Supplementary Figures

Supplementary Fig. S1. Comparison of *in situ* sequencing techniques. In both methods, a barcode mRNA is first reverse transcribed into a cDNA. In BaristaSeq (*left*), the barcode portion is then copied into a padlock probe intermediate, which is circularized and used as a template for rolling circle amplification. In FISSEQ (*right*), the cDNA is directly circularized and used as a template for rolling circle amplification.

Supplementary Fig. S2. Optimization of BaristaSeq for brain slices. (A) Amplification of barcodes in brain slices in the indicated reaction chambers. Scale bars = 100 μm. (B) Merged images of rolonies (yellow) generated in barcoded brain slices and the residual GFP signals (cyan) with the indicated time of pepsin treatment. Scale bars = 100 μm. (C) Comparison of barcode amplicons generated using BaristaSeq (a), the original padlock method (b), and FISSEQ (c). Scale bars = 50 μm. (D) Sequencing images of cycles 2, 4, and 6 of barcoded brain slices sequenced using SOLiD sequencing chemistry (*top*) and using Illumina sequencing chemistry (*bottom*). Imaging conditions were kept constant throughout each sequencing run. Scale bar = 100 μm. (E) Average S/N of Illumina (red) and SOLiD (blue) sequencing *in situ* over cycles. Error bars indicate the standard error for the S/N for pixels. (F-H) Sequencing quality and signal intensity of individual base calls (F), mean signal intensity over cycles (G), and the fraction of the bases over cycles (H) are plotted.

Supplementary Fig. S3. Validation of BARseq using retrograde tracing. (A) Representative image of a brain slice double labeled with barcodes (cyan) and CTB (magenta) from the contralateral auditory cortex. Scale bar = 100 μm. (B) Histograms of the number of barcode molecules for each neuron recovered in the olfactory bulb (OB, blue) and in the contralateral auditory cortex (AudC). Both axes were drawn on log scales. The vertical dashed line indicates the noise threshold used in the experiment (5 molecules).

Supplementary Fig. S4. Histogram of the minimal hamming distance between barcodes recovered from MAPseq of brain XC9 and other barcodes of the same brain.

Supplementary Fig. S5. Filtering projection data using non-negative matrix factorization. (A) Positive (green) or negative (red) Pearson correlation coefficients among projections to the indicated areas in all neurons. (B) The average projection pattern of all neurons sampled (center) is decomposed into six projection modules (Basis 1-6). (C) The projection patterns of two example neurons (left) are filtered using projection modules (right). The weight for each module is labeled to the left of each module. (D) Comparison of the original projection strengths (blue) and the filtered projection strengths (red) for the two neurons shown in (C). (E) The fraction of variance explained (y-axis) using NMF (blue), individual projections (red), and PCA (black). (F) The fraction of neurons that remains in the same class-level clusters (y-axis) when filtering the projection data with the indicated number of projection modules (x-axis) compared to the clusters without filtering.

Supplementary Fig. S6. Hierarchical clustering of projection neurons in the mouse auditory cortex. (A) The workflow of the hierarchical clustering. (B) The distribution of the maximum cluster probability for individual neurons when classified using all 11 projection areas (a) or 10 projection areas (b-l). For classification using 10 projection areas, the unused projection area is labeled on top of each graph. (C) The fraction of well-classified neurons in each subclass. The subclass labels correspond to those in Fig. 3G and the class the subclasses belong to are labeled

below. The dotted line indicate 80% well-classified neurons. (D) Comparison of clusters obtained using k-means (upper row), spectral clustering (middle row), and Louvain community detection (lower row) at the indicated hierarchies. All clusters were color coded onto a t-SNE plot generated using all data. The colors are randomly assigned to individual clusters. (E) Single-cell projection patterns sorted by cluster identities. Each row represent a barcode and each column represent projection strengths to the indicated brain area.

Supplementary Fig. S7. Validating BARseq projection patterns using retrograde tracing. (A) Triple retrograde tracing of neurons projecting to the rostral striatum (CTB-647), the caudal striatum (CTB-488), and the tectum (RetroBeads). (B) Representative image of the triple retrograde labeling in the auditory cortex showing neurons projecting to the rostral striatum (magenta), the caudal striatum (cyan), and the tectum (yellow). Scale bar = 100 μm. (C) Venn diagram showing the number of neurons projecting to each of the three areas.

Supplementary Fig. S8. Laminar distribution of projection neurons. (A)(B) Representative images of FISH against Cux2 (A) and Fezf2 (B) in two adjacent slices. Scale bars = 50 μm. (C) Violin plots of the laminar distribution of all BARseq neurons (All) and those with (Proj) or without (Non-proj) detected projections.

Supplementary Fig. S9. Laminar distribution of projection neuron subclasses. (A) Differences in normalized entropy (x-axis) of individual subclasses between the two brains are plotted against the negative logarithm of the p values (y-axis). The subclasses were color-coded according to their class-level divisions as indicated. The p values were shown without multiple testing correction. The red vertical dashed line indicate no difference in entropy, and the black horizontal dashed line indicate significance level after Bonferroni correction. (B) Differences in mean laminar locations (x-axis) of individual subclasses between the two brains are plotted against the negative logarithm of the p values (y-axis). The subclasses were color-coded according to their class-level divisions as indicated. The p values were shown without multiple testing correction. The red vertical dashed line indicate no difference in the mean laminar locations, and the black horizontal dashed line indicate significance level after Bonferroni correction. (C)(D)(E) Histograms of the strengths of tectal projections (C), thalamic projections (D), and striatal projections (E) of the corticotectal neurons in L5 (blue) and L6 (red). $p < 0.0005$ after Bonferroni correction for the two distributions in (C), and $p > 0.05$ for the two distributions in (D) and (E). p values were obtained using bootstrap ks test. (F)(G) Pearson correlation coefficients among the projection targets of the ITc (F) and ITi (G) neurons. Projections to the thalamus and the tectum were not shown because they were the main targets of the PT and CT neurons, not the IT neurons. Only statistically significant correlations were shown. The fact that projections were correlated within ITi and ITc suggest that projections were structured within each class.

Supplementary Fig. S10. BARseq can link projection patterns with gene expression and *in vivo* functional two photon imaging. (A) *In situ* sequencing of barcodes and endogenous genes. *Left*: Barcodes (magenta) and endogenous mRNAs (yellow) are both amplified for *in situ* sequencing to correlate neuronal projections with gene expression. *Right*: Barcodes (*top*) and mRNAs (*bottom*) are sequenced sequentially to avoid interference during base-calling. Scale bars = 100 μm. (B) *in vivo* two photon images of GFP-positive neurons imaged in live animals are registered to *ex vivo* images of the same neurons in brain slices. Such registration potentially allows correlation of *in vivo* functional imaging with BARseq. Scale bar = 50 μm.

Supplementary Table 1. Comparison between MAPseq and retrograde tracing experiment. Each row represent a single neuron recovered from BaristaSeq with visible GFP signal from the barcodes and good sequencing quality (quality score > 0.75). The first four columns indicate the raw barcode counts in the olfactory bulb (OB), contralateral auditory cortex (c1), the cortical area surrounding the contralateral auditory cortex with CTB signals visible to the naked eyes (c2), and an even larger cortical area surrounding the tracer area with CTB signals visible under the microscope (c3). The rest of the columns indicate whether the cell projects contralaterally based on CTB and/or BARseq

Supplementary Table 2. The number of barcodes sequenced per brain from the projection sites, the number of cells sequenced per brain from the auditory cortex, and the number of BaristaSeq *in situ* barcodes matching barcodes at the projection sites with or without quality filtering were indicated for each brain. *In XC14, the auditory cortex was sequenced *in vitro*.

Supplementary Table 3. Normalized layer boundary positions determined in three pairs of slices across the auditory cortex. In each slice, the thickness of the cortex was normalized to that in the BARseq brains, and the boundary positions were scaled accordingly.

Supplementary Note 1. Optimization of BaristaSeq for brain slices

We tested three commercially available reaction chambers that were physically compatible with our samples (Supp. Fig. S2A), and found that the HybriWell-FL sealing system was the only system that did not inhibit rolony formation (Supp. Fig. S2Ab). The ImmEdge hydrophobic barrier pen also produced good amplification (Supp. Fig. S2Ac), but the HybriWell-FL system offered better control of liquid evaporation during heating steps and easier handling. Therefore, we used the HybriWell-FL system for BaristaSeq on brain slices.

RNAs in fixed tissues are less accessible to *in situ* enzymatic reactions than those in cell culture are. This reduced accessibility is likely due to excessive protein crosslinking caused by the increased formaldehyde fixation time needed to fix brain tissues adequately. We therefore added a pepsin digestion step before reverse transcription to increase accessibility of fixed RNAs (Supp. Fig. S2B). The pepsin step also reduced the GFP signal from the cells (cyan in Supp. Fig. S2B), which may interfere with sequencing signals. We found that 3 mins of 0.2% pepsin digestion at room temperature greatly increased rolony formation (Supp. Fig. S2Bb) compared to no pepsin treatment (Supp. Fig. S2Ba), whereas 5 mins of pepsin digestion caused excessive tissue loss (Supp. Fig. S2Bc). We therefore used 3 mins of pepsin digestion for BaristaSeq in brain slices. These optimizations for BaristaSeq allowed us to amplify barcodes efficiently in barcoded neurons in brain slices (Fig. 2A). The optimized BaristaSeq protocol (Supp. Fig. S2Ca) greatly outperformed the original padlock probe-based technique[7] (Supp. Fig. S2Cb) and FISSEQ[6] (Supp. Fig. S2Cc) in amplifying barcodes in brain slices.

To sequence the barcodes using Illumina sequencing chemistry, we based our sequencing protocol on the HiSeq recipe files and adjusted the incubation times to account for the heat transfer of the reaction chambers. We also increased the number of washes after the incorporation step compared to the original BaristaSeq protocol to counteract the increased background staining in tissue slices. This increased number of washes was essential for reducing the background signals, especially for long sequencing runs. We compared this optimized Illumina sequencing *in situ* to sequencing by ligation (SOLiD) used by other sequencing methods[6,7]. We sequenced the first six bases of barcodes in brain slices (Supp. Fig. S2D). The signal-to-noise ratios averaged over all six cycles were 39 ± 4 for Illumina sequencing (Supp. Fig. S2E), ~10-fold higher than that of SOLiD sequencing (4 ± 1), probably due to the improved washing conditions and higher background in tissues using SOLiD.

Supplementary Note 2. BARseq for the auditory cortex projections

In each brain, we collected 11 target areas, including four ipsilateral cortical areas (motor, orbitofrontal, visual, and somatosensory), two contralateral cortical areas (visual and auditory), three subcortical areas (rostral and caudal striatum and the amygdala), the thalamus, and the tectum. Care was taken to avoid major axonal tracts through the thalamus and to leave buffering areas between adjacent areas. Example images of the actual collected areas are available at Dryad (see *Data and software availability* in *Methods*). These areas covered all major brain areas to which the auditory cortex projects, as determined by conventional bulk GFP tracing experiments[16]. We also collected the olfactory bulb as a negative control to which the auditory cortex does not project.

The 30-nt barcodes were sequenced fully at each projection site using conventional Next-Gen Sequencing, and 15 bases were sequenced *in situ* using BaristaSeq at the injection sites. The 15 bases read length *in situ* was sufficient to distinguish unambiguously all infected barcodes allowing one mismatch. For the XC9 brain, barcodes recovered through MAPseq had a mean hamming distance of 4.5 ± 0.7 (mean ± stdev; Supp. Fig. S3). Only one pair (0.04%) out of 4841 barcodes had a hamming distance of 1 and 10 pairs (0.4%) out of 4841 had a hamming distance of 2. Because

the sequencing experiment in Fig. 2 showed only a single error for 51 barcodes, each sequenced 25 bases, our sequencing error rate was approximately $1/(51 \times 25) = 0.08\%$. Therefore, assuming that sequencing errors have no bias toward a particular base, the probability of matching an *in situ* barcode to the wrong MAPseq barcode, while allowing one mismatch, is $\frac{2}{4841} \times 0.08\% \div 3 = 1e-7$. The probability of an *in situ* barcode matching to two MAPseq barcodes is $\frac{20}{4841} \times 0.08\% \times 2 \div 3 + \frac{2}{4841} = 4e-4$. Although we cannot detect false positive matches, an ambivalent match could be detected. In the XC9 data, however, no ambivalent match between the *in situ* barcodes and the MAPseq barcodes have occurred.

In addition, XC9 had three pairs of barcodes whose first 15 bases were the same. These appeared to have arisen from amplification errors in homopolymer stretches of the same barcode rather than different barcodes, because each pair had a single in-del and had almost identical projection patterns. These three pairs were not recovered *in situ* and thus did not affect the analyses.

Similarly, out of 13581 total sequences, XC28 had 5 pairs of barcodes within one mismatch and 106 pairs of barcodes within two mismatches for the first 15 bases. No XC28 barcodes had identical sequences in the first 15 bases. The probability of a wrong match in XC28 is $\frac{10}{13581} \times 0.08\% \div 3 = 2e-7$, and the probability of an ambiguous match in XC28 is $\frac{212}{13581} \times 0.08\% \times 2 \div 3 + \frac{10}{13581} = 7e-4$. No actual ambivalent match was seen in XC28. Therefore, allowing one mismatch for a 15-base sequence is sufficient to match barcodes in the somas to those at the projection sites unambiguously for both brains.

We rejected barcodes obtained from highly deformed tissues and cells outside of the cortex, and matched the remaining *in situ* barcodes to those in the target areas. We filtered "orphan barcodes," i.e. any barcode sequences recovered at a projection target for which the corresponding sequence was not recovered at the injection site. These orphan barcodes were likely from barcoded cells outside of the dissected injection site due to the diffusion of the virus, and therefore could reflect cell types in neighboring cortical areas. We performed further analyses on 6391 neurons with high-quality projection data.

Supplementary Note 3. The diversity of binary projection patterns

Out of the 12 areas we sampled, one was a negative control (the olfactory bulb). The total number of possible projection patterns should be the random combinations of projections to 11 areas, or $2^{11} = 2048$. These patterns include one with no projection to any area, which would have been filtered out in our dataset. Therefore, we would have been able to detect 2047 binary projection patterns at most. The 264 patterns actually observed is thus a significant fraction (13%) of all possible patterns.

Supplementary Note 4. Hierarchical clustering of projection neurons

To cluster the projection data, we first filtered the data using non-negative matrix factorization[24]. We noticed that projections to different brain areas were correlated (Supp. Fig. S5A). Such correlation suggests that cortical projections are not organized randomly, which is consistent with the known differences among classes of projection neurons. Because these correlations likely reflect high-order structures in the organization of projections, we want to preserve such correlations among projection areas while reducing the noise in the projection data. We therefore used non-negative matrix factorization to group correlated projections into *k* sets of projections, or

projection "modules" (Supp. Fig. S5B). The projection patterns of individual neurons can thus be approximated by a weighted sum of the projection modules (Supp. Fig. S5C, D).

We tested varying the module number k for filtering. Reducing the number of modules increases the amount of noise that can be removed, but could also potentially remove structures in the data important for clustering. NMF explained more variance of the data with higher k values, but the increase slows at $k \geq 5$ (Supp. Fig. S5E). However the first two levels of clustering were not robust at $k = 5$ (Supp. Fig. S5F). We therefore used $k = 6$ for our filtering, but other k values produced similar clustering results.

After filtering the data, we performed a divisive hierarchical clustering (Supp. Fig. S6A). At each hierarchy, a group of neurons was split into two subgroups using k-means clustering. We only kept the subgroups when the divisions were statistically significant and when each subgroup contained more than 1% of all neurons. This process was repeated for the newly generated subgroups, until no more significant splits were found. We then used random forest to find the probability that neurons belong to each cluster by doing pair-wise comparison among all pairs of clusters. This probabilistic cluster calling revealed that the majority of neurons (5968/6391, or 93% of all neurons) were assigned to a dominant cluster with high probability (>98% probability), but a small set of neurons (421/6391, or 7% of all neurons) were assigned to two clusters at around 50% probability each (Supp. Fig. S6Ba). These results indicate that the majority of neurons were unambiguously assigned to a single cluster.

Although BARseq has a low false-negative rate (~10%), such a false-negative rate may accumulate for subclasses with multiple projections, leading to higher rate in misclassification. To examine the extent of such misclassification, we used random forest to classify neurons probabilistically using 10 out of the 11 projections, thus simulating the effect of a projection being uninformative (Supp. Fig. S6Bb-l). This analysis revealed similar distribution of neurons with the majority being well-classified (i.e. >98% probability for the dominant cluster) and a smaller fraction being ambiguously assigned to two clusters (~50% probability for the dominant cluster). A third small group was visible that corresponds to neurons that were ambiguously assigned to three clusters when some projections were not used (~33% probability for the dominant cluster, Supp. Fig. S6Be-h, j). Projections to the caudal striatum and to the amygdala seemed to be more critical for clustering than the other projections. In contrast, the defining projections for the major classes, including the contralateral projections, corticothalamic projections, and the corticotectal projections, appeared less critical for classification. This result probably reflects the fact that major classes have different projection patterns in addition to the defining projections, whereas subclasses within a class were more similar.

We then considered a neuron ambivalently classified if it was assigned to two or more clusters using all 11 projections, or assigned to the wrong cluster using any of the 10 projections if the resulting cluster was consistent with a dropped projection rather than a false-positive projection. This resulted in 5682/6391 (89%) well-classified neurons and 709/6391 (11%) ambivalent neurons. These estimates represent an upper bound on the number of ambivalent neurons, because it did not take into consideration the actual low false-negative rate of BARseq. The ambivalent neurons were concentrated in a few ITi subclasses (Supp. Fig. S6C), indicating that these subclasses may have resulted from misclassification of other subclasses due to false negative projections.

We also compared our clustering to graph based clustering using Louvain community detection[25] and hierarchical clustering using spectral clustering[27]. Louvain community detection identified 2-4 clusters at each hierarchy, and therefore did not fully correspond to the clusters obtained by bifurcation only at any hierarchical level. However, the resulting clusters from both methods, especially high-level nodes, were similar to those obtained using k-means (Supp. Fig. S6D). We chose to base all further analyses on clustering using k-means, because the major classes were better separated than using spectral clustering and the imposed bifurcation was easier to interpret than the clusters produced by Louvain community detection.

Supplementary Note 5. Subclasses of cortical projection neurons

Our clustering produced nine subclasses of contralaterally projecting intratelencephalic (ITc) neurons, ten subclasses of ipsilaterally projecting intratelencephalic (ITi) neurons, four subclasses of pyramidal tract (PT) neurons, and two subclasses of corticothalamic (CT) neurons (Fig. 3G). Most divisions appeared to be based on whether the neurons project to the striatum, the ipsilateral sensory cortices, and the amygdala. The projections to the orbitofrontal cortex and the motor cortex appeared to have little effects on the clustering, probably because these projections were usually weak and very few neurons projected to these areas (Supp. Fig. S6E, Fig. 3D).

We identified a cluster of neurons (Leaf 14, Fig. 3G) that appeared to be CT neurons projecting to the striatum, but not to the tectum. Layer 6 CT neurons, however, usually do not project to the striatum[10]. The apparent striatal projections could be caused by contamination by fibers passing through the striatum. Alternatively, these neurons could be PT neurons in layer 5 and deep layer 6 whose subcerebral projections were missed due to either weak projections in the tectum or projections to other targets rather than the tectum. Our analyses of projection neurons *in situ* support the latter hypothesis. We observed a total of 590 corticothalamic neurons that did not project to the tectum or the striatum, and 505 that did project to the striatum. However, only 10 of the latter group was obtained in the *in situ* sequenced brains, compared to 229 of the former group. Therefore, most of the striatum and thalamus projecting neurons were from the conventional MAPseq brain (XC14). Because we only sequenced neurons at the center of each injection site *in situ* (XC9 and XC28), but collected a much larger injection site that may have included neighboring cortical areas in the conventional MAPseq brain (XC14), these neurons were rare in the auditory cortex, and were more likely in neighboring cortical areas, where PT neurons could project to other targets. Furthermore, the 10 neurons that projected to the striatum had a laminar profile similar to that of PT neurons, but different from those of the layer 6 CT neurons (Supp. Fig. 4C). These results suggest that most of the striatum projecting "corticothalamic" neurons were likely PT neurons in layer 5 and deep layer 6 in neighboring cortical areas.

Supplementary Note 6. Laminar distribution of projection neurons

To estimate the boundaries of cortical layers, we performed FISH against two known layer-specific marker genes, Cux2[18] (Supp. Fig. S8A), and Fezf2 (Supp. Fig. S8B). Cux2 was strongly expressed in L2/3 and only sporadically in other layers; Fezf2 was strongly expressed in L5 and weakly in L6. Because L4 is poorly defined in the auditory cortex[29], we omitted L4 and defined only the remaining two borders (Supp. Table S3). We defined the L2/3 and L5 border as below the Cux2 band and above the strong Fezf2 band, and defined the L5 and L6 border as between the strong and weak bands of Fezf2. To account variations in cortex thickness and sample preparation, we examined three slices spanning 800 μm in the auditory cortex, normalized all cortical thickness to

1200 μm (i.e. the same cortical thickness as the BARseq brains), and calculated the mean positions of layer boundaries (Supp. Table S3), The L2/3 and L5 border defined by Fezf2 agreed with that defined by Cux2. Based on these measurements, we defined the L2/3 and L5 border to be at 590 μm and the L5 and L6 border to be at 830 μm. These borders were used for the BARseq analysis when layer identities were involved (Fig. 4A, C).

We saw few projection neurons in superficial L2/3 in our dataset. This is partially due to smaller number of neurons labeled near the cortical surface, and partially due to an enrichment of neurons without detectable projections in superficial L2/3 (Supp. Fig. S8C). Neurons in superficial L2/3 (or L2) of the auditory cortex are known to project local and not contralaterally[30]. Because we did not sample neighboring cortical areas, these locally projecting ITi neurons would show as non-projecting neurons in BARseq.

Supplementary Note 7. The spatial organization of subclasses of projection neurons

We examined whether clustering resulted in subclasses that were more restrictive in laminar locations. One natural measure of spatial compactness is the standard deviation of the spatial distribution of neurons within a class, but such a measure yields spuriously high values for multimodal distributions. We therefore examined the entropy (normalized to fall between 0 and 1) of the laminar distribution of all nodes and leaves in the clustering, a measure which is insensitive to the shape of the distribution. We did not see a reduction in the mean cluster entropy with more divisions ($p = 0.94$ for cluster hierarchy 2-7, one-way ANOVA), especially for the ITc subclasses (blue dots). We saw no significant difference in the entropy of the laminar distributions of the subclasses between the two brains (Fig. 4B; $p > 0.05$ by random sampling after Bonferroni correction; Supp. Fig. S9A), suggesting that the two brains were consistent. The lack of laminar restriction was also not due to misalignment between the two brains, because the average laminar locations of neurons from each brain were similar for all but one subclass (Supp. Fig. S9B; $p > 0.05$ after Bonferroni correction, Mann-Whitney U test). The one exception was a CT subclass that were on average 50 μm deeper in the XC28 than in XC9 (Supp. Fig. S9B; $p < 0.0005$ after Bonferroni correction), but this could be explained by more deep L6 neurons being labeled in XC28 than in XC9 (XC9 and XC28 had 165 and 132 L6 labeled neurons with laminar depths < 1000 μm, respectively, compared to 56 and 129 L6 neurons with laminar depths > 1000 μm; $p < 10^{-7}$, fisher's exact test). Therefore, subclasses were generally not more restrictive in laminar distribution than classes.

We further investigated the projection patterns of corticotectal PT neurons in L5 and L6. PT neurons in L5 and L6 of the auditory cortex have distinct morphology and physiological properties[20], and thus likely belong to different classes. However, we failed to identify a PT subclass restricted to either L5 or L6 (Fig. 4C). Corticotectal neurons mainly projected to the tectum, the thalamus, and the caudal striatum. All L5 (185 / 185) and L6 (80 / 80) corticotectal PT neurons as defined by clustering projected to the thalamus. In addition, similar fractions of neurons projected to the caudal striatum (43 / 185 = 23% for L5 and 24 / 80 = 30% for L6, $p = 0.3$ using fisher's exact test). To ensure that such results were not biased by our clustering, we also looked in all neurons that projected to the tectum regardless of their classification by clustering. Similarly, 98% (183 / 187) and 98% (65 / 66) of L5 and L6 corticotectal neurons, respectively, projected to the thalamus. Because these projection probabilities were likely limited by the sensitivity of BARseq (92%), virtually all corticotectal neurons project to the thalamus regardless of their laminar origins. For corticostriatal projections, 25% (46 / 187) and 27% (18 / 66) of L5 and L6 neurons with tectal projections, respectively, projected to the caudal striatum ($p = 0.7$ using fisher's

exact test). These results indicate that the L5 and L6 corticotectal neurons have similar projection probabilities to their targets.

We then examined the projection strengths of single corticotectal neurons in L5 and L6. In contrast to the projection probabilities, corticotectal projections were stronger in L5 than those in L6 [Supp. Fig. S9C; 11.7 ± 2.2 (mean $\pm$ stdev), $N = 80$ for L6 neurons and 13.8 ± 1.6, $N = 185$ for L5 neurons, $p < 0.0001$ using bootstrap ks test]. Projections to both the thalamus and the striatum were indistinguishable between the two groups (Supp. Fig. S9D, E, $p = 0.5$ for both corticothalamic projections and corticostriatal projections using bootstrap ks test). The distribution of the strengths of corticotectal projections, however, overlap significantly between the two groups. These results were consistent with the notion that projections are not organized by laminae.