Area-specific temporal control of corticospinal motor neuron differentiation by COUP-TFI

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Transcription factors with gradients of expression in neocortical progenitors give rise to distinct motor and sensory cortical areas by controlling the area-specific differentiation of distinct neuronal subtypes. However, the molecular mechanisms underlying this area-restricted control are still unclear. Here, we show that COUP-TFI controls the timing of birth and specification of corticospinal motor neurons (CSMN) in somatosensory cortex via repression of a CSMN differentiation program. Loss of COUP-TFI function causes an area-specific premature generation of neurons with cardinal features of CSMN, which project to subcerebral structures, including the spinal cord. Concurrently, genuine CSMN differentiate imprecisely and do not project beyond the pons, together resulting in impaired skilled motor function in adult mice with cortical COUP-TFI loss-of-function. Our findings indicate that COUP-TFI exerts critical areal and temporal control over the precise differentiation of CSMN during corticogenesis, thereby enabling the area-specific functional features of motor and sensory areas to arise.

The fate of neurons and lamination in cortical architectures in each specific area determines their function: the adult primary motor cortex contains a large number of CSMN and has a thick layer V; the primary somatosensory area is characterized by a thick layer IV, where the neurons that receive relayed sensory inputs are located (10). The area-specific differences in neuronal fate and cortical architecture have been thought to result from late postmitotic events, e.g., selective postnatal pruning of axons (11), and premitotic events, such as the timing, rate, and duration of proliferation of precursors producing distinct projection neuron subtypes (12–16). As a striking illustration of such processes, CSMN are generated at a higher rate in the developing motor cortex than in sensory areas in mice (12), but the molecular mechanisms that control this area-specific differential production of CSMN are not known. The transcription factor COUP-TFI is particularly interesting in this regard, because it is expressed at different levels in presumptive sensory and motor cortices, and could thus underlie the striking cytoarchitectural differences between these two cortical areas (17, 18). Using cortex-specific conditional loss-of-function of COUP-TFI, we have previously demonstrated that this transcription factor is critical for areal patterning by acting in sensory cortex to repress frontal/motor cortical area identity (17). COUP-TFI has also been shown to regulate neuronal differentiation (19) and, together with COUP-TFI, to control the timing of the switch of progenitor cells from neurogenesis to gliogenesis in the developing cortex (20). Given the dual function of COUP-TFI in neuronal and areal specification, we hypothesized that COUP-TFI might control sensory area formation by repressing a “motorizing” genetic program of differentiation in neurons of the somatosensory cortex.

We find this to be the case, and show that in the absence of COUP-TFI function, CSMN are born prematurely in somatosensory cortex, at a time when layer VI corticothalamic neurons are normally born. Layer V is expanded at the expense of layer VI, with a corresponding redistribution of neurons expressing CSMN-specific genes and projecting to the spinal cord. In the context of an aberrantly expanded motor cortex and a corticospinal tract consisting largely of the axons of abnormally specified corticothalamic neurons, adult COUP-TFI conditional mutant mice exhibit impaired fine motor skills, reinforcing the necessity for precision in both areal and temporal control of CSMN differentiation. Our results indicate a critical role for COUP-TFI in controlling the emergence of the area-specific cytoarchitectural and functional features of sensory and motor cortical areas during corticogenesis, via specific areal and temporal repression of a CSMN differentiation program in corticofugal neurons of the somatosensory cortex.

Results

COUP-TFI Regulates the Laminar Cytoarchitecture and the Molecular Identities of Corticofugal Neurons in Somatosensory Cortex. COUP-TFI is expressed in the caudal-most region of the telencephalic anlage as early as embryonic day (E) 9.5 before neurogenesis starts, and this high caudo-lateral to low rostro-medial expression pattern becomes very prominent at E13.5 during the peak period of CSMN production (Fig. S1). To investigate whether COUP-TFI might restrict the generation or specification of layer V CSMN in somatosensory and motor cortices, and could thus underlie the striking cytoarchitectural differences between these two cortical areas (17, 18). Using cortex-specific conditional loss-of-function of COUP-TFI, we have previously demonstrated that this transcription factor is critical for areal patterning by acting in sensory cortex to repress frontal/motor cortical area identity (17). COUP-TFI has also been shown to regulate neuronal differentiation (19) and, together with COUP-TFI, to control the timing of the switch of progenitor cells from neurogenesis to gliogenesis in the developing cortex (20). Given the dual function of COUP-TFI in neuronal and areal specification, we hypothesized that COUP-TFI might control sensory area formation by repressing a “motorizing” genetic program of differentiation in neurons of the somatosensory cortex.

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to sensory cortex, we analyzed the expression of selected CSMN-specific genes in the frontal/motor (motor) and parietal/somatosensory (S1) cortices in wild type (WT) and in the frontal motor and parietal/somatosensory motorized (mS1) area in COUP-TFI conditional mutant (CKO) mice (Fig. 1). The transcription factors Fezf2 and CTIP2 are specifically expressed at high levels by CSMN and related subcerebral projection neurons in layer V, and at much lower levels by corticothalamic neurons in layer VI (4, 6, 21). In WT mice, both Fezf2 and CTIP2 delineate a much broader and denser layer V in the motor cortex than in S1 (Fig. 1 A, C, D, G, and H), reflecting area-specific differences in CSMN generation and differentiation (Fig. S2). In sharp contrast, in COUP-TFI CKO cortex, there is a dramatic increase in the number of high-level Fezf2- and CTIP2-expressing neurons in layer VI, most pronounced in mS1 and visible as an area-specific thickening of layer V (Fig. 1 B, F, and J). Interestingly, CTIP2 expression is strikingly increased in the most superficial layer VI neurons, called “mVI” throughout this study. We next used area-specific markers, such as Crim1, FOXP2, and Igfbp4, which, in WT cortex, are expressed in neurons of layer V in motor, but not in S1 cortex (4, 6) (Fig. 1 K, L, O, P, S, and T). These markers are ectopically expressed in layer V in mS1 (Fig. 1 N, R, and V), further confirming that loss of COUP-TFI function imparts motor-like characteristics to neurons in parietal cortex. FOXP2 and Igfbp4 are also expressed by layer VI neurons (4) (Fig. 1 O, P, S, and T), and their expression is strikingly reduced or abolished in layer VI (Fig. 1 Q, R, U, and V), further indicating that a subset of layer VI neurons differentiate abnormally in the absence of COUP-TFI function. Abnormal cytoarchitecture is confirmed by cresyl-violet histological analysis of COUP-TFI CKO cortex, which shows an expansion and abnormal morphology of layer V neurons in sensory areas, and a decrease in thickness of layer VI in both sensory and motor areas of COUP-TFI CKO mice (Fig. S3).

Taken together, these results indicate that, in the absence of COUP-TFI function, the number of neurons expressing high levels of CSMN markers in layer V of the motorized S1 is dramatically increased, at the expense of layer VI neurons. Importantly, in the “genuine,” occipitally misplaced S1 and V1 mutant cortical areas, the pattern of CTIP2-expressing neurons is comparable to corresponding areas of WT animals (Fig. S4). Therefore, our data strongly suggest that COUP-TFI acts in an area-restricted manner on the differentiation of the two main classes of corticofugal neurons: corticothalamic and corticospinal motor neurons.

We next assessed expression of FOXP2, TBR1 (expressed by layer VI neurons, including corticothalamic neurons) (22), and CTIP2 (strongly expressed only in CSMN) (4), to further investigate potential interactions in the differentiation pathways of corticothalamic neurons and CSMN in the absence of COUP-TFI function (Fig. 2). In P8 WT mice, FOXP2/TBR1 and CTIP2 are expressed by distinct subsets of neurons in layers VI and V, respectively, with only rare layer V and VI neurons co-expressing both FOXP2 and TBR1 or CTIP2 (Fig. 2A, G, H, P, and Q) (6). In striking contrast, the proportion of neurons co-expressing both FOXP2 and CTIP2 (Fig. 2F, G, and H) or TBR1 and CTIP2 (Fig. 2K, P, and Q) is increased in layers V and VI of COUP-TFI CKO mice, (Fig. 2D) (FOXP2+CTIP2+; layer V: WT, 4.1 ± 1.2%; KO, 15.0 ± 0.3%; P = 0.01; layer VI: WT, 13.4 ± 3.4%; KO, 47.2 ± 7.8%; P = 0.05) (Fig. 2E) (TBR1+CTIP2+; layer V: WT, 1.3 ± 0.3%; KO, 6.9 ± 1.5%; P = 0.02; layer VI: WT, 7.1 ± 0.9%; KO, 21.4 ± 0.7%; P = 0.0002), indicating abnormal acquisition of mixed corticothalamic and CSMN identity by corticofugal neurons. Taken together, these data indicate that loss of COUP-TFI function leads to a failure of corticothalamic neurons and CSMN to differentiate along segregated molecular pathways, resulting in a large number of neurons with mixed corticothalamic and CSMN identities.

Next, we investigated the temporal course of expression of the neuron subtype-specific markers Fezf2, CTIP2, and TBR1 in WT and KO cortex at E13.5 (the peak time of birth of CSMN) and at E16.5, when generation of corticofugal neurons is terminated. In the absence of COUP-TFI function, there is an expansion of the Fezf2- and CTIP2-positive populations at E13.5, which is matched with a reduction of the Thr1-expressing cells (Fig. S5). This altered balance between Fezf2/CTIP2- and TBR1-positive populations is still present at E16.5, indicating that COUP-TFI is normally involved in the distinct differentiation of CSMN and corticothalamic neurons from early stages of corticogenesis, in accordance with its high expression levels in presumptive corticofugal neurons at prenatal stages (Fig. S1). Taken together, this suggests that in S1 cortex, COUP-TFI normally represses a CSMN differentiation program during generation of layer VI corticothalamic neurons and that, in the absence of COUP-TFI function, presumptive corticothalamic neurons abnormally display cardinal molecular features of CSMN differentiation.

**Abnormal Expression of CSMN-Specific Genes Motorizes Layer VI Corticothalamic Neurons.** We next investigated whether the abnormal expression of the transcription factors Fezf2 and CTIP2 directs the differentiation of neurons normally destined to become corticothalamic neurons into CSMN, and results in a shift in their axonal projections to subcerebral targets instead of to the thalamus. We retrogradely-labeled subcerebrally-projecting neurons from the cerebral peduncle via ultrasound-guided microinjections of FluoroGold in P2 mice, and performed the analysis at P6 (Fig. 3A and B). In striking contrast to WT mice, in which subcerebral projection neurons are sharply confined to layer V (Fig. 3 E and G), the position of these neurons in COUP-TFI CKO mice includes mVI of mS1, where abnormal high CTIP2-expressing neurons are located (Fig. 3 F, H, and I). This finding demonstrates that abnormal expression levels of CSMN-specific control genes in presumptive corticothalamic neurons initiate central features of CSMN differentiation, including subcerebral axonal targeting. Strikingly, retrograde labeling from the spinal cord (Fig. 3 J) reveals that these abnormal subcerebral projection
neurons of mVI are the dominant corticofugal neuron population able to successfully send axonal projections to more caudal targets in the cervical spinal cord of COUP-TFI CKO mice (Fig. 3 N and P’); indeed, in layer V, genuine CSMN, which abnormally express high levels of Fez2, CTIP2, FOXP2, and TBR1 (Figs. 1 and 2), send axons which reach the cerebral peduncle, but not the spinal cord. This finding indicates that transcriptional dysregulation in genuine CSMN in the absence of COUP-TFI function results in abnormal differentiation of CSMN.

Motorized Layer VI Neurons Project to Cervical, Thoracic, and Lumbar Spinal Cord. To better investigate the entire trajectories of subcerebral axons, we crossed WT and COUP-TFI CKO mutants with "CST-YFP" mice, which express YFP in corticofugal neurons (23) (Fig. 4). At P7, the trajectory of subcerebral projections is largely unaffected by loss of COUP-TFI function (Fig. 4 A–M’). However, COUP-TFI CKO mice develop a detectable decrease in corticolumbar projections by P21 (Fig. 4 N and O and Fig. S6), suggesting abnormal degeneration or area-specific pruning at later stages. Remarkably, this corticospinal connectivity primarily reflects axonal projections of the mispecified mVI neurons of the abnormally expanded motorized cortex, because the axons of genuine CSMN in layer V largely do not reach the cervical cord (Fig. 3 N–P’).

Taken together, these data indicate that COUP-TFI normally controls CSMN differentiation and cortical efferent connectivity, and that loss of COUP-TFI strikingly enables a subset of late-born corticothalamic neurons to establish corticospinal projections to cervical, thoracic, and lumbar spinal cord segments.

COUP-TFI Controls the Area-Specific Timing of CSMN Specification During Genesis of Corticofugal Neurons. Our data indicate that lack of COUP-TFI predominantly affects the latest-born (i.e. most

Fig. 2. Abnormal molecular specification of corticofugal neurons in COUP-TFI CKO parietal cortex. (A and B) Double immunofluorescence against CTIP2 and FOXP2 and higher magnification views (C–H’) in layers V and VI. (I and K) Double immunofluorescence against CTIP2 and TBR1 and higher magnification views (L–O’) in layers V and VI. Arrows indicate neuron that co-express both markers in WT and COUP-TFI CKO P8 parietal cortices. (I and K) Quantification of number of double-positive cells per total of cells in layers V and VI indicates a significant difference between WT and CKO cortices. Error bars represent SEM. Student’s t test, *P ≤ 0.05, **P ≤ 0.01. [Scale bars: 100 μm (A, B, J, and K); 20 μm (C–H’ and L–O’).]

Fig. 3. Corticofugal neurons in superficial layer VI send axons to subcerebral targets in COUP-TFI CKO brains. Sagittal schematic views (A and J) and ultrasonographic image (B) of a mouse brain showing FluoroGold (FG) injection in the cerebral peduncle (CeP and arrowhead in B) and cervical spinal cord (SC). (C, D, K, and L) Retrogradely labeled coronal hemisections of P7 brains, with locations of the higher-magnification panels. (E–I) FG injection into the CeP shows abnormally located retrogradely labeled neurons in superficial layer VI (red arrowheads in F), which are also positive for CTIP2. (M–P’) FG injection (green in M) into the SC labels abnormally located retrogradely labeled neurons exclusively in superficial layer VI. These neurons express CTIP2 (arrowheads in O–P’). WM, white matter. [Scale bars: 0.5 mm (B–D, K, and L); 50 μm (E–H, M, and N); 20 μm (I–P’ and O–P’).]
superficially located in layer VI) corticothalamic neurons, which are generated immediately before CSMN, raising the possibility that during corticogenesis COUP-TFI acts to control the timing of the transition between corticothalamic and corticospinal motor neuron generation. Thus, we first determined the date of birth of corticofugal neurons in COUP-TFI CKO mice by injecting BrdU from E11.5 to E13.5, the normal birth dates of corticofugal neurons (7, 24), and found that the laminar distribution of BrdU birth-dated cells is not distinguishable between WT and COUP-TFI CKO mice at P0 (Fig. S7). This indicates that the migration of corticofugal neurons is unaffected by the absence of COUP-TFI function. Next, we examined whether loss of COUP-TFI increased the probability of E12.5 BrdU birth-dated corticofugal neurons to strongly express CTIP2, taken as a bona fide index of CNSM differentiation (Fig. 5A–D). Loss of COUP-TFI function leads to a 2-fold increase in the number of E12.5-born neurons that strongly express CTIP2 in layer mVI (Fig. 5E) (WT, 43 ± 7%; CKO, 93 ± 2%; n = 3; P = 0.02), indicating that COUP-TFI normally acts to restrict CNSM specification during corticothalamic neurogenesis in S1 cortex. Strikingly, this control is exerted in an area-specific manner, because, in frontal/motor cortex, E12.5-born neurons are not more likely to strongly express CTIP2 in the absence of COUP-TFI function (Fig. 5E) (WT, 65 ± 6%; CKO, 83 ± 4%; n = 3; P = 0.07). Importantly, this area-specific premature generation of CNSM is limited to the time when corticofugal neurons are generated (Fig. S8). Together, these data strongly suggest that COUP-

TFI normally acts to control the area-specific timing of the transition between corticothalamic and CNSM specification by setting the onset of CNSM differentiation to appropriate time points of corticogenesis in the S1 cortex.

**COUP-TFI CKO Mice Have Impaired Skilled Motor Behavior.** We next investigated how the increase in motor area size (17), and the reassignment of the corticospinal connectivity to layer VI corticofugal neurons, might affect sensorimotor function in COUP-TFI adult mutant mice. We first investigated sensorimotor function by using an adhesive patch removal task (25), in which the mouse has to remove a piece of adhesive patch placed on each hindpaw. We next investigated sensorimotor function by using an adhesive patch removal task (25), in which the mouse has to remove a piece of adhesive patch placed on each hindpaw. We find that COUP-TFI CKO mice are significantly less efficient in removing the patches than WT mice (Fig. 6A) (F(1/19) = 13.635; P = 0.001; see also SI Methods). This decreased performance reflects impairment in motor function rather than reduced tactile perception, as COUP-TFI CKO mice readily detect the presence of the patch (as indicated by similar latencies in the first attempt to remove the patch) (Fig. 6B) (F(1/19) = 0.145; P = 0.7). Once the patch is detected, COUP-TFI CKO mice make significantly more removal attempts than WT mice (Fig. 6C) (F(1/19) = 21.382; P < 0.001), suggesting specific impairment in fine motor control.

![Fig. 4](image_url) Cortico-subcerebral connectivity in COUP-TFI CKO mice. Schematic sagittal (A and A') and coronal (A) views of the brain indicating location of the immunofluorescence photomicrographs shown in B to G along the corticospinal corticofugal pathways (green) and obtained in CST-YFP WT and COUP-TFI CKO mice. At P7, corticofugal axons in WT and COUP-TFI CKO mice fasciculate compactly within the cerebral peduncles (arrowheads in B and C), the pons (arrowheads in D and E), and tectum (arrowheads in F and G), and decussate normally in the medulla oblongata (H, I, and open arrowhead in D and E). Within the spinal cord (U–O), the corticospinal tract is compactly bundled within the dorsal funiculus (Dors funic) in the cervical cord (A–M), even though the CST appears normal at P7 (U–M). Even though the CST appears normal at P7 (U–M), there is a variable defect in the number of lumbar projecting CST axons in COUP-TFI CKO mice at P21 (N, O). [Scale bars: 250 μm (B–K); 100 μm (L–O).]

![Fig. 5](image_url) Loss of COUP-TFI function causes abnormal timing of specification of subcerebral projection neurons in S1 cortex. Coronal sections of P0 WT (A–A') and COUP-TFI CKO (C–C') parietal cortices after BrdU injection at E12.5, immunostained for BrdU and CTIP2. Higher magnification views of layers V (B and D) and VI (B' and D') indicate a higher number of E12.5 double-labeled neurons in superficial layer VI of COUP-TFI CKO brains (arrowheads in D'). (E) Graphical representation of the percentage of BrdU-birthdated neurons expressing high levels of CTIP2 in superficial layer VI of WT and COUP-TFI CKO parietal and motor cortices. Pink circles indicate values for individual experiments (n = 3). CP, cortical plate. [Scale bars: 50 μm (A–A' and C–C'); 40 μm (B, B', D, and D').]
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CSMN in layer V do not project to the spinal cord in COUP-TFI CKO mice; however, these mice remarkably establish corticospinal projections to all segments of the spinal cord. Our retrograde tracings from the spinal cord demonstrate that the tract originates largely from the corticothalamic neurons in layer mVI within the abnormally expanded motorized cortex (17). Thus, these misspecified neurons may not be integrated into appropriate cortical motor neuronal networks, and therefore are unable to contribute to fine motor control. Interestingly, COUP-TFI CKO mice largely reproduce behavioral defects observed in rats after either specific corticospinal lesions or motor or somatosensory cortical ischämia (26, 27, 33, 34), emphasizing that dysregulation of precise CSMN development leads to deficits of high-level motor function and behavior.

**Methods**

**Mice.** COUP-TFI CKO mice were generated and genotyped as shown previously (17). For genetic labeling of the entire corticospinal motor tract, COUP-TFI CKO mice were crossed with Thy1-STOP-YFP mouse (23) (kind gift of J. Sanes). All experiments were conducted following guidelines of the Institutional Animal Care and Use Committee, Cardarelli Hospital, Naples, Italy, and in accordance with institutional and federal guidelines of the Massachusetts General Hospital IACUC.

**Immunocytochemistry, In Situ Hybridization, and Histology.** Brains were treated and processed for free-floating and standard immunofluorescence protocols as described (4, 17). FYP detection was amplified with a GFP-specific antibody (1:1000 Chemicon) and whole-mount in situ hybridization and nonradioactive in situ hybridization were performed as described (17). Antisense RNA probes were labeled using a DIG-RNA labeling kit (Roche). For Nissl staining, sections were stained with 0.5% cresyl violet, as described (4).

**Retrograde Labeling.** Subcerebrally projecting neurons were retrogradely labeled via FluoroGold injections into the cerebral peduncle or spinal cord, at P2 and P3 under ultrasound guidance (Vevo 660, VisualSonics), as described (4). Injected mice were collected at P6 or P7 and processed for immunocytochemistry. Each experiment was repeated at least three times and showed reproducible results.

**Brdu Birth Dating.** Timed pregnant females received a single i.p. injection of BrdU (50 mg/kg) at E11.5, E12.5, E13.5, or E15.5. Pups were collected at birth, processed for BrdU immunocytochemistry, and quantified as described (6).

**Behavioral Analysis.** For the adhesive patch removal task, an adhesive patch was placed on the dorsal surface of each hindpaw, then mice were released in the testing cage and observed for 240 s. Animals underwent three consecutive trials, with an intertrial interval of 60 min. The skilled reaching task was adapted in mice from studies in rats (27). The task consisted of three phases: habituation, unskilled reaching, and skilled reaching. More details of both tasks are available in SI Methods.

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