Corticospinal populations broadcast complex motor signals to coordinated spinal and striatal circuits

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Many models of motor control emphasize the role of sensorimotor cortex in movement, principally through the projections that corticospinal neurons (CSNs) make to the spinal cord. Additionally, CSNs possess expansive supraspinal axon collaterals, the functional organization of which is largely unknown. Using anatomical and electrophysiological circuit-mapping techniques in the mouse, we reveal dorsolateral striatum as the preeminent target of CSN collateral innervation. We found that this innervation is biased so that CSNs targeting different striatal pathways show biased targeting of spinal cord circuits. Contrary to more conventional perspectives, CSNs encode not only individual movements, but also information related to the onset and offset of motor sequences. Furthermore, similar activity patterns are broadcast by CSN populations targeting different striatal circuits. Our results reveal a logic of coordinated connectivity between forebrain and spinal circuits, where separate CSN modules broadcast similarly complex information to downstream circuits, suggesting that differences in postsynaptic connectivity dictate motor specificity.

CSNs, the principle output of sensorimotor cortex, relay motor command signals to the spinal cord, where their main axons synapse on several classes of interneurons involved in patterning motor output and shaping sensory feedback. Corticospinal output is topographically organized: CSNs within regions that control specific body parts preferentially innervate spinal segments containing the circuits that control the muscles for those body parts. CSNs arising from motor regions of isocortex form synapses spread across intermediate and ventral laminae of the spinal gray, home to premotor spinal interneurons; while sensory cortical CSNs have terminal fields mostly restricted to superficial regions of spinal cord, home to the dorsal horn interneurons that regulate peripheral sensory feedback. Partly because of this anatomy, motor cortical CSNs have been hypothesized to control the activity of individual muscles or muscle synergies through their direct projections to the spinal cord. Concordantly, many CSNs are active at discrete phases of movement or during the recruitment of single muscles. However, CSNs often display complex activity patterns that do not closely correspond to muscle output, suggesting their role in movement may be nuanced and task dependent. For instance, some pyramidal tract-projecting neurons in nonhuman primates act as mirror neurons, displaying activity that is modulated during both execution of a forelimb movement and the passive observation of an experimenter’s forelimb movement. Complex activity in corticospinal populations has led to hypotheses that CSNs subserve diverse modulatory control over spinal cord, including motor inhibition, plasticity of spinal circuits, reafference suppression and state-dependent gain control. This complexity of corticospinal function is further reflected in peculiarities of corticospinal organization. Foremost, CSNs give rise to axon collaterals that project to a broad range of brain structures, affording them remarkable—yet largely uncharted—influence over nearly all levels of the motor control neuraxis. Despite the widespread supraspinal collateralizations of CSNs, a detailed characterization of their anatomical and functional organization has been elusive. Therefore, we sought to (1) comprehensively map the structural organization of CSNs and their brain-wide axon collaterals, (2) uncover synaptic and circuit principles that mediate coordinated spinal and supraspinal connectivity, and (3) characterize the behaviorally relevant activity of CSNs with identified cell-type-specific supraspinal targets.

Uncertainty surrounding the anatomical and electrophysiological properties of corticospinal output is partly the result of difficulties in capturing, mapping and monitoring large populations of CSNs, particularly during complex skilled behaviors. In this study, we overcame these challenges by first using a suite of intersectional anatomical methods to map the input and output organization of CSNs. While CSNs formed widespread synapses in many supraspinal structures, we discovered that the striatum is the preeminent target of collateral innervation. The striatum is composed of two distinct populations of spiny projection neurons (SPNs) defined in part by the expression of dopamine receptor type 1 (D1) or type 2 (D2). Using anatomical and electrophysiological circuit-mapping techniques, we showed that separate populations of CSNs synapse on D1 and D2 SPNs, that more synapses are formed on D1 SPNs than D2 SPNs, and that this biased connectivity translates to distinct innervation of the cervical spinal cord.

D1 and D2 SPNs are both necessary for the learned sequencing of body movements, and encode sequence-related information, but to different degrees. Therefore, we leveraged calcium imaging during a striatal-dependent lever-press sequence behavior, identifying neurons that linearly encoded muscle activity as well as ones that encoded higher-order features of behavior, including sequence onset and offset. Combining these experiments with a new use of in vivo transsynaptic tracing revealed that both muscle-related and higher-order activity are transmitted to both D1 and D2 striatal SPNs. Our results support a model wherein CSNs broadcast copies of muscle-correlated and non-muscle-correlated information throughout the nervous system, and differences in postsynaptic connectivity determine motor specificity.
Corticospinal collaterals project prominently to the striatum

CSNs collateralize throughout the brain, but the degree to which CSNs innervate each target structure is unclear. We combined transverse viral expression of fluorescent markers with unbiased anatomical reconstruction to quantify collateral innervation of brain regions by CSNs. First, we labeled inputs to the spinal cord by injecting an adeno-associated virus, transported in retrograde, encoding Cre recombinase fused to a red fluorescent protein (AAV-retro-Cre-RFP) into right forelimb cortical regions C3–C7 (ref. 39). In the same animals, we injected a Cre-dependent AAV encoding a green fluorescent protein (AAV-FLEX-GFP) into forelimb-control regions by CSNs. Anatomical characterization of corticospinal neurons. a, Schematic illustrating the viral injection sites in motor cortex and the spinal cord, and their relative positions in the nervous system. Dashed lines indicate the position of representative images to follow. b–l, Confocal micrographs of transverse sections throughout the brain, illustrating GFP+ CSNs and their axonal projections (green), along with all spinal inputs made to express Cre.RFP (red). Some ROIs are boxed by dashed lines and include: primary motor cortex (M1), DLS, zona incerta (ZI), midbrain reticular nucleus (MRN), superior colliculus (SC), pons (P), periaqueductal gray (PAG), pontine reticular nuclei (PRN), inferior colliculus (IC), gigantocellular reticular nucleus (GRN) and intermediate reticular nucleus (IRN). DAPI staining is in blue. Representative of N = 3. j–l, 3D reconstructions of spinal inputs (j), CSN somata (k) and CSN neurites (l) throughout the brain. Colors correspond to major brain divisions in which they reside. Note that the most caudal portion of brain stem is not included in these analyses. m, Correlation analyses of mesoscopic animal-to-animal CSN somata locations (left) and CSN neurite locations (right). CC, correlation coefficient. n, Top brain regions to which CSNs project, measured as the fraction of all bins of neurites found within those brain structures, excluding sensorimotor cortex and fiber tracts. GP, globus pallidus; RSA, retrosplenial area; PG, pontine gray; SN, substantia nigra; H, hypothalamus; TRN, tegmental reticular nucleus; APN, anterior pretectal nucleus; IRN, intermediate reticular nucleus; My, medulla; RN, red nucleus; VIS, visual areas; PBN, parabrachial nucleus; VM, ventromedial nucleus of the thalamus; Cg, cingulate; RT, reticular nucleus of the thalamus; VTA, ventral tegmental area. N = 3 mice. o, A high-magnification micrograph of DLS. Representative of N = 3. p, Imaris 3D reconstruction of the DLS (dark green) and CSN axonal labeling in the DLS (red) superimposed over a 3D projection of GFP labeling. Representative of N = 3. Error bars are the s.e.m.
Fig. 2 | Identifying brain-wide inputs to CSNs. a, Strategy to use intersectional transsynaptic tracing to label inputs to CSNs. b–d, Identification of starter cells (arrowheads) through coexpression of tdTomato (b) and Cre (c). Overlay image is shown in d. Representative of N = 3. e–j, Example confocal micrographs of tdTomato labeling throughout the brain. DAPI staining is in blue. Representative of N = 3. k, 3D reconstruction of tdTomato+ neurons, color coded by brain group. l, Correlation analysis of somata population positions across animals. m, Quantification of the top brain regions giving rise to neurons that form synapses on CSNs. The pie chart indicates the major brain groups providing input to CSNs. The inset image displays neuronal labeling in subdivisions of the thalamus. N = 3 mice. Error bars are the s.e.m. ACA, anterior cingulate area; A1A, agranular insular area; CL, central lateral nucleus of the thalamus; ORB, orbital area; VAL, ventral anterior-lateral complex of the thalamus; VP, ventral posterior complex of the thalamus; VPL, ventral posterolateral nucleus of the thalamus.

Brainstem regions implicated in motor control (Extended Data Fig. 2f–l). Consistently, all of the regions targeted by these neurons the largest fraction of axons was found in the DLS (Extended Data Fig. 2f; N = 3).

CSNs synapse on a range of interneurons with distinct roles in motor control and sensory processing34. We wondered whether CSNs also innervate interneuron subtypes with well-defined functions, such as the GAD2-expressing neurons responsible for presynaptic inhibition of sensory afferents35, as well as Chx10-expressing propriospinal excitationary neurons and dorsal somatostatin (SST)-expressing mechanoreceptive neurons. We used an intersectional approach to drive expression of two viral constructs in spinal interneurons expressing GAD2, Chx10 or SST: one encoding the avian receptor for EnVa glycoprotein (AAV-FLEX-TVA), and the other encoding the rabies glycoprotein necessary for transsynaptic spread (AAV-FLEX-N2cG; Extended Data Fig. 2j). We then injected the spinal cord with the improved pseudotyped, G-deficient CVS-N2c rabies construct expressing FlpO recombinase fused to mCherry (EnVa-N2cG-FlpO.mCherry). This construct infects neurons expressing TVA, and from a subset of those also expressing N2cG, infects and expresses FlpO in presynaptic input neurons, in turn driving expression of GFP through Flp-mediated recombination (see Extended Data Fig. 3 for validation of transsynaptic rabies constructs)36. We then mapped the position of GFP-labeled neurons constituting all of the spinal interneuron subtypes tested (GAD2: 585 ± 316, N = 3; Chx10: 592 ± 425, N = 4; SST: 869 ± 187, N = 2). Importantly, CSNs that synapsed on each interneuron subtype of interest all formed consistently widespread axonal arborizations in the DLS (Extended Data Fig. 2k–m).

We followed our output mapping experiments by determining the sources of input to CSNs. We first used an intersectional approach to drive expression of TVA and N2cG in CSNs (Fig. 2a). Two weeks later, we injected motor cortex with EnVa-N2cG-tdTomaTo, resulting in expression of tdTomaTo in synaptic inputs to CSNs (Fig. 2b–j)36. Using anatomical reconstructions, we found that isocortical regions such as S1 and M2 provide the main source of input to CSNs (Fig. 2k–m; N = 3). Interestingly, thalamic regions dominated non-cortical input to CSNs, including regions that are thought to serve as relays for the output of the basal ganglia (Fig. 2m)37. These anatomical experiments reveal the structural complexity of CSNs, and highlight their unique capacity to influence diverse brain regions involved in motor control, most notably the main input to the basal ganglia, the striatum.

We next sought to determine if CSNs are unique in their capacity to directly modulate both the spinal cord and the striatum. We injected AAV-retro-GFP into cervical spinal cord and AAa-retro-tdTomato into the contralateral DLS (Fig. 3a). Using anatomical reconstruction, we then identified the brain-wide sources of input to the DLS and spinal cord. The only structures that contained appreciable numbers of both DLS inputs and spinal inputs were in the isocortex (Fig. 3b–d; N = 4). Within the isocortex, DLS-projecting and spinal cord-projecting neurons may be interspersed, or there may be neurons that simultaneously project to both structures. To disambiguate these possibilities, we marked neurons that were positive for both GFP and tdTomato, and confirmed that nearly all cells projecting to both DLS and cervical spinal cord were located in the sensorimotor cortex (Fig. 3e–h; N = 4).

CSNs form biased synapses onto distinct striatal pathways

To understand how CSN axon collaterals influence the brain, it is critical to understand the cell types that these axons innervate. Within the striatum, CSNs could synapse on two interspersed populations of SPNs, defined in part by expression of either dopamine receptor 1 or 2 (D1 or D2 SPNs). Previous research revealed that corticopontine neurons (that is, PT neurons) drive larger currents in D1 SPNs than D2 SPNs, despite that D1 SPNs, on average, are larger than D2 SPNs and have lower input resistances24,40. Yet, PT neurons are a diverse population encompassing both corticobulbar and functionally heterogeneous CSNs41,42. Additionally, the synaptic mechanisms underlying differential connectivity between PT neurons and striatal SPNs is unclear. Therefore, we combined an intersectional optogenetic expression strategy with whole-cell
voltage-clamp recordings to characterize synapses arising exclusively from cervical spinal cord-projecting CSNs. First, we expressed channelrhodopsin-2 (ChR2) in CSNs by injecting AAV-retro-ChR2 tdTomato into the cervical spinal cord of adult D1-tdTomato or D2-GFP reporter mice (Fig. 4a). Weeks later, we made targeted whole-cell recordings from D1 and D2 SPNs in brain slices, identified in part by the presence or absence of reporter gene expression in cell bodies visualized under differential interference contrast (DIC) optics (Fig. 4b–h). Recordings were made from neighboring (within 50 µm) D1 and D2 SPNs in sequence (N = 3, n = 12 pairs), or simultaneously (N = 3, n = 8 pairs), and were pooled for further analysis (N = 6, n = 20). Brief (10 ms) photostimulation of ChR2-expressing CSN axons drove excitatory postsynaptic currents (EPSCs) in both D1 and D2 SPNs when measured at membrane holding potentials of −70 mV (Fig. 4i). Comparing ChR2-evoked current and charge revealed that CSN collaterals drive larger responses in D1 SPNs when compared to D2 SPNs (Figs. 4j–l; 33.44 ± 5.69 peak current amplitude (pA) for D1, 17.79 ± 3.67 pA for D2, d.f. = 19, t = 3.3067, P = 0.0037, d = 0.7314; 7.17 ± 1.12 nC for D1, 3.90 ± 0.87 nC for D2, d.f. = 19, t = 3.021 P = 0.006, d = 0.7641). Repeating these experiments using stimulation of intratelencephalic (IT) axon collaterals resulted in equivalently sized EPSCs in D1 and D2 neurons, suggesting biased innervation might be unique to CSNs (Extended Data Fig. 4). Our results comport with previous studies of PT versus IT innervation of SPNs, and build on those results by finding that cervical CSNs in particular are biased in their innervation of D1 SPNs over D2 SPNs43.

Results from the above experiments could be explained by CSNs forming either larger synapses onto D1 SPNs than D2 SPNs, or more numerous similarly sized synapses. To disambiguate between these possibilities, we replaced extracellular calcium with the divalent cation strontium, which desynchronizes neurotransmitter release from the presynaptic neuron (Fig. 4m)44. We reasoned that measuring the amplitude of isolated miniature EPSCs (mEPSCs) evoked by

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Fig. 3 | CSNs are likely to be the only neurons that project to both DLS and spinal cord. a, Experimental strategy to label inputs to the DLS in red and inputs to spinal cord in green. b, Photomicrographs of labeling throughout the brain. The boxed region indicates sensorimotor cortex, which is highlighted in c–e. c, 3D reconstructions of cell body positions from two vantages. The DLS injection site is indicated, which corresponds to a bolus of red cell bodies. d, Quantification of brain regions with inputs to the DLS (red bars) and spinal cord (green bars). e, Confocal photomicrograph from sensorimotor cortex showing dual labeling. f, High-magnification view from the inset in e. Arrowheads indicate double-positive cells. g, 3D view of double-positive neurons. h, The brain regions with double-positive neurons. Primary somatosensory areas: SS pul (upper limb), SS pl (lower limb), SS pr (trunk), SS p (unassigned), SS pbfd (barrel field); VISa, anterior area; rSPag, retrosplenial area, agranular part; AUDd, dorsal auditory area; ACAd, anterior cingulate area, dorsal part; AUDv, ventral auditory area; AL, basolateral amygdalar nucleus; PL, prelimbic area; Alp, agranular insular area; ECT, ectorhinal area; MD, mediodorsal nucleus of the thalamus; ENT, entorhinal area; TEa, temporal association area; VISC, visceral area. N = 4. Error bars are the s.e.m.
photostimulation would allow us to infer the size of single synapses made by CSN axons on SPNs\textsuperscript{45}. To this end, the averages of mEPSCs recorded from D1 or D2 SPNs were indistinguishable, implying that CSNs form similarly sized synapses on both populations (Fig. 4n–p; 4.47±0.51 pA for D1, n=5; 4.45±0.40 pA for D2, n=8; d.f. = 11, t = 0.0366, P = 0.97, N = 5). Stimulation of IT axons also resulted
in equivalently sized mEPSCs in D1 and D2 SPNs (Extended Data Fig. 4i–l). By extension, we concluded that CSNs, on average, form more synapses on D1 SPNs than on D2 SPNs. Together, these electrophysiological experiments reveal a synaptic and circuit basis by which CSNs interact with two distinct pathways of the basal ganglia.

### CSNsD1 and CSNsD2 synapse in distinct spinal compartments

The CSNs that synapse on D1 or D2 SPNs could belong to the same population, or could belong to at least partially distinct populations. Furthermore, if they represent at least partially distinct populations, CSNs that innervate D1 or D2 SPNs could project into different regions of the spinal cord and synapse onto different spinal interneurons. To address these possibilities, we used rabies tracing to map the spinal projections of CSNsD1 and CSNsD2 into the DLS of D1-Cre or A2a-Cre mice, we injected a cocktail of AAV-FLEX-TVA and AAV-FLEX-N2cG. We later injected EnVa-N2cAG-tdTomato into the same site, labeling inputs to D1 or D2 SPNs with tdTomato (Fig. 5a). Because the input to the DLS that projects to spinal cord is sensorimotor cortex (Fig. 3), we concluded that axons found in the spinal cord arose from CSNs. We then took high-resolution confocal images throughout the cervical spinal cord, visualizing antibody-enhanced tdTomato labeling, along with coexpression of vGlut1 to identify presynaptic boutons (Fig. 5b).

**Fig. 5 | Mapping the distribution of spinal synapses from CSNsDLS.** a. Experimental strategy to transynaptically label CSNsD1 and CSNsD2. b. Photomicrograph of tdTomato-labeled CSNs with identified synapses on striatal SPNs (left), and the synapses formed by these neurons in spinal cord (right). tdTomato+ synapses are identified by coincident expression of vGlut1 (cyan; inset). Arrowheads indicate vGlut1+ boutons. The green arrow indicates the central canal. Fluorescent Nissl stain is shown in gray. Representative of N = 10: c2, n = 22,664; c3, n = 30,418; c4, n = 26,091; c5, n = 30,479; c6, n = 23,963; c7, n = 23,571. d, e. Contour plots illustrating the relative distribution of synapses arising from CSNsD1 (d) and CSNsD2 (e), N = 5 for each genotype; n = 69,636 each. f. The difference between contour plots in d and e. g. Random resampling analysis. The dataset was resampled with different sample sizes (color, n = 4,000:8,000), and statistical analysis was repeated many times. h. Statistical differences between the spatial distribution of CSNsD1 and CSNsD2 synapses. N = 5 for each genotype (Methods). i. Experimental strategy to transynaptically label CSNsD1 and CSNsD2 and measure close appositions on spinal cell types. j. Contour plot illustrating the relative distribution of GAD2 cell bodies for one mouse; n = 531. k. Photomicrograph showing GAD2 neurons (purple), CSNsD2 axons (red) and vGlut1 immunolabeling (cyan) in cervical spinal cord, C6 segment. The corticospinal tract is visible in the upper left corner. Representative of N = 3. l. High-magnification view showing vGlut1+ CSNsD1 varicosities closely apposed to a GAD2+ neuron, indicated with arrowheads. Representative of N = 3. m, n. The number of close appositions on GAD2 neurons (m) or CB neurons (n) divided by the total number of vGlut1+ CSNsD1 or CSNsD2 varicosities in the field of view. N = 3 for each genotype for GAD2, N = 3 for CSNsD1, and N = 4 for CSNsD2 for CB. Significance is measured by an unpaired two-sided t-test. P = 0.0345 for m. P = 0.029 for n. Error bars are the s.e.m.
throughout the spinal gray, with the densest innervation in inter-
mediate and superficial laminae. Still, we identified vGlut1+ bout-
tons deep within the ventral horn, including those that were closely
apposed to putative Renshaw neurons, highlighting their capacity
to intimately influence motor output (Extended Data Fig. 5a–g)46,47.

We next separately analyzed the distribution of synapses arising
from CSNsD1 and CSNsD2. While both populations of neurons formed synapses throughout cervical spinal cord, CSNsD1 synapses were biased to more rostral coordinates, home to interneurons that modulate sensory feedback (for example, SST interneurons in Extended Data Fig. 2j), while CSNsD2 synapses were skewed to the ventral regions of spinal cord, where there is an enrichment of premotor interneuron populations (for example, Chx10 interneurons; Fig. 5d–f and Extended Data Fig. 5h; 147.41 ± 0.79 µm for D2 versus 188.17 ± 0.78 µm for D1, dorsoventral relative to the central
canal; d.f. = 139,266, t = 36.72, P = 1.08 × 10−295, d = 0.20)48. Random
subsampleting revealed these results were statistically robust (Fig. 5g;
that is, 1,100 of 69,636 resampled coordinates, median of repeated
t-tests, dorsoventral: P = 2.37 × 10−4). Next, we performed regional
comparative statistics, revealing highly distributed regions of spinal
gray with statistically significant innervation differences (Fig. 5h and Extended Data Fig. 5i,j). These data reveal that CSNsD1 and CSNsD2 are at least partially distinct populations, with biased spinal projection patterns.

These results raise the question of whether CSNsD1 and CSNsD2 are biased in their innervation of spinal interneuron subtypes. We reasoned that a stringent test for this possibility is to measure rela-
tive innervation of an interneuron species found in a region with equivalent innervation density by CSNsD1 and CSNsD2. Our experi-
ments using transsynaptic tracing from spinal interneurons showed that GAD2-expressing putative GABAergic neurons are innervated by CSNsD1 (Extended Data Fig. 2j–m), making this population a prime candidate for study, because a group of these neurons are enriched in intermediate laminae of cervical spinal cord where we found equi-
valent innervation by CSNsD1 and CSNsD2 (compare Fig. 5i with Fig. 5j). We therefore generated double transgenic mice that (1) express Cre recombinase in D1 or D2 striatal SPNs and (2) express GFP in GAD2-expressing spinal interneurons49. We then used rabies virus to express tdTomato in CSNs that innervate either D1 or D2 SPNs, and quantified the number of vGlut1+ CSNs or CSN-α-opioid receptor
on the somata and proximal dendrites of GAD2 interneurons, normal-
ized by the total number of vGlut1+ corticospinal synapses in that region of interest (ROI; Fig. 5i–l). These experiments revealed that GAD2 neurons receive more appositions from CSNsD1 compared to CSNsD2 (Fig. 5m; 0.0024 ± 0.0000218 appositions/total CSN boutons for CSNsD1, 0.0011 ± 0.0000283 for CSNsD2, d.f. = 4, t = 3.15, P = 0.0345, d = 2.57). We questioned whether increased innervation of interneurons by CSNsD1 is a general feature, or if other interneu-
ron types receive different patterns of innervation. To test this, we analyzed innervation of calbindin 28k (CB)-immunolabeled inter-
neurons, many of which are found in the same intermediate regions as GAD2-expressing neurons. In contrast to GAD2 neurons, CB
neurons received more synapses from CSNsD3 compared to CSNsD2 (Fig. 5n; 0.000556 ± 0.0000336 appositions/total CSN boutons for
CSNsD1, 0.000340 ± 0.0000535 for CSNsD2, d.f. = 5, t = 3.034, P = 0.029, d = 2.42). Altogether, these results reveal coordinated connection between CSNs and cell types in the striatum and spinal
cord.

Corticospinal neurons signal both single movements and sequences

The anatomical complexities of CSNs—particularly their promi-
nent projections to the striatum—inspired us to characterize their activity during a behavioral task relevant to basal ganglia. Previous research showed that the striatum is necessary for sequential lever-press behaviors, and that many striatal SPNs are selectively active around the onset or offset of sequences, while other neurons fire throughout execution28,29. We sought to determine whether inputs to striatum from CSNs display similar encoding properties during sequence performance, or if these striatal activity patterns arise downstream of the cortex. Water-restricted mice were trained to depress a narrow lever positioned in front of their right forepaw
four times in succession to receive a water reward (Fig. 6a). Mice were acclimated to handling and head fixation, and rewarded for each lever press they made. After 7 d of this training, mice were then rewarded only after every fourth lever press. Next, the maxi-
mum sequence length that issued reward was lowered first to 3 s, then 2 s. Mice learn this task successfully, as evidenced by the rapid execution of grouped lever presses, and the increased performance of four press sequences and decrease of two press sequences (Fig. 6b–d; two-way analysis of variance (ANOVA), F = 84.77, d.f. = 9, P = 0, post hoc t-test, P = 0.0284 and P = 0.026, respectively, N = 8). Across training, both the inter-press interval (speed) and coefficient of variation (variability) of the inter-press interval for lever-press sequences decreased and stabilized (Fig. 6e and Extended Data Fig. 6a,b). To analyze muscle activity with high resolution, we implanted electrodes made for recording electromyographic (EMG) signals into four forelimb muscles. To monitor the activity of CSNs, we injected AAV-retro-GCaMP6f into the right cervical spinal cord of D1-Cre or A2a-Cre mice (Extended Data Fig. 6c), and implanted a cranial window over the left forelimb motor cortex. Two-photon (2p) imaging was used to record activity in dendritic trunks of CSNs approximately 300 µm below the pial surface (Fig. 6f,g). These dendritic signals are highly correlated with and faster than somatic calcium activity30,31. Calcium signals were extracted using constrained nonnegative matrix factorization (CNMF) and highly correlated (rho > 0.8) processes were treated as belonging to the same neuron to minimize overrepresentation by branching dendrites (Fig. 6h)32,33. To overcome the temporal limitations of calcium transients, we used deconvolution developed with CNMF to convert calcium signals to an event signal, which we aligned to lever-press sequences34. Event-triggered averaging of both raw fluo-
rescence and CNMF-extracted signal revealed deconvolved activity corre-
sponds well to underlying fluorescence signals (Extended Data Fig. 6d–h), Z-score deconvolved activity from one mouse (Extended Data Fig. 7a,b) and across all mice (Fig. 6i) was visibly faster than calcium activity, and there was a strong trend for neuronal activity to be enhanced around lever pressing (Extended Data Fig. 7c). Heat maps of z-scored activity aligned to a single lever press revealed a temporal distribution of peak responses (Extended Data Fig. 7d). Binning neurons by the time of their peak responses revealed most neurons were active immediately around lever pressing (Extended Data Fig. 7e; median time to peak response −93 ms), and the responses of these neurons were larger than those active further in time relative to lever pressing (Extended Data Fig. 7f).

Is CSN activity linearly related to motor output, or can CSNs dis-
play sequence-level activity similar to what is seen in striatal SPNs? To answer this question, we identified and grouped sequences of lever presses of one, two, three or four presses. We used time warping to standardize the inter-press interval within sequences to 200 ms, allowing us to preserve temporal resolution when averaging across trials (Extended Data Fig. 7g–i and Methods). Aligning activity from the total population of neurons (n = 2,374, N = 8) to lever-press sequences revealed that, on average, CSN activity scales in duration to lever-press sequences of increasing length (Fig. 6j; see Extended Data Fig. 7j,k for calcium signal). Yet, when plotted individually, the top three principal components (PCs) of z-scored CSN activity each displayed unique activity signatures. The component accounting for the most variance was elevated in activity throughout sequence execution, while the next two PCs were active most strongly at the onset or offset of sequence (Fig. 6k). This result motivated us to characterize the activity patterns of single
neurons. We aligned time-warped z-scored deconvolved events of single neurons to four lever-press sequences. Doing so, we found a heterogenous population of neurons, including those with activity around the first or final press in a sequence, and neurons that were active around each press in a sequence (Fig. 6l–n), all of which were intermingled in the same fields of view. Aligning z-scored event rates to each press in a sequence recapitulated these results (Extended Data Fig. 8a).

Motivated by these results, we next aligned binned z-scored events to time-warped lever-press sequences, and identified neurons with significant modulation at different windows of the lever-press sequence. We identified 8.26% and 16.04% of CSNs responding at the onset and offset of the sequence, respectively, as well as a larger population of neurons with activity sustained throughout sequence execution (Fig. 6o; 29.18%). We further identified a population of neurons with activity significantly suppressed relative to baseline.
(8.45%), as well as population of neurons that did not meet criteria for significant modulation (38.41%). Averaging the z-scored activity of categorized neurons revealed the relative stereotypy of these responses, and this was recapitulated by inspecting the timing of peak responses of categorized neurons (Extended Data Fig. 8b,c).

Corticospinal neurons encode muscle- and non-muscle-correlated activity

Muscle activity may change from lever press to press, raising the possibility that the variability we observed in neuronal activity could be due to differential recruitment of musculature at the onset or offset of sequences, or preferential correlation with individual muscles. To directly address these possibilities, we analyzed the EMG activity of biceps and triceps during behavior and in relation to neuronal activity (Fig. 6p–q and Extended Data Fig. 9). First, aligning all biceps activity to local peaks in triceps activity revealed a robust alternation of the activity between these two antagonist muscles (Extended Data Fig. 9a). Biceps and triceps activity alternated preceding lever press, consistent with their flexor and extensor identity (Extended Data Fig. 9b). Importantly, biceps and triceps activity strongly alternated during sequences, and the amplitude of EMG activity preceding lever-press events was similar throughout the sequence (Fig. 6p–q; biceps: one-way ANOVA, d.f. = 3, F = 0.98, P = 0.4199; triceps: one-way ANOVA, d.f. = 3, F = 1.1, P = 0.3684, N = 8; Extended Data Fig. 9c). We leveraged our EMG dataset by correlating CSN event rate to biceps and triceps activity during concatenated periods of behavioral quiescence or concatenated lever-press sequences. On average, CSNs were more correlated with triceps activity than biceps during random periods of activity and quiescence, but this preference was lost when correlating neural activity with only concatenated lever-press sequences, controlling for the number of samples in each condition (Extended Data Fig. 9d). We next measured the correlation between average time-warped biceps and triceps EMG activity and average time-warped events of onset, offset, sustained and suppressed neurons during lever-press sequences. Sustained neurons were more correlated with biceps and triceps activity than onset and offset CSNs, and suppressed neurons were anticorrelated with muscle activity (Fig. 6r). While some individual neurons were more strongly correlated with biceps or triceps EMG activity, on average no group of neurons was consistently more correlated with one muscle over the other, suggesting encoding of muscle identity cannot explain sequence encoding properties of CSNs (Fig. 6r,s and Extended Data Fig. 9e,f). Finally, we addressed the possibility that onset neurons were encoding gross body movements that preceded the onset of lever-press sequences. We measured the variance of ROIs encompassing various body parts in videos of trained animals performing the task, and showed that before arm movement onset, there were no overt body, mouth or nose movements (Extended Data Fig. 9g–l). These data indicate that, in general, muscle output is not the exclusive determinant of CSN activity during skilled motor sequences.

Diverse corticospinal neuron activity is broadcast to both striatal pathways

Our results thus far show CSNs can encode sequence-level activity similar to what is observed in striatal SPNs. Although similar proportions of D1 and D2 SPNs display onset and offset signals, more D1 than D2 SPNs show activity that is sustained through sequences, while more D2 than D1 SPNs are suppressed during sequences. Therefore, it is plausible to hypothesize that CSNs that synapse onto D1 SPNs are more likely to be sustained neurons, while CSNs that synapse on D2 SPNs are more likely to be suppressed neurons. To address this possibility, we combined our 2p calcium imaging experiments with transsynaptic rabies tracing from D1 or D2 SPNs. In the same mice as above (that is, D1-Cre, N = 4 or A2a-Cre, N = 4), we injected AAV-FLEX-N2eG and AAV-FLEX-TVA into the DLS before cranial window implantation (Fig. 7a). After all functional calcium imaging data were acquired, EnVA-N2eG-tdTomato was injected into the same location of the DLS (Fig. 7b–d). Ten days following rabies injection, we took structural images of GCaMP labeling and z-stacks of tdTomato labeling (Fig. 7e and Extended Data Fig. 10). We then used three-dimensional (3D) reconstruction to improve detection of tdTomato+ dendrites at the functional imaging plane, and generated binary masks from this dataset. We then used the GCaMP structural reference images to align masks of rabies labeling to the functional imaging dataset. This approach allowed us to identify CSNsD1 and CSNsD2 post hoc, avoiding any deleterious effect rabies expression has on response properties. Because our anatomical experiments revealed that CSNsD1 and CSNsD2 are at least partially nonoverlapping populations, we reasoned that this approach would allow us to distinguish large encoding differences between groups. We first analyzed neuronal activity in CSNs with confirmed synapses in the striatum, and found that it scaled in duration with lever-press sequences, similar to general CSNs (Fig. 7f). Do CSNsD1 and CSNsD2 comprise similar proportions of onset, offset, sustained and suppressed neurons? We applied our classification scheme to rabies-labeled CSNs, and compared these data to unlabeled neurons from the same mice to control for potential differences in rabies expression across animals. Surprisingly, we found similar proportions of onset, offset, sustained and suppressed neurons in tdTomato-positive neurons compared to tdTomato-negative CSNs, along with no apparent enrichment of any response type when comparing CSNsD1 and CSNsD2 (Fig. 7g). These results support a model whereby the complex information encoded by CSNs during motor sequences is transmitted in a balanced fashion to both D1 and D2 SPNs, suggesting that the different encoding properties of D1 versus D2 SPNs emerge from differences in the synaptic properties, intrinsic connectivity and neuromodulatory receptors of the target neurons.

Discussion

The results presented here reveal that CSNs encode information that is diverse in its relationship to behavioral output, from muscle-related activity to higher-order sequence-related information in the form of onset or offset responses. Our results further uncover synaptic and circuit principles governing the communication of these neural signals between spinal and basal ganglia circuits. Populations of CSNs preferentially synapsing onto D1 SPNs have different spinal projection patterns than CSNs preferentially synapsing onto D2 SPNs, and are biased in their connectivity to different spinal interneuron cell types. Surprisingly, these different CSN populations broadcast similar information to downstream circuits, suggesting that motor specificity emerges from the cell types innervated and differences in connectivity at these postsynaptic sites.

We first used a suite of anatomical tools to map the brain-wide organization of inputs to the spinal cord, and identified isocortical regions—particularly the sensorimotor cortex—as containing the most spinal cord-projecting neurons across all mapped structures. In addition to sensorimotor cortex, we identified many midbrain and hindbrain inputs, including structures with well-defined roles in modulating premotor circuits. Notably, the AAV-retro and rabies-based tracing methods we used do not necessarily reflect the strength of connectivity across these circuits, and differences in viral tropism may contribute to differences in labeling of brain structures. Still, our approach to using these improved viral tools revealed details of corticospinal organization that have been elusive. First, we found that CSN collaterals project most prominently to the DLS, and that CSNsD1, CSNsD2 and CSNsD3 comprise a diverse population originating in both the motor and somatosensory cortex. An interesting future direction would be to characterize functional or anatomical differences between motor cortical and somatosensory cortical CSNsD1, CSNsD2 and CSNsD3.
Our results are at odds with some studies suggesting that corticospinal and corticostriatal populations are largely nonoverlapping, including antidromic stimulation-based experiments\(^1\). This may be because individual CSNs have terminal fields that occupy a small region of the striatum and form relatively few synapses, meaning that the striatal stimulating electrode likely does not effectively drive individual CSNs that have terminal fields that occupy a small brain regions. Future studies will be useful in addressing this possibility. Within the striatum, CSNs drive larger responses in D1 SPNs than D2 SPNs, even though the lower input resistances of D1 SPNs should intuitively result in smaller postsynaptic currents\(^2\). This synaptic capacity to differentially regulate spinal circuits through segregated interneuron populations. Consistent with this, we found CSNs D2 provide roughly twice as much input to GAD2 neurons compared to CSNs D1, an anatomical organization that may be important for the regulation of proprioceptive sensory feedback\(^3\,^4\). Ramón y Cajal, using highly effective Golgi stains, made note of substantial supraspinal corticospinal innervation in his landmark studies\(^5\,^6\). And while some anatomical studies have de-emphasized the spinal cord: CSNs that synapse on either D1 or D2 SPNs form distinct terminal fields in the cervical spinal cord, revealing their capacity to differentially regulate spinal circuits through segregated interneuron populations. Consistent with this, we found CSNs D2, provide roughly twice as much input to GAD2 neurons compared to CSNs D1, an anatomical organization that may be important for the regulation of proprioceptive sensory feedback\(^3\,^4\).

Because of their widespread projections to the DLS, we sought to characterize what information CSNs relate to basal ganglia. An important feature of the basal ganglia is that it is necessary for the performance of learned sequences of body movements\(^5\,^6\). Striatal SPNs encode features of movement sequences in their spiking activity, including neurons that encode the onset or offset of sequences, as well as neurons that are active around each individual movement in a sequence. Indeed, more D1 than D2 SPNs show activity that is sustained through sequence execution, while more D2 than D1 SPNs are suppressed during sequences. Because CSNs provide input to the striatum, we reasoned that the activity properties of SPNs may be mirrored in the activity properties of their presynaptic CSN inputs. This led us to measure the sequence-related activity of CSNs, and then to measure whether CSNs that synapse on D1 or D2 SPNs differentially encode sequence activity. Using calcium imaging during a skilled head-fixed lever-press sequence behavior, we first showed that the activity of many CSNs is closely related to muscle activity, which we measured use high-resolution EMG techniques.
Remarkably, a substantial proportion of both CSNs showed activity that was not highly correlated with individual muscle activity, but instead was correlated with either sequence onset or sequence offset. Finally, a new combination of 2p calcium imaging and transsynaptic rabies tracing revealed CSNGLDK encode lever-press sequences in a similar fashion to the broader CSN population. Moreover, onset, offset and sustained activity levels were found in equal proportions of CSNGDS1 and CSNGDS2. While some fraction of CSNs may synapse on both D1 and D2 SPNs, we found there are at least partially non-overlapping populations of CSNGDS1 and CSNGDS2. Because of this, we reason that our experimental approach would likely detect large differences in how these populations encode movement. These results raise the question of how D1 and D2 SPNs differentially represent sequence information, particularly why more D1 CSNs are sustained, while more D2 CSNs are suppressed. One possibility is that CSNs act in a broadcasting capacity, transmitting efference copies of sequence-related performance to both the spinal cord and the striatum. The bias of CSNs to synapse on D1 SPNs over D2 SPNs, combined with the state-dependent effects of dopaminergic feedback and local connectivity of striatal circuits, could then amplify sustained activity in D1 and suppress sustained activity in D2 SPNs. Another possibility is that there are differences in sequence encoding in other populations of striatal inputs such as IT neurons or thalamic neurons. An interesting question is whether more complex behavioral information that is encoded in striatal SPNs is similarly encoded in CSNs presynaptic to those neurons. Future studies using our transsynaptic rabies tagging approach will be useful to address such possibilities.

In summary, our results unravel an organizational logic where separate populations of CSNs that synapse onto D1 and D2 SPNs form biased projection patterns and cell-type-specific connectivity in the spinal cord. However, CSNs targeting different striatal and spinal circuits broadcast similar movement-related information, including both neural signals closely related to motor output as well as signals related to higher-order features of behavior. These anatomical and functional circuit features suggest that motor specificity arises from the translation of corticospinal movement-related information into divergent circuits in the basal ganglia and spinal cord. These differences in postsynaptic connectivity presumably act alongside various other neuronal inputs and downstream executive circuits to ultimately translate behavioral intent to action.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41593-021-00939-w.

Received: 2 November 2020; Accepted: 10 September 2021; Published online: 4 November 2021

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Methods

A full list of resources and reagents can be found in Supplementary Table 1.

Experimental model and animals. All experiments and procedures were performed according to National Institutes of Health (NIH) guidelines and approved by the Institutional Animal Care and Use Committee of Columbia University. Adult mice of both sexes, aged 2–6 months, were used for all experiments, including slice electrophysiology. The strains used were: C57BL/6J (Jackson Laboratories, 000664), B6.Cg-Tg(Drd1a-tdTomato)6Calak/J (Jackson laboratories, 0016204), Tg(Drd2-EGFP)S118Gsat/Mmucd (MMRRC, 000230), Tg(Drd1a-cre)E2Y17Gsat/Mmucd (Jackson Laboratories, 023778), B6.FVB(Cg)-Tg(Adora2a-cre)KG139Gsat/Mmucd (MMRRC, 036158), Chx10-Cre (Custom Jessell Laboratory), B6.Cg-Ssttm2.1(Cre)Zjh/Mwdr (Jackson Laboratories, 028864), Gad2tm2(Zjh)/Zjh (Jackson Laboratories, 010802) and Gad2-FP (GAD65-FP, G. Szabo). Mice used for behavioral experiments were individually housed, and all mice were kept under a 12 h-light–dark cycle.

Stereotoxic viral injections. Analgesia in the form of subcutaneous injection of carprofen (5 mg per kg body weight) or buprenorphine SR (0.5–1 mg per kg body weight) was administrated on the day of the surgery, along with bupivacaine (2 mg per kg body weight). Mice were anesthetized with isoflurane and placed in a stereotaxic holder (Leica). A midline incision was made to expose the skull, and a craniotomy was made over the injection site. To label CNSs with GFP, 100 nl of AAV-FLEX-GFP was injected into each of two sites of motor cortex, 1.5 mm lateral to the midline and 0.5 and 1.0 mm rostral to bregma, approximately 700 μm below the pial surface. Care was made to ensure there was no efflux of virus by stabilizing the skull and waiting 10 min after penetration before injecting. AAV-retro-Cre.mCherry was then injected into the spinal cord (see below). For transgenic rabies tracing from striatal SPNs, 40 nl of a 1:1 mixture of AAV-FLEX-N2cG and AAV-FLEX-TV A.mCherry was injected into spinal cord between C3 and C7. Next, 15 nl of virus was injected along the dorsoventral axis every 50 μm between 1.2 mm and 0.1 mm below the surface of the cord, totaling 1,035 nl per segment. Following all injections, the skin was sutured closed and animals were closely monitored during recovery.

Spinal cord viral injections. Analgesia in the form of subcutaneous injection of carprofen (5 mg per kg body weight) or buprenorphine SR (0.5–1 mg per kg body weight) was administrated on the day of the surgery, along with bupivacaine (2 mg per kg body weight). Mice were anesthetized with isoflurane and placed in a stereotaxic holder (Leica). A midline incision was made to expose the spinal column. The musculature overlaying the column was resected, and a metal clip attached to a spinal clamp was used to secure the T2 process and minimize spinal cord movement. The tail was gently stretched with another spinal clamp to separate the vertebral bodies. A surgical microscope was used to sever the meninges, exposing the spinal cord. A pulled glass pipette was filled with virus, and a Nanoject III was used to make multiple small-volume injections across into the spinal cord, with parameters that depended on the experiment and reagents used. For injections of AAV-retro-GCaMP6f AAV-retro-Cre.mCherry, AAV-retro-ChR2.tDTomato, AAV-retro-GFP or AAV-retro-ENaC, one penetration was made into each segment of the spinal cord between C3 and C7. Twenty injections of 10 nl each were made into the center of the spinal gray, for a total volume of 200 nl per spinal segment. For injections of AAV-FLEX-N2cG or AAV-FLEX-TV A.mCherry, two penetrations were made into each segment of the spinal cord between C3 and C7. In total, 10 nl of virus was injected along the dorsoventral axis every 50 μm between 1.2 mm and 0.1 mm below the surface of the cord, totaling 460 nl per segment. For injections of EnVAGA-N2cG-FlpO.mCherry, three penetrations were made into each segment of the spinal cord between C3 and C7. Next, 15 nl of virus was injected along the dorsoventral axis every 50 μm between 1.2 mm and 0.1 mm below the surface of the cord, totaling 1,035 nl per segment. Following all injections, the skin was sutured closed and animals were closely monitored during recovery.

Slice electrophysiology and optogenetic photostimulation. Mice were deeply anesthetized with isoflurane and transcardially perfused with an ice-cold carbogenated high-magnesium (10 mM) artificial cerebrospinal fluid (ACSF). The brain was removed from the skull, and glued to the stage of a vibrating microtome (Leica). Next, 300–μm coronal brain slices were cut in a bath of ice-cold, slushy, carbogenated low-calium ACSF. Slices were incubated for 15–30 min in a 37°C bath of normal ACSF containing 24 mM NaCl, 2.7 mM KCl, 1.25 mM NaHCO3, 3 mM MgSO4, 26 mM NaH2PO4, 1.25 mM NaHCO3, 18 mM glucose and 0.79 mM sodium ascorbate. Slices were then transitioned to room temperature, where they remained for the duration of the experiment. Patch electrodes (3–6 MΩ) were filled with either a potassium glutamate-based internal solution (155 mM potassium glutamate, 2 mM MgCl2, 0.5 mM potassium magnesium ATP, 9.5 mM sodium GTP, 10 mM HEPES, 10 mM phosphocreatine and 0.15% Neurobiotin) or a cesium/QX-314-based internal solution (5 mM QX-314, 2 mM ATP magnesium salt, 0.3 mM GTP sodium salt, 10 mM phosphocreatine, 0.2 mM EGTA, 2 mM MgCl2, 5 mM NaCl, 10 mM HEPES, 120 mM cesium methanesulfonate and 0.15% Neurobiotin). All recordings were made using a Multiclamp 700B amplifier, filtered at 1–10 kHz (Digitax 11A). Resistance was always <35 MΩ and was compensated up to 90%. Neurons were targeted with DIC microscopy and epifluorescence when appropriate. For simultaneous recordings, pairs of neighboring SPNs (within 50 μm of each other) were identified first by morphology using DIC imaging. The cellular identity of targeted neurons was confirmed through expression or lack of expression of transgenically targeted fluorescent reporters. For experiments exploiting potassium glutamate-based internal solutions, neurons were further identified through intrinsic electrophysiological properties, including excitability and current/voltage transformation. In a subset of experiments, cell morphology was visualized through 405 nm excitation of tdTomato. All imaging was performed using a Leica SP8 confocal microscope. Fluorescein 488 sodium salt. ChR2-expressing axons were photostimulated using 10 ms pulses of 473-nm LED light (CoolLED) delivered through a ×10 objective centered over the recording site. Brain slices were histologically processed to visualize Neurobiotin-filled cells through streptavidin-Alexa Fluor processing.

Histology and confocal imaging. Mice were deeply anesthetized with isoflurane and transcardially perfused with PBS followed by ice-cold 4% paraformaldehyde. Brains and spinal cords were postfixed overnight in 4% paraformaldehyde, and then cryopreserved in a 30% sucrose solution for 3–4 days at 4°C. Brains and spinal cords were embedded in Optimal Cutting Temperature Compound (Tissue Tek), and 70 μm coronal sections were cut on a cryostat. Tissue was rinsed several times in PBS, then permeabilized in PBS containing 0.2% Triton X-100 (PBST). For imaging synapses in spinal cord, tissue sections were first permeabilized in 1% PBST to aid in antibody penetration. Immunostaining was performed with primary antibodies diluted at 1:1,000 for 3–4 days at 4°C, and with secondary antibodies at a 1:1,000 dilution overnight at 4°C. Counterstains of DAPI or Neurotrace were included in the secondary antibody incubation at a dilution of 1:1,000. Brain and spinal cord slices mounted to slides were briefly incubated with TrueBlack diluted in 70% ethanol to quench lipofuscin and background autofluorescence. Confocal imaging was performed on a Zeiss 710 or Zeiss 880 using a ×10, ×20, ×40, ×63 or ×100 objective. For mapping the distribution of spinal synapses arising from CNSNcs, high-resolution YZ-stitched images were acquired using a ×40 water immersion high-numerical NA objective. Imaris was used to identify tDtomato+ axons that colocalized with vGlut1 expression. Synaptic boutons were then marked with spots, and the coordinates of these spots were measured relative to the center of the central canal.

Slide scanning and anatomical reconstructions. Coronal sections (70 μm) were serially mounted on slides, and were treated with TrueBlack diluted in 70% ethanol to quench lipofuscin and background autofluorescence. Sections were imaged using an AZ100 automated slide scanning microscope equipped with a ×4 0.4-NA objective (Nikon). Image processing and analysis using BrainJ proceeded as previously described. Briefly, brain sections were aligned and registered using two-dimensional (2D) rigid-body registration. A seven-pixeloline-ball filter was used on all images to reduce background signal and a machine-learning pixel classification approach using iLastik was used to identify cell bodies and neuronal processes. To this end, several background-subtracted images were imported into iLastik (separately for each fluorescence channel) to generate a large sample of manually variable morphological characteristics representative of cell bodies, neurites and background fluorescence features were selected to train the algorithm across all images. Probabilistic assignment of image features was continuously checked with a live preview feature to ensure accuracy. The algorithms were then used to generate probability images for each fluorescence channel of each brain section image, and the resulting images were processed for segmentation of cell bodies and neurites. To map the location of these structures to an annotated brain atlas, 3D image registration was performed using Elastix relative to a reference brain. The coordinates of detected cells and processes were then projected into the Allen Brain Atlas Common Coordinate Framework. Visualizations of the data were performed in ImageJ and Imaris, and subsequent analyses were performed in MATLAB using custom software.

Electrophysiological electrode and headpost implantation. EMG electrodes were fabricated as previously described. Two pieces of insulated braided stainless-steel wire were knotted, and 0.5-mm portions of insulation were stripped from each wire just below the knot so that exposed contact sites were separated by 0.5 mm.
The portions of wire with contact sites were twisted, and the ends secured in a crimped hypodermic needle to permit easy insertion into targeted muscle groups. The opposing strands were soldered to a miniature connector. This process was repeated three times to produce a total of four differential recording electrodes that could be implanted into four muscles.

Analgesia in the form of subcutaneous injection of carprofen (5 mg per kg body weight) or buprenorphine SR (0.5–1 mg per kg body weight) was administered on the day of the surgery, along with bupivacaine (2 mg per kg body weight). Mice were anesthetized with isoflurane and placed in a stereotaxic frame. Hair was carefully shaved from the right forelimb, neck and head, and the skin was thoroughly cleaned. Incisions were made over the neck and forelimb, and the electrode assembly was snaked through these sites so that the miniature connector was positioned near the head and the individual recording electrodes positioned near the biceps, triceps, extensor digitorum communis and palmaris longus. Electrodes were implanted in each muscle by passing each needle and wire through targeted muscle groups until the knot was abutted to the muscle entry point. The tag ends of wire were then knotted by the exit point, thus securing the contact sites within the muscle. Forelimb incisions were closed with sutures, and the headpost implantation proceeded. The scalp was removed to expose the cranium, and fascia was cleared using a scalpel and saline irrigation. A custom 3D-printed plastic headpost was affixed to the cranium using Metabond dental cement (Parkell), and reference points were marked to facilitate the implantation of a cranial window. Finally, the miniature connector for the EMG electrode assembly was cemented to the caudal edge of the headpost, and the skin overlying the neck was closed with sutures.

Cranial window implantation. Analgesia in the form of subcutaneous injection of buprenorphine SR (0.5–1 mg per kg body weight) was administered on the day of the surgery, along with bupivacaine (2 mg per kg body weight) and the anti-inflammatory dexamethasone (2 mg per kg body weight). Mice were secured in a stereotaxic frame (David Kopf Instruments), and the headpost forks designed to clamp the custom headpost. The custom cranial window was composed of two semicircular pieces of glass coverslip (200-μm thick; Tower Optical), fused together and then to a 4-mm round no. 1 coverslip (Warner Instruments) with optical cement (Norland Optical Adhesive 61). A craniotomy the shape of the insertable coverslips was made over forelimb motor cortex, and the window was implanted so that the semicircular plug was gently pressing on the brain. The entire assembly was secured using Metabond.

Behavior. Behavioral training occurred in parallel using behavioral chambers equipped with custom-molded and assembled components. Mice were head-fixed using 3D-printed hard plastic forks that clamped around a custom plastic headpost cemented to the cranium. The body rested in an opaque plastic tube, and the left forelimb was allowed to rest on a movable perch. The right forelimb was positioned over a milled plastic lever that had a small counterweight. Lever pressures were reported as the counterweighted arm passed through an infrared beam. Water rewards were dispensed through a blunt needle positioned ~3 mm from the mouth so that beads of water reward were reachable by licking. Water reward was calibrated regularly by adjusting the length of the TTL pulse sent to a solenoid valve. Behavioral assays were controlled using software written for and deployed with pyControl (https://pycontrol.readthedocs.io/en/latest/). Performance was continuously monitored and recorded with an infrared camera for each mouse.

Mice were accustomed to handling for several days, and then placed on a water-restriction schedule using established guidelines. Weight, appearance and general health was monitored daily, and supplemental water was administered of identified synapses were binned to 20 μm for each mouse. Sets of nine (3×3) bins were selected using a sliding window of one bin in each mediolateral and dorsoventral direction, and the values of those bins were concatenated across mice for each genotype. For each sliding window position, an unpaired two-tail t-test with an alpha value of 0.05 was used to measure significance, and the resulting P value was assigned to the center of that sliding window. The sliding window was then shifted by one bin, and the process was repeated. To quantify corticospinal projections on GAD2 or CB neurons, high-resolution tiled z-stacks acquired at 10 kHz alongside 2p imaging data using Prairie View. EMG signals were amplified and filtered (250–20,000 Hz) with a differential amplifier (MA102 with MA103S preamplifiers, University of Cologne electronics laboratory). These signals were acquired at 10 kHz alongside 2p imaging data using Prairie View. EMG signals were downsampled to 1 kHz, high-pass filtered at 40 Hz, rectified and convolved with a Gaussian that had a standard deviation of 10 ms.

Statistics and reproducibility. No statistical methods were used to predetermine sample size, but our sample sizes are similar to those reported in previous publications. No data were excluded from the analyses. Data for measuring the distribution of spinal synapses were subsampled and randomized. Randomization was performed and for other experiments. When quantifying spinal synapses. Blinding was not used for other experiments, but automated analyses were used to limit experimenter bias. Data distribution was assumed to be normal, but this was not formally tested. Animals (within genotype pools) were randomly assigned to experimental groups.

Automated anatomical reconstruction. Analysis of slice scanning data was performed using MATLAB. Data were output from the Brain toolbox in the pipeline of the large file containing measurements of neurite labeling and cell body count from each region in the Allen Brain Atlas Common Coordinate Framework. These measurements used hierarchical unbiased analysis and generated the required shape and volume data. For measurements from higher-order brain regions (that is, isocortex), measurements from descend region identified by the Allen Brain Atlas application programming interface were grouped. In the Allen Institute Mouse Common Coordinate Framework, these are referred to as ‘major divisions’.

Slice electrophysiology. Analysis of slice electrophysiology data was performed in MATLAB and in Clampfit (Molecular Devices). Tests of significance were performed using paired t-tests with an alpha value of 0.05. Amplitude and charge were measured from a 200-ms window following stimulus onset relative to a baseline period 250 ms before the onset of the stimulus. To measure the amplitude of miniature EPSCs evoked through optogenetic stimulation of CSNs using strontium-containing ACSF, an mEPSC template was created in Clampfit. This template was used to search for mEPSCs. Each mEPSC was measured for improved signal-to-noise ratio, higher-order brain regions (that is, isocortex), measurements from descend region identified by the Allen Brain Atlas application programming interface were grouped. In the Allen Institute Mouse Common Coordinate Framework, these are referred to as ‘major divisions’.

Distribution of spinal synapses. For resampling analysis, subsets of dorsoventral and mediolateral synaptic positions (from 100 to 8,000 samples) were randomly selected from the population, and this was repeated to generate 10,000 random datasets for each subsampled group. Statistical significance was measured for each group across genotype using a two-tailed unpaired t-test. The result was plotted in a histogram to illustrate the frequency of calculated P values measured for each subsample recorded with the number of identified synapses in the spatial distribution (that is, clusters) of spinal synapses from each genotype; the coordinates of identified synapses were binned to 20 μm for each mouse. Sets of nine (3×3) bins were selected using a sliding window of one bin in each mediolateral and dorsoventral direction, and the values of those bins were concatenated across mice for each genotype. For each sliding window position, an unpaired two-tail t-test with an alpha value of 0.05 was used to measure significance, and the resulting P value was assigned to the center of that sliding window. The sliding window was then shifted by one bin, and the process was repeated. To quantify corticospinal projections on GAD2 or CB neurons, high-resolution tiled z-stacks acquired using Prairie View. EMG signals were amplified and filtered (250–20,000 Hz) with a differential amplifier (MA102 with MA103S preamplifiers, University of Cologne electronics laboratory). These signals were acquired at 10 kHz alongside 2p imaging data using Prairie View. EMG signals were downsampled to 1 kHz, high-pass filtered at 40 Hz, rectified and convolved with a Gaussian that had a standard deviation of 10 ms.

Cadmium imaging. Calcium imaging analysis was performed using NCMF. First, raw imaging datasets (~10 min each) were motion corrected using rigid, nonrigid registration. Registered datasets were then processed in NCMF using an autoregressive process p of 2. Analysis was also performed using a p of 0 to replicate results, although these data are not included in this study. Output of the
CNMF was in the form of $\Delta F/F$ and deconvolved events. Signals were up-sampled to match the sampling rate of EMG data and z-scored for further analysis. Time warping was used to standardize the inter-press interval of lever-press trials. To this end, a lever-press sequence time-series template was created that comprised six time anchors, four of which corresponded to a lever-press sequence as well as two anchors corresponding to pre-trial and post-trial time periods. The inter-press interval for this template was standardized to 200 ms. For each behavioral trial, the real-time interval was measured between lever pressing, and linear resampling was performed as needed to either increase or decrease the number of samples within this window to match the 200-ms template. Neurons were classified by their response properties as follows. For each neuron, trials of four lever-press sequences were identified and time warped. A baseline period was defined as the first 250 ms of each trial (beginning 1.5 s before the first lever press). Each trial (excluding the baseline period) was segmented into bins of ten samples in length, and the bins with mean activity that was significantly different than baseline (measured using a within-trial paired two-tail t-test with an alpha of 0.05) were marked. Within this group, bins with mean activity greater than 2.5 standard deviations of baseline were then identified as positively modulated, and bins with mean activity less than that of baseline were identified as negatively modulated. We then identified significantly modulated bins in each of nine time periods that spanned the trial (excluding the baseline period). The rationale for analyzing short bins was that brief deviations in activity could be overlooked or diluted if averaging across longer time windows. Neurons with significant and positively modulated activity in one or more of periods 1–4 and zero in periods 6–9 were classified as onset. Neurons with significant and positively modulated activity in one or more of periods 6–9 and zero in periods 1–4 were classified as offset. Neurons with significant and positively modulated activity in two or more of periods 3–7 were marked as sustained. Neurons with significant and negatively modulated activity in two or more of periods 3–7 were classified as suppressed. Neurons that met none of these criteria were marked as weak.

To mark CNSs co-labeled with tdTomato through rabies infection, we used a 3D reconstruction approach to improve identification of red fluorescent neurites. Around 1 week to 10 d after rabies injection, z-stacks of tdTomato fluorescence were acquired at 1,040 nm. These tdTomato z-stacks were imported into Imaris, and binary masks were generated using the Surface function. The binary stack was then re-sliced to generate one binary mask at the same z plane used for functional imaging of GCaMP. This mask was registered to the functional dataset using shift parameters derived from registration of reference GCaMP fluorescence images. We then identified tdTomato pixels that fell within the spatial boundaries of GCaMP ROIs, and summed these pixels, which were weighted by how close they were to the center of the ROI. This number was divided by the total tdTomato pixels within that structure, yielding a value that reflected (1) the proximity of the tdTomato process to the center of the GCaMP ROI, and (2) the degree to which the tdTomato structure was overlapping with the GCaMP ROI. If this value was greater than 60% of the sum of weighted GCaMP ROI pixels divided by the total number of those pixels, that ROI was marked as tdTomato positive.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Data that support the findings of this study are available from the corresponding authors upon reasonable request.

**Code availability**

Custom code used in this study is available from the corresponding authors upon reasonable request.

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**Acknowledgements**

We thank K. Fidelin and V. Athalye for feedback on this manuscript. We thank H. Rodrigues for designing and constructing behavioral equipment. We thank S. Brenner-Morton for custom antibodies, and S. Fageiry and K. Ritala for custom viral constructs. We thank Zuckerman Institute’s Cellular Imaging platform for instrument use and technical advice. We are grateful for technical assistance from L. Hammond, G. Martins, M. Correia, C. Warriner, A. Miri and K. MacArthur. We thank I. Marcelo for time-warping code. Imaging was performed with support from the Zuckerman Institute’s Cellular Imaging platform. We thank T. Jessell for inspiring this research and for providing critical feedback. R.M.C. was funded by the NIH (5U19NS104649) and the Simons-Emory International Consortium on Motor Control. A.N. was a Howard Hughes Medical Institute Fellow of the Helen Hay Whitney Foundation and is currently supported by a NIH Pathway to Independence Award (1K99NS118053-01).

**Author contributions**

A.N. and R.M.C. designed experiments, interpreted data and wrote the manuscript. A.N. performed experiments and analyzed data. B.A. assisted in collecting and analyzing anatomical tracing data.

**Competing interests**

The authors declare no competing interests.

**Additional information**

Extended data is available for this paper at https://doi.org/10.1038/s41593-021-00939-w.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41593-021-00939-w.

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**Peer review information Nature Neuroscience** thanks Bernardo Sabatini and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Mapping brainwide inputs to the spinal cord. (a) Illustration of approach to visualize inputs to cervical spinal cord. (b) 3D reconstruction of inputs to spinal cord. Colors correspond to major brain divisions. (c) The top brain regions that project to spinal cord, determined by the relative fraction of total somata. Notable regions are indicated by colored bars. Insets illustrate exemplar brain regions with substantial labeling. Dashed boxes are colored to correspond to notable brain regions. The inset pie chart shows the major brain structures (‘major divisions’ as classified by the Allen Institute Mouse Common Coordinate Framework; see Methods) projecting to cervical spinal cord. N = 3. (d-f) Micrographs of CSN axons expressing GFP (green) in transverse cross-sections of cervical (D), thoracic (E), and lumbar (F) spinal cord. The insets are high magnification images of GFP+ bulbous varicosities from different laminae of cervical (7Sp/8Sp), thoracic (7Sp/1CI), and lumbar (4Sp) segments. Neuronal processes expressing Cre.RFP are in red. Representative of N = 3. (g) Quantification of cortical inputs to spinal cord (RFP+), divided by cortical region and laminae. The inset photomicrograph illustrates the L5b positioning of CSNs. Note that the Allen Brain Atlas classification did not subdivide L5 into L5a and L5b, and the position of corticospinal somata fell around the boundary between L5 and L6a. N = 3. (h) Experimental strategy, same as Fig. 1a. (i) The cortical regions giving rise to corticospinal somata (GFP+). Dark green bars represent the major regions; light green bars represent subdivisions of cortical regions. N = 3. (j) Major brain regions containing GFP+ neurites. This includes dendritic processes in sensorimotor cortex. Grouping the many brain regions comprising these major structures reveals the intense innervation of several subcortical structures, including brainstem. (k) Photomicrograph of brainstem labeling by CSNs. N = 3. (l) Experimental strategy to label synapses arising from CSNs. (m) Synaptophysin GFP (green) labeling in the brain. N = 3. (n) Synaptophysin GFP (green) and FlpO (red) labeling in motor cortex. N = 3. (o) Synaptophysin GFP (green) labeling in DLS. N = 3. (p) Top brain regions to which CSNs project, excluding sensorimotor cortex and fiber tracts. N = 2. Error bars are SEM.
Extended Data Fig. 2 | Mapping the brain wide targets of CSNsDLS. (a) Experimental strategy to label corticospinal neurons that project to striatum (CSNsDLS). (b) Photomicrographs exemplifying the cortical distribution CSNsDLS. Representative of N = 3. (c) Sagittal Z projection of raw fluorescence aligned to atlas space. Representative of N = 3. (d) 3D reconstruction of CSNsDLS cell bodies. Representative of N = 3. (e) Quantification of cortical regions contributing to the total population of CSNsDLS, compared to experiments from Fig. 1 targeting primarily the motor cortical population of CSNs (M-CSNs). Asterisks indicate statistically significant differences in innervation between M-CSNs and CSNsDLS (*: p < 0.05, **: p < 0.005, 2-way ANOVA with post-hoc t-test). (f) Quantification of brain regions targeted by CSNsDLS, compared to data from Fig. 1. Note that – despite the differences in experimental strategy – DLS is a primary target of CSNsDLS. N = 3. (g) 3D reconstruction of CSNsDLS projections throughout the brain, colored by targeted brain region. (h) Photomicrograph showing CSNsDLS axon labeling in the brainstem. Representative of N = 3. (i) Higher magnification inset from (H). Representative of N = 4. (j) Experimental strategy to drive expression of GFP in corticospinal neurons that form synapses on identified spinal cell types. (k) 3D reconstruction of axons from CSNsDLS, color coded by brain region. (l) Confocal micrograph exemplifying CSNsDLS axon labeling in DLS. (m) Quantification of brain structures that receive substantial input from CSN subtypes. N = 4, Chx10, N = 2, SST, N = 3, GAD2. Error bars are SEM.
Extended Data Fig. 3 | Controls for transsynaptic tracing experiments. (a) Experimental strategy to confirm Cre-dependent expression of AAVs-FLEX encoding rabies glycoprotein and TVA-mCherry in DLS, as well as dependence of EnVA-N2cΔG-tdTomato infection on expression of TVA. The AAVs were injected into wild type mice, followed by injection of rabies. (b-d) Photomicrographs illustrating the absence of any mCherry or tdTomato labeling in the brain. Representative of N = 3. (e) Experimental strategy to confirm Cre-dependent expression of AAVs-FLEX encoding rabies glycoprotein and TVA-mCherry in the spinal cord, as well as dependence of EnVA-N2cΔG-FlpO.mCherry infection on expression of TVA. (f-g) Photomicrographs illustrating the absence of any mCherry or tdTomato labeling in the spinal cord or brain. Representative of N = 3. (h) Experimental strategy to confirm dependency of transsynaptic spread on rabies glycoprotein, in the DLS. AAV-FLEX-TVA-mCherry was injected into DLS of D1-Cre mice. (i-k) Injecting EnVA-N2cΔG-tdTomato led to local tdTomato expression, but no expression in presynaptic inputs to DLS. Representative of N = 1. (l) Experimental strategy to confirm dependency of transsynaptic spread on rabies glycoprotein, in the spinal cord. AAV-FLEX-TVA-mCherry was injected into spinal cord of GAD2-Cre mice. (m-o) Injecting EnVA-N2cΔG-FlpO.mCherry led to local mCherry expression, but no expression in presynaptic inputs to spinal cord. Representative of N = 3.
Extended Data Fig. 4 | Synaptic organization of intratelencephalic corticostriatal projections. (a) Schematic illustrating the experimental strategy. Retrogradely-transported and expressed AAV encoding Chr2.tdtomato was injected into contralateral DLS or M1. D1 and D2 SPNs were targeted for simultaneous recording. (b) Photomicrograph of Chr2.tdtomato (red) and D2-GFP (green) labeling in a brain slice. (c) High magnification image of the boxed region from (B). Note the expansive axonal plexus. (d) DIC image of a D1+ (magenta) and D1-SPNs targeted for simultaneous whole cell recording. The dashed lines indicate the location of recording electrodes. (e) Superimposed current-clamp voltage recordings from an SPN following optogenetic stimulation of IT corticostriatal axons, highlighting the potency of this projection. (f) Grand average response of all D1 (blue) and D2 (orange) SPNs to optogenetic stimulation of IT corticostriatal neurons. (g-h) Pairwise comparison of Chr2-evoked amplitude (G) and charge (H) in D1 versus D2 SPNs. N = 3 animals, n = 7 cells. Paired t-test. (i-j) Trial average of mEPSCs evoked from an example D1 (I) and D2 (J) SPN. Individual trials are in grey. (k) Average mEPSC amplitudes in D1 versus D2 SPNs. N = 2 animals, n = 7 cells. (l) Distribution of all mEPSCs ordered by mEPSC peak current, recorded in D1 (blue) or D2 (orange) SPNs. The inset is an overlay of the average mEPSC from D1 and D2 SPNs. Error bars are SEM. Shaded areas are SEM. The horizontal dashes in the box plots in (G), (H), and (K) represent the median. The dots in (G) and (H) indicate the means. The bottom and top edges of all boxes indicate the 25th and 75th percentiles, respectively, while the whiskers indicate the minima and maxima, excluding outliers.
Extended Data Fig. 5 | Distribution of spinal synapses arising from CSNs

(a) The same confocal micrograph from Fig. 3b. The ventral horn inset is shown in (b), with vGlut1+ varicosities indicated with arrowheads. (c) Confocal micrograph of immunolabeled calbindin-expressing interneurons located below motor pools in cervical spinal cord (putative Renshaw neurons). (d-g) High magnification 63x single optical section images showing two vGlut1+ synapses from a rabies-labeled CSN in close apposition to the proximal dendrite belonging to a calbindin+ interneuron. (h) Quantification of the mean mediolateral and dorsoventral position of CSNsD1 (blue) and CSNsD2 (orange). N = 5 each, unpaired two-sided t-test, p = 1.08 × 10−293. (i) Raw CSN spinal synapse data from three example mice. The position of each dot corresponds to a vGlut1+ axonal varicosity. (j) Raw data is spatially binned for each mouse. A sliding window is used to group local bins, and the density of labeling within these groups is compared across genotypes of mice.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | Analysis of behavior and deconvolution. (a) Rate of reward across training. The broken X axis indicates where training changed from Phase 1 (one press issues reward) to Phase 2 (four press sequences issue reward). The green vertical line indicates the day at which the maximum rewarded inter-press interval (IPI) for four lever press sequences is limited to 3 seconds. Before this line (days 8-10), reward was issued every four presses, regardless of IPI. The purple vertical line indicates the day at which the maximum IPI for four lever press sequences to lead to reward is limited to 2 seconds. (b) The inter-press interval of lever press sequences across Phase 2 of training. (c) Confocal micrograph of the spinal cord injection site. (d) Signals extracted from CSNs from one mouse for a portion of a session. Raw fluorescence signal in blue. Calcium signal derived using CNMF is in green. Deconvolved event rate is in orange. Units are a.u. (e) A higher magnification view of signals extracted from one neuron. (f) Histogram depicting the number of deconvolved events as a function of their amplitude. (g) Average of raw fluorescence signal triggered by deconvolved events, aligned to event time. The shade of purple corresponds to the size of the associated deconvolved event. (h) Average of CNMF-derived calcium signal triggered by deconvolved events, aligned to event time. The shade of red corresponds to the size of the associated deconvolved event. Shaded area is SEM.
Extended Data Fig. 7 | Imaging the activity of CSNs during behavior. (a) Trial-averaged calcium activity aligned to lever press for neurons from a single mouse. (b) Same data as (A), but for deconvolved events. (c) Z scored deconvolved events of neurons at baseline versus at lever press. (d) Z scored activity of neurons aligned to single lever press events. (e) Histogram of the times of peak activity relative to lever press, for all neurons. (f) Average activity traces for neurons with peak activity that falls within different bins of time relative to lever press. (g–j) Illustration of time warping procedure for four press sequences. Dots indicate lever press times, as well as time anchors used for pre- and post-trial alignment (six time points per trial). (h) Illustration of how time is either dilated or contracted to match a template sequence. (j–k) Z scored calcium activity before (J) and after (K) time warping, to illustrate the utility of time warping for resolving latent features in unaligned activity. Note the emergence in (K) of peaks in activity corresponding to individual lever press events. This is the same dataset as Fig. 6j, but is calcium signal, instead of deconvolved events. Shaded area is SEM.
Extended Data Fig. 8 | Classification of CSN activity during lever press sequences. (a) The same neurons as (Fig. 6l-n), instead displaying the average of events aligned to first, second, third, or forth press in the sequence. (b) The average activity of neurons belonging to each activity profile, aligned to four lever press sequence onset. (c) Histogram of the times of peak activity for CSNs with categorized activity profiles, aligned to lever press sequence onset. Shaded area is SEM.
Extended Data Fig. 9 | See next page for caption.
Extended Data Fig. 9 | Analysis of EMG during behavior and CSN activity correlations to EMG. (a) Example recording of biceps and triceps muscle activity from one mouse. Biceps EMG is aligned to peaks in triceps EMG. (b) Average EMG activity for four forelimb muscles aligned to single lever presses. (c) Average EMG activity for four forelimb muscles aligned to lever press sequences. (d) Correlation of CSN activity to biceps versus triceps EMG during concatenated random segments of behavior and rest (grey) or concatenated lever press sequences (purple), matched in duration. Paired two-sided t-tests, concatenated random segments: \( p = 6.66 \times 10^{-27} \); concatenated lever press sequences: \( p = 0.722 \), \( N = 8 \), \( n = 2252 \). (e) Correlation of trial-averaged CSN activity with biceps or triceps EMG. Neurons with correlations biased to triceps or biceps are colorized in red or green, respectively. (f) Average lever press sequence-related activity of CSNs highly correlated to triceps (red) or biceps (green) EMG. Activity from neurons with similar correlation coefficients is in grey. (G-L) Analysis of body movements during behavior. (g) A video still with highlighted regions of interest used for quantifying body movements. (h–k) Images of variance from the same vantage as (G) revealing different body parts moving during the behavior. (l) Variance from different body regions of interest aligned to the onset of arm movement sequences. Notice the absence of overt body movements before arm movement. Shaded area is SEM.
Extended Data Fig. 10 | Method to identify CSNs with identified striatal synapses. (a) Exemplar photomicrograph of CSNs expressing GCaMP (green), and corticostriatal neurons marked with tdTomato (red). Representative of N = 8. (b) Cartoon depiction of fluorescent expression possibilities, viewed from an X-Z perspective. (c) Cartoon depiction of fluorescent expression possibilities, viewed from an X-Y perspective. (d) Two example possibilities for overlapping green and red fluorescence, one constituting a double-positive (top) and one rejected from being a double-positive (bottom).
Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
- Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
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- Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
Software used to collect data included PrairieView 5.4, Clampex 10, pyControl 1.6, ZEN Black, and NIS Elements 5.21. Custom programs were used in pyControl to acquire behavioral data.

Data analysis
All statistical analyses were performed using MATLAB 2018a. Data processing and analysis was performed in MATLAB 2018a, ImageJ 2.1.0, Clampfit 10. Custom programs were used in MATLAB to process and analyze data. Clampfit 10 was used to identify and collate miniature EPSCs.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

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Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

The data that support the findings of this study are available from the corresponding author upon reasonable request.
Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | No statistical methods were used to predetermine sample sizes. However, all anatomical observations were repeated using multiple animals, similar to previous studies (i.e. Nelson and Mooney, Neuron 2016). Electrophysiological, behavioral, and calcium imaging experiments used sample sizes that were comparable to previous studies (i.e. Nelson and Mooney, Neuron 2016). |
| Data exclusions | No data was excluded from this study. |
| Replication | All attempts to replicate data were successful. Anatomical results were replicated in multiple mice using automated analyses. Slice electrophysiological experiments were performed using several mice and were consistent across animals. Behavioral and calcium imaging experiments were replicated across mice within experimental cohorts. |
| Randomization | Except for random subsampling of anatomical data, randomization was not used during analysis. Animals (within genotype pools) were randomly assigned to experimental groups. Within animal controls were performed when possible, including for experiments combining calcium imaging and transsynaptic rabies tracing. |
| Blinding | Identical confocal imaging parameters were used for collecting anatomical synapse distribution data. Investigators analyzing synapse distribution were blinded to genotype and group allocation during data analyses. Blinding was not used for other analyses, but automated analyses used were not subject to experimenter bias. |

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

| n/a | Involved in the study |
| --- | --- |
| ✔️ | Antibodies |
| ✔️ | Eukaryotic cell lines |
| ✔️ | Palaeontology and archaeology |
| ✔️ | Animals and other organisms |
| ✔️ | Human research participants |
| ✔️ | Clinical data |
| ✔️ | Dual use research of concern |

### Methods

| n/a | Involved in the study |
| --- | --- |
| ✔️ | ChIP-seq |
| ✔️ | Flow cytometry |
| ✔️ | MRI-based neuroimaging |

### Antibodies

Antibodies were validated by examining vendor statistics and controls when available (Takara 632496: Vendor certificate of analysis from Western blot analysis; Biortby orb116138: Vendor validation images of Western blot analysis; MilliporeSigma 69050-3: Vendor validation images of Western blot analysis; Jackson ImmunoResearch AffiniPure secondary antibodies were validated by the vendor using immunoelectrophoresis and/or ELISA; Columbia University custom antibodies for EGFP and dsRed were made against full length purified protein, and colocalize with visible protein in tissue. They did not stain tissue not expressing fluorescent protein. Additionally, images were carefully examined for background staining and nonspecific labeling, which was not present for any of the reagents used in this study. Several of the antibodies used in this study have been used in previous publications (Swant CB38: Airaksinen M.S., et al, (1997), PNAS 94(4) : 1488-1493, absence of staining in Calbindin D-28k knock-out mice; ThermoFisher N21483: Neurosci Lett 184, 169 (1995), ThermoFisher S32351: Proc Natl Acad Sci USA 71, 3537 (1974), Columbia University custom antibody for vGlut1: Betley et al. (2009), Cell 139(1):161-74).|
Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | Adult mice of both sexes, aged between 2-6 months were used for all experiments, including slice electrophysiology. The strains used were: C57BL6/J, Jackson Laboratories #000664, B6.Cg-Tg(Drd1a-ttdTomato)6Calak/J, Jackson Laboratories #016204, Tg(Drd2-EGFP)S118Gsat/Mmnc, MMRRC #000230, Tg(Drd1a-cre)EY217Gsat/Mmuucd, Jackson Laboratories #030778, B6.FVB(Cg)-Tg(Adora2a-cre)KG139Gsat/Mmuucd, MMRRC #036158, Tg(Drd1a-cre)KG139Gsat/Mmuucd, MMRRC #036158, Chx10-Cre, Custom Jessell Laboratory, B6.Cg-Ssttm2.1(cre)Zjh/MwarJ, Jackson Laboratories #028864, Gad2tm2(cre)Zjh/J, Jackson Laboratories #010802, GAD2-GFP (GAD65-GFP), Gábor Szabó. Mice used for behavioral experiments were individually housed, and all mice were kept under a 12 hour light/dark cycle. Temperature was kept at 72 degrees F and 40% humidity. |
| Wild animals | No wild animals were used in this study. |
| Field-collected samples | No field-collected samples were used in this study. |
| Ethics oversight | All experiments and procedures were performed according to NIH guidelines and approved by the Institutional Animal Care and Use Committee of Columbia University. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.