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 laminar cytoarchitecture 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. The area-specific differences in neuronal fate and cytoarchitecture 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 somato-
In sensory areas, and a decrease in thickness of layer VI in both cortices. [Scale bars: 50 μm (A and B); 100 μm (C–V).]

**Fig. 1.** Increased expression of molecular hallmarks of CSMN in corticofugal neurons of S1 cortex in COUP-TFI CKO mice. (A and B) Coronal sections and (C–V) higher magnification views of frontal (M) and parietal (S1/mS1) cortices of WT and COUP-TFI CKO P8 brains indicate abnormal expression levels of the CSMN markers Fezf2 (A–F), CTIP2 (G–J), Crim1 (K–N), FOXp2 (O–R), and ligp2p4 (S–V) in layer V and radial expansion of these markers toward superficial layer VI (mVI) in mS1 of COUP-TFI CKO cortices. Note that expression of FOXp2 is reduced (Q and R) and expression of ligp2p4 is abolished (U and V) in layer VI in both areas of COUP-TFI CKO cortices. [Scale bars: 50 μm (A and B); 100 μm (C–V).]
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 Fezf2, 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 cortico-lumbar 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 misspecified 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
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, as well as the reassignment of the corticospinal connectivity to layer VI corticospinal neurons, might affect sensorimotor function in 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′); 10 μm (L–O)].

TFI normally acts to control the area-specific timing of the transition between corticothalamic and CSMN specification by setting the onset of CSMN 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 corticothalamic 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 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.
Our study has con-

tered mice, as indicated by a strikingly lower rate of pellet recovery

C mice in the test, *p = 0.005) (see also Tomassy et al.

Several studies have demonstrated that the fate speci-

COUP-TFI CKO mice are indistinguishable (Fig. 6

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Discussion

In this study, we show that COUP-TFI plays a critical role in regu-

Corticospinal Motor Neuron Specification. Our study has con-

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Finally, because COUP-TFI and Fezf2 are both expressed in postmitotic neurons, it is possible that abnormal differentiation of CSMN and corticothalamic neurons in the absence of COUP-TFI occurs at a postsomatic level, as recently shown for other cortical transcriptional regulators (8, 30, 31, 32).

COUP-TFI-Dependent Precision of Corticospinal Neuron Differentiation Is Critical for Skilled Motor Behavior. In the present study, we found that, in the absence of COUP-TFI, skilled motor function related to the sensorimotor cortex and corticospinal motor tract is substantially impaired. These behavioral data indicate that precise complement and connectivity of the distinct corticofugal neuronal populations is critical for proper motor function (27, 33). Genuine
CNSM 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 ischemia (26, 27, 33, 34), emphasizing that dysregulation of precise CNSM 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). YFP detection was amplified with a GFP-specific antibody (1:1000 Chemicon) through 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). Immunocytochemically processed tissues were counterstained with 0.01% cresyl violet or 0.1% toluidine blue.

**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 31 Methods.

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