



Figures and figure supplements

Anatomical and single-cell transcriptional profiling of the murine habenular complex

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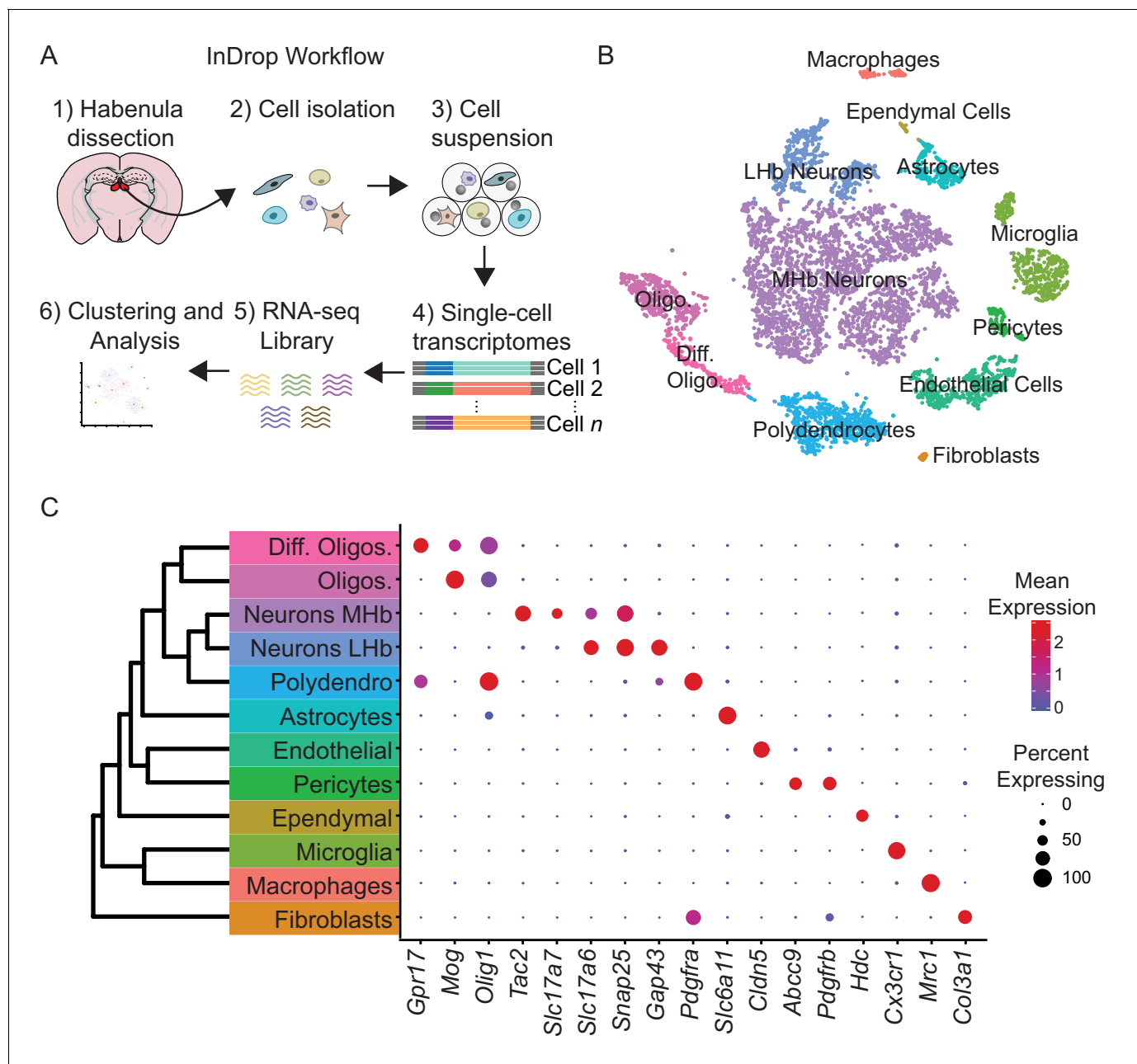


Figure 1. High-throughput single-cell transcriptomic profiling of the habenula. (A) Schematic for scRNA-seq using the inDrop platform. Tissue containing the habenula was microdissected from acute coronal brain slices prepared from adult mice (1). Tissue chunks were digested in a cocktail of proteases and followed by trituration and filtration to obtain a cell suspension (2). Single cells were encapsulated using a droplet-based microfluidic device (3) for cell barcoding and mRNA capture (4). RNA sequencing (5) and bioinformatics analysis followed (6). (B) t-SNE plot of the processed dataset containing 7,506 cells from six 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 enriched genes used to identify each major cell class. The color of each dot (blue to red) indicates the relative log-scaled expression of each gene, whereas the dot size indicates the fraction of cells expressing the gene.

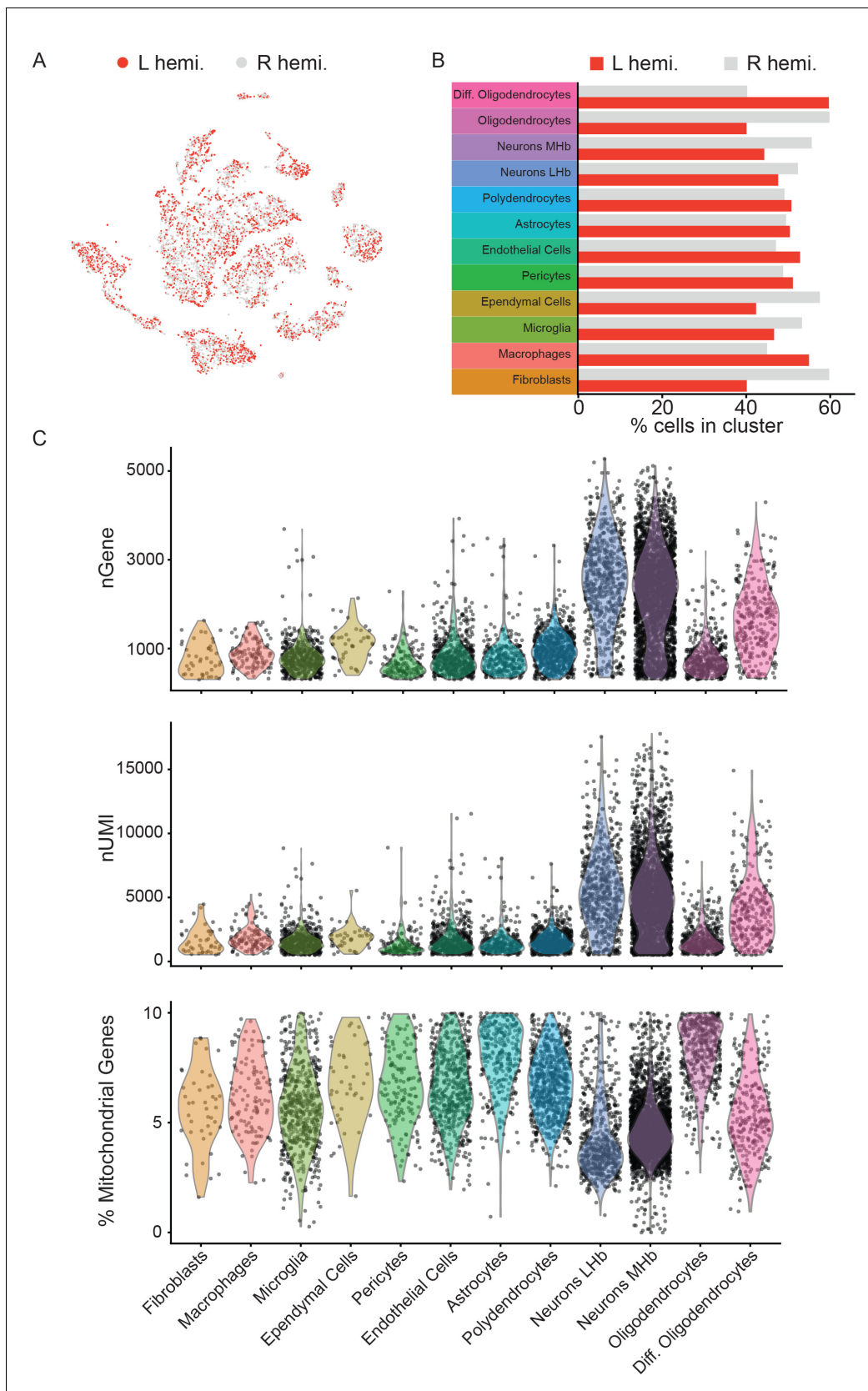


Figure 1—figure supplement 1. Comparison of cell-type composition across hemispheres and gene diversity, mitochondrial genes, and UMIs across cell types. (A) t-SNE plot of the dataset with cells color-coded by the hemisphere from which the sample was acquired. (B) Bar plots showing the percentage of cells in each cell type cluster for L and R hemispheres. (C) Violin plots showing the distribution of nGene, nUMI, and % Mitochondrial Genes across cell types.

Figure 1—figure supplement 1 continued on next page

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percentage of cells in each hemisphere that are categorized into each of the 12 major cell types. (C) Violin plots of the number of genes (top), unique molecular identifiers (UMIs, middle), and percentage of mitochondrial genes per cell (bottom) for each of the 12 cell types. Each point represents a single cell and filled area is a probability distribution of all the cells in that category.

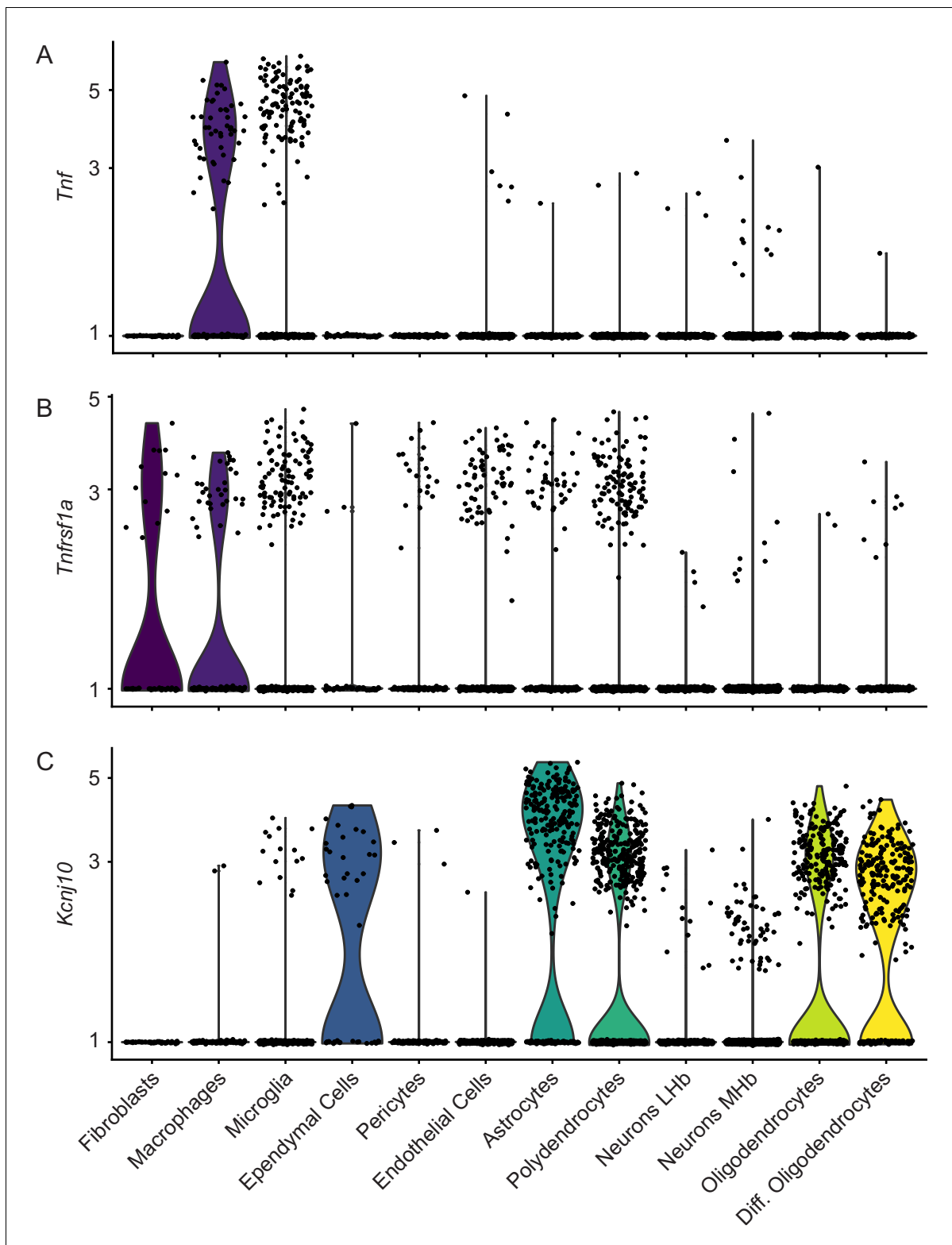


Figure 1—figure supplement 2. Expression of genes known to be important for habenular microglial and astrocytic function. Violin plots of gene expression (scaled and log normalized) for *Tnf* (A), *Tnfrsf1a* (TNF-receptor) (B), and *Kcnj10* (Kir4.1) (C). Each point represents a single cell and the filled area is a probability distribution of all the cells in that category.

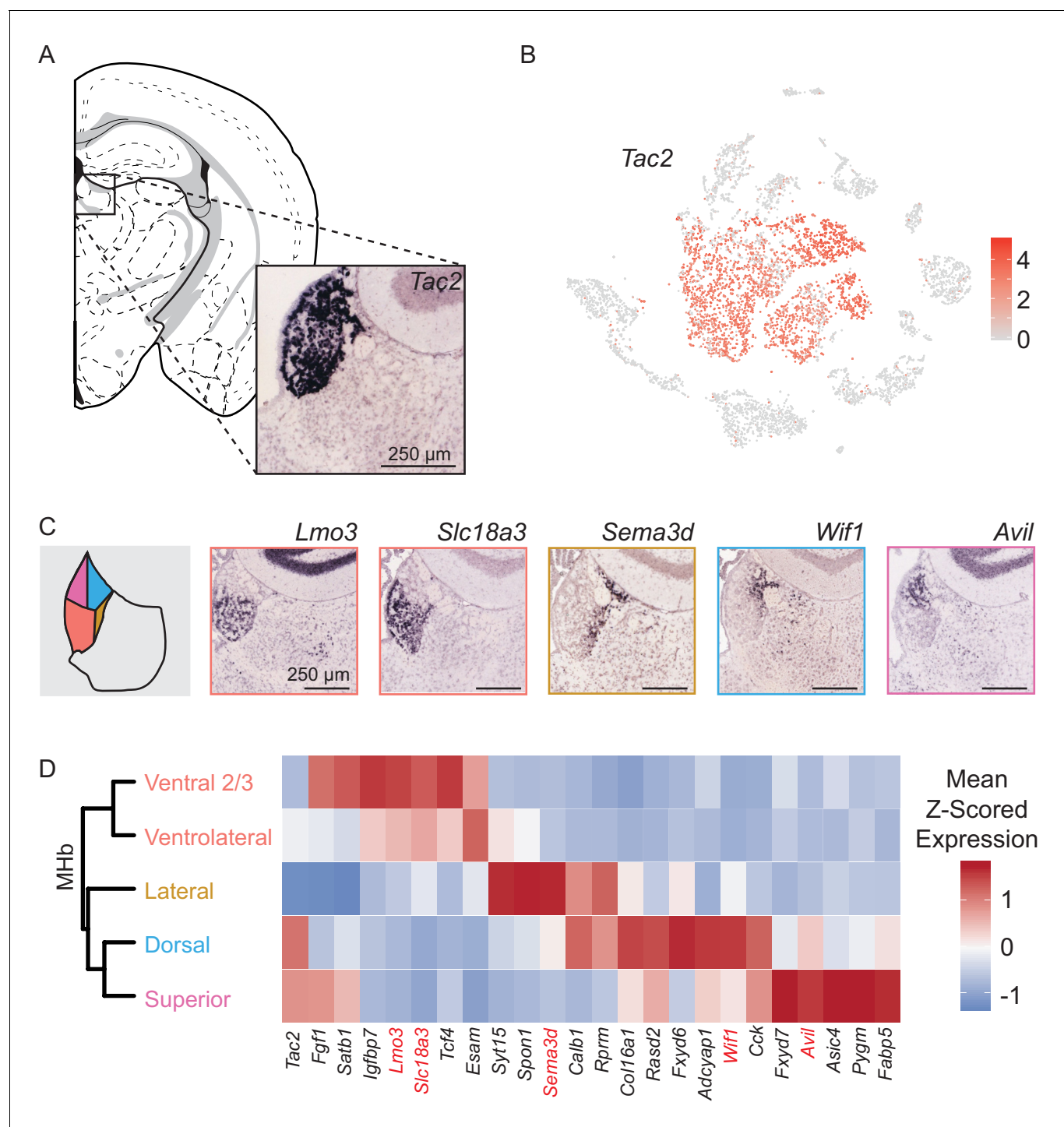


Figure 2. MHb neuron subtypes can be distinguished transcriptionally. (A) Location of MHb and ISH of *Tac2* expression from the Allen Institute Database. *Tac2* expression is restricted to cells in the MHb in this region. (B) *Tac2* serves as an excellent marker for MHb neurons in the dataset of SCTs (Scale on right shows normalized (log) gene expression.) (C) Left: Illustration showing patterns of gene expression observed for DEGs using the Allen Institute Database. Right: Sample ISH images from the Allen Institute Database showing selected differentially expressed genes for distinct transcriptionally defined neuronal subtypes in MHb. (D) Left: Dendrogram with MHb subtype labels corresponding to clusters shown in (Figure 2—figure supplement 1C). Right: Heatmap showing the relative expression (mean z-scored) of selected genes that are enriched in each MHb neuron subtype. Spatial distributions of enriched genes highlighted in (C) are labeled in red.

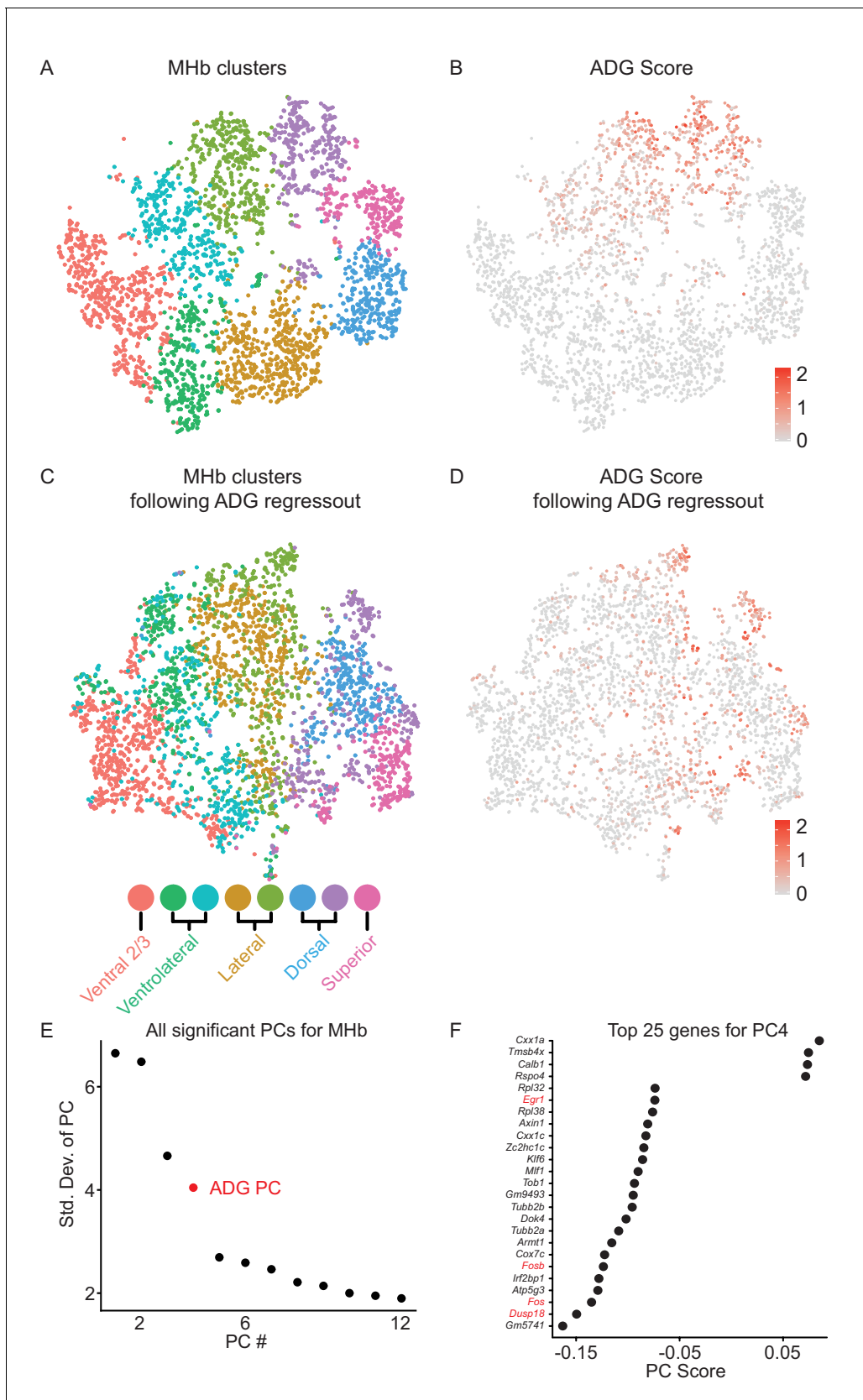


Figure 2—figure supplement 1. Subclustering of MHb neurons before and after subtraction of heterogeneity introduced by elevated expression of activity-dependent genes (ADGs). (A) t-SNE plot of subclustered MHb neurons extracted from cells in **Figure 1B**. (B) t-SNE plot showing three clusters

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Figure 2—figure supplement 1 continued

of cells (top) that expressed elevated levels of several ADGs (*Fos*, *Fosb*, *Egr1*, *Junb*, *Dusp18*, etc.). (C) t-SNE plot after regressing out the principle component (PC) that included many of the ADGs shown in (B). Cells from clusters that were high in ADG expression were now intermingled with clusters that we defined by the spatial location of their DEGs (See also **Figure 2C and D**). (D) t-SNE plot showing ADG score following regressing out of the PC containing ADGs. (E) All 12 statistically significant PCs for the MHB neuron clusters shown above. PC number 4 (red) contained several ADGs. (F) The top 25 genes associated with PC4 (the ADG PC) contained several known ADGs highlighted in red.

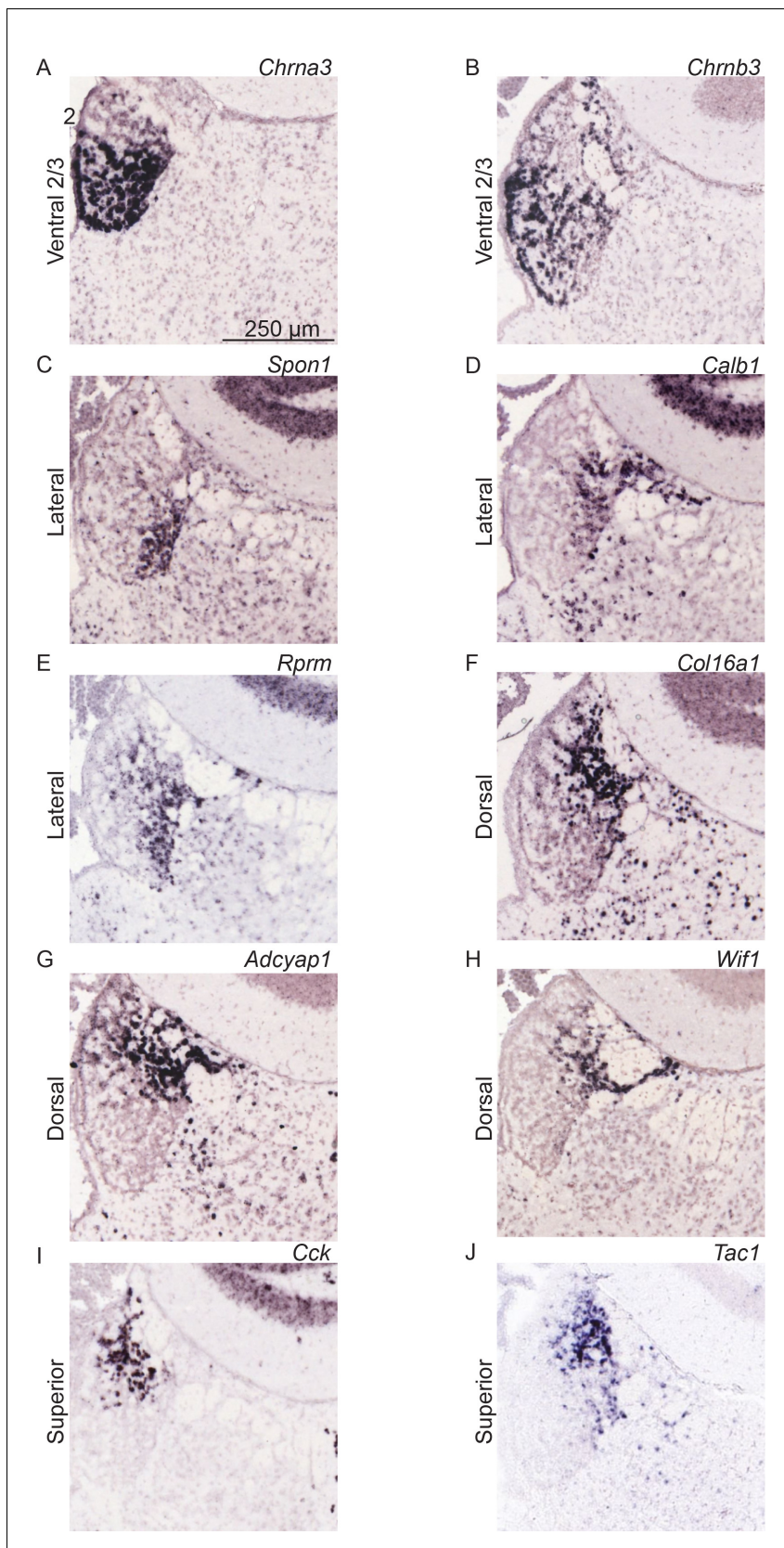


Figure 2—figure supplement 2. Sample ISH images showing spatial distribution of selected differentially expressed genes in MHB. (A–J) Sample ISH images from the Allen Institute Database showing selected differentially expressed genes for distinct transcriptionally defined neuronal subtypes in MHB. Gene name is in the upper right of each image and subregion where gene is enriched is on the left. Scale bar = 250 μ m.

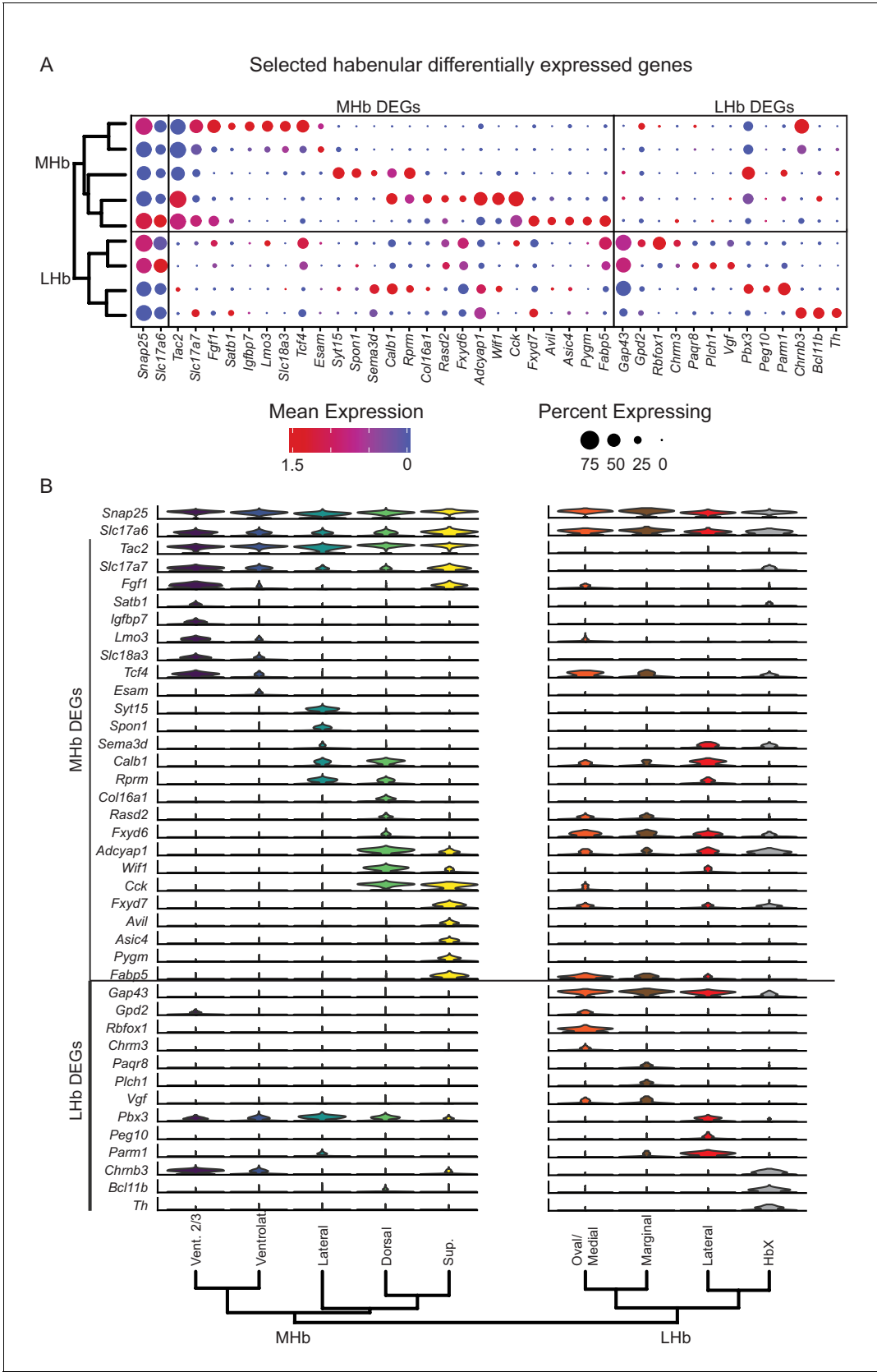


Figure 2—figure supplement 3. Differentially expressed genes define distinct habenular subtypes. (A) Left: Dendrogram for subclustering of all neurons shown in **Figures 2** and **3**. Right: Dot plot displaying expression of example differentially expressed genes used to identify each subtype of **Figure 2—figure supplement 3** continued on next page

Figure 2—figure supplement 3 continued

habenula neuron. The color of each dot (blue to red) indicates the relative expression of each gene, whereas the dot size indicates the fraction of cells expressing the gene. (B) Violin plots of expression level for select MHb and LHb DEGs. The Y-axis maximum for all plots is 5 (\log_2 transformed and scaled) and minimum is 1. Colored region of the plot shows the probability distribution of all neurons in the labeled cluster. Only representative genes are shown – for entire list of DEGs see **Supplementary file 3** and **5**.

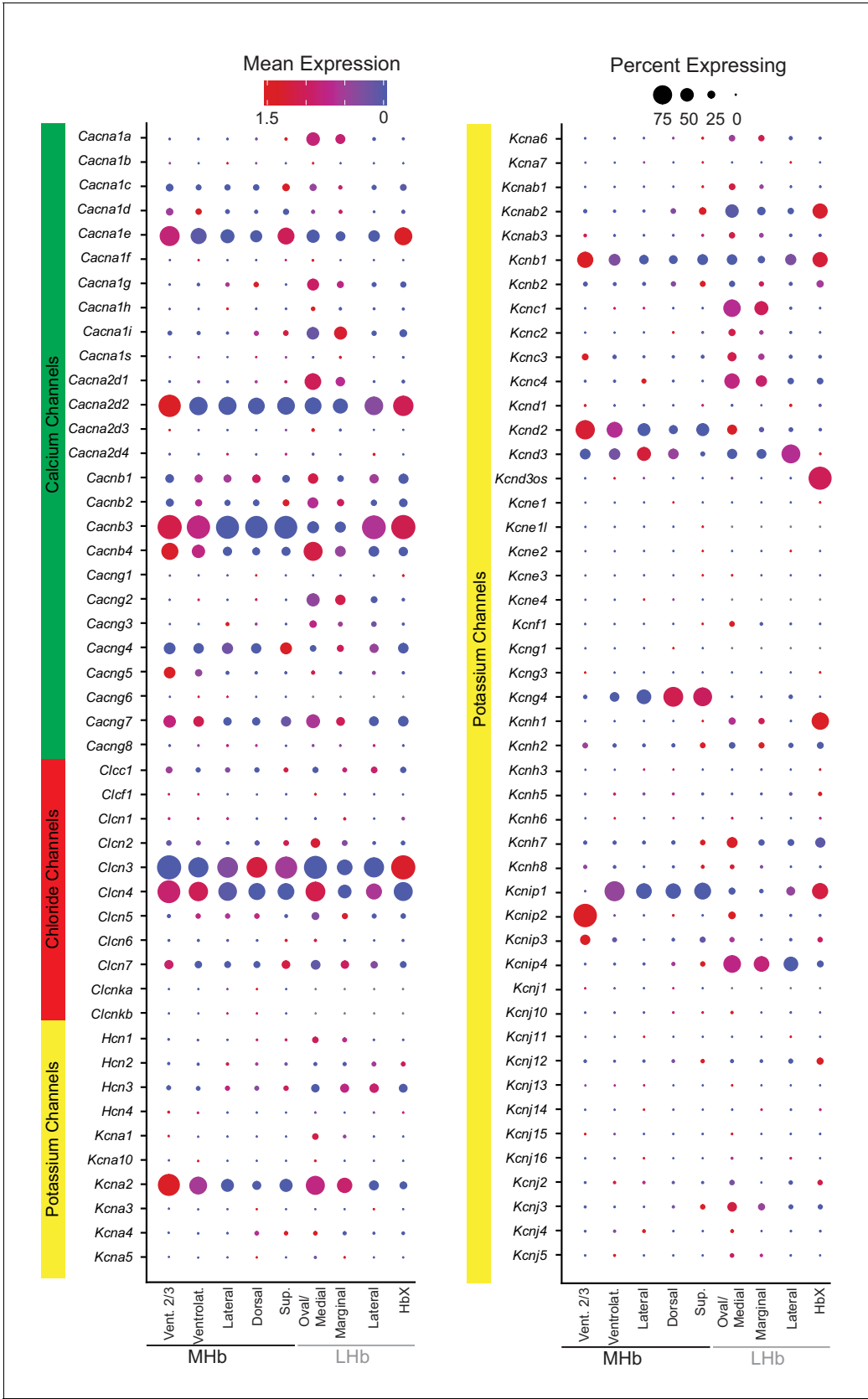


Figure 2—figure supplement 4. Ion channel diversity in MHb and LHb neuronal subtypes. Dot plot displaying the expression of all ion channels detected in MHb and LHb neurons. The color of each dot (blue to red) indicates the relative expression of each gene, whereas the dot size indicates the fraction of cells expressing the gene.

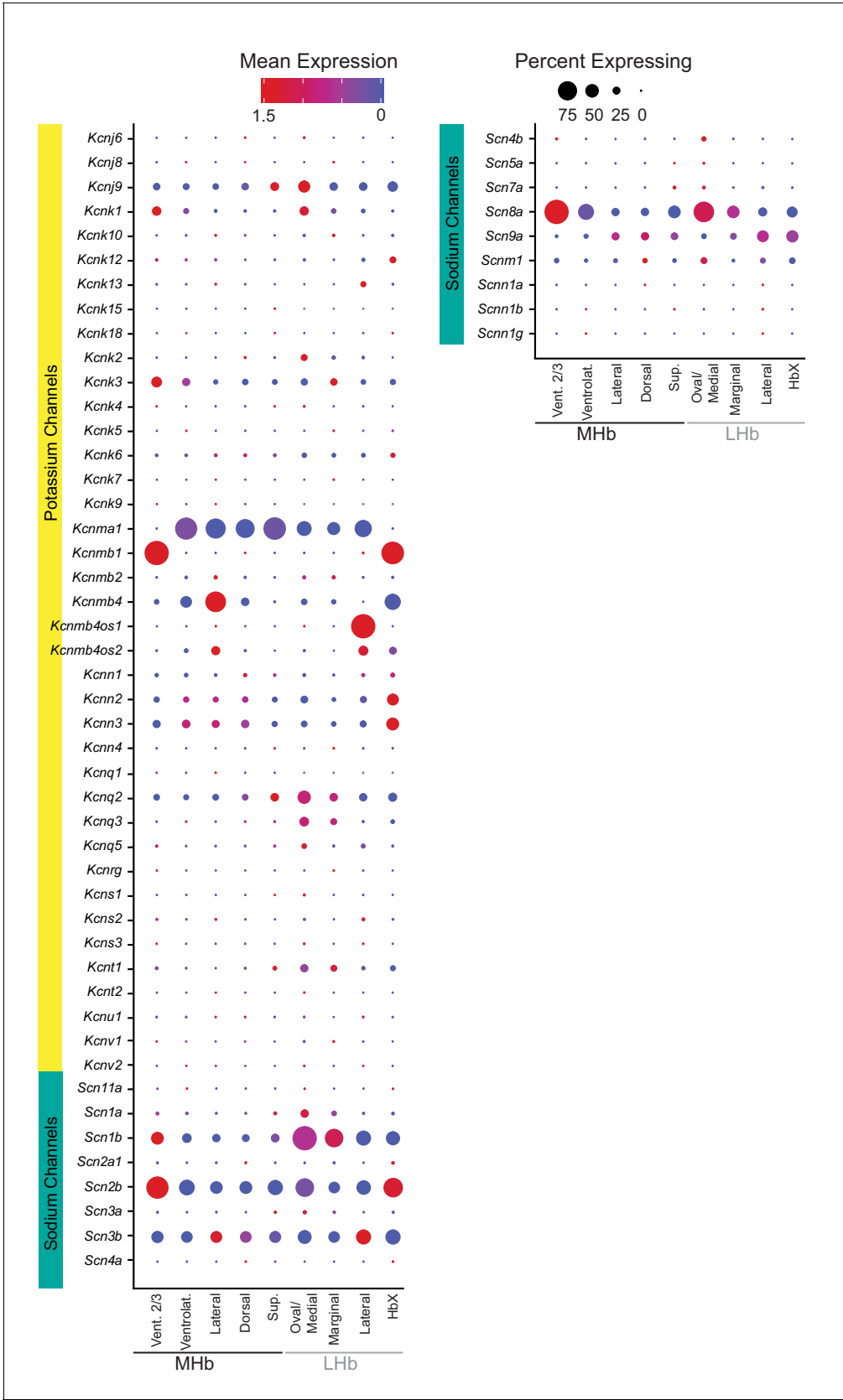


Figure 2—figure supplement 5. Ion channel diversity in MHb and LHb neuronal subtypes (part 2). Dot plot displaying the expression of all ion channels detected in MHb and LHb neurons. The color of each dot (blue to red) indicates the relative expression of each gene, whereas the dot size indicates the fraction of cells expressing the gene.

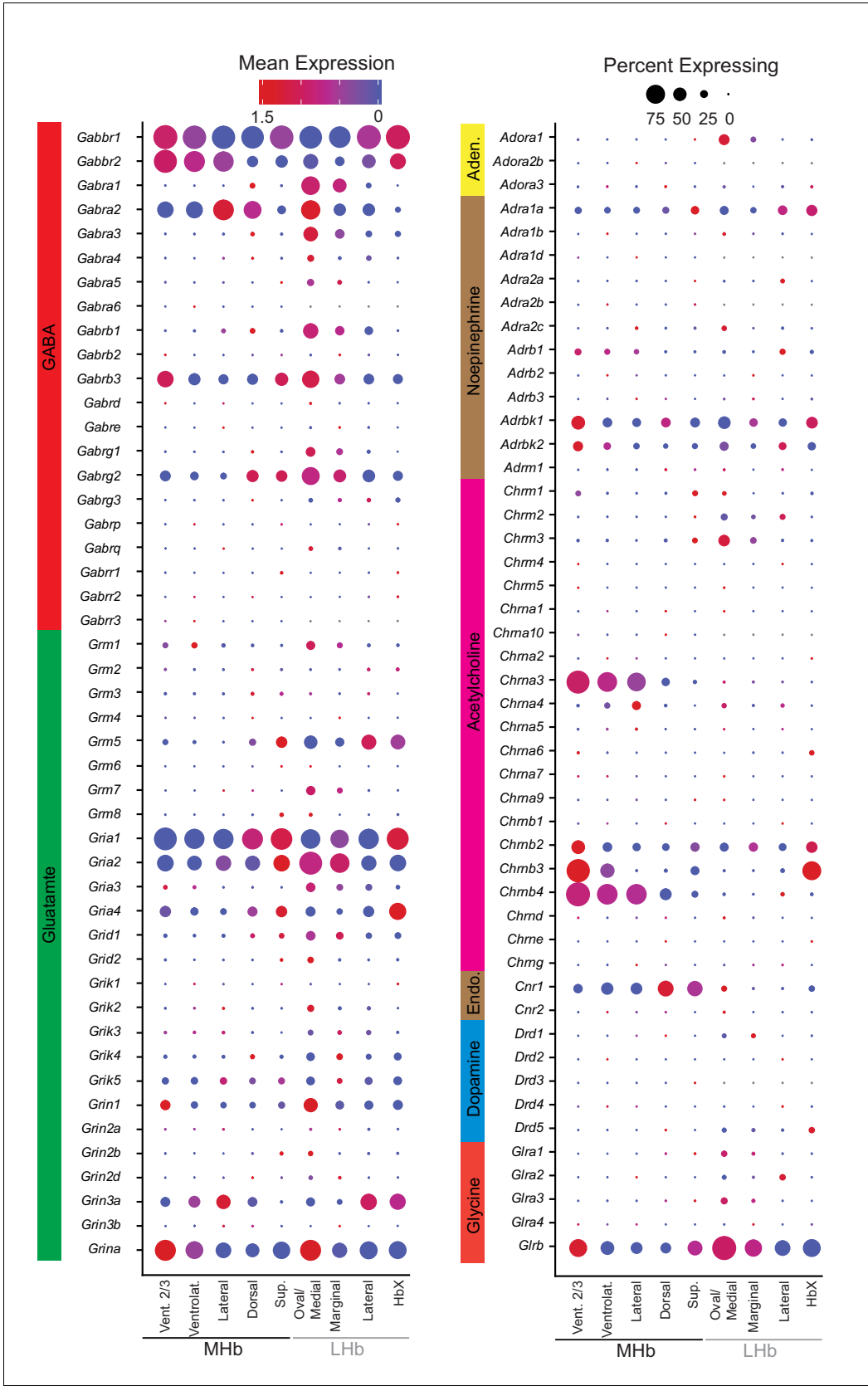


Figure 2—figure supplement 6. Neurotransmitter receptor diversity in MHb and LHb neuronal subtypes. Dot plot displaying gene expression of all small molecule neurotransmitter receptors detected in MHb and LHb neurons. The color of each dot (blue to red) indicates the relative expression of each gene, whereas the dot size indicates the fraction of cells expressing the gene.

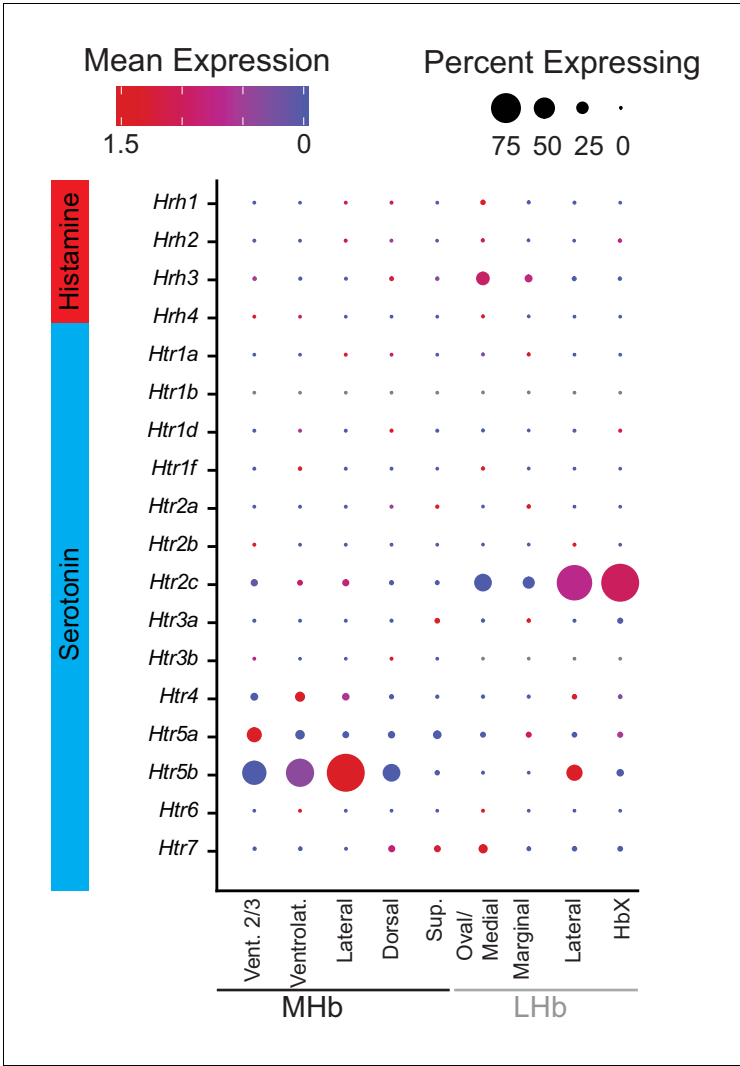


Figure 2—figure supplement 7. Neurotransmitter receptor diversity in MHb and LHb neuronal subtypes (part 2). Dot plot displaying gene expression of all small molecule neurotransmitter receptors detected in MHb and LHb neurons. The color of each dot (blue to red) indicates the relative expression of each gene, whereas the dot size indicates the fraction of cells expressing the gene.

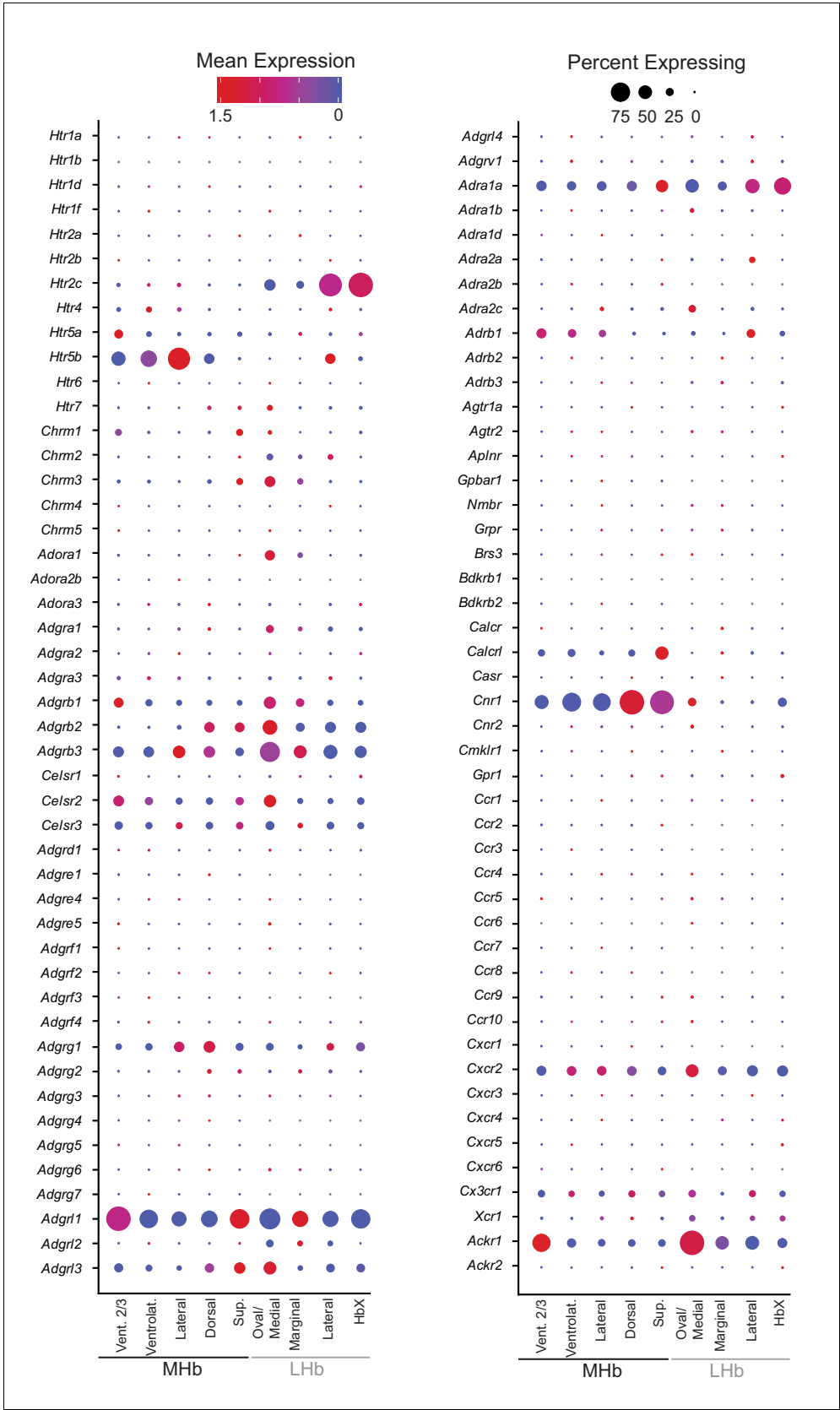


Figure 2—figure supplement 8. GPCR diversity in MHb and LHb neuronal subtypes. Dot plot displaying gene expression of all GPCRs detected in MHb and LHb neurons (Harding et al., 2018; Regard et al., 2008). Genes are grouped and listed alphabetically by receptor family name (Adenosine Figure 2—figure supplement 8 continued on next page

Figure 2—figure supplement 8 continued

Receptors, Somatostatin Receptors, etc.). The color of each dot (blue to red) indicates the relative expression of each gene whereas the dot size indicates the fraction of cells expressing the gene.

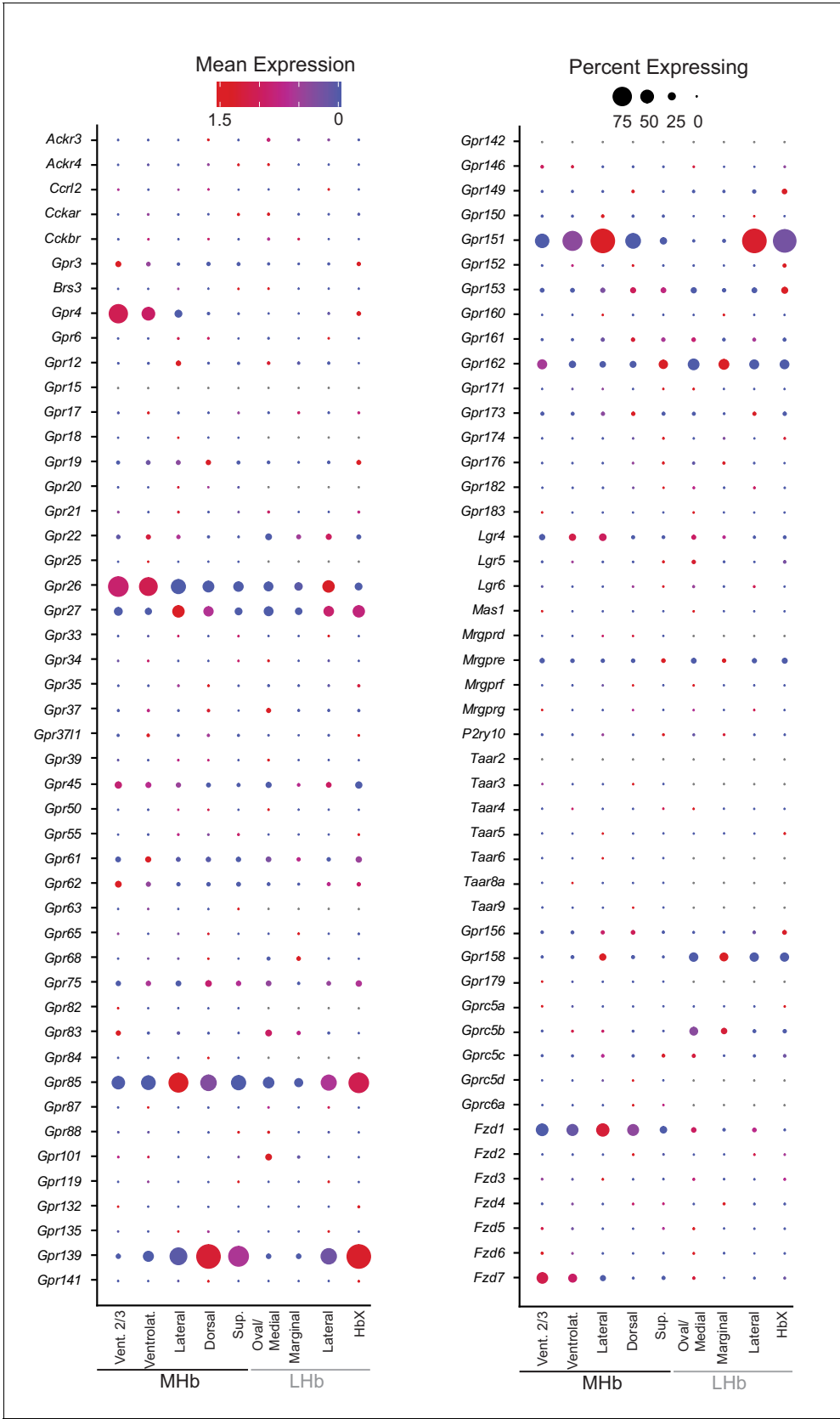


Figure 2—figure supplement 9. GPCR diversity in MHb and LHb neuronal subtypes (part 2). Dot plot displaying gene expression of all GPCRs detected in MHb and LHb neurons (Harding et al., 2018; Regard et al., 2008). Genes are grouped and listed alphabetically by receptor family name

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Figure 2—figure supplement 9 continued

(Adenosine Receptors, Somatostatin Receptors, etc.). The color of each dot (blue to red) indicates the relative expression of each gene whereas the dot size indicates the fraction of cells expressing the gene.



Figure 2—figure supplement 10. GPCR diversity in MHb and LHb neuronal subtypes (part 3). Dot plot displaying gene expression of all GPCRs detected in MHb and LHb neurons (Harding et al., 2018; Regard et al., 2008). Genes are grouped and listed alphabetically by receptor family name. Figure 2—figure supplement 10 continued on next page

Figure 2—figure supplement 10 continued

(Adenosine Receptors, Somatostatin Receptors, etc.). The color of each dot (blue to red) indicates the relative expression of each gene whereas the dot size indicates the fraction of cells expressing the gene.

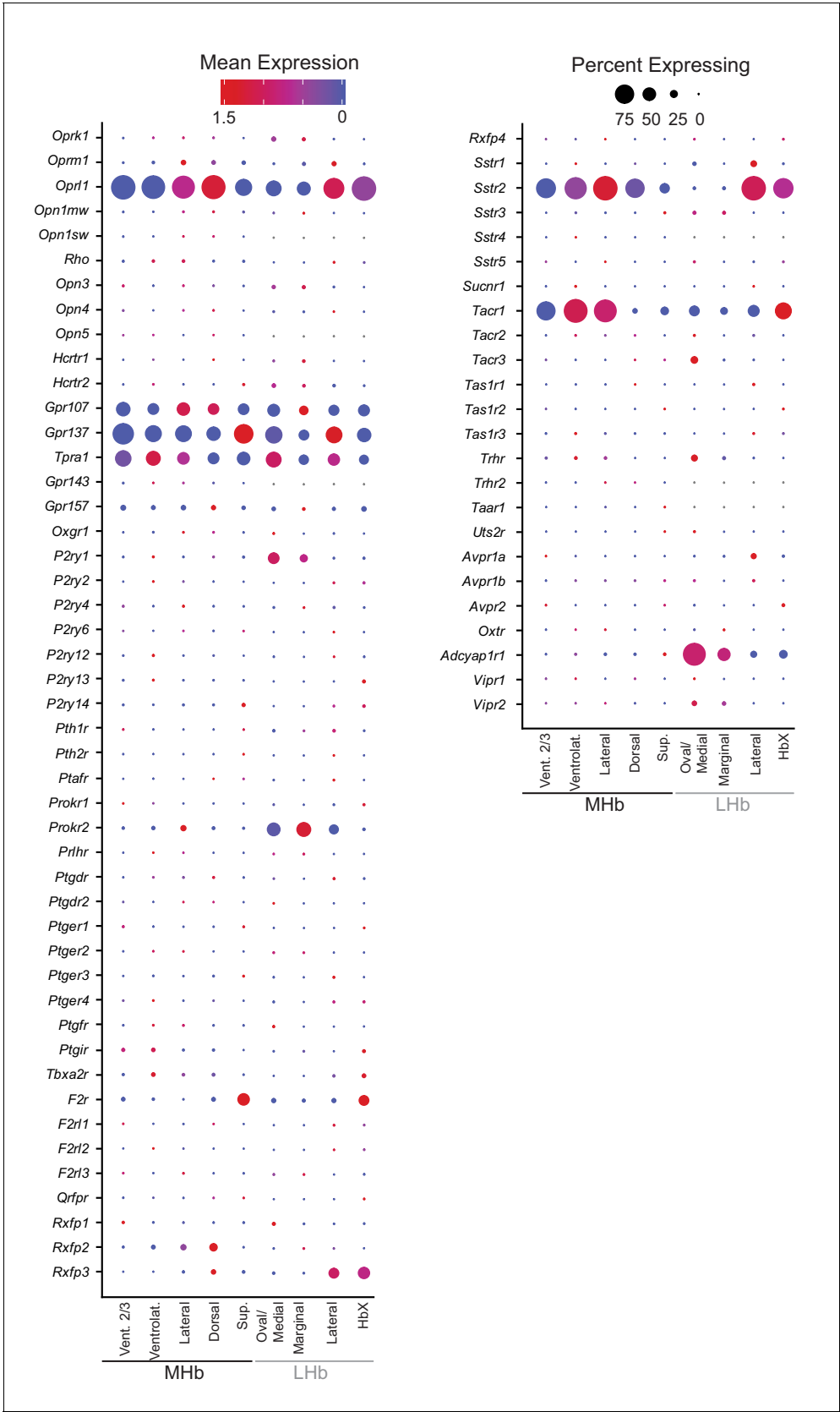


Figure 2—figure supplement 11. GPCR diversity in MHb and LHb neuronal subtypes (part 4). Dot plot displaying gene expression of all GPCRs detected in MHb and LHb neurons (Harding et al., 2018; Regard et al., 2008). Genes are grouped and listed alphabetically by receptor family name

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(Adenosine Receptors, Somatostatin Receptors, etc.). The color of each dot (blue to red) indicates the relative expression of each gene whereas the dot size indicates the fraction of cells expressing the gene.

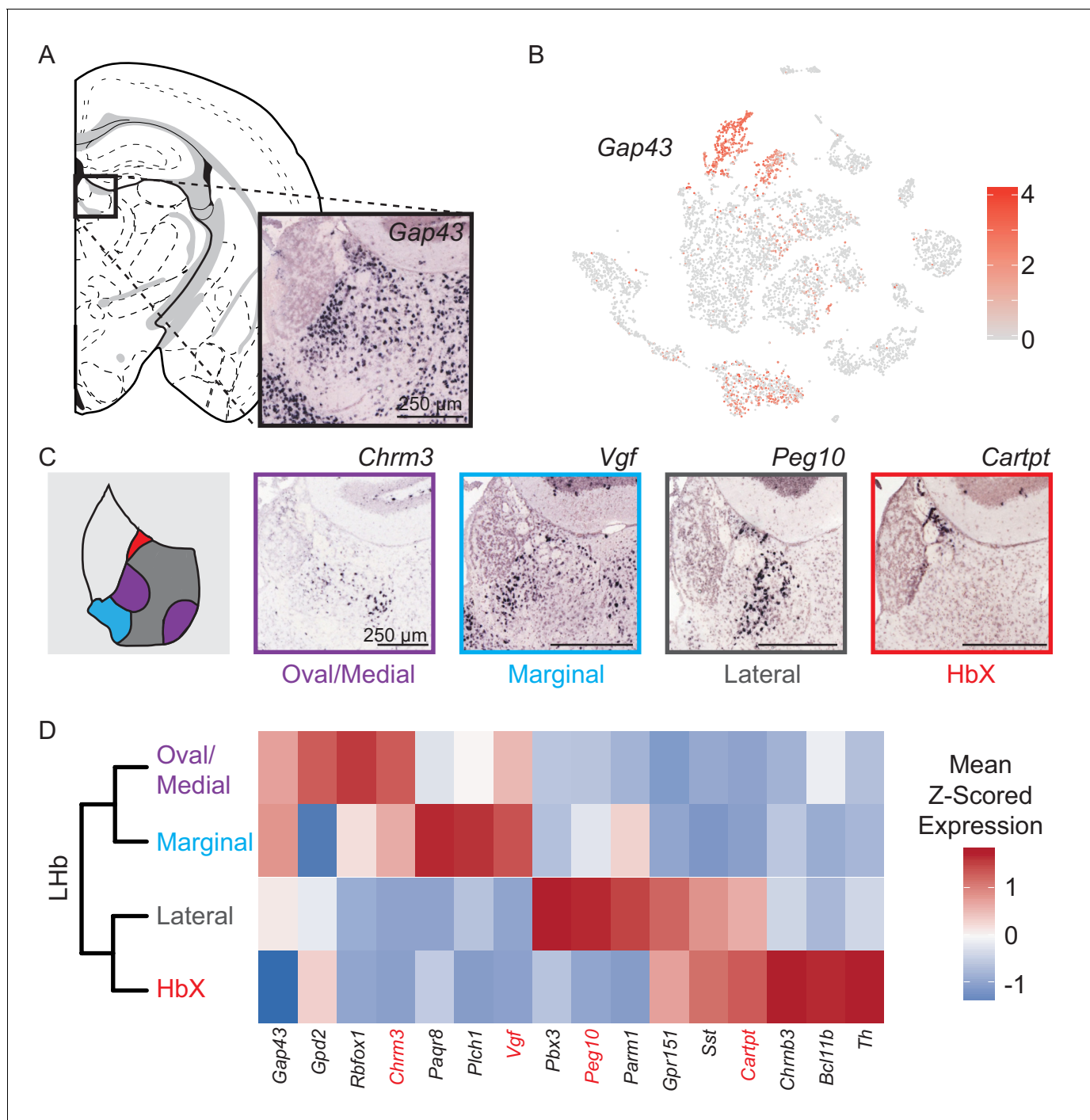


Figure 3. Characterization of genes differentially expressed between LHb neuron subtypes. (A) Location of LHb clusters and ISH *Gap43* expression from the Allen Institute Database. *Gap43* is highly expressed in neurons of the LHb and surrounding thalamus in this region, but excluded from MHb neurons. (B) *Gap43* serves as an excellent marker for LHb neurons in the dataset of single-cell transcriptomes (Scale on right shows normalized (log) gene expression.) (C) Left: Illustration showing patterns of gene expression observed for DEGs. Right: Sample ISH images from the Allen Institute Database showing selected differentially expressed genes for distinct transcriptionally defined neuronal subtypes in LHb. (D) Left: Dendrogram with LHb neuron labels corresponding spatial locations of differentially expressed genes within the LHb. Right: Heatmap showing the relative expression of selected genes that are enriched in each LHb neuron subtype. Spatial distributions of enriched genes highlighted in (C) are labeled in red.

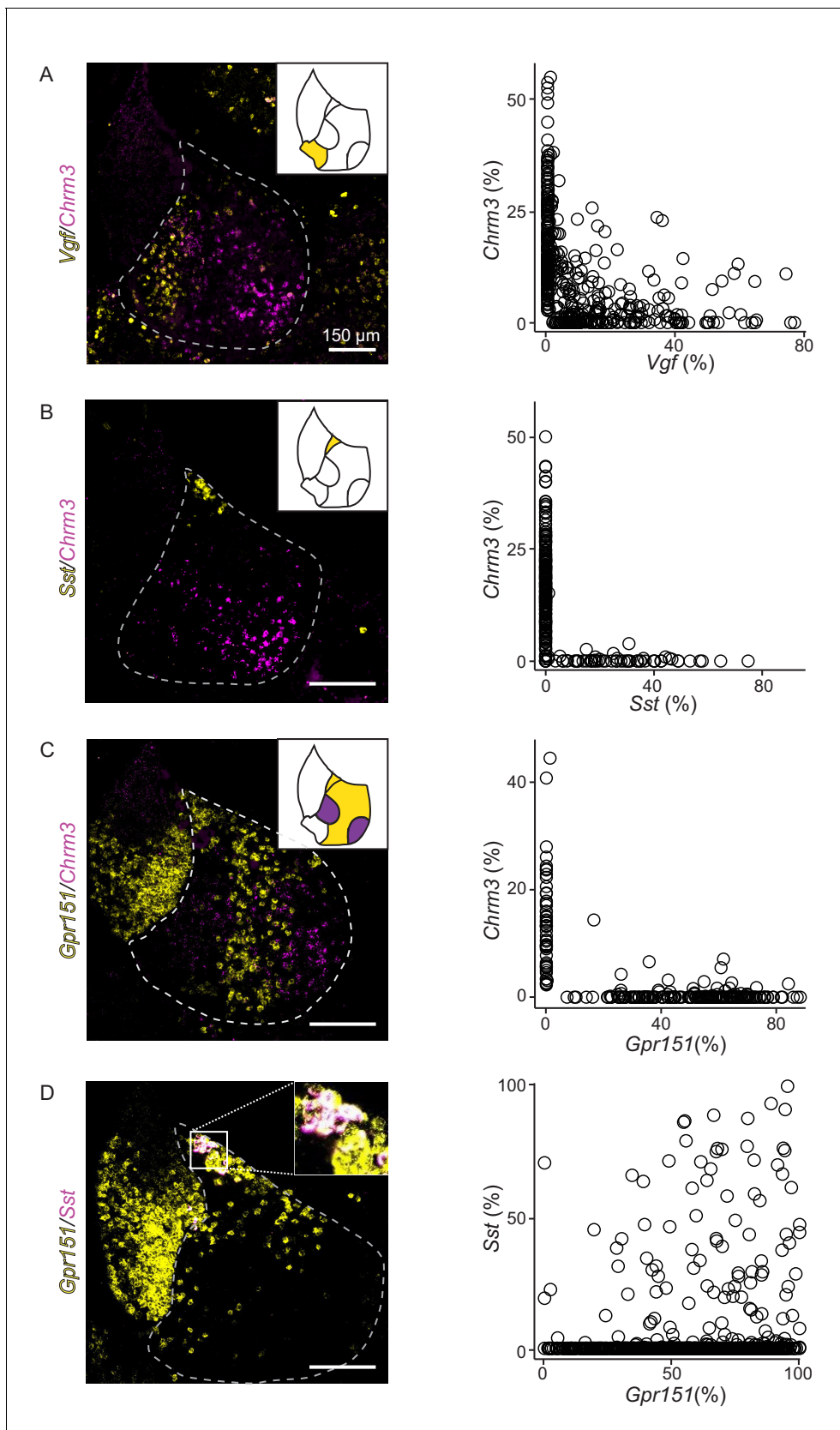


Figure 4. FISH confirms that differentially expressed genes from LHb subclusters are nonoverlapping and confined to specific spatial locations of LHb. (A) Left: Sample FISH of two differentially expressed LHb genes (*Vgf* (yellow) and *Chrm3* (magenta)), with distinct spatial profiles (LHb outlined with gray). Figure 4 continued on next page

Figure 4 continued

dashed line). Right: Quantification of fluorescence coverage of single cells for FISH of *Vgf* and *Chrm3* in LHb neurons (n = 444 cells, three mice). (B) Left: Sample FISH of two differentially expressed LHb genes (*Sst* (yellow) and *Chrm3* (magenta)), with distinct spatial profiles. Right: Quantification of fluorescence coverage of single cells for FISH of *Sst* and *Chrm3* in LHb neurons (n = 252 cells, three mice). (C) Left: Sample FISH of two differentially expressed LHb genes (*Gpr151* (yellow) and *Chrm3* (magenta)), with distinct spatial profiles (illustrated in upper right inset), LHb outlined in gray dashed line. Right: Quantification of fluorescence coverage of single cells for FISH of *Gpr151* and *Chrm3* in LHb neurons (n = 240 cells, three mice). (D) Left: Sample FISH of two differentially expressed LHb genes (*Sst* (yellow) and *Gpr151* (magenta)), with similar spatial profiles (both expressed in HbX region). Inset of overlapping *Sst* and *Gpr151* expression in HbX. Right: Quantification of fluorescence coverage of single cells for FISH of *Sst* and *Gpr151* in LHb neurons (n = 112 cells, three mice).

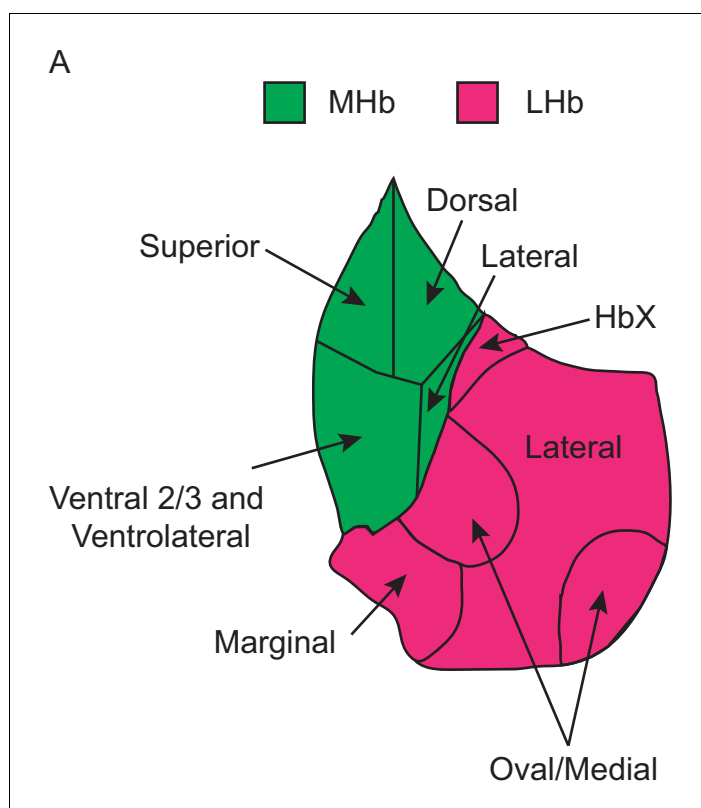


Figure 4—figure supplement 1. A map of habenula subregions based on single-cell transcriptomic profiling. (A) Habenular subregions are outlined in black, MHb subregions are green and LHb subregions are magenta. The location of borders is a rough estimate of a boundary between transcriptionally defined neuronal subtypes that have been confirmed with FISH and previous literature. Importantly, this map depicts the region of habenula that is located in the middle of the anterior/posterior axis of the nucleus and does not apply to the anterior and posterior poles where subregions are less well defined.

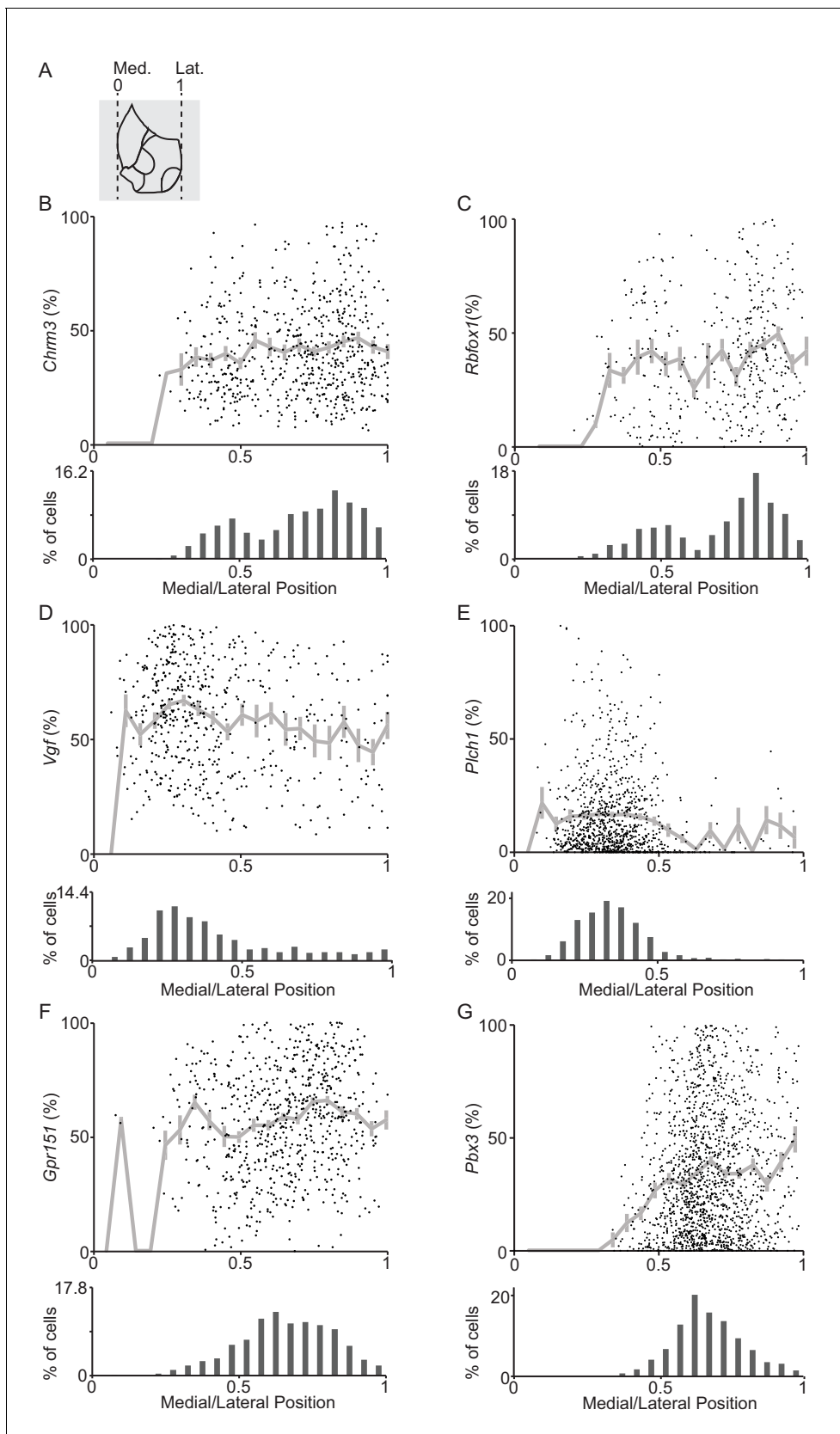


Figure 4—figure supplement 2. Spatial distribution of gene expression patterns along the medial/lateral axis for selected LHb DEGs. (A) Illustration of LHb subregions depicting the medial (0) and lateral (1) edges of the habenula used to describe the spatial distribution of gene expression. (B,C) (Top) Figure 4—figure supplement 2 continued on next page

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Expression levels of *Chrm3* (B) or *Rbfox1* (C) in individual LHb neurons along the medial/lateral axis. Gray line is the mean expression level and vertical bars are the SEM for each bin (bin size 0.05). (Bottom) Histogram showing the distribution of the density of *Chrm3*+ (B) or *Rbfox1*+ (C) neurons along the medial lateral axis of LHb (*Chrm3*: n = 616 cells/3 mice, *Rbfox1*: n = 392 cells/3 mice). (D,E) (Top) Expression levels of *Vgf* (D) or *Plch1* (E) in individual LHb neurons along the medial/lateral axis. Gray line is the mean expression level and vertical bars are the SEM for each bin (bin size 0.05). (Bottom) Histogram showing the distribution of the density of *Vgf*+ (D) or *Plch1*+ (E) neurons along the medial lateral axis of LHb (*Vgf*: n = 535 cells/3 mice, *Plch1*: n = 961 cells/3 mice). (F,G) (Top) Expression levels of *Gpr151* (F) or *Pbx3* (G) in individual LHb neurons along the medial/lateral axis. Gray line is the mean expression level and vertical bars are the SEM for each bin (bin size 0.05). (Bottom) Histogram showing the distribution of the density of *Gpr151*+ (F) or *Pbx3*+ (G) neurons along the medial lateral axis of LHb (*Gpr151*: n = 1122 cells/3 mice, *Pbx3*: n = 1507 cells/3 mice).

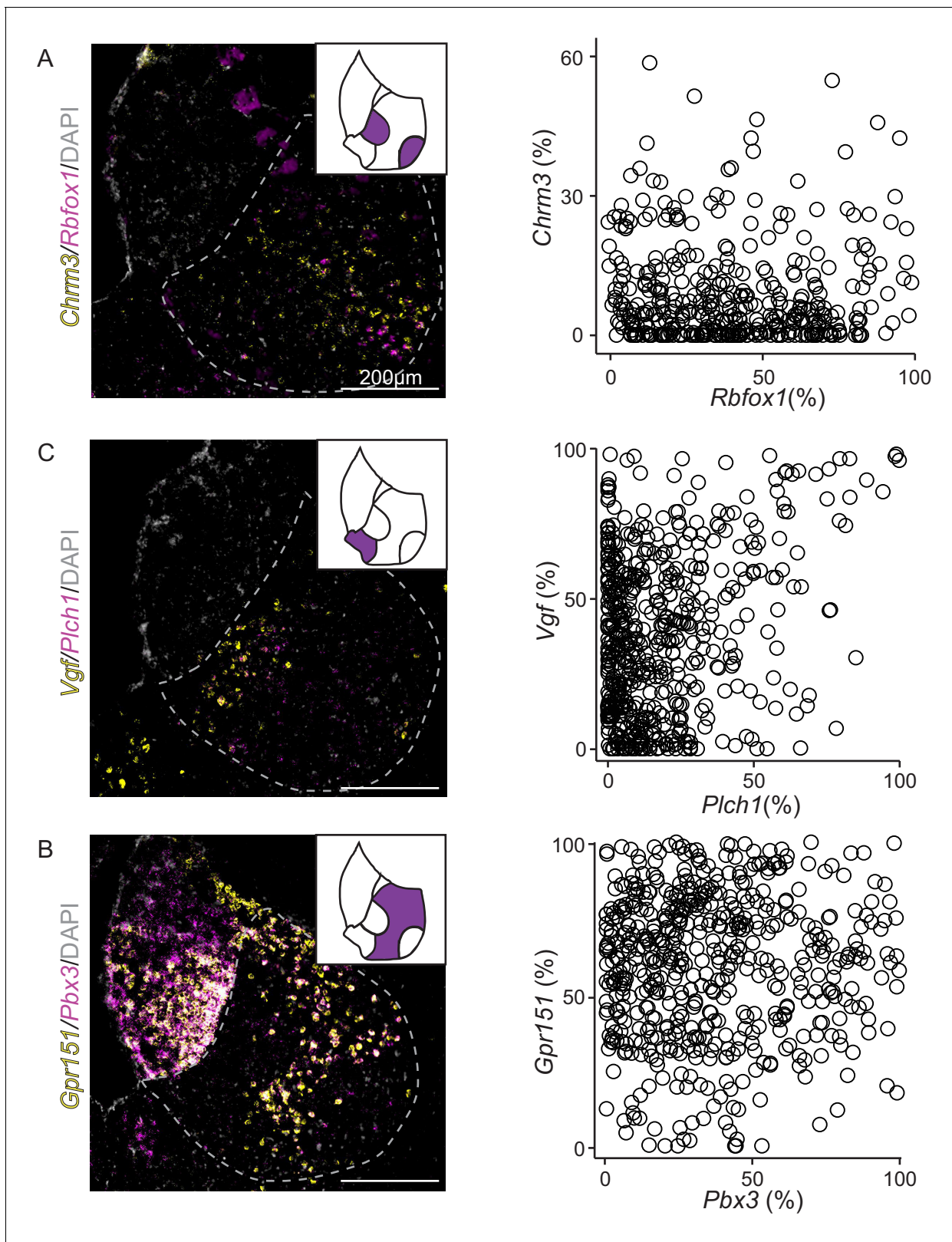


Figure 4—figure supplement 3. FISH confirms that differentially expressed genes from the same LHb subcluster are overlapping and confined to similar spatial locations of LHb. (A) Left: Sample FISH of two differentially expressed LHb genes (*Chrm3* (yellow) and *Rbfox1* (magenta)), from the same cluster (Oval/Medial) (LHb outlined with gray dashed line). Right: Quantification of fluorescence coverage of single cells for FISH of *Chrm3* and *Rbfox1*. Figure 4—figure supplement 3 continued on next page

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in LHb neurons (n = 392 cells, three mice). **(B)** Left: Sample FISH of two differentially expressed LHb genes (*Vgf* (yellow) and *Plch1* (magenta)), from the same cluster (Marginal) (LHb outlined with gray dashed line). Right: Quantification of fluorescence coverage of single cells for FISH of *Vgf* and *Plch1* in LHb neurons (n = 561 cells, three mice). **(C)** Left: Sample FISH of two differentially expressed LHb genes (*Gpr151* (yellow) and *Pbx3* (magenta)), from the same cluster (Lateral) (LHb outlined with gray dashed line). Right: Quantification of fluorescence coverage of single cells for FISH of *Gpr151* and *Pbx3* in LHb neurons (n = 546 cells, three mice).

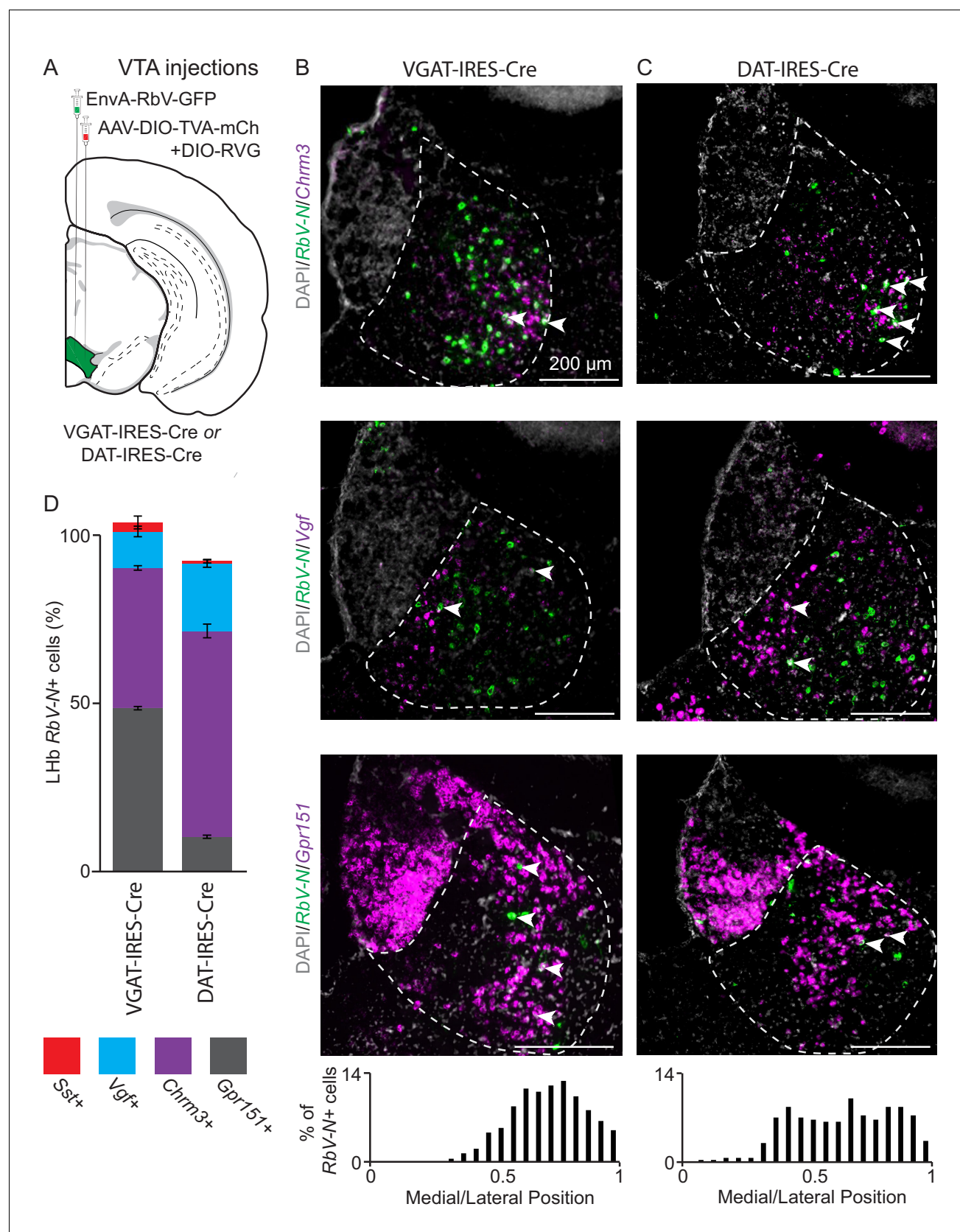


Figure 5. Distinct LHb neuron subtypes prefer different downstream targets, but all subtypes target both the VTA. (A) Location of site for AAV helper viruses (AAV-FLEX-TVA-mCh and AAV-FLEX-RVG) and pseudotyped rabies virus (EnvA-RbV-GFP) injection into VTA. (B) Sample habenula FISH images

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Figure 5 continued

for *RbV-N* and *Chrm3* (top), *Vgf* (middle), or *Gpr151* (bottom) following viral injection into the VTA of a VGAT-IRES-Cre mouse. Arrow heads show *RbV-N*⁺ cells coexpressing the DEG in each image. Histogram at bottom shows the distribution of the density of *RbV-N*⁺ neurons along the medial lateral axis of LHb (n = 822 cells/4 animals). (C) Sample habenula FISH images for *RbV-N* and *Chrm3* (top), *Vgf* (middle), or *Gpr151* (bottom) following viral injection into the VTA of a DAT-IRES-Cre mouse. Arrow heads show *RbV-N*⁺ cells coexpressing the DEG in each image. Histogram at bottom shows the distribution of the density of *RbV-N*⁺ neurons along the medial lateral axis of LHb (n = 299 cells/3 animals). (D) Quantification of the proportion of *RbV-N* labeled neurons that overlapped with the enriched genes for distinct LHb neuron subtypes (VGAT-IRES-Cre n = 1430 cells/ four mice and DAT-IRES-Cre n = 549/3 mice). Filled rectangles are the mean and error bars are \pm SEM, see **Supplementary file 6** for statistical comparisons.

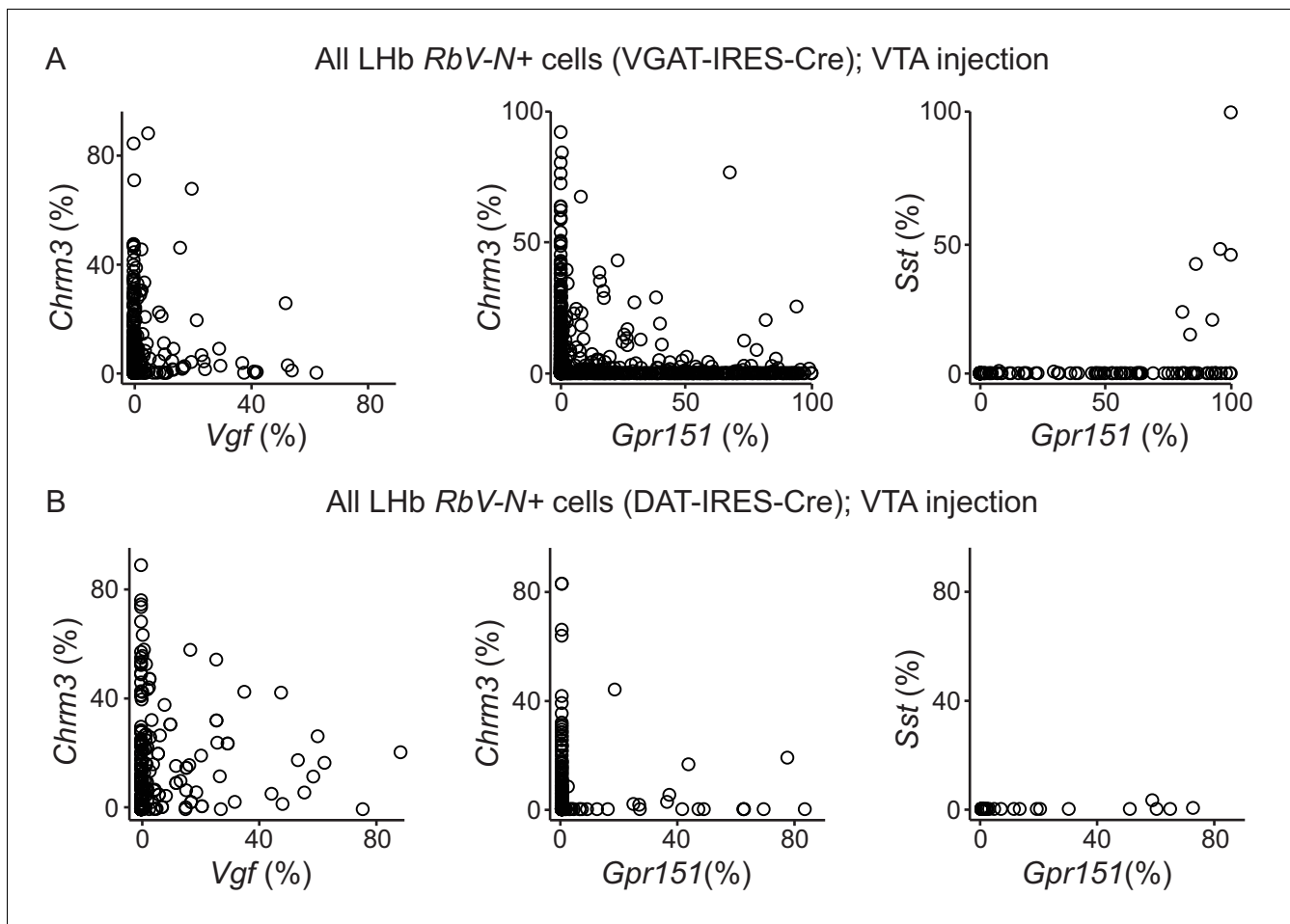


Figure 5—figure supplement 1. Cells from all four LHb subtypes project to both VGAT-IRES-Cre+ and DAT-IRES-Cre+ cells in the VTA. (A) Quantification of fluorescence coverage of single cells for FISH of selected enriched genes in LHb neurons that were positive for *RbV-N* following monosynaptic retrograde tracing from VGAT-IRES-Cre+ neurons in the VTA (left: $n = 521$ cells, four mice; center: $n = 742$ cells, four mice; right: $n = 167$ cells, two mice). (B) Quantification of fluorescence coverage of single cells for FISH of selected enriched genes in LHb neurons that were positive for *RbV-N* following monosynaptic retrograde tracing from DAT-IRES-Cre+ neurons in the VTA (left: $n = 233$ cells, three mice; center: $n = 233$ cells, three mice; right: $n = 103$ cells, three mice).

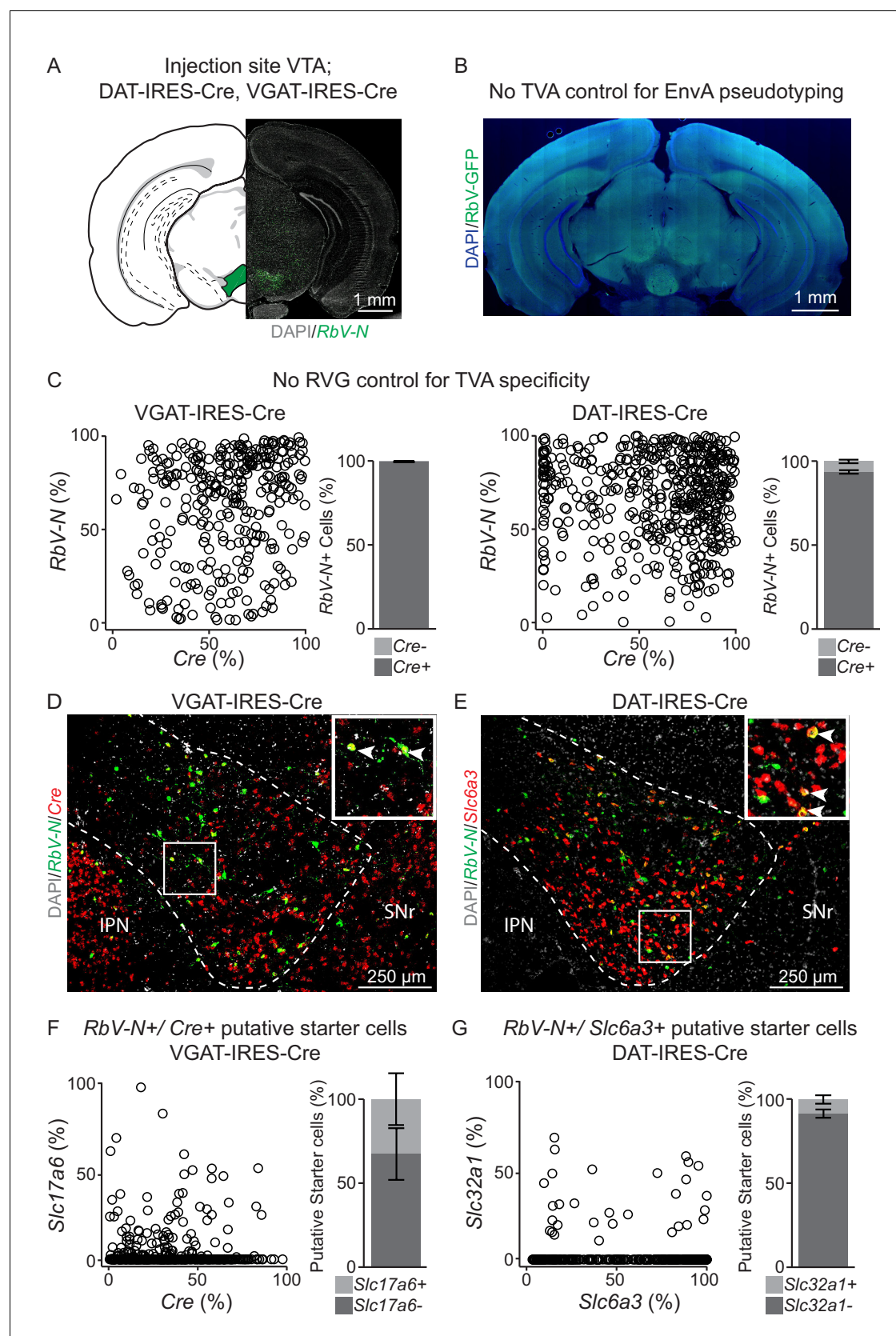


Figure 5—figure supplement 2. Quantification and genetic characterization of VTA starter cells from monosynaptic retrograde tracing and controls for rabies virus. (A) Left: Coronal section of injection site into VTA and starter cells location for Cre-dependent monosynaptic retrograde-tracing Figure 5—figure supplement 2 continued on next page

Figure 5—figure supplement 2 continued

experiments. Right: FISH for *RbV-N* to demonstrate the location of rabies infected cells in the VTA. (B) Negative control for EnvA pseudotyping of the rabies virus (EnvA-RbV-GFP) showing a coronal section following injection of EnvA-RbV-GFP into the VTA without prior infection by AAV-TVA-mCh. Without co injecting AAV-DIO-TVA-mCh, EnvA-RbV-GFP cannot infect neurons, thus no GFP expression. (C) Controls for specificity of starter cell populations. EnvA-RbV-GFP was injected into the VTA of VGAT-IRES-Cre (left) and DAT-IRES-Cre (right) mice following injection of AAV-DIO-TVA-mCh, but not AAV-DIO-RVG. Therefore, rabies virus could not spread from initially infected neurons. Left: Quantification of fluorescence coverage of *RbV-N+* cells in VGAT-IRES-Cre mice, almost all (99%) infected cells expressed detectable levels of *Cre* demonstrating specificity of the AAV-DIO-TVA virus ($n = 319$ cells, two mice). Right: Quantification of fluorescence coverage of *RbV-N+* cells in DAT-IRES-Cre mice, almost all infected cells (95%) expressed detectable levels of *Cre* demonstrating specificity of the AAV-DIO-TVA virus ($n = 522$ cells, two mice). (D) Sample VTA FISH image of starter cell location for monosynaptic retrograde tracing experiments performed in VGAT-IRES-Cre mice. Inset depicts two putative starter cells (arrowheads) that express *RbV-N* (green) and *Cre* (red). VTA is outlined by white dashed line. (E) Sample VTA FISH image of starter cell location for monosynaptic retrograde tracing experiments performed in DAT-IRES-Cre mice. Inset depicts three putative starter cells (arrowheads) that express *RbV-N* (green) and *Cre* (red). VTA is outlined by white dashed line. (F) Left: Quantification of fluorescence coverage of single putative starter cells (*Cre+* and *RbV-N+*) for FISH of *Cre* and *Slc17a6* in VTA neurons of the VGAT-IRES-Cre animals ($n = 567$ cells, four mice). Right: The proportion of putative starter cells that expressed *Slc17a6*. There is a subset of VGAT-IRES-Cre+ neurons in the VTA that co-express *Slc17a6* (Root et al., 2014). (G) Left: Quantification of fluorescence coverage of single putative starter cells (*Slc6a3+* and *RbV-N+*) for FISH of *Slc6a3* and *Slc32a1* in VTA neurons of the DAT-IRES-Cre animals ($n = 566$ cells, three mice). Right: The proportion of putative starter cells that expressed *Slc32a1*. Note: Approximately 12% (451/3754 cells/4 mice) of *Slc32a1+* VTA neurons were starter cells in VGAT-IRES-Cre retrograde labeling experiments. In DAT-IRES-Cre experiments, approximately 18% (468/2598 cells/3 mice) of *Slc6a3+* VTA neurons were starter cells. Filled rectangles represent the mean and error bars are \pm SEM, Abbreviations: IPN - interpeduncular nucleus, SNr - substantia nigra reticulata.

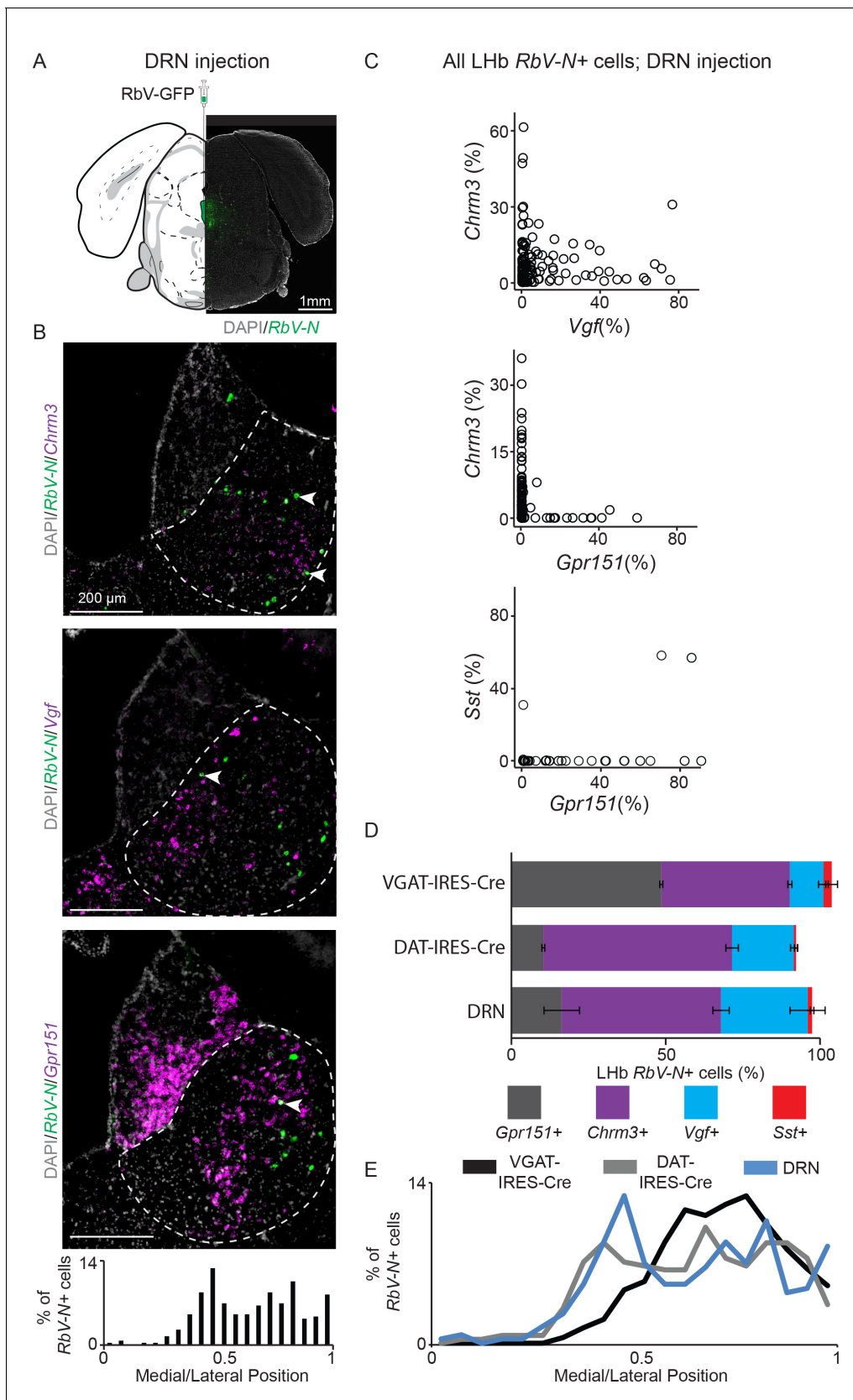


Figure 5—figure supplement 3. Quantification and genetic characterization of LHb cells that project to the DRN using a nonpseudotyped (G-deleted) rabies virus. (A) Location of site for nonpseudotyped (G-deleted) rabies virus (RbV-GFP) injection into DRN. (B) Sample habenula FISH images for RbV-N
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and *Chrm3* (top), *Vgf* (middle), or *Gpr151* (bottom) following viral injection into the DRN. Arrow heads show *RbV-N+* cells coexpressing the DEG in each image. Histogram shows the distribution of the density of *RbV-N+* neurons along the medial lateral axis of LHb ($n = 274$ cells/4 animals). (C) Quantification of fluorescence coverage of single cells for FISH of selected enriched genes in LHb neurons that were positive for *RbV-N* following injection of *RbV-GFP* into the DRN (top: $n = 163$ cells, 3 mice; middle: $n = 133$ cells, 3 mice; bottom: $n = 169$ cells, 3 mice). (D) Quantification of the proportion of *RbV-N*-labeled neurons that overlapped with the enriched genes for distinct LHb neuron subtypes (VGAT-IRES-Cre $n = 1430$ cells/4 mice, DAT-IRES-Cre $n = 549/3$ mice, and DRN $n = 465/3$ mice). Filled rectangles are the mean and error bars are \pm SEM, see **Supplementary file 6** for statistical comparisons. (E) Histogram showing the medial lateral distribution of *RbV-N+* cells labeled in all three retrograde labeling experiments for comparison (bin size = 0.05).