

# **Pyramidal cell types and circuit organization of the mouse insular cortex reveal functional specializations**

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## **Supplementary Information**

### Supplementary Notes

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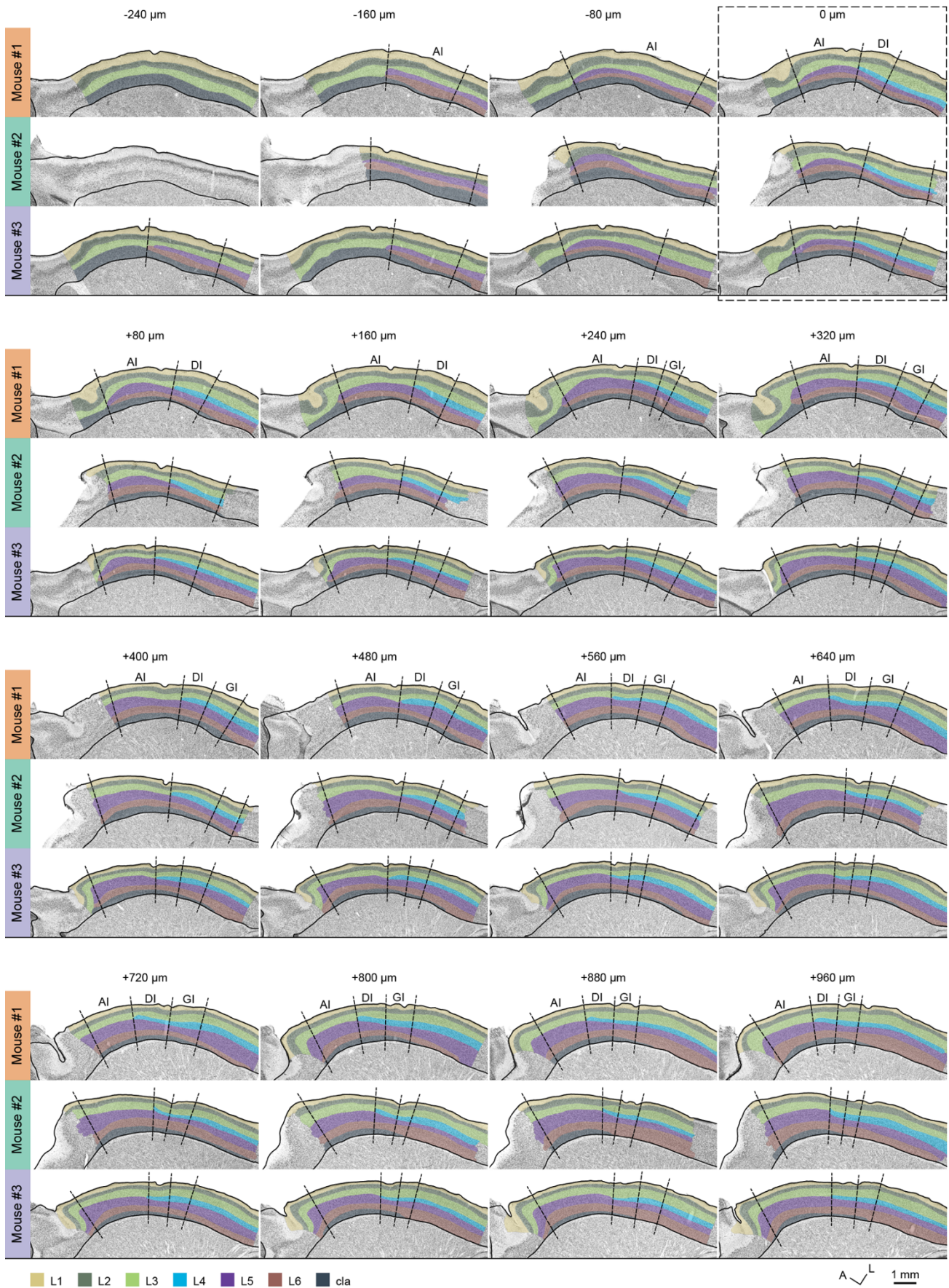
## Supplementary Notes

### Cross-modal associations between cell types and potential molecular contributors

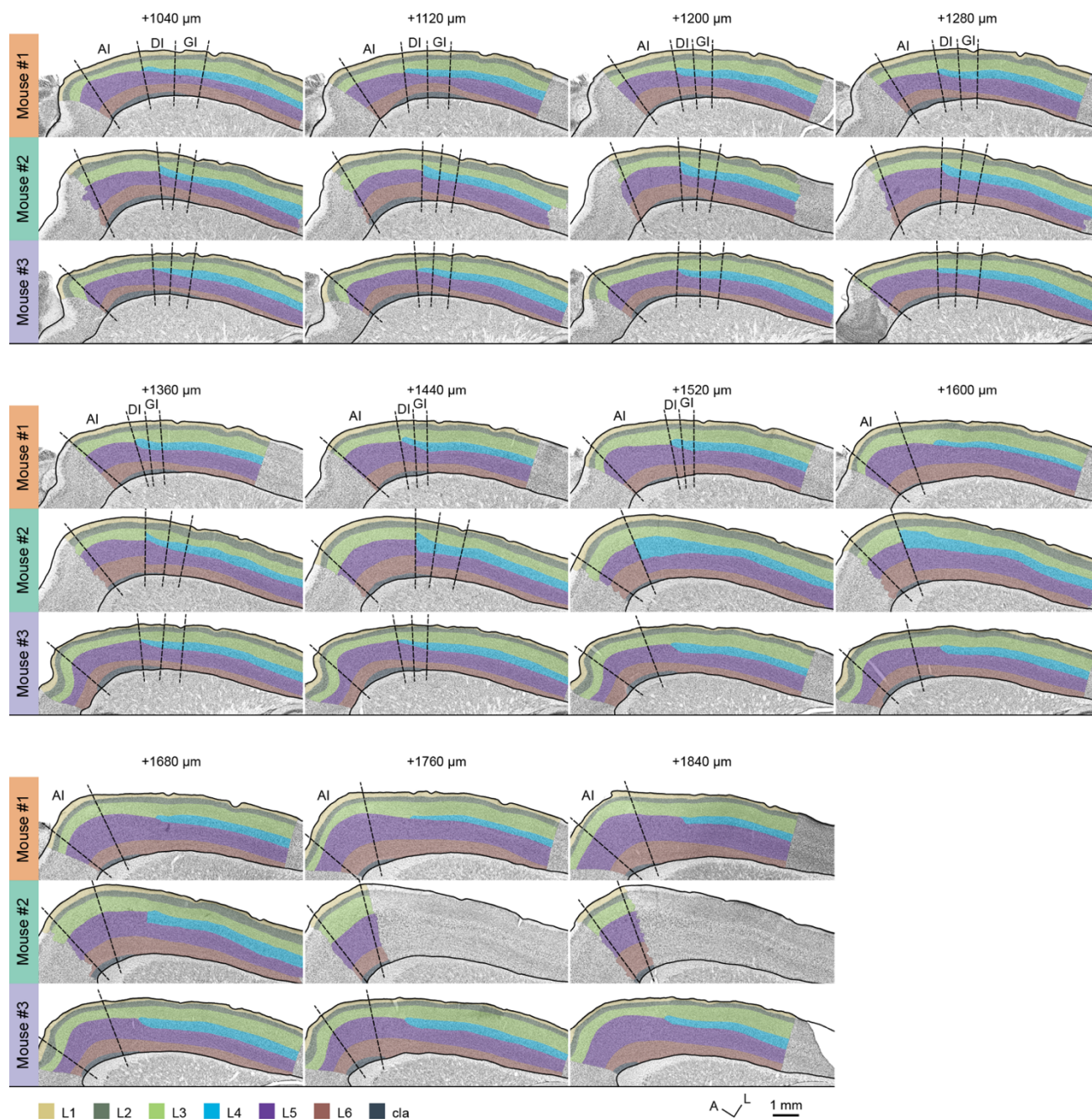
Despite heterogeneous cross-modal associations between M-types and E- or I-profiles, groups of M-types occupied confined regions in the electrical and input feature spaces. For example, all L5b morphologies displayed large voltage sags with either burst or regular spiking behaviors (Fig. 6b). Such cross-modal associations were rooted in specific biological parameters: the more elaborate the distal dendritic arbor, the larger the voltage sags (Fig. 6d). This points to the functional significance of cellular structures, because distal dendrites preferentially host HCN channels,<sup>53</sup> and the regulation of HCN channel density is an important factor for synaptic integration at the dendritic level.<sup>93</sup> Further, such physiological properties can be dynamic and modulated by neuromodulators (e.g., dopamine, acetylcholine).<sup>60,94</sup> Additionally, cell type-specific modulation of input strength may fine-tune local input distributions.<sup>60,94</sup> Therefore, E- and I-profiles obtained from ‘naïve’ mice in the present work may reflect baseline electrical and input states from which cells can transition in response to neuromodulation.

### IC subregions defined by topographical distributions of M-types

Our dorsoanterior-AI coordinates generally corroborate with the empirically defined anterior IC,<sup>10,13</sup> whereas our ventroposterior-AI coordinates are anteriorly-shifted relative to the empirical posterior IC.<sup>11</sup> This might be due to the technical constraints for empirically determined IC subregion—often from *in vivo* studies—in separating the AI/DI/GI, because the AI’s center of mass is more anterior compared to the entire IC.

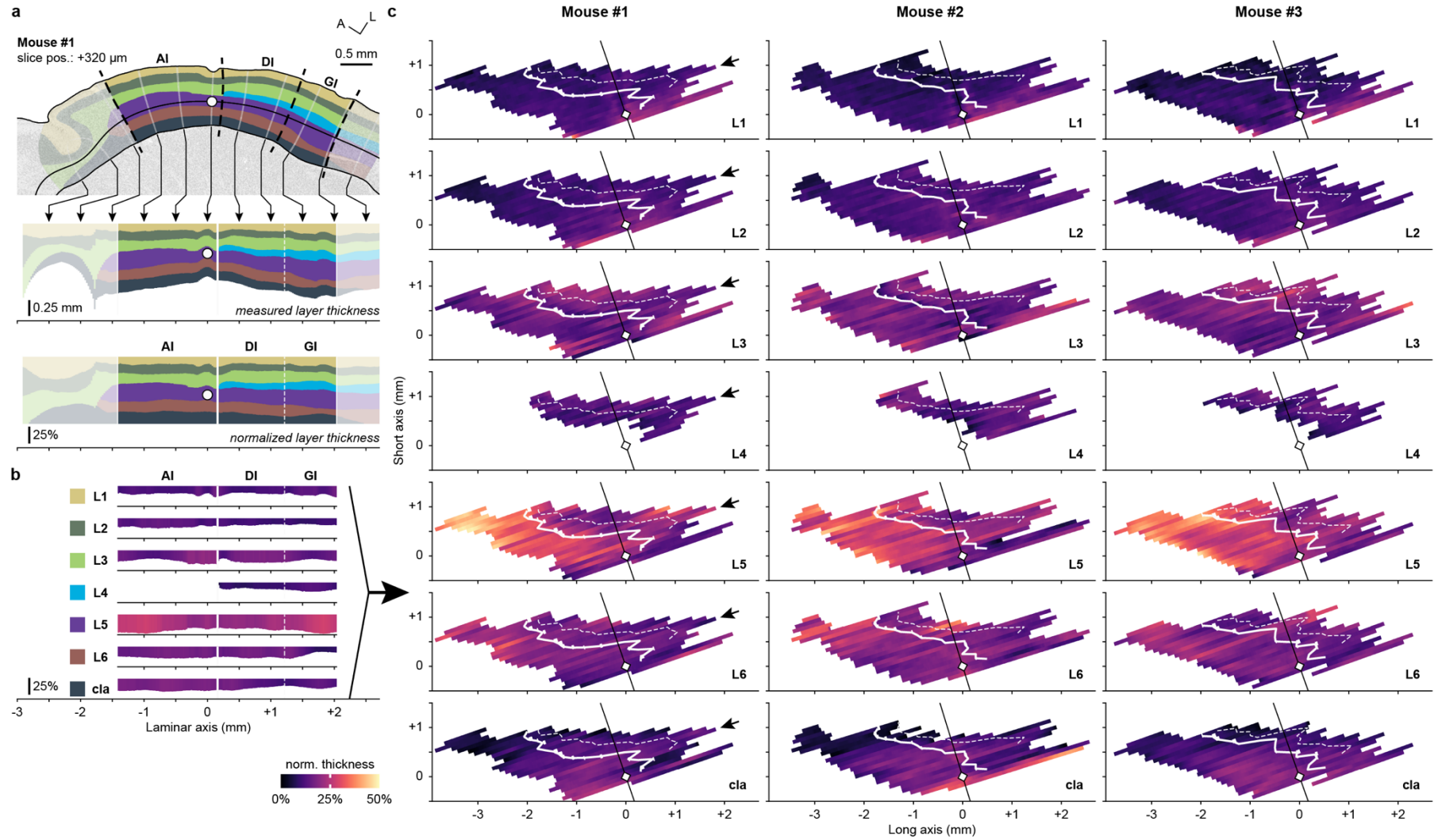






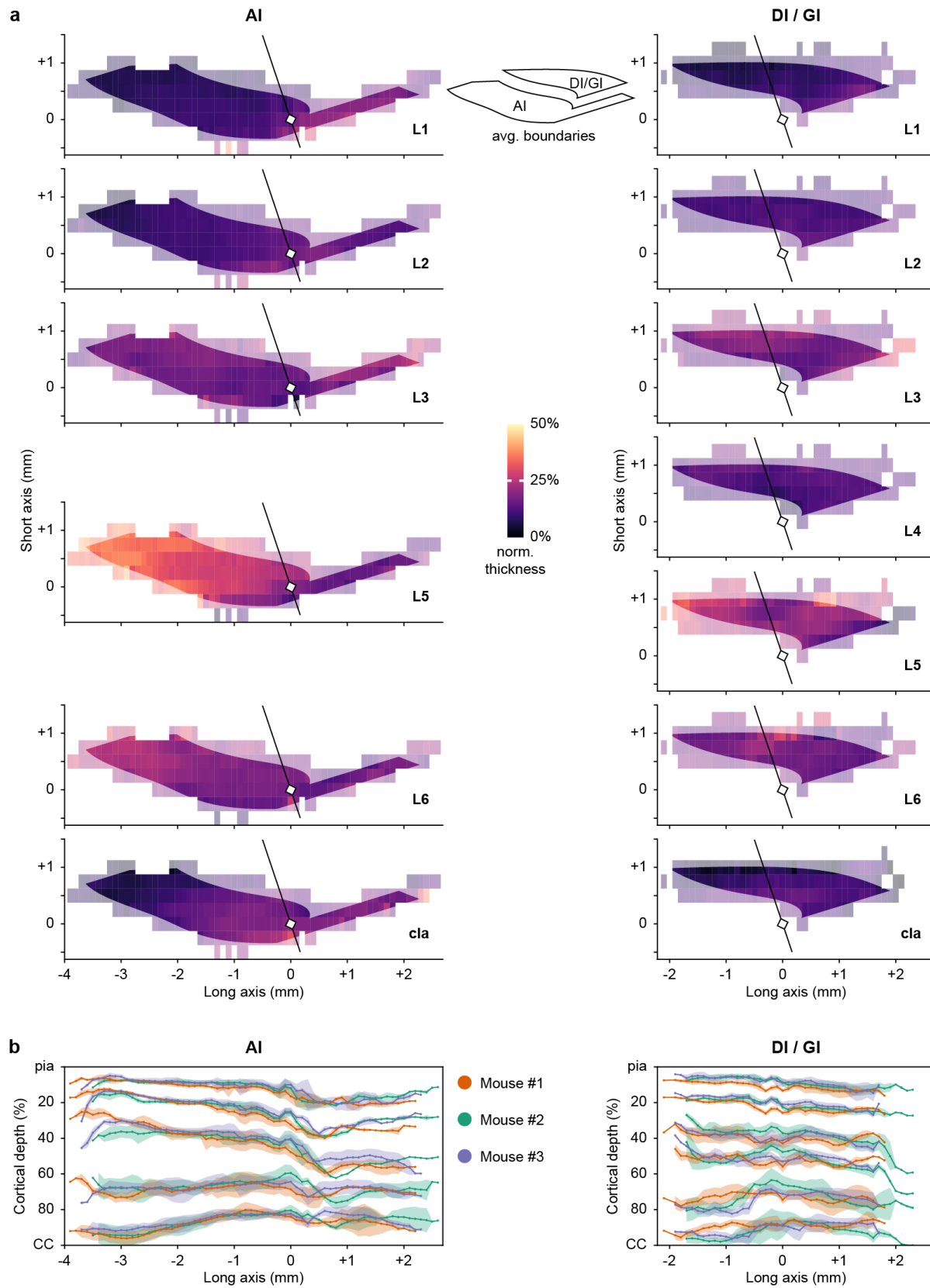
**Supplementary Fig. 1: Complete sets of consensus manual annotations of the insular cortex.** An alternating (every other) series of 40 μm-thick horizontal sections collected from three *PV-Cre<sup>+/+</sup>;Ai9<sup>+/+</sup>* mice processed for Nissl histology. Overlay shows the average consensus from two independent expert annotators demarcating the locations of all cortical layers within and adjacent to the insular cortex, as well as the boundaries of its subregions. Sections from each animal are arranged in rows along the ventral-to-dorsal cutting axis (top-left to bottom-right) and aligned across animals based on the ventral-most section in which

layer 4 (L4) is conspicuous (dashed box, top row, 4<sup>th</sup> column). The position of each section relative to this L4 anchor along the ventral-to-dorsal cutting axis is indicated above each column in  $\mu\text{m}$ . A, anterior; L, lateral; AI, agranular insula; DI, dysgranular insula; GI, granular insula; cla, claustrum; L1-L6, cortical layers 1-6.



Supplementary Fig. 2: See next page for caption.

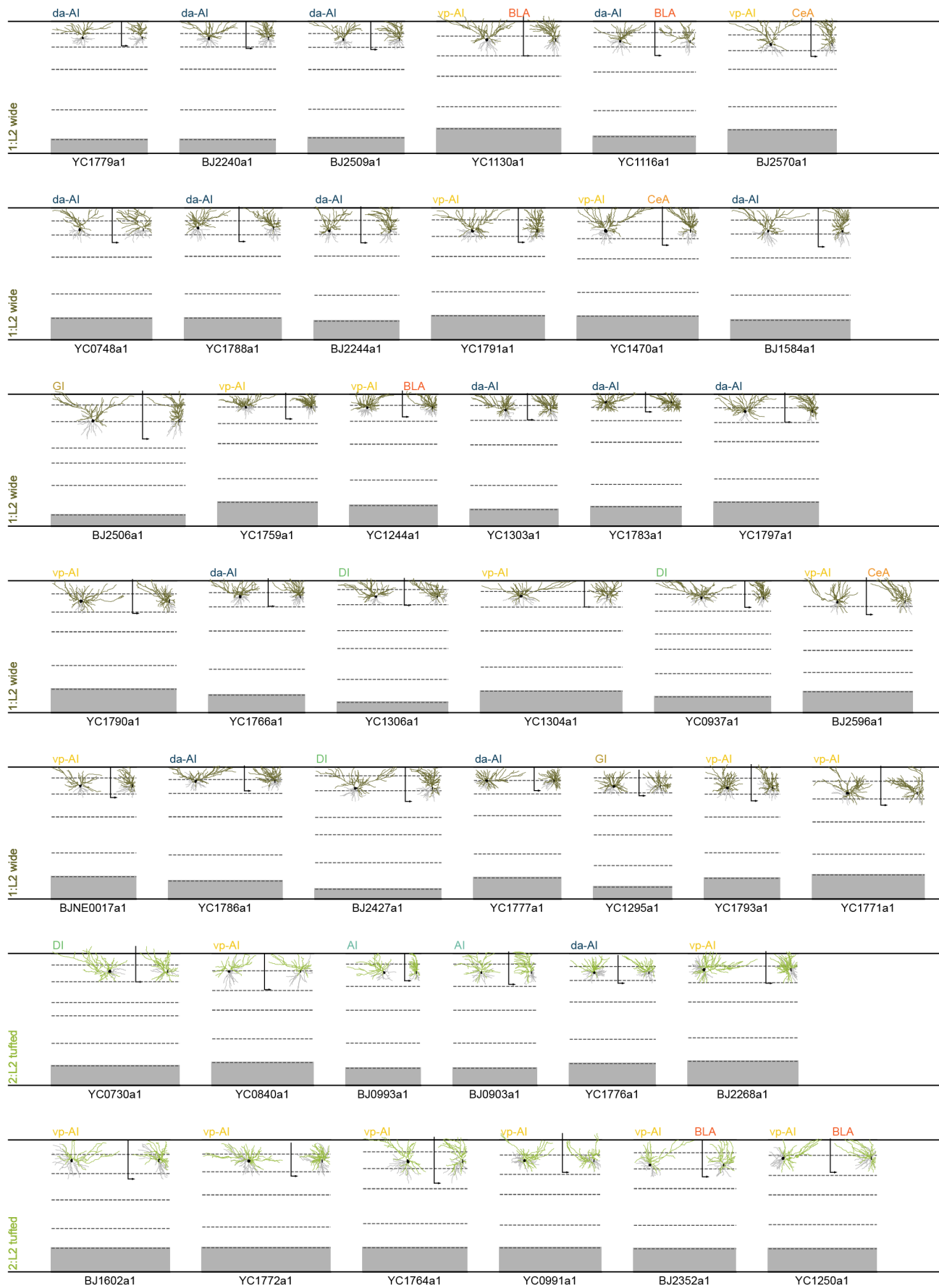
**Supplementary Fig. 2: Quantification of insular cortex layer thickness.** **a**, Example process of defining layer boundaries and calculating relative cortical depth. The layers were first manually annotated (top; Fig. 1c and Supplementary Fig. 1). The section was then ‘flattened’ according to streamlines (middle; Fig. 1d) and normalized by cortical thickness (bottom; Extended Data Figs. 1c,e). Arrows (displayed at 0.5 mm intervals) show the relative mapping of 25- $\mu$ m wide streamline bins from the physical section (top) to a ‘flattened’ laminar axis plot (middle/bottom). White circles: MCA-derived coordinate origin. **b**, Visual representations of the change in normalized thickness for each layer from (**a**) along the laminar axis. Luminance indicates normalized cortical thickness. **c**, 2D flatmaps of relative layer thickness for each reference mouse, rotated as in Extended Data Fig. 1f. White diamond: MCA origin; black line: best-fit plane to MCA; thick white line: AI/DI border; thin dashed white line: DI/GI border; arrowheads: row in Mouse #1 corresponding to data shown in (**a** and **b**). A, anterior; L, lateral; AI, agranular insula; DI, dysgranular insula; GI, granular insula; MCA, middle cerebral artery; cla, claustrum.

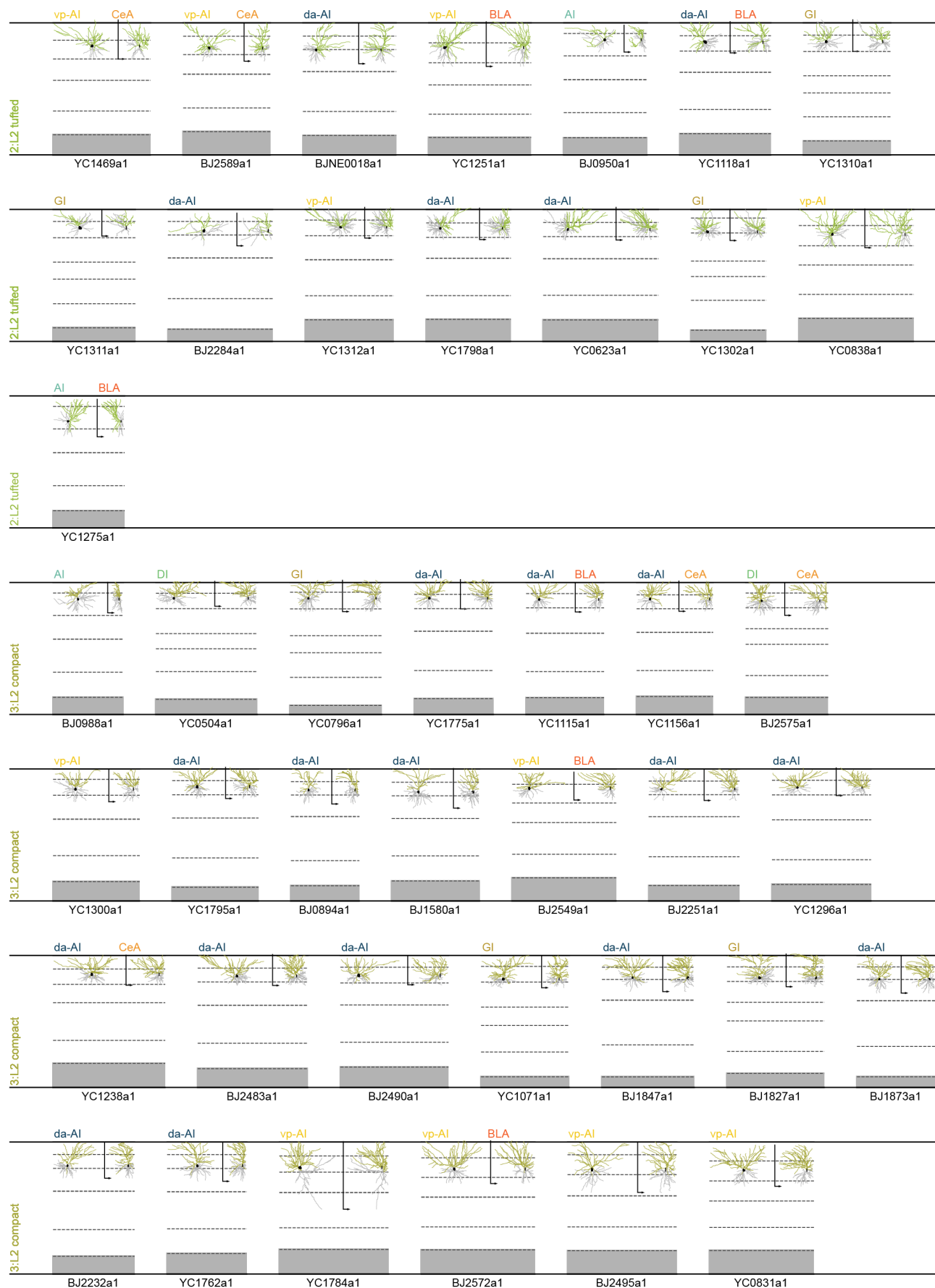


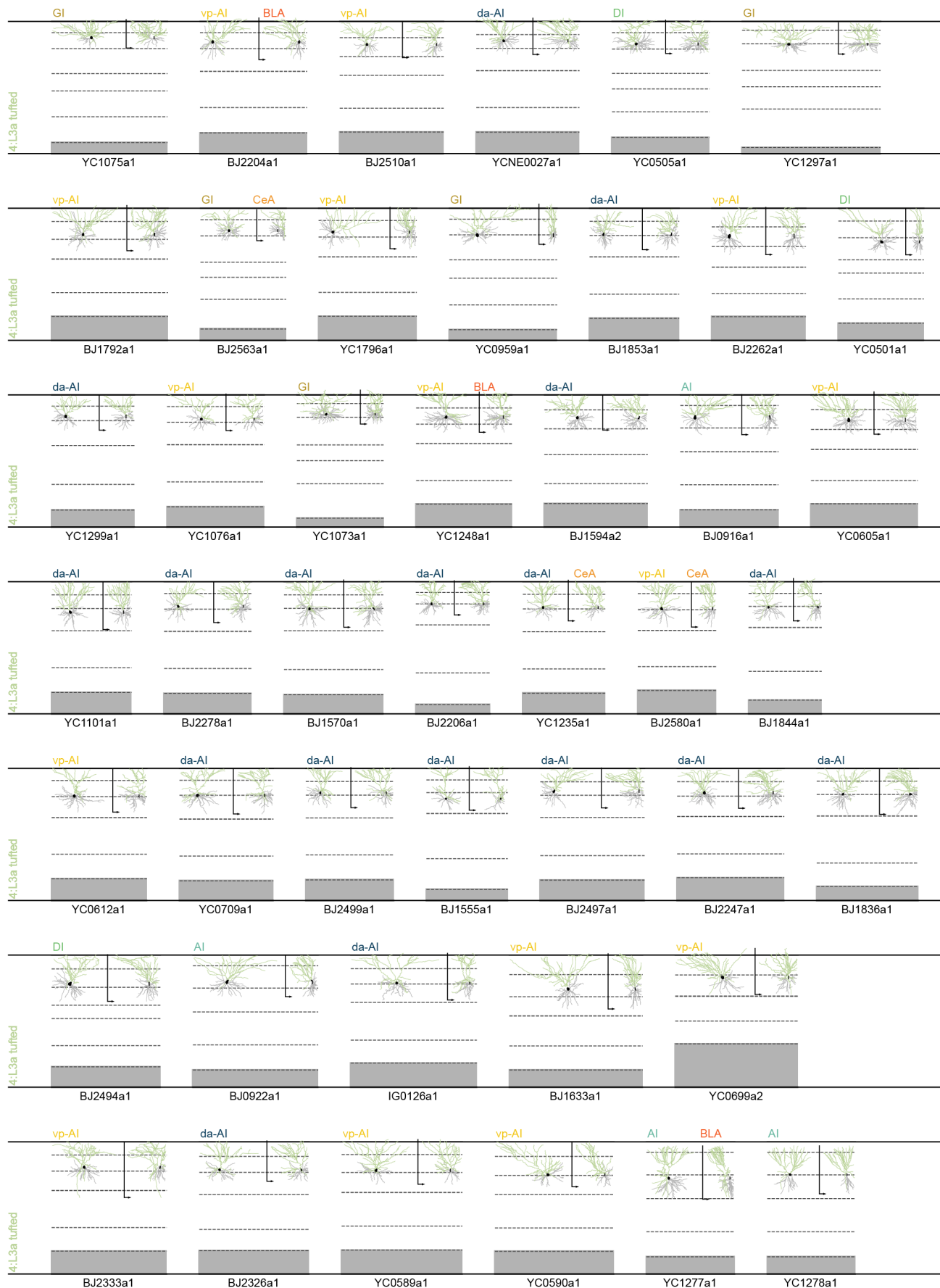
**Supplementary Fig. 3:** See next page for caption.

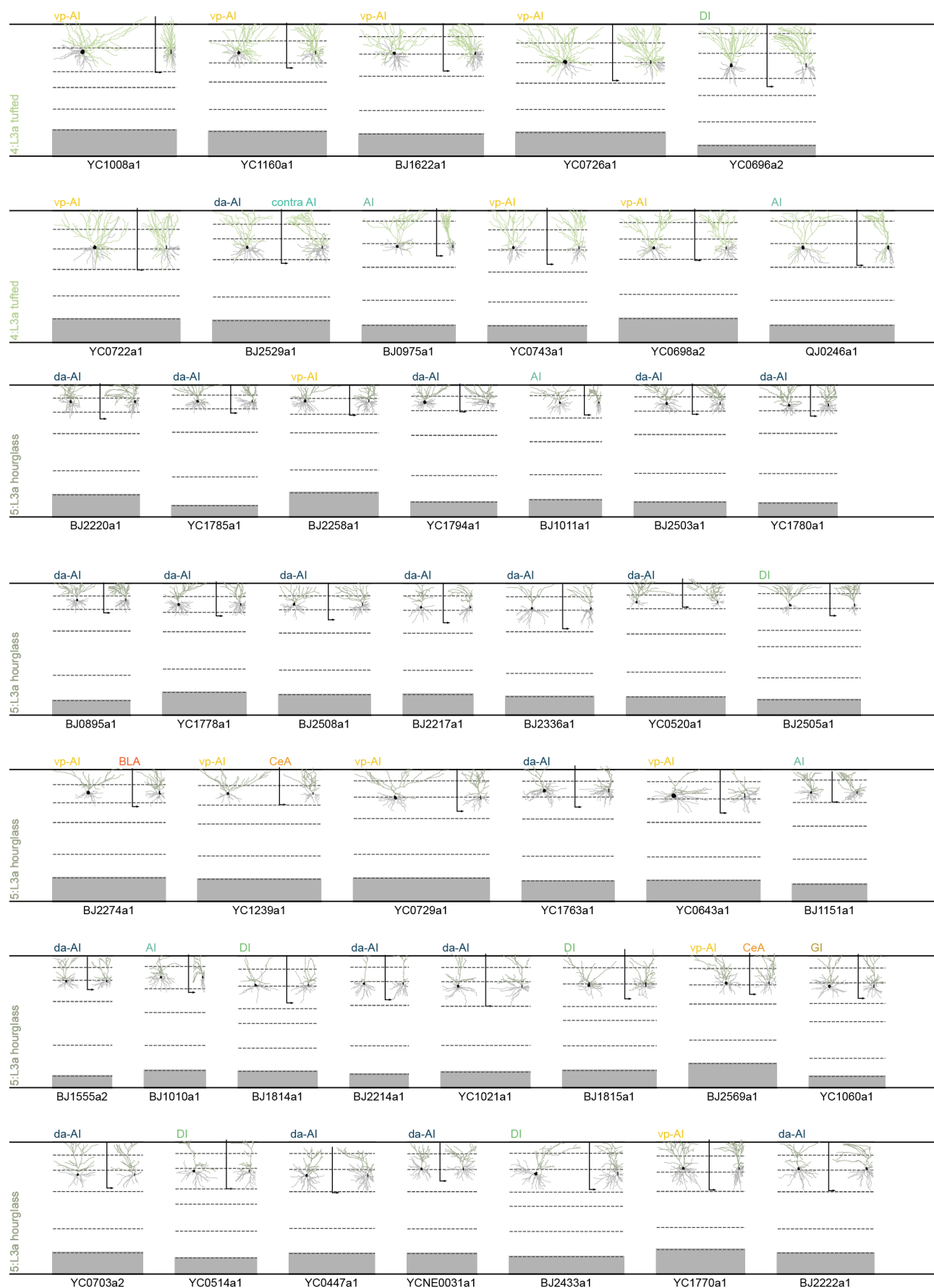


**Supplementary Fig. 3: Mean layer thickness across the insular cortex. a**, 2D flatmaps showing mean normalized layer thickness for AI (left) and DI/GI (right), respectively. Values from individual flatmaps (Supplementary Fig. 2c) were binned at 100- $\mu$ m and 250- $\mu$ m intervals along the long and short axes, respectively, relative to the MCA origin (white diamond). Luminance indicates normalized cortical thickness. Shaded areas: the average boundary of each subregion (Extended Data Fig. 1g); black line: best-fit plane to MCA. **b**, Mean normalized position of layer boundaries (Supplementary Fig. 2) binned along the flatmap long axis separately for AI (left) and DI/GI (right) for each mouse. Shaded regions: standard deviation. AI, agranular insula; DI, dysgranular insula; GI, granular insula; MCA, middle cerebral artery; cla, claustrum; CC, corpus callosum.

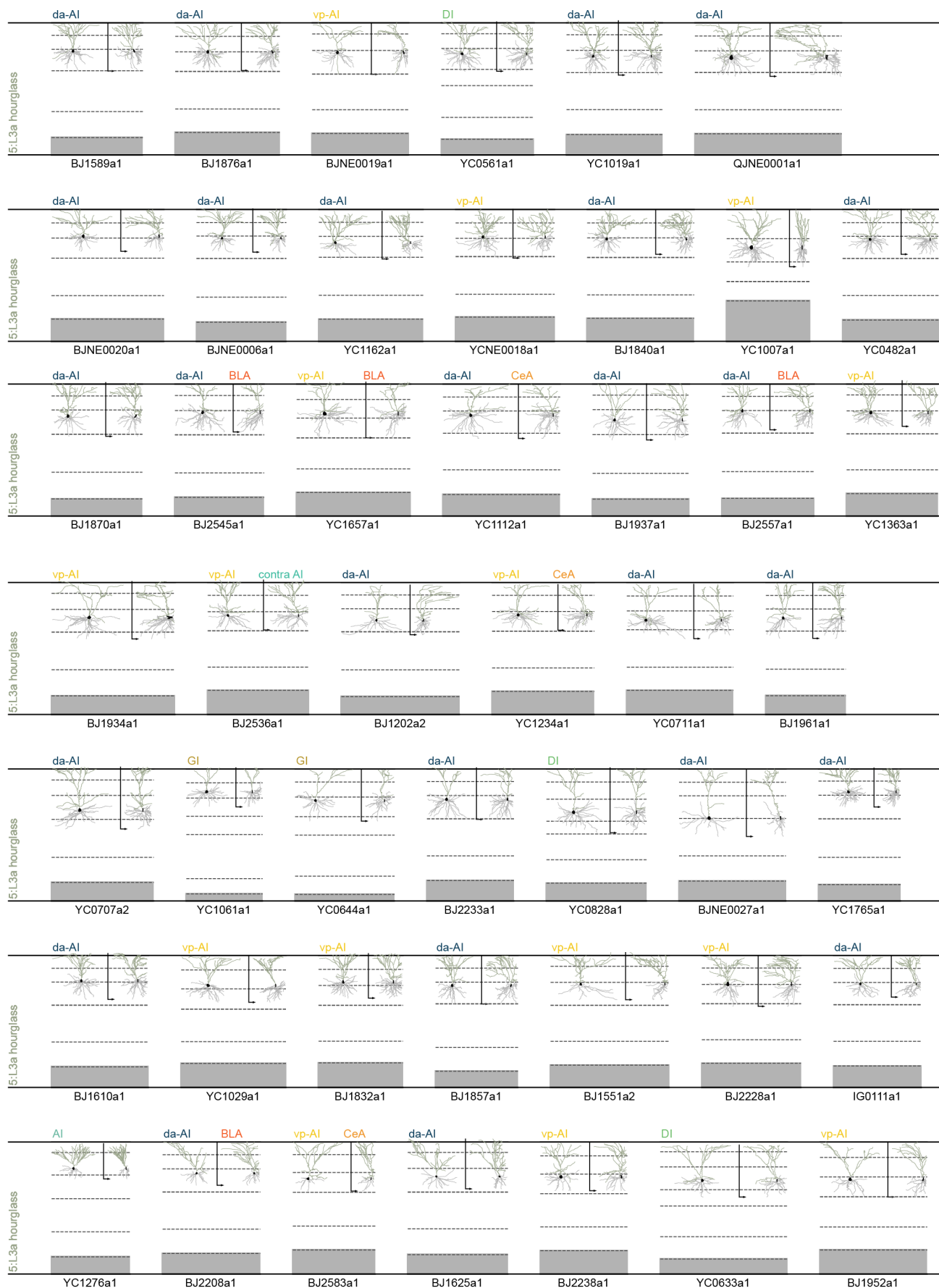


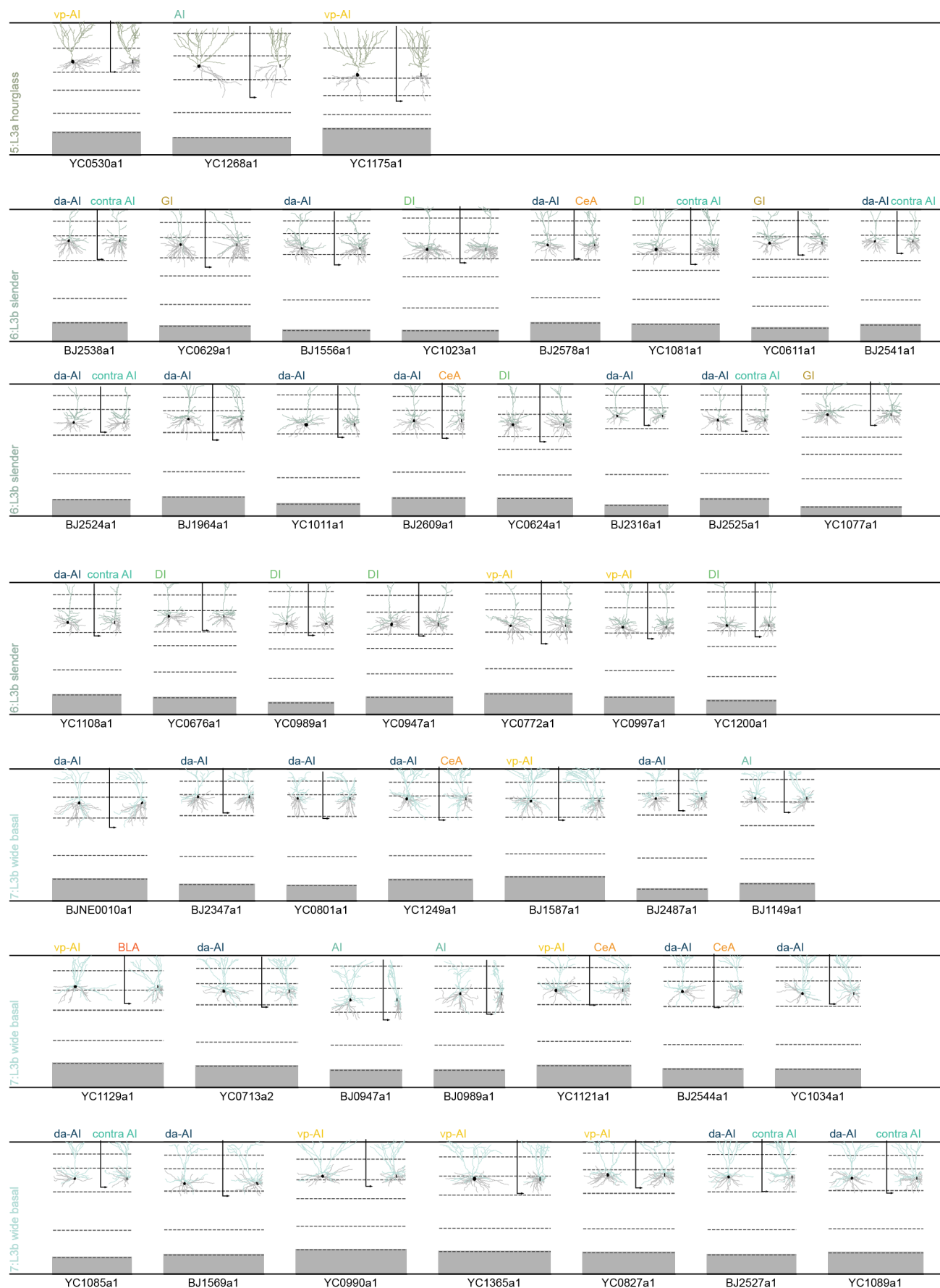


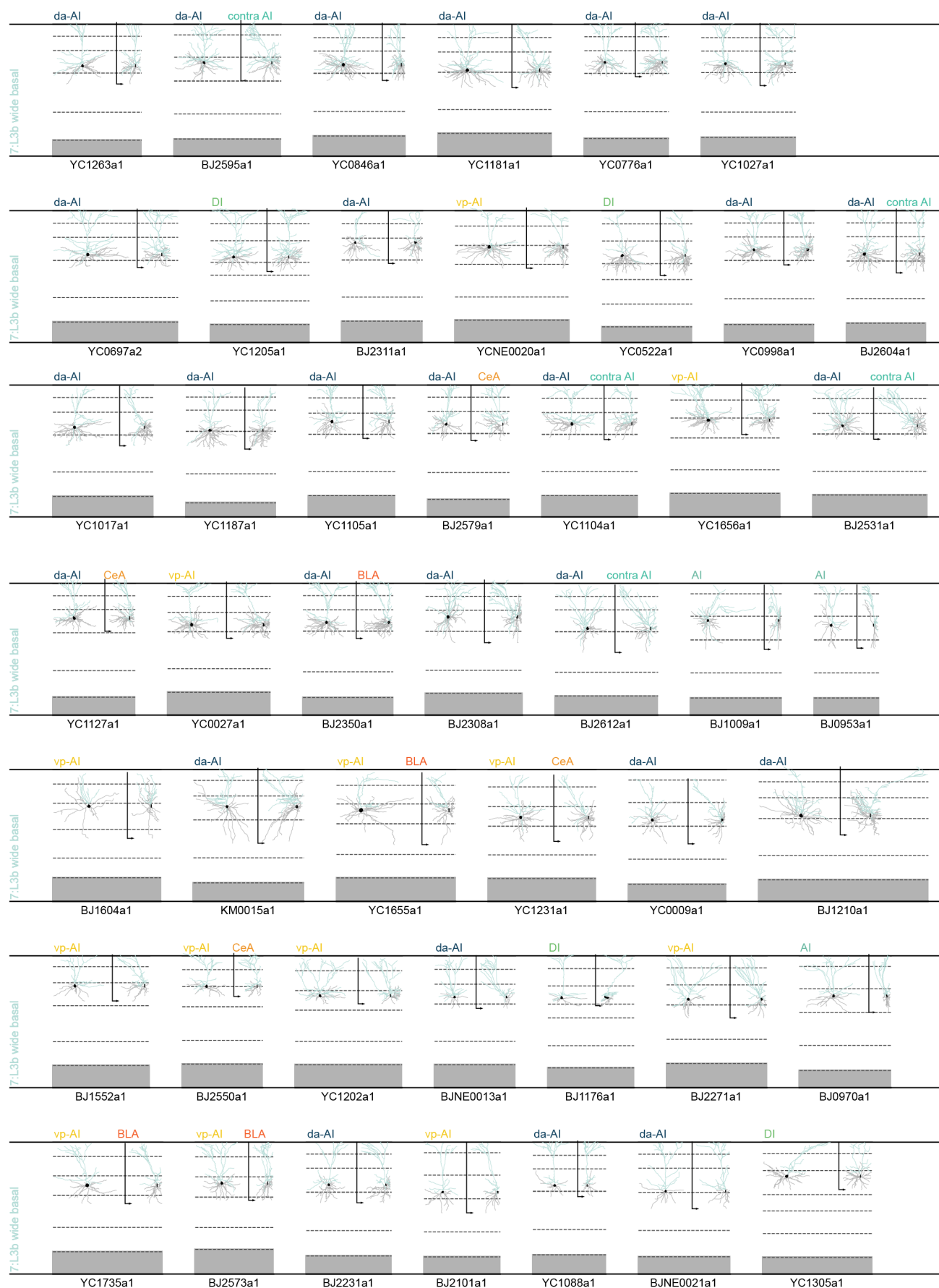


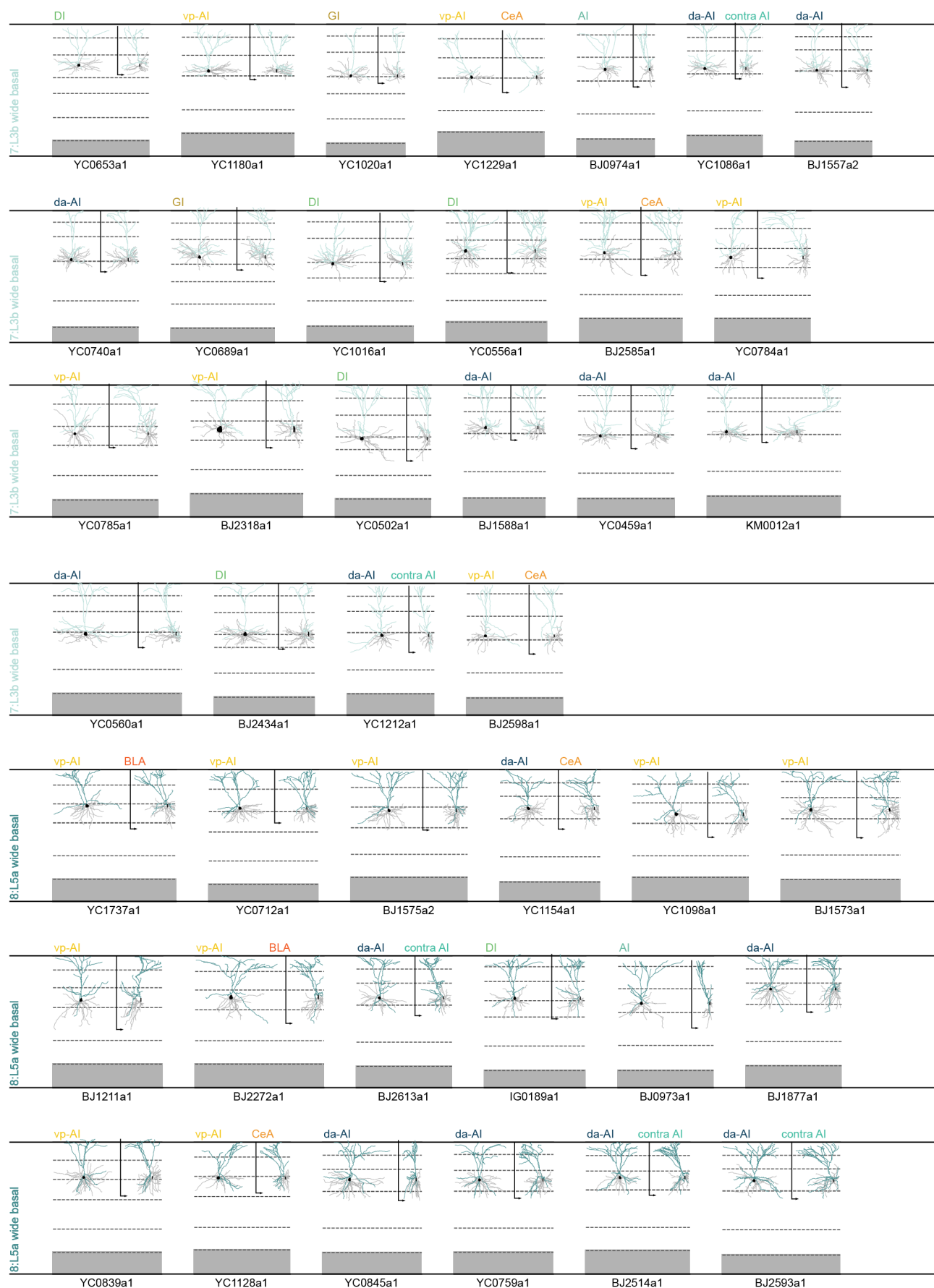


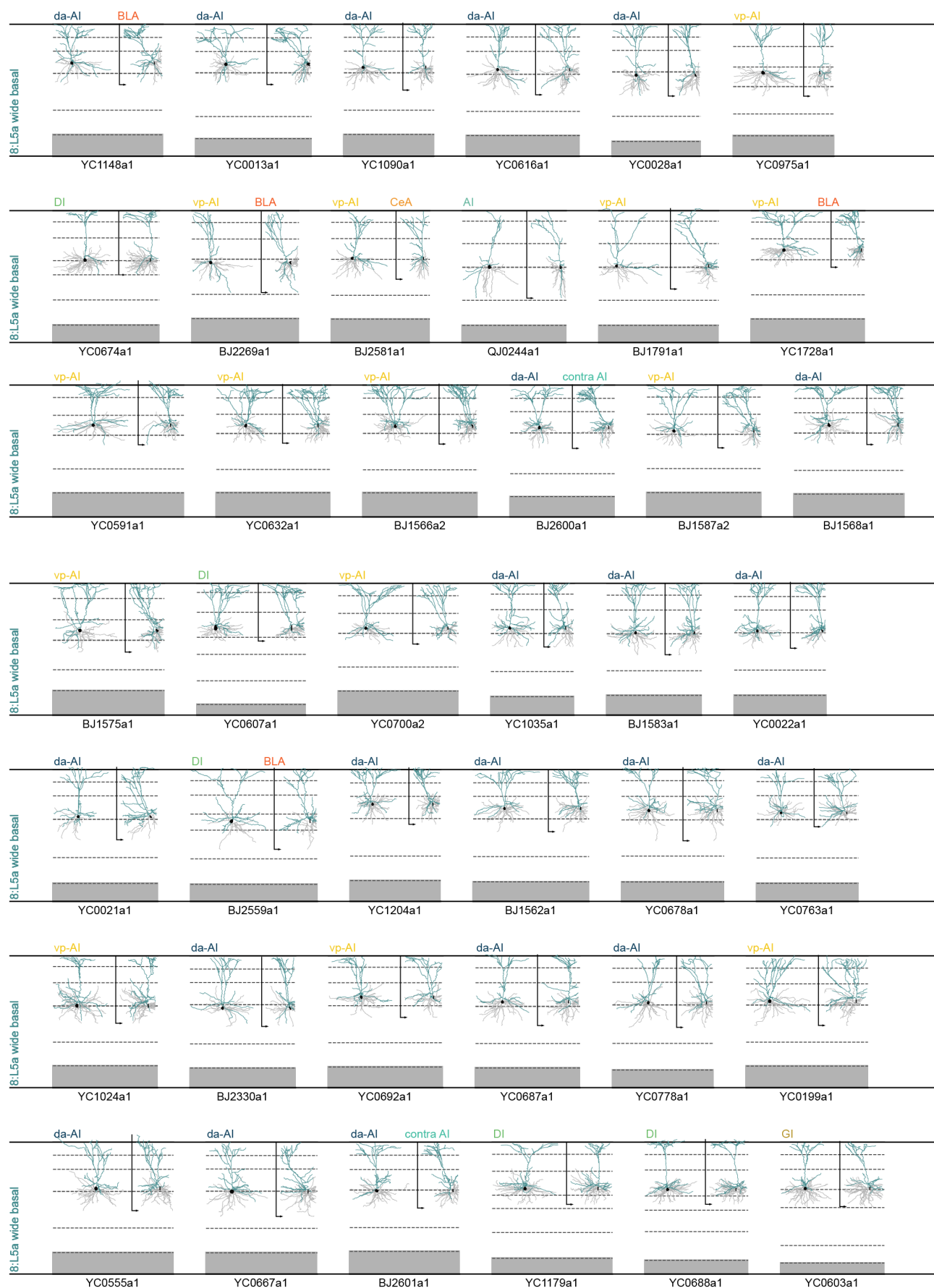




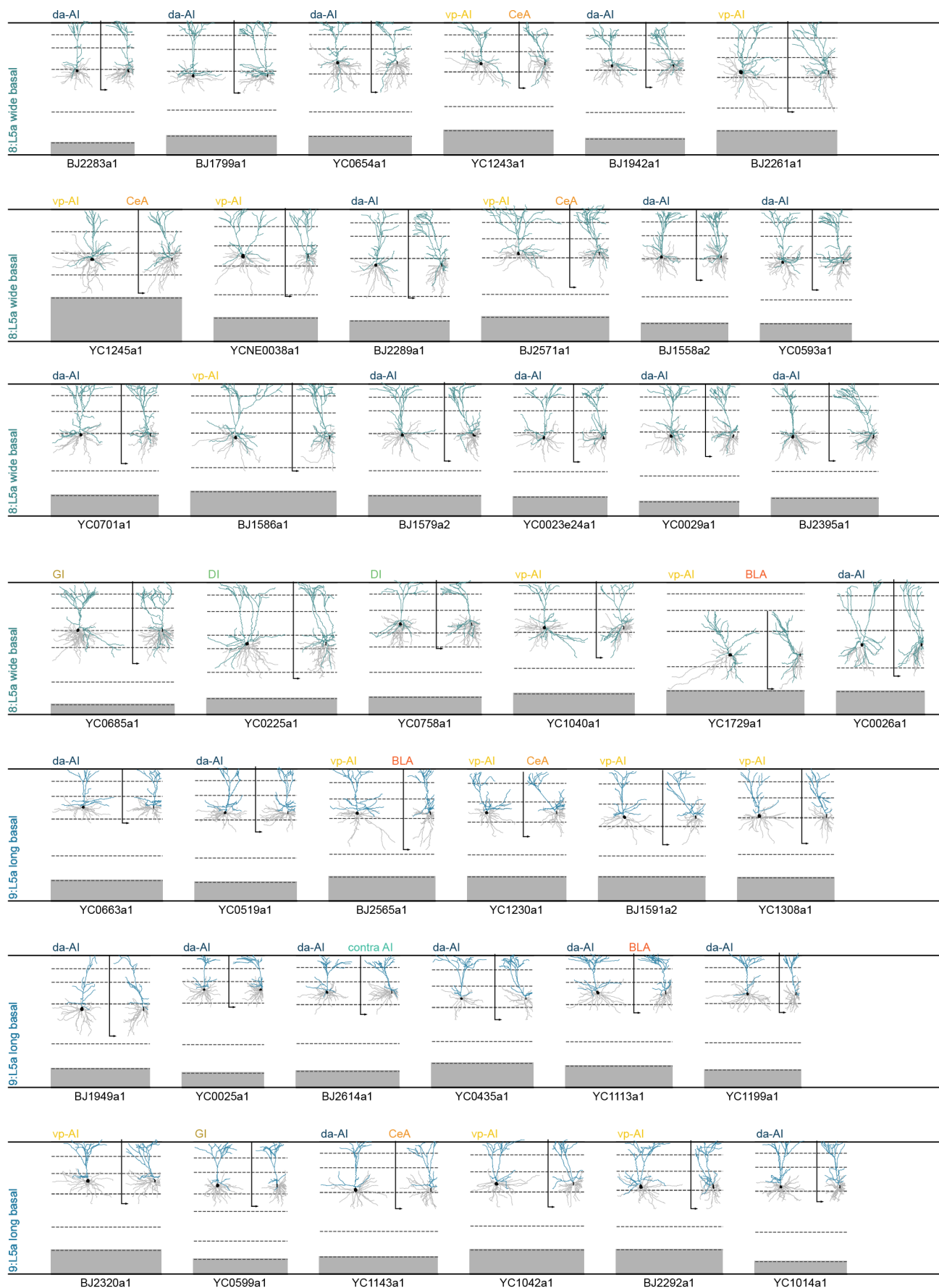




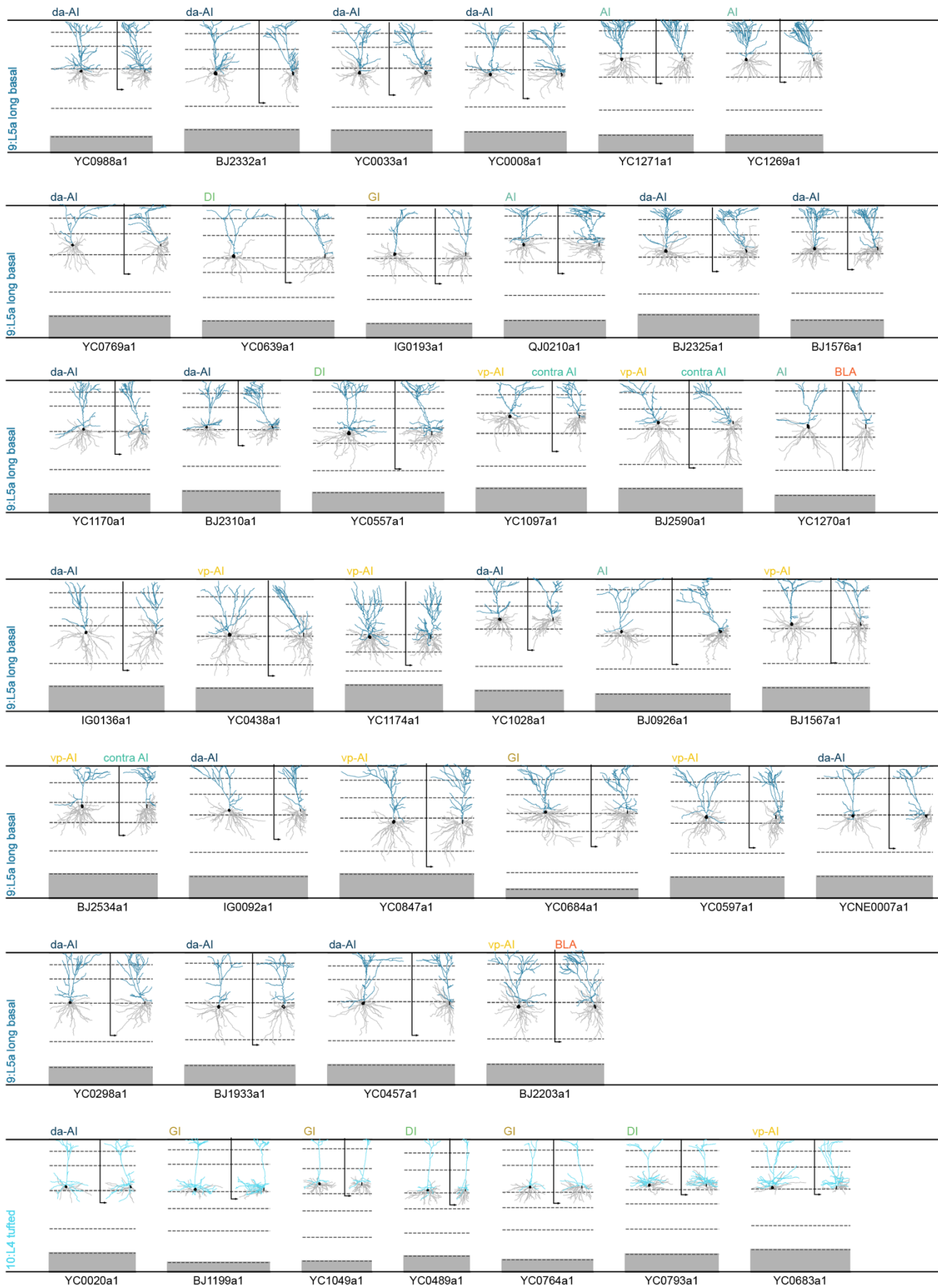


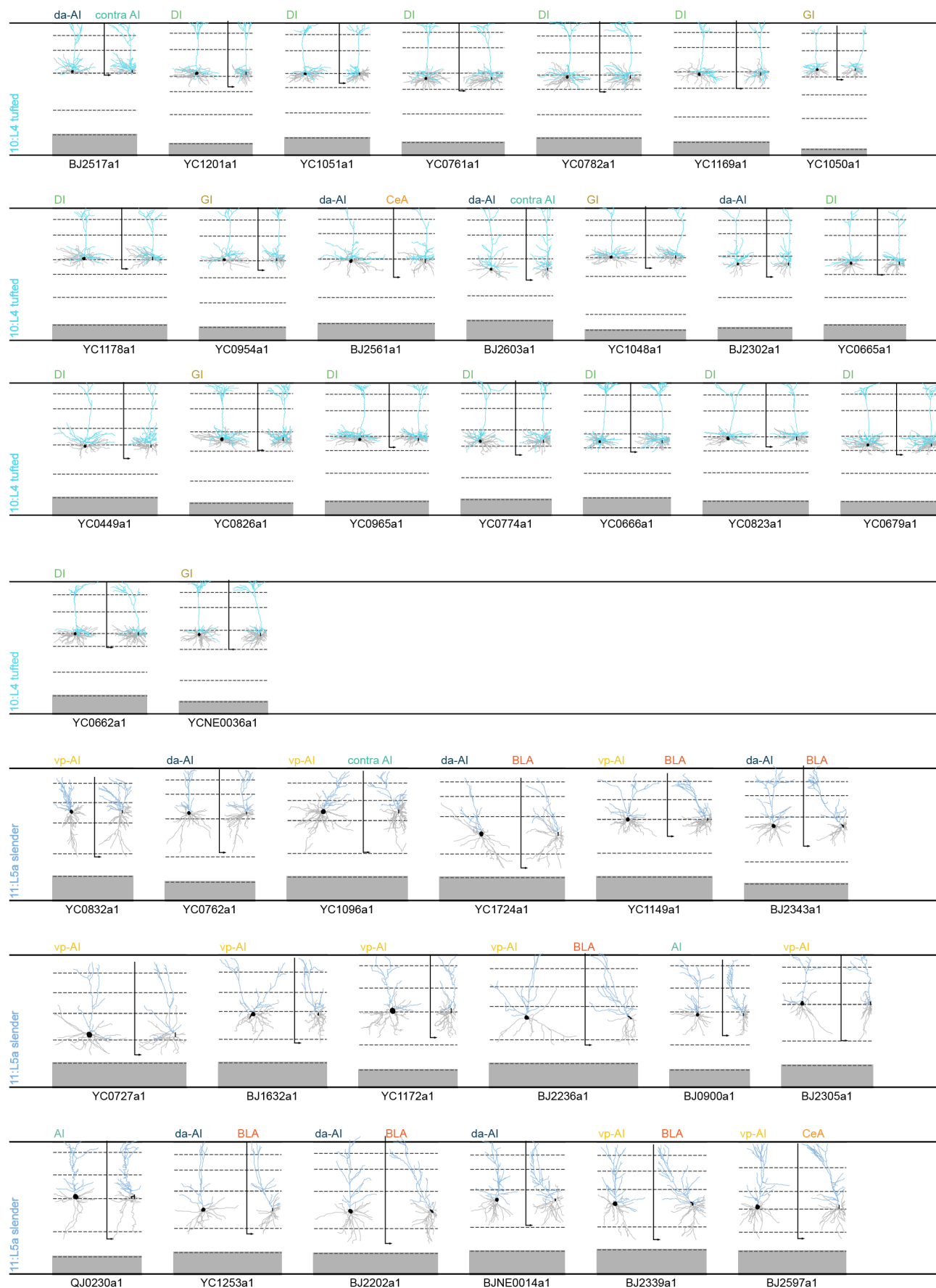


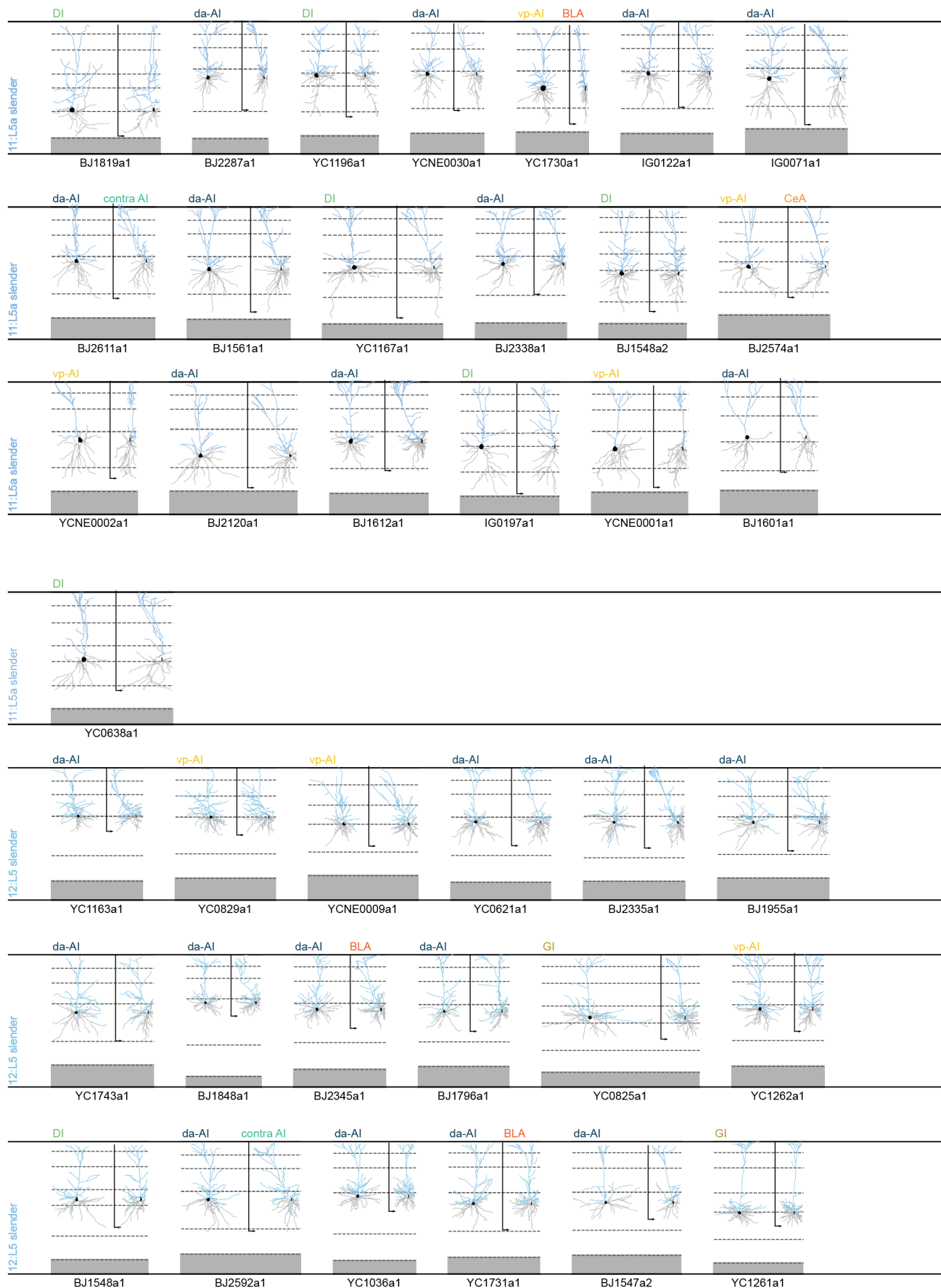


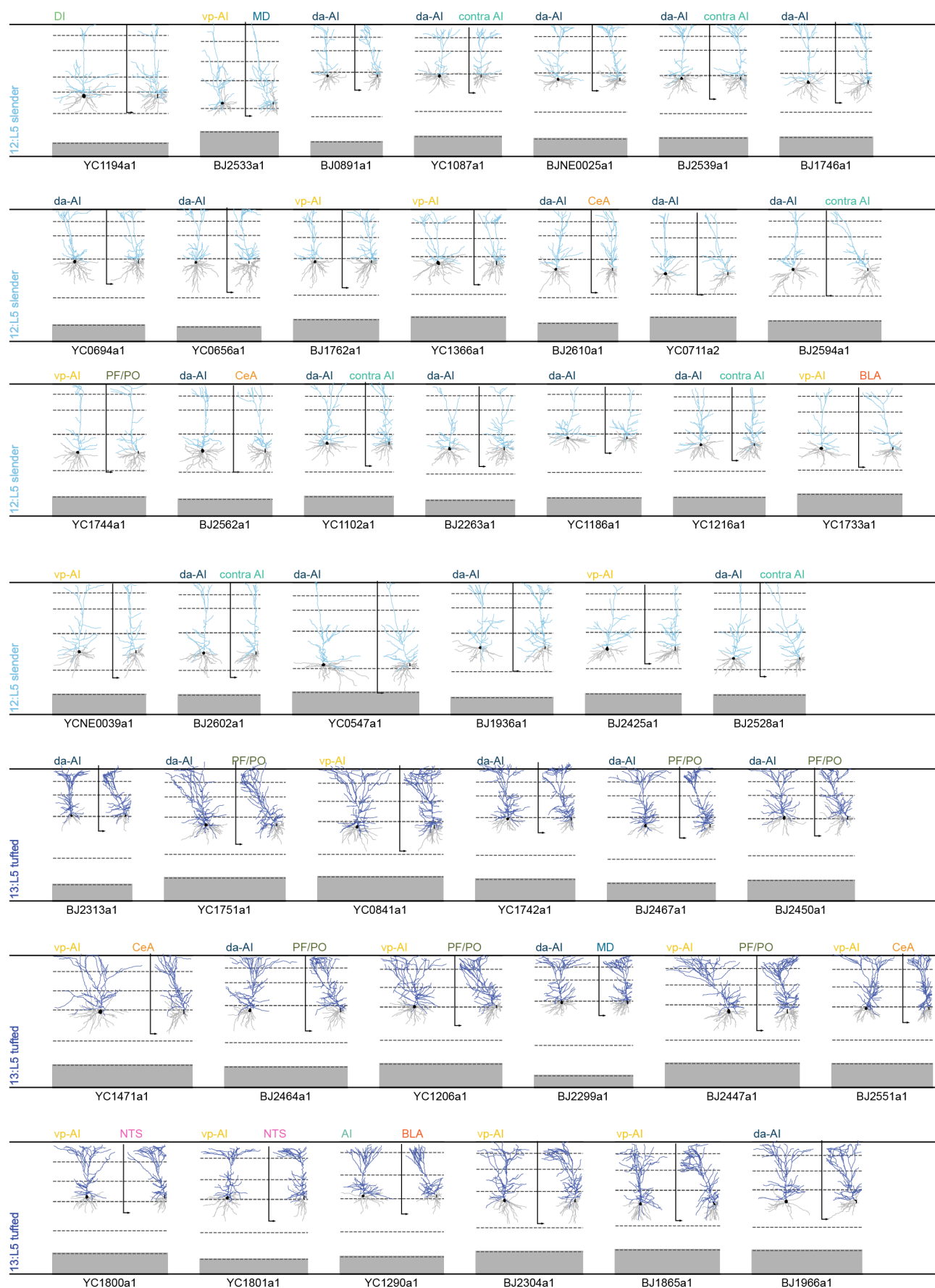


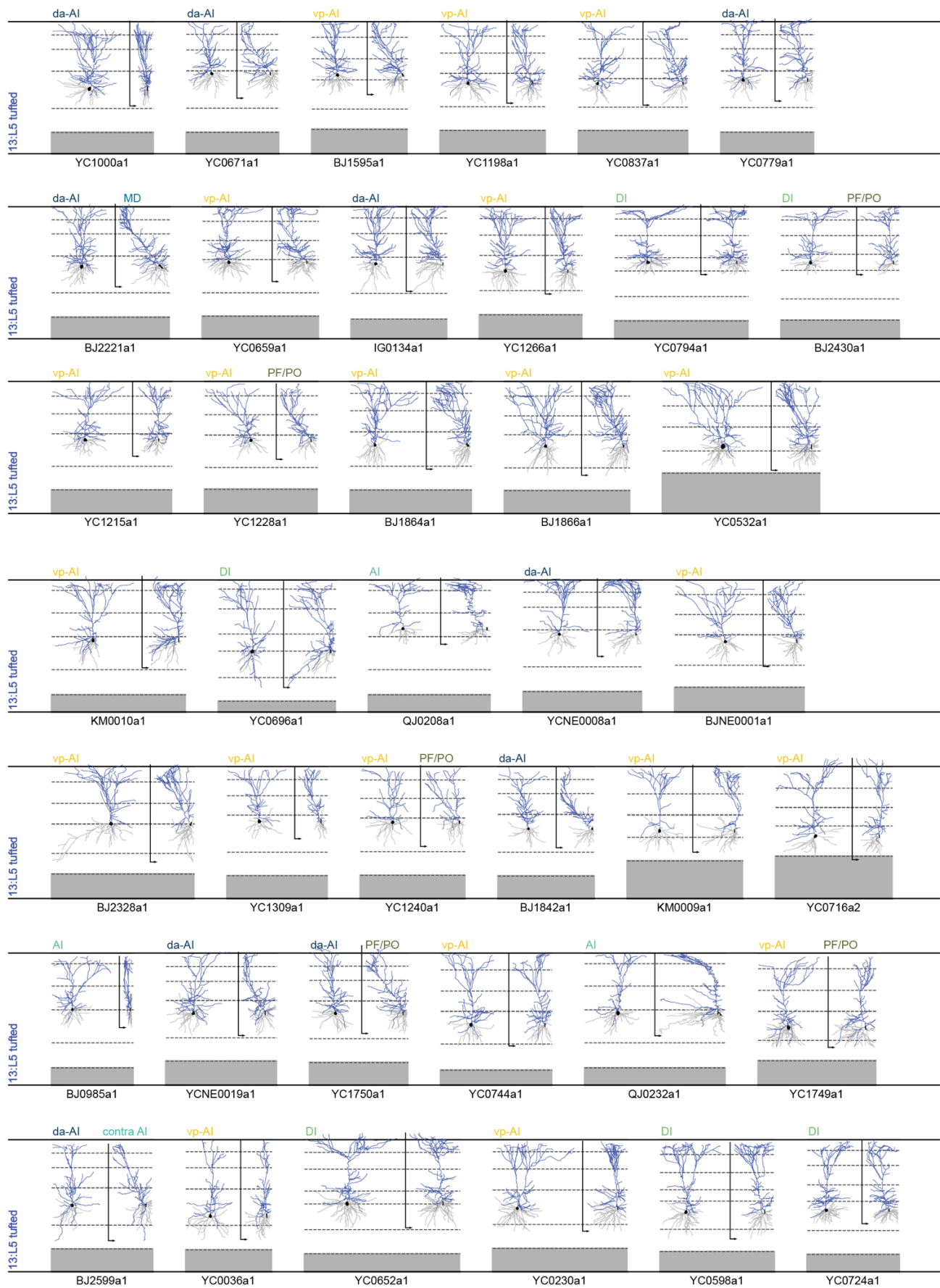




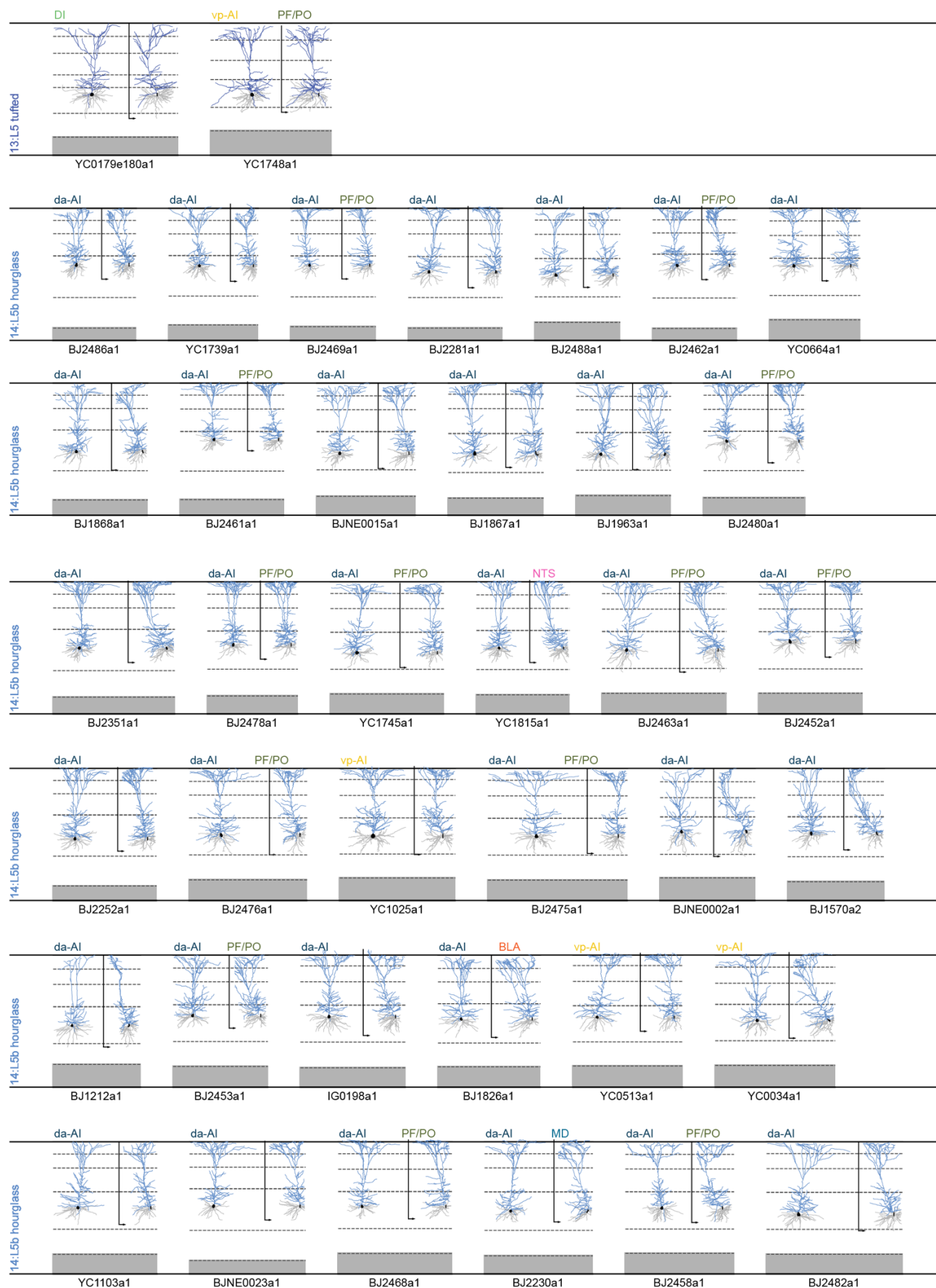




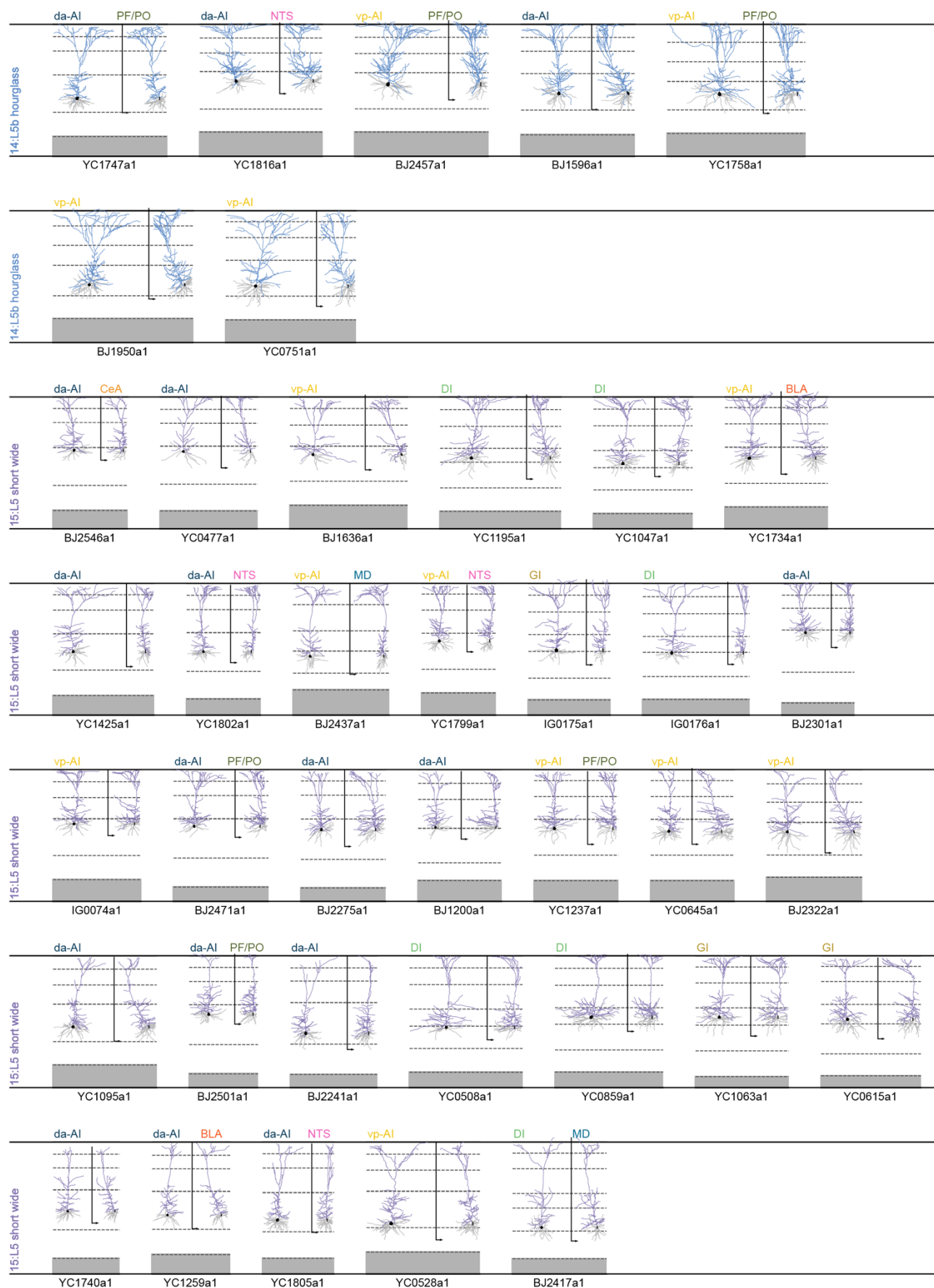


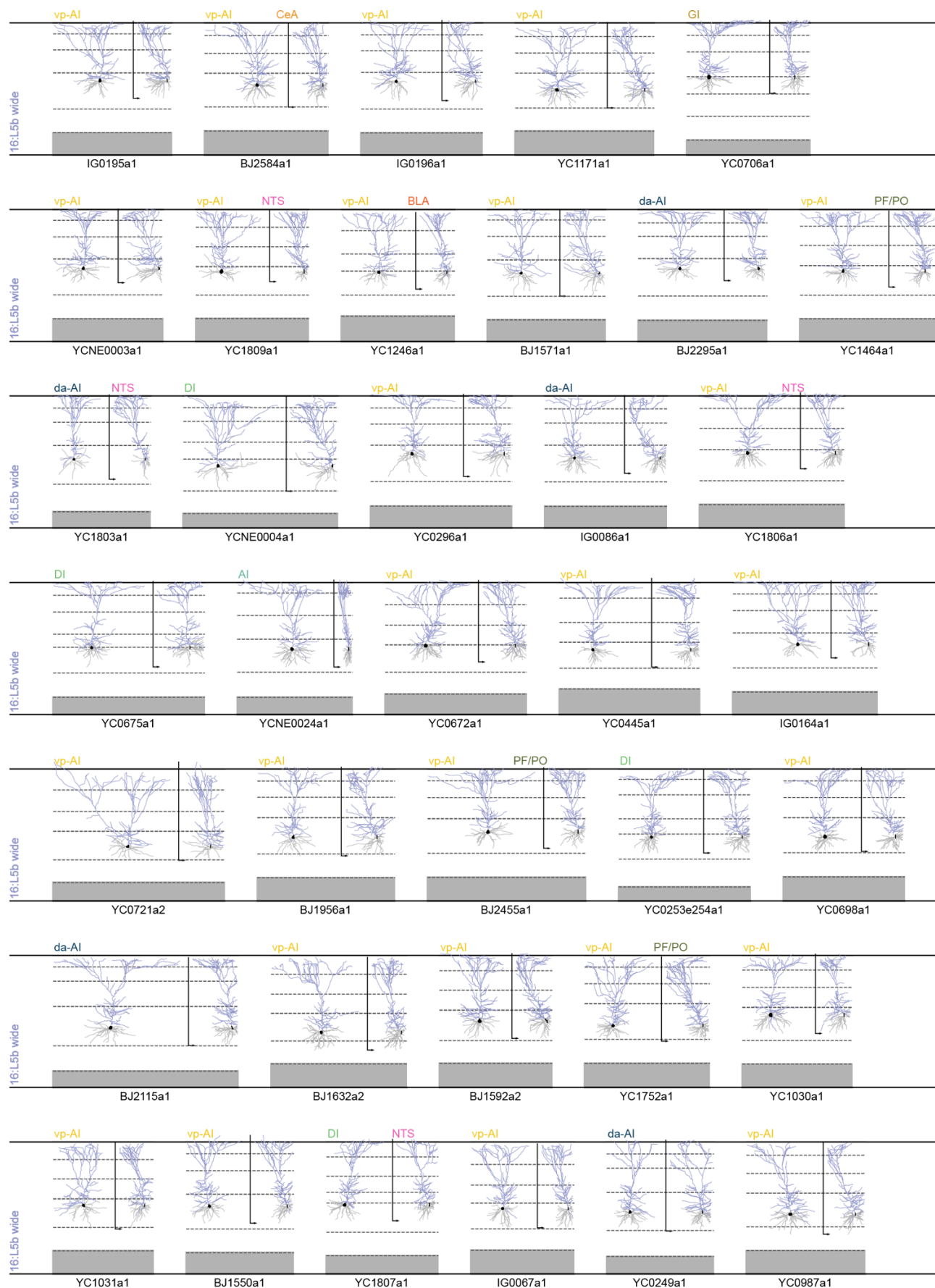


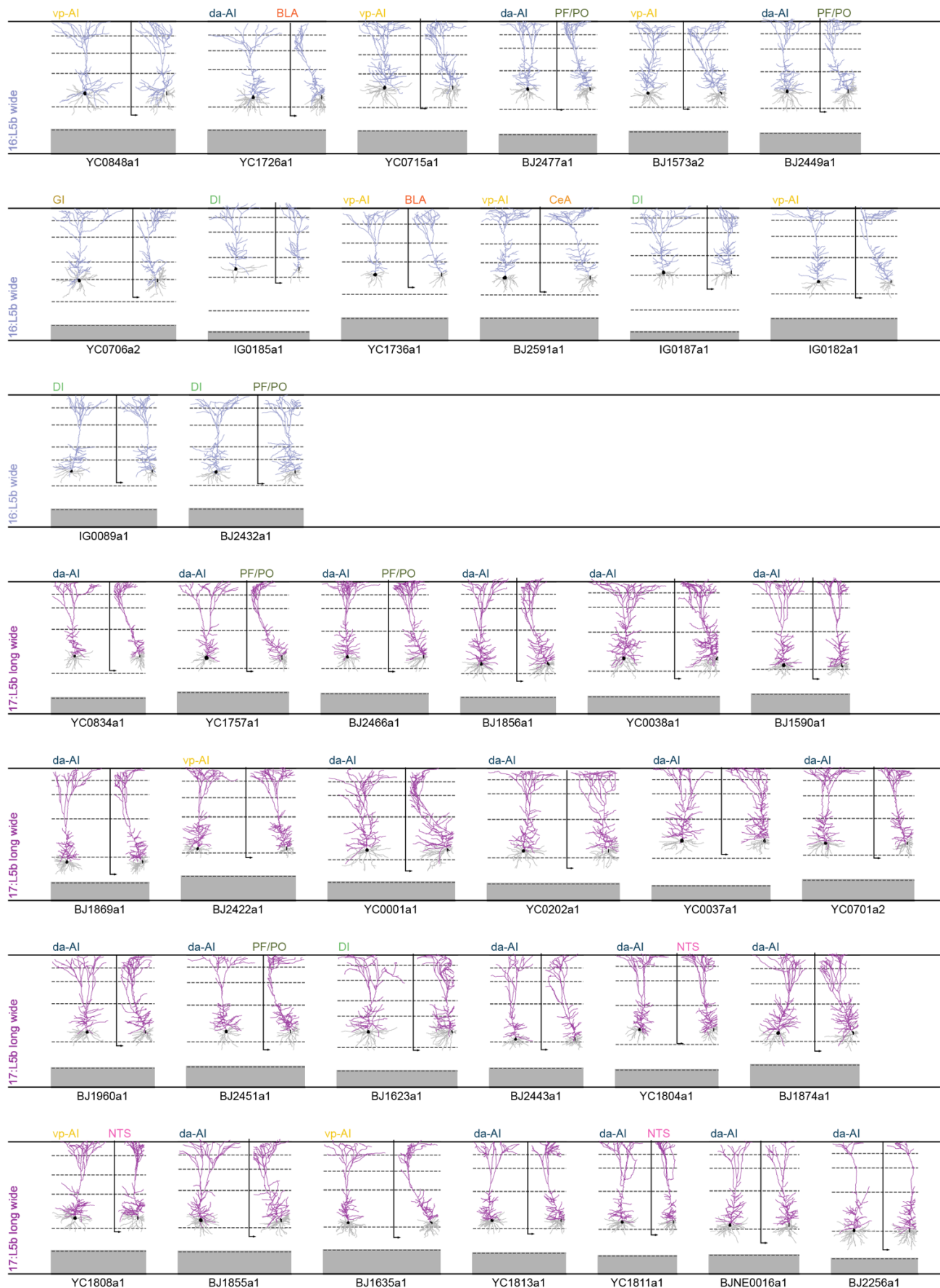


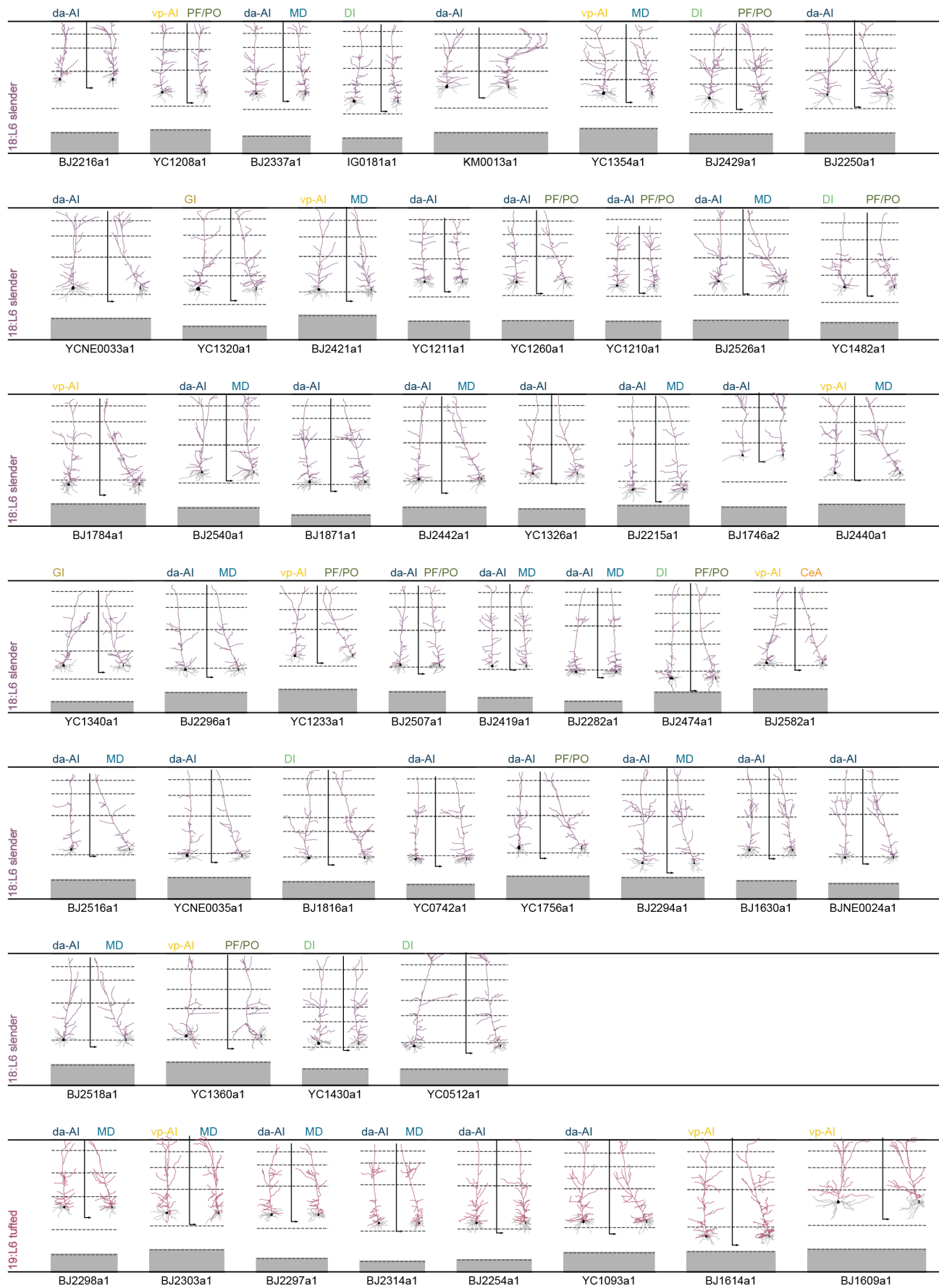




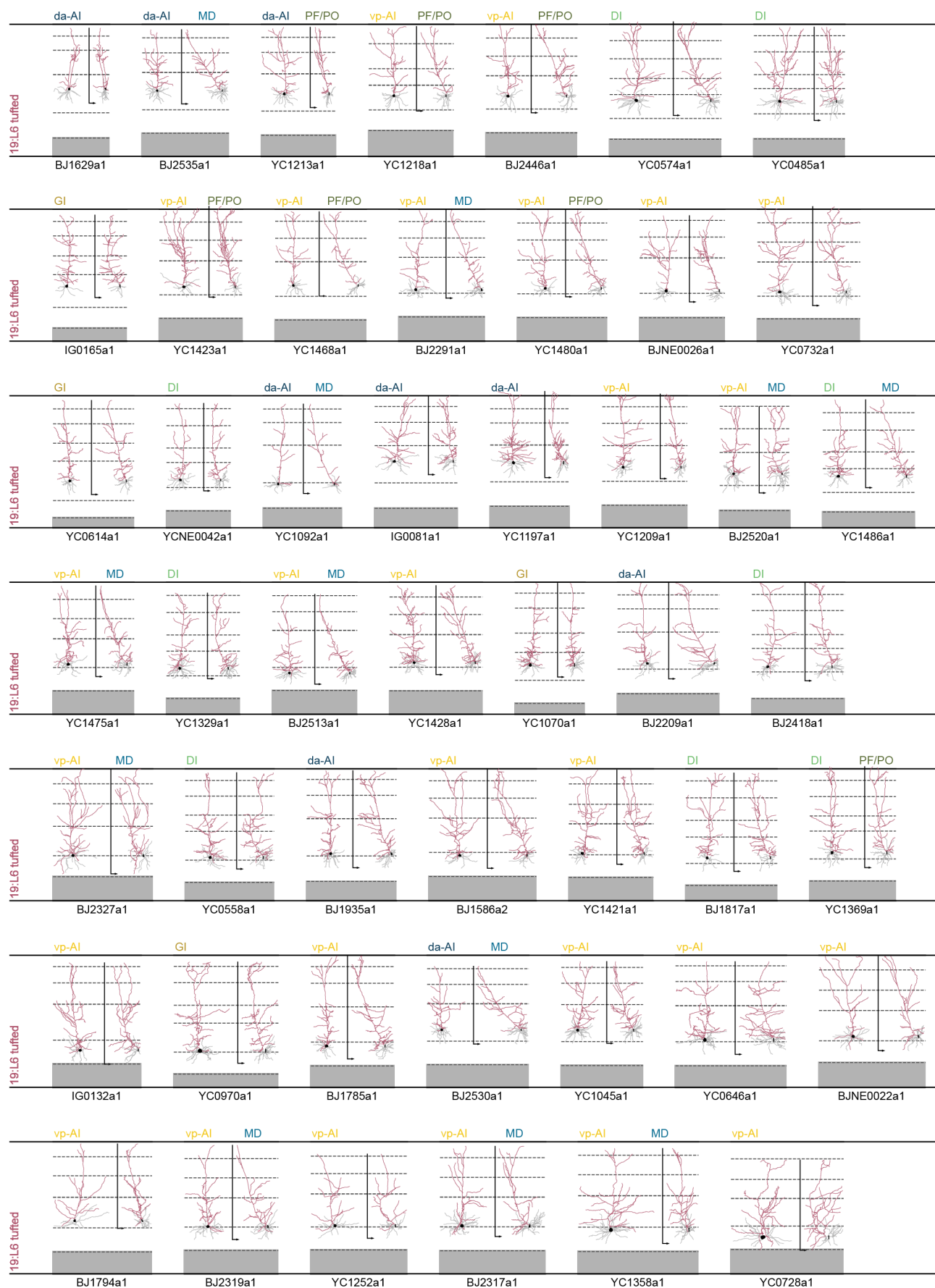




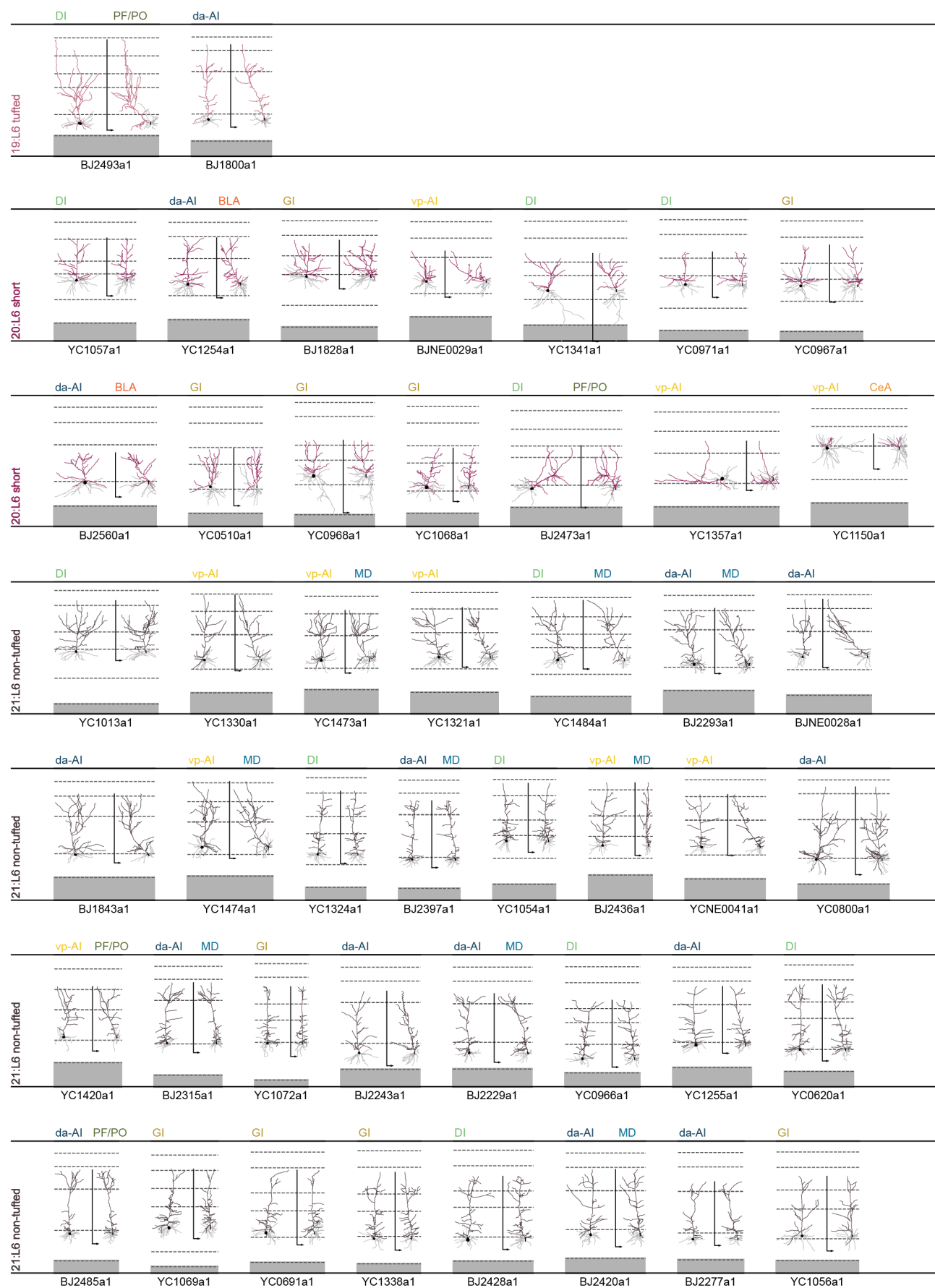


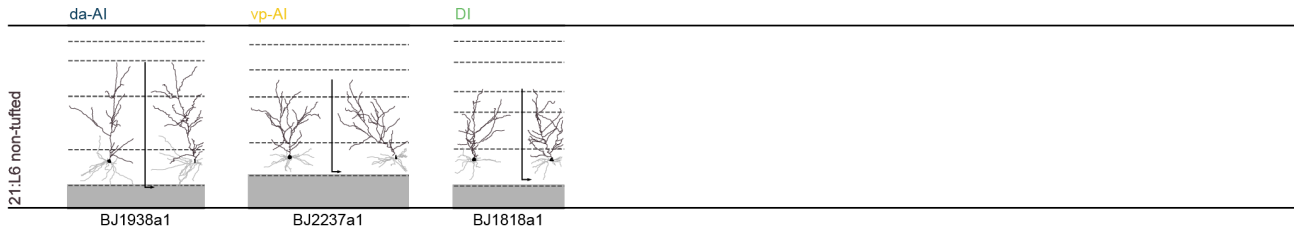




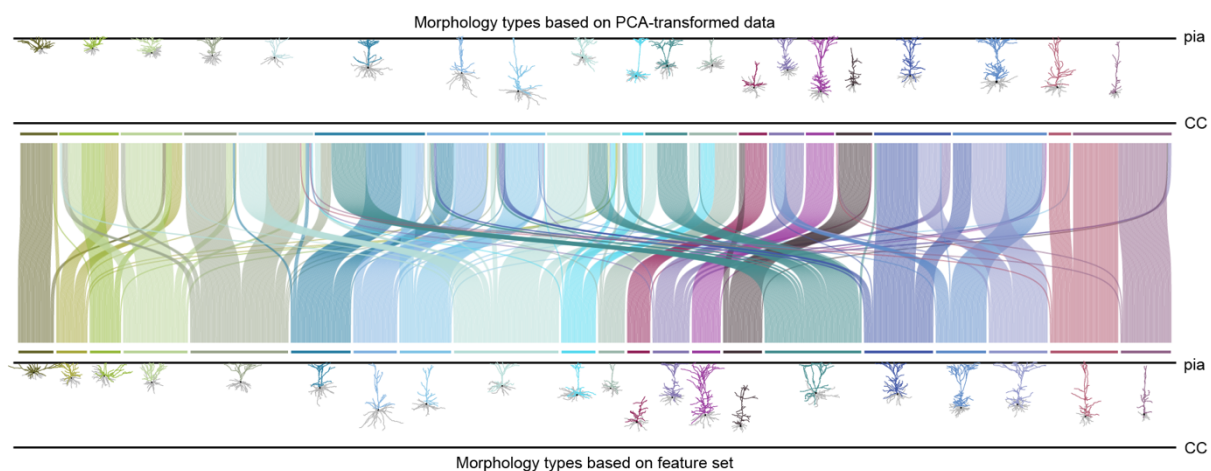






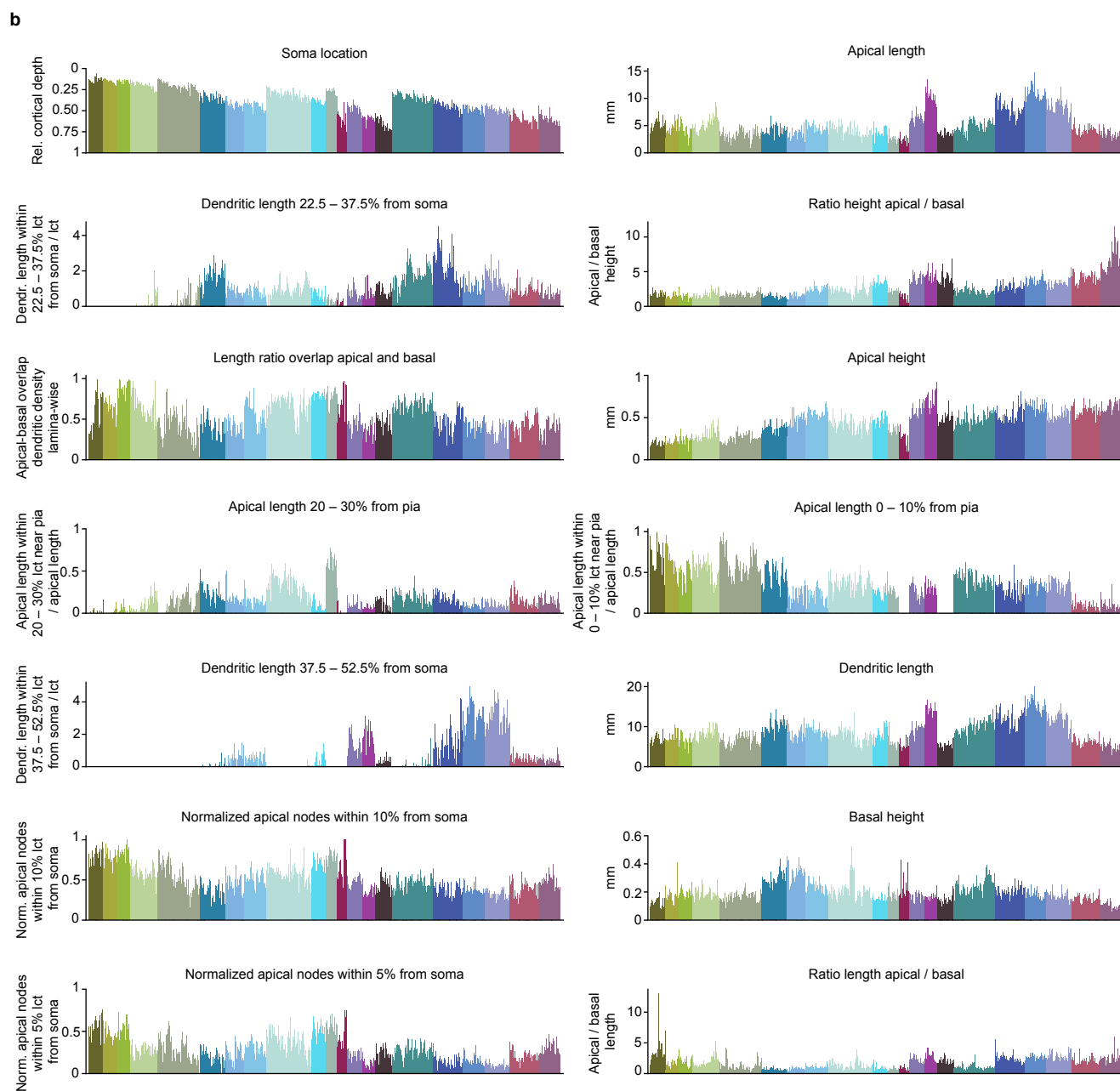
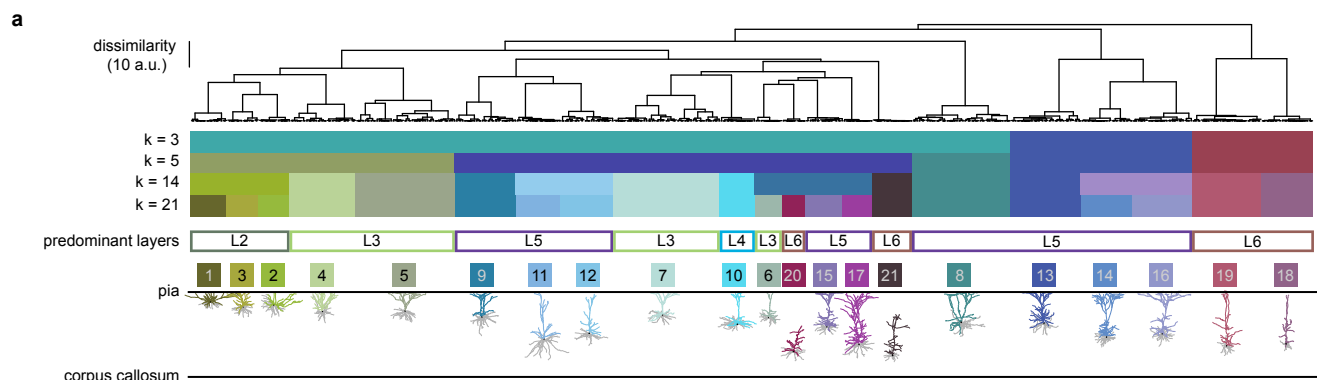


**Supplementary Fig. 4: Overview of 958 reconstructed dendritic morphologies.** Apical (colored) and basal (gray) dendrites shown in the *en face* (left) and side-view (right) planes for each reconstructed morphology that passed quality control—these morphologies were used for iCCA and as the classifier training dataset (Methods). Dimensions are scaled to the local cortical thickness. Dashed lines demarcate layer boundaries between pia (top) and corpus callosum (bottom) for L1, L2, L3, L4<sup>#</sup>, L5, L6, and claustrum (gray zone), as determined by mean local layer thickness following registration to the Nissl atlas (Extended Data Figs. 1h and 2, Supplementary Fig. 3). Corresponding insular subregion allocations (top-left) and projection targets (top-right, if determined) are shown as text. Individual morphologies are sorted within each M-type based on cluster analysis dendrogram. <sup>#</sup>: L4 is only shown when located in the (dys)granular insula. da-AI: dorsoanterior agranular insula; vp-AI: ventroposterior agranular insula; AI<sup>\*</sup>: agranular insula; DI: dysgranular insula; GI: granular insula; BLA: basolateral amygdala; CeA: central amygdala; contra AI: contralateral agranular insula; MD: mediodorsal thalamus; NTS: nucleus tractus solitarius; PF/PO: parafascicular/posterior thalamus. <sup>\*</sup>: 39 cells were recorded in acute coronal brain slices and thus not assigned to AI subregions.

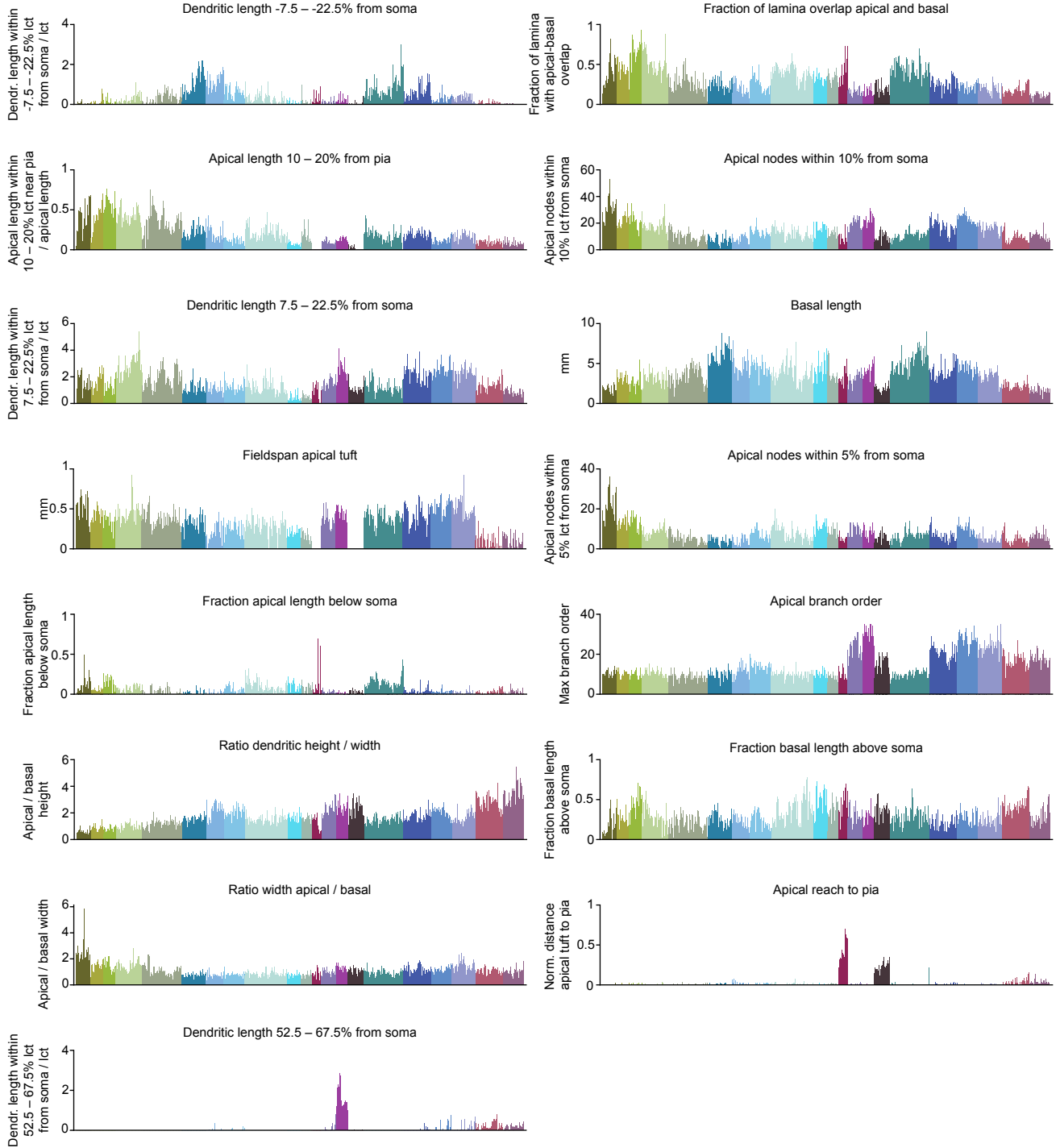


**Supplementary Fig. 5: Comparison of M-type allocations between two iCCA approaches.**

Representative morphologies for each cluster based on the PCA-transformed dataset (top row) or the best-performing iCCA unique feature set (bottom row) and cell-wise correspondence between the two analysis approaches (adjusted Rand index = 0.36).



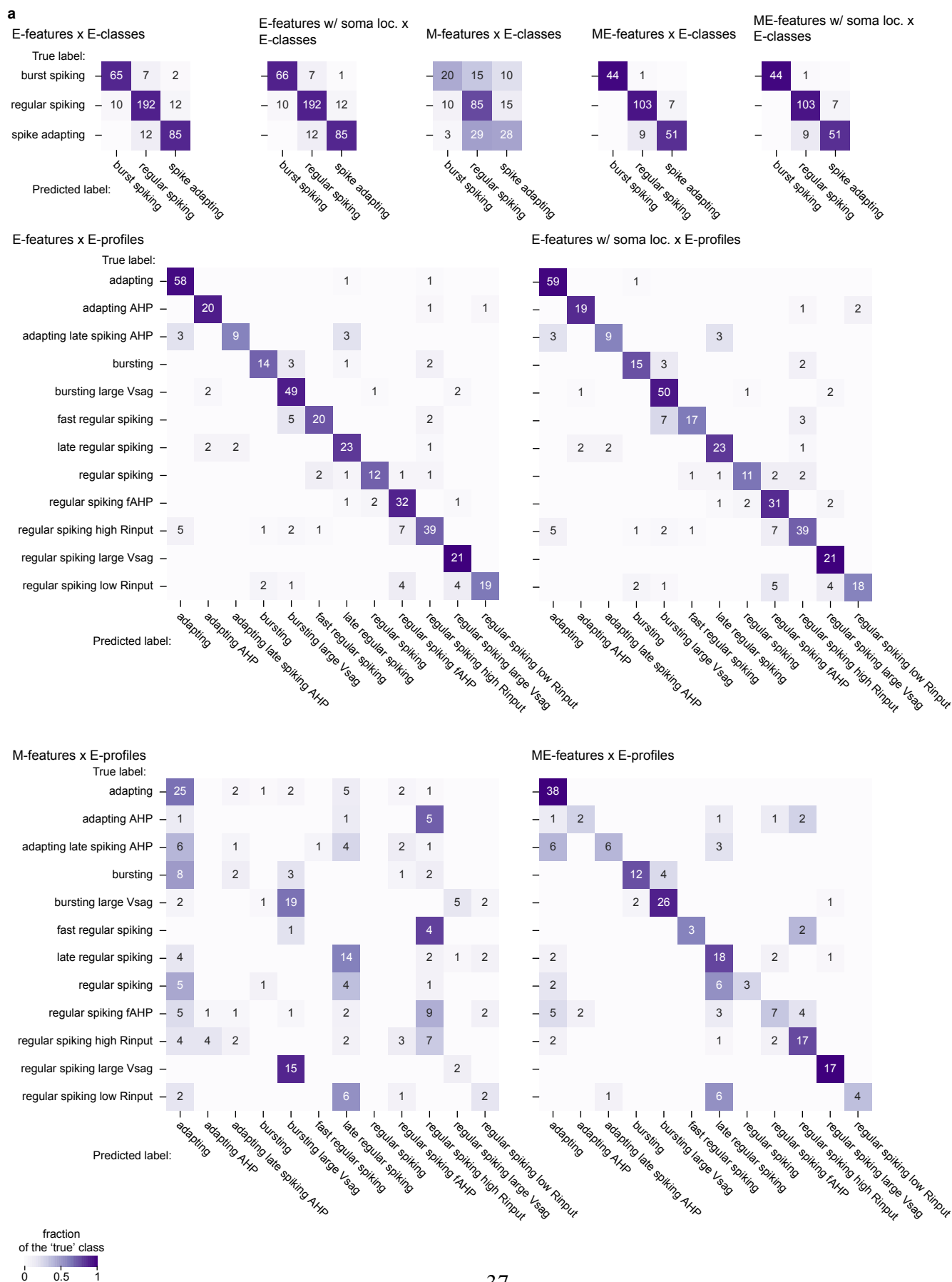
b (continued)



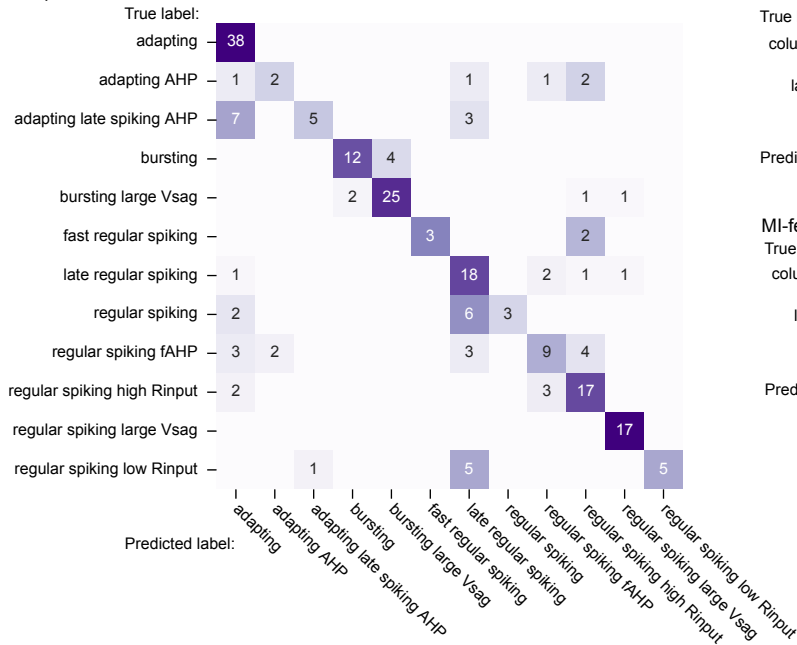
**Supplementary Fig. 6: Distinct features of M-types.** a, Consensus cluster dendrogram cut at different levels ( $k = 3, 5, 14, 21$  clusters) indicated by color coding below the dendrogram to show how M-types relate hierarchically. The representative morphologies (bottom) were aligned to  $k = 21$  clusters, same as in Fig. 2a.



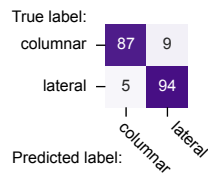
Layer allocation was based on the predominant layer. **b**, All 29 features used for the iCCA, sorted by random forest classifier-derived Gini-index (Extended Data Fig. 4a). Histogram bars represent individual cells (n = 958) and are sorted in the same sequence as the dendrogram in (**a**), color-coded based on corresponding M-types.



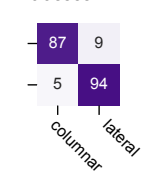
ME-features w/ soma loc. x E-profiles



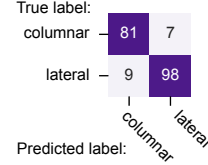
I-features x I-classes



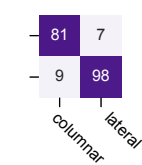
I-features w/ soma loc. x I-classes



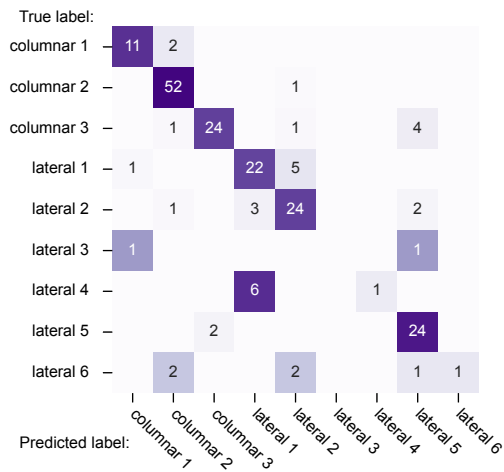
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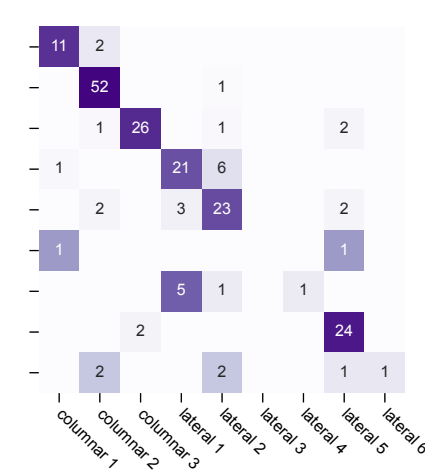
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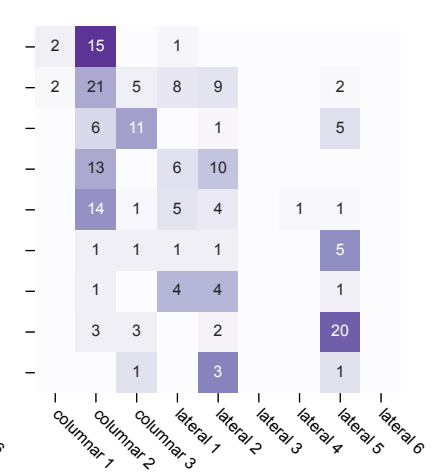
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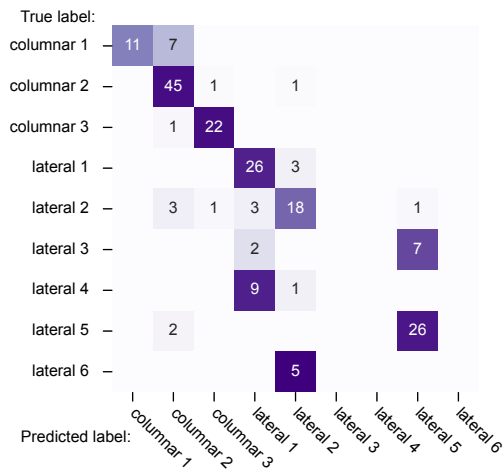
I-features w/ soma loc. x I-profiles



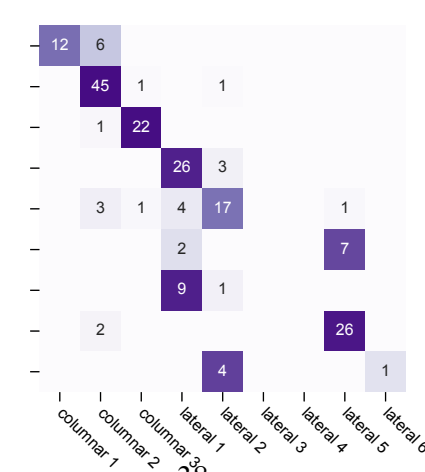
M-features x I-profiles



MI-features x I-profiles



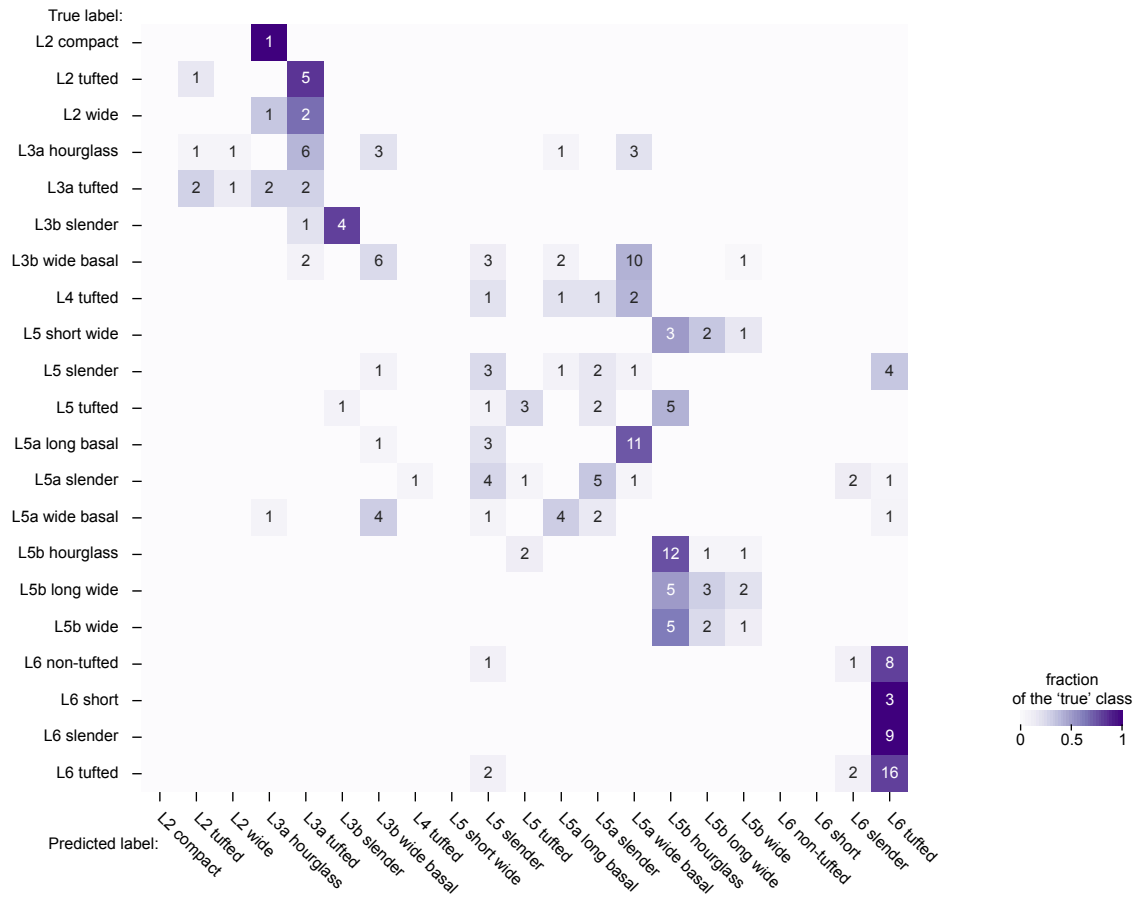
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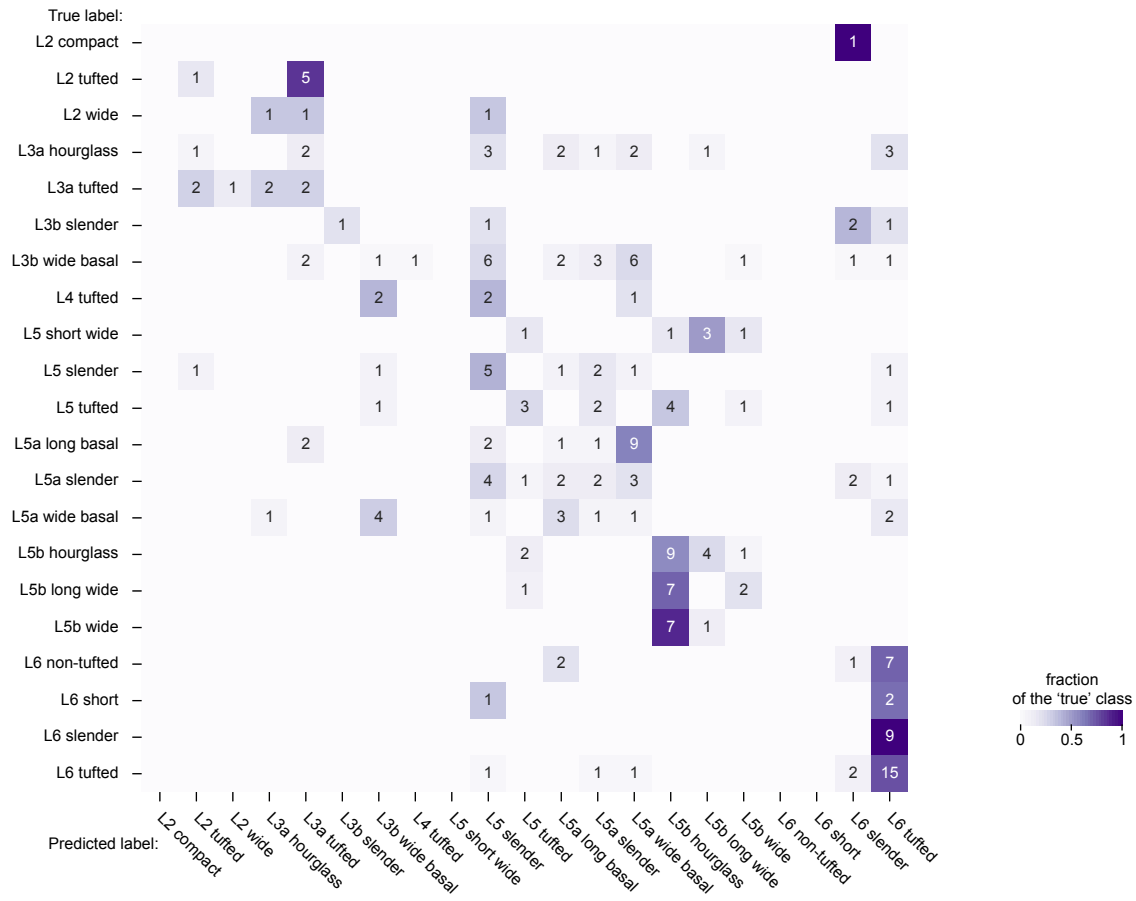
fraction  
of the 'true' class

0 0.5 1

E-features w/ soma loc. x M-types

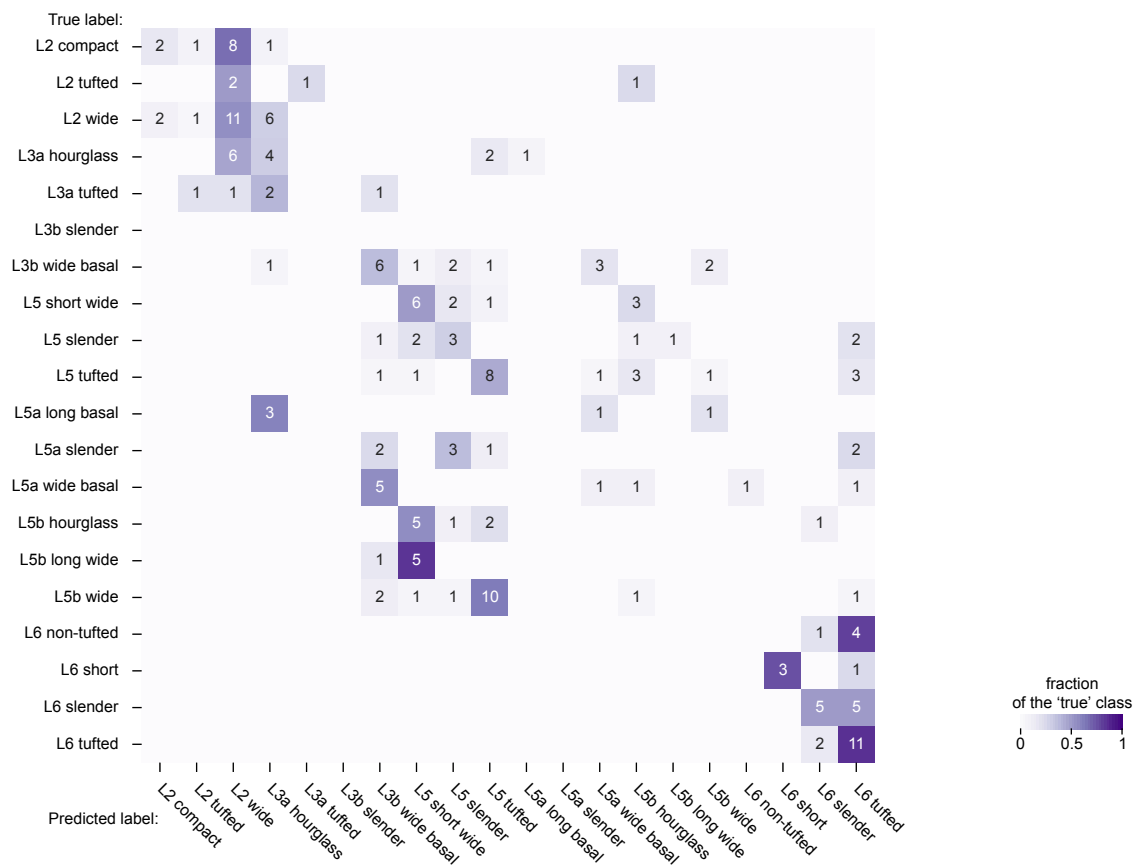


E-features x M-types

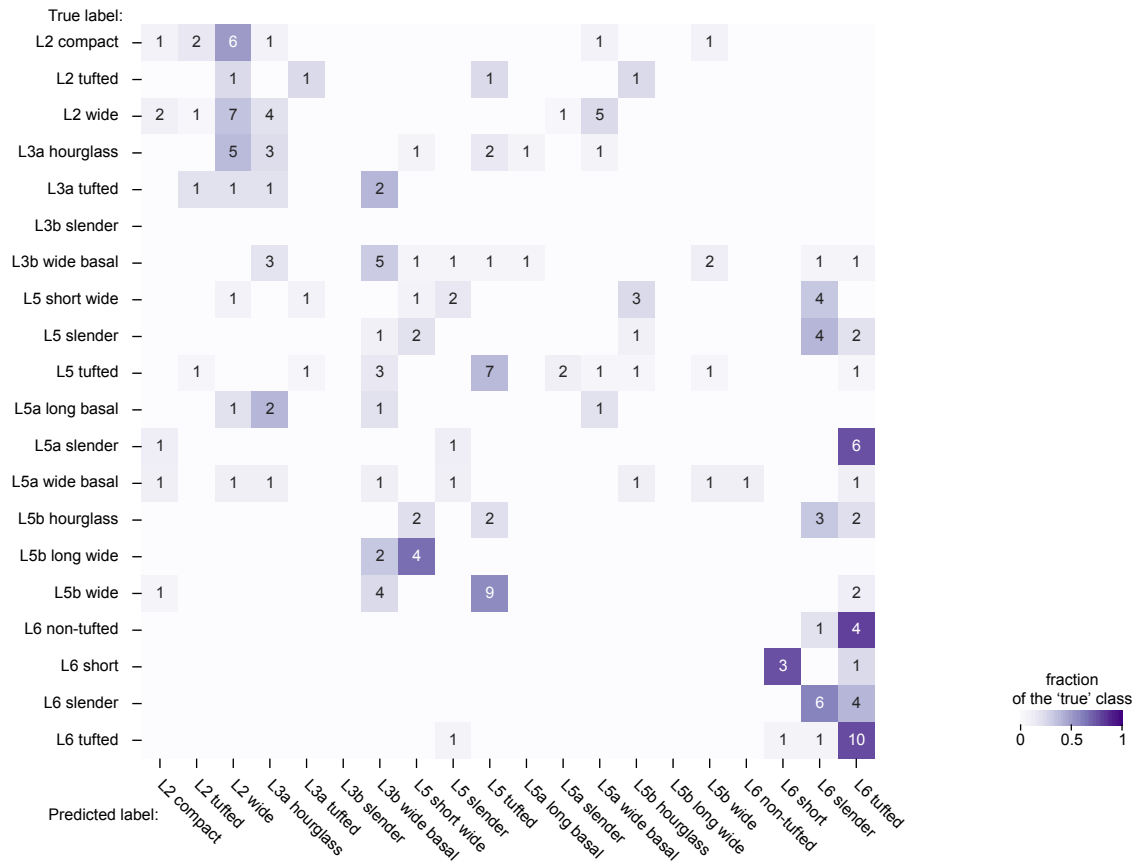




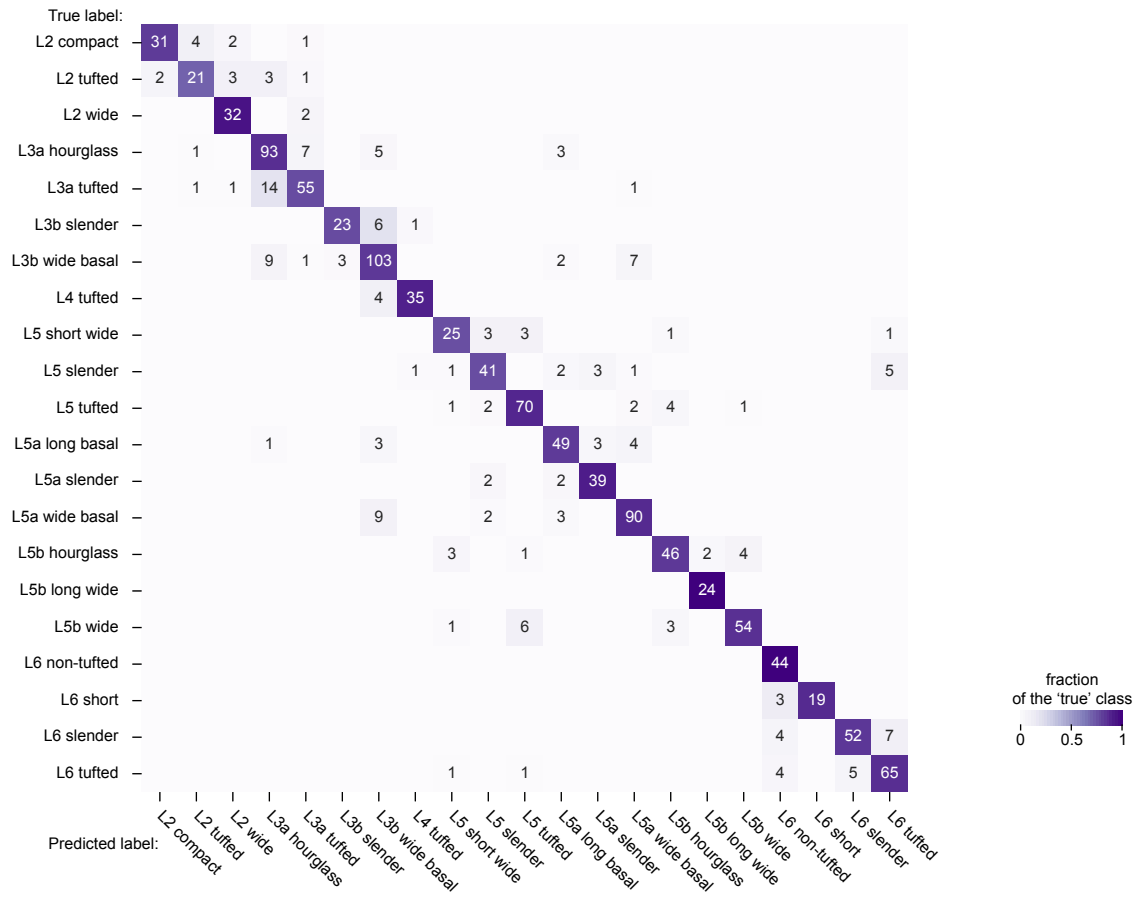
I-features w/ soma loc. x M-types



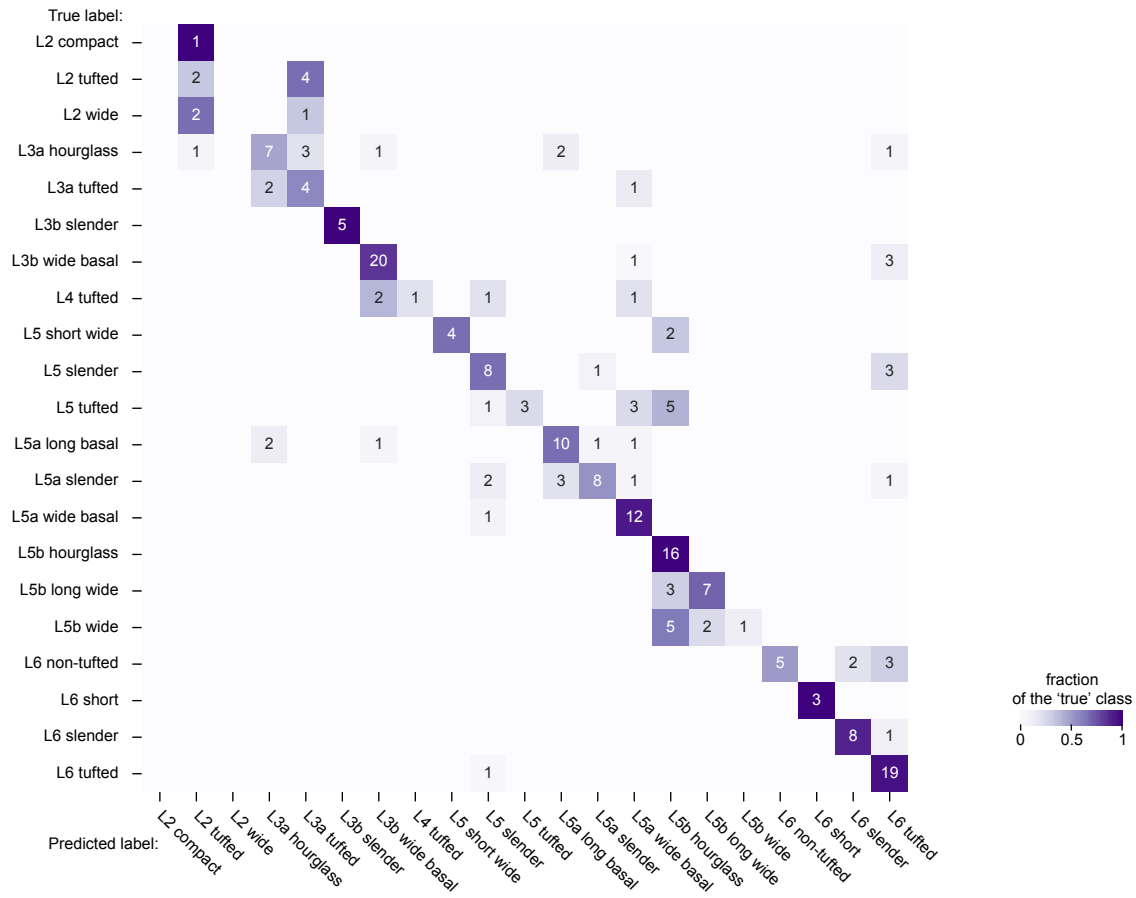
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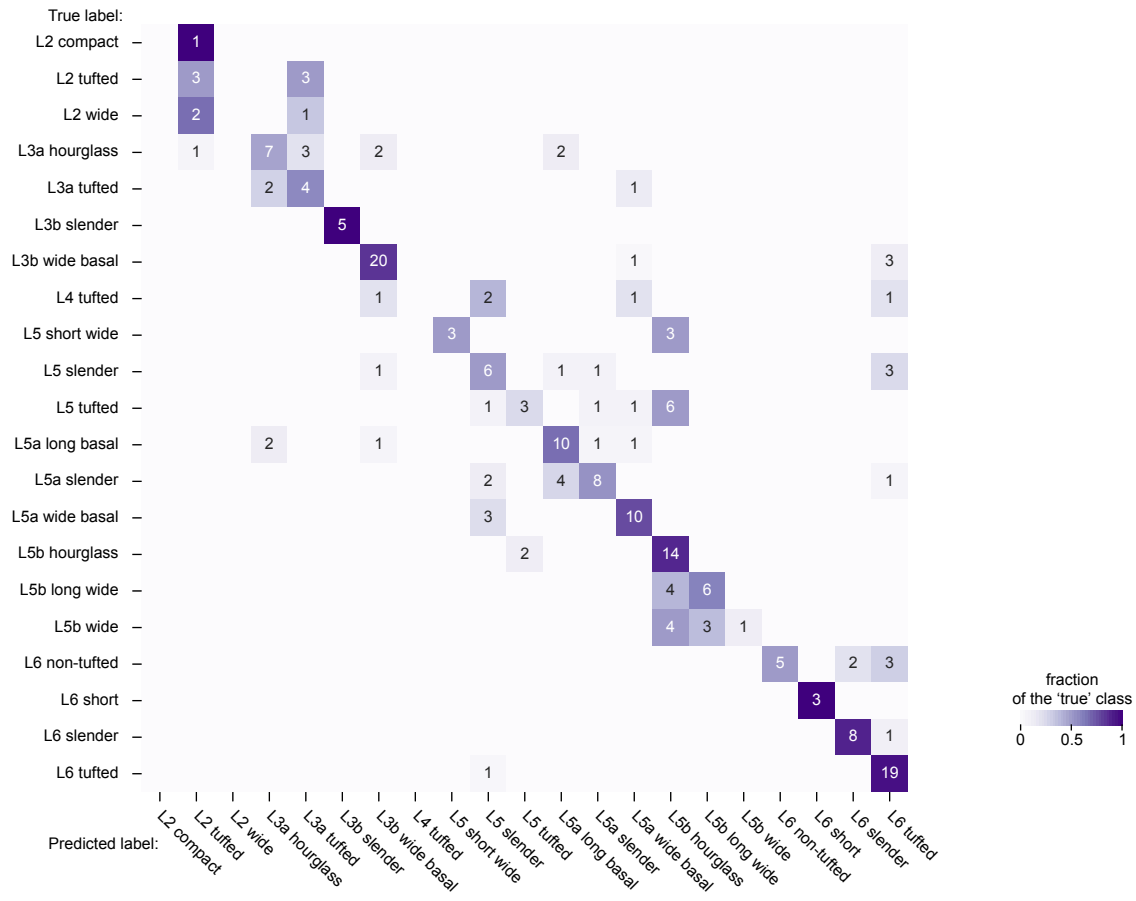
M-features x M-types



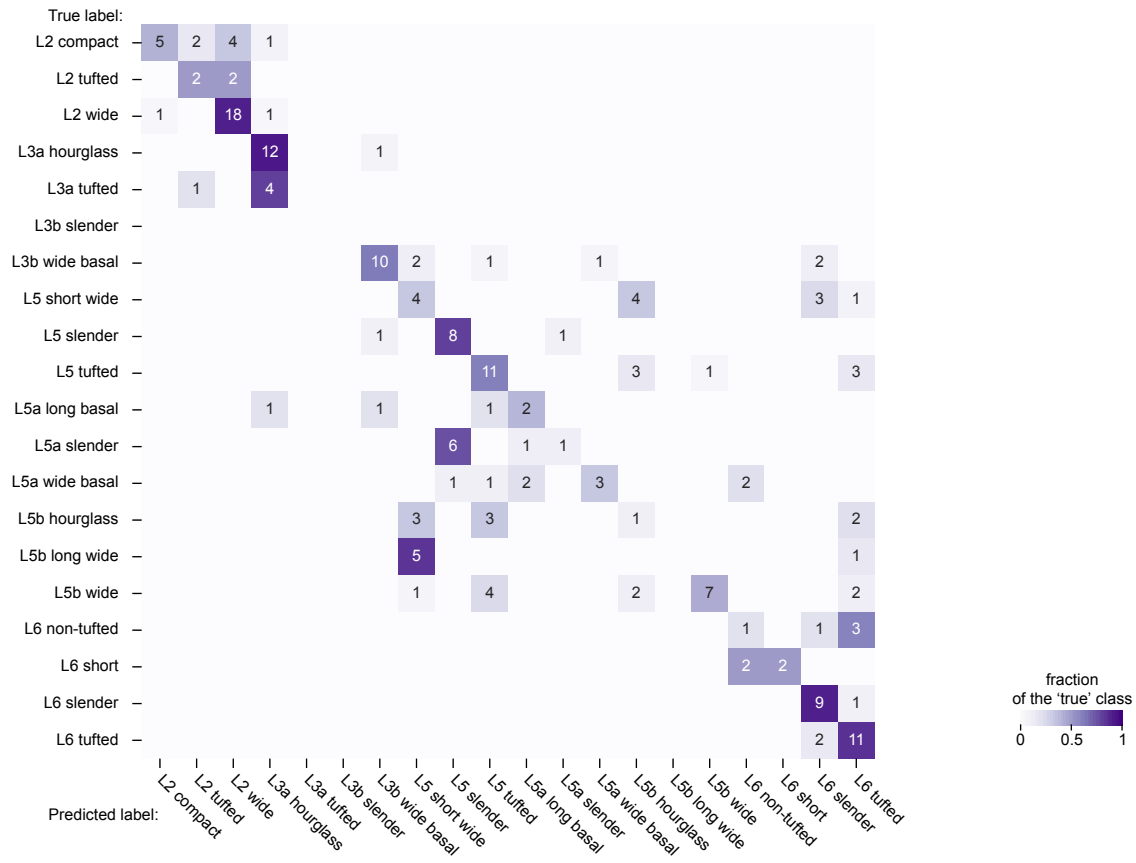
ME-features x M-types



ME-features w/ soma loc. x M-types

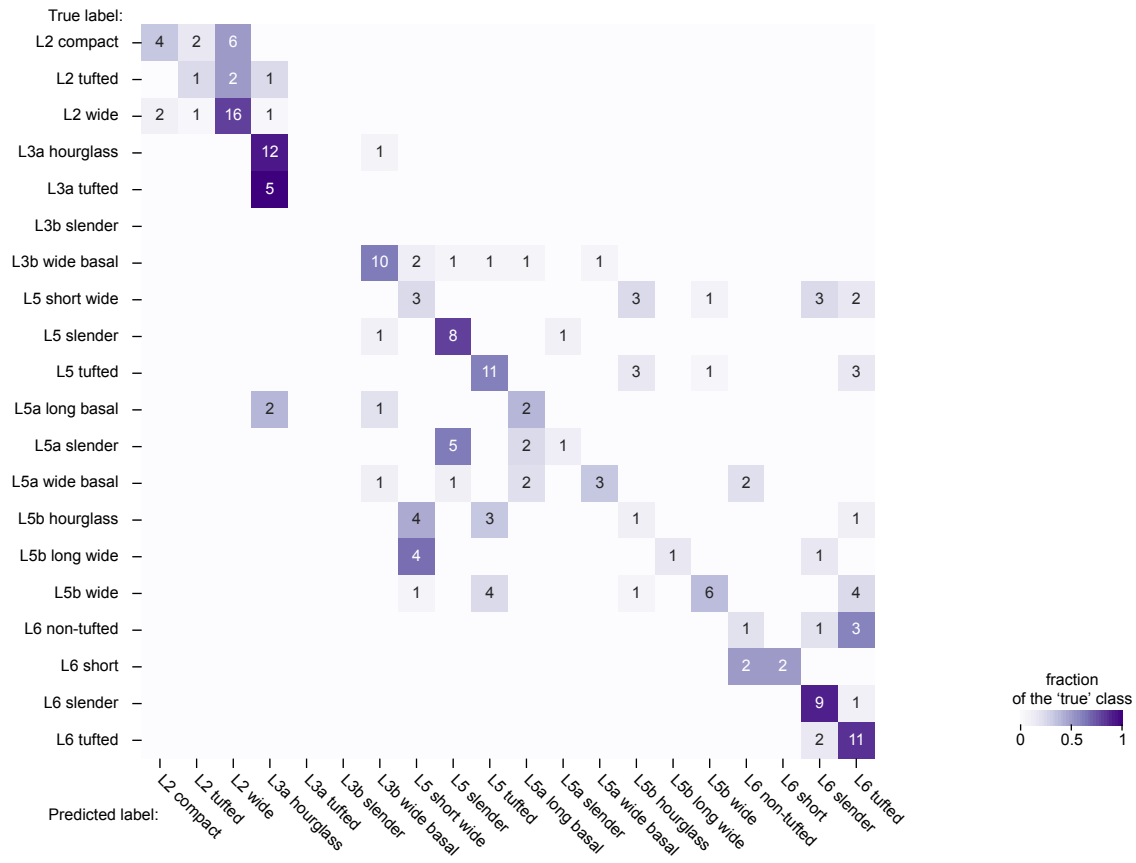


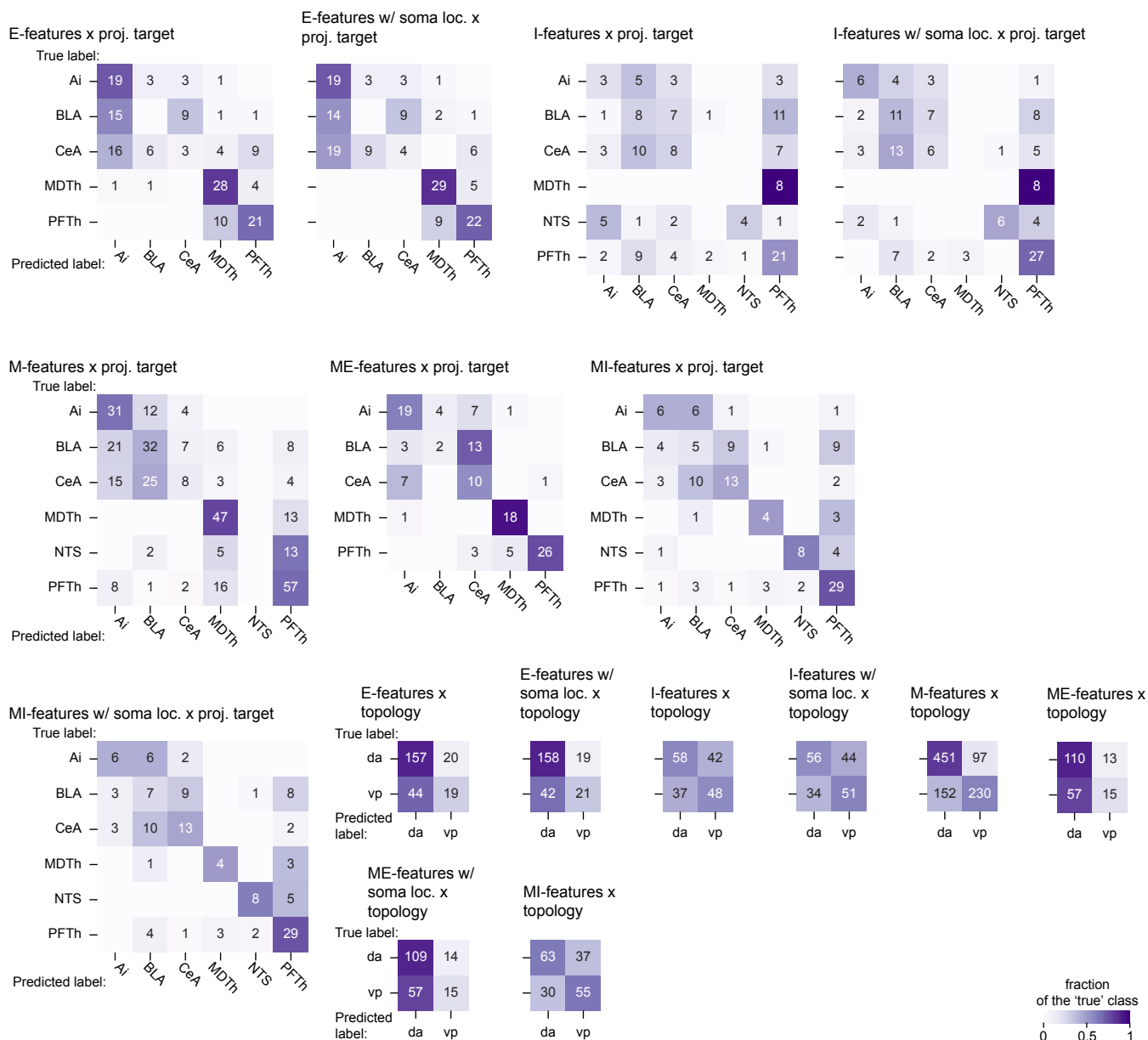
MI-features x M-types





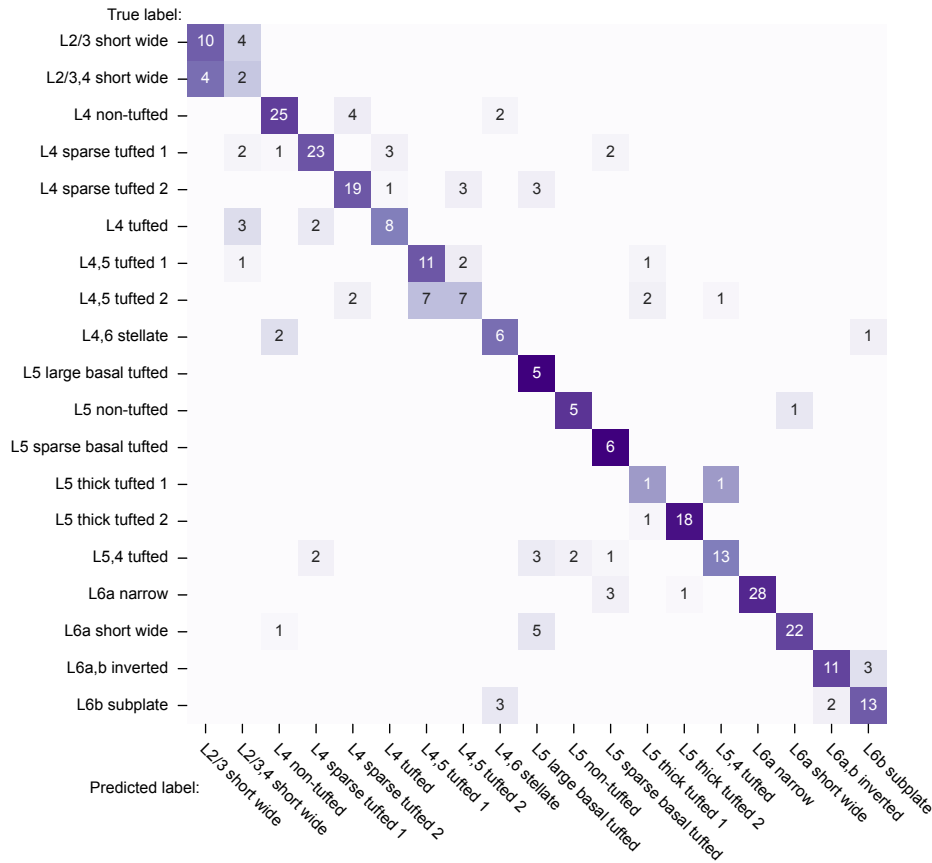
MI-features w/ soma loc. x M-types



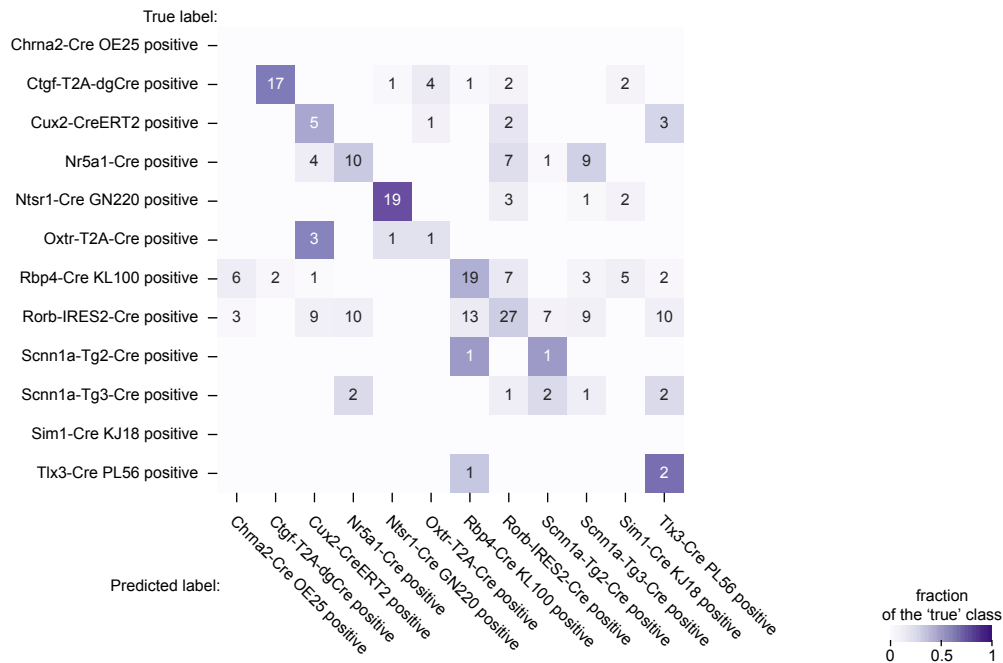


b

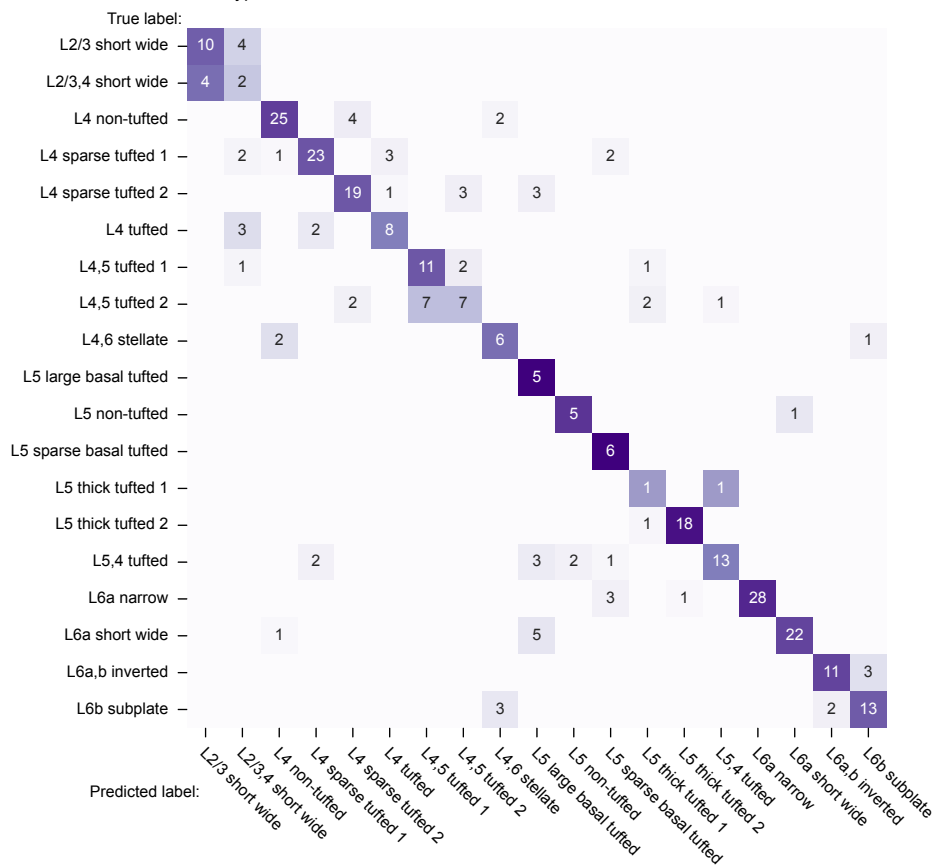
V1 data: z-scored M-features x M-types



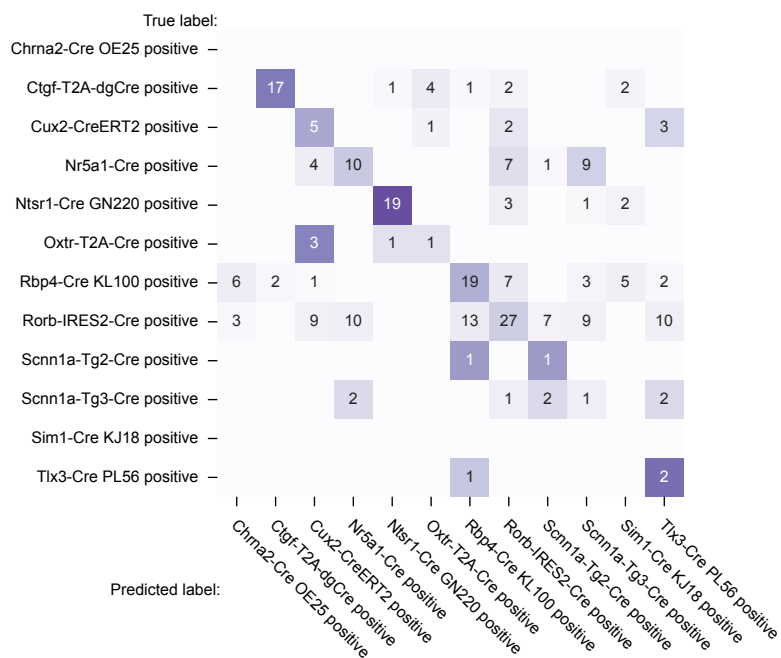
V1 data: z-scored M-features x Cre-drivers



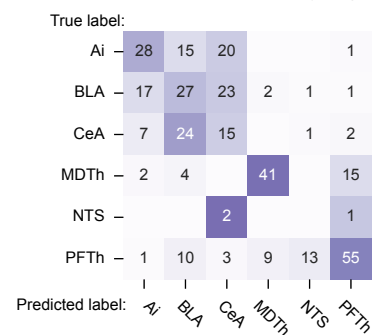
V1 data: M-features x M-types



V1 data: M-features x Cre-drivers



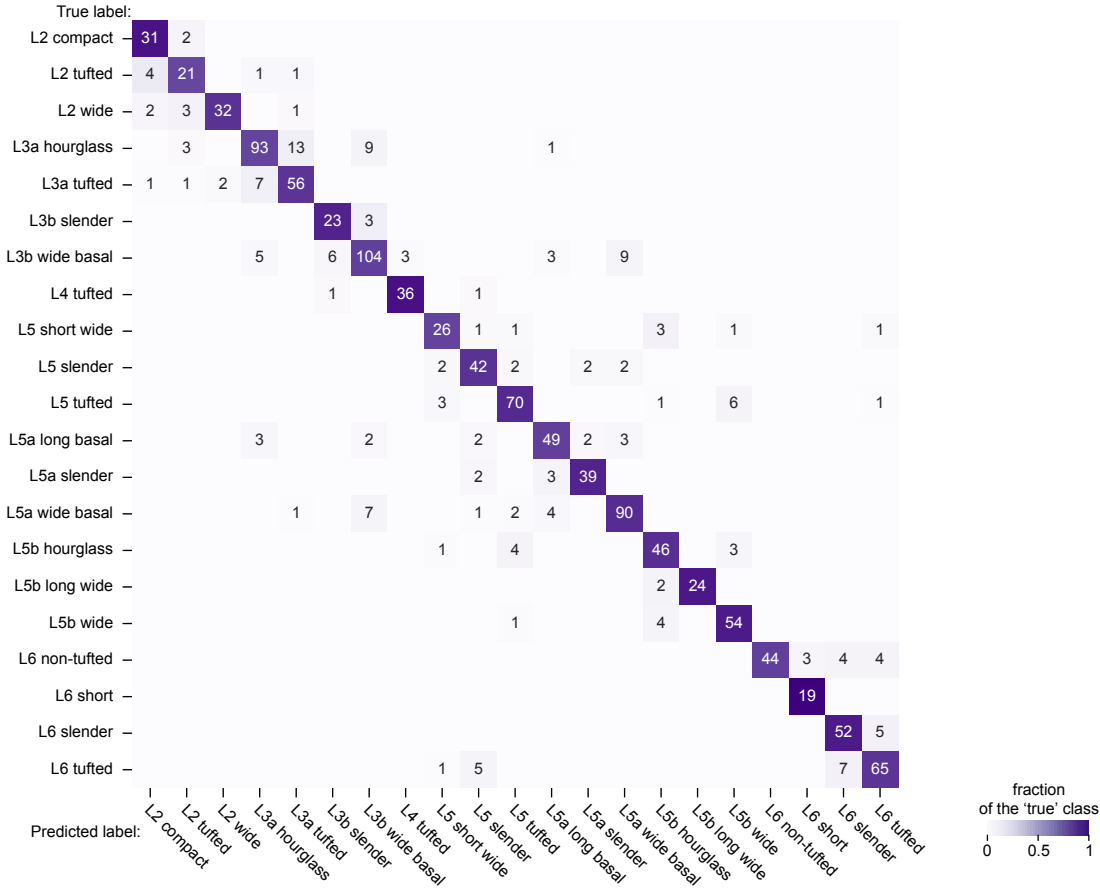
Insula data: z-scored M-features x proj. target



fraction  
of the 'true' class

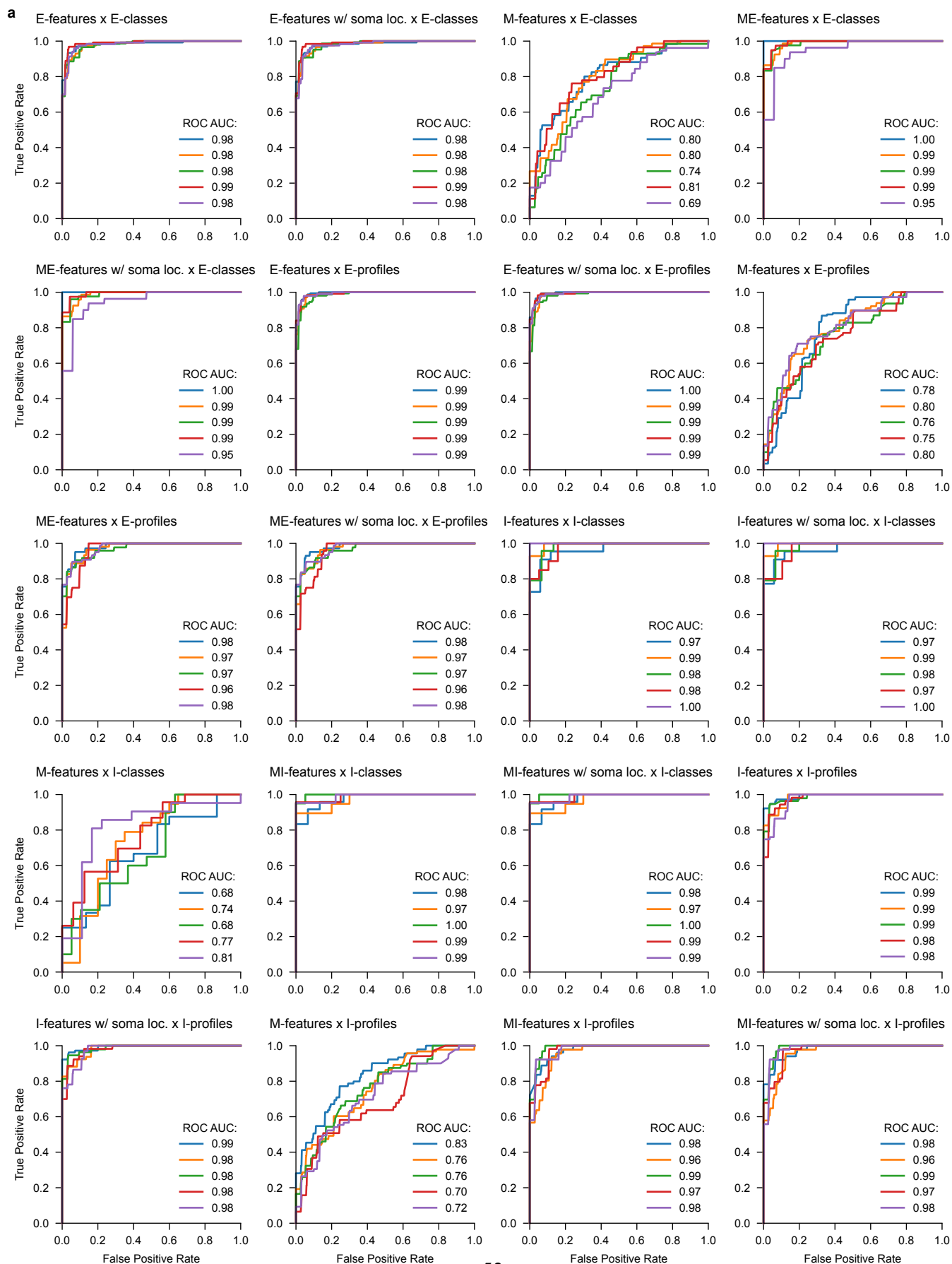
0 0.5 1

Insula data: z-scored M-features x M-types

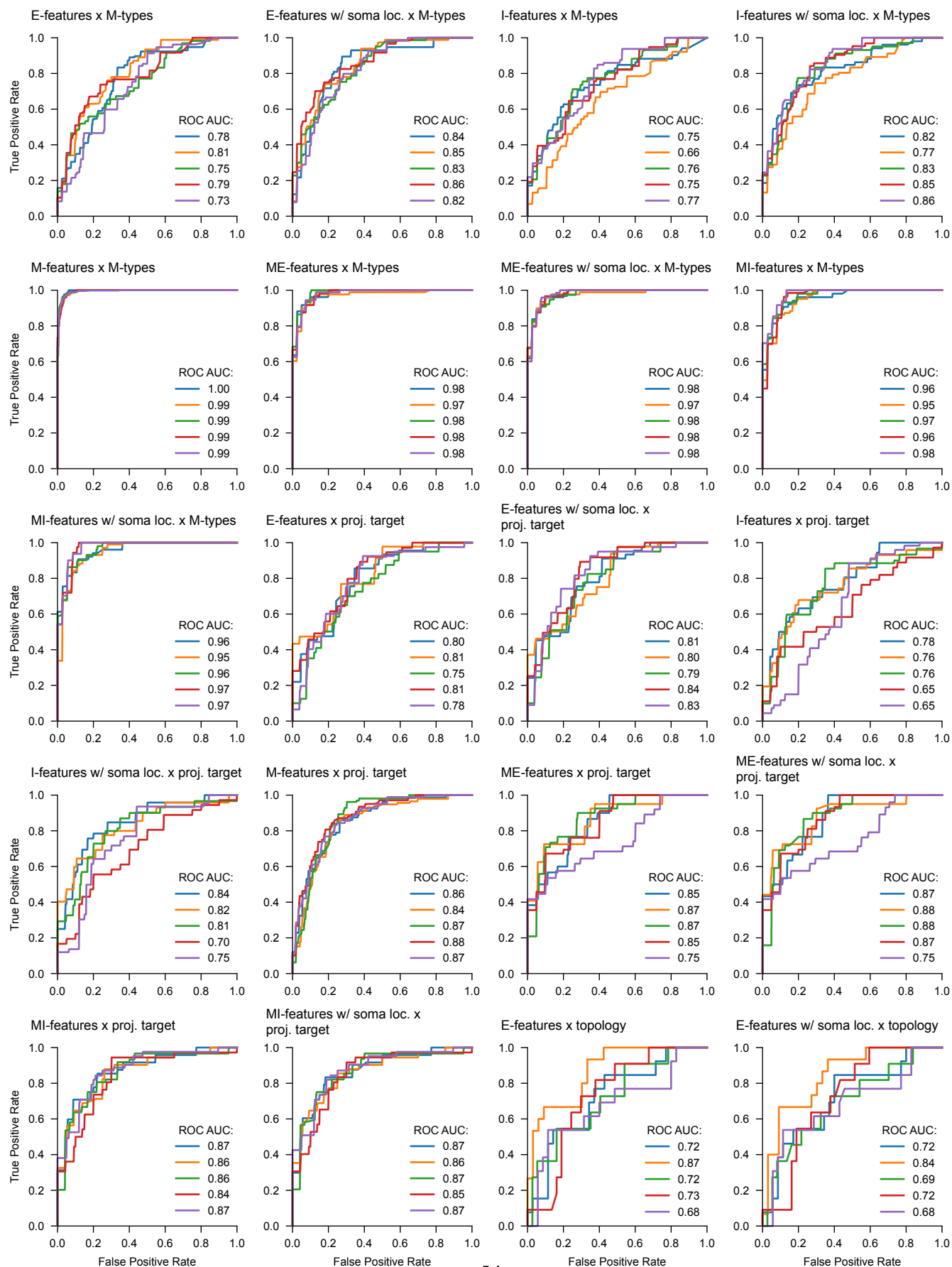


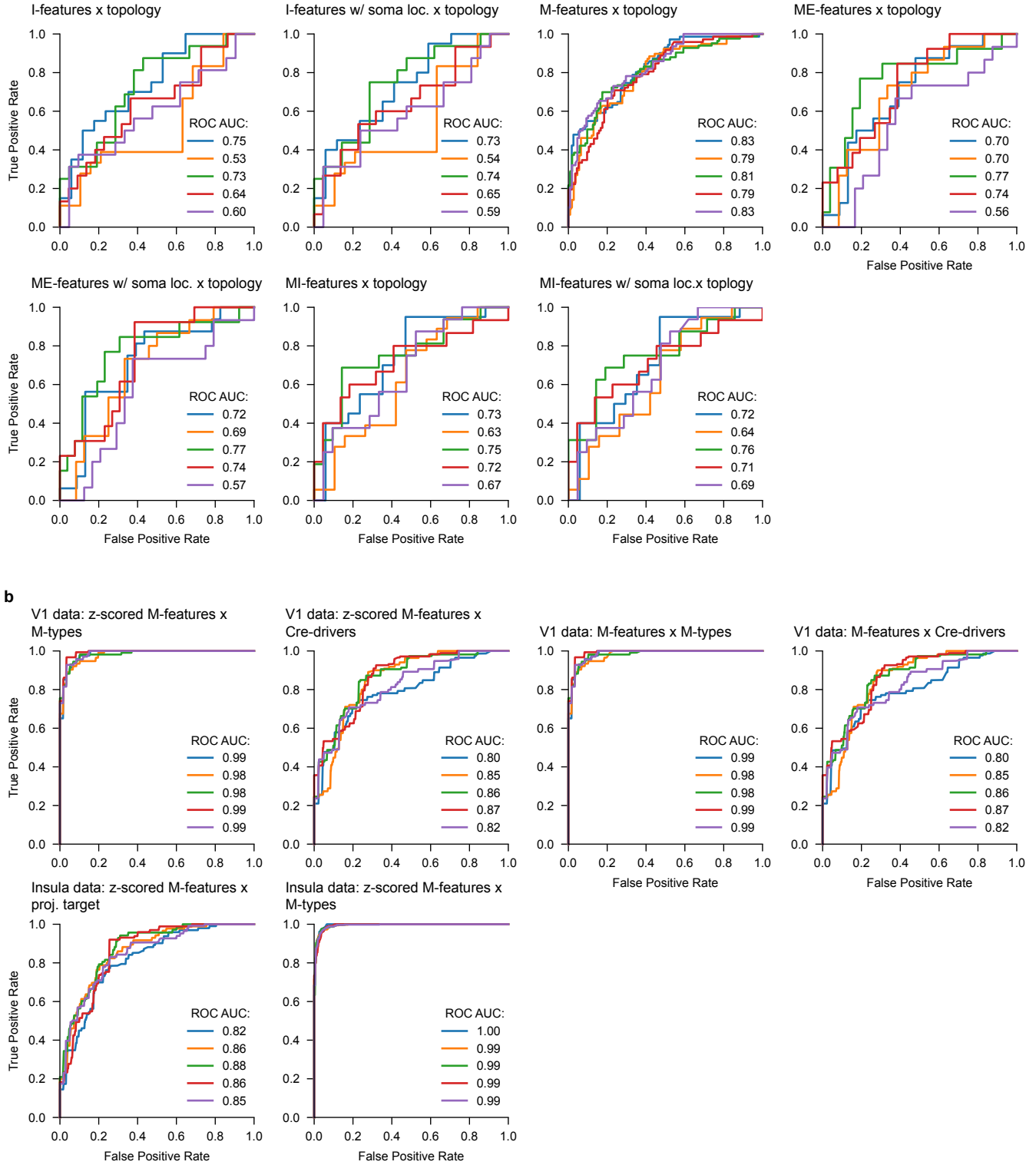
**Supplementary Fig. 7: Confusion matrices of trained random forest classifiers.** **a**, Performances of classifiers trained on untransformed insular data. Subpanel order matches order of comparisons shown in Fig. 7a. **b**, Performances of classifiers trained on z-score transformed V1 data, untransformed V1 data, and z-score transformed insula data (V1 data from Gouwens et al.<sup>33</sup>). For (**a** and **b**), number of observations displayed in purple-hued boxes were accumulated over five independent instances of classifier training for each type of classifier, e.g., to predict electric (E)-class based on E-features (see Methods). For example, in the case of E-class predicted by E-features,  $n = 77$  cells per classifier instance accumulated to a total of  $n = 385$  cells displayed in the confusion matrix. The performance of each classifier instance was assessed with the withheld dataset (25%). Purple luminance: percentage of neurons correctly predicted by classifiers for a given type, class, projection target, or subregion. da: dorsoanterior; vp: ventroposterior; BLA: basolateral

amygdala; CeA: central amygdala; contra AI: contralateral agranular insula; MD: mediodorsal thalamus;  
NTS: nucleus tractus solitarius; PF/PO: parafascicular/posterior thalamus; loc.: location; proj.: projection.





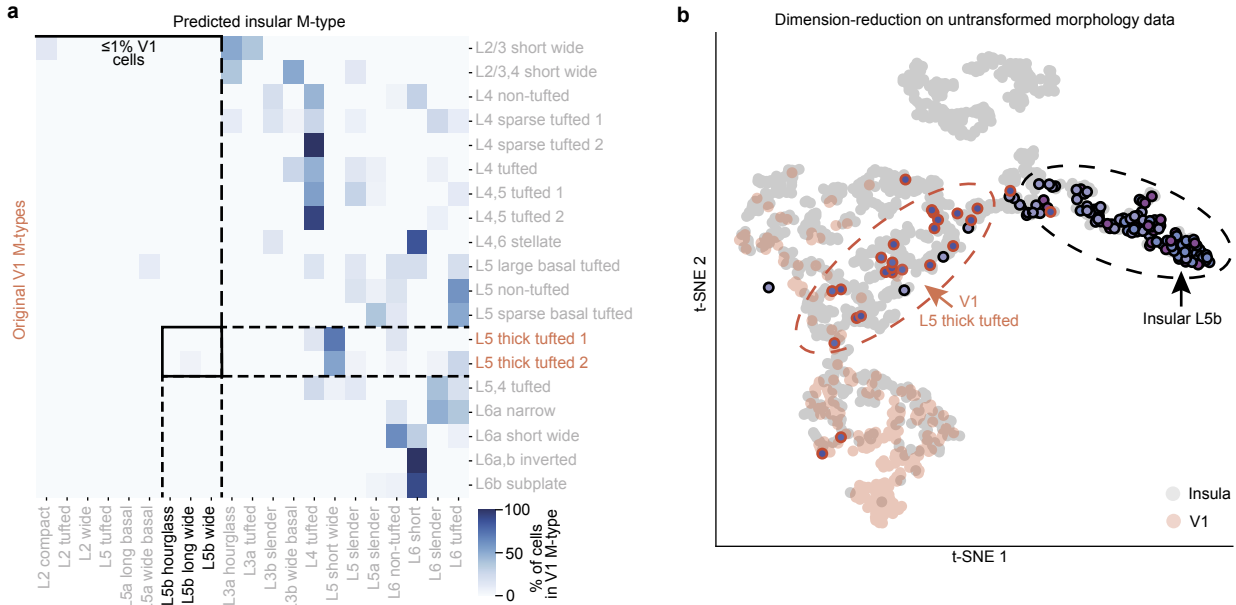




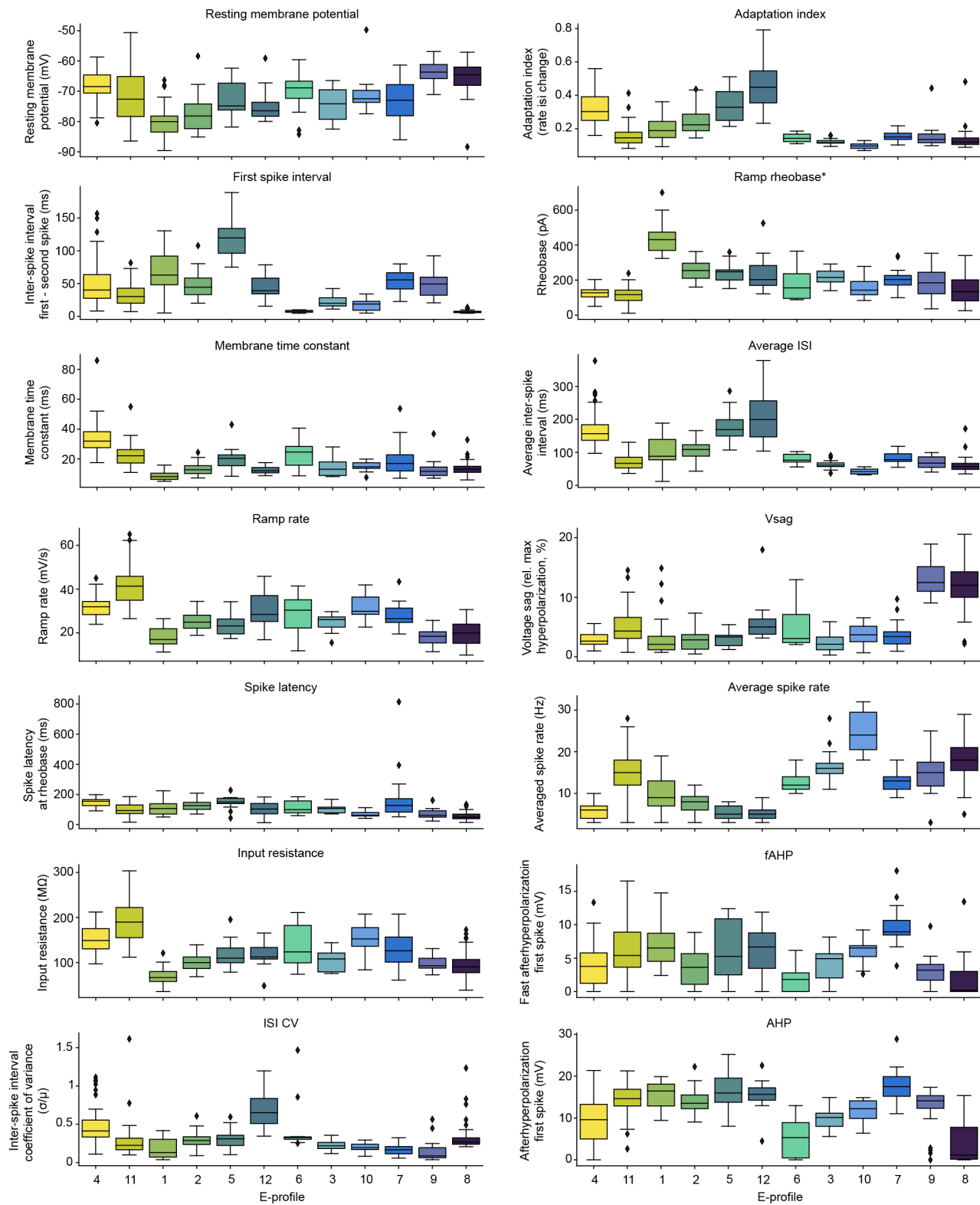
**Supplementary Fig. 8: Receiver-operating characteristic (ROC) and total area under the curve (AUC)**

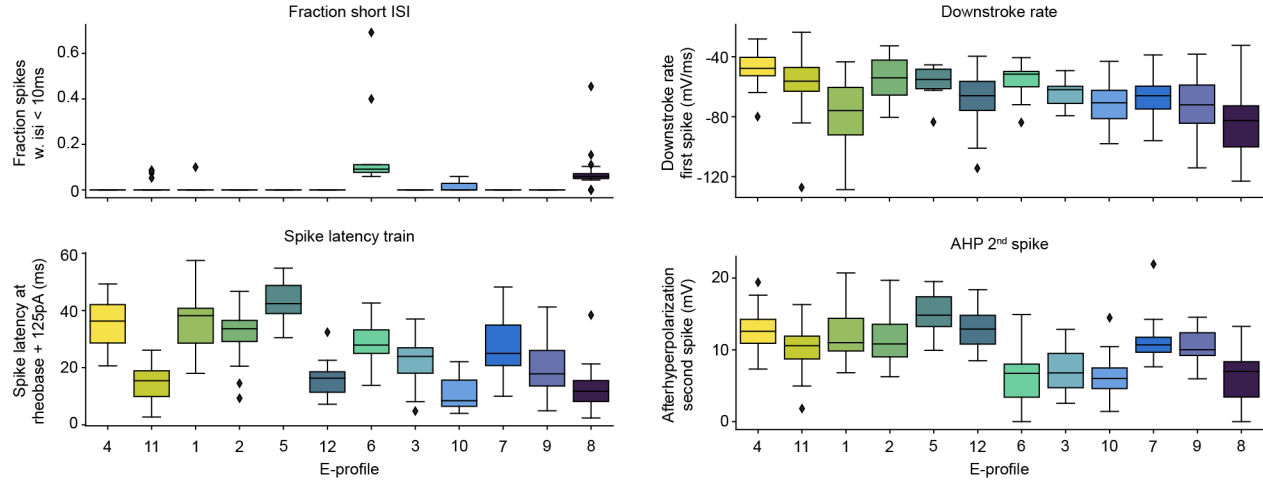
**performances of trained forest classifiers. a and b, The ROC and AUC displayed for each independent**

instance of classifier training for each type of classifier (using a macro-averaged one-vs.-rest scheme, Methods). **a**, performances of classifiers trained on untransformed insular data. Subpanel order matches order of comparisons shown in Fig. 7a. **b**, performances of classifiers trained on untransformed V1 data, z-score transformed insular, and z-score transformed V1 data (V1 data from Gouwens et al.<sup>33</sup>).



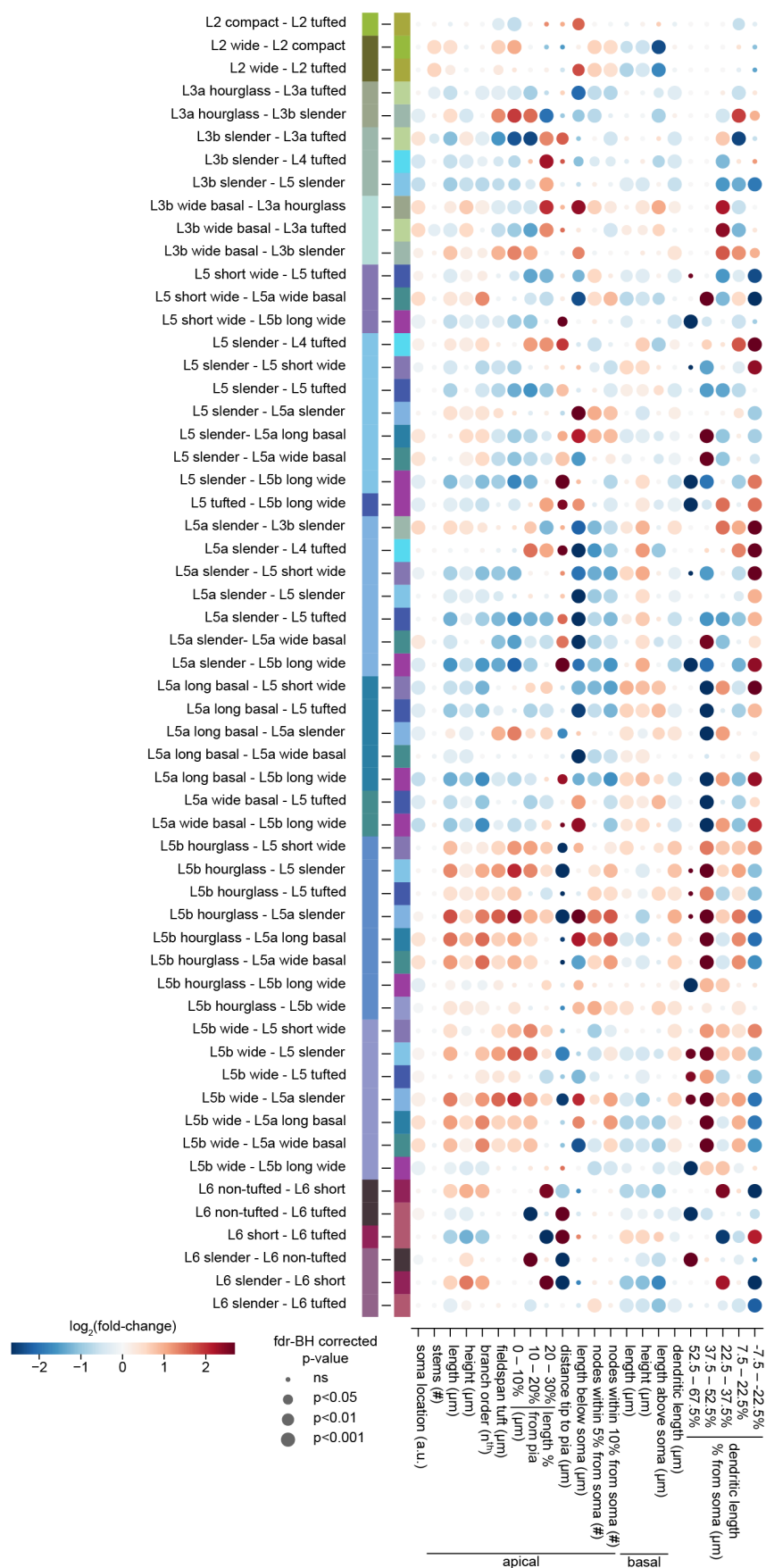
**Supplementary Fig. 9: No insular L5b M-type equivalents were found in V1.** **a**, Matching of V1 M-types to insular M-types using classifiers trained on untransformed insular data. The black rectangle highlights the absence of L5b M-type allocations to the largest V1 M-type neurons ('L5 thick tufted 1' and 'L5 thick tufted 2'). Blue luminance: percentage of cells from a given V1 M-type that are allocated to an insular M-type by the classifier. **b**, t-SNE visualization of the untransformed morphology data from both the insular (grey filled-circles) and V1 (red filled-circles) datasets. The largest M-types from both datasets are primarily located in non-overlapping regions of the t-SNE plot (red dashed ellipse: V1 L5 thick tufted M-types; black dashed ellipse: insular L5b M-types). V1 data from Gouwens et al.<sup>33</sup>



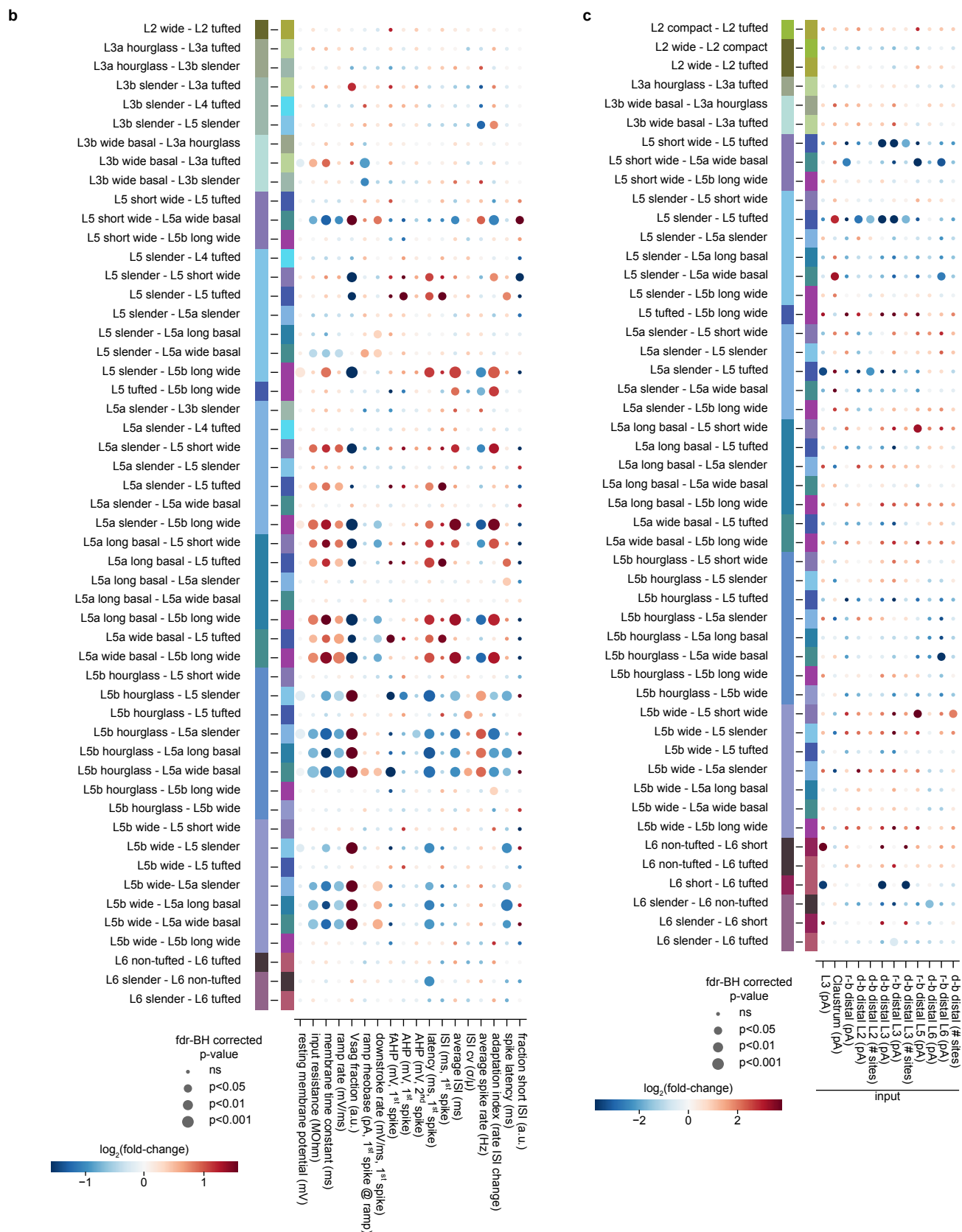


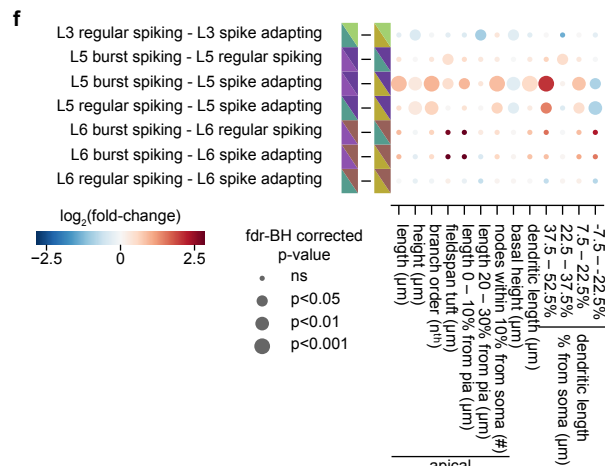
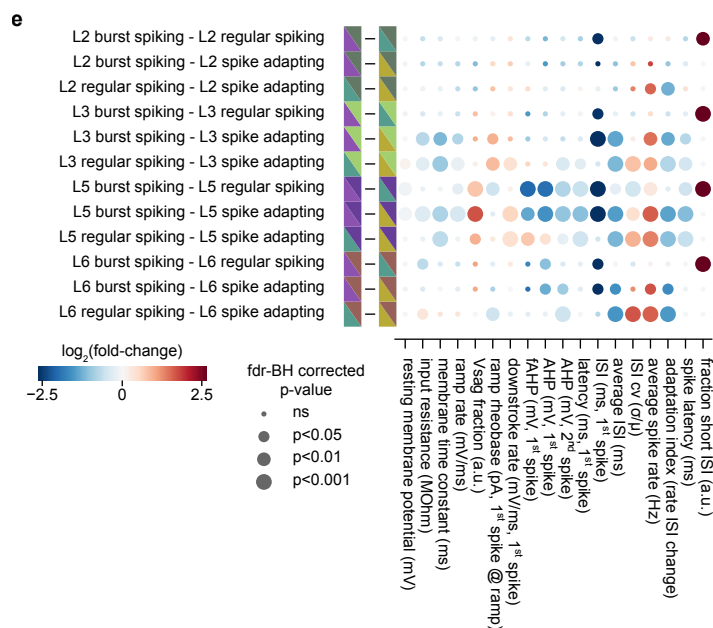
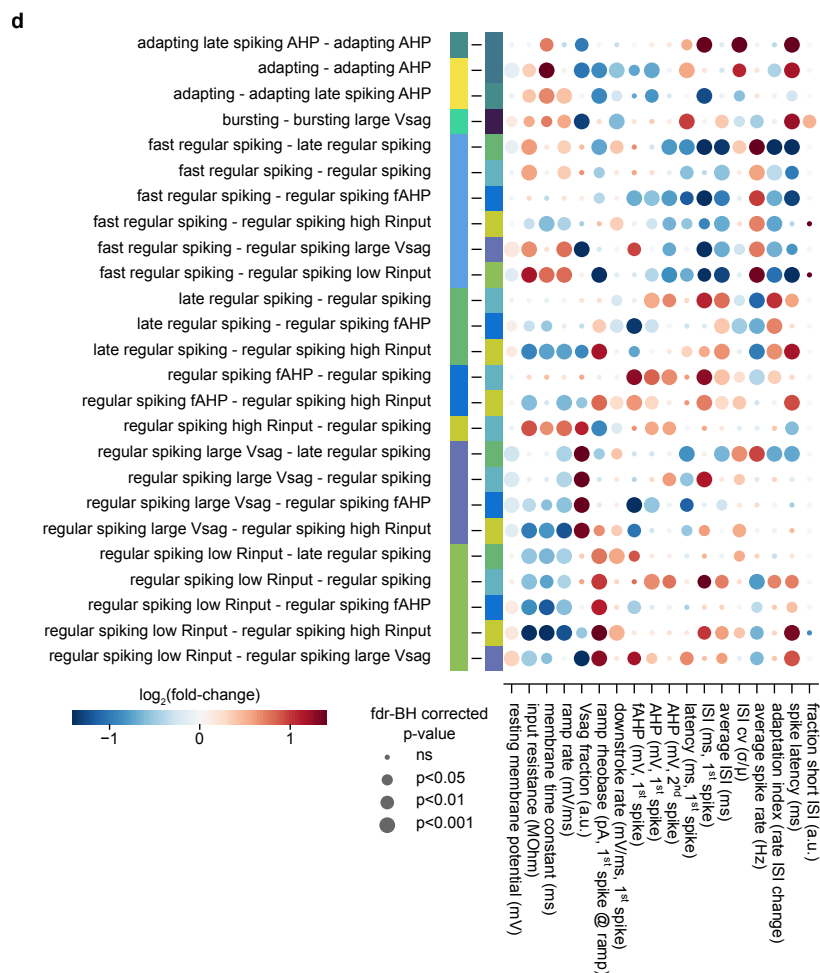
**Supplementary Fig. 10: Distinct features of E-profiles.** All 16 electrical features used for the iCCA, as well as resting membrane potential and adaptation index. \*: Current required to elicit the first action potential during ramping depolarizing current injection. Boxplots are sorted in sequence as shown in the dendrogram from Fig. 4a. Number of observations (n) for all groups provided in Supplementary Table 14. Boxplots use the following convention: center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range; diamonds, outliers.

**a**

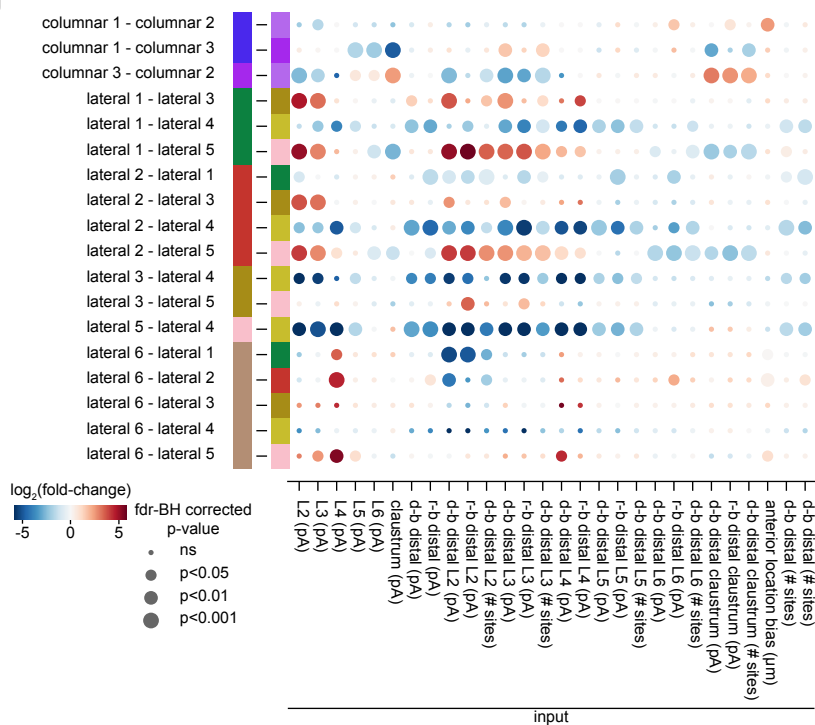




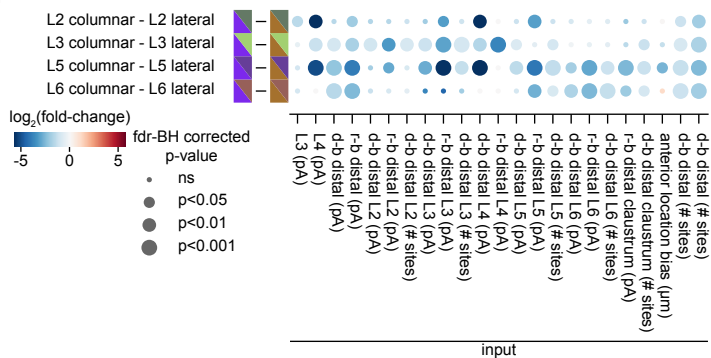




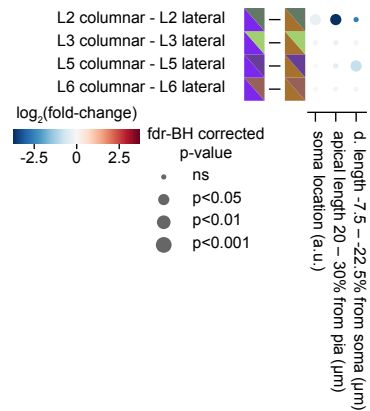
g

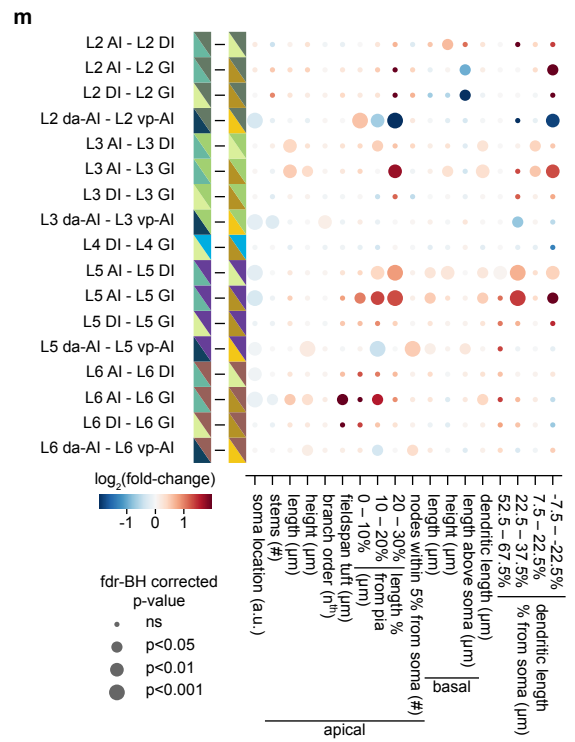
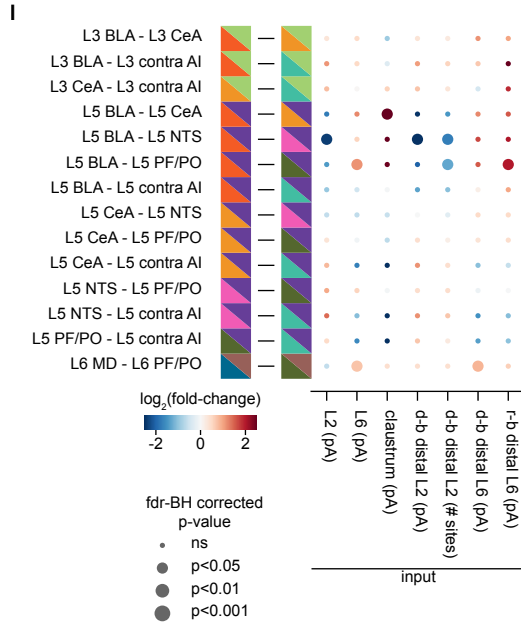
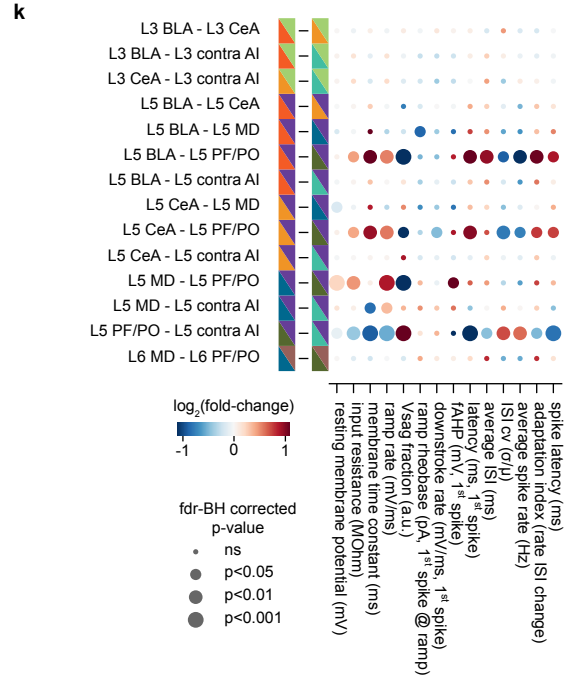
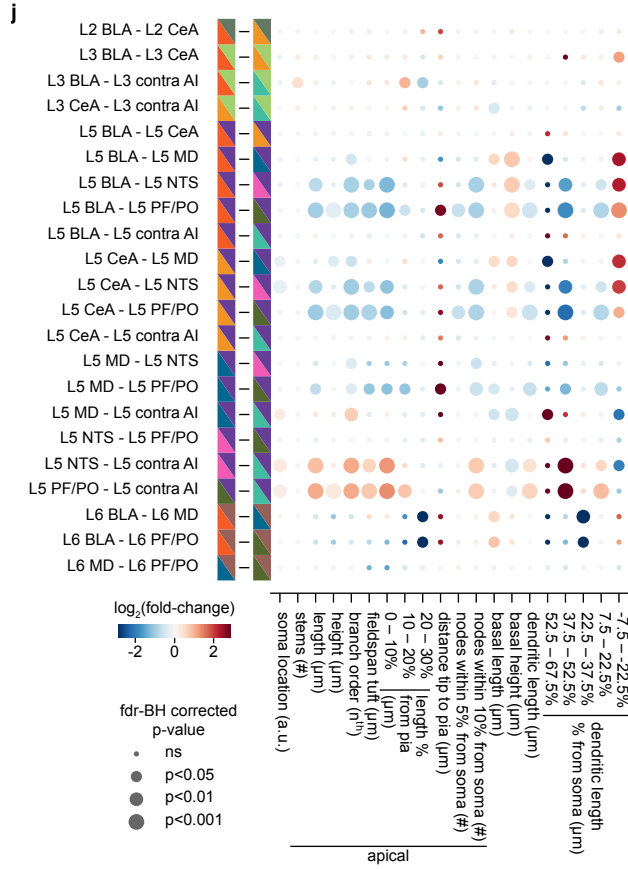


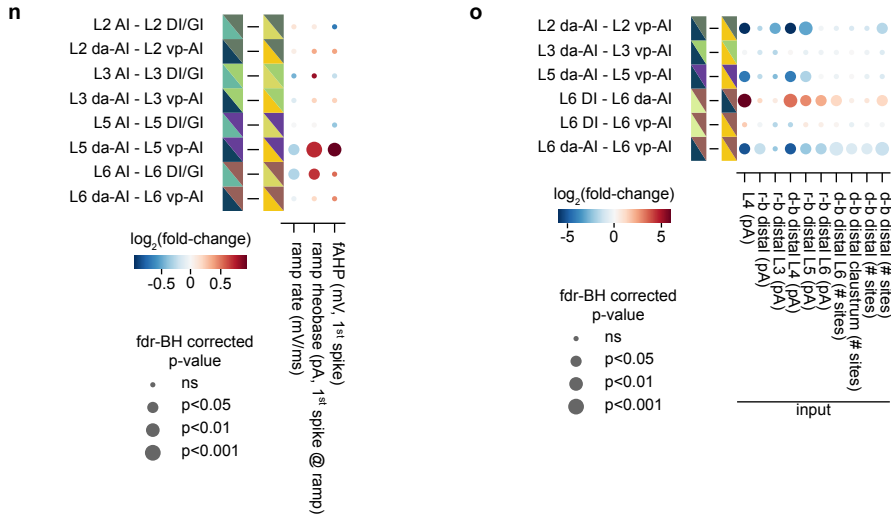
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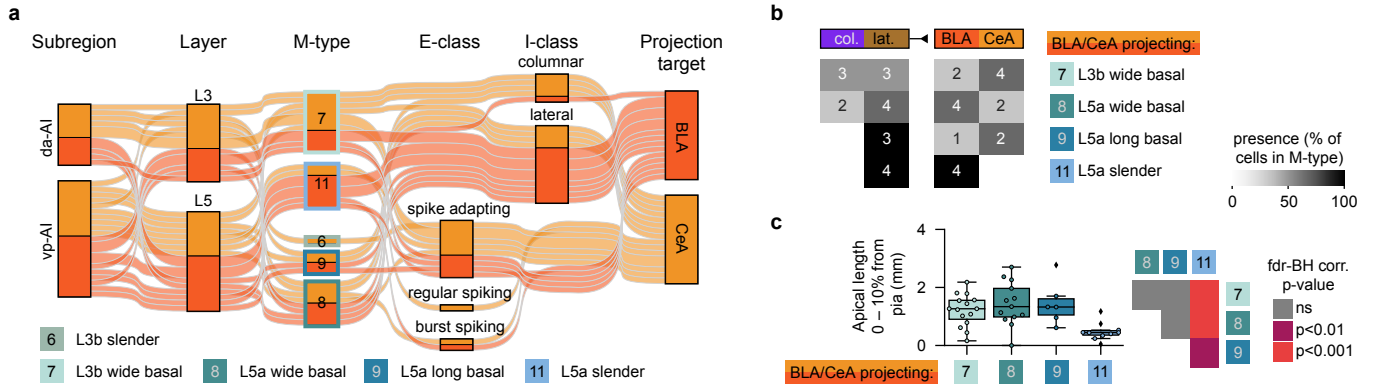
i



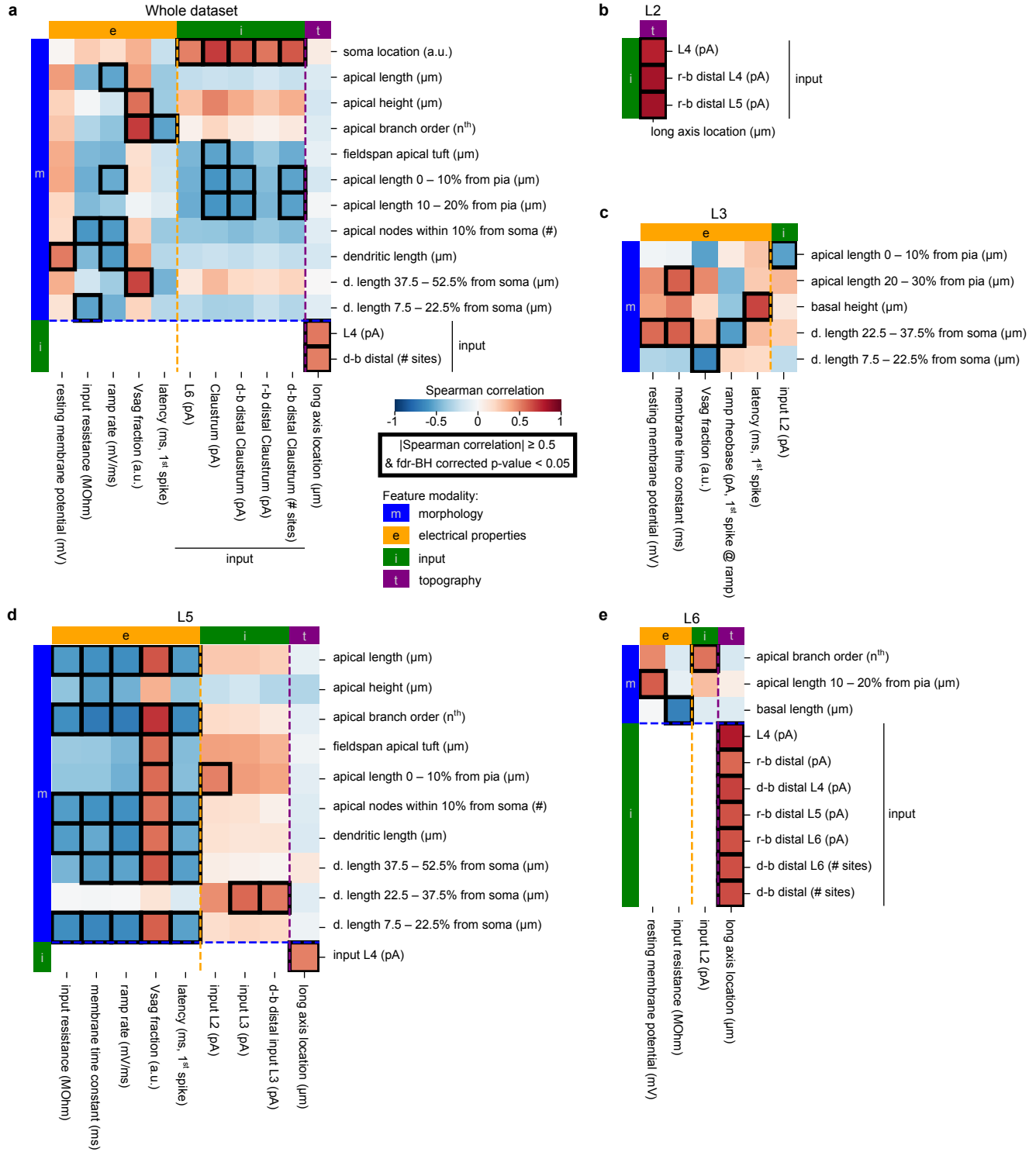




**Supplementary Fig. 11: Informative features for pair-wise comparisons between cell types across all modalities.** **a–o**, Pair-wise cross-modal feature comparisons between cell types ( $n \geq 3$  cells per type) of morphological (**a–c**), electrical (**d–f**), input (**g–i**), and topographical (**m–o**) modalities. In each row, the left and right cell types are color-coded as defined earlier (Figs. 1c, 2a, 3b, 3c, 4a, and 5d). For cell types based on the intersection of two modalities (**h–o**), e.g., layer allocation and input class (**h**), colors corresponding to each modality are shown in triangles. All features used for the iCCA are included (Extended Data Figs. 4a, 6b, and 8b). Bubble plots show fold difference calculated as  $\log_2$  of mean feature value of the left cell type divided by that of the right cell type. Red luminance: the mean value of the left cell type  $>$  mean value of the right cell type; blue luminance: the reverse. All comparisons were statistically tested using a Mann-Whitney U two-sided test with false-discovery rate Benjamini-Hochberg corrections (fdr-BH corr.), indicated by four bubble sizes. Only features with a significant difference between any cell type pair are shown. For distal input features, the distal input border was defined by boundaries based on either 95% dendritic density (density-based, d-b) or 95% maximum dendritic reach (reach-based, r-b). Number of observations and exact p-values are provided in Supplementary Table 16.



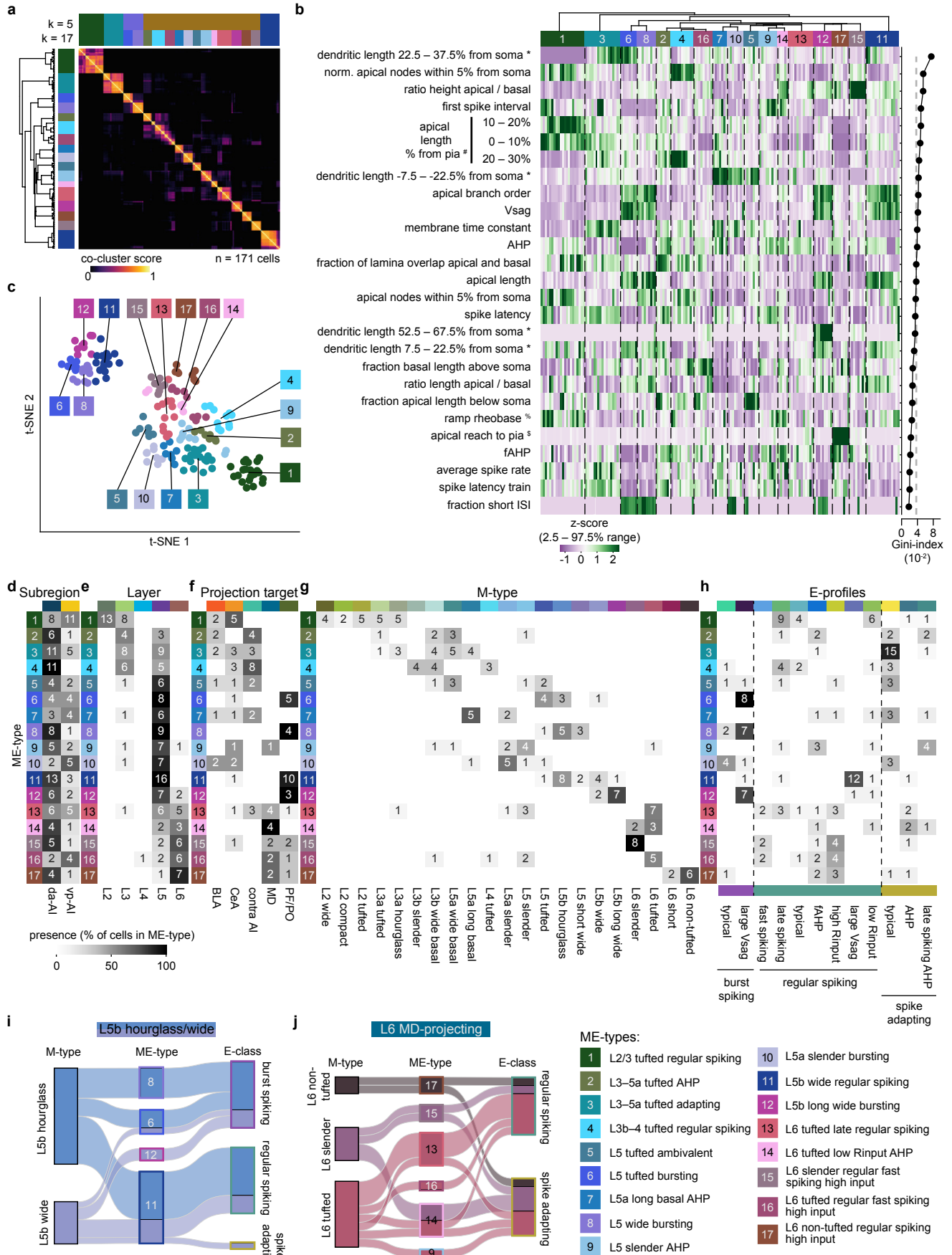
**Supplementary Fig. 12: Informative features for cell type identification.** **a**, Parallel categories diagram of L3b and L5a amygdala-projecting neurons and their allocations to subregions, M-types, E-classes, and I-classes. **b**, Contingency table of the same subset of M-types in **(a)** versus input classes or projection targets. Grayscale luminance: the percentage of cells within each M-type allocated to a given input class or projection target. Numbers in gray boxes: n of observed cells (when n > 0). **c**, Quantification of apical length within 10% cortical thickness bin from pia (left) and the corresponding heatmap of p-values from the pair-wise statistical comparisons between the same subset of M-types in **(a)** (right, Kruskal-Wallis test  $H(3) = 14.08$ ,  $p = 0.003$ , with post hoc Dunn's two-sided comparisons with false-discovery rate Benjamini-Hochberg correction (fdr-BH corr.)). Circles: individual observations. Diamonds: outliers. Number of observations (n) for all groups and exact p-values provided in Supplementary Table 17.



**Supplementary Fig. 13: Cross-modal feature correlations.** a–e, Feature pair-wise Spearman correlation between morphology (m, blue), electrical (e, yellow), input (i, green), and topographic (t, purple) features. Spearman correlations were computed based on the whole dataset (a), or subsets based on layer allocation of

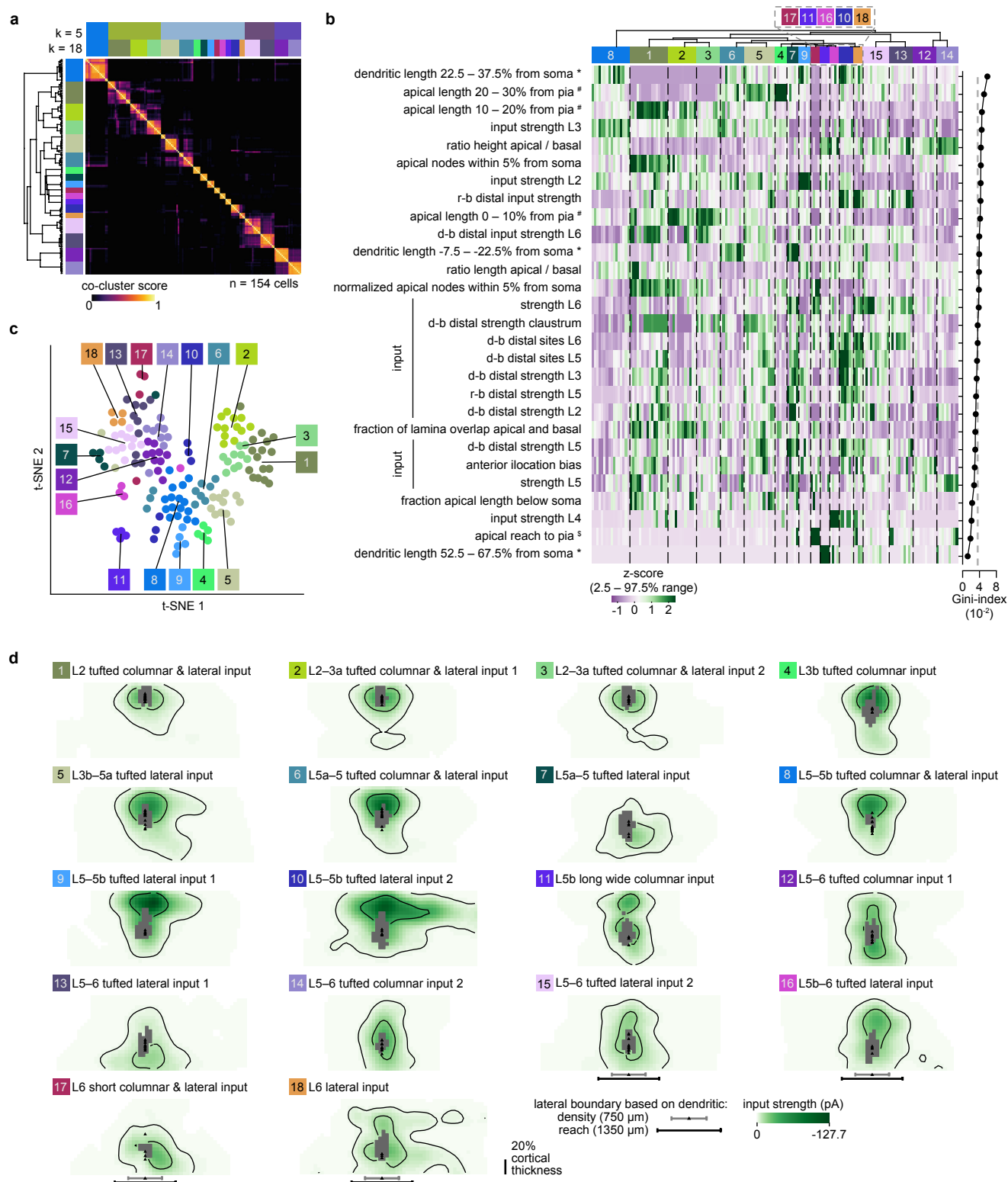
the individual cells (**b–e**). Note that topology (t, long axis location) correlated with input (i), but not morphology (m) or electrical (e), features in the whole dataset and layer subsets (L2, L5, and L6) (**a**, **b**, **d**, and **e**). Feature pairs with an absolute Spearman correlation  $\geq 0.5$  and false-discovery rate Benjamini-Hochberg (fdr-BH) corrected p-value  $< 0.05$  are boxed. Only feature pairs yielding significant correlations between any two modalities are shown (Methods, whole dataset:  $n = 25$ , L2:  $n = 3$ , L3:  $n = 7$ , L5:  $n = 36$ , L6:  $n = 10$ ). For lateral input features, the lateral border was defined by boundaries based on either 95% dendritic density (density-based, d-b) or 95% maximum dendritic reach (reach-based, r-b).

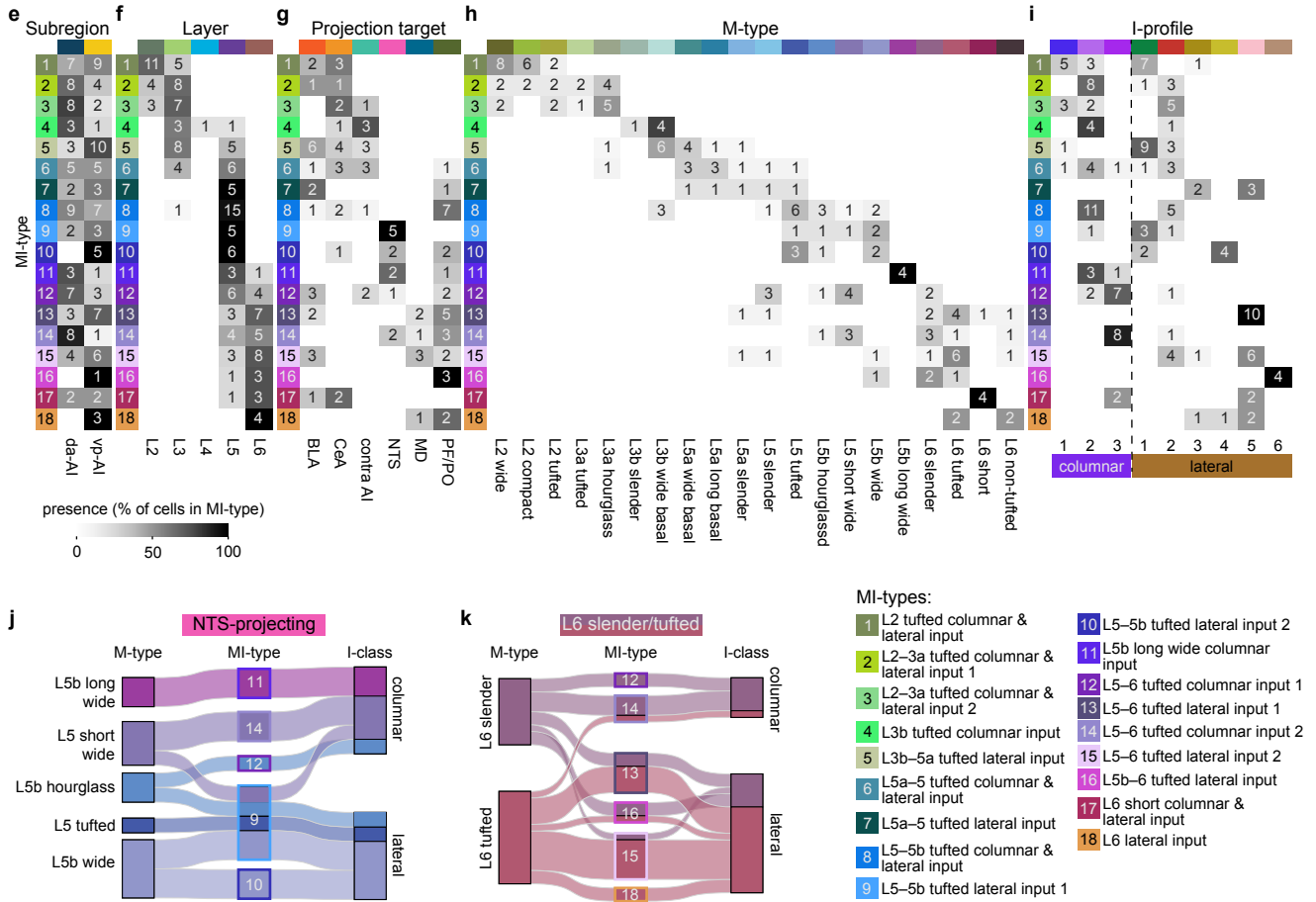




**Supplementary Fig. 14: Analysis of combined morpho-electrical features and subsequent cell groups.**

**a**, Hierarchical cluster analysis (HCA) on pair-wise co-cluster scores across 171 cells using both morphological and electrical features. Dendrogram-based grouping is color-coded for 17 clusters ( $k = 17$ ) based on optimal silhouette score, as well as for 5 clusters ( $k = 5$ ). **b**, Z-scored electrical and morphological features (left), ranked by random forest classifier Gini-index (right, gray dashed line indicates equal feature contribution [1/27 features]). \*: Dendritic length located within a 15% local cortical thickness bin positioned relative to the soma, normalized to local cortical thickness. #: Apical length within a 10% local cortical thickness bin positioned relative to the pia, normalized to total apical length. %: Current required to elicit the first action potential during ramping depolarizing current injection. \$: Vertical distance between pia and closest apical dendrite, normalized to local cortical thickness. **c**, t-SNE distribution of morpho-electrical types (ME-types). For (**b** and **c**), colored boxes with numbers: ME-types color-coded for  $k = 17$  in (**a**). **d–h**, Contingency table of cell allocations to subregion (**d**), layer (**e**), projection target (**f**), M-type (**g**), or E-profile (**h**). Note in (**f**), most ME-types only contain IT (e.g., BLA-, CeA-, or contra AI-projecting) or ET (e.g., MD- or PF/PO thalamus-projecting) neurons, suggesting IT and ET neurons (Fig. 6f) can be readily resolved using ME-types. Grayscale luminance: the percentage of cells in each ME-type allocated to a given modality. Numbers in gray boxes:  $n$  of observed cells (when  $n > 0$ ). **i** and **j**, Parallel categories diagrams of *L5b hourglass* (M-type 14) and *L5b wide* (M-type 16) neurons (**i**;  $n = 23$  cells) and L6 MD-projecting neurons (**j**;  $n = 14$  cells) across M-types, ME-types, and E-classes. Several M-types and E-classes can be sub-specified into multiple ME-types. ME-type legend (bottom right) is shared across all panels.





**Supplementary Fig. 15: Analysis of combined morpho-input features and subsequent cell groups.** **a**, Hierarchical cluster analysis (HCA) on pair-wise co-cluster scores across 154 cells using both morphological and input features. Dendrogram-based grouping is color-coded for 18 clusters ( $k = 18$ ) based on optimal silhouette score, as well as for 5 clusters ( $k = 5$ ). **b**, Z-scored morphological and input features (left), ranked by random forest classifier Gini-index (right, gray dashed line indicates equal feature contribution [1/28 features]). For distal input features, the border was defined by boundaries based on either 95% dendritic density (density-based, d-b) or 95% maximum dendritic reach, (reach-based, r-b). \*: Dendritic length located within a 15% local cortical thickness bin positioned relative to the soma, normalized to local cortical thickness. #: Apical length within a 10% local cortical thickness bin positioned relative to the pia, normalized to total apical length. §: Vertical distance between pia and closest apical dendrite, normalized to local cortical thickness. **c**, t-SNE distribution of morpho-input types (MI-types). **d**, Average input maps grouped by MI-

types, aligned by pia, formatted as in Fig. 5b. Maps are vertically interpolated and normalized to the cortical thickness. Gray and black lateral whisker bars: lateral boundary thresholds, used during feature extraction, based on dendritic density (750  $\mu\text{m}$ ) or reach (1350  $\mu\text{m}$ ), respectively; gray pixels: direct response sites; black triangles: soma locations. For **(b–d)**, colored boxes with numbers: MI-types color-coded for  $k = 18$  in **(a)**. **e–i**, Contingency tables of MI-types versus subregion **(e)**, layer **(f)**, projection target **(g)**, M-type **(h)** and I-profile **(i)**. Grayscale luminance: the percentage of cells in each MI-type allocated to a given aforementioned modality. Numbers in gray boxes:  $n$  of observed cells (when  $n > 0$ ). **j**, Parallel categories diagrams of NTS-projecting neurons ( $n = 12$ ). **k**, Parallel categories diagrams of *L6 slender* (M-type 18) and *L6 tufted* (M-type 19) neurons ( $n = 24$ ). Note in **(i–k)**, a majority of MI-types (12/18) retain predominance in either columnar or lateral input classes. MI-type legend (bottom right) is shared across all panels.

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