Online Methods

Animals. Wild-type C57Bl/6J mice (Jackson Laboratories) were used in accordance with the animal welfare guidelines and regulations of Northwestern University, National

Institutes of Health, and Society for Neuroscience. Acute brain slice experiments were performed using mice of either gender at a postnatal age of 22 to 28 days.

Retrograde labeling. To label corticostriatal neurons, stereotaxic injections were made into the dorsolateral striatum at coordinates 0.0 mm posterior, 2.0 mm lateral, and 2.5 mm ventral to bregma. Mice were deeply anesthetized by inhaled isoflurane. A small craniotomy was opened over the left or right parietal area 2.0 mm posterior and 3.5 mm lateral to bregma. A 5 μL capacity borosilicate glass pipette (VWR), pulled to a shallow taper (length >1 cm, tip diameter ~30 μm) was advanced into the dorsolateral striatum at an angle ~17° off the sagittal plane and ~48° off the horizontal plane. A small volume (~25 nL) of undiluted solution with red or green fluorescent latex microspheres (RetroBeads, Lumafluor) was pressure-injected (Picospritzer III, Parker Hannifin). The pipette was kept in place for several minutes prior to extracting it. Acute brain slice experiments were performed 1–7 days later and inspected to confirm accurate placement of the dorsoalateral striatal injection. These injections gave a characteristic distribution of labeling in the contralateral motor-frontal cortex consistent with the anatomy of the crossed corticostriatal projection [49]. In contrast, injections in adjacent striatal zones resulted in different labeling patterns, indicating that the labeling we observed was specific to the striatal injection location and not due to nonspecific labeling along the track of the injection pipette. To label corticospinal neurons, beads were injected into the spinal cord at the cervical level 2, ~1 mm lateral to the midline. Methods were otherwise as for corticostriatal neurons.

Slices. Off-coronal brain slices containing motor-frontal cortex were prepared as previously described [3]. The slice angle was pitched rostrally by ~10° for alignment with the radial axis of the cortex, yielding slices in which the apical dendrites of layer 5 pyramidal neurons were parallel to the cut surfaces. Slices were cut in chilled choline-based solution (in mM: 110 choline chloride, 25 NaHCO3, 25 D-glucose, 11.6 sodium

ascorbate, 7 MgSO4, 3.1 sodium pyruvate, 2.5 KCl, 1.25 NaH2PO4, and 0.5 CaCl2), allowed to recover in 35 °C ACSF (in mM: 127 NaCl, 25 NaHCO3, 25 D-glucose, 2.5 KCl, 1 MgCl2, 2 CaCl2, and 1.25 NaH2PO4) for 30 minutes and maintained at 21–22 °C thereafter.

Slice morphometry. For each neuron in mapping experiments, a bright-field video image was captured at the time of mapping. Offline, these images were analyzed by marking, along a line extending from the pia to the white matter along the radial axis of the cortex, the locations of the pia and major laminar borders. In preliminary experiments, the location of the border between layer 5A and B was established on the basis of comparison to the same border in adjacent somatosensory cortex, and analysis of the optical density profile [3].

Fluorescence imaging and analysis. Cortical slices were visualized under epifluorescence optics, and fluorescence intensity analyses were performed using custom routines in Matlab. Images were rotated to align the pia horizontally, and a region of interest (ROI) spanning the entire cortical thickness and containing labeled neurons in the motor-frontal cortex was selected. The pixel intensities in this ROI were averaged along the rows, yielding a profile representing the average pixel intensities along the vertical axis, showing the radial distribution of fluorescence in the cortex. Next, we performed background subtraction to reduce the autofluorescence signal, by fitting a polynomial to the non-fluorescent portions of the profile and subtracting a calculated background profile from the initial profile.

Electrophysiology. Whole-cell patch recordings were made from pyramidal neurons as described previously [3]. Potassium-based intracellular solution (128 mM KCH_3SO_3, 4 mM $MgCl_2$, 10 mM HEPES, 10 mM phosphocreatine, 4 mM ATP, 0.4 mM GTP, 3 mM ascorbate) contained fluorescent dye (50 μM Alexa-350, -488, or -594; Invitrogen) to facilitate post-recording inspection of neurons, to verify intactness of apical dendrites.

The bath solution for photostimulation studies contained elevated concentrations of divalent cations (4 mM Ca^{2+} and 4 mM Mg^{2+}) and an NMDA receptor antagonist (5 μM CPP; Tocris), to dampen neuronal excitability. Recordings were performed at 21 °C. Voltages were not corrected for liquid junction potential. Recordings were monitored for series resistance (inclusion criterion: <40 MΩ; mean: ~25 MΩ). Recordings in voltage-clamp mode were made at a holding potential of –70 mV.

Glutamate uncaging and laser scanning photostimulation (LSPS). LSPS mapping followed previously described methods and mapping parameters [3]. Photostimuli were 1.0 msec in duration and 20 mW at the specimen plane. Map grids had 100-μm spacing of rows and columns. Grids were aligned to a standard orientation: the top row of the grid was flush with the pia, and the soma was horizontally centered in the middle of the grid.

Analysis of input maps and generation of laminar connectivity matrices and side view plots. Methods for the construction of synaptic input maps and laminar connectivity matrices have been described in detail [3]. For each neuron, a synaptic input map was constructed on the basis of the mean current over a 0–50 msec poststimulus time window. Maps were pooled by projection class and sorted by laminar position, and the data were projected onto a single presynaptic-postsynaptic plane by averaging along the rows (the horizontal dimension in the input maps), producing a laminar connectivity matrix in terms of pre- and postsynaptic laminar location. Along the postsynaptic dimension, the data were averaged in 16 bins spanning the full thickness of the cortex (from pia to white matter). 'Side view' plots (**Figs. 1c** and **2c**) were generated by averaging along map rows; i.e., each individual map was converted to a single vector.

Flavin autofluorescence (FA) imaging. *In vitro* FA imaging was performed following published methods [18, 19]. The microscope optics were modified in two ways for compatibility with caged glutamate photolysis (see below), as described [19]. First, the

dichroic mirror in the GFP filter set was replaced by one with band-pass transmission around the wavelength of the ultraviolet (UV) laser used for photolysis. Second, the emission filter was removed from the GFP filter cube and placed directly in front of the video camera. Image series were captured by triggering a cooled CCD video camera (Retiga 2000-R, Q-Imaging). Movies of FA signals were made by normalizing the images to the baseline fluorescence, calculated from a 2.5-sec baseline interval prior to stimulation, yielding movies of $\Delta F/F$. Displayed images are of the $\Delta F/F$ at 1–2 sec post-stimulus, around the time of maximal response. Stimulation parameters were identical to those used for LSPS mapping experiments except that the stimulus duration was increased to 10 msec in order to observe adequate FA signal intensities. For each neuron, FA movies were repeated ten times and averaged. Simultaneous whole-cell recording from a pyramidal neuron located at layer 5A/B border was performed to verify that postsynaptic neurons were not synaptically driven to spike under these conditions.

In utero **electroporation (IUEP).** IUEP-mediated transfection of plasmids into neuronal precursor cells was performed using methods optimized for transfecting layer 2/3 neurons in mouse motor cortex [50]. Plasmids encoding a ChR2-mVenus fusion protein driven by the CAG promoter (for details of the construct see [21]) were prepared using endonuclease-free plasmid amplification kits (Qiagen). Pregnant mice were deeply anesthetized by inhaled isoflurane and a laparotomy was performed to externalize the uterus. For each embryo, a glass pipette, sharpened with a beveller (Narishige), was advanced into the right lateral ventricle and ~1 μL of 1 μg μL^{-1} plasmid-containing solution dyed with Fast Green (Sigma) was injected. Tweezer-style electrodes were oriented across the cranium to favor transfection of progenitor neurons in the motor-frontal area of cortex, and electroporation was performed with 5 pulses, each at 50 V for 50 msec with a 1 sec inter-pulse interval. Transfection was performed at embryonic day (E) 16, yielding a pattern of expression restricted to layer 2/3

pyramidal neurons in the motor-frontal cortex of the right hemisphere, as described previously [20-22].

Channelrhodopsin-assisted circuit mapping (CRACM). Brain slices were prepared from mice transfected with plasmids coding for ChR2 and mVenus by IUEP [21]. To excite ChR2 we used the same ultraviolet (UV; wavelength 355 nm) laser used for LSPS. To avoid synaptic driving, we used the same conditions as for glutamate-LSPS, including CPP to block NMDA receptor currents and 4 mm Ca^{2+} and Mg^{2+} to dampen excitability. In preliminary control experiments, we recorded excitation profiles (**Supplementary Fig. 2**) from non-ChR2 expressing layer 2/3 (n = 3) and layer 5 (n = 5) cells to test for synaptic driving; even at the highest laser powers (>30 mW, many times greater than used for mapping experiments) the postsynaptic neurons did not spike, indicating no synaptic driving. In other control experiments, we recorded excitation profiles over a range of laser powers (**Supplementary Fig. 2**) to determine the optimal range Because of variability both in the transfection efficiency (i.e., number of ChR2-expressing neurons per animal) and in ChR2 expression levels (i.e., number of ChR2 molecules per transfected neuron), for each slice we empirically determined a suitable laser power, which was then used for all cells recorded in the same slice. Specifically, we first patched a corticostriatal neuron in layer 5A, adjusted the laser power to give inputs of ~100 pA peak amplitude following stimulation at perisomatic sites, and then obtained CRACM maps at this power level (typically 4–5 mW). Next, we patched a second corticostriatal neuron in the slice, located in layer 5B, and acquired CRACM maps using identical power settings as for the first neuron. The same approach was used for contralateral layer 2/3 ChR2 input mapping, but in many cases it was not possible to evoke ~100 pA peak perisomatic EPSCs. Therefore the laser power was adjusted so as to evoke ~25 pA peak perisomatic EPSCs for neurons in layer 5A and this stimulation intensity was used for CRACM maps of pairs of neurons in the same slice.

Software for data acquisition and analysis. *Ephus* software was used for hardware control and data acquisition (http://openwiki.janelia.org). Imaging and electrophysiological data were analyzed off-line using custom Matlab routines.

Statistical analysis. Unless stated otherwise, unpaired *t*-tests were used to compare group means, with significance defined as $P < 0.05$.

SI Guide (Supplementary Information Titles)

Filename: Supplementary Figures.pdf

Contents:

Supplementary Fig. 1. Axons of upper layer 2/3 neurons project to the layer 5A/B border.

Supplementary Fig. 2. Characterization and calibration of ChR2 photostimulation.