

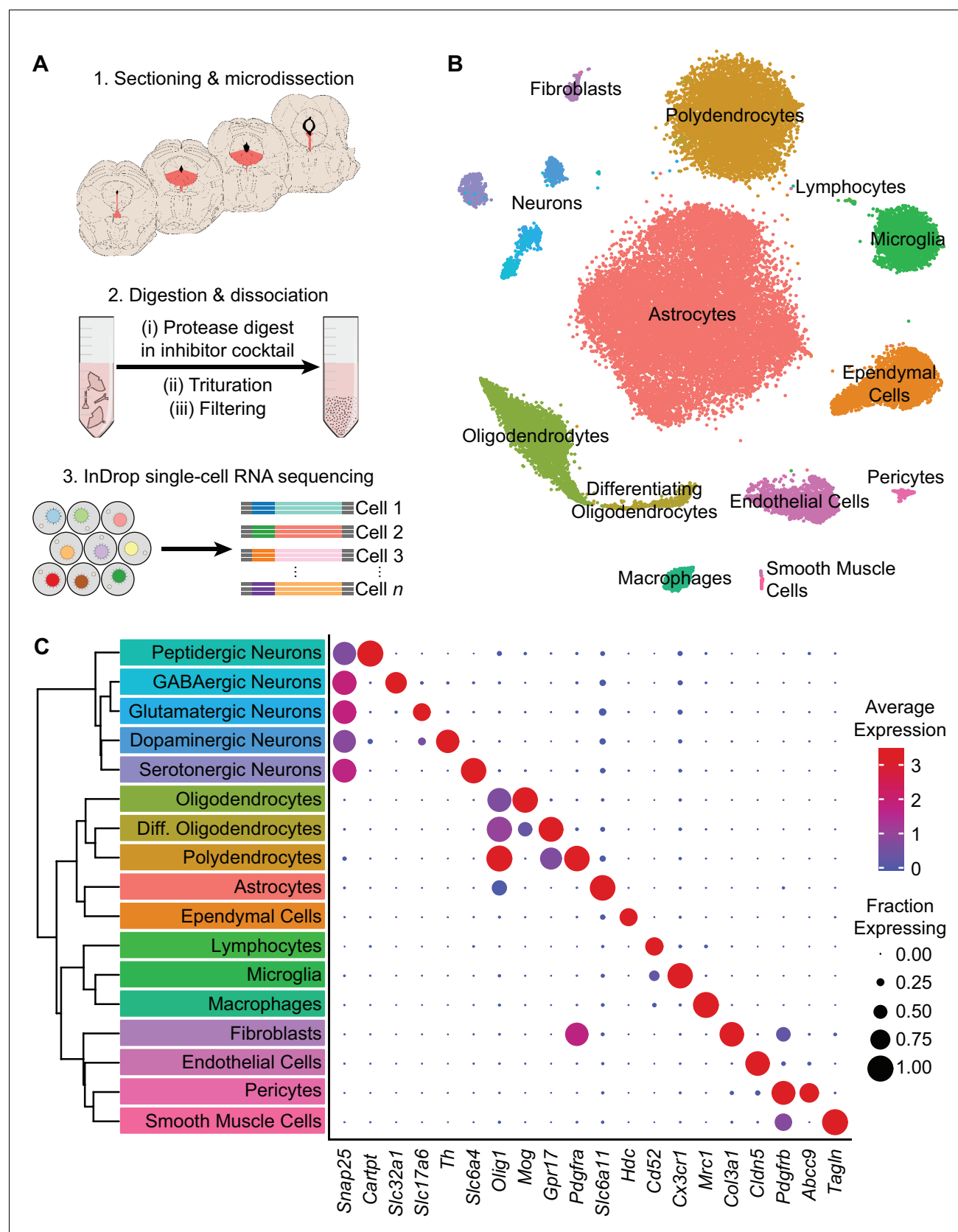


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## Figures and figure supplements

Molecular and anatomical organization of the dorsal raphe nucleus

**Kee Wui Huang et al**



**Figure 1.** High-throughput single cell transcriptomic profiling of the DRN. (A) Schematic for scRNA-seq using the inDrop platform. (1) Tissue containing the DRN and surrounding regions was microdissected from acute coronal brain slices prepared from adult C57BL/6J mice. (2) Tissue chunks were

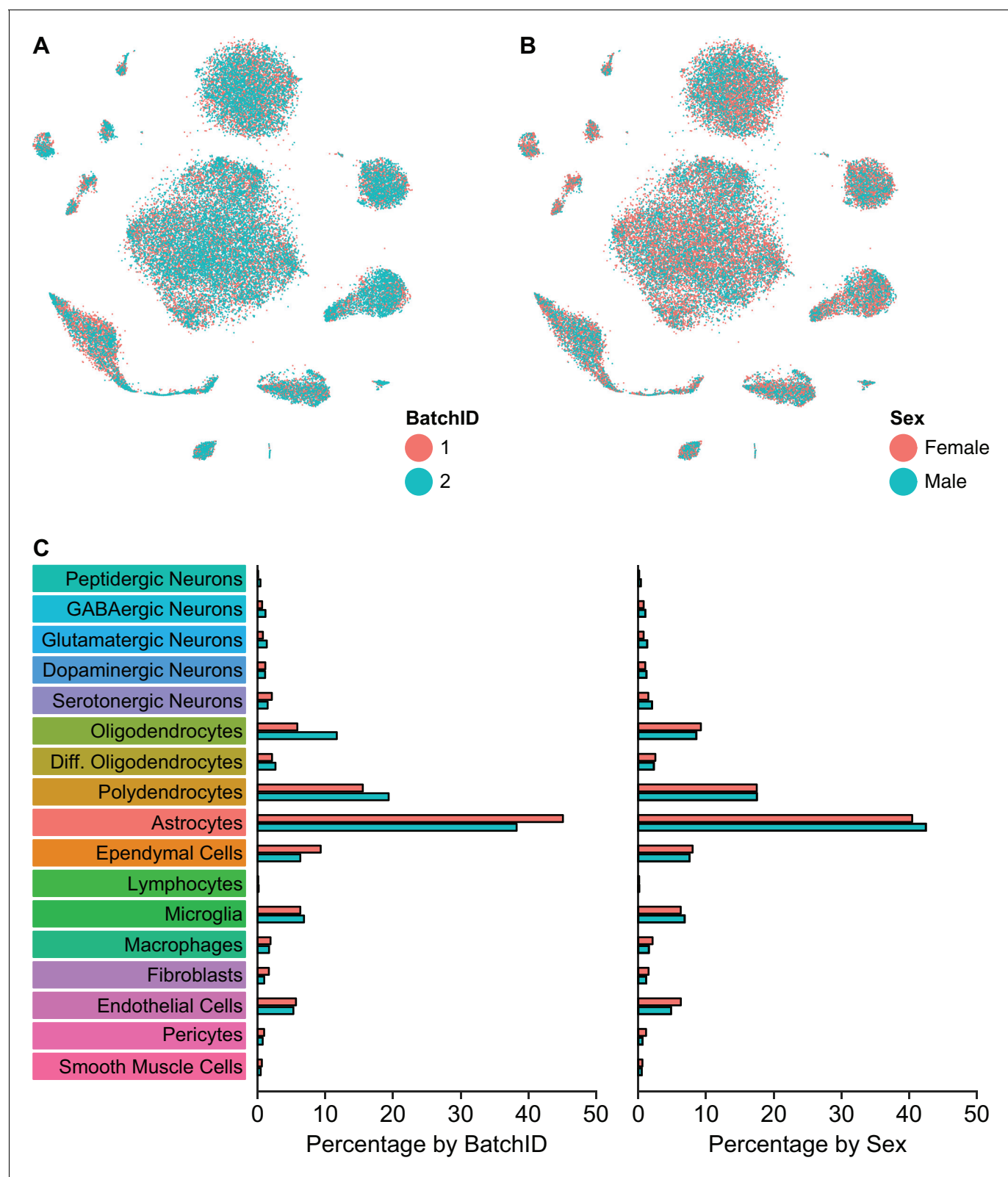
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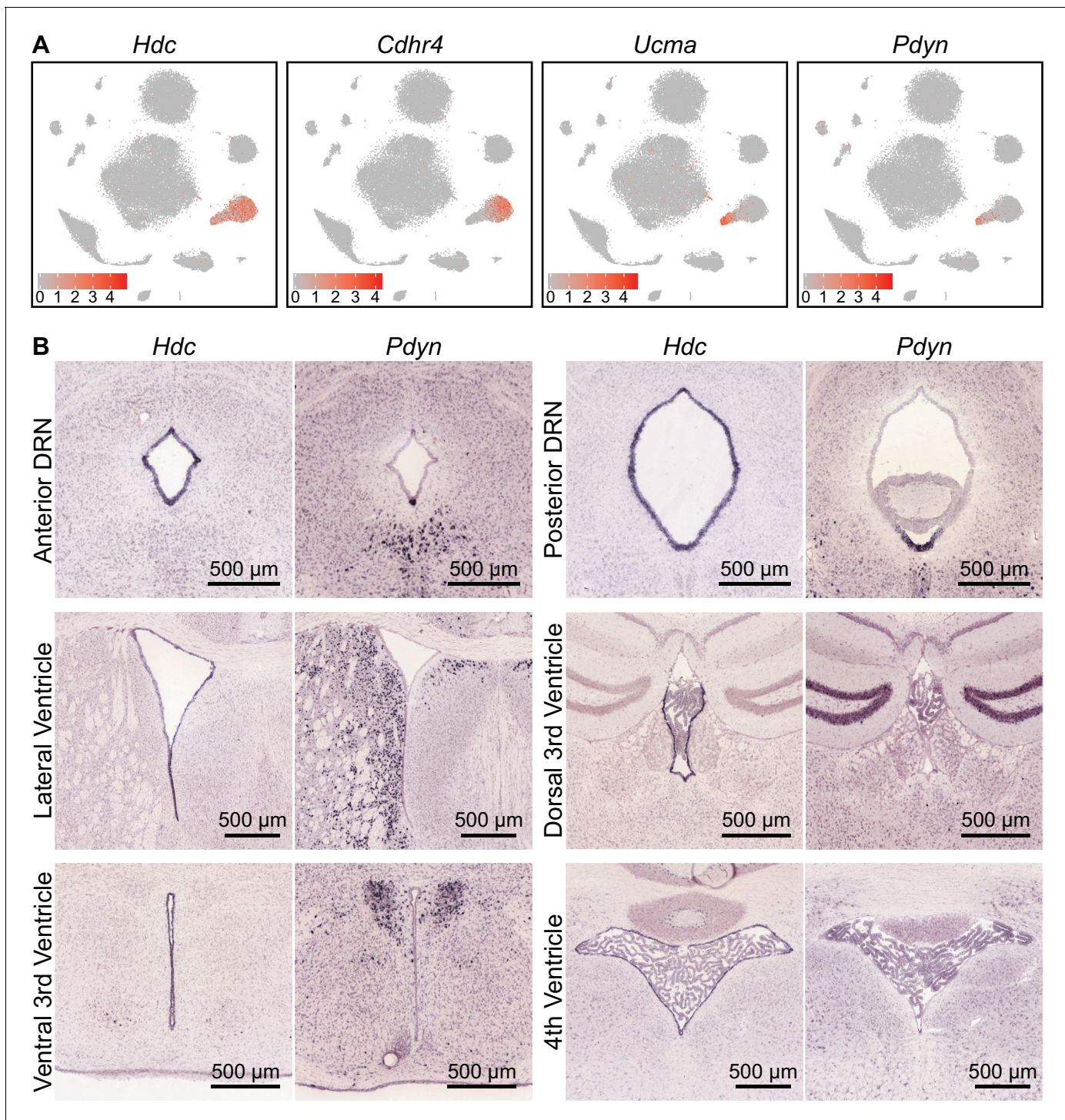
digested in a cocktail of proteases and inhibitors of neuronal spiking, transcription, and translation. Digestion was followed by trituration and filtration to obtain a cell suspension. (3) Single cells were encapsulated using a droplet-based microfluidic device for cell barcoding and RNA capture. (B) t-SNE plot of the processed dataset containing 39,411 cells from eight animals. Cells are color-coded according to the cluster labels shown in (C). (C) Left: Dendrogram with cell class labels corresponding to clusters shown in (B). Right: Dot plot displaying expression of example genes used to identify each major cell class. The color of each dot (blue to red) indicates the average expression of each gene whereas the dot size indicates the fraction of cells expressing the gene. Additional genes used for identifying cell classes/types are listed in **Supplementary file 1**.

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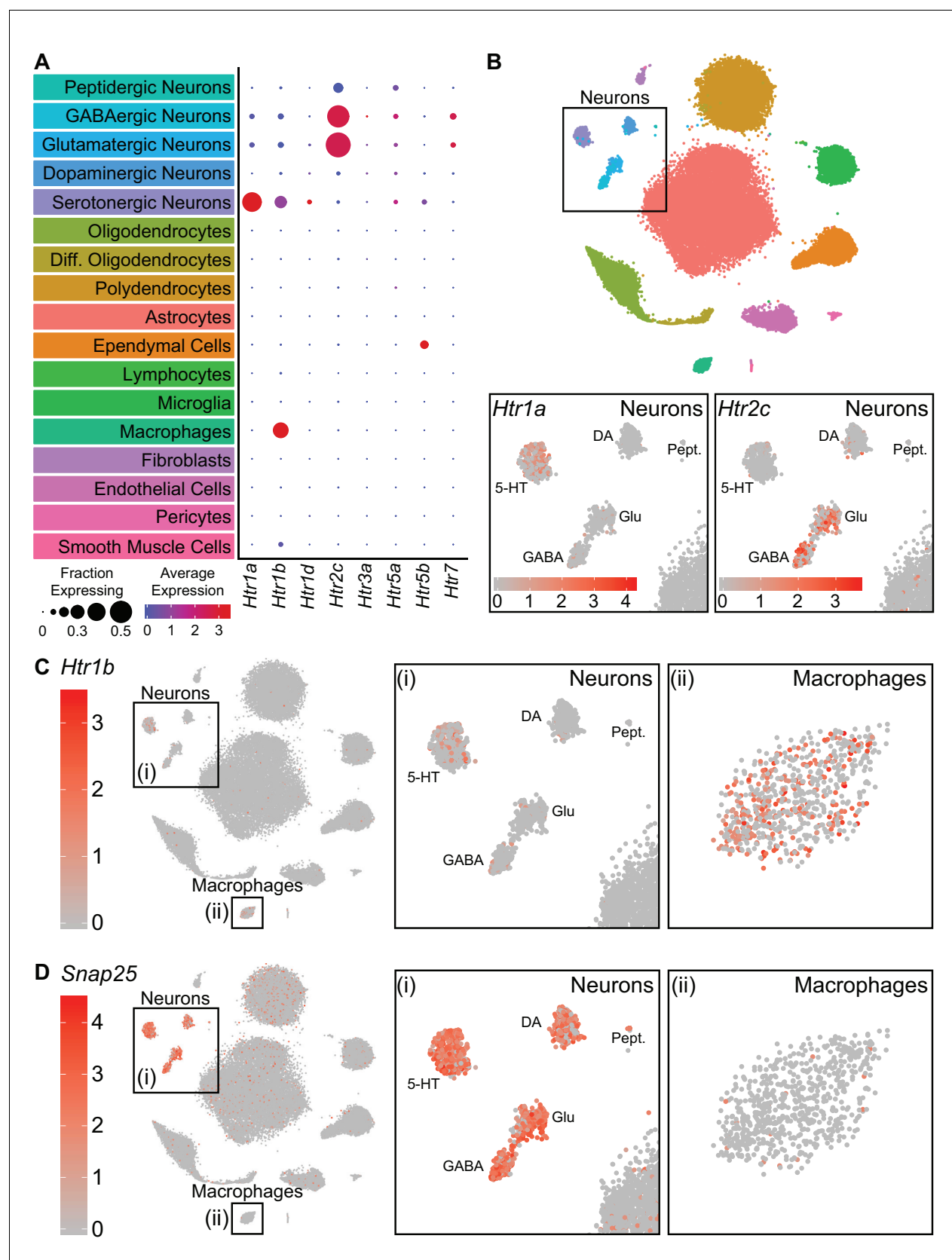
**Figure 1—figure supplement 1.** Comparison of cell type composition across sample batches and sex. (A) t-SNE plot of the dataset with cells color-coded by experiment batch. All clusters contain cells from both batches. (B) t-SNE plot of the dataset with cells color-coded by the sex of the mouse from which the sample was acquired. (C) Bar plots showing the percentage of cells in each batch (left) or sex (right) that are categorized into each of the 17 major cell types.

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**Figure 1—figure supplement 2.** Ependymal cells lining the ventral cerebral aqueduct are of several distinct subtypes. (A) t-SNE plots of the dataset, with cells color-coded by their expression of genes identifying different subsets of ependymal cell. *Hdc* is expressed in all ependymal cells, whereas genes such as *Cdhr4*, *Ucma*, and *Pdyn* are expressed in distinct subsets. (B) Images of coronal ISH from the Allen Brain Atlas showing expression of *Hdc* and *Pdyn* at various parts of the ventricular system. *Hdc* is expressed by ependymal cells lining most of the ventricular system. *Pdyn* expression is specific to the cells lining the ventromedial part of the posterior ventricular system, where it is highly expressed in the cerebral aqueduct, but not the lateral ventricles or 3<sup>rd</sup> ventricle.

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**Figure 2.** Serotonin receptors are expressed in both neurons and non-neuronal cells. (A) Dot plots showing expression of the serotonin receptors present in the scRNA-seq dataset in each major cell class. *Htr1a* is expressed specifically in 5-HT neurons, whereas *Htr1b* is expressed in both 5-HT

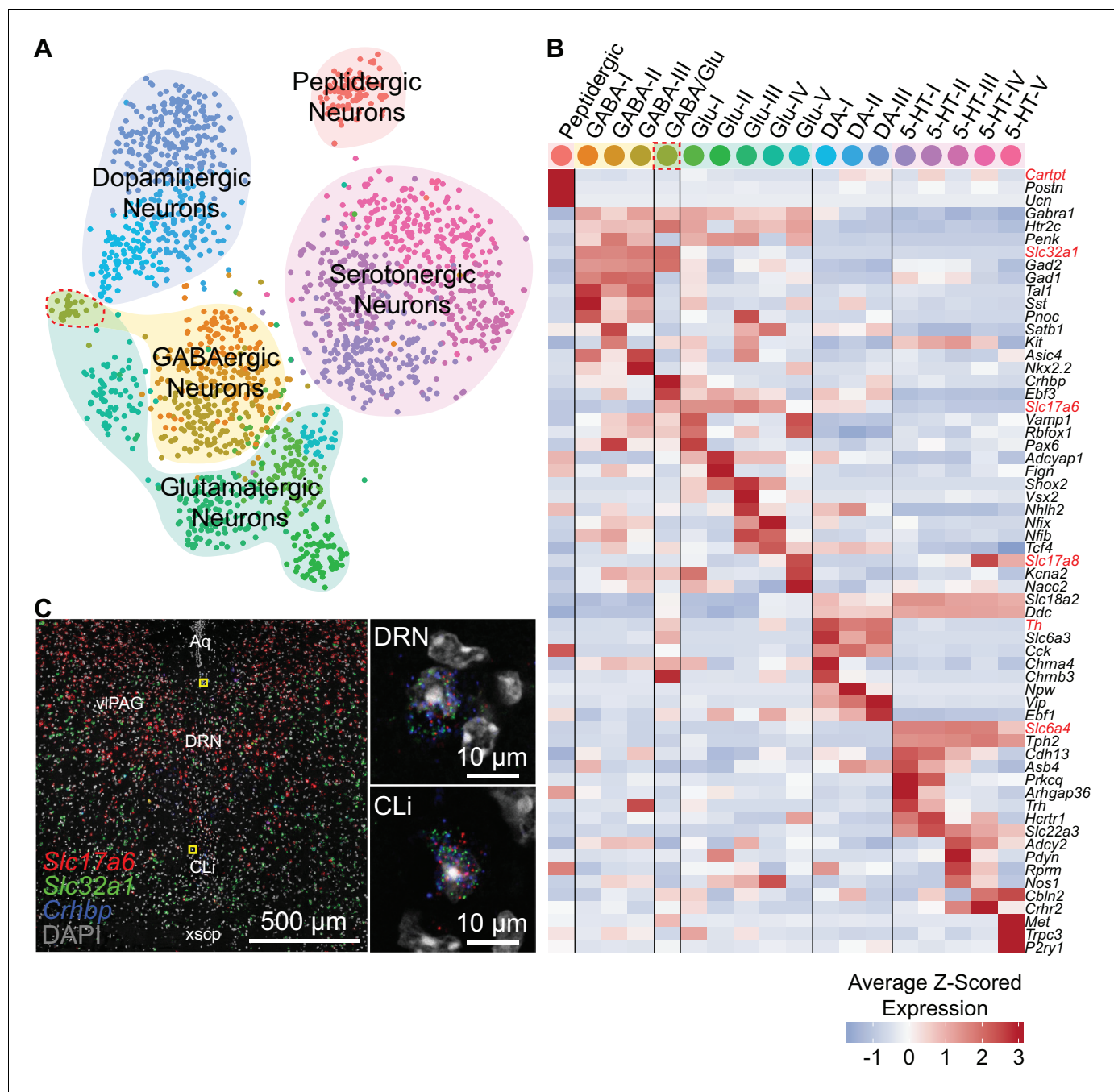
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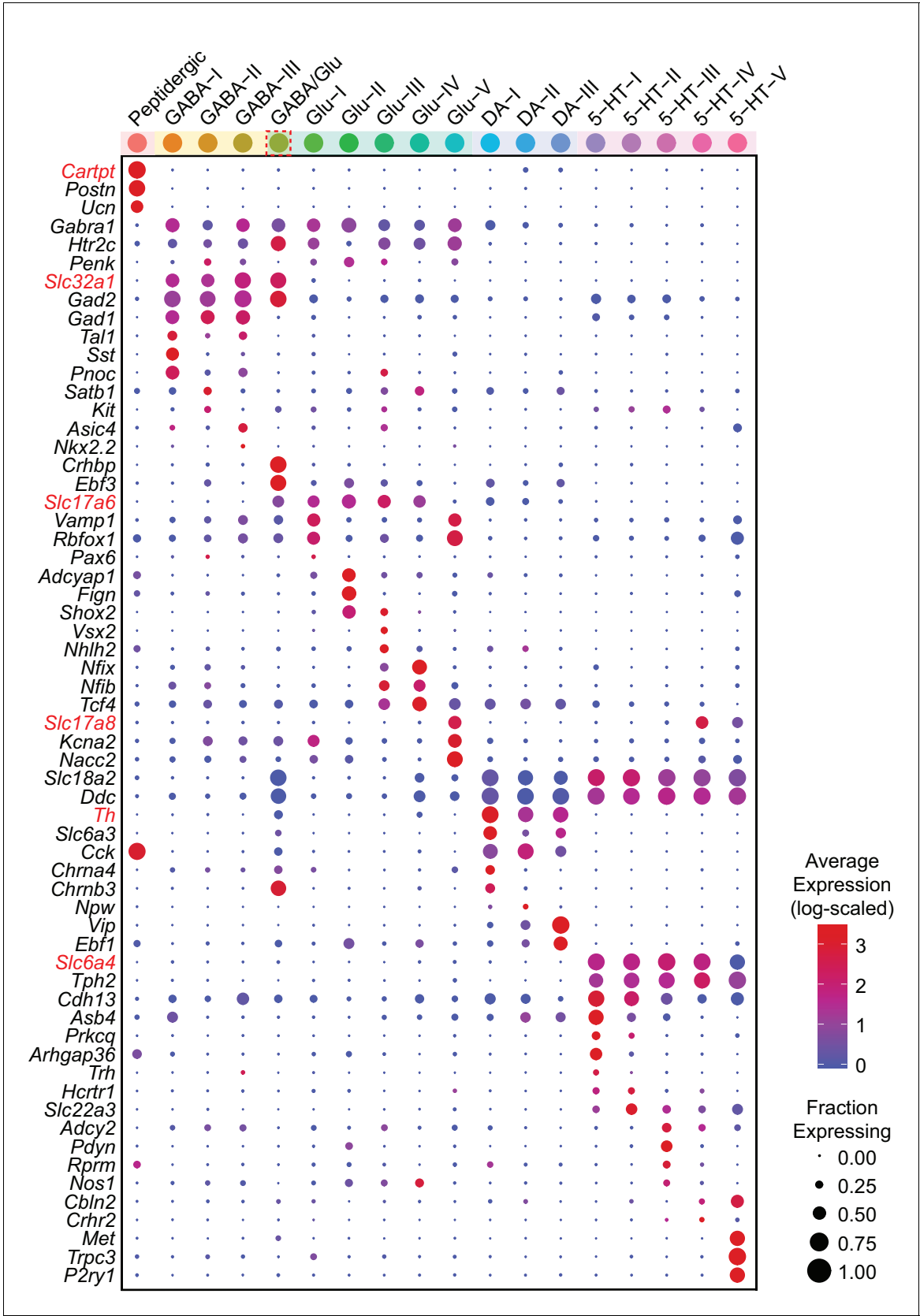
neurons and perivascular macrophages. *Htr2c* is expressed in both GABAergic and glutamatergic neurons. *Htr5b* is expressed in ependymal cells and a subset of 5-HT neurons. *Htr7* is expressed in subsets of GABAergic and glutamatergic neurons. The remaining 5-HT receptors were detected at low levels. (B) t-SNE plots with the region containing neuronal clusters enlarged to show expression of *Htr1a* (bottom, left) or *Htr2c* (bottom, right) in individual cells. Cells are color-coded by expression of each transcript. Scale bars are in log-normalized UMI-filtered counts for the respective transcripts. (C) t-SNE plots with cells color-coded by their expression of *Htr1b*. Regions containing neuronal or perivascular macrophage clusters are enlarged. (D) t-SNE plots with cells color-coded by their expression of the neuron-specific transcript *Snap25*. Regions containing neuronal or perivascular macrophage clusters are enlarged. *Snap25* transcripts are abundant in all neuronal clusters but not in perivascular macrophages, suggesting that *Htr1b* transcripts in perivascular macrophages are unlikely to be a result of engulfment of neuronal debris.

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**Figure 3.** Neuron subtypes can be distinguished transcriptionally. (A) t-SNE plot of 2,041 neurons. The main neuron types were defined by the expression of genes necessary to make or release specific neurotransmitters or neuropeptides, and are highlighted by the shaded regions. Several subtypes of each class of neuron, designated by the cell color, can be contained in each shaded region. Color codes for subtype identities correspond to column headers in (B). The cluster outlined in red corresponds to cells expressing both glutamatergic and GABAergic markers. (B) Heatmap showing the cluster average Z-scored expression of selected genes that are enriched in each neuron subtype. Genes used to identify major neuronal cell types by their neurotransmitters are labeled in red. (C) Fluorescent in situ hybridization validating the existence of cells expressing both GABAergic and glutamatergic markers. Cells expressing *Slc32a1*, *Slc17a6*, and *Crhbp* were found in anterior regions of the DRN in the caudal linear nucleus (CLi) and dorsomedial DRN in close proximity to the cerebral aqueduct. The regions indicated by the yellow boxes are shown enlarged on the right.

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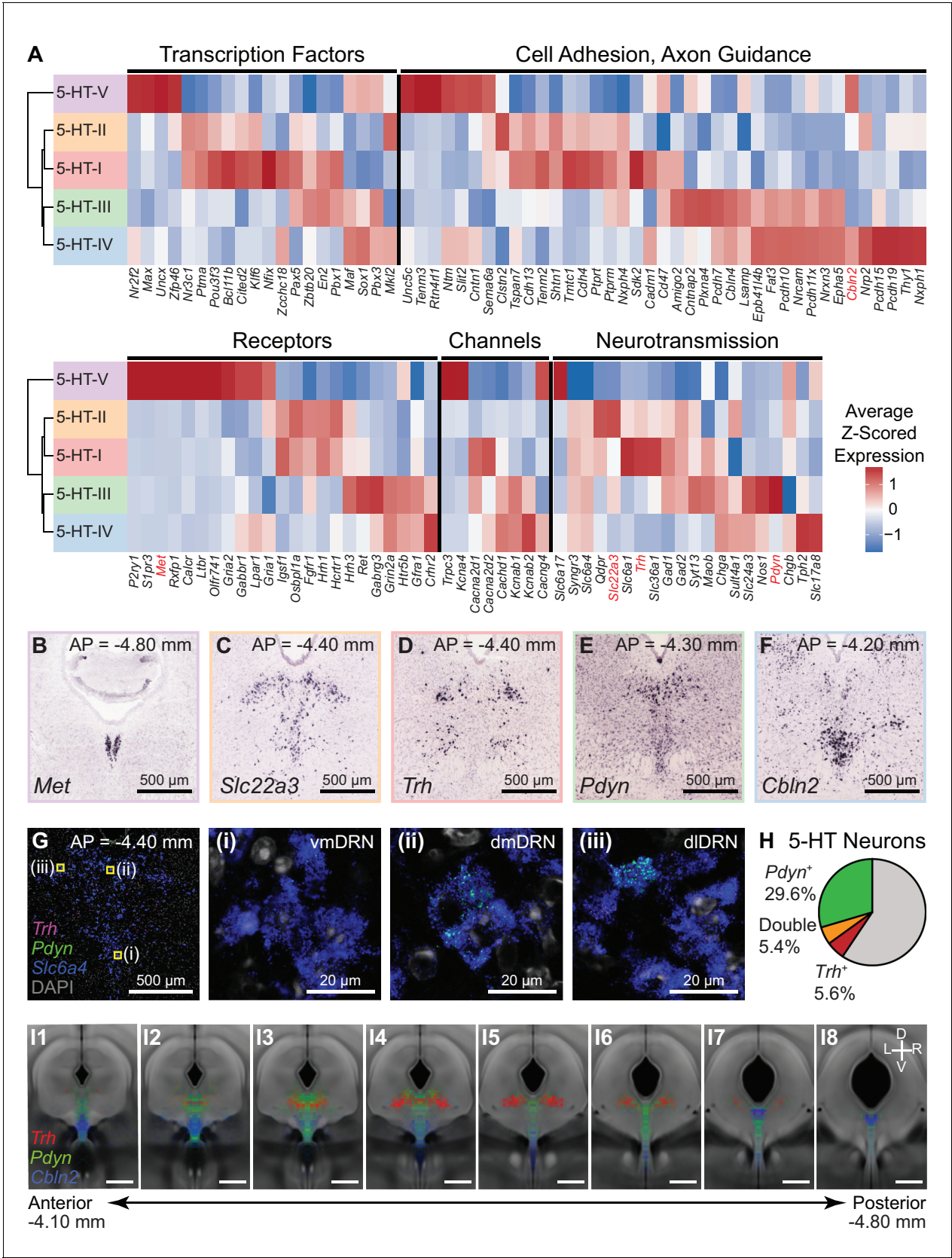
**Figure 3—figure supplement 1.** Average log-scaled expression of genes differentially expressed between neuron subtypes. Dot plot showing the log-scaled expression for each gene shown in Figure 3B. The color of each dot represents the average log-transformed UMI counts for each gene after Figure 3—figure supplement 1 continued on next page

Figure 3—figure supplement 1 continued

normalizing for the total transcript count per cell, while the size of the dot represents the fraction of cells in each neuron subtype cluster in which transcripts for that gene were detected.

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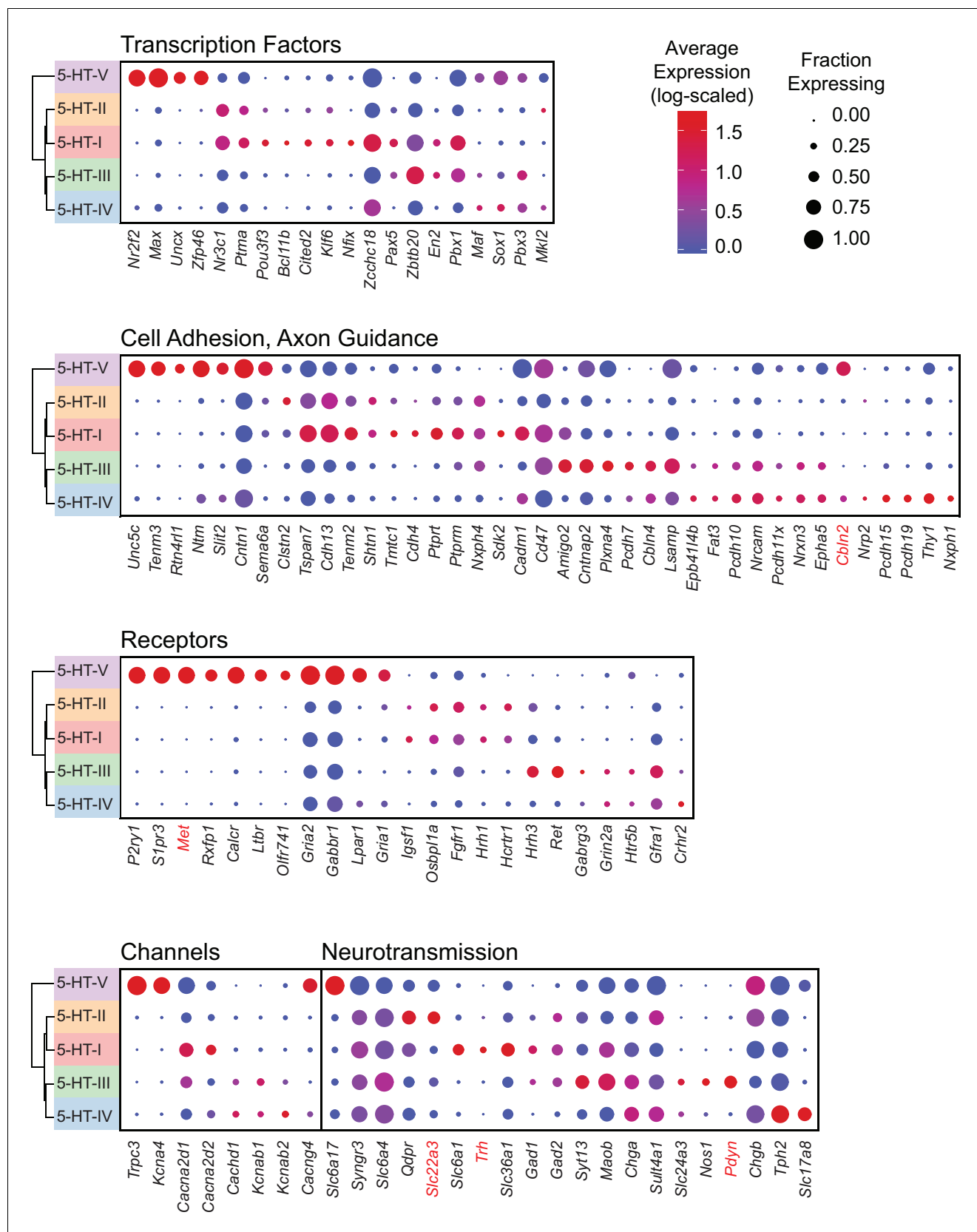


**Figure 4.** Characterization of genes differentially expressed between serotonergic neuron subtypes. **(A)** Z-scored expression heatmaps of selected genes with functional annotations that are differentially expressed between DRN 5-HT neuron subtypes. A total of 333 differentially expressed (DE) Figure 4 continued on next page

## Figure 4 continued

genes were found (5% false discovery rate, Benjamini-Hochberg correction). A large proportion of DE genes encode for cell surface and transmembrane proteins. Genes shown are categorized into five groups: transcription factors, cell adhesion and axon guidance molecules, transmembrane receptors, ion channels, and neurotransmission-related genes. (B-F) In situ hybridizations from the Allen Brain Atlas showing the expression patterns of selected DE genes enriched in each of the five 5-HT neuron subtypes. Many DE genes are not expressed uniformly throughout the DRN and are instead spatially heterogeneous along the anterior-posterior, medial-lateral, and dorsal-ventral axes, suggesting that 5-HT neuron subtypes are differentially distributed across spatial domains. (G) Confocal images from a representative DRN coronal section (AP = -4.40 mm) stained for mRNA transcripts of *Trh* (magenta), *Pdyn* (green), and *Slc6a4* (blue) using RNAscope single molecule fluorescent in situ hybridization (FISH). Regions marked in yellow are enlarged in images (i)-(iii) and show differential expression of *Trh* and *Pdyn* in three DRN subregions. *Trh* and *Pdyn* expression is low in the ventromedial DRN (vmDRN), whereas 5-HT neurons expressing high levels of *Trh* or *Pdyn* can be found in the dorsomedial DRN (dmDRN) and dorsolateral DRN (dlDRN). Cells expressing both *Pdyn* and *Trh* were occasionally observed in the regions bordering dmDRN and dlDRN. (H) Proportion of 5-HT neurons (*Slc6a4*<sup>+</sup>) containing *Pdyn* or *Trh* transcripts (minimum of 3 puncta). *Trh* transcripts were detected in a small proportion of *Pdyn*<sup>+</sup> 5-HT neurons. (I) Probability distribution maps of *Slc6a4*<sup>+</sup> 5-HT neurons containing transcripts for *Pdyn* (green), *Trh* (red), or *Cbln2* (blue) across multiple FISH experiments. Cells of each category were separately mapped to a reference atlas and counted in 50  $\mu$ m x 50  $\mu$ m bins. Bin counts were normalized to the total cell count for each category to obtain the probability maps that are displayed as heatmaps overlaid on the average coronal Nissl template from the Allen Brain Atlas. *Pdyn*-expressing 5-HT neurons are enriched along the midline, whereas *Trh*-expressing 5-HT neurons are enriched in dorsolateral regions. *Cbln2*-expressing 5-HT neurons are distributed more ventrally, and are also found in the posterior B6 tail of the DRN (panel I7). Positions of *Slc6a4*<sup>+</sup> cells are not shown. Scale bars: 500  $\mu$ m, unless otherwise stated.

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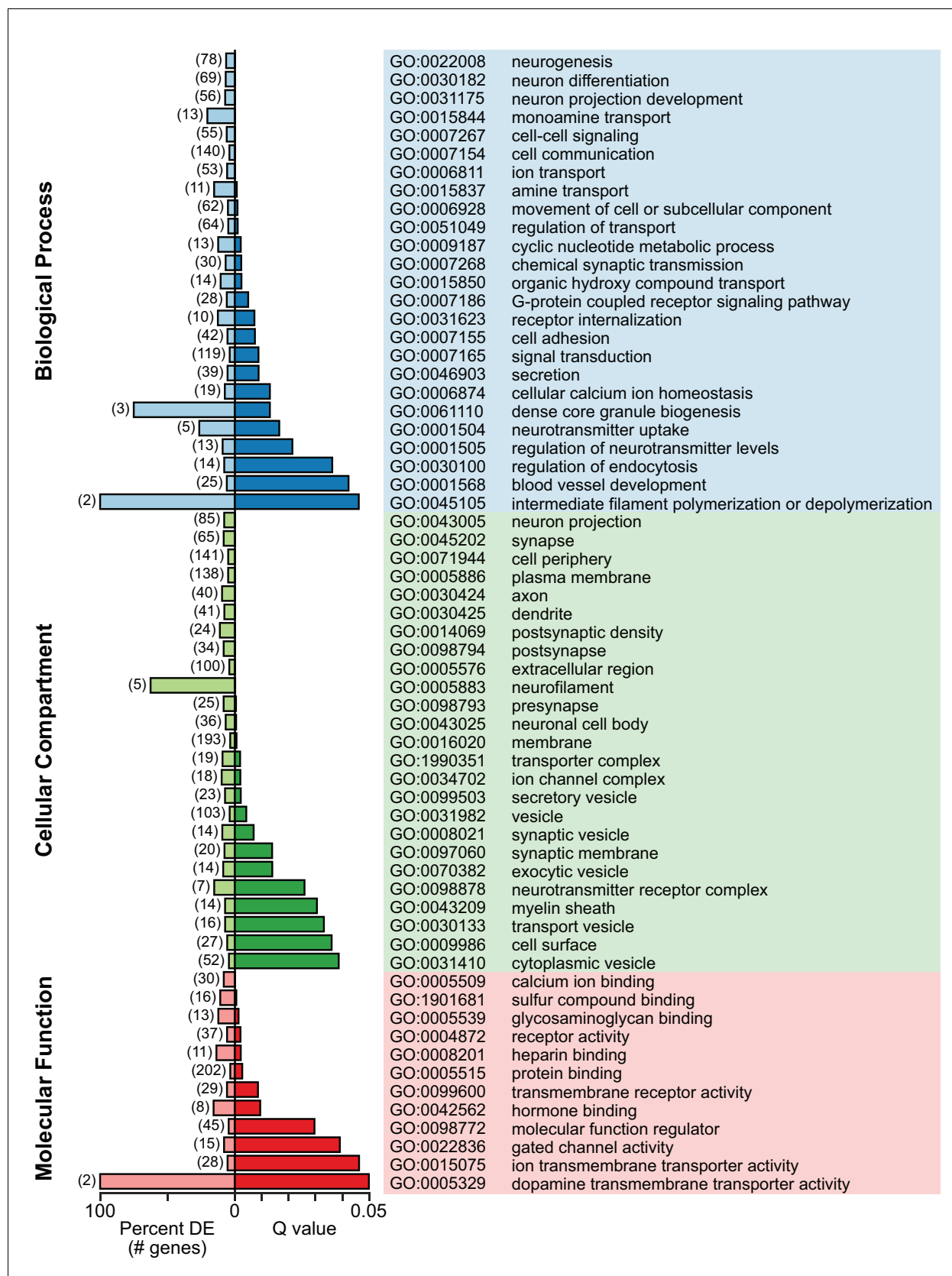


**Figure 4—figure supplement 1.** Average log-scaled expression of genes differentially expressed between 5-HT neuron subtypes. Dot plot showing the log-scaled expression for each gene shown in **Figure 4A**. Genes are sorted into four separate dot plots based on their functional grouping. The Figure 4—figure supplement 1 continued on next page

*Figure 4—figure supplement 1 continued*

color of each dot represents the average log-transformed UMI counts for each gene after normalizing for the total transcript count per cell, while the size of the dot represents the fraction of cells in each 5-HT neuron subtype cluster in which transcripts for that gene were detected.

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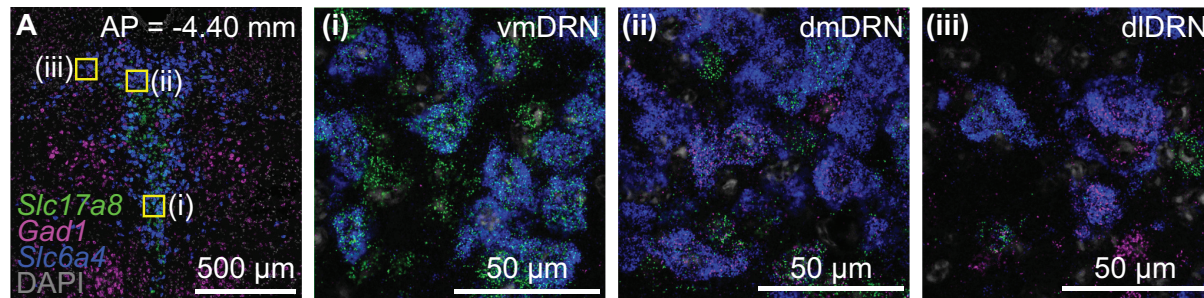
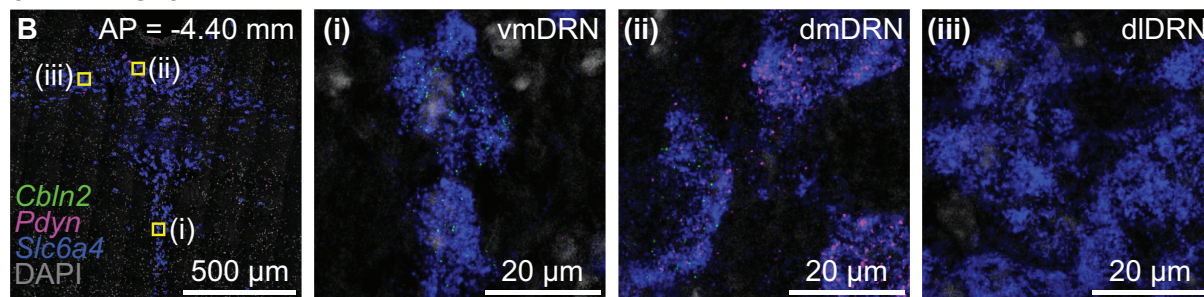
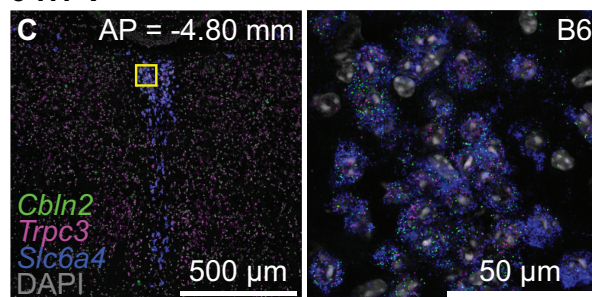
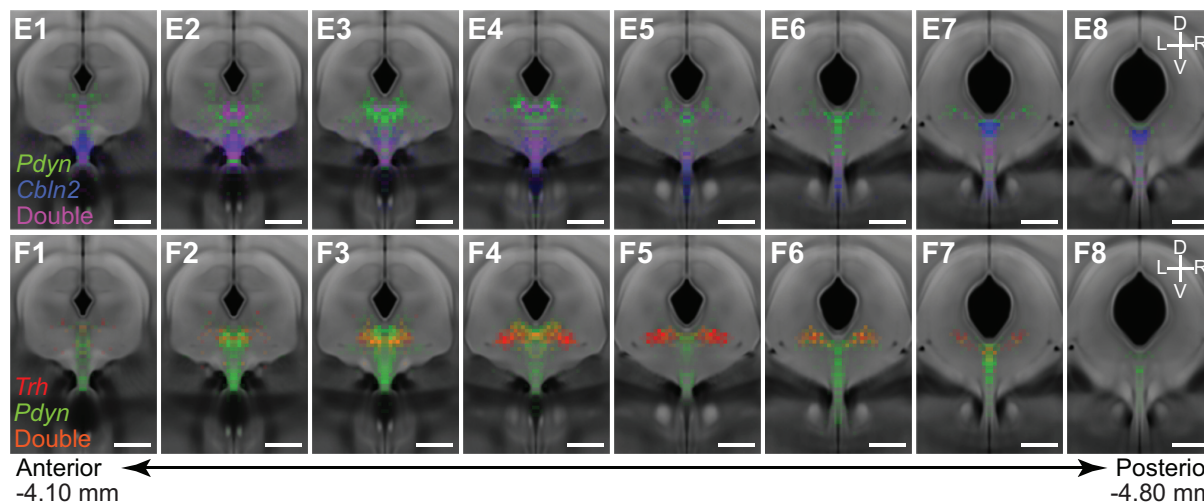
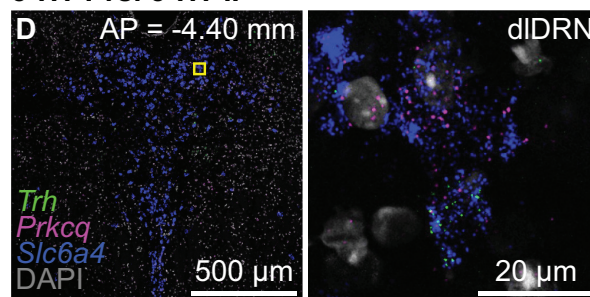
**Figure 4—figure supplement 2.** Enriched gene ontology terms associated with genes differentially expressed between 5-HT neuron subtypes. Selected gene ontology (GO) terms that were significantly enriched ( $Q < 0.05$ , Benjamini-Hochberg correction) were sorted by ontology category and Figure 4—figure supplement 2 continued on next page

*Figure 4—figure supplement 2 continued*

arranged in order of increasing Q values. Q values and the proportion of differentially expressed genes associated with each GO term, expressed as a percentage of genes in the background list associated with the GO term, are shown as bar plots. The majority of genes differentially expressed between 5-HT neuron subtypes were membrane proteins (GO:0016020, 193 of 333 genes) that included transporters, cell adhesion molecules, receptors, and synaptic proteins.

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**5-HT-I/II vs. 5-HT-III vs. 5-HT-IV****5-HT-III vs. 5-HT-IV****5-HT-V****5-HT-I vs. 5-HT-II**

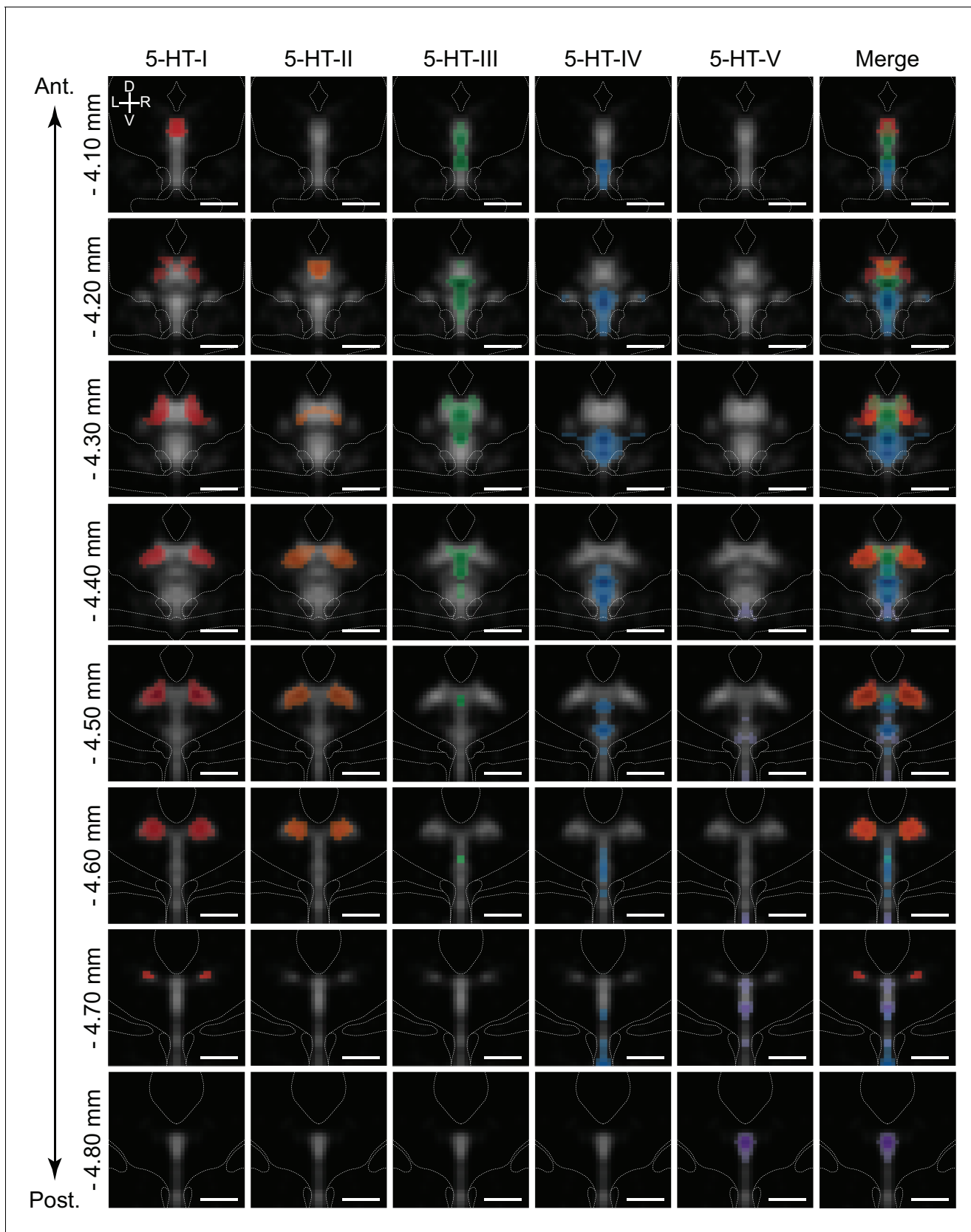
**Figure 4—figure supplement 3.** Additional validation of differential gene expression between 5-HT neuron subtypes using *FISH*. (A-D) Confocal images from representative DRN coronal sections stained for transcripts of interest using multiplexed single molecule *FISH*. All sections were stained  
Figure 4—figure supplement 3 continued on next page

## Figure 4—figure supplement 3 continued

for *Slc6a4* (blue) to identify 5-HT neurons, and counterstained using DAPI (gray). For panels A and B, regions outlined in yellow are enlarged in the images on the right labeled (i), (ii), and (iii), which correspond to the ventromedial DRN (vmDRN), dorsomedial DRN (dmDRN), and dorsolateral DRN (dlDRN), respectively. (A) 5-HT neurons expressing *Slc17a8* (green) are enriched along the midline in both vmDRN and dmDRN. 5-HT neurons expressing *Gad1* (magenta) were found in dmDRN and dlDRN. (B) *Cbln2* (green) is expressed in 5-HT neurons along the midline, but is largely absent from dlDRN. 5-HT neurons expressing both *Cbln2* and *Pdyn* were also found in both vmDRN and dmDRN. 5-HT neurons in dmDRN expressed higher levels of *Pdyn* compared to *Pdyn*<sup>+</sup> 5-HT neurons in vmDRN. (C) Putative 5-HT-V neurons expressing high levels of both *Cbln2* (green) and *Trpc3* (magenta) were found in the posterior B6 subregion of the DRN, which is enlarged in the image on the right. Cells co-expressing *Cbln2* and *Trpc3* also showed reduced staining for *Slc6a4* transcripts compared to 5-HT neurons in other subregions, consistent with reduced expression of *Slc6a4* in the 5-HT-V cluster from the scRNA-seq data. (D) Putative 5-HT-I neurons expressing *Trh* (green) are a subset of *Prkcq*<sup>+</sup> (magenta) 5-HT neurons. *Prkcq*<sup>+</sup>;*Trh*<sup>+</sup> 5-HT neurons are putative 5-HT-II neurons. (E) Spatial distribution of 5-HT neurons from a single *FISH* experiment co-staining for *Cbln2*, *Pdyn*, and *Slc6a4* (*n* = 5,806 cells, 3 mice). The locations of 5-HT neurons positive for *Cbln2* only (blue; *n* = 1,926 cells), *Pdyn* only (green; *n* = 1,048 cells), or double-positive for both transcripts (magenta; *n* = 1,517 cells) are displayed as probability heatmaps overlaid on the coronal averaged Nissl template. (F) Probability heatmaps from a separate *FISH* experiment co-staining for *Trh*, *Pdyn*, and *Slc6a4* (*n* = 6304 cells, 3 mice). Heatmaps show the spatial distribution of 5-HT neurons positive for *Trh* only (red; *n* = 355 cells), *Pdyn* only (green; *n* = 1,867 cells), or double-positive for both transcripts (orange; *n* = 339 cells). Scale bars: 500  $\mu$ m, unless otherwise stated.

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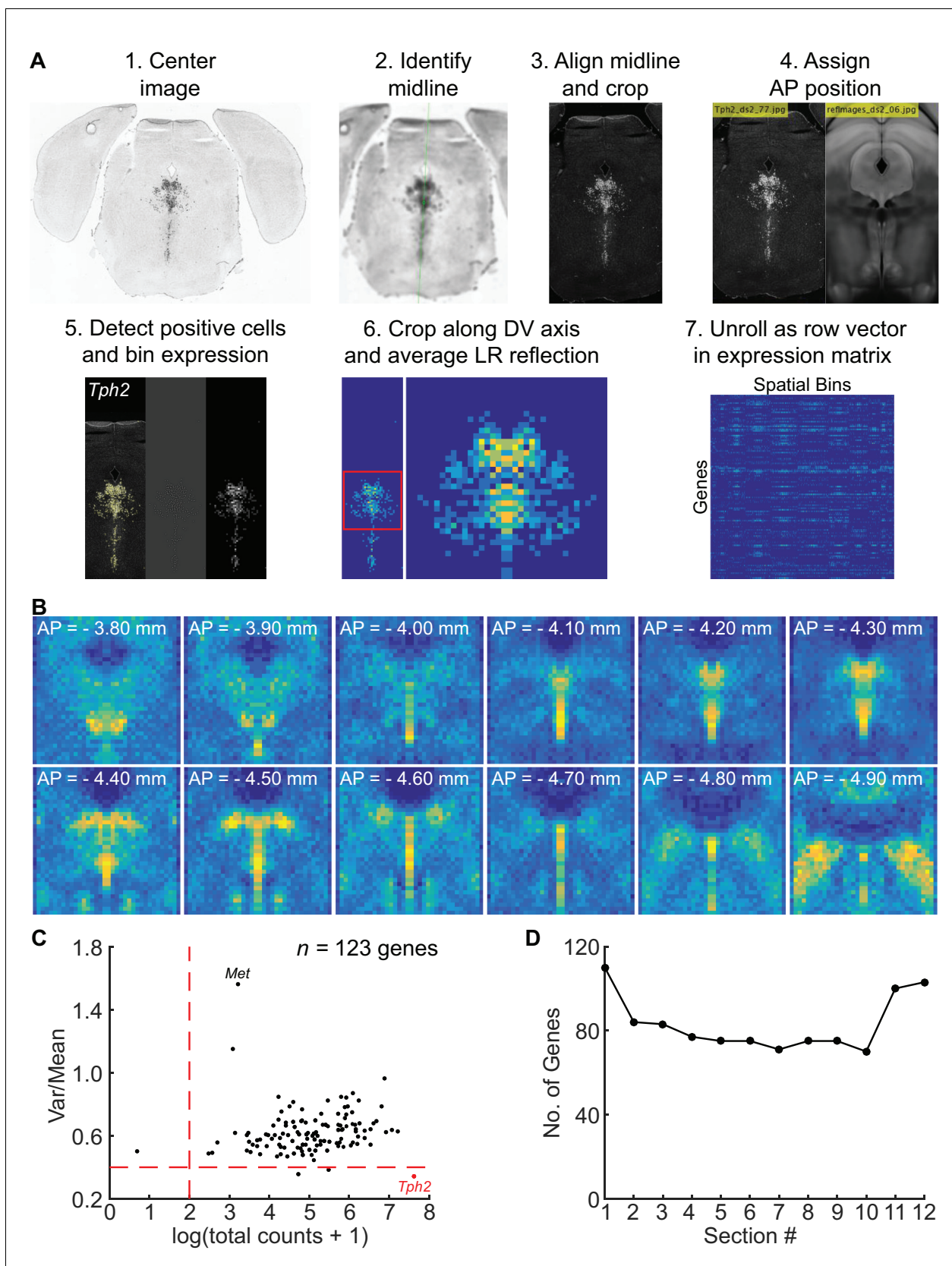
**Figure 5.** 5-HT neuron subtypes are distributed in distinct but overlapping spatial domains. Inferred spatial distributions of 5-HT neuron subtypes displayed as a map of correlation coefficients between profiles of gene expression of each subtype and each spatial bin. Each column shows the

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*Figure 5 continued*

correlation map for each 5-HT neuron subtype at different coronal sections along the anterior-posterior axis of the DRN (rows). Only bins with positive correlation coefficients greater than 0.1 are shown, and maps were smoothed using a 2D Gaussian filter (1SD) for display. Correlation maps are overlaid on the averaged *Tph2* expression (grayscale, based on *ISH* data from the Allen Brain Atlas). Anatomical landmarks visible in the averaged Nissl template, such as the cerebral aqueduct and fiber tracts, are outlined and overlaid on each coronal image. Correlation maps for subtypes 5-HT-I and 5-HT-II suggest that they are largely intermingled in the dorsolateral DRN, with some overlap in the anterior DRN with 5-HT-III in the dorsomedial DRN. 5-HT-IV was enriched in the ventral regions of the DRN, while 5-HT-V was highly restricted to the posterior B6 tail of the DRN that was highly enriched in expression of the 5-HT-V enriched gene *Met*. Scale bars: 500  $\mu$ m.

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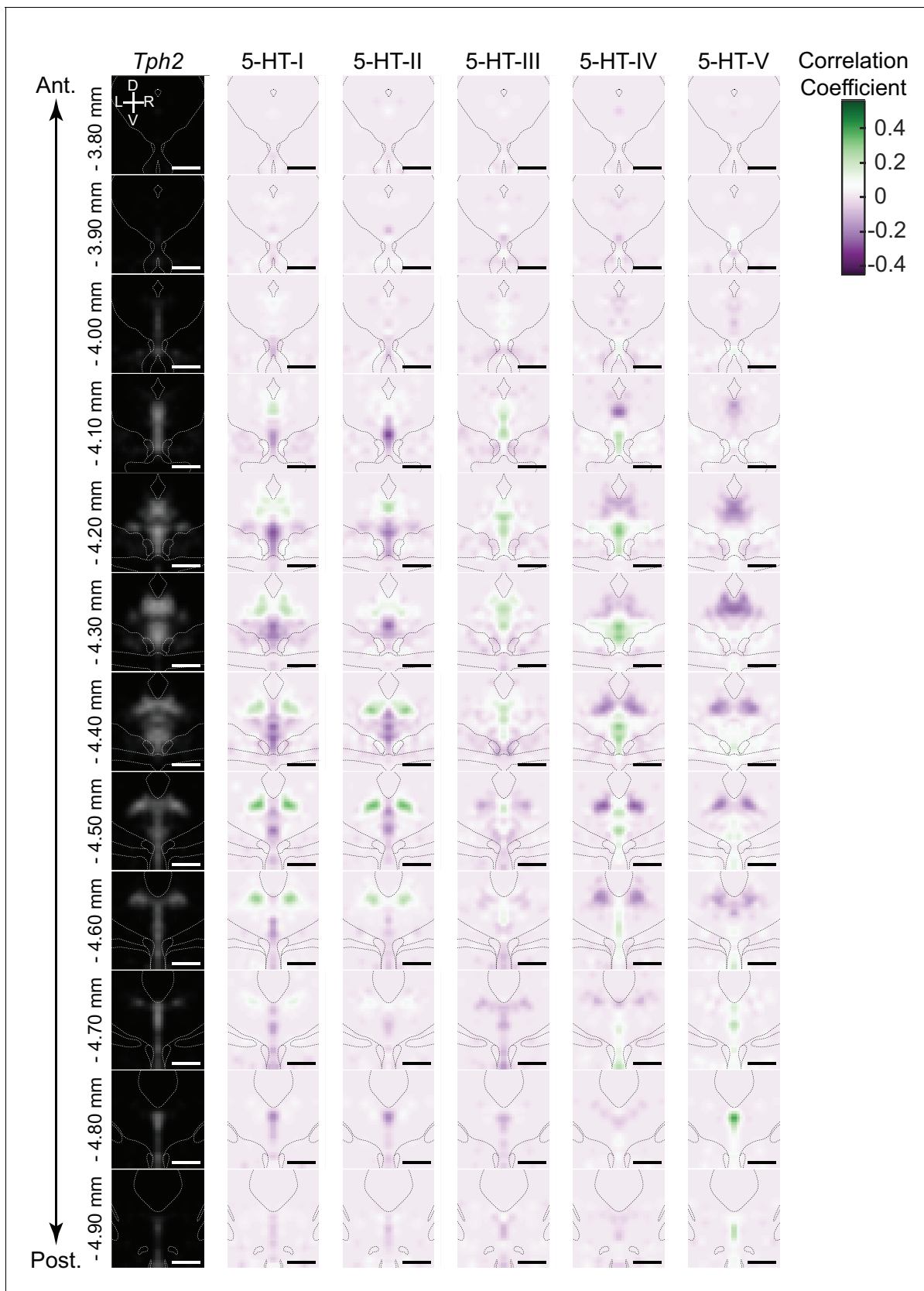
**Figure 5—figure supplement 1.** Processing of ISH images for spatial correlation analysis. (A) Overview of workflow for processing ISH images from the Allen Brain Atlas to generate the spatial expression matrix. Coronal images spanning the DRN in each ISH experiment were downsampled and

Figure 5—figure supplement 1 continued on next page

## Figure 5—figure supplement 1 continued

centered on the brain slice. The midline in each slice was identified and the image was rotated to align the midline vertically. Rotated images were cropped and assigned to corresponding positions along the anterior-posterior axis relative to the Allen Brain Atlas coronal template. Cells positive for the *ISH* signal were identified using a spot detection algorithm and counted in  $50 \times 50 \mu\text{m}$  bins to generate 2D histograms for each image. 2D histograms, representing the spatial expression profile of the probed gene in a given coronal section, were aligned and cropped along the dorsal-ventral axis to retain only the region containing the DRN (36 X bins  $\times$  36 Y bins). Cropped histograms were averaged with the left-right reflection and combined with the other histograms generated from other images in the same experiment image series to generate the volume data (36 X bins  $\times$  36 Y bins  $\times$  12 images) including NaN values for missing data. Volume data for each gene was unrolled into a single row vector (of 15,552 entries), and row vectors were concatenated to generate the spatial expression matrix for spatial correlation analysis. **(B)** Sum of 2D histograms generated by the workflow in (A), displayed as a series of 12 coronal sections showing the summed count of cells detected across all *ISH* images analyzed. Anatomical landmarks such as the cerebral aqueduct, fiber tracts, periaqueductal gray, and DRN are easily discerned from the images of the histograms, suggesting that the histograms were well aligned and have good coverage of the DRN. **(C)** Scatter plot of the total expression and mean-normalized variance for genes in the spatial expression matrix within the *Tph2*<sup>+</sup> region. Each dot represents 1 of the 123 genes in the expression matrix. Genes with low spatial variability within the masked region, such as *Tph2* (marked red), and low total expression were excluded from the spatial correlation analysis. *Met*, which showed the most spatial variability since its expression is restricted to the posterior cluster, is also marked on the plot. **(D)** Plot of the number of genes used for the spatial correlation analysis at each of the 12 coronal sections (genes per 2D histogram). Genes with missing data at a given coronal section (i.e. no image or damaged slice) were not included in the count. Approximately 80 genes on average were used for calculating the correlation coefficient for each spatial bin.

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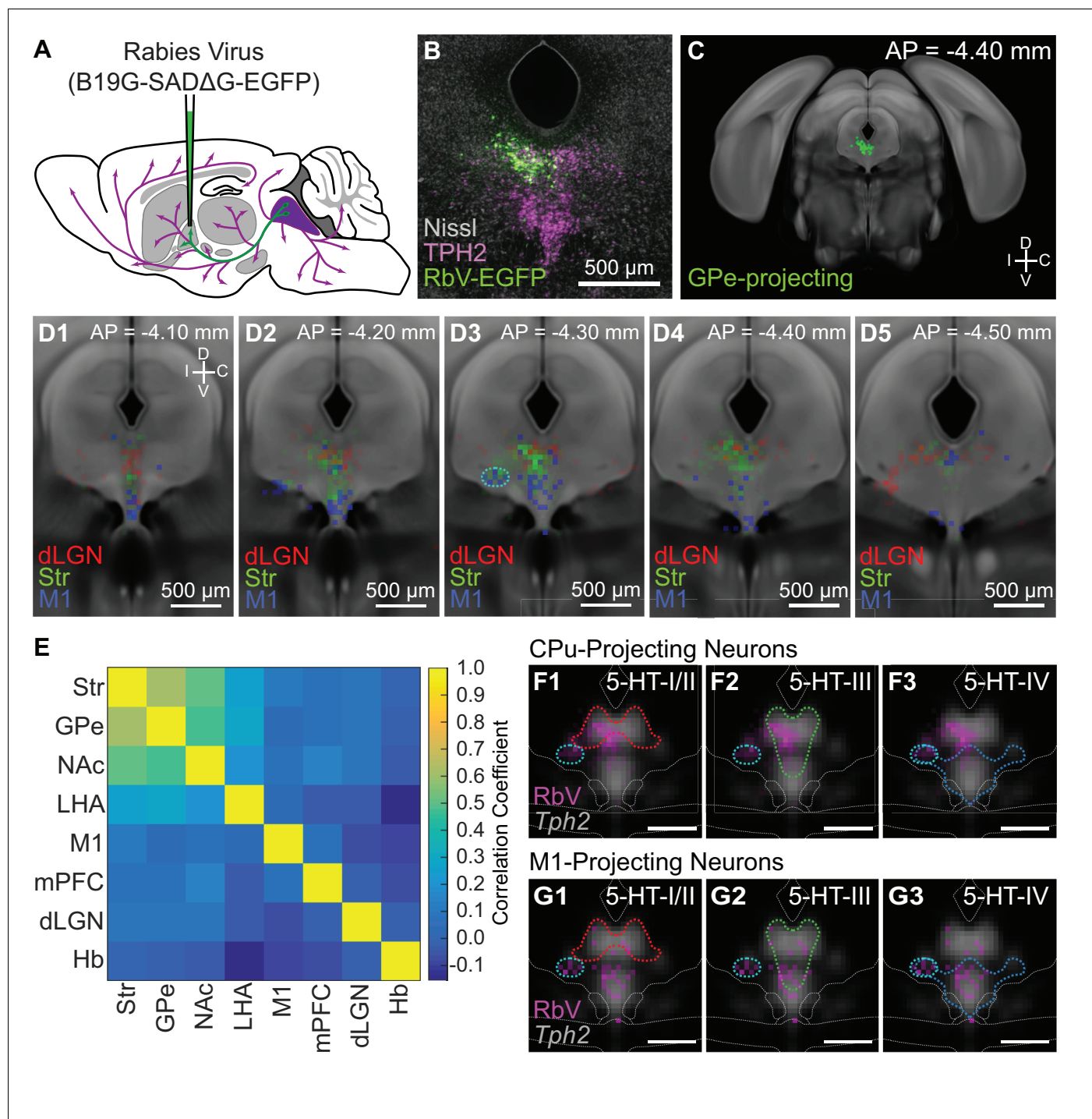


**Figure 5—figure supplement 2.** Correlation coefficient maps for DRN 5-HT neuron subtypes. Spatial correlation maps displayed as heatmaps arranged in columns for each 5-HT neuron subtype. Heatmaps correspond to coronal sections, and are arranged vertically in order along the anterior-posterior axis. Figure 5—figure supplement 2 continued on next page

*Figure 5—figure supplement 2 continued*

posterior axis. The averaged *Tph2* ISH signal is included in the left-most column as a reference for the DRN outline. Anatomical landmarks visible in the averaged Nissl template, such as the cerebral aqueduct and fiber tracts, are outlined and overlaid on each coronal image. Spatial bins in the heatmaps with positive correlations (green) indicate the most likely locations of each 5-HT neuron subtype. Spatial correlation maps have been smoothed using a 2D Gaussian filter (1SD) for visualization. Since only differentially expressed genes were used for this analysis, negative correlations are also seen. These reflect the locations of 5-HT neurons of other subtypes from which, by definition, the differentially expressed genes that define one subtype are excluded. Scale bars: 500  $\mu$ m.

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**Figure 6.** Projection-defined DRN neuron subpopulations overlap with multiple 5-HT subtypes. (A) Schematic of retrograde tracing experiments in which unpseudotyped rabies viruses (SADΔG B19 stain) were used to infect and label neurons whose axons innervate the virus-injected target region (GPe in this example). (B) Image of a coronal section of the DRN showing rabies-labeled, GPe-projecting neurons. Brains were fixed 7 days post-injection for histology, and 5-HT neurons in the DRN were visualized using immunohistochemistry against TPH2. Sections were counterstained using a fluorescent Nissl stain (Neurotrace). (C) The location of all rabies-labeled cells within the DRN and vPAG, such as the GPe-projecting cells in (B), were manually registered onto a coronal template derived from the averaged Allen Brain Atlas coronal Nissl stain. Labeled neurons outside of this region of interest, such as the PPN and dorsal PAG, were not included. (D1-5) Distributions of Str-projecting (green), M1-projecting (blue), and dLGN-projecting (red) subpopulations, displayed as a probability density map (50  $\mu$ m x 50  $\mu$ m bins) overlaid on the coronal template. Coronal sections shown are centered in panel D3 at the maximum density of the Str-projecting population. Str- and M1-projecting populations were largely separated along the dorsal-ventral axis, with some overlap. Co-labeled neurons projecting to both Str and M1 were typically found in the ventrolateral DRN, outlined in cyan. Figure 6 continued on next page

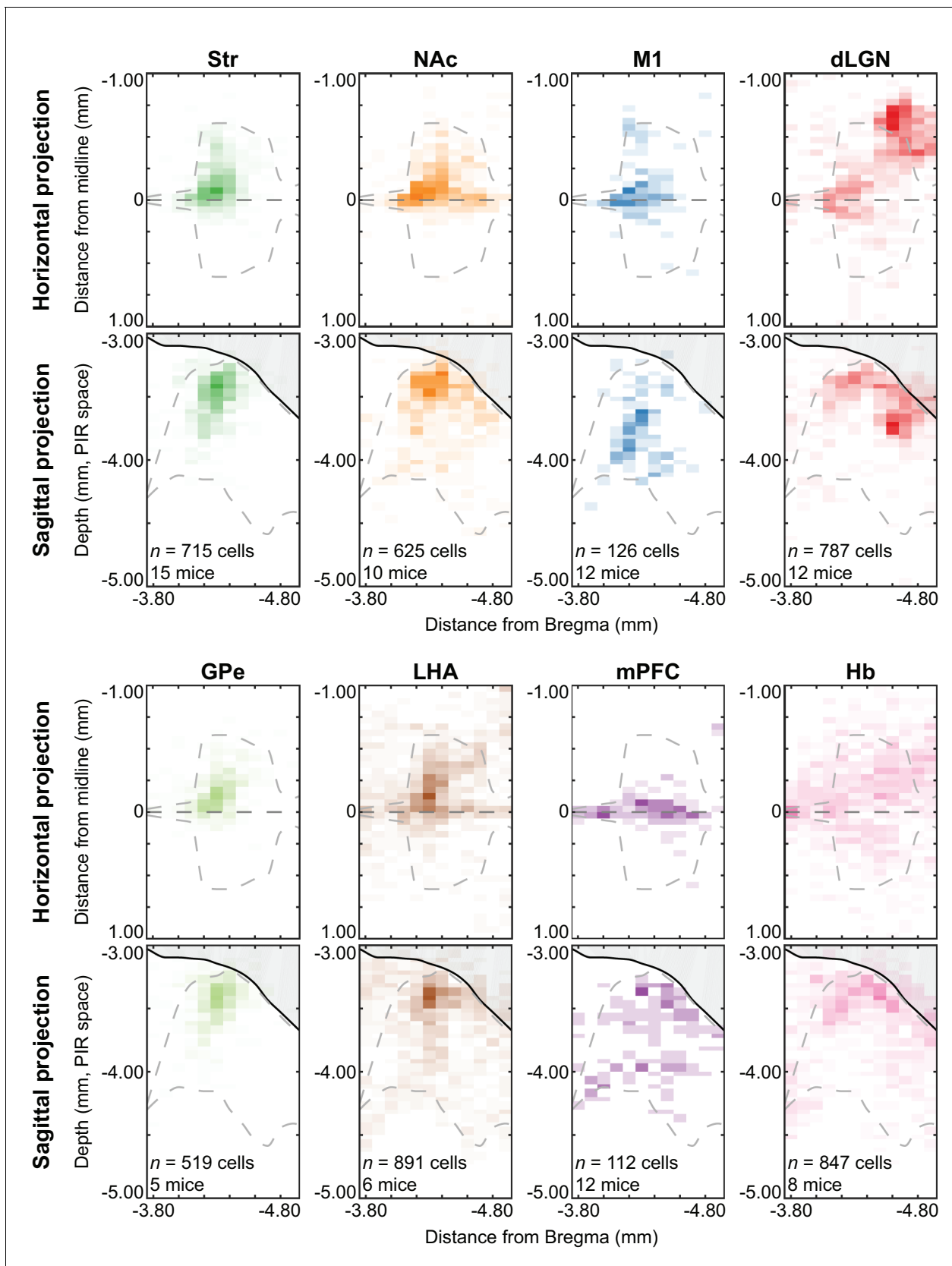


*Figure 6 continued*

in D3, and the ventromedial DRN. (E) Pairwise correlations between the distributions of projection-defined subpopulations. Correlations between the Str-projecting subpopulation were higher with other subpopulations that also innervate nuclei of the basal ganglia, compared with subpopulations innervating functionally distinct targets such as M1 or dLGN. (F-G) Overlap between the distributions of neurons innervating Str (F) or M1 (G) and the 5-HT neuron subtype distributions at AP = −4.30 mm. Distributions of each projection-defined subpopulation are represented as probability maps (magenta) overlaid on the averaged *Tph2* expression. Subtype distributions are represented as color-coded outlines corresponding to the colors used in **Figure 4** for each subtype. Str-projecting neurons largely fell into regions occupied by both 5-HT-III and 5-HT-IV subtypes, suggesting molecular heterogeneity within this projection-defined population. M1-projecting neurons were more confined to the ventral DRN, overlapping with the distribution of the 5-HT-IV subtype. Scale bars: 500  $\mu$ m.

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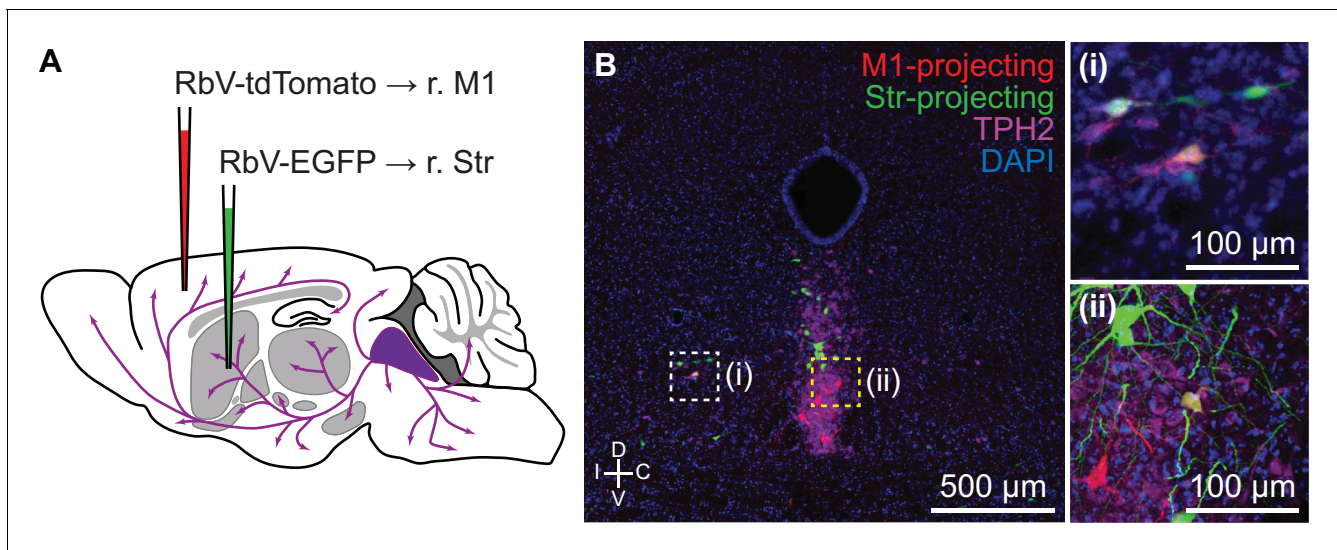


**Figure 6—figure supplement 1.** Spatial distributions of projection-defined subpopulations. Distributions of all projection-defined subpopulations in **Figure 5** displayed as probability density maps. Distributions for each subpopulation were projected onto either the horizontal or sagittal plane (50 × 50 μm). **Figure 6—figure supplement 1 continued on next page**

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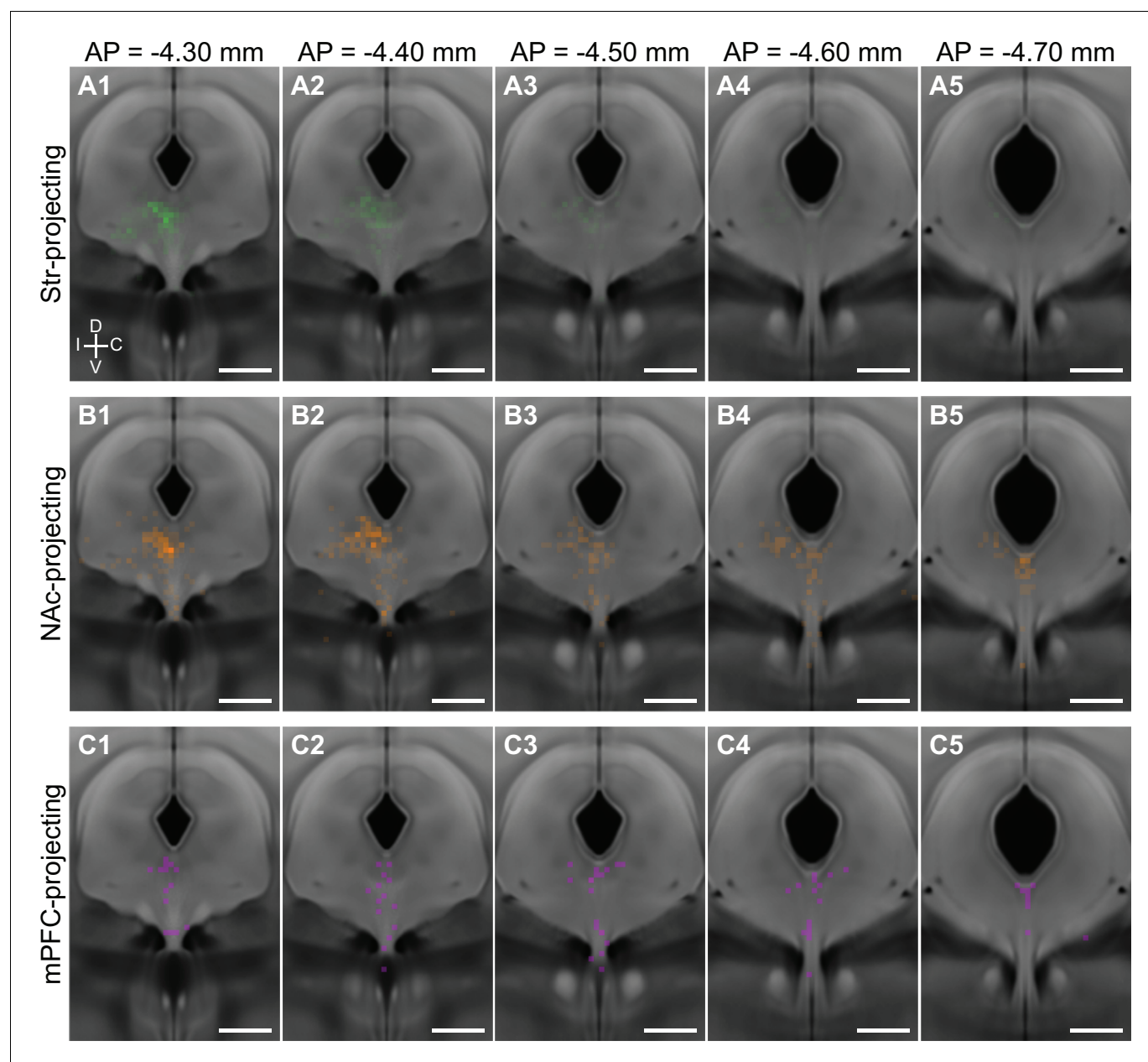
100  $\mu\text{m}$  bins). The midline in horizontal projections is indicated by a straight gray line at 0 mm, and distances from the midline are negative for the hemisphere ipsilateral to the injection site. The region occupied by the cerebral aqueduct is shaded in solid gray in sagittal projections, and the depth indicated is relative to the origin of the PIR space in the Allen Brain Atlas. The flattened projection of the region containing *Tph2*<sup>+</sup> cells is outlined by the gray dashed line in each image. Str-projecting and GPe-projecting subpopulations were the most similar, whereas the Str-projecting and NAc-projecting subpopulations were mostly overlapping in the anterior DRN but differed in the posterior DRN. Cells outside of the ventrolateral periaqueductal gray and DRN, such as cells in the pedunclopontine nucleus or dorsal periaqueductal gray, were excluded from the probability maps. Abbreviations: Str: striatum; GPe: globus pallidus, external; NAc: nucleus accumbens; LHA: lateral hypothalamic area; M1: primary motor cortex; mPFC: medial prefrontal cortex; dLGN: dorsal lateral geniculate nucleus; Hb: habenula.

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**Figure 6—figure supplement 2.** Ventrolateral DRN neurons send axon collaterals to both striatum and motor cortex. (A) Schematic of double retrograde tracing experiment using unpseudotyped rabies viruses. RbV-tdTomato was injected into primary motor cortex, and RbV-EGFP was injected into the ipsilateral striatum. Tissue collection for histology was performed 7 days post-injection. (B) Representative fluorescent image of a coronal section through the anterior DRN containing both M1- and Str-projecting neurons. 5-HT neurons were labeled by an antibody stain for TPH2. Neurons innervating both M1 and Str were co-labeled by RbV-tdTomato and RbV-EGFP, and were typically found in the ventrolateral DRN and the dorsal border of the ventromedial DRN. Inset (i) is a confocal image of the ventrolateral DRN region in (B) marked by the white dashed box, and inset (ii) is a confocal image of the ventromedial DRN region in (B) marked by the yellow dashed box. Both insets are centered on TPH2<sup>+</sup> cells co-labeled by RbV-tdTomato and RbV-EGFP.

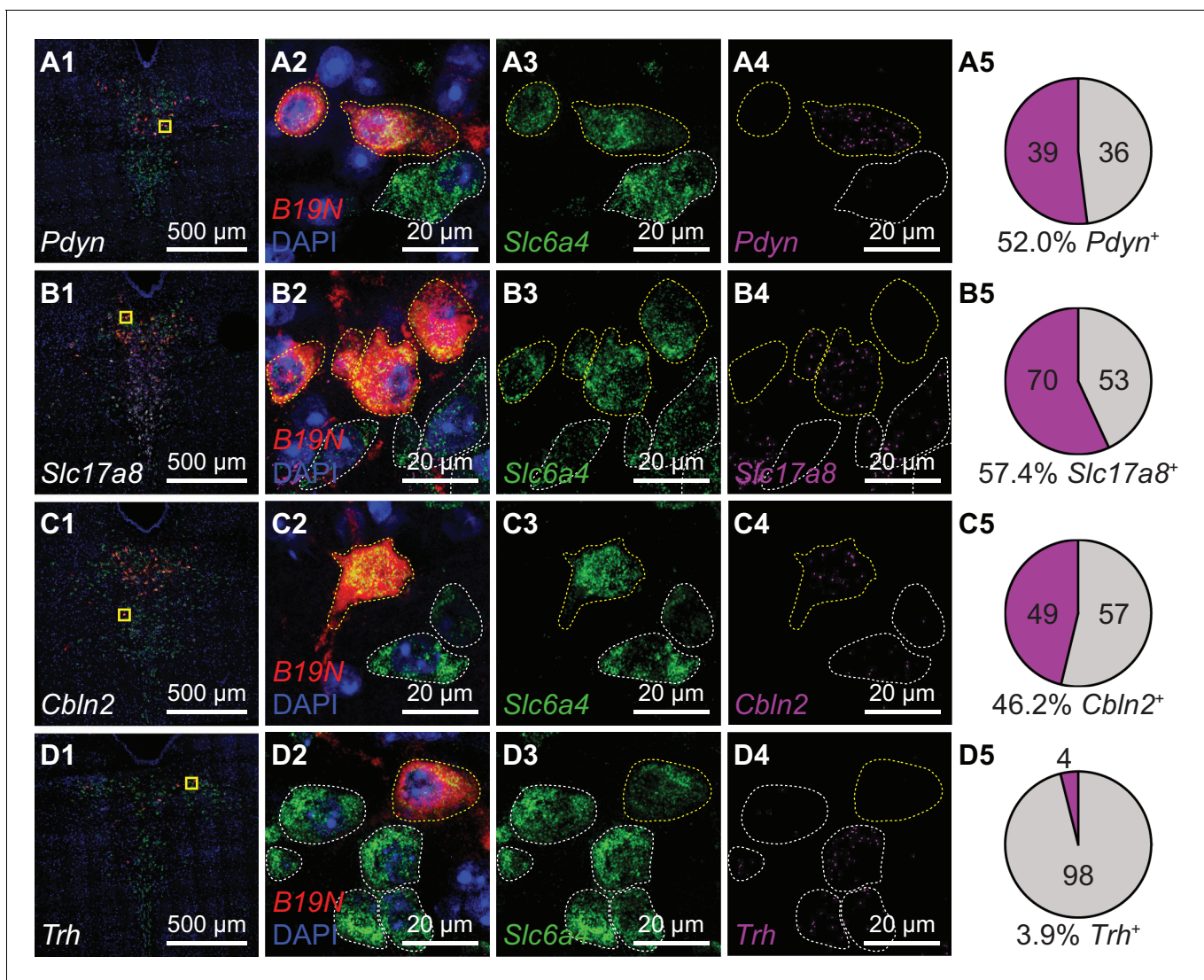
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**Figure 6—figure supplement 3.** Caudal DRN B7 neurons innervate ventral striatum and prefrontal cortex. Distributions of Str-projecting (A), NAc-projecting (B), and mPFC-projecting (C) neurons displayed as probability density maps overlaid on the Allen Brain Atlas coronal template. Str-projecting and NAc-projecting are both found in the dorsomedial DRN at anterior sections (columns 1–2). Str-projecting neurons are more restricted to anterior sections of the DRN, whereas the NAc-projecting neurons are also found in posterior sections of the DRN (columns 3–5) where they overlap with the distribution of mPFC-projecting neurons.

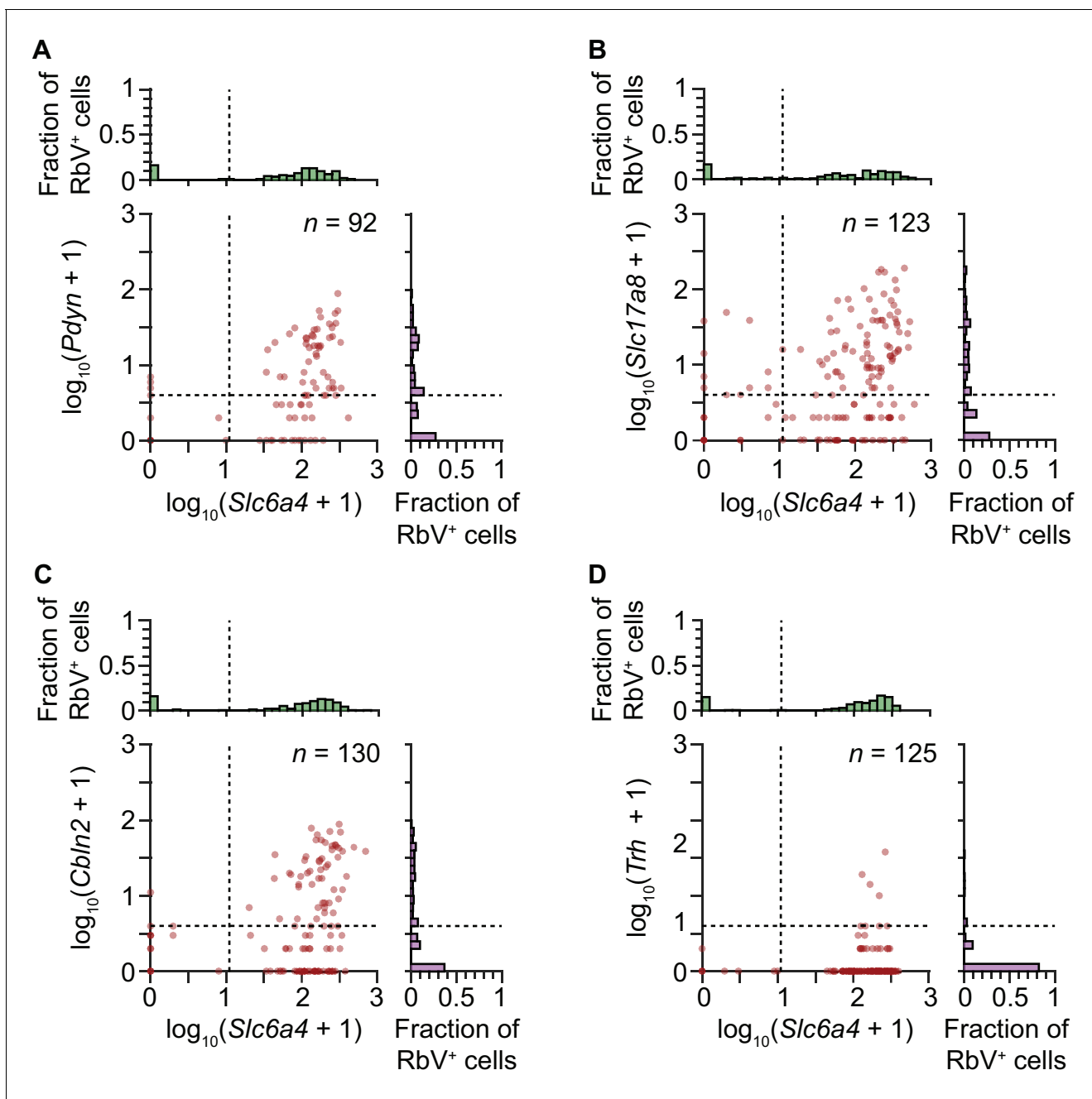
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**Figure 7.** Striatum receives inputs from multiple DRN 5-HT neuron subtypes. Multiplexed *FISH* was used to identify rabies-infected Str-projecting 5-HT neurons in the DRN and quantify their expression of 5-HT subtype enriched genes. Rabies-infected neurons are identified by expression of the virus transcript *B19N*. Confocal images of the DRN from representative coronal sections are shown in column 1, and regions marked in yellow are expanded and separated by channel in columns 2–4. In columns 2–4, rabies-infected cells (*B19N*<sup>+</sup>) are outlined in yellow, whereas uninfected 5-HT neurons are outlined in white. The proportions of DRN 5-HT neurons that express each subtype-enriched gene are summarized in pie charts in column 5. Approximately half of the Str-projecting 5-HT neurons express the 5-HT-III enriched gene *Pdyn* (row A), and a similar proportion express 5-HT-IV enriched genes such as *Slc17a8* (row B) and *Cbln2* (row C). Most Str-projecting 5-HT neurons do not express the 5-HT-I enriched gene *Trh* (row D).

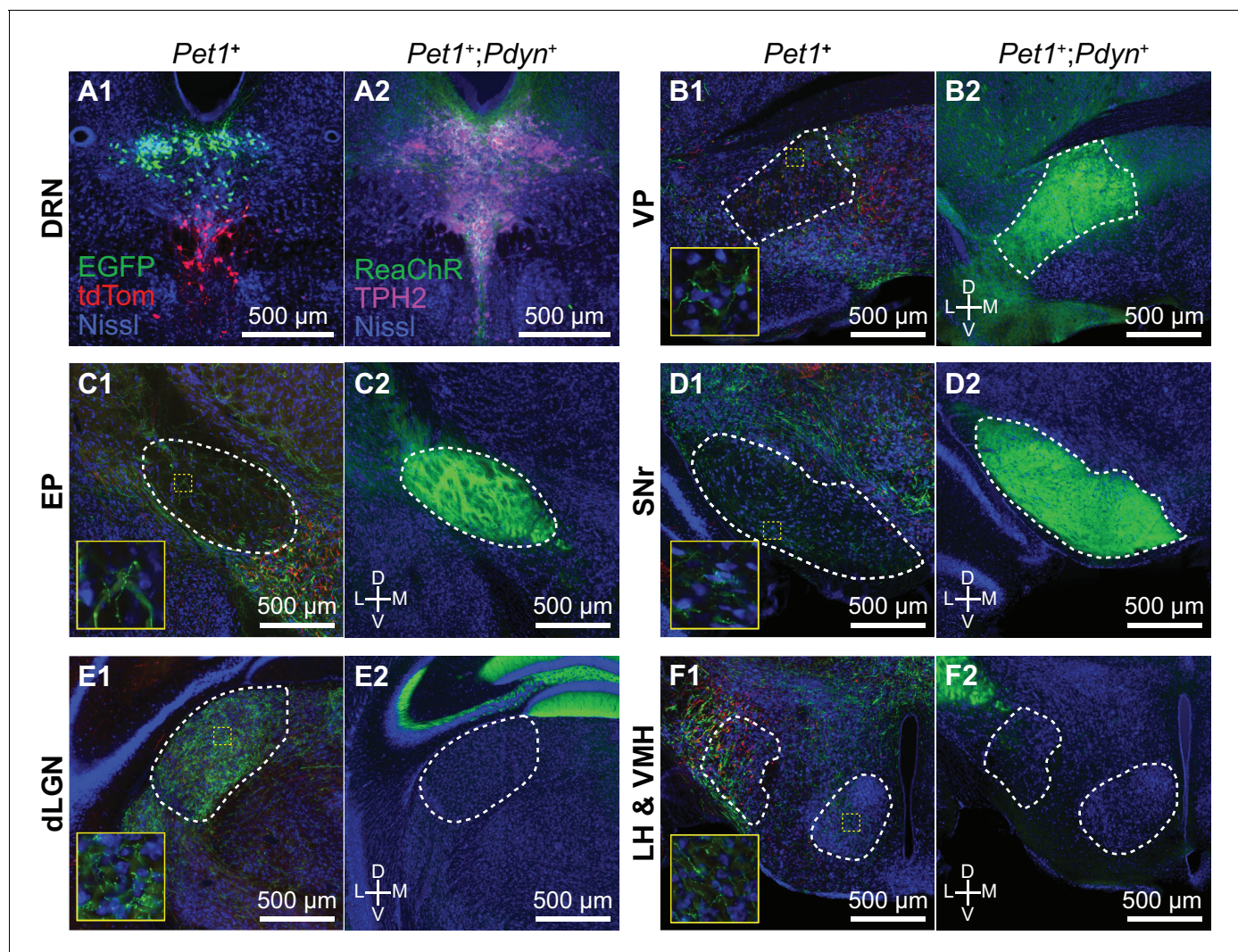
DOI: <https://doi.org/10.7554/eLife.46464.019>



**Figure 7—figure supplement 1.** Quantification of RNAscope puncta in Str-projecting neurons. Scatter plots with marginal histograms showing the quantification of the number of single molecule *FISH* puncta (RNAscope) in each Str-projecting cell. Each dot represents a single RbV-labeled cell. RbV-labeled cells were categorized based on expression of *Slc6a4* and one of four 5-HT subtype enriched genes: *Pdyn* (A) for 5-HT-III, *Slc17a8* (B) and *Cbln2* (C) for 5-HT-IV, and *Trh* (D) for 5-HT-I. Dashed lines in the scatter plots and marginal histograms indicate the thresholds used to binarize expression. Separate RNAscope experiments were performed for each 5-HT subtype enriched gene. All RbV-labeled cells in the analyzed images were included, including some cells in the pedunculopontine nucleus, and the number of RbV-labeled cells in each experiment is indicated in the plot.

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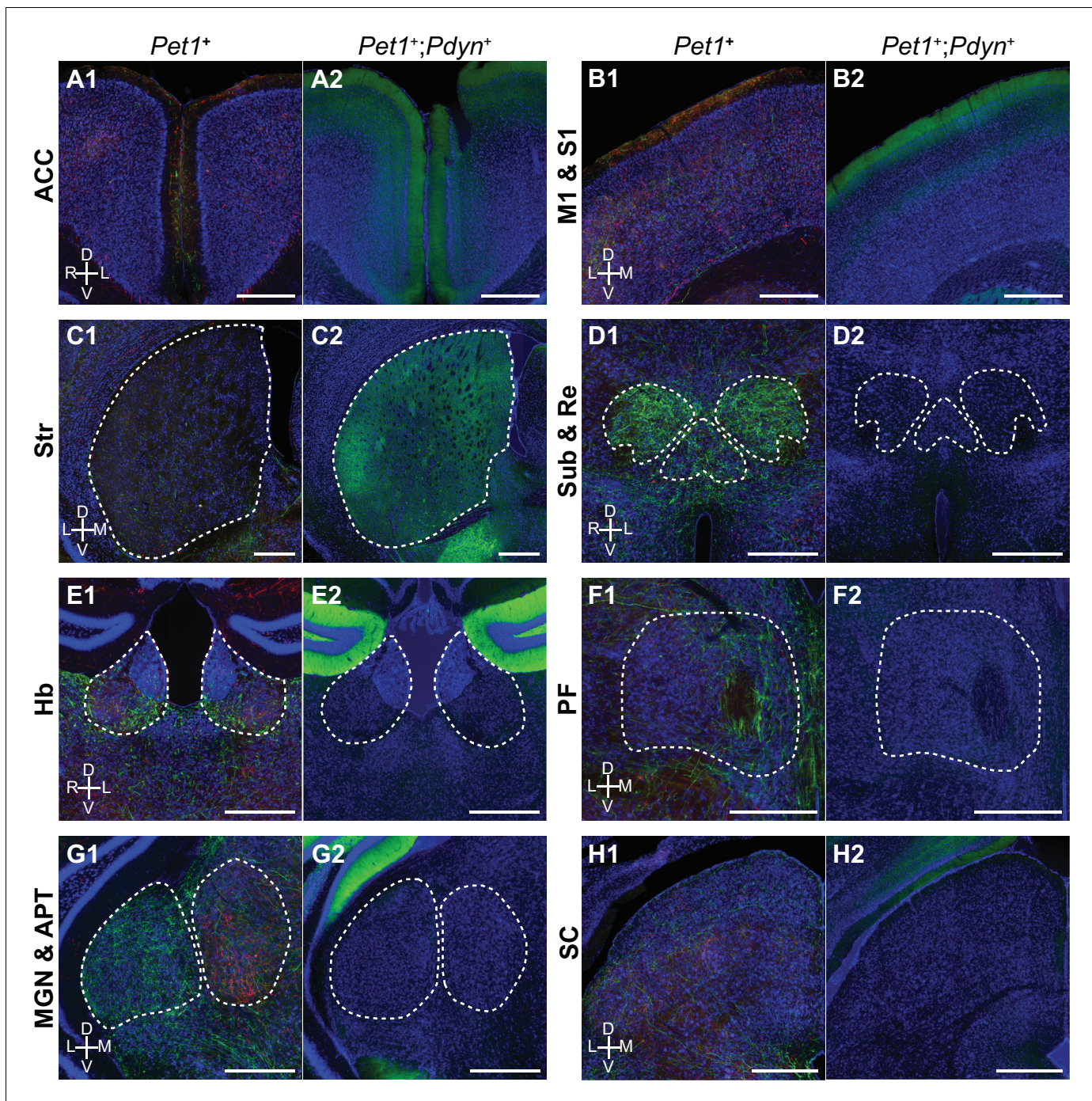




**Figure 8.** *Pdyn*<sup>+</sup> 5-HT neurons target basal ganglia circuits. (A) Conditional anterograde tracing from subsets of 5-HT neurons. Anterograde tracing from *Pet1*<sup>+</sup> DRN neurons was performed by injecting AAV2/1-CAG-FLEX-EGFP into the dorsal DRN and AAV2/1-CAG-FLEX-tdTomato into the ventral DRN of *ePet1*-Cre mice. A coronal section from the DRN injection site containing *Pet1*<sup>+</sup> cells expressing either EGFP or tdTomato is shown on the left in (A1). *Pdyn*<sup>+</sup>; *Pet1*<sup>+</sup> neurons were labeled by intersectional genetic targeting with a Cre- and Flp-dependent reporter line *R26-LSL-FSF-ReaChR:mCitrine* crossed to *Pdyn*-IRES-Cre; *Pet1*-Flpe mice. *Pdyn*<sup>+</sup>; *Pet1*<sup>+</sup> expressing ReaChR:mCitrine in the DRN are shown on the right in (A2). 5-HT neurons are labeled by immunostaining for TPH2. (B–F) Axonal projections of *Pdyn*<sup>+</sup>; *Pet1*<sup>+</sup> neurons were compared to projections of DRN *Pet1*<sup>+</sup> neurons using separate approaches for conditional anterograde tracing. *Pdyn*<sup>+</sup>; *Pet1*<sup>+</sup> neurons densely innervate many output nuclei of the basal ganglia, including VP (B2), EP (C2), and SNr (D2), which are also structures innervated by DRN *Pet1*<sup>+</sup> neurons (B1, C1, and D1). *Pdyn*<sup>+</sup>; *Pet1*<sup>+</sup> axons were not found in structures that were functionally distant from basal ganglia circuits, such as dLGN (E2) and hypothalamic nuclei (F2), although these structures received dense inputs from DRN *Pet1*<sup>+</sup> neurons (E1 and F1). Regions outlined in yellow in B1 – F1 are enlarged in the insets showing EGFP<sup>+</sup>/tdTomato<sup>+</sup> axon fibers in each region. Abbreviations: DRN – dorsal raphe nucleus. VP – ventral pallidum. EP – entopeduncular nucleus. SNr – substantia nigra pars reticulata. dLGN – dorsal lateral geniculate nucleus. LH – lateral hypothalamus. VMH – ventromedial hypothalamus. All tissue sections are counterstained with fluorescent Neurotrace (Nissl).

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**Figure 8—figure supplement 1.** *Pet1*<sup>+</sup>;*Pdyn*<sup>+</sup> neurons innervate a subset of regions targeted by DRN *Pet1*<sup>+</sup> neurons. Images of cortical and subcortical regions in the forebrain from conditional anterograde tracing of axons from DRN *Pet1*<sup>+</sup> neurons and *Pdyn*<sup>+</sup>;*Pet1*<sup>+</sup> neurons. Axons from *Pdyn*<sup>+</sup>;*Pet1*<sup>+</sup> neurons were also found in frontal and prefrontal cortical regions (A-B), but were absent from thalamic nuclei that were innervated by DRN *Pet1*<sup>+</sup> neurons (D-G) and subcortical regions outside of the basal ganglia, such as SC (H). Within Str, *Pdyn*<sup>+</sup>;*Pet1*<sup>+</sup> axons were not uniformly distributed, with the highest density in the ventrolateral regions of Str. Abbreviations: ACC: anterior cingulate cortex; M1: primary motor cortex; S1: primary somatosensory cortex; Str: striatum; Sub: submedial thalamic nucleus; Re: nucleus reuniens; Hb: habenula; PF: parafascicular thalamic nucleus; MG: medial geniculate nucleus; APT: anterior pretectal nucleus; SC: superior colliculus. Scale bars: 500  $\mu$ m.

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