12.5 send axons to the cerebral peduncle instead of to the thalamus, indicating that CThPN located in the upper segment of what would otherwise be layer VI are fate-converted to SCPN (Figure 4).

The population of CThPN located in the most superficial portion of layer VI, near the interface between layer V and layer VI, is particularly vulnerable to switching fate from corticothalamic to subcerebral due to ectopic expression of SCPN genetic controls (Lai et al., 2008; Tomassy et al., 2010). In the absence of *Ctip1*, all layer VI neurons express higher-than-normal levels of *Fezf2* and *Ctip2* (asterisks in Figure 3B, 3D); however, only those neurons located in the most superficial portion of layer VI cease to express CThPN controls (Figure 3G–L) and consequently project subcerebrally (Figure 4A–F). 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, and for allocation of correct proportions 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 CPN, CThPN, and subplate neurons. 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 superficial-layer CPN (low); and *Sox5* by CThPN (high) and SCPN (low) (Arlotta et al., 2005; Hevner et al., 2001; Lai et al., 2008; McKenna et al., 2011; Molyneaux et al., 2005; Woodworth et al., 2012). *Ctip1*, in contrast, is specific only in its exclusion from CSMN (Figure 1D–I). This widespread and non-subtype-specific

expression is most likely responsible for *Ctip1* not being identified as a candidate in microarray-based screens for genes involved in subtype specification (Arlotta et al., 2005; Chen et al., 2005a; 2005b). The data presented here regarding *Ctip1* function suggest that further unidentified controls over subtype development might expand or contract populations of neurons by action in developmentally, and likely evolutionarily, related populations.

Abnormalities of commissural neurons

In the absence of *Ctip1* function, fewer deep-layer CPN project through the corpus callosum (Figure 5I–K), and, instead, many deep-layer neurons in somatosensory cortex project to the contralateral hemisphere via the anterior commissure (Figure 5L–R). This re-routing might be necessary because the superficial-layer neurons in cingulate cortex that would normally pioneer the callosal projection instead aberrantly project through the pyramidal tract (Figure 5A–H), impairing pathfinding by deep-layer CPN. Alternatively, more AC-projecting neurons might be specified in the absence of *Ctip1* function, reflecting a previously unidentified balance between specification of CC-projecting and AC-projecting commissural neurons controlled, at least in part, by *Ctip1*. We do not exclude the possibility that *Ctip1* might regulate expression of axon guidance molecules necessary for pathfinding by CPN. *Ctip1* might regulate pathfinding in SCPN and CThPN, as well, particularly in light of aberrant subplate neuron gene expression and axon extension in null mice (Figure S4).

In contrast with these striking abnormalities of deep-layer CPN targeting, *Ctip1*$^{fl/fl}$*;Emx1-Cre* superficial-layer CPN neither express SCPN-specific controls (Figure 3A–F) nor project through the cerebral peduncle (Figure 4A–B), except in cingulate cortex (Figure 5A–H). Although *Ctip1* is expressed at high levels by superficial-layer CPN (Figure 1C), these neurons appear to be specified largely normally in the absence of *Ctip1* (Figure 3M–R), although many of their axons do not successfully cross the corpus callosum (Figure S5I–K). These data are consistent with the hypothesis that superficial-layer and deep-layer CPN are substantially different from each other molecularly and with regard to their developmental origins, perhaps reflecting distinct evolutionary events of projection neuron diversification (Arlotta et al., 2008; Fame et al., 2011; Molyneaux et al., 2009). *Ctip1* is expressed by both superficial-layer and deep-layer CPN, but is necessary for subtype specification only for deep-layer CPN, which appear to bear a closer relationship to concurrently-generated corticofugal projection neurons (Greig et al., 2013; Sohur et al., 2012). Loss of *Ctip1* function contrasts starkly with the loss-of-function phenotype observed for another important negative regulator of SCPN development, *Satb2*, in which large numbers of both superficial-layer and deep-layer neurons aberrantly express SCPN controls such as CTIP2 (Alcamo et al., 2008; Britanova et al., 2008; Srinivasan et al., 2012). This contrast indicates that an increasingly rich set of molecular regulators controls both novel and potentially subtle, but likely functionally critical, aspects of precise projection neuron development.

Ctip1 and *Ctip2* interact cross-repressively in developing cortex

Ctip1 and *Ctip2*, paralogous and highly similar transcription factors, are initially co-expressed in developing cortex (Figure S3A–B), but later are expressed in a complementary fashion, with CTIP1 expressed by CPN and CThPN, and CTIP2 expressed by SCPN (Figure 1D–F, Figure S3C–D). Expression of CTIP2 increases in the absence of *Ctip1*, and

expression of CTIP1 increases in the absence of *Ctip2* (Figure S3E–J). Furthermore, overexpression of either *Ctip1* or *Ctip2* at E12.5 *in vivo* is sufficient to repress expression of the other (Figure 7B–C, Figure S7A, S7C). From these data, we conclude that *Ctip1* and *Ctip2* cross-repressively interact to control the development of cortical projection neurons. *Ctip1* and *Ctip2* exhibit similar patterns of exclusive expression in development of other cell types; *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).

Our model of *Ctip1* function is at odds with that of Cánovas et al. (Cánovas et al., 2015), who recently proposed that CTIP1 is extensively co-expressed with CTIP2 and is a positive control over SCPN development through repression of TBR1. In direct contrast, we demonstrate that expression of TBR1 and other characteristic CThPN genes is reduced in the absence of *Ctip1* (Figure 3G–L, Figure 4M–O), and that expression of CTIP2 is increased (Figure 3A–B), leading to more early-born neurons becoming SCPN (Figure 4A–F). These discrepancies likely result from different loss-of-function approaches, as our investigation uses null and conditional null mouse lines, rather than modest knockdown of *Ctip1* using mosaic shRNA electroporation in wild-type animals.

Ctip1 as a candidate human intellectual disability gene

CTIP1 is highly conserved from mouse to human, with only three amino acid substitutions (99.6% identity) between the longest mouse and human CTIP1 isoforms, and *Ctip1* and its promoter are located within one of the most ultraconserved non-coding region-rich stretches of the human genome (Sandelin et al., 2004). CTIP1 is expressed in fetal human brain (Satterwhite et al., 2001), although the subtype specificity of its expression in human brain is not known. Intriguingly, *Ctip1* is one of three protein-coding genes consistently deleted in 2p15-p16.1 microdeletion syndrome, which is characterized by intellectual disability, microcephaly, and frequently by autistic behavior (Hancarova et al., 2013), and a patient with a *de novo Ctip1* microdeletion has been reported with severe speech sound disorder and intellectual delay (Peter et al., 2014). These phenotypes are consistent with functions of *Ctip1* in the subtype-specific differentiation and migration of neocortical projection neurons, suggesting that *Ctip1* also functions critically in organizing the human neocortex.

Ctip1 couples development of projection neuron subtypes and cortical areas

Although subtype and area development are typically analyzed as independent developmental vectors in the emergence of the mature neocortex, the two are, in fact, highly interdependent, since the proportion and identity of neuron subtypes varies significantly by cortical area (Brodmann, 1909; Cajal, 1899). Molecular programs controlling subtype and area differentiation likely interact at multiple nodes; here, we identify *Ctip1* as a central node. *Ctip1* directs the proportional allocation of subcerebral and corticothalamic projection neurons in sensory cortex, and, in the absence of *Ctip1*, sensory cortical areas are transformed, with subtype compositions, gene expression, and output connectivity that is usually present in motor areas (Figure 3; Figure 4; Greig et al., 2016). Strikingly, the expression (Figure 1, Figure S1) and functions (Figure S3) of *Ctip1* in postmitotic neurons indicate that this regulation of subtype composition in distinct cortical areas occurs

postmitotically. Therefore, the final non-uniform distribution of cortical projection neuron subtypes across cortical areas is based, at least in part, not on differences in subtype production, but in subtype differentiation.

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 cingulate cortex neurons, allowing these populations to pioneer callosal projections. Finally, *Ctip1* enables the proper migration and laminar positioning of superficial-layer callosal projection neurons, permitting these neurons to populate correct laminar locations. Identification of further fine transcriptional controls over the precise differentiation, diversity, positioning, and connectivity of neocortical projection neurons will increasingly illuminate the staggering organizational and functional complexity of the mature neocortex, and provide insight into overt and subtle dysgenesis that can result in human cognitive, sensory, motor, and integrative disorders.

EXPERIMENTAL PROCEDURES

Animals

All mouse studies were approved by the Harvard University IACUC, and were performed in accordance with institutional and federal guidelines. The date of vaginal plug detection was designated E0.5, and the day of birth as P0. Unless noted otherwise, all experiments with *Ctip1*$^{fl/fl}$*;Emx1-Cre* were controlled with *Ctip1*$^{wt/wt}$*;Emx1-Cre*, and *Ctip1*$^{-/-}$ with *Ctip1*$^{wt/wt}$, although no abnormal phenotypes were observed in *Ctip1*$^{-/wt}$ or *Ctip1*$^{fl/wt}$*;Emx1-Cre* heterozygotes. Sources of mouse lines used are described in Supplemental Information.

Immunocytochemistry and *in situ* hybridization

Mice were transcardially perfused with 4% paraformaldehyde, and brains were dissected and post-fixed at 4°C overnight. Tissue was sectioned at 50μm on a vibrating microtome (Leica). Non-specific binding was blocked by incubating tissue and antibodies in 8% goat serum/0.3% bovine serum albumin in phosphate-buffered saline. For DAPI staining, tissue was mounted in DAPI-Fluoromount-G (SouthernBiotech). Antibodies and *in situ* probes used are described in Supplemental Information.

BrdU birthdating

Timed pregnant females were intraperitoneally injected with bromodeoxyuridine (50 mg/kg in PBS) at E11.5, E12.5, E13.5, E14.5, or E15.5. Tissue was collected at P4 and processed for BrdU immunocytochemistry (Magavi and Macklis, 2008).

For analysis of BrdU-labeled SCPN, BrdU labeling was performed as above at E12.5, and littermate pairs were retrogradely labeled from the cerebral peduncle as described below. For

acute BrdU administration, timed pregnant females were intraperitoneally injected with BrdU (75 mg/kg) at E14.5, and embryos were collected 30 minutes later.

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; coding sequence from NM_001242934); or IRES-*nEgfp* (nuclear EGFP) (control) or *Ctip1 XL*-IRES-*nEgfp*, *Ctip2*-IRES-*nEgfp*, or *Fezf2*-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 pups electroporated in somatosensory cortex 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 lox-STOP-lox-*Egfp* and *Cre* in $Ctip1^{wt/wt}$ (control) or $Ctip1^{fl/fl}$ (experimental) embryos. Plasmids were electroporated at age indicated in figure (E12.5, E14.5, or E15.5) and brains were collected either 72 hours later (Figure 6, Figure S6) or at P4 (Figure S5). Electroporation conditions were described previously (Molyneaux et al., 2005).

Retrograde labeling

Projection neurons were labeled from their axon termini under ultrasound guidance by pressure injection of Alexa fluorophore-conjugated cholera toxin B (Invitrogen). SCPN were labeled by injection into cerebral peduncle at P1, CThPN were labeled by injection into sensory thalamic nuclei (VPM and VPL) at P1, CSMN were labeled by injection into cervical spinal cord at P2, and CPN were labeled by injection into contralateral corpus callosum at P3. Anterior commissure-projecting neurons were labeled by injection into contralateral anterior commissure at P1. Tissue for all injections was collected at P4, and processed as for immunocytochemistry, above.

Quantification and statistics

Littermate pairs of experimental and control mice were collected at age indicated in figure and processed as for immunocytochemistry, above. Anatomically matched sections from each mouse (generally at the level of the anterior commissure, unless otherwise noted) were selected, and single confocal slices of somatosensory cortex were imaged. Cells positive for indicated marker(s) (retrograde label, protein by immunocytochemistry, BrdU label, GFP by electroporation) were counted within a box of pre-defined size spanning the radial thickness of cortex, with the same size box applied to wild-type and mutant images. Except where specifically noted (Figure 2, Figure S2), data are not corrected for differences in cortical thickness between wild-type and mutant animals (Table S1). Statistical analyses were conducted using unpaired two-tailed t-tests in Microsoft Excel, with a significance threshold of $p<0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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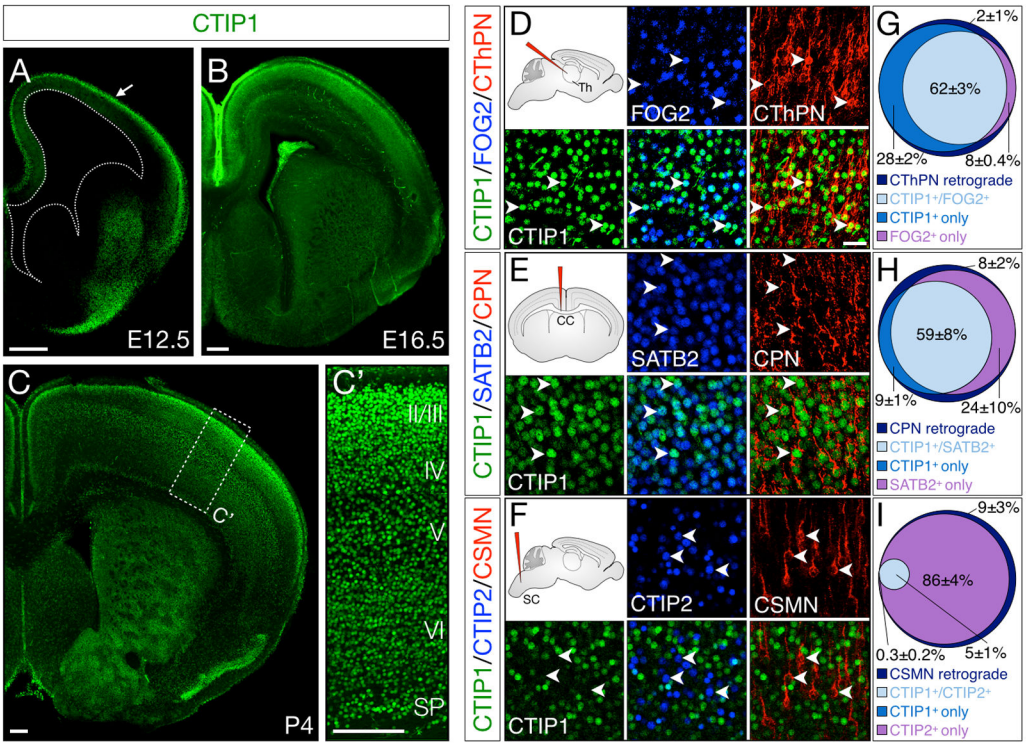

Figure 1. *Ctip1* is expressed by postmitotic corticothalamic and callosal projection neurons, but is excluded from corticospinal motor neurons

(A–C′) CTIP1 immunocytochemistry on coronal brain sections. CTIP1 is expressed by newly-postmitotic projection neurons in the cortical plate beginning around E12.5 (A, arrow) and continuing through E16.5 (B) and P4 (C). At P4, CTIP1 is expressed by neurons in all layers of primary sensory cortex (C′).

(D–I) At P4, CTIP1 is expressed by CThPN and CPN, but not by CSMN. CTIP1 co-localizes with FOG2, as well as with cholera toxin B (CTB) retrograde label after injection into thalamus (D). CPN express both CTIP1 and SATB2, and are retrogradely labeled by injection of CTB into the corpus callosum (E). CSMN are retrogradely labeled by CTB injection into the cervical spinal cord, and express CTIP2, but not CTIP1 (F). Quantification, n=4 (G–I).

CC, corpus callosum; CPN, callosal projection neurons; CSMN, corticospinal motor neurons; CThPN, corticothalamic projection neurons; SC, spinal cord; SP, subplate; Th, thalamus.

Scale bars: 200um (A–C), 40um (D–F). Data are represented as mean ± SEM.

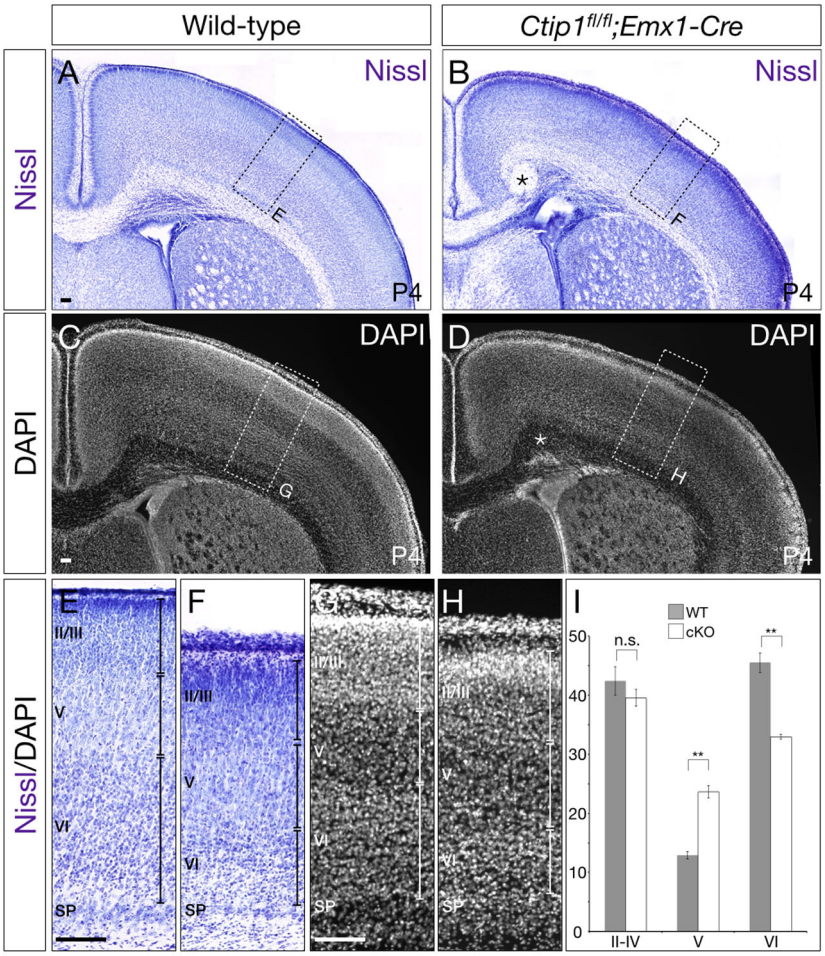

Figure 2. In the absence of neocortical *Ctip1*, cortical layer V is expanded at the expense of cortical layer VI

(A–H) Nissl staining and DAPI staining of cortex in wild-type (A, C) and *Ctip1*$^{fl/fl}$*;Emx1-Cre* cortical conditional null (B, D) mice at P4. Layer V is expanded in conditional null cortex (F, H) compared with wild-type (E, G). Asterisk in B and D marks the location of a Probst bundle.

(I) Quantification of layer thickness performed on Nissl-stained tissue, with each layer presented as a percentage of total cortical thickness (n=5).

Scale bars: 100um. Data are represented as mean ± SEM.

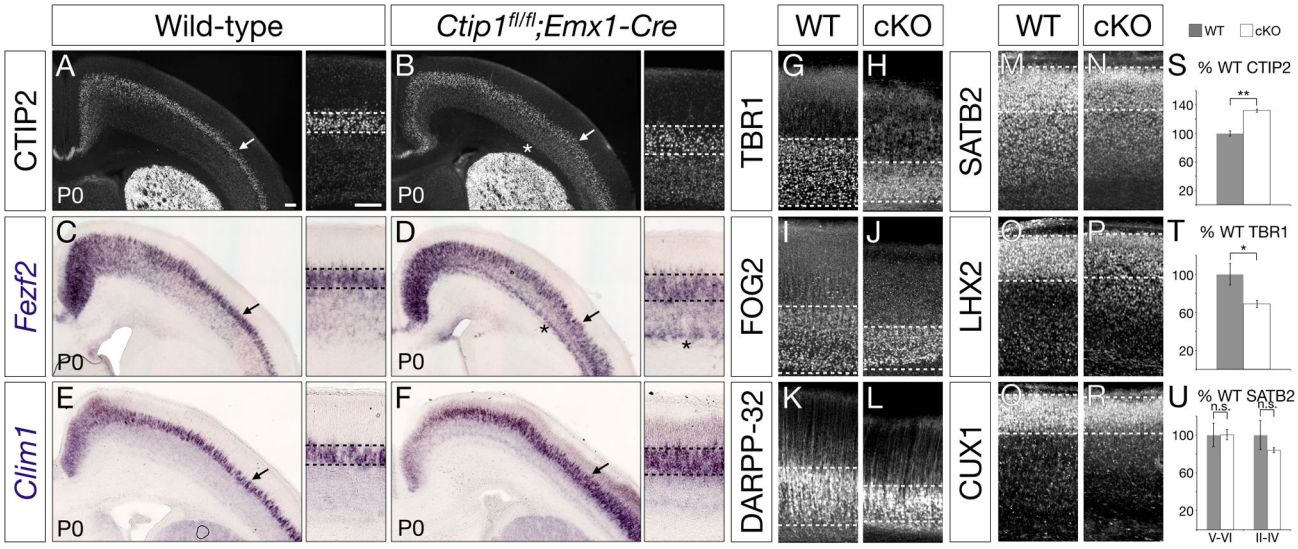

Figure 3. More neurons in *Ctip1*$^{fl/fl}$*;Emx1-Cre* cortex adopt a subcerebral projection neuron identity, and fewer adopt a corticothalamic projection neuron identity

(A–F) In the absence of *Ctip1* function, expression of SCPN marker and control genes CTIP2, *Fezf2*, and *Clim1* increases at P0, especially in somatosensory cortex (arrows in A–F). Dashed lines denote superficial and deep limits of gene expression.

(G–L) In tandem, expression of CThPN marker and control genes TBR1, FOG2, and DARPP-32 is reduced at P0. Dashed lines denote superficial and deep limits of gene expression. Even layer VI neurons that continue to express CThPN identity genes (H, J, L) express aberrantly high levels of CTIP2 and *Fezf2* (asterisks in B, D), suggesting mixed CThPN/SCPN identity.

(M–R) CPN marker and control genes SATB2, LHX2, and CUX1 are expressed normally at P0, although boundaries between superficial and deep layers are more difficult to discern. Dashed lines denote superficial and deep limits of gene expression.

(S–U) Quantification of the number of neurons expressing CTIP2 (S), TBR1 (T), and SATB2 (U) in conditional null cortex, presented as a percentage of wild-type neurons, n=3 for each marker.

*, $p<0.05$; **, $p<0.01$; n.s., not significant

Scale bars: 100um. Data are represented as mean ± SEM.

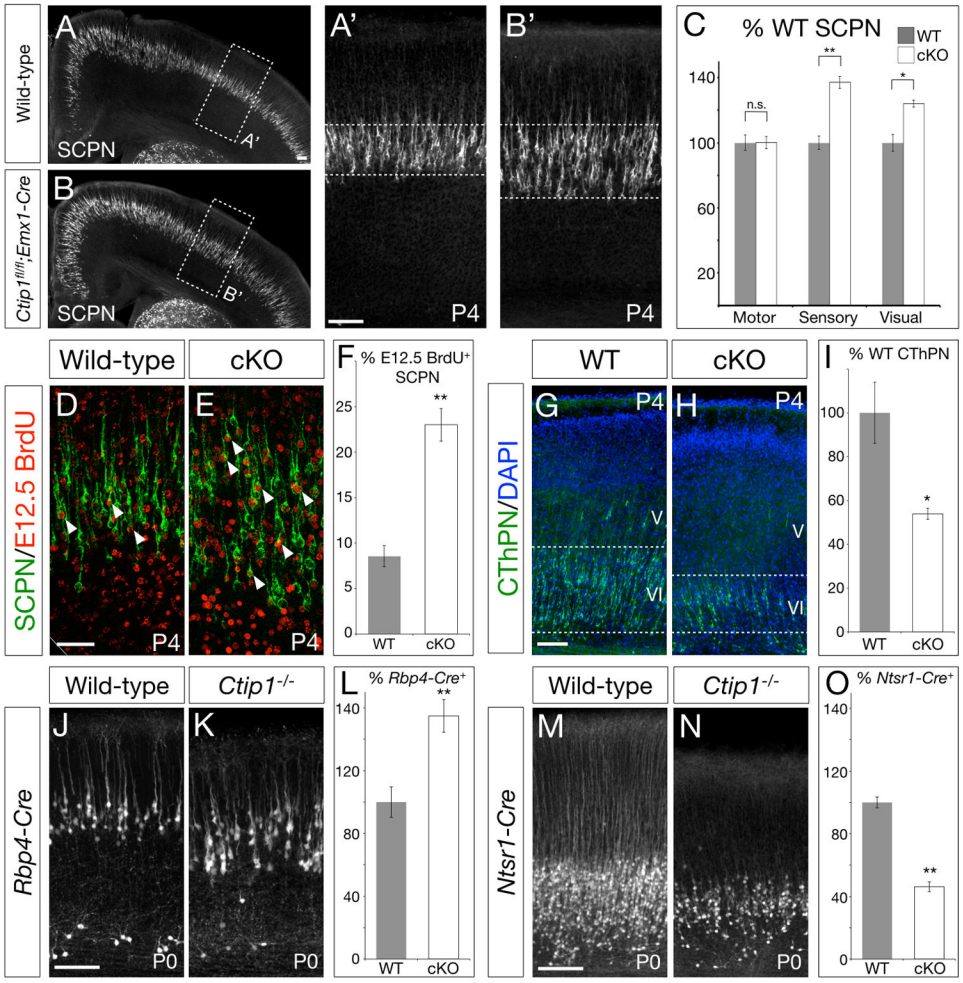

Figure 4. More neurons in *Ctip1*$^{fl/fl}$*;Emx1-Cre* somatosensory and visual cortex project toward subcerebral targets, and fewer project toward thalamic targets

(A–B) More neurons in *Ctip1* conditional null somatosensory cortex (B, B′) are retrogradely labeled by injection of cholera toxin B (CTB) into the cerebral peduncle than in wild-type cortex (A, A′).

(C) Quantification of retrogradely-labeled SCPN by area, presented as a percentage of wild-type SCPN in each area, n=3.

(D–F) Additional SCPN in *Ctip1*$^{fl/fl}$*;Emx1-Cre* brains are aberrantly differentiated from E12.5-born neurons. More neurons labeled by injection of BrdU at E12.5 are co-labeled by injection of CTB into the cerebral peduncle in conditional null cortex (E) compared with wild-type cortex (D). Arrowheads mark representative co-labeled neurons. Quantification, n=4 (F).

(G–I) Compared with wild-type (G), fewer CThPN are retrogradely labeled in somatosensory cortex of *Ctip1*$^{fl/fl}$*;Emx1-Cre* mutants (H) following CTB injection into sensory thalamic nuclei. Quantification (I).

(J–O) *Ctip1* null cortex contains more mature SCPN and fewer mature CThPN than wild-type. More neurons are marked by SCPN-specific *Rbp4-Cre* in *Ctip1*$^{-/-}$ P0 cortex (K) compared with wild-type (J) (n=4). In contrast, fewer neurons are marked by CThPN-

specific *Ntsr1-Cre* in null P0 cortex (N) compared with wild-type (M) (n=3). Quantification (L, O). Recombination by *Rbp4-Cre* and *Ntsr1-Cre* is reported by a *Rosa26R-tdTomato*^{fl} allele.

n.s., not significant; *, $p<0.05$; **, $p<0.01$

Scale bars: 100um (A–B, G–H, J–K, M–N), 50um (D–E). Data are represented as mean ± SEM.

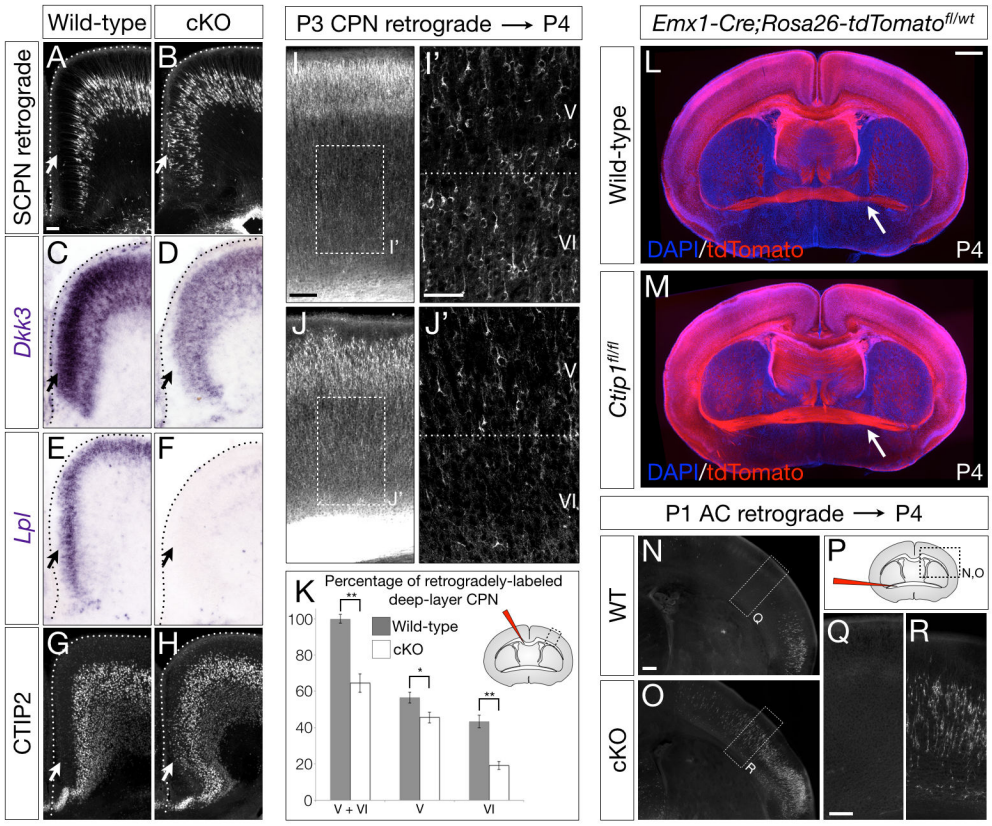

Figure 5. Cingulate CPN fail to pioneer the callosum in the absence of *Ctip1* function, impairing projections of deep-layer CPN

(A–H) Superficial-layer neurons in *Ctip1*$^{fl/fl}$*;Emx1-Cre* cingulate cortex aberrantly project through the cerebral peduncle (A–B), fail to express cingulate CPN genes *Dkk3* (C–D) and *Lpl* (E–F), and instead express CTIP2 (G–H).

(I–K) Failure of cingulate CPN to pioneer the corpus callosum results in a reduction of retrogradely-labeled deep-layer CPN in *Ctip1*$^{fl/fl}$*;Emx1-Cre* cortex. Fewer CPN are retrogradely labeled in P4 *Ctip1*$^{fl/fl}$*;Emx1-Cre* layers V and VI (J-J′) compared with wild-type (I–I′). Quantification of I–J as a percentage of wild-type deep-layer CPN, n=3 (K). Layer VI CPN are more severely affected than layer V CPN. Schematic of injection site, upper right corner.

(L–R) More neurons in *Ctip1* conditional null brains aberrantly project to the contralateral hemisphere via the anterior commissure pathway. The anterior commissure is abnormally enlarged in P4 *Ctip1*$^{fl/fl}$*;Rosa26R-tdTomato*$^{fl/wt}$*;Emx1-Cre* brains (arrow in M) compared with *Ctip1*$^{wt/wt}$*;Rosa26R-tdTomato*$^{fl/wt}$*;Emx1-Cre* (wild-type) (arrow in L). Retrograde labeling from the contralateral hemisphere at P1 reveals that additional AC-projecting neurons in *Ctip1* conditional null brains are aberrantly located in somatosensory cortex, an area where AC-projecting neurons are not normally located (N–O, Q–R). Schematic of injection site (P).

*, p<0.05; **, p<0.01

Scale bars: 100um (A–J), 50um (I′–J′), 500um (L–M), 200um (N–R). Data are represented as mean ± SEM.

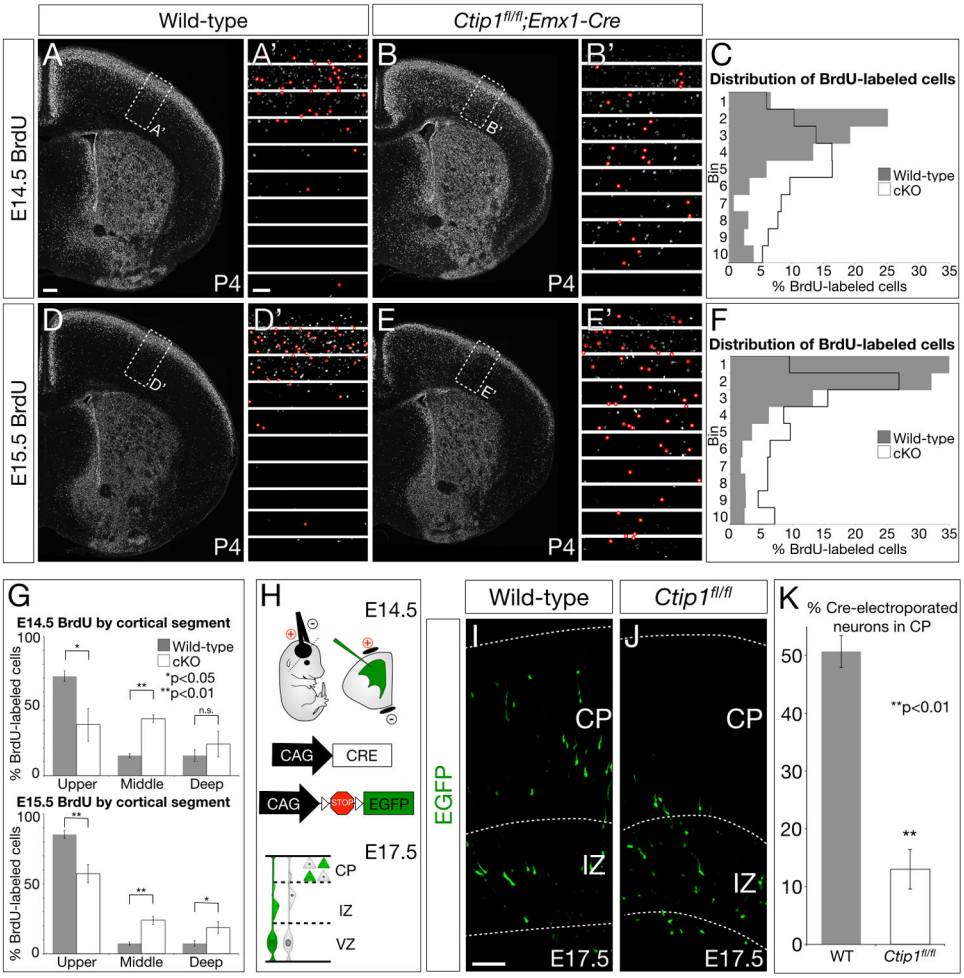

Figure 6. In the absence of neocortical *Ctip1*, superficial-layer projection neurons migrate aberrantly and become inappropriately positioned in cortex

(A–F) Wild-type neurons labeled by BrdU injection at E14.5 (A–A′) or E15.5 (D–D′) are primarily located in superficial layers, while *Ctip1*$^{fl/fl}$*;Emx1-Cre* neurons labeled by BrdU at E14.5 (B–B′) or E15.5 (E–E′) are frequently ectopically located in deep layers. The overall laminar distribution of labeled neurons (marked in red in A′–B′, D′–E′) is strikingly abnormal (C, F). Bin 1 is the most superficial bin, and Bin 10 is the deepest.

(G) Quantification of A–F, with bins 1–4 grouped as “upper” cortical segment, 5–7 as “middle”, and 8–10 as “deep”.

(H–K) Sparse electroporation of *Cre* at E14.5 causes *Ctip1*$^{fl/fl}$ neurons to be delayed in the intermediate zone rather than migrate into the cortical plate at E17.5. Schematic of experimental approach (H). Wild-type neurons electroporated with *Cre* (I) are significantly more likely than electroporated *Ctip1*$^{fl/fl}$ neurons (J) to have migrated into the cortical plate by E17.5 (quantification, K).

CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone; *, p<0.05; **, p<0.01

Scale bars: 200um (A–B, D–E), 50um (A′–B′, C′–D′, I–J). Data are represented as mean ± SEM.

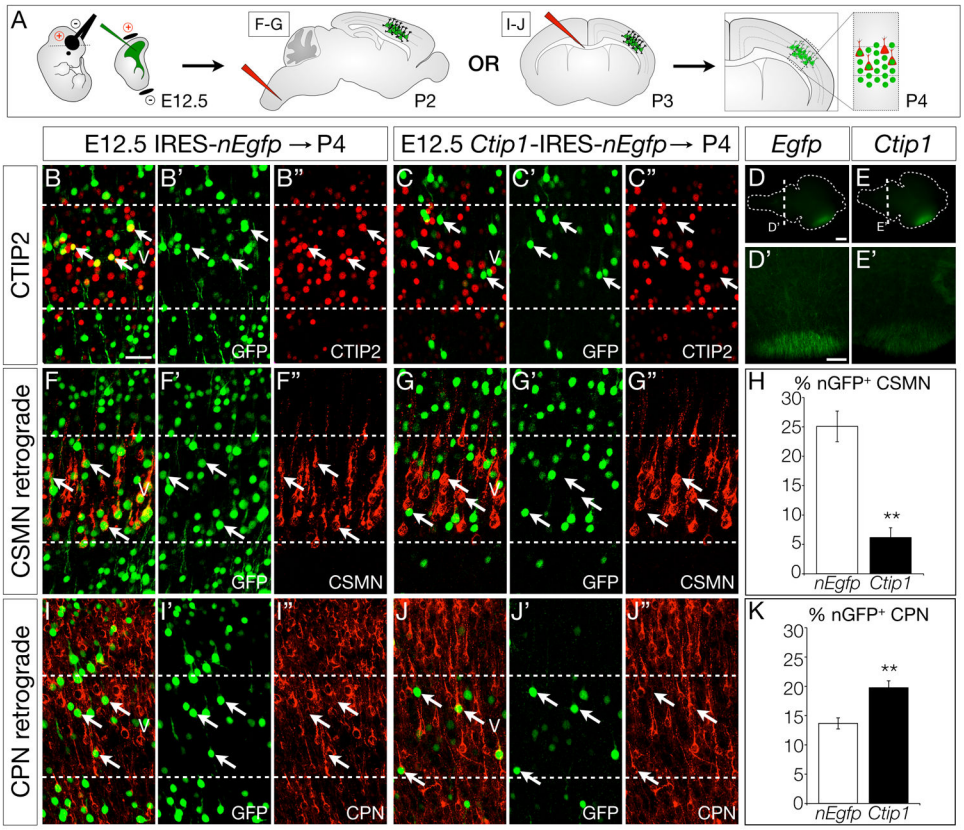

Figure 7. CTIP1 misexpression *in vivo* represses SCPN identity and projection to the spinal cord

(A) Schematic of experimental approach. Wild-type embryos were electroporated at E12.5, and retrograde labeling from spinal cord (F–G) or from the contralateral cortical hemisphere (I–J) was performed on electroporated pups at P2 or P3, respectively. Brains were collected at P4 and somatosensory cortex was imaged and analyzed.

(B–C) Many neurons electroporated with control nuclear *Egfp* (*nEgfp*) at E12.5 express CTIP2 (B–B″; 39%), while few neurons electroporated with *Ctip1*-IRES-*nEgfp* express CTIP2 (C–C″; 7%), n = 3.

(D–K) Misexpression of *Ctip1* causes E12.5-born neurons to redirect their axons away from the brainstem and spinal cord, toward targets on the contralateral cortical hemisphere. Neurons electroporated with *Ctip1*-IRES-*Egfp* at E12.5 send few axons to the brainstem (wholemount electroporated brains in D–E, coronal brainstem sections in D′–E′). Fewer neurons electroporated at E12.5 with *Ctip1*-IRES-*nEgfp* are retrogradely labeled by spinal cord injection at P2 than those electroporated with control *nEgfp* (F–G; quantification in H, n = 3), while more *Ctip1*-electroporated neurons than control *nEgfp*-electroporated neurons are retrogradely labeled by injection into the corpus callosum (I–J; quantification in K, n = 3).

**, p<0.01

Scale bars: 50um (B–C, F–G, I–J), 1mm (D–E), 100um (D′–E′). Data are represented as mean ± SEM.