

Axonal Barcode Analysis of Pyramidal Tract Projections from Mouse Forelimb M1 and M2

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Forelimb-related areas of the motor cortex communicate directly to downstream areas in the brainstem and spinal cord via axons that project to and through the pyramidal tract (PT). To better understand the diversity of the brainstem branching patterns of these pyramidal tract projections, we used MAPseq, a molecular barcode technique for population-scale sampling with single-axon resolution. In experiments using mice of both sexes, we first confirmed prior results demonstrating the basic efficacy of axonal barcode identification of primary motor cortex (M1) PT-type axons, including corticobulbar (CBULB) and corticospinal (CSPI) subclasses. We then used multiplexed MAPseq to analyze projections from M1 and M2 (caudal and rostral forelimb areas). The four basic axon subclasses comprising these projections (M1-CSPI, M1-CBULB, M2-CSPI, M2-CBULB) showed a complex mix of differences and similarities in their brainstem projection profiles. This included relatively abundant branching by all classes in the dorsal midbrain, by M2 subclasses in the pons, and by CSPI subclasses in the dorsal medulla. Cluster analysis showed graded distributions of the basic subclasses within the PT class. Clusters were of diversely mixed subclass composition and showed distinct rostrocaudal and/or dorsomedial projection biases. Exemplifying these patterns was a subcluster likely enriched in corticocuneate branches. Overall, the results indicate high yet systematic PT axon diversity at the level of brainstem branching patterns; projections of M1 and M2 appear qualitatively similar, yet with quantitative differences in subclasses and clusters.

Key words: axon branching; barcode; corticobulbar; corticospinal; motor cortex; pyramidal tract

Significance Statement

Axons of the PT class of cortical projection neurons, which includes corticospinal and corticobulbar neurons, anatomically link motor cortex to brainstem and spinal cord circuits. Both of these subclasses can form branches to brainstem destinations along the way, but the extent and diversity of these branching patterns is incompletely understood. Here, we used MAPseq to tag PT axons with individual molecular barcodes for high-throughput quantification of branching patterns across the brainstem. The results reveal diverse, complex, yet systematic branching patterns of corticospinal and corticobulbar neurons arising from two motor cortex areas, M1 and M2.

Introduction

Sensorimotor functions of the forelimb rely on multiregional circuits linking the motor cortex, brainstem nuclei, cervical spinal

cord, and more (Arber and Costa, 2018). Axonal projections from the primary motor cortex (M1) to the brainstem and spinal cord travel via the pyramidal tract (PT), running along the ventral pons and medulla. These projections constitute a mix of two basic subclasses of PT-type axons, (1) corticospinal (CSPI) axons, which travel through the pyramidal tract en route to the spinal cord and may or may not send branches to the brainstem along the way, and, (2) noncorticospinal corticobulbar (CBULB) axons, which terminate at various levels within the brainstem without reaching the spinal cord. The diversity and complexity of PT axon projections is a long-standing and controversial area of motor systems research (Shepherd, 2014; Smith et al., 2014). Prior studies have characterized many features of the branching patterns of PT axons, including those of M1. The MouseLight project has generated many single-axon reconstructions of mouse motor cortex PT axons (Economo et al., 2016; Winnubst et al., 2019). A recent large-scale multiomics project has also

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applied this approach as well as conventional bulk labeling methods, molecular barcoding, and related anatomic techniques to characterize motor cortex projections (BRAIN Initiative Cell Census Network (BICCN), 2021; Muñoz-Castañeda et al., 2021).

Here, we sought to build on these recent advances by using MAPseq, a molecular barcoding method for characterizing anatomic projections with single-axon resolution (Kebschull et al., 2016), to further explore its utility for analyzing brainstem projections of axons arising from PT neurons in motor cortex (BRAIN Initiative Cell Census Network (BICCN), 2021; Muñoz-Castañeda et al., 2021). In MAPseq, as described in detail in the original study (Kebschull et al., 2016), projection neurons in a region of interest—in this case, motor cortex areas—are individually labeled with a unique genetic identifier by injecting a barcode library consisting of a collection of Sindbis virus particles, each carrying a random short RNA sequence (barcode). On average, each neuron is labeled with one virion and thus one barcode, and because the number of unique barcodes in the library ($\sim 10^7$) vastly exceeds the number of neurons infected ($\sim 10^3$), degenerate barcoding (i.e., the same barcode being expressed in multiple neurons) is vanishingly rare. Neurons rapidly express high levels of barcodes (as mRNA), which specifically bind to MAPP-nL, a modified presynaptic protein, and are transported into axon terminals; as a result, barcodes distribute throughout axons and their branching arbors. Tissue samples are collected at the injection site and in multiple downstream regions expected to receive the axonal projections containing the barcodes. Samples are sequenced to determine barcode abundances. The resulting datasets reveal the anatomic profiles of axonal projections at a population scale and with single-neuron (i.e., single axon) cellular resolution; the anatomic resolution, which is relatively low compared with microscopy methods, is set by the sampling strategy, that is, the volumes (and particular sizes and shapes) of the dissected tissue samples.

Questions guiding our application of MAPseq to analyze motor cortex projections to the pyramidal tract included the following: What is the overall pattern of PT projections to major brainstem regions (midbrain, pons, and medulla)? To what extent are these differentiated for the CSPI versus CBULB subclasses? How do they compare for projections arising from different motor cortical areas? We first focused on PT projections from forelimb M1 (i.e., caudal forelimb area) to confirm the efficacy of identifying top-level projection classes using MAPseq. We then examined in greater detail the PT projections to the brainstem from both forelimb M1 and the anterior secondary motor cortex, M2 [i.e., rostral forelimb area (RFA)], which contains a smaller collection of CSPI neurons (Nudo and Masterton, 1990). Our studies confirm the efficacy of applying MAPseq to analyze subcerebral projections from the cortex and identify basic features of the brainstem branching patterns of CSPI and CBULB projections from M1 and M2.

Materials and Methods

Animals. Wild-type C57Bl/6j adult (median age 3 months) mice were used in accordance with the guidelines of the Northwestern University Animal Care and Use Committee and National Institutes of Health. Mice were housed with *ad libitum* access to food and water on a 12 h light/dark cycle. Mice of both sexes were used in approximately equal numbers. Animal numbers were underpowered for detecting sex-dependent differences, which were not expected for the anatomic projections under study.

Surgery and injections. Viral injections into brain and spinal cord targets were made following standard procedures as previously described

(Yamawaki et al., 2021). Stereotaxic coordinates for the forelimb subregion of M1 were 0.0 mm anterior and 1.5 mm lateral, relative to bregma, as previously determined based on retrograde labeling of cervically projecting corticospinal neurons in relation to the localization of the laterally adjacent forelimb S1 (forelimb subfield of primary somatosensory cortex; Ueno et al., 2018; Yamawaki et al., 2021). Coordinates for M2 were 2.0 mm anterior and 1.25 mm lateral, based on the localization of corticospinal neurons in the M2/RFA by retrograde labeling from the cervical spinal cord. Spinal cord injections were made at cervical level 6 (C-6). Sindbis injections were performed at biosafety level 2, and mice were housed at this level until being killed for tissue harvesting. Adeno-associated virus (AAV) injections were performed at biosafety level 1.

MAPseq labeling and sampling. The MAPseq Core staff at Cold Spring Harbor Laboratory (CSHL) provided the barcode viral library and performed sample processing and sequencing. MAPseq experimental design and protocols followed methods as originally published (Kebschull et al., 2016) and available on-line at <https://www.protocols.io/view/mapseq-multiplexed-analysis-of-projections-by-sequ-bism9nc96>.

Aliquots of the high-diversity ($\sim 10^7$ or more) Sindbis-packaged barcode library were prepared at CSHL, shipped, and stored at -80°C until use. Stereotaxic injections of the virus were performed as described above, injecting 100 nl twice, at depths of 0.4 and 0.8 mm. Pilot studies were initially performed using fluorescence imaging of the GFP expression of the Sindbis library to verify the efficacy of transfecting hundreds to thousands of cortical neurons at the injection site and expression in the pyramidal tract and other projections.

At 44–48 h postinjection, mice were killed by perfusion fixation (4% paraformaldehyde) and decapitation (at $\sim\text{C1/C2}$ level). The brain and spinal cord (to at least the low thoracic level) were rapidly dissected out. Tissue samples were postfixed overnight, then washed with PBS. The spinal cord was further dissected to collect upper ($\sim\text{C2–5}$) and lower ($\sim\text{C5–8}$) cervical samples in separate tubes, which were quick frozen on dry ice. The brains (with attached rostralmost cervical cord) were quick frozen on dry ice and stored at -80°C in an embedding compound (Tissue Plus O.C.T. Compound, Fisher) mold. Brains were mounted for rostro-to-caudal coronal cryosectioning at 0.3 mm thickness (Leica CM1850UV Cryostat). With this orientation, the first sample collected was the olfactory bulbs, serving as a negative control (i.e., lacking known projections from motor cortex). Tissue was further sectioned and discarded until reaching the level of the injection site in forelimb M1. This section was dissected (using a scalpel blade) to collect samples of the M1 injection site and the contralateral forelimb M1. We then continued cutting and collecting coronal sections of the remaining brain tissue at 0.3 mm per section. Sections caudal to the level of the decussation were pooled with the upper cervical spinal sample. After every third section, a thin (~ 0.05 mm) section was collected for *post hoc* microscopic inspection and registration to anatomic coordinates. Multiple measures, such as cleaning with ethanol and RNaseZap, only using clean blades, and frequently changing gloves, were taken throughout the cryosectioning process to avoid sample cross-contamination and RNase exposure.

In the experiments involving M1 and M2 injections, we followed the same procedure, but for each brainstem-containing section a horizontal cut was made to separately sample the ventralmost portion containing the main PT-containing white-matter tract (i.e., cerebral peduncles in the more rostral sections and the pyramidal tract itself in the more caudal sections) and the larger dorsal portion containing brainstem neuropil. In this experiment the spinal sample included all the cervical cord in a single tube.

MAPseq sequencing and processing. Samples were shipped on dry ice to CSHL for sequencing and basic analysis following standard protocols developed by the Zador lab at CSHL, as described in the original (Kebschull et al., 2016) and more recent studies (Mathis et al., 2021). Briefly, sequencing involved (1) extracting total RNA, (2) adding spike-in RNA to enable corrections for varying efficiencies at subsequent steps, and (3) tagging each individual barcode mRNA with a random 12 nucleotide unique molecular identifier (UMI) to enable precise counting of barcode cDNA molecules and with an 8 nucleotide sample-specific identifier (SSI) to enable sample multiplexing in the sequencing run. Reverse transcription and amplification of each mRNA sequence (barcode+SSI+UMI) in each

sample yielded a cDNA library that was sequenced (Illumina Next Generation Sequencing). Barcode sequences were processed, including sorting according to SSIs, application of threshold criteria to eliminate low-read molecules likely to be PCR/sequencing errors, and normalization steps to account for varying reverse transcription and PCR efficiencies and thereby compensate for variations during sequencing library preparation.

MAPseq analysis of PT projections. The resulting barcode matrices were then further analyzed using MATLAB (MathWorks) functions as described (see below, Results) to parse the barcodes and assign them to major classes and subclasses of cortical projection neurons. In the multiplexed M1/M2 MAPseq experiments, we assigned barcodes to either area, based either on their exclusive presence in only one of the two areas (~85% of barcodes) or, for barcodes detected in both areas (~15% of barcodes), on their relative abundance at each injection site; as barcode abundances at the less-abundant site were extremely low (<0.5% on average), area assignments were unambiguous. Similarly, for parsing of barcodes to intratelencephalic (IT) and PT classes, the vast majority (>90%) could be assigned based on their exclusive presence in class-defining territories (contralateral cortex for IT, brainstem/spinal samples for PT); the remaining barcodes were assigned based on relative abundance. Barcode matrices were clustered by hierarchical agglomerative clustering using Euclidean distance and Ward's linkage with the cluster function in MATLAB after first row normalizing the data to correct for absolute differences in barcode abundance between samples. The number of clusters was chosen automatically by the *evalclusters* function in MATLAB using the Calinski–Harabasz criterion, which gives the number of clusters that maximizes the ratio of between-cluster variance to within-cluster variance. Barcode matrices were reduced to two dimensions for visualization using Uniform Manifold Approximation and Projection (UMAP; McInnes et al., 2018) with all default parameters.

Confocal imaging. Mice that were previously injected with AAVs in the motor cortex and/or spinal cord underwent perfusion fixation. AAVs used included the following: AAV1-CAG-GFP (catalog #37825, Addgene), AAV1-CAG-tdTomato (catalog #59462, Addgene), AAV5-FLEX-tdTomato (catalog #28306, Addgene), AAV5-pCAG-FLEX-EGFP-WPRE (catalog #51502, Addgene), AAVretro.hSyn.Cre.WPRE.hGH (catalog #105553, Addgene), AAVretro-CAG-tdTomato (catalog #59462, Addgene). The brain was dissected out with the upper cervical spinal cord attached. Coronal sections were cut at 0.15 mm thickness on a vibratome (Microm), mounted, and coverslipped on glass slides. Sections were imaged under a confocal microscope (CSU-W1, Nikon) equipped for multicolor fluorescence imaging, with tiling and stitching as necessary. Images were further inspected and analyzed using ImageJ software.

Experimental design and statistical analysis. The experimental design followed principles previously established for MAPseq analysis of axonal projections in the original (Kebuschull et al., 2016) and subsequent studies (Han et al., 2018; Klingler et al., 2021; Muñoz-Castañeda et al., 2021). Sample sizes were not predetermined by power analysis. The animal numbers are similar to those in prior MAPseq studies. The parametric or nonparametric statistical tests used, measures of central tendency and dispersion, sample sizes, controls for multiple comparisons, and related statistical information are provided in the main text. Statistical analyses were performed using standard MATLAB functions.

Results

Experiment 1: MAPseq identification of pyramidal tract projections from forelimb M1

To confirm the basic efficacy of MAPseq for analysis of mouse M1 projections (Muñoz-Castañeda et al., 2021), we designed an experiment in which we injected forelimb M1 using stereotaxic coordinates based on anatomic landmarks (Ueno et al., 2018; Yamawaki et al., 2021), dissected and sequenced downstream areas with a focus on the pyramidal tract projections, and analyzed the barcode matrices to verify that we could identify in our samples the expected major classes of M1 projection neurons. This experiment builds on that performed as part of the BRAIN Initiative Cell Census Network (BICCN) characterization of

mouse motor cortex projections (BRAIN Initiative Cell Census Network (BICCN), 2021; Muñoz-Castañeda et al., 2021) but focuses primarily on the subcerebral pyramidal tract components. In designing this experiment we took into consideration the anatomy of PT projections, as imaged based on bulk and intersectional labeling methods, to label the pyramidal tract projections broadly and the CSPI axons specifically (see above, Materials and Methods). In coronal sections (Fig. 1A–C), we observed the expected basic features of PT projections. Proceeding caudally from the injection site, the main white-matter pathway of the M1 projection was easily followed through the internal capsule and cerebral peduncles, continuing from the level of the pons as the pyramidal tract until its decussation and further continuation in the spinal cord. Labeling in brainstem neuropil was generally sparser, although stronger labeling was seen in dorsal midbrain regions. Labeling of PT axons was generally stronger than for CSPI axons, consistent with expectations (i.e., as the latter are a subset of the former), although differences in labeling intensity also likely reflect technical factors, such as the labeling strategies (non-Cre-dependent vs intersectional).

Guided by this anatomic characterization, we similarly performed MAPseq analysis of the same brainstem projections from forelimb M1. We injected forelimb M1 with a highly diverse Sindbis-packaged barcode library, waited 44–48 h, perfusion fixed the animals, and collected cryosectioned samples of the brain and spinal cord (see above, Material and Methods; Fig. 2A). The series of coronal sections included sequential 0.3 mm sections from the thalamus to spinal cord. Each section constituted an individual sample and was processed as a whole volume of tissue (i.e., not further dissected; accordingly, the anatomic resolution of the sampling strategy was 0.3 mm in the rostrocaudal axis). After every third section, an ~50 μ m section was taken for bright-field microscopic imaging and anatomic registration. This sampling strategy was chosen to sample the major projections of forelimb M1, particularly including axons projecting to and through the brainstem, with identification of the approximate rostrocaudal level of each slice.

Samples were processed and sequenced, yielding a barcode matrix representing barcode abundances in each sample. That is, each row in the matrix corresponds to the unique barcode of a single labeled neuron, each column corresponds to one of the harvested tissue samples, and each entry represents the abundance of one particular barcode in one particular tissue sample. Standard MAPseq controls were included to verify low false-positive and false-negative rates (see above, Materials and Methods).

Barcode matrices were then analyzed to identify projection classes and subclasses, as shown in the example (Fig. 2B). Our strategy was to parse the barcodes, each of which represents the axonal projection of a single forelimb M1 neuron, into major top-level classes of M1 projection neurons. For this, we considered the known axonal branching patterns of IT, PT, and corticothalamic (CT) projection neurons. In the series of coronal sections, we identified those containing cortex, thalamus (including the ventrolateral and other motor-related nuclei), brainstem (including midbrain, pons, and/or medulla), and/or spinal cord tissue. In some cases these were overlapping (e.g., more rostral sections might contain cortex, thalamus, and midbrain).

Barcodes were parsed based on their presence or absence in key regions. Contralaterally (callosally) projecting IT (cIT) neurons were identified as barcodes present in the contralateral M1 sample. This group of cIT neurons constituted a large fraction of

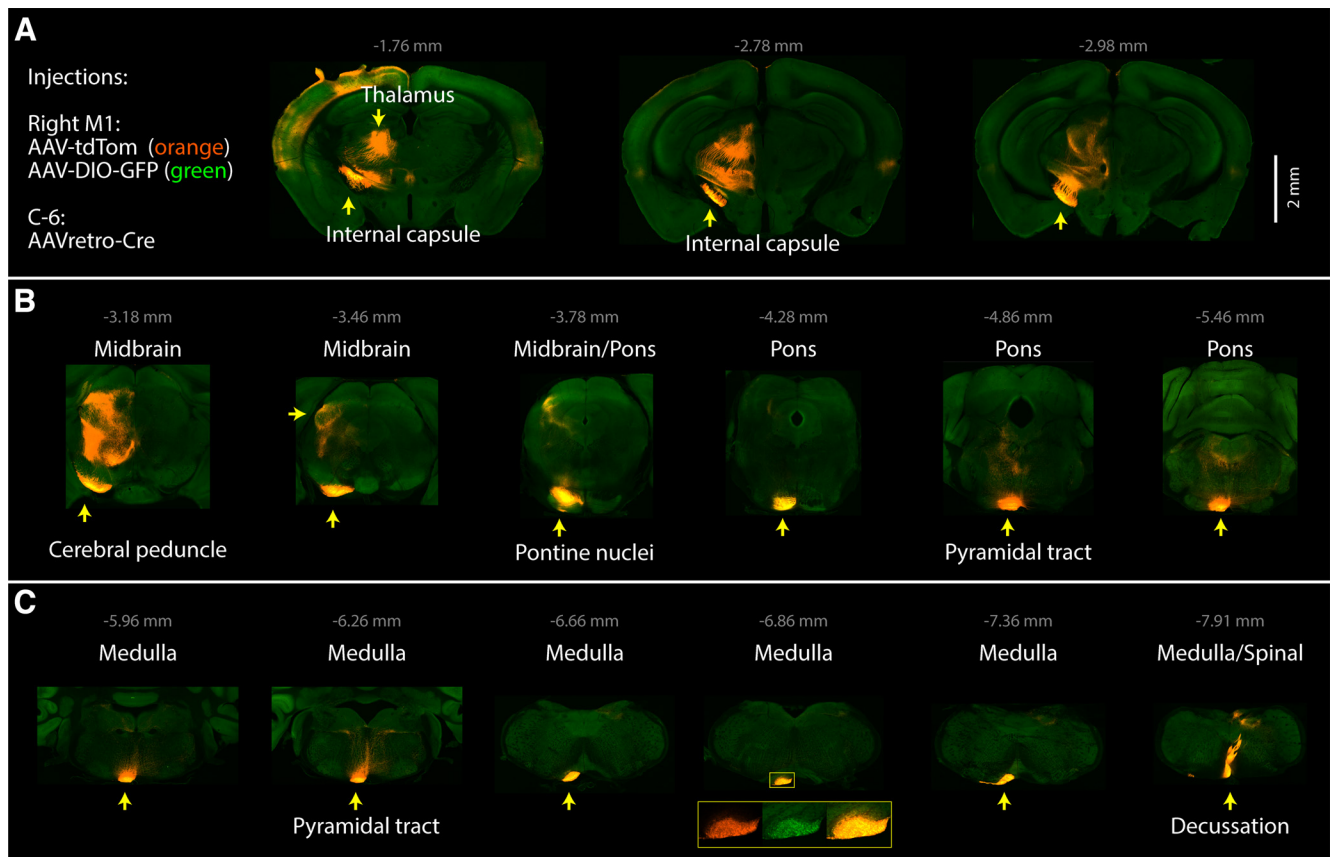


Figure 1. Anatomical overview of pyramidal tract projections from forelimb M1. Example series of coronal sections, rostral to caudal, from a mouse injected to label all cortical neurons, including PT neurons in general in one color (red, labeled by AAV-tdTomato) and CSPI neurons in particular in another color (green, intersectionally labeled by M1 injection of AAV-DIO-GFP plus C6 spinal injection of AAVretro-Cre). Upward-pointing arrows generally mark the trajectory of the PT axons as they descend via the internal capsule, cerebral peduncle, and pyramidal tract toward their decussation at the base of the brainstem. **A**, Labeling in sections at the level of the thalamus and internal capsule (left) through the rostral midbrain (right). Far left image, Cortical labeling in somatosensory areas is shown. **B**, Labeling in sections from the level of the midbrain and cerebral peduncles (left) through the pons (right). Horizontal arrow marks labeling in the dorsal midbrain. **C**, Labeling in sections from the rostral medulla (left) through the caudal medulla and decussation of the pyramids (right). Inset, Zoomed-in view of the pyramidal tract labeling, including the separate red and green as well as the merged view.

the total, as expected because mouse IT neurons are the most abundant top-level class of projection neurons and typically form callosal projections. Confirming the efficacy of the parsing, barcodes of cIT neurons were present only in samples containing cortex.

To identify the PT neurons among the remaining non-cIT neurons, we first identified any barcodes found in spinal cord samples as CSPI neurons. Any remaining barcodes found in brainstem sections, specifically in sections caudal to cortex, were then identified as CBULB neurons. (Note that we are using “cortico-bulbar” in the sense of non-CSPI PT neurons, without implying that CSPI neurons lack branches to brainstem regions.) These criteria were expected to capture all PT-type neurons projecting caudally at least as far as the pons, which should include the vast majority of the PT class of neurons (Brodal, 1981; Winnubst et al., 2019). Although some PT-type neurons terminating at levels rostral to the pons might be missed by these criteria, such neurons appeared to be relatively scarce, as further discussed below.

The remaining set of barcodes comprised a mix of non-cIT and non-PT neurons. These were assumed to include mainly CT neurons (projecting to thalamus only) and IT neurons projecting only ipsilaterally.

Having assigned the barcoded axons to these major classes, we then sorted the barcodes of each class according to length (i.e., based on the caudalmost extent of their projections),

resulting in an overall matrix of barcodes organized as a function of projection classes and rostrocaudal length (Fig. 2B).

For an overview of the resulting patterns as a function of projection class, we calculated barcode abundance for each group based on the sums and means in each sample (Fig. 2C). These rostrocaudal profiles showed qualitative features expected from the criteria used for parsing, including restriction of barcodes for cIT neurons to cortex-containing sections and distribution of the barcodes of PT neurons to and through the brainstem, for CBULB and CSPI neurons, respectively.

Further analyses focused on these CBULB and CSPI axons and their patterns of distribution across the brainstem-containing sections (Fig. 2B, bottom, blue boxed region; shown in greater detail in Fig. 2D). In addition to the overall sums and means (Fig. 2C), we calculated the means normalized to the total per axon (Fig. 2E). These profiles showed a prominent peak corresponding to the upper midbrain and a smaller peak at the level of the pontine nuclei. The CBULB axons appeared to vary considerably in their lengths, a histogram of which showed a peak just caudal to the pons (Fig. 2E).

The example shown confirms and illustrates the efficacy of MAPseq for population-scale single-axon analysis of PT projections from forelimb M1. Experiments with additional mice yielded broadly similar findings, as illustrated in a second example (Fig. 2F,G). Barcoded forelimb M1 axons ($N = 3$ animals; $n =$

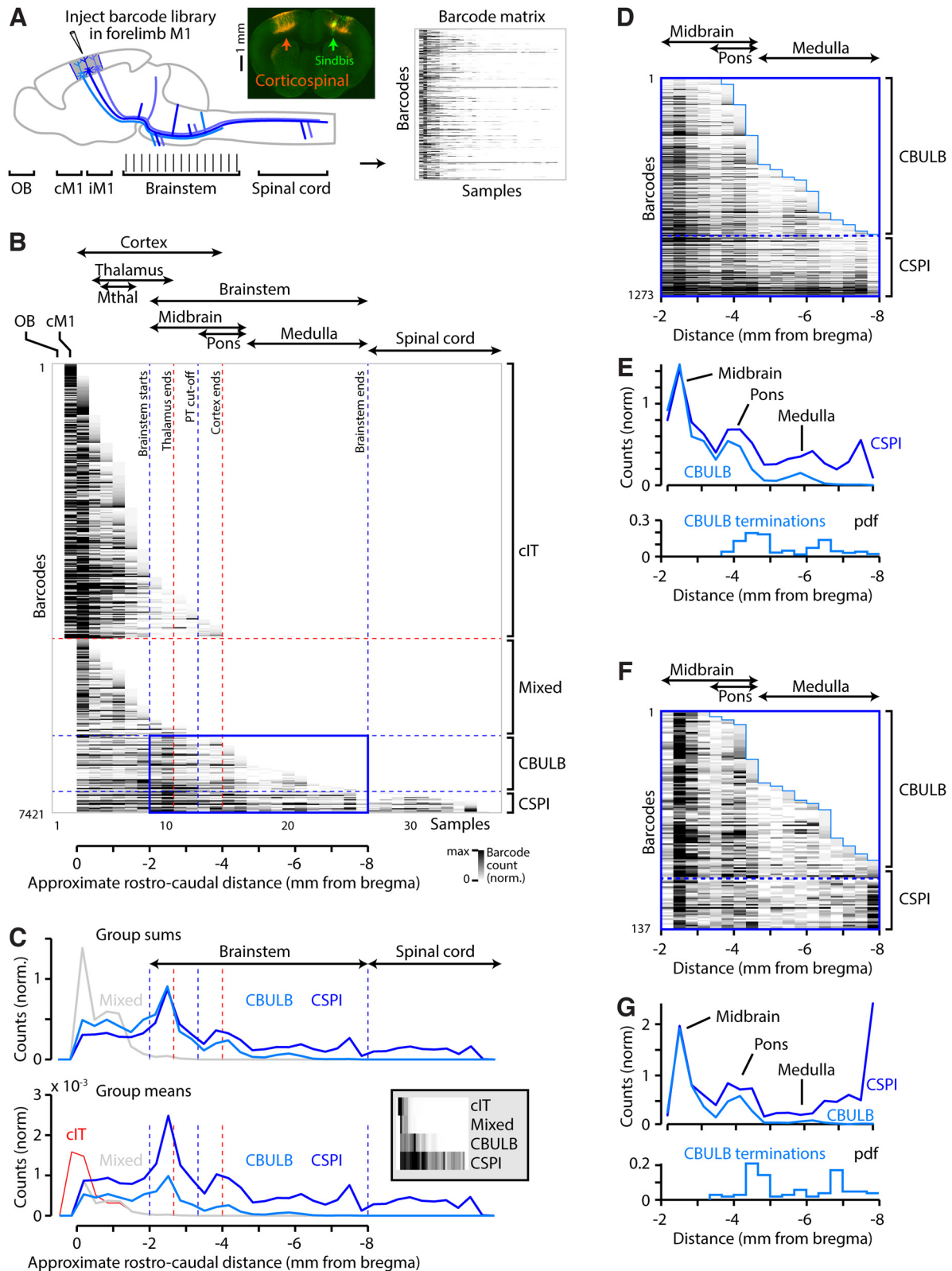


Figure 2. MAPseq identification of pyramidal tract projections from forelimb M1. **A**, Left, Schematic illustrating the injection and dissection strategy for collecting coronal sections and additional samples for MAPseq analysis. Middle, Example image shows Sindbis-GFP labeling of cortical neurons at the forelimb M1 injection site (green) and AAVretro-tdTomato labeling of corticospinal neurons (orange) by bilateral spinal cord injection. Right, Thumbnail image of the barcode matrix generated by sequencing, showing barcode counts across samples before parsing and sorting. **B**, Barcode matrix parsed into major projection classes and subclasses and sorted by length (i.e., by the caudalmost section of each barcode). Top, Arrows indicate the rostrocaudal extent of major brain regions and spinal cord. Horizontal lines demarcate the projection classes. Vertical lines indicate key anatomic landmarks used for parsing. Bottom, Blue boxed region indicates the PT-related region of interest (PT subset of barcodes, brainstem subset of sections). OB, Olfactory bulb; Mthal, motor thalamus. Barcode abundances are shown on a grayscale. **C**, Rostrocaudal profiles for the major classes, for the total (top, sums) and average (bottom) barcode counts per group. Inset, Same data as in the average plots, shown as a grayscale image. **D**, Expanded view of the PT region of interest (blue box), showing CBULB and CSPI axons (blue dashed line) sorted by their lengths (light blue staircase line). **E**, Top, Plot of CSPI and CBULB

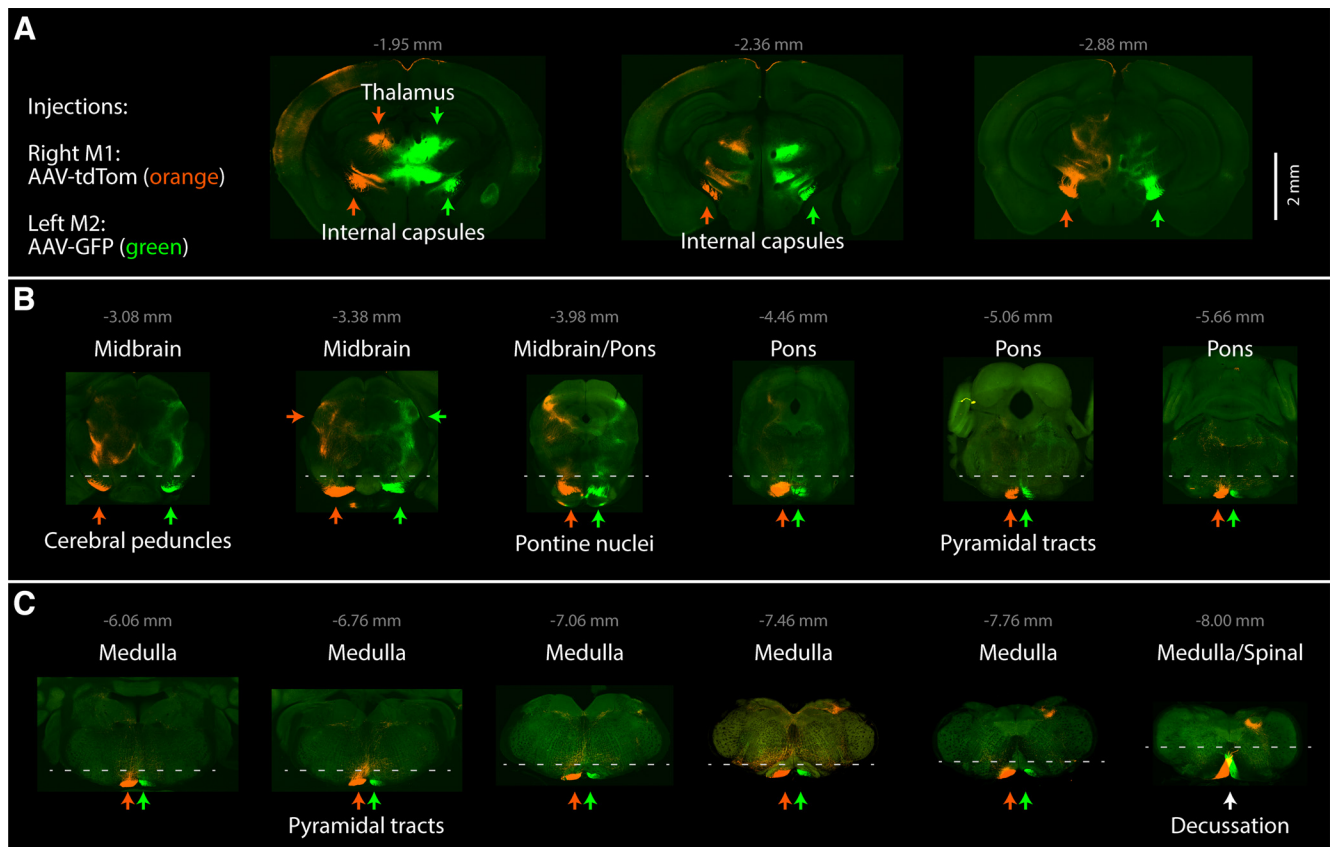


Figure 3. Anatomical overview of pyramidal tract projections from both forelimb M1 and M2/RFA. Example series of coronal sections, ordered from rostral to caudal, from a mouse injected to label PT projections from both the forelimb M1 (orange, labeled by AAV-tdTomato) and contralateral M2/RFA (green, labeled by AAV-GFP). Upward-pointing arrows generally mark the trajectory of the PT axons as they descend via the internal capsule, cerebral peduncle, and pyramidal tract toward their decussation at the base of the brainstem. **A**, Labeling in sections at the level of the thalamus and internal capsules (left and middle) through the rostral midbrain (right). **B**, Labeling in sections from the level of the midbrain and cerebral peduncles (left) through the pons (right). Horizontal dashed lines indicate the approximate level where cuts were made to dissect the coronal sections into dorsal and ventral samples. Horizontal arrows mark labeling in the dorsal midbrain. **C**, Labeling in sections from the rostral medulla (left) through the caudal medulla and decussation of the pyramids (right).

2808 axons/brain on average) were parsed into cIT, mixed, and PT classes. The PT axons were $17 \pm 4\%$ of the total (mean $\pm$ SEM). The PT axons included CSPI and CBULB subclasses (CSPI fraction of PT axons, $37 \pm 11\%$; CBULB fraction, $63 \pm 11\%$). Nearly all CBULB axons (99%) extended caudally at least to the level of the pontine nuclei.

Experiment 2: multiplexed MAPseq analysis of pyramidal tract projections from forelimb M1 and M2

A second MAPseq experiment was performed to further characterize the pyramidal tract projections from M1 and to characterize M2 projections as well. We again in parallel studies also imaged the broad features of the anatomic organization of these projections from M1 and M2 (Fig. 3A–C). The M1 and M2 projection patterns, although differing considerably in cortical and thalamic regions, were broadly similar across midbrain, pontine, and medullary regions. The main white-matter trajectories of the PT axons could readily be followed along the cerebral peduncles, pyramidal tracts, decussation, and spinal cord.

Aided by this anatomic overview of these projections, we proceeded with MAPseq experiments and analysis. The rostrocaudal profiles in the preceding MAPseq analyses indicated peaks corresponding to the midbrain, pons, and medulla, for both PT subclasses. We sought to resolve what part of the signal in each brainstem section comes from the primary axons traveling to and through the pyramidal tract itself, and what part comes from branches in the neuropil. Therefore, in this second set of MAPseq experiments (Fig. 4A; $N = 3$ mice), we dissected the brainstem coronal sections by making a horizontal cut to separate the ventral part containing the main white-matter tracts (cerebral peduncles or pyramidal tracts) along with ventral axon branches from the dorsal part containing dorsal branches. Taking advantage of the multiplexing capability of MAPseq (Kebschull et al., 2016), in the same animals we injected both forelimb M1 (as before) and the anterior M2 (RFA) in the contralateral hemisphere. We pooled the dorsal versus ventral subsections dissected from the series of coronal sections across all the major regions of interest, spanning (rostrocaudally) the caudal midbrain, pons, rostral medulla, caudal medulla, and decussation (spinomedullary junction); as before, we also included spinal cord samples for distinguishing CSPI versus CBULB projections.

To analyze the barcode matrix, we first parsed it into M1 and M2 submatrices (Fig. 4A). We then analyzed these using a similar approach as above, modified to assess barcode counts in the

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barcode counts across brainstem sections, normalized to the sum of barcode counts. Bottom, Histogram of the terminations of CBULB barcodes, defined as the distalmost section containing barcode (plus one), plotted as a probability density function (pdf). **F**, **G**, Same as **D** and **E** for a second example experiment.

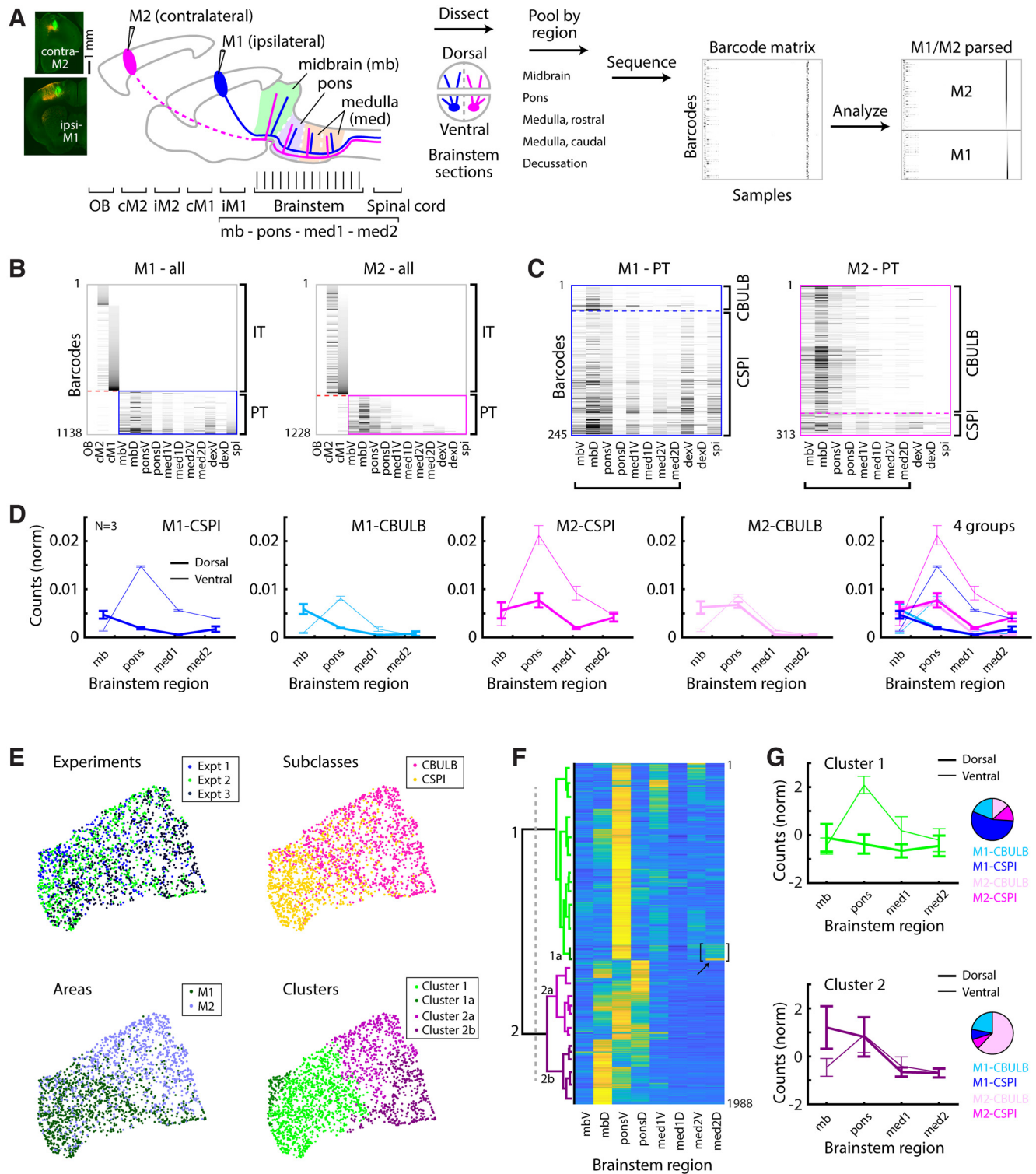


Figure 4. Multiplexed MAPseq analysis of pyramidal tract projections from forelimb M1 and M2. **A**, Left, Schematic of multiplexed MAPseq strategy for analyzing M1 and M2 pyramidal tract projections. Brainstem sections were cut in two to sample the bottom (ventral) part, containing the main white-matter projections (i.e., pyramidal tract) and top (dorsal) part containing brainstem branches of PT axons. Far left, Example images of M2 (top) and forelimb M1 (bottom) show Sindbis-GFP labeling of cortical neurons at the injection site (green) and AAVretro-tdTomato labeling of corticospinal neurons (red) by bilateral spinal cord injection. Right, Thumbnail image of the barcode matrix generated by sequencing, showing barcode counts across samples. An initial parsing was done to identify barcodes originating from M1 versus M2, yielding two submatrices that were then further analyzed. **B**, Parsing and analysis of the M1 (left) and M2 (right) submatrices (see above, Materials and Methods) into IT (above the red dotted line) versus PT projections (bottom, blue boxed region indicates the brainstem-spanning region of interest). **C**, The resulting collection of PT barcodes was further subdivided into CBULB and CSPI submatrices. OB, Olfactory bulb; mbV, ventral midbrain; D, dorsal midbrain; med1 and 2, rostral and caudal medulla; dex, decussation; spi, spinal cord. Subsequent analyses focused on the brainstem regions of interest (horizontal bar at bottom). **D**, Group averages ($N = 3$ mice; mean $\pm$ SEM) for the brainstem projection profiles of M1-CSPI (blue), M1-CBULB (light blue), M2-CSPI (magenta), and M2-CBULB (light magenta) axons, plotted for dorsal (thick lines) and ventral (thin lines) subsections. **E**, UMAP plots, color coded by experiment (animal), axon subclass type (CBULB, CSPI), cortical region of origin (M1, M2), and cluster. **F**, Hierarchical agglomerative clustering of barcode values. Left, Dendrogram showing hierarchical relationship between data points based on Ward's linkage of Euclidean distances. Dashed line indicates height at which to cut the dendrogram

Table 1. Multiplexed M1 and M2 experiments: summary data

Expt	Overall	M1					M2				
		Total	clT	PT	CBULB	CSPI	Total	clT	PT	CBULB	CSPI
B27	2048	1138	598	245	42	203	1228	892	313	267	46
B29	4308	3131	2286	527	216	311	1748	1257	238	169	69
B31	3240	1396	496	336	146	190	2428	2042	366	263	103
Mean	3199	1888	1127	369	135	235	1801	1397	306	233	73
SD	1131	1084	1005	144	88	66	602	588	64	55	29
SEM	653	626	580	83	51	38	347	339	37	32	17

The numbers of barcodes (i.e., neurons) of the various classes and subclasses in the projections arising from the two cortical areas are shown for each experiment. Expt refers to the experiment identifier.

dorsal and ventral subsections of each region. As shown in the example (Fig. 4B,C), we again parsed IT-type from PT-type projections based on the presence of a barcode in the contralateral cortex for the former and in the brainstem for the latter (Fig. 4B), and we parsed CSPI versus CBULB based on the presence or absence of a barcode in the spinal cord sample (Fig. 4C). Consistent with the greater abundance of CSPI neurons in M1 than M2 (Nudo and Masterton, 1990), in the pyramidal tract projections from M1 there were more CSPI than CBULB axons, whereas in the M2 projections there were more CBULB than CSPI axons [CSPI proportion, defined as CSPI/(CSPI + CBULB), for M1, 0.66 ± 0.08 , mean $\pm$ SEM; for M2, 0.24 ± 0.05 ; $N = 3$ mice; $p = 0.01$, t test]. Additional data and summary statistics pertaining to the basic parsing are also provided (Table 1).

Subsequent analyses focused on the brainstem-spanning portion of the matrices (i.e., from caudal midbrain to caudal medulla). We separated the M1 and M2 data into their dorsal and ventral subsections, calculated the region-total averages for the CSPI and CBULB axons for M1 and M2, and averaged across animals ($N = 3$ mice) to obtain a collection of rostrocaudal brainstem profiles for the four subclasses (M1-CSPI, M1-CBULB, M2-CSPI, M2-CBULB; Fig. 4D). Several qualitative features stood out, including relatively high barcode values in the ventral pons and dorsal midbrain, consistent with imaging findings showing abundant axon branching in these regions (Fig. 3). Statistical comparisons (based on rank sum tests, corrected for multiple comparisons; Table 2) showed that in the caudal midbrain, no consistent differences were observed across experiments, but in other regions a variety of differences were found (p values of 0.001 or less for all significant differences), including the following: (1) In the pons, both CSPI and CBULB axons from M2 were consistently more abundant than their M1 counterparts (3/3 experiments); (2) in the rostral medulla, consistent differences (3/3) included more M2-CSPI compared with either M2-CBULB or M1-CSPI; and (3) in the caudal medulla, CSPI axons were consistently (3/3) greater than CBULB for both areas.

We also analyzed the same datasets using dimensionality-reduction and clustering methods as a way to assess PT axon subtypes independent of their known subclass (CBULB, CSPI) and area-of-origin (M1, M2) identities. In UMAP plots, all PT

Table 2. Multiplexed M1 and M2 experiments: statistical testing

Region	Comparison	B27	B29	B31
Midbrain	M1, CBULB vs CSPI	0.34437	3.77E-09	0.000541
	M2, CBULB vs CSPI	0.12336	0.009552	0.82642
	CBULB, M1 vs M2	0.05091	0.031223	0.030679
	CSPI, M1 vs M2	0.26791	0.77821	4.12E-06
Pons	M1, CBULB vs CSPI	0.51153	0.17575	0.1473
	M2, CBULB vs CSPI	0.84668	0.9495	0.002687
	CBULB, M1 vs M2	6.74E-10	1.03E-24	2.48E-25
	CSPI, M1 vs M2	2.11E-10	6.85E-15	4.34E-22
Medulla	M1, CBULB vs CSPI	0.26895	0.067312	0.03518
	M2, CBULB vs CSPI	1.11E-09	7.25E-11	8.45E-20
	CBULB, M1 vs M2	0.44225	0.80854	0.002286
	CSPI, M1 vs M2	2.86E-08	2.75E-08	5.62E-20
Medulla	M1, CBULB vs CSPI	0.001194	2.61E-30	2.09E-06
	M2, CBULB vs CSPI	5.50E-25	1.87E-19	9.60E-36
	CBULB, M1 vs M2	0.000781	0.18668	0.7501
	CSPI, M1 vs M2	6.51E-09	0.058168	2.95E-18

Table entries show the p values for within-animal comparisons as indicated for each experiment (identified as B27, B29, and B31; $N = 3$ total), based on rank sum tests. Bold text indicates significant differences following multiple-comparison correction within each experiment ($p < 0.05/16$). Bold italic indicates multiple comparisons across all three experiments ($p < 0.05/48$). Asterisks (*) mark where significant differences at the most stringent level were consistently found across animals (3/3).

axons occupied a single territory (Fig. 4E) containing the axons from all the experiments, with overlapping distributions of subclass and area of origin. Cluster analysis identified two top-level clusters (Fig. 4F), which were further analyzed by plotting the brainstem profiles of these clusters (Fig. 4G) and determining their subclass and area composition (Table 3). Cluster 1 axons, constituting more than half of all PT axons, projected relatively weakly to dorsal subsections and strongly to the ventral subsections of the pons and rostral medulla. Most Cluster 1 axons were CSPI (69%), most came from M1 (74%), and indeed roughly half were M1-CSPI axons (55%). Axons in Cluster 2 had relatively low abundance in medullary samples, but more prominent branching throughout the pons and especially the dorsal midbrain. Most Cluster 2 axons were CBULB (84%), most were of M2 origin (70%), and indeed most were M2-CBULB axons (62%). Cluster 2 axons tended to project in an inverse manner to the dorsal pons versus midbrain ($R^2 = 0.4$), with Cluster 2a axons having relatively stronger dorsal pons projections and Cluster 2b axons having stronger dorsal midbrain projections. However, both of these clusters displayed considerable heterogeneity, particularly across dorsal midbrain and dorsal and ventral pons. In summary, Cluster 1 axons thus tended to follow a pontomedullary profile with a ventral bias, with M1 and CSPI enrichment; Cluster 2 axons tended to follow a pontomesencephalic profile, with M2 and CBULB enrichment.

The primary focus in these analyses was on identifying and characterizing these top-level clusters. However, cluster analysis also identified multiple subclusters within each of these, and although additional studies will be needed to characterize these systematically, we chose one as an example for exploring how PT branching observed by barcode analysis may relate to PT branching observed by conventional axon imaging. Within the Cluster 1 group, we noted that one cluster, Cluster 1a, had particularly strong projections to the dorsal caudal medulla subsections (Fig. 4F). The average brainstem projection profile of Cluster 1a axons differed greatly from that of the other axons in Cluster 1 in the strength of these dorsal caudal medulla branches ($p = 10^{-53}$, rank sum test, $n = 89$ Cluster 1a axons, $n = 1060$ non-1a Cluster 1 axons; Fig. 5A–C). The subclass composition was primarily M1-CSPI with smaller contributions from each of the three other

to maximize the ratio of between- to within-cluster variances. Right, Data from C, row normalized to mean zero and 1 SD, then ordered by cluster assignment in the dendrogram. Brackets with arrow mark the particularly strong projections to the dorsal caudal medulla subsections characteristic of Cluster 1a. G, Brainstem profiles of the same data, showing averages for each cluster (median $\pm$ median absolute deviation), for dorsal (thick lines) and ventral (thin lines) subsections. Right, Pie charts indicate the fractions within each cluster of different axon subclasses (CBULB, CSPI) and cortical areas of origin (M1, M2).

Table 3. Multiplexed M1 and M2 experiments: composition of main clusters

	Subclass		Area		Area and subclass				
	All PT	CSPI	CBULB	M1	M2	M1-CSPI	M1-CBULB	M2-CSPI	M2-CBULB
Cluster 1	58%	69%	31%	74%	26%	55%	19%	13%	13%
Cluster 2	42%	16%	84%	30%	70%	8%	22%	8%	62%

Table entries show the percentages for axons of each type for the two main clusters.

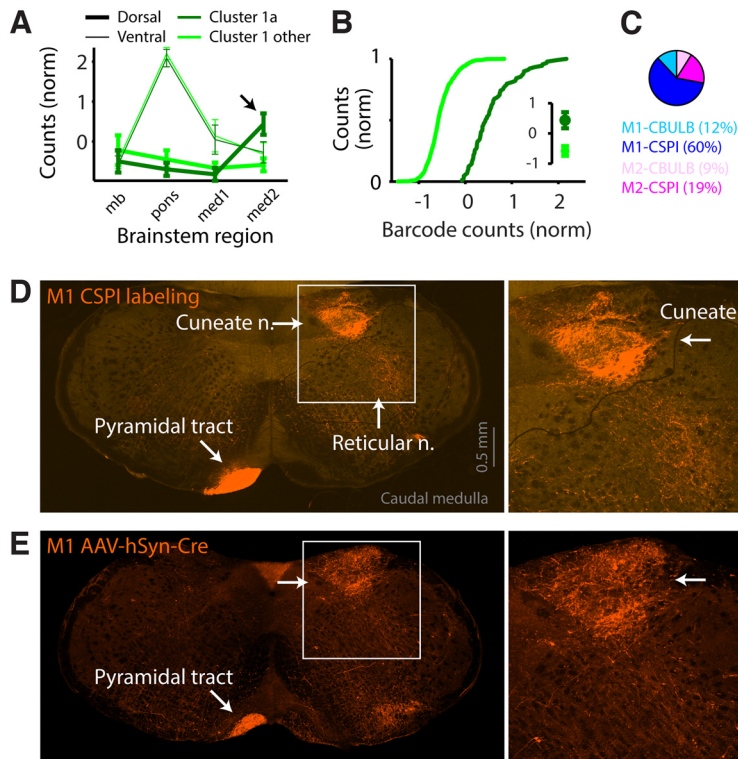


Figure 5. PT branches to the cuneate nucleus. **A**, Brainstem profiles of the data for Cluster 1 axons, separated into Cluster 1a and non-1a axons (Fig. 4E,F), showing averages for each cluster (median $\pm$ median absolute deviation), for dorsal (thick lines) and ventral (thin lines) subsections. **B**, Plot of the cumulative distributions of barcode counts in the dorsal caudal medulla samples for Cluster 1a (dark green) and other (light green) Cluster 1 axons. **C**, Pie chart of the fractions in Cluster 1a of different axon subclasses (CBULB, CSPI) and cortical areas of origin (M1, M2). **D**, Confocal images of the caudal medulla showing labeling pattern in a WT mouse with intersectionally labeled forelimb M1 CSPI axons, with prominent labeling present in the cuneate nucleus (boxed area, shown at higher magnification on the right). **E**, Same, for an Ai14 mouse that was injected in forelimb M1 with AAV-hSyn-Cre, also showing prominent labeling in the cuneate nucleus.

subclasses (60% M1-CSPI, 12% M1-CBULB, 19% M2-CSPI, 9% M2-CBULB; Fig. 5C). To explore potential anatomic correlates, we injected mice to intersectionally label CSPI neurons (Cre-dependent AAV in M1, AAVretro-Cre in cervical spinal cord). Inspection of coronal sections containing the dorsal caudal medulla showed particularly strong labeling in and around the cuneate nucleus (Fig. 5D), with additional branching observed in the reticular nucleus and other regions. Cuneate/pericuneate labeling was observed in multiple animals injected in M1 and/or M2, including intersectionally labeled CSPI neurons (cervically injected AAVretro-Cre plus cortically injected AAV-Flex-GFP/RFP; $N = 4$ injected in M1, $N = 2$ injected in M2). Similar labeling was observed following AAV-hSyn-Cre injections in Ai14 mice (mCherry reporter line; $N = 2$ mice injected in M1), a method for anterograde transsynaptic/transneuronal labeling of postsynaptic neurons (Zingg et al., 2017; Yamawaki et al., 2021). These imaging results showing strong corticospinal labeling in the dorsal caudal

medulla in and around the cuneate nucleus are thus consistent with the MAPseq results showing prominent projections to this region associated with one subcluster in particular, as well as with prior evidence for CSPI branching to the cuneate (Canedo, 1997).

Discussion

The results confirm and extend previous characterizations, particularly a prior MAPseq analysis (Muñoz-Castañeda et al., 2021) as well as single-axon reconstructions (Kita and Kita, 2012; Economo et al., 2018; Winnubst et al., 2019; Muñoz-Castañeda et al., 2021), of the projections from mouse motor cortex to and through the pyramidal tract. Our confirmatory observations include the efficacy of MAPseq for identifying major motor cortex projection neuron classes (IT, PT) and subclasses (CBULB, CSPI), and the general pattern of M1 projections to and through the brainstem en route to spinal cord, including the tendency for axons of PT neurons to extend caudally at least to the level of the pons. Our results also illuminate basic features of brainstem projections from M2, which appear broadly similar to those from M1 for both the CSPI and CBULB subclasses of PT neurons. Common to both areas and both subclasses were an abundance of corticotectal branches.

Among several technical considerations, an important one that limits our study relates to the spatial resolution of sampling. Although MAPseq provides single-cell resolution for identifying the axonal projections of individual neurons, its resolution for analyzing the spatial distributions of axonal branches is set by the anatomic sampling strategy. Here, we chose to focus on the rostrocaudal dimension and therefore cut coronal sections and either analyzed each (experiment 1) or cut them into dorsal and ventral portions for pooling by brainstem region (experiment 2). Additional experiments will be needed for finer spatial resolution, such as by microdissecting brainstem nuclei. Another limitation with our sampling was that midbrain sampling was limited to the caudalmost regions of the midbrain because of a focus on the pyramidal tract spanning regions (pons and medulla) and the technical difficulty of excluding thalamus and other regions from rostralmost midbrain samples.

In addition to the projections from M1, we also characterized those from M2. Our approach, using multiplexed MAPseq, illuminates several features of the brainstem projections arising from these two territories of motor cortex. The main finding was the broad similarity of M1 and M2 projections across the midbrain, pons, and medulla for both CBULB and CSPI subclasses of PT axons. The similarities of brainstem branching patterns contrasts with the many dissimilarities of M1 and M2 projections at the cortical, striatal, and thalamic levels, as characterized in

prior studies (Harris et al., 2019). Nevertheless, it is important to emphasize again that the approach used here focused on relatively coarse sampling rather than the fine details of branching at the level of specific brainstem nuclei.

Our findings confirm the prominence of corticotectal and other midbrain branches of the axons of PT neurons, which have been previously observed in anatomic (Alloway et al., 2010) and connectomic studies (Winnubst et al., 2019; Muñoz-Castañeda et al., 2021). These were among the most abundant branches of M1 and M2 brainstem projections of PT neurons. Of note, the MAPseq data additionally showed this pattern to hold for both the CBULB and CSPI subclasses of PT axons. Indeed, this ability to identify CBULB versus CSPI axons is among the distinct advantages of MAPseq. In contrast, the intersectional viral methods we used for anatomic labeling were able to selectively label CSPI but not CBULB axons. The specific cellular targets within the dorsal midbrain of these corticotectal branches remain to be determined but could involve tectospinal pathways, for example. The presence of such abundant branches off the primary axons of PT neurons exemplifies how the functions of these axons presumably arise from their entire output (i.e., all branches), not just the branches used for labeling or of special interest (e.g., CSPI).

Hierarchical clustering, used in addition to characterizing PT projection patterns in terms of the four projectionally and areally defined PT subclasses (M1-CSPI, etc.), showed that clusters did not correspond to subclasses in a simple one-to-one manner. Instead, clusters were of mixed subclass composition and vice versa. These findings indicate that differences among motor cortex PT axons, that is, between M1 and M2 and between CSPI and CBULB, are generally quantitative (i.e., composition differing in proportions of subclasses/clusters) rather than qualitative (unique/specific subclasses/clusters). Similarly, all PT axons were distributed within a single zone in UMAP space. The brainstem branching profiles of the two top-level clusters showed broad biases to different brainstem regions, with Cluster 1 relatively more ventrally and caudally biased and Cluster 2 more dorsally and rostrally biased. A crucial point is that cluster identification depends on which projection territories are included/excluded for analysis. For example, different results would be expected if clustering is based on other or additional territories such as striatum and thalamus, which also receive PT branches. Therefore, clusters identified here, based on sampling brainstem regions, do not necessarily correspond to clusters identified using other criteria.

These findings add to a growing knowledge about the diversity of PT projections. Our results confirm that PT axons generally extend at least to the pons, with a subset continuing caudally in the pyramidal tract and a further subset extending beyond the decussation as CSPI axons (Brodal, 1981). Our findings are consistent with single-axon tracings and full reconstructions showing that at least in rodents PT axons extend multiple branches off the main trunk in patterns that can vary considerably from axon to axon (Kita and Kita, 2012; Winnubst et al., 2019; Muñoz-Castañeda et al., 2021).

Previous studies have identified distinct subtypes of PT axons. In particular, motor cortex PT axon subtypes that preferentially branch to thalamus versus medulla have been characterized (Economio et al., 2018). Similar results were obtained in a large-scale multiomics analysis of M1 neurons (BRAIN Initiative Cell Census Network (BICCN), 2021; Muñoz-Castañeda et al., 2021). Our findings are broadly consistent with these studies. For example, it was previously observed that PT axons constitute a single

but heterogeneous top-level group based on clustering and dimensionality reduction methods (Muñoz-Castañeda et al., 2021); similarly, we found that PT axons occupied a single UMAP zone. Among the BICCN studies, our barcoding (MAPseq) results are most directly comparable to the barcoding (BARseq) results of Muñoz-Castañeda et al. (2021), which identified two top-level PT clusters having either strong or weak spinal projections, similar to our Clusters 1 and 2, respectively. Additional studies (BRAIN Initiative Cell Census Network (BICCN), 2021), extending prior findings (Economio et al., 2018), further defined two top-level PT classes as either medulla- versus non-medulla-projecting (and identified marker genes *Slco2a1* and *Npnt*, respectively), again similar to our Clusters 1 and 2, respectively. However, at a finer-grained level of further PT subtypes, comparison of our results to BICCN subtypes is more challenging and speculative. For example, our Cluster 2a and Cluster 2b axons tended to follow corticopontine and cortico-midbrain patterns, respectively, and thus potentially correspond to the corticopontine and corticotectal motifs described on the basis of full single-axon reconstructions (Muñoz-Castañeda et al., 2021). However, these clusters displayed considerable heterogeneity, and most of the axons were of M2 origin; moreover, our sampling strategy lacked thalamic and striatal samples, which would be expected to influence clustering results.

Spinal cord injury and multiple sclerosis are among the many neurologic conditions commonly affecting the axonal projections from the motor cortex to and through the pyramidal tract, and there is much interest in the potential for branch plasticity of CBULB and CSPI axons to mediate functional recovery (Ghosh et al., 2010). Our results suggest the potential utility of MAPseq and related new barcoding methods for quantitatively investigating axon branch plasticity at the single-axon level in disease models.

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