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The gene expression landscape of the human locus coeruleus revealed by single-nucleus and spatially-resolved transcriptomics

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Abstract

Norepinephrine (NE) neurons in the locus coeruleus (LC) project widely throughout the central nervous system, playing critical roles in arousal and mood, as well as various components of cognition including attention, learning, and memory. The LC-NE system is also implicated in multiple neurological and neuropsychiatric disorders. Importantly, LC-NE neurons are highly sensitive to degeneration in both Alzheimer's and Parkinson's disease. Despite the clinical importance of the brain region and the prominent role of LC-NE neurons in a variety of brain and behavioral functions, a detailed molecular characterization of the LC is lacking. Here, we used a combination of spatially-resolved transcriptomics and single-nucleus RNA-sequencing to characterize the molecular landscape of the LC region and the transcriptomic profile of LC-NE neurons in the human brain. We provide a freely accessible resource of these data in web-accessible formats.

eLife assessment

This is a valuable initial study of cell type and spatially resolved gene expression in and around the locus coeruleus, the primary source of the neuromodulator norepinephrine in the human brain. The data are generated with cutting-edge techniques, and the work lays the foundation for future descriptive and experimental approaches to understand the contribution of the locus coeruleus to healthy brain function and disease. However, due to small sample size and the need for additional confirmatory data, the data only incompletely support the main conclusions presented here. With the strengthening of the analyses, this paper, and the associated web application, will be of great interest to neuroscientists working on arousal-based behaviors and neurological and neuropsychiatric phenotypes.

Introduction

The locus coeruleus (LC) is a small bilateral nucleus located in the dorsal pons of the brainstem, which serves as the brain's primary site for production of the neuromodulator norepinephrine (NE). NE-producing neurons in the LC project widely to many regions of the central nervous system to modulate a variety of highly divergent functions including attention, arousal, and mood [(1)–(4)]. The LC, translated as “blue spot”, comprises merely 3,000 NE neurons in the rodent (~1500-1600 each side of the brainstem) (1), and estimates in the human LC range from 19,000-46,000 total NE neurons (5). Despite its prominent involvement in a number of critical brain functions and its unique capacity to synthesize NE, the LC's small size and deep positioning within the brainstem has rendered it relatively intractable to a comprehensive cellular, molecular, and physiological characterization.

The LC plays important roles in core behavioral and physiological brain function across the lifespan and in disease. For example, there is strong evidence for age-related cell loss in the LC (6; 7), and the LC-NE system is implicated in multiple neuropsychiatric and neurological disorders (4; 8). The LC is one of the earliest sites of degeneration in Alzheimer's disease (AD) and Parkinson's disease (PD), and profound loss of LC-NE neurons is evident with disease progression [(8)–(10)]. Moreover, maintaining the neural density of LC-NE neurons prevents cognitive decline during aging (11). In addition, primary neuropathologies for AD (hyperphosphorylated tau) and PD (alpha-synuclein) can be detected in the LC prior to other brain regions [(12)–(15)]. However, the molecular mechanisms rendering LC-NE neurons

particularly vulnerable to age-related decline and neurodegeneration are not well-understood. In addition to its role in aging, the LC-NE system plays a critical role in mediating sustained attention, and its dysregulation is associated with attention-deficit hyperactivity disorder (ADHD) (16; 17). Of note, the NE reuptake inhibitor atomoxetine is the first non-stimulant medication that is FDA-approved for ADHD [(18)–(20)]. Better understanding the gene expression landscape of the LC and surrounding region and delineating the molecular profile of LC-NE neurons in the human brain could facilitate the ability to target these neurons for disease prevention or manipulate their function for treatment.

The recent development of single-nucleus RNA-sequencing (snRNA-seq) and spatially-resolved transcriptomics (SRT) technological platforms provides an opportunity to investigate transcriptome-wide gene expression scale with cellular and spatial resolution (21; 22). SRT has recently been used to characterize transcriptome-wide gene expression within defined neuroanatomy of cortical regions in the postmortem human brain (22), while snRNA-seq has been used to investigate specialized cell types in a number of postmortem human brain regions including medium spiny neurons in the nucleus accumbens and dopaminergic neurons in the midbrain (21; 23). Importantly, snRNA-seq and SRT provide complementary views: snRNA-seq identifies transcriptome-wide gene expression within individual nuclei, while SRT captures transcriptome-wide gene expression in all cellular compartments (including the nucleus, cytoplasm, and cell processes) while retaining the spatial coordinates of these measurements. While not all SRT platforms achieve single-cell resolution, depending on the technological platform and tissue cell density, spatial gene expression has been resolved at, for example, ~1-10 cells per spatial measurement location with a diameter of 55 μm in the human brain (22). These platforms have been successfully used in tandem to spatially map single-nucleus gene expression in several regions of both neurotypical and pathological tissues in the human brain including the dorsolateral prefrontal cortex (22) and the dopaminergic substantia nigra (21).

In this report, we characterize the gene expression signature of the LC and surrounding region at spatial resolution, and identify and characterize a population of NE neurons at single-nucleus resolution in the neurotypical adult human brain. In addition to NE neurons, we identify a population of 5-hydroxytryptamine (5-HT, serotonin) neurons, which have not previously been characterized at the molecular level in human brain samples (24). We observe expression of cholinergic marker genes within NE neurons, and confirm that this is due to co-expression within individual cells using fluorescent labeling with high-resolution imaging. We compare our findings from the human LC and adjacent region to molecular profiles of LC and peri-LC neurons that were previously characterized in rodents using alternative technological platforms [(25)–(27)], and observe partial conservation of LC-associated genes across these species.

Results

Experimental design and study overview of postmortem human LC

We selected 5 neurotypical adult human brain donors to characterize transcriptome-wide gene expression within the LC at spatial and single-nucleus resolution using the 10x Genomics Visium SRT (28) and 10x Genomics Chromium snRNA-seq (29) platforms (see **Supplementary Table 1** for donor demographic details). In each tissue sample, the LC was first visually identified by neuroanatomical landmarks and presence of neurons containing the pigment neuromelanin on transverse slabs of the pons (**Figure 1A**). Prior to SRT and snRNA-seq assays, we ensured that the tissue blocks encompassed the LC by probing for

known LC marker genes (30). Specifically, we cut 10 μ m cryosections from tissue blocks from each donor and probed for the presence of a pan-neuronal marker gene (*SNAP25*) and two NE neuron-specific marker genes (*TH* and *SLC6A2*) by multiplexed single-molecule fluorescence *in situ* hybridization (smFISH) using RNAscope (31; 32) (Figure 1B). Robust mRNA signal from these markers, visualized as puncta on imaged tissue sections, was used as a quality control measure in all tissue blocks prior to proceeding with inclusion in the study and performing SRT and snRNA-seq assays.

Resource	Data	Format	Link
Shiny [42] web app	Visium SRT	Interactive web app	https://libd.shinyapps.io/locus-c_Visium/
iSEE [43] web app	snRNA-seq	Interactive web app	https://libd.shinyapps.io/locus-c_snRNA-seq/
R/Bioconductor ExperimentHub data package	Visium SRT and snRNA-seq	Downloadable R/Bioconductor objects in SpatialExperiment [44] and SingleCellExperiment [33] formats	https://bioconductor.org/packages/WeberDivechaLCdata (available in Bioconductor release version 3.16 from Nov 2, 2022 onwards)

Table 1:

Summary of data resources providing access to datasets described in this manuscript.

All datasets described in this manuscript are freely accessible in the form of interactive web apps and downloadable R/Bioconductor objects.

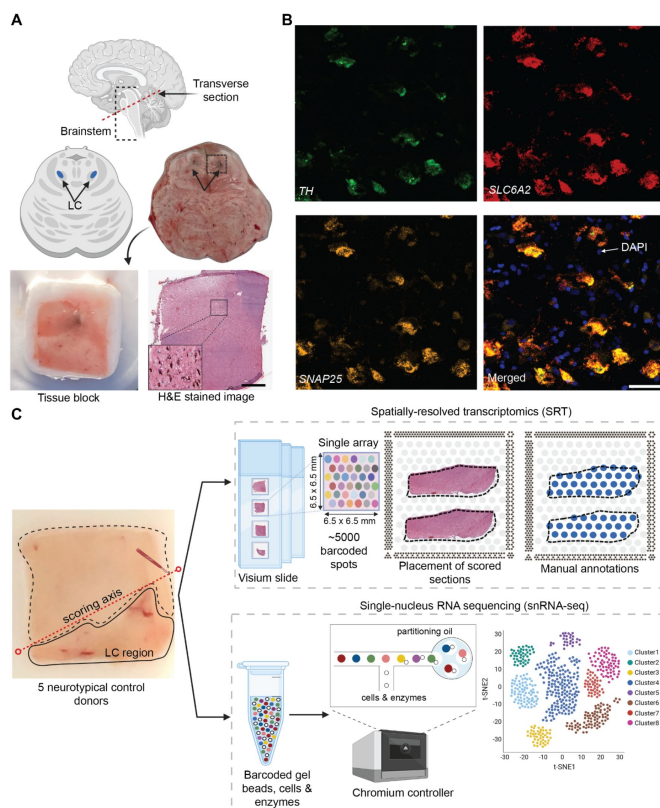


Figure 1:

Experimental design to measure the landscape of gene expression in the postmortem human locus coeruleus (LC) using spatially-resolved transcriptomics (SRT) and single-nucleus RNA-sequencing (snRNA-seq).

(A) Brainstem dissections at the level of the LC were conducted to collect tissue blocks from 5 neurotypical adult human brain donors. (B) Inclusion of the LC within the tissue sample block was validated using RNAscope (31; 32) for a pan-neuronal marker gene (*SNAP25*) and two NE neuron-specific marker genes (*TH* and *SLC6A2*). High-resolution H&E stained histology images were acquired prior to SRT and snRNA-seq assays (scale bars: 2 mm in H&E stained image; 20 μ m in RNAscope images). (C) Prior to collecting tissue sections for SRT and snRNA-seq assays, tissue blocks were scored to enrich for the NE neuron-containing regions. For each sample, the LC region was manually annotated by visually identifying NE neurons in the H&E stained tissue sections. 100 μ m tissue sections from 3 of the same donors were used for snRNA-seq assays, which included FANS-based neuronal enrichment prior to library preparation to enrich for neuronal populations.

For tissue blocks included in the study, we cut additional 10 μ m tissue sections, which were used for gene expression profiling at spatial resolution using the 10x Genomics Visium SRT platform (28) (Figure 1C). Fresh-frozen tissue sections were placed onto each of four capture areas per Visium slide, where each capture area contains approximately 5,000 expression spots (spatial measurement locations with diameter 55 μ m and 100 μ m center-to-center, where transcripts are captured) laid out in a honeycomb pattern. Spatial barcodes unique to each spot are incorporated during reverse transcription, thus allowing the spatial coordinates of the gene expression measurements to be identified (28). Visium slides were stained with hematoxylin and eosin (H&E), followed by high-resolution acquisition of histology images prior to on-slide cDNA synthesis, completion of the Visium assay, and sequencing. For our study, 10 μ m tissue sections from the LC-containing tissue blocks were collected from the 5 brain donors, with assays completed on 2-4 tissue sections per donor. Given the small size of the LC compared to the area of the array, tissue blocks were scored to fit 2-3 tissue sections from the same donor onto a single capture area to maximize the use of

the Visium slides, resulting in a total of $N=9$ Visium capture areas (hereafter referred to as samples).

For 3 of the 5 donors, we cut additional 100 μm sections from the same tissue blocks to profile transcriptome-wide gene expression at single-nucleus resolution with the 10x Genomics Chromium single cell 3' gene expression platform (29) ([Figure 1C](#)). Prior to collecting tissue sections, the tissue blocks were scored to enrich for NE neuron-containing regions. Neuronal enrichment was employed with fluorescence-activated nuclear sorting (FANS) prior to library preparation to enhance capture of neuronal population diversity, and snRNA-seq assays were subsequently completed. **Supplementary Table 1** provides a summary of SRT and snRNA-seq sample information and demographic characteristics of the donors.

Spatial gene expression in the human LC

After applying the 10x Genomics Visium SRT platform (28), we executed several analyses to characterize transcriptome-wide gene expression at spatial resolution within the human LC. First, we manually annotated spots within regions identified as containing LC-NE neurons, based on pigmentation, cell size, and morphology from the H&E stained histology images ([Figure 2A](#) and [Supplementary Figure 1](#)). Next, we performed additional sample-level quality control (QC) on the initial $N=9$ Visium capture areas (hereafter referred to as samples) by visualizing expression of two NE neuron-specific marker genes (*TH* and *SLC6A2*) ([Figure 2B](#)), which identified one sample (Br5459_LC_round2) without clear expression of these markers ([Supplementary Figure 2A-B](#)). This sample was excluded from subsequent analyses, leaving $N=8$ samples from 4 out of the 5 donors. For the $N=8$ Visium samples, the annotated regions were highly enriched in the expression of the NE neuron marker genes (*TH* and *SLC6A2*) ([Figure 2C](#) and [Supplementary Figure 2C](#)), confirming that these samples captured dense regions of LC-NE neurons within the annotated regions. We performed spot-level QC to remove low-quality spots based on QC metrics previously applied to SRT data (22; 33; 34) (**Methods**). Due to the large differences in read depth between samples ([Supplementary Figure 3A](#), **Supplementary Table 1**, **Methods**), we performed spot-level QC independently within each sample. After filtering low-expressed genes (**Methods**), this resulted in a total of 12,827 genes and 20,380 spots across the $N=8$ samples used for downstream analyses ([Supplementary Figure 3B](#)).

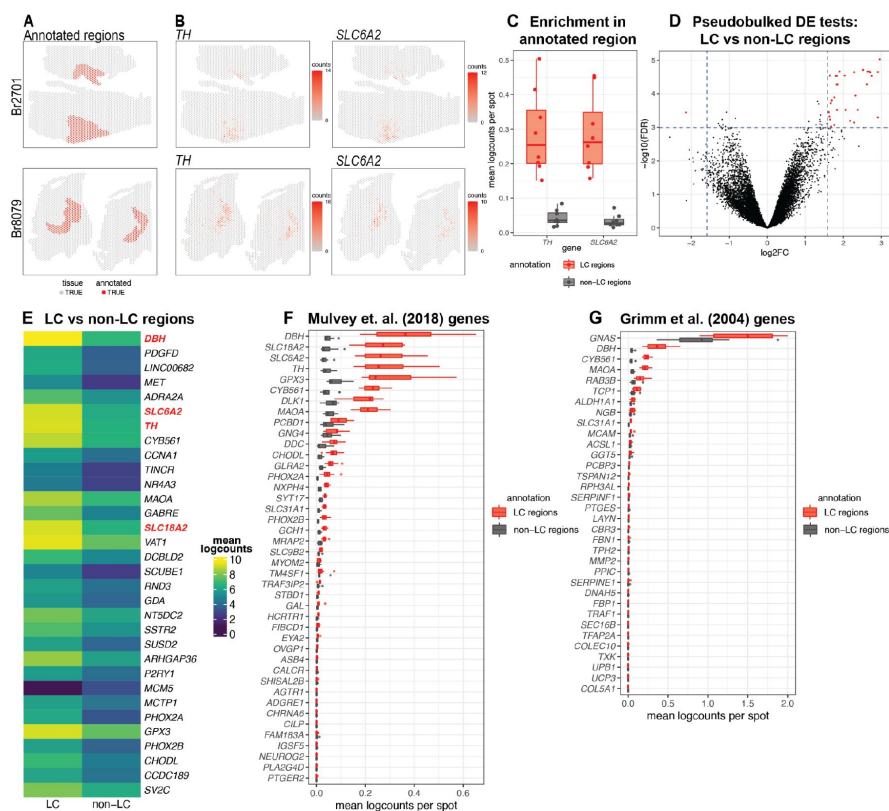


Figure 2:

Spatial gene expression in the human LC using SRT.

(A) Spots within manually annotated LC regions containing NE neurons (red) and non-LC regions (gray), which were identified based on pigmentation, cell size, and morphology from the H&E stained histology images, from donors Br2701 (top row) and Br8079 (bottom row). (B) Expression of two NE neuron-specific marker genes (*TH* and *SLC6A2*). Color scale indicates unique molecular identifier (UMI) counts per spot. Additional samples corresponding to A and B are shown in [Supplementary Figures 1, 2A-B](#). (C) Boxplots illustrating the enrichment in expression of two NE neuron-specific marker genes (*TH* and *SLC6A2*) in manually annotated

LC regions compared to non-LC regions in the $N=8$ Visium samples. Values show mean log-transformed normalized counts (logcounts) per spot within the regions per sample. Additional details are shown in [Supplementary Figure 2C](#). (D) Volcano plot resulting from differential expression (DE) testing between the pseudobulked manually annotated LC and non-LC regions, which identified 32 highly significant genes (red) at a false discovery rate (FDR) significance threshold of 10^{-3} and expression fold-change (FC) threshold of 3 (dashed blue lines). Horizontal axis is shown on log2 scale and vertical axis on log10 scale. Additional details and results for 437 statistically significant genes identified at an FDR threshold of 0.05 and an FC threshold of 2 are shown in [Supplementary Figure 7](#) and [Supplementary Table 2](#). (E) Average expression in manually annotated LC and non-LC regions for the 32 genes from D. Color scale shows logcounts in the pseudobulked LC and non-LC regions averaged across $N=8$ Visium samples. Genes are ordered in descending order by FDR ([Supplementary Table 2](#)). (F-G) Cross-species comparison showing expression of human ortholog genes for LC-associated genes identified in the rodent LC (25; 26) using alternative experimental technologies. Boxplots show mean logcounts per spot in the manually annotated LC and non-LC regions per sample in the human data.

To investigate whether the LC regions could be annotated in a data-driven manner, we applied a spatially-aware unsupervised clustering algorithm (BayesSpace (35)) after applying a batch integration tool (Harmony (36)) to remove sample-specific technical variation in the molecular measurements ([Supplementary Figure 4](#)). The spatially-aware clustering using $k=5$ clusters identified one cluster that overlapped with the manually annotated LC regions in several samples. However, the proportion of overlapping spots between the manually annotated LC region and this data-driven cluster (cluster 4, colored red in [Supplementary Figure 5A](#)) was relatively low and varied across samples. We quantitatively evaluated the clustering performance by calculating the precision, recall, F1 score, and adjusted Rand index (ARI) for this cluster in each sample (see [Methods](#) for definitions). We found that while precision was >0.8 in 3 out of 8 samples, recall was <0.4 in all samples, the F1 score was <0.6 in all samples, and the ARI was <0.5 in all samples ([Supplementary Figure 5B](#)). Therefore, we judged that the data-driven spatial domains identified from BayesSpace were not sufficiently reliable to use for the downstream analyses, and instead proceeded with the

histology-driven manual annotations for all further analyses. In addition, we note that using the manual annotations avoids potential issues due to inflated false discoveries resulting from circularity when performing differential gene expression testing between sets of cells or spots defined by unsupervised clustering, when the same genes are used for both clustering and differential testing (37). Next, in addition to the manually annotated LC regions, we also manually annotated a set of individual spots that overlapped with NE neuron cell bodies identified within the LC regions, based on pigmentation, cell size, and morphology from the H&E histology images ([Supplementary Figure 6A](#)). However, we observed relatively low overlap between spots with expression of NE neuron marker genes and this second set of annotated individual spots. For example, out of 706 annotated spots, only 331 spots had ≥ 2 observed UMI counts of *TH* ([Supplementary Figure 6B](#)). We hypothesize that this may be due to technical factors including sampling variability in the gene expression measurements, partial overlap between spots and cell bodies, potential diffusion of mRNA molecules between spots, as well as biological variability in the expression of these marker genes. Therefore, we instead used the LC region-level manual annotations for all further analyses.

Next, to identify expressed genes associated with the LC regions, we performed differential expression (DE) testing between the manually annotated LC and non-LC regions by pseudobulking spots, defined as aggregating UMI counts from the combined spots within the annotated LC and non-LC regions in each sample (22). This analysis identified 32 highly significant genes at a false discovery rate (FDR) threshold of 10^{-3} and expression fold-change (FC) threshold of 3 ([Figure 2D](#) and [Supplementary Figure 7A](#)). This includes known NE neuron marker genes including *DBH* (the top-ranked gene by FDR within this set), *SLC6A2* (ranked 6th), *TH* (ranked 7th), and *SLC18A2* (ranked 14th). Out of the 32 genes, 31 were elevated in expression within the LC regions, while one (*MCM5*) was depleted. The set includes one long noncoding RNA (*LINC00682*), while the remaining 31 genes are protein-coding genes ([Figure 2E](#) and [Supplementary Table 2](#)). Alternatively, using standard significance thresholds of $\text{FDR} < 0.05$ and expression $\text{FC} > 2$, we identified a total of 437 statistically significant genes ([Supplementary Figure 7B](#) and [Supplementary Table 2](#)).

As a second approach to identify genes associated with LC-NE neurons in an unsupervised manner, we applied a method to identify spatially variable genes (SVGs), nnSVG (38). This method ranks genes in terms of the strength in the spatial correlation in their expression patterns across the tissue areas. We ran nnSVG within each contiguous tissue area containing an annotated LC region for the $N=8$ Visium samples (total 13 tissue areas, where each Visium sample contains 1-3 tissue areas) and combined the lists of top-ranked SVGs for the multiple tissue areas by averaging the ranks per gene. In this analysis, we found that a subset of the top-ranked SVGs (11 out of the top 50) were highly-ranked in samples from only one donor (Br8079), which we determined was due to the inclusion of a section of the choroid plexus adjacent to the LC in these samples (based on expression of choroid plexus marker genes including *CAPS* and *CRLF1*) ([Supplementary Figure 8A-C](#)). In order to focus on LC-associated SVGs that were replicated across samples, we excluded the choroid plexus-associated genes by calculating an overall average ranking of SVGs that were each included within the top 100 SVGs in at least 10 out of the 13 tissue areas, which identified a list of 32 highly-ranked, replicated LC-associated SVGs. These genes included known NE neuron marker genes (*DBH*, *TH*, *SLC6A2*, and *SLC18A2*) as well as mitochondrial genes ([Supplementary Figure 8D](#)).

We also compared the expression of LC-associated genes previously identified in the rodent LC from two separate studies. The first study used translating ribosomal affinity purification sequencing (TRAP-seq) using an *SLC6A2* bacTRAP mouse line to identify gene expression profiles of the transcriptome of LC neurons (25). The second study used microarrays to assess gene expression patterns from laser-capture microdissection of individual cells in tissue sections of the rat LC (26). We converted the lists of rodent LC-associated genes from these studies to human orthologs and calculated average expression for each gene within the

manually annotated LC and non-LC regions. A small number of genes from both studies were highly associated with the manually annotated LC regions in the human data, including *DBH*, *TH*, and *SLC6A2* from (25), and *DBH* and *GNAS* from (26). However, the majority of the genes from both studies were expressed at low levels in the human data, which may reflect species-specific differences in biological function of these genes as well as differences due to the experimental technologies employed (Figure 2F-G).

Single-nucleus gene expression of NE neurons in the human LC

To add cellular resolution to our spatial analyses, we characterized gene expression in the human LC and surrounding region at single-nucleus resolution using the 10x Genomics Chromium single cell 3' gene expression platform (29) in 3 of the same neurotypical adult donors from the SRT analyses.

Samples were enriched for NE neurons by scoring tissue blocks for the LC region and performing FACS to enhance capture of neurons. After raw data processing, doublet removal using *scDblFinder* (39), and standard QC and filtering, we obtained a total of 20,191 nuclei across the 3 samples (7,957, 3,015, and 9,219 nuclei respectively from donors Br2701, Br6522, and Br8079) (see **Supplementary Table 1** for additional details). For nucleus-level QC processing, we used standard QC metrics including the sum of UMI counts and detected genes (33) (see **Methods** for additional details). We observed an unexpectedly high proportion of mitochondrial reads in nuclei with expression of NE neuron marker genes (*DBH*, *TH*, and *SLC6A2*), which represented our rare population of interest, and hence we did not remove nuclei based on proportion of mitochondrial reads (Supplementary Figures 9-10).

We identified NE neuron nuclei in the snRNA-seq data by applying an unsupervised clustering workflow adapted from workflows used for snRNA-seq data in the human brain (23), using a two-stage clustering algorithm consisting of high-resolution *k*-means and graph-based clustering that provides sensitivity to identify rare cell populations (33). The unsupervised clustering workflow identified 30 clusters, including clusters representing major neuronal and non-neuronal cell populations, which we labeled based on expression of known marker genes (Figure 3A-B). This included a cluster of NE neurons consisting of 295 nuclei (168, 4, and 123 nuclei from donors Br2701, Br6522, and Br8079, respectively), which we identified based on expression of NE neuron marker genes (*DBH*, *TH*, and *SLC6A2*). In addition to the NE neuron cluster, we identified clusters representing excitatory neurons, inhibitory neurons, astrocytes, endothelial and mural cells, macrophages and microglia, oligodendrocytes, and oligodendrocyte precursor cells (OPCs), as well as several clusters with ambiguous expression profiles including pan-neuronal marker genes (*SNAP25* and *SYT1*), which may represent damaged neuronal nuclei (Figure 3A-B and Supplementary Figure 9).

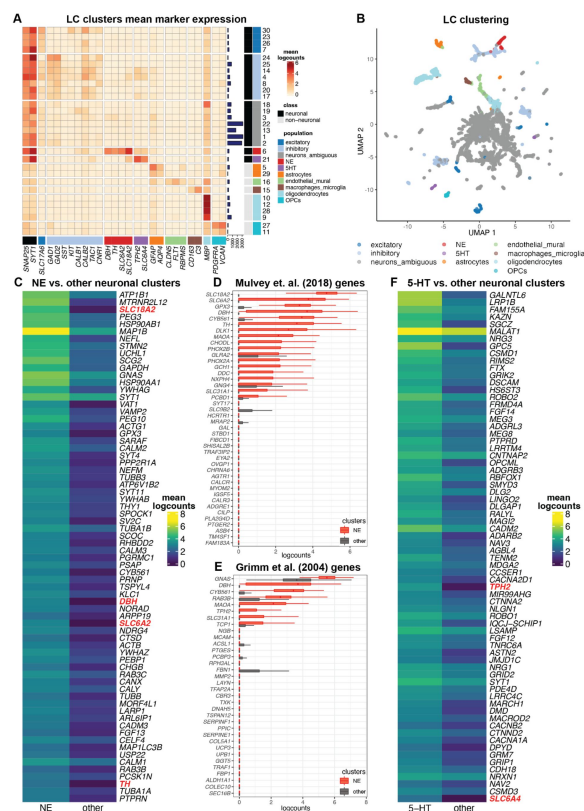


Figure 3:

Single-nucleus gene expression in the human LC using snRNA-seq.

We applied an unsupervised clustering workflow to identify cell populations in the snRNA-seq data. **(A)** Unsupervised clustering identified 30 clusters representing populations including NE neurons (red), 5-HT neurons (purple), and other major neuronal and non-neuronal cell populations (additional colors). Marker genes (columns) were used to identify clusters (rows). Cluster IDs are shown in labels on the right, and numbers of nuclei per cluster are shown in horizontal bars on the right. Heatmap values represent mean logcounts per cluster. **(B)** UMAP representation of nuclei, with colors matching cell populations from heatmap. **(C)** DE testing between neuronal clusters identified a total of 327 statistically significant genes with elevated expression in the NE neuron cluster, at an FDR threshold of 0.05 and FC threshold of 2. Heatmap displays the top 70 genes, ranked in descending order by FDR, excluding mitochondrial genes, with NE neuron marker genes described in text highlighted in red. The full list of 327 genes including mitochondrial genes is provided in **Supplementary Table 4**. Heatmap values represent mean logcounts in the NE neuron cluster and mean logcounts per cluster averaged across all other neuronal clusters. **(D-E)** Cross-species comparison showing expression of human ortholog genes for LC-associated genes identified in the rodent LC (25; 26) using alternative experimental technologies. Boxplots show logcounts per nucleus in the NE neuron cluster and all other neuronal clusters. Boxplot whiskers extend to 1.5 times interquartile range, and outliers are not shown. **(F)** DE testing between neuronal clusters identified a

5-HT neuron cluster, at an FDR threshold of 0.05 and FC threshold of 2. Heatmap displays the top 70 genes, ranked in descending order by FDR, with 5-HT neuron marker genes described in text highlighted in red. The full list of 361 genes is provided in **Supplementary Table 5**.

To validate the unsupervised clustering, we also applied a supervised strategy to identify NE neuron nuclei by simply thresholding on expression of NE neuron marker genes (selecting nuclei with ≥ 1 UMI counts of both *DBH* and *TH*). As described above, we noted a higher than expected proportion of mitochondrial reads in nuclei with expression of *DBH* and *TH*, and did not filter on this parameter during QC processing, in order to retain these nuclei (**Supplementary Figure 10A-B**). This supervised approach identified 332 NE neuron nuclei (173, 4, and 155 nuclei from donors Br2701, Br6522, and Br8079, respectively), including 188 out of the 295 NE neuron nuclei identified by unsupervised clustering (**Supplementary Figure 10C**). We hypothesized that the differences for nuclei that did not agree between the two approaches were due to sampling variability in the snRNA-seq measurements for these two marker genes. To confirm this, we used an alternative method (smFISH RNAscope (32)) to assess co-localization of three NE neuron marker genes (*DBH*, *TH*, and *SLC6A2*) within individual cells on additional tissue sections from one additional independent donor (Br8689). Visualization of high-magnification confocal images demonstrated clear co-localization of these three marker genes within individual cells (**Supplementary Figure 11**). Since the unsupervised clustering is based on expression of a large number of genes and is therefore less sensitive to sampling variability for individual genes, we used the unsupervised clustering results for all further downstream analyses.

We performed DE testing between the neuronal clusters and identified 327 statistically significant genes with elevated expression in the NE neuron cluster, compared to all other neuronal clusters captured in this region, at an FDR threshold of 0.05 and FC threshold of 2. These genes include known NE neuron marker genes (*DBH*, *TH*, *SLC6A2*, and *SLC18A2*) as well as the 13 protein-coding mitochondrial genes, which are highly expressed in large, metabolically active NE neurons (**Figure 3C**, **Supplementary Figure 12A**, and **Supplementary Table 4**). Compared to the LC-associated genes identified in the SRT samples, differences are expected since the snRNA-seq data contains measurements from nuclei at single-nucleus resolution, while the SRT samples contain reads from nuclei, cytoplasm, and cell processes from multiple cell populations within the annotated LC regions.

To compare with previous results in rodents, we evaluated the expression of the rodent LC marker genes from (25; 26) in the NE neuron cluster compared to all other neuronal clusters in the human snRNA-seq data (**Figure 3D-E**). Consistent with the SRT samples, we observed that several genes were conserved across species. However, compared to the SRT samples, we observed relatively higher expression of the conserved genes within the NE neuron cluster, which is expected since the NE neuron cluster contains reads from individual nuclei from this population only.

We note that a recent publication using snRNA-seq in mice found that LC-NE neurons were highly enriched for *Calca*, *Cartpt*, *Gal*, and *Calcr* in addition to canonical NE neuron marker genes (27). In the human data, we noted significant enrichment of *GAL* and *CARTPT* in DE testing between the manually annotated LC and non-LC regions in the SRT samples (**Supplementary Table 2**). While visualization of the snRNA-seq clustering suggests that *CARTPT* is expressed in the NE neuron cluster in the snRNA-seq data (**Supplementary Figure 13**), it was not identified as statistically significant in the DE testing between the NE cluster compared to all other neuronal clusters (**Supplementary Table 4**). For *CALCA* and *CALCR*, we observed no enrichment in the annotated LC regions in the SRT samples, nor in

the NE neuron cluster in the snRNA-seq data ([Supplementary Tables 2, 4 and Supplementary Figure 13](#)).

Identification of 5-HT neurons and diversity of inhibitory neuron subpopulations in single-nucleus data

In addition to NE neurons, we identified a cluster of likely 5-hydroxytryptamine (5-HT, serotonin) neurons in the unsupervised clustering analyses of the snRNA-seq data ([Figure 3A-B](#)) based on expression of 5-HT neuron marker genes (*TPH2* and *SLC6A4*) (40). This cluster consisted of 186 nuclei (145, 28, and 13 nuclei from donors Br2701, Br6522, and Br8079, respectively). DE testing between the neuronal clusters identified 361 statistically significant genes with elevated expression in the 5-HT neuron cluster, compared to all other neuronal clusters captured in this region, at an FDR threshold of 0.05 and FC threshold of 2. These genes included the 5-HT neuron marker genes *TPH2* and *SLC6A4* ([Figure 3F](#), [Supplementary Figure 12B](#), and [Supplementary Table 5](#)). To investigate the spatial distribution of this population, we visualized the spatial expression of the 5-HT neuron marker genes *TPH2* and *SLC6A4* in the $N=9$ initial Visium samples, which showed that this population was distributed across both the LC and non-LC regions ([Supplementary Figure 14A-B](#)). Similarly, we did not observe significant spatial enrichment of *TPH2* and *SLC6A4* expression within the manually annotated LC regions ([Supplementary Figure 14C](#)). To further confirm this finding, we applied RNAscope (32) to visualize expression of an NE neuron marker gene (*TH*) and 5-HT neuron marker genes (*TPH2* and *SLC6A4*) within additional tissue sections from donor Br6522, which demonstrated that the NE and 5-HT marker genes were expressed within distinct cells and these neuronal populations were not localized within the same regions ([Supplementary Figure 15](#)).

We also investigated the diversity of inhibitory neuronal subpopulations within the snRNA-seq data from the human LC and surrounding region by applying a secondary round of unsupervised clustering to the inhibitory neuron nuclei identified in the first round of clustering. This identified 14 clusters representing inhibitory neuronal subpopulations, which varied in their expression of several inhibitory neuronal marker genes including *CALB1*, *CALB2*, *TAC1*, *CNR1*, and *VIP* (additional marker genes shown in [Supplementary Figures 13](#), [16](#)). In addition, similar to recently published results from mice (27), we found that expression of neuropeptides *PNO*C, *TAC1*, and *PENK* varied across the individual inhibitory neuronal populations ([Supplementary Figure 13](#)).

In order to integrate the single-nucleus and spatial data, we also applied a spot-level deconvolution algorithm, cell2location (41), to map the spatial coordinates of NE and 5-HT neurons within the Visium samples. This algorithm integrates the snRNA-seq and SRT data by estimating the cell abundance of the snRNA-seq populations, which are used as reference populations at cellular resolution, at each spatial location (spot) in the Visium samples. This successfully mapped the NE neuron population from the snRNA-seq data to the manually annotated LC regions in the Visium samples ([Supplementary Figure 17A](#)). Similarly, for the 5-HT neurons, this population was mapped to the regions where this population was previously identified based on expression of marker genes ([Supplementary Figure 17B](#)). However, the estimated absolute cell abundance of these neuronal populations per spot was higher than expected, which may be due to their relatively large size and high transcriptional activity, especially for NE neurons, compared to other neuronal and non-neuronal cell populations.

Co-expression of cholinergic marker genes within NE neurons

We observed expression of cholinergic marker genes, including *SLC5A7*, which encodes the high-affinity choline transporter, and *ACHE*, within the NE neuron cluster in the snRNA-seq

data ([Supplementary Figure 13](#)). Because this result was unexpected, we experimentally confirmed co-expression of *SLC5A7* transcripts with transcripts for NE neuron marker genes in individual cells using RNAscope (32) on independent tissue sections from donors Br6522 and Br8079. We used RNAscope probes for *SLC5A7* and *TH* (NE neuron marker), and imaged stained sections at 63x magnification to generate high-resolution images, which allowed us to definitively localize expression of individual transcripts within cell bodies. This confirmed co-expression of *SLC5A7* and *TH* in individual cells in a tissue section from donor Br8079 ([Supplementary Figure 18](#)), validating that these transcripts are expressed within the same cells. To further investigate the spatial distribution of the cholinergic marker genes, we visualized expression of *SLC5A7* and *ACHE* in the Visium samples, which showed that these genes were expressed both within and outside the annotated LC regions ([Supplementary Figure 19](#)).

Interactive and accessible data resources

We provide freely accessible data resources containing all datasets described in this manuscript, in the form of both interactive web-accessible and downloadable resources ([Table 1](#)). The interactive resources can be explored in a web browser via a Shiny (42) app for the Visium SRT data and an Isee (43) app for the snRNA-seq data ([Supplementary Figure 20](#)). The data resources are also available from an R/Bioconductor ExperimentHub data package as downloadable objects in the SpatialExperiment (44) and SingleCellExperiment (33) formats, which can be loaded in an R session and contain meta-data including manual annotation labels and cluster labels.

Discussion

Due to the small size and inaccessibility of the LC within the brainstem, this region has been relatively understudied in the human brain, despite its involvement in numerous functions and disease mechanisms. Our dataset provides the first transcriptome-wide characterization of the gene expression landscape of the human locus coeruleus (LC) using spatially-resolved transcriptomics (SRT) and single-nucleus RNA-sequencing (snRNA-seq). Analysis of these data identified a population of norepinephrine (NE) neurons as well as a population of 5-hydroxytryptamine (5-HT, serotonin) neurons, and spatially localized them within the LC and surrounding region. We evaluated expression of previously known marker genes for these populations and identified novel sets of significant differentially expressed (DE) genes, and assessed how their expression varies in space across the neuroanatomy of the region. We compared our findings from the human LC to molecular profiles of LC and peri-LC neurons that were previously characterized in rodents using alternative technological platforms, which confirmed partial conservation of LC-associated genes across these species. Finally, we validated our results using smFISH RNAscope to assess co-localization of marker genes on independent tissue sections.

Identifying genes whose expression is enriched in NE neurons is important because the LC-NE system is implicated in multiple neuropsychiatric and neurological disorders (4; 8), and prominent loss of NE cells in the LC occurs in neurodegenerative disorders [(8)–(10)]. Unbiased analysis of the snRNA-seq and SRT data identified a number of genes that are enriched in the human LC region and in LC-NE neurons themselves. As expected, these analyses validated enrichment of genes involved in NE synthesis and packaging (*TH*, *SLC18A2*, *DBH*) as well as NE reuptake (*SLC6A2*). We also noted expression selectively in LC-NE neurons of a number of genes whose expression is altered in animal models or in human disease in the LC (*SSTR2*, *PHOX2A*, *PHOX2A*) (45; 46). We identified LC enrichment of a number of genes that have been associated at the cellular level with apoptosis, cell loss, or pathology in the context of neurodegeneration (*RND3*, *P2RY1*) [(47)–(50)]. We also noted

enrichment of *NT5DC2* in LC, a gene which has been associated with attention-deficit hyperactivity disorder (ADHD) and regulates catecholamine synthesis *in vitro* (51; 52). Localization of these genes to the LC in humans, and LC-NE neurons in particular, may provide important biological insights about physiological function of these neurons and provide context about underlying mechanistic links between these genes and disease risk. Future work using the transcriptome-wide molecular expression profiles of NE neurons at single-nucleus resolution and the LC region at spatial resolution generated here could investigate associations with individual genes and gene sets from genome-wide association studies (GWAS) for these disorders as well as genes more generally associated with aging-related processes.

Our study has several limitations. The SRT data using the 10x Genomics Visium platform captures around 1-10 cells per measurement location in the human brain, and future studies could apply a higher-resolution platform to characterize expression at single-cell or sub-cellular spatial resolution. In the single-nucleus data, we identified a relatively small number of NE neurons, which may be related to technical factors that affect the recovery of this population due to their relatively large size and fragility. These technical factors may have also contributed to the unexpectedly high proportion of mitochondrial reads that we observed in NE neurons in the snRNA-seq data. While mitochondrial reads are not expected in the nuclear compartment, recent studies reported contamination of nuclear preparations in snRNA-seq data with ambient mRNAs from abundant cellular transcripts (53). Given the relatively elevated energy demand and increased metabolic activity of NE neurons, higher than expected mitochondrial contamination in the nuclear preparation of LC tissue may be plausible. Because NE neurons were the population of highest interest to profile in this dataset, we opted not to perform QC filtering on the proportion of mitochondrial reads, in order to retain this population. Further optimizing technical procedures for cell sorting and cryosectioning to avoid cellular damage, as well as for straining for large cells, could enhance recovery of this population for future, larger-scale snRNA-seq studies in this brain region.

Since the identification of 5-HT neurons in the single-nucleus data was an unexpected finding, the experimental procedures were not designed to optimally recover this population, and the precise anatomical origin of the 5-HT neurons recovered in this dataset is not entirely clear. It is possible that these cells were close to the borders of the LC dissections, residing within the dorsal raphe nucleus, which is neuroanatomically adjacent to the LC. Supporting this hypothesis, RNAscope data in [Supplementary Figure 15](#) from an independent tissue section shows that *TPH2* and *SLC6A4* expression appears to be distinct from the LC region containing a high density of NE cells. However, there is some evidence for expression of serotonergic markers within the LC region in rodents (26; 54; 55), and our SRT data does support this possibility in the human brain, although further characterization is needed. Comprehensively understanding the full molecular diversity of 5-HT neurons in the human brain would require dissections that systematically sample across the extent of the dorsal raphe nucleus. Similarly, the identification of cholinergic marker gene expression, particularly the robust expression of *SLC5A7* within NE neurons, was unexpected. While previous studies have identified small populations of cholinergic interneurons within or adjacent to the LC in rodents (27), analysis of our data did not classify any of the other neuronal populations as cholinergic per se.

However, both the SRT and RNAscope data ([Supplementary Figure 18](#)) supports the hypothesis that expression of cholinergic markers occurs in NE cells themselves, as well as in sparse populations of cholinergic neurons adjacent to the LC region that do not express NE marker genes. We note that our snRNA-seq data may be underpowered to fully identify and classify these sparse populations, and future experiments designed to specifically investigate this finding in more detail could lead to a better understanding of cholinergic signaling within the LC of the human brain. Similarly, our snRNA-seq may be underpowered to perform gene set enrichment studies (56) to identify whether the NE and 5-HT neurons

harbor aggregated genetic risk for psychiatric disorders, but these data could be aggregated with future snRNA-seq data generated from the LC to address this question. To facilitate further exploration of these data, we provide a freely accessible data resource that includes the snRNA-seq and SRT data in both web-based and R-based formats, as well as reproducible code for the computational analysis workflows for the snRNA-seq and SRT data.

Materials and Methods

Postmortem human brain tissue samples for RNAscope, SRT, and snRNA-seq assays

Brain donations in the Lieber Institute for Brain Development (LIBD) Human Brain Repository were collected from the Office of the Chief Medical Examiner of the State of Maryland under the Maryland Department of Health's IRB protocol #12-24, and from the Western Michigan University Homer Stryker MD School of Medicine, Department of Pathology, and the Department of Pathology, University of North Dakota School of Medicine and Health Sciences, both under WCG IRB protocol #20111080. Clinical characterization, diagnoses, and macro- and microscopic neuropathological examinations were performed on all samples using a standardized paradigm, and subjects with evidence of macro- or microscopic neuropathology were excluded. Details of tissue acquisition, handling, processing, dissection, clinical characterization, diagnoses, neuropathological examinations, RNA extraction, and quality control measures have been described previously (57; 58). We obtained tissue blocks from 5 male neurotypical brain donors of European ancestry. To select tissue blocks for study inclusion, we identified the LC in transverse slabs of the pons from fresh-frozen human brain. The LC was identified through visual inspection of the slab, based on neuroanatomical landmarks and the presence of neuromelanin pigmentation. For each donor, a tissue block was dissected from the dorsal aspect of the pons, centered around the LC, using a dental drill. The tissue block was taken at the level of the motor trigeminal nucleus and superior cerebellar peduncle. Tissue blocks were kept at -80 °C until sectioning for experiments. We cut 10 µm tissue sections for performing SRT assays using the 10x Genomics Visium SRT platform (28). High-resolution images of the H&E stained histology were acquired prior to on-slide cDNA synthesis and completing the Visium assays. Assays were performed on 2-4 tissue sections collected from each of the 5 donors, and the tissue blocks were scored to fit 2-3 tissue sections from the same donor onto a single Visium capture area to maximize the use of the Visium slides. This resulted in a total of $N=9$ Visium capture areas (hereafter referred to as samples) in the SRT dataset. For 3 of the 5 donors, we cut additional 100 µm cryosections for snRNA-seq assays using the 10x Genomics Chromium snRNA-seq platform (29). **Supplementary Table 1** provides information on brain donor demographics as well as sample information for the SRT and snRNA-seq datasets.

Multiplexed smFISH using RNAscope

For RNAscope experiments, tissue blocks were sectioned at 10 µm and single-molecule fluorescent *in situ* hybridization assays were performed with RNAscope technology (32) using the Fluorescent Multiplex Kit v.2 and 4-plex Ancillary Kit (catalog no. 323100, 323120 ACD) according to the manufacturer's instructions. Briefly, 10 µm tissue sections (2-4 sections per donor) were fixed with 10% neutral buffered formalin solution (catalog no. HT501128, Sigma-Aldrich) for 30 min at room temperature, series dehydrated in increasing concentrations of ethanol (50%, 70%, 100%, and 100%), pretreated with hydrogen peroxide for 10 min at room temperature and treated with protease IV for 30 min. For QC experiments to confirm LC inclusion in the tissue block (**Figure 1B** showing example for additional independent donor Br8689), tissue sections were incubated with 3 different

probes (2 LC-NE neuron markers and one pan-neuronal marker): *SLC6A2* (catalog no. 526801-C1, Advanced Cell Diagnostics) encoding the norepinephrine transporter, *TH* (catalog no. 441651-C2, Advanced Cell Diagnostics) encoding tyrosine hydroxylase, and *SNAP25* (catalog no. 518851-C3, Advanced Cell Diagnostics). To confirm co-expression of LC-NE marker genes within individual cells ([Supplementary Figure 11](#)), we used *SLC6A2* (catalog no. 526801-C1, Advanced Cell Diagnostics), *TH* (catalog no. 441651-C2, Advanced Cell Diagnostics), and *DBH* (catalog no. 545791-C3, Advanced Cell Diagnostics) encoding dopamine beta-hydroxylase. To localize serotonergic and cholinergic markers within the LC ([Supplementary Figure 15](#)), we used *TH* (catalog no. 441651-C2, Advanced Cell Diagnostics) encoding tyrosine hydroxylase, *TPH2* (catalog no. 471451-C1, Advanced Cell Diagnostics) encoding tryptophan hydroxylase 2, *SLC6A4* (catalog no. 604741-C3, Advanced Cell Diagnostics) encoding the serotonin transporter, and *SLC5A7* (catalog no. 564671-C4, Advanced Cell Diagnostics) encoding the high-affinity choline transporter. After probe labeling, sections were stored overnight in 4x saline-sodium citrate buffer (catalog no. 10128-690, VWR). After amplification steps (AMP1-3), probes were fluorescently labeled with Opal Dyes 520, 570, and 690 (catalog no. FP1487001KT, FP1488001KT, and FP1497001KT, Akoya Biosciences; 1:500 dilutions for all the dyes) and counter-stained with DAPI (4',6-diamidino-2-phenylindole) to label cell nuclei. Lambda stacks were acquired in z-series using a Zeiss LSM780 confocal microscope equipped with 20x, 0.8 numerical aperture (NA) and 63x, 1.4 NA objectives, a GaAsP spectral detector, and 405-, 488-, 561- and 633-nm lasers. All lambda stacks were acquired with the same imaging settings and laser power intensities.

After image acquisition, lambda stacks in z-series were linearly unmixed using Zen Black (weighted; no autoscale) using reference emission spectral profiles previously created in Zen for the dotdotdot software (git hash v.4e1350b) (31), stitched, maximum intensity projected, and saved as Carl Zeiss Image (.czi) files.

Visium SRT with H&E staining data generation and sequencing

Tissue blocks were embedded in OCT medium and cryosectioned at 10 µm on a cryostat (Leica Biosystems). Briefly, Visium Gene Expression Slides were cooled inside the cryostat and tissue sections were then adhered to the slides. Tissue sections were fixed with methanol and then stained with hematoxylin and eosin (H&E) according to manufacturer's staining and imaging instructions (User guide CG000160 Rev C). Images of the H&E stained slides were acquired using a CS2 slide scanner (Leica Biosystems) equipped with a color camera and a 20x, 0.75 NA objective and saved as a Tagged Image File (.tif). Following H&E staining and acquisition of images, slides were processed for the Visium assay according to manufacturer's reagent instructions (Visium Gene Expression protocol User guide CG000239, Rev D) as previously described (22). In brief, the workflow includes permeabilization of the tissue to allow access to mRNA, followed by reverse transcription, removal of cDNA from the slide, and library construction. Tissue permeabilization experiments were conducted on a single LC sample used in the study and an optimal permeabilization time of 18 minutes was identified and used for all sections across donors. Sequencing libraries were quality controlled and then sequenced on the MiSeq, NextSeq, or NovaSeq Illumina platforms. Sequencing details and summary statistics for each sample are reported in [Supplementary Table 1](#).

snRNA-seq data generation and sequencing

Following SRT data collection, three tissue blocks (Br6522, Br8079, and Br2701) were used for snRNA-seq. Prior to tissue collection for snRNA-seq assays, tissue blocks were further scored based on the RNAscope and SRT data to enrich tissue collection to the localized site of LC-NE neurons. After scoring the tissue blocks, samples were sectioned at 100 µm and collected in cryotubes. The sample collected for Br6522 contained 10 sections, weighing 51

mg, and the samples from Br8079 and Br2701 each contained 15 sections, weighing 60.9 mg and 78.9 mg, respectively. These samples were processed following a modified version of the ‘Frankenstein’ nuclei isolation protocol as previously described (23). Specifically, chilled EZ lysis buffer (MilliporeSigma) was added to the LoBind microcentrifuge tube (Eppendorf) containing cryosections, and the tissue was gently broken up, on ice, via pipetting. This lysate was transferred to a chilled dounce, rinsing the tube with additional EZ lysis buffer. The tissue was then homogenized using part A and B pestles, respectively, for ~10 strokes, each, and the homogenate was strained through a 70 µm cell strainer. After lysis, the samples were centrifuged at 500 g at 4 °C for 5 min, supernatant was removed, then the sample was resuspended in EZ lysis buffer. Following a 5 min incubation, samples were centrifuged again. After supernatant removal, wash/resuspension buffer (PBS, 1% BSA, and 0.2 U/uL RNasin), was gently added to the pellet for buffer interchange. After a 5 min incubation, each pellet was again washed, resuspended and centrifuged three times.

For staining, each pellet was resuspended in a wash/resuspension buffer with 3% BSA, and stained with AF488-conjugated anti-NeuN antibody (MiliporeSigma, catalog no. MAB377X), for 30 min on ice with frequent, gentle mixing. After incubation, these samples were washed with 1 mL wash/resuspension buffer, then centrifuged, and after the supernatant was aspirated, the pellet was resuspended in wash/resuspension buffer with propidium iodide (PI), then strained through a 35 µm cell filter attached to a FACS tube. Each sample was then sorted on a Bio-Rad S3e Cell Sorter on ‘Purity’ mode into a 10x Genomics reverse transcription mix, without enzyme. A range of 5637-9000 nuclei were sorted for each sample, aiming for an enrichment of ~60% singlet, NeuN+ nuclei. Then the 10x Chromium reaction was completed following the Chromium Next GEM Single Cell 3’ Reagent Kits v3.1 (Dual Index) revision A protocol, provided by the manufacturer (10x Genomics) to generate libraries for sequencing. The number of sequencing reads and platform used per sample are shown in **Supplementary Table 1**.

Analysis of Visium SRT data

This section describes additional details on the computational analyses of the Visium SRT data that are not included in the main text.

Manual alignment of the H&E stained histology images to the expression spot grid was performed using the 10x Genomics Loupe Browser software (v. 5.1.0). The raw sequencing data files (FASTQ files) for the sequenced library samples were processed using the 10x Genomics Space Ranger software (v. 1.3.0) (59) using the human genome reference transcriptome version GRCh38 2020-A (July 7, 2020) provided by 10x Genomics. Sequencing summary statistics for each sample are provided in **Supplementary Table 1**.

For spot-level QC filtering, we removed outlier spots that were more than 3 median absolute deviations (MADs) above or below the median sum of UMI counts or the median number of detected genes per spot (33). We did not use the proportion of mitochondrial reads per spot for spot-level QC filtering, since we observed a high proportion of mitochondrial reads in the NE nuclei in the snRNA-seq data (for more details, see **Results** and **Supplementary Figure 9**), so using this QC metric would risk removing the rare population of NE nuclei of interest in the snRNA-seq data. Therefore, for consistency with the snRNA-seq analyses, we did not use the proportion of mitochondrial reads for spot-level QC filtering in the SRT data. Due to the large differences in read depth between samples (e.g. median sum of UMI counts ranged from 118 for sample Br6522_LC_2_round1 to 2,252 for sample Br6522_LC_round3; see **Supplementary Figure 3A** and **Supplementary Table 1** for additional details), we performed spot-level QC independently within each sample. The spot-level QC filtering identified a total of 287 low-quality spots (1.4% out of 20,667 total spots) from the $N=8$ Visium samples that passed sample-level QC. These spots were removed from subsequent analyses

([Supplementary Figure 3B](#)). For gene-level QC filtering, we removed low-expressed genes with a total of less than 80 UMI counts summed across the $N=8$ Visium samples.

For the evaluation of the spatially-aware clustering with BayesSpace (35) to identify the LC regions in a data-driven manner, precision is defined as the proportion of spots in the selected cluster that are from the true annotated LC region, recall is defined as the proportion of spots in the true annotated LC region that are in the selected cluster, F1 score is defined as the harmonic mean of precision and recall (values ranging from 0 to 1, with 1 representing perfect accuracy), and the adjusted Rand index is defined as the percentage of correct assignments, adjusted for chance (values ranging from 0 for random assignment to 1 for perfect assignment).

For the pseudobulked DE testing, we aggregated the reads within the LC and non-LC regions using the scater package (60), and then used the limma package (61) to calculate empirical Bayes moderated DE tests.

Analysis of snRNA-seq data

This section describes additional details on the computational analyses of the snRNA-seq data that are not included in the main text.

We aligned sequencing reads using the 10x Genomics Cell Ranger software (62) (version 6.1.1, cellranger count, with option `--include-introns`), using the human genome reference transcriptome version GRCh38 2020-A (July 7, 2020) provided by 10x Genomics. We called nuclei (distinguishing barcodes containing nuclei from empty droplets) using Cell Ranger (“filtered” outputs), which recovered 8,979, 3,220, and 10,585 barcodes for donors Br2701, Br6522, and Br8079, respectively. We applied scDblFinder (39) using default parameters to computationally identify and remove doublets, which removed 1,022, 205, and 1,366 barcodes identified as doublets for donors Br2701, Br6522, and Br8079, respectively.

We performed nucleus-level QC processing by defining low-quality nuclei as nuclei with outlier values more than 3 median absolute deviations (MADs) above or below the median sum of UMI counts or the median number of detected genes (33), which did not identify any low-quality nuclei, so all nuclei were retained. We did not use the proportion of mitochondrial reads for QC processing, since we observed a high proportion of mitochondrial reads in nuclei with expression of NE neuron markers (*DBH* and *TH*) (for more details, see **Results** and [Supplementary Figure 10](#)). Therefore, QC filtering on the proportion of mitochondrial reads would risk removing the rare population of NE neuron nuclei of interest. We performed gene-level QC filtering by removing low-expressed genes with less than 30 UMI counts summed across all nuclei. After doublet removal and QC processing, we obtained a total of 7,957, 3,015, and 9,219 nuclei from donors Br2701, Br6522, and Br8079, respectively.

For the unsupervised clustering, we used a two-stage clustering algorithm consisting of high-resolution k -means and graph-based clustering that provides sensitivity to identify rare cell populations (33). For the first round of clustering (results in [Figure 3A-B](#), [Supplementary Figure 13](#)), we used 2,000 clusters for the k -means step, and 10 nearest neighbors and Walktrap clustering for the graph-based step. For the secondary clustering of inhibitory neurons ([Supplementary Figure 16](#)), we used 1,000 clusters for the k -means step, and 10 nearest neighbors and Walktrap clustering for the graph-based step. We did not perform any batch integration prior to clustering, since batch integration algorithms may strongly affect rare populations but these algorithms have not yet been independently evaluated on datasets with rare populations (<1% of cells). We calculated highly variable genes, log-transformed normalized counts (logcounts), and performed dimensionality reduction using the scater and scanr packages (34; 60).

For the DE testing, we performed pairwise DE testing between all neuronal clusters, using the `findMarkers()` function from the `scrn` package (34). We tested for genes with $\log_2$ -fold-changes ($\log_2FC$) significantly greater than 1 ($lfc = 1$, $direction = "up"$) to identify genes with elevated expression in any of the neuronal clusters compared to all other neuronal clusters.

For the spot-level deconvolution using `cell2location` (41), we used the following parameters for human brain data from the Visium platform: `detection_alpha = 20`, `N_cells_per_location = 3`.

Supporting information

supplements/514241_file02.xlsx Supplementary Table 1
 supplements/514241_file03.csv Supplementary Table 2
 supplements/514241_file04.csv Supplementary Table 3
 supplements/514241_file05.csv Supplementary Table 4
 supplements/514241_file06.csv Supplementary Table 5

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Supplementary Tables

Supplementary Table 1: Summary of experimental design, sample information, and donor demographic details. Information includes the types of assays performed, donor demographic details, sample IDs, number of Visium tissue areas per sample, and sequencing summary statistics for each sample. The table is provided as an .xlsx file.

Supplementary Table 2: Differential expression (DE) testing results in pseudobulked Visium SRT data. Columns include gene ID, gene name, mean log-transformed normalized counts ($\log$ counts) in manually annotated LC and non-LC regions (" mean_logcounts_LC " and " $\text{mean_logcounts_nonLC}$ "), $\log_2$ fold change ($\log_2FC$), p-value, false discovery rate (FDR), and columns identifying significant ($FDR < 0.05$ and $FC > 2$) and highly significant ($FDR < 10^{-3}$ and $FC > 3$) genes. The table is provided as a .csv file.

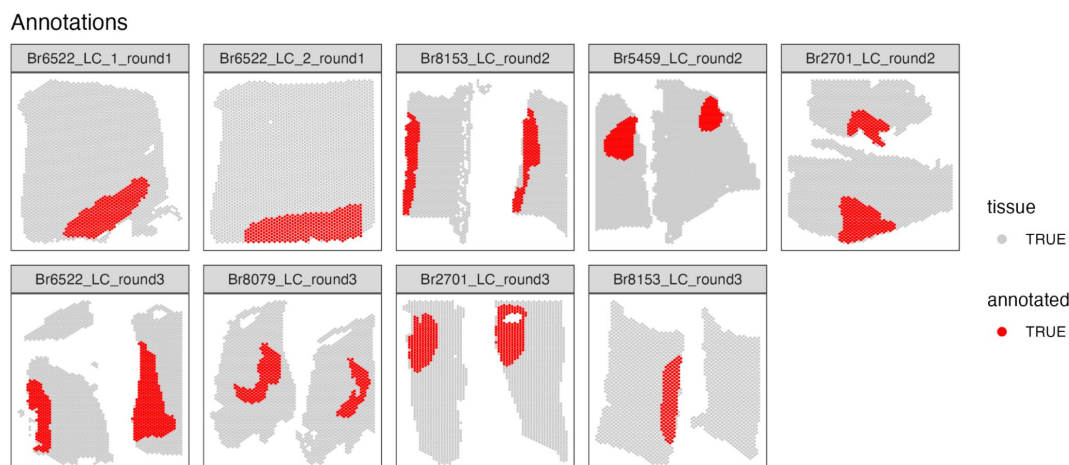
Supplementary Table 3: Spatially variable genes (SVGs) in Visium SRT data. Results for SVGs identified using `nnSVG` in Visium SRT data. Columns include gene ID, gene name, overall rank of SVGs identified in replicated tissue areas (" $\text{replicated_overall_rank}$ ", i.e. top LC-associated SVGs; see **Results**), overall rank of identified SVGs according to average rank across tissue areas (" overall_rank ", i.e. including choroid plexus-associated SVGs from one donor; see **Results**), average rank of identified SVGs across individual tissue areas

(“average_rank”), number of times (tissue areas) identified within top 100 SVGs (“n_withinTop100”), and ranks within each individual tissue area. The table is provided as a .csv file.

Supplementary Table 4: Differential expression (DE) testing results for NE neuron cluster in snRNA-seq data. DE testing results comparing NE neuron cluster against all other neuronal clusters in snRNA-seq data. Columns include gene ID, gene name, sum of UMI counts across all nuclei (“sum_gene”), average log-transformed normalized counts (logcounts) within the NE neuron cluster (“self_average”), average of average logcounts within all other neuronal clusters (“other_average”), combined p-value, false discovery rate (FDR), summary $\log_2$ fold change in the pairwise comparison with the lowest p-value (“summary_logFC”), and column identifying significant (FDR < 0.05 and FC > 2) genes. The table is provided as a .csv file.

Supplementary Table 5: Differential expression (DE) testing results for 5-HT neuron cluster in snRNA-seq data. DE testing results comparing 5-HT neuron cluster against all other neuronal clusters in snRNA-seq data. Columns include gene ID, gene name, sum of UMI counts across all nuclei (“sum_gene”), average log-transformed normalized counts (logcounts) within the 5-HT neuron cluster (“self_average”), average of average logcounts within all other neuronal clusters (“other_average”), combined p-value, false discovery rate (FDR), summary $\log_2$ fold change in the pairwise comparison with the lowest p-value (“summary_logFC”), and column identifying significant (FDR < 0.05 and FC > 2) genes. The table is provided as a .csv file.

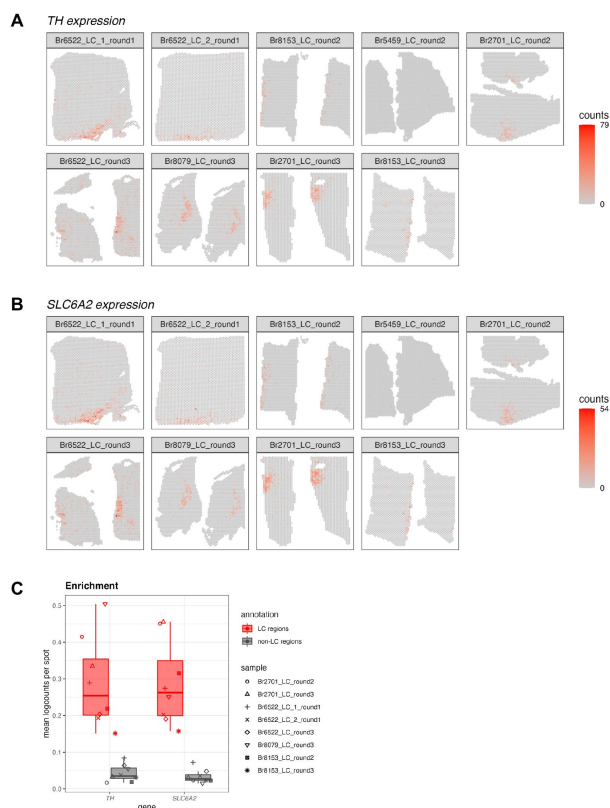
Supplementary Figures



Supplementary Figure 1:

Spot-plot visualizations of manually annotated Visium spots within regions identified as containing LC-NE neurons in SRT data.

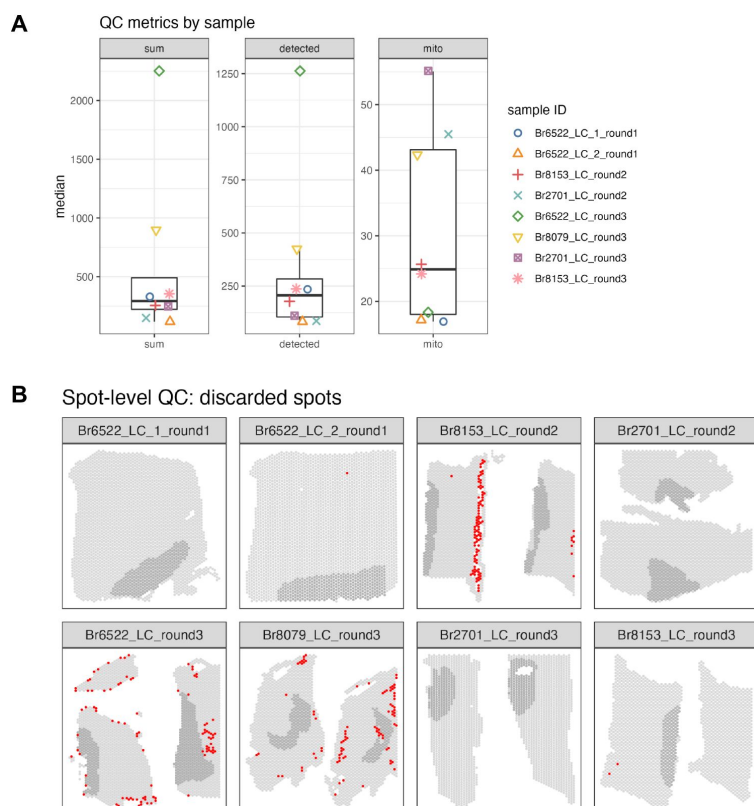
For each of the $N=9$ Visium capture areas (hereafter referred to as samples), the spots were manually annotated as being within the LC regions (red) or within the non-LC regions (gray) based on spots containing NE neurons, which were identified by pigmentation, cell size, and morphology on the H&E stained histology images.



Supplementary Figure 2:

Spatial expression of two NE neuron-specific marker genes in Visium samples for quality control (QC) in SRT data.

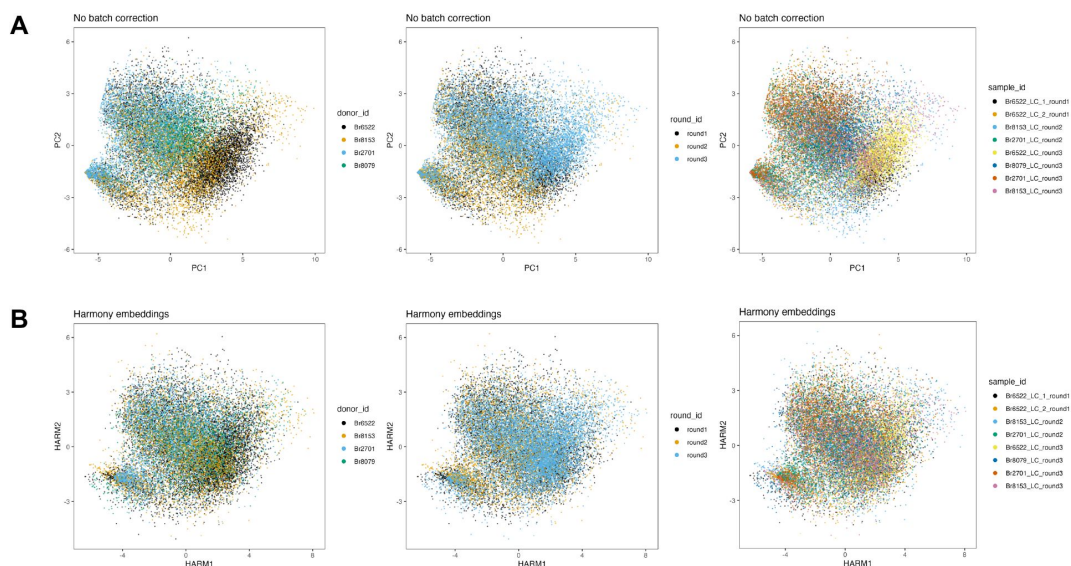
(A-B) Spot-plot visualizations of NE neuron marker gene expression (*TH* and *SLC6A2*, **A** and **B**, respectively) in the $N=9$ Visium samples. Color scale shows UMI counts per spot. One sample (Br5459_LC_round2) did not show clear expression of the NE neuron marker genes. This sample was excluded from subsequent analyses, leaving $N=8$ Visium capture areas (samples) from 4 out of the 5 donors. (**C**) Enrichment of NE neuron marker gene expression (*TH* and *SLC6A2*) within manually annotated LC regions compared to non-LC regions in the $N=8$ Visium samples. Boxplots show values as mean log-transformed normalized counts (logcounts) per spot within each region per sample, with samples represented by shapes.



Supplementary Figure 3:

Spot-level quality control (QC) data visualizations for Visium samples in SRT data.

(A) QC metrics, medians per sample (from left to right: sum of UMI counts per spot, number of detected genes per spot, and proportion of mitochondrial reads per spot). Boxplots show median for each QC metric per sample, with samples represented by shapes. (B) Applying thresholds of 3 median absolute deviations (MADs) to the sum of UMI counts and number of detected genes for each sample identified a total of 287 low-quality spots (red) (1.4% out of 20,667 total spots), which were removed from subsequent analyses. We did not use the proportion of mitochondrial reads for spot-level QC filtering (see **Methods** for more details).

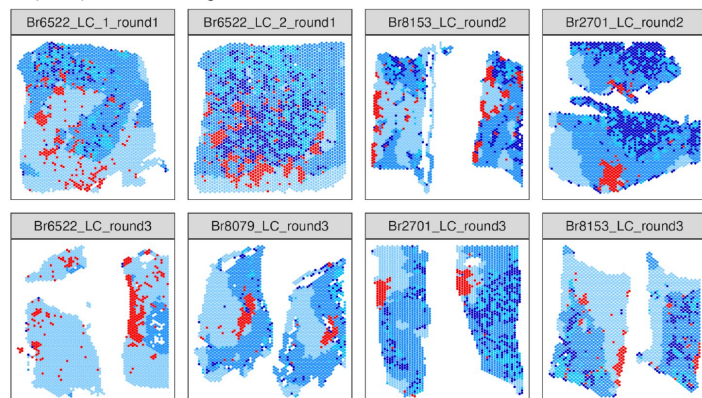


Supplementary Figure 4:

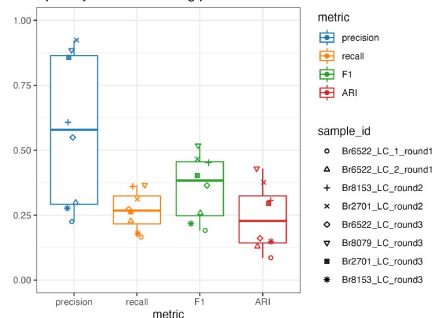
Dimensionality reduction embeddings before and after batch integration across Visium samples in SRT data.

We applied a batch integration tool (Harmony (36)) to remove technical variation in the molecular measurements between the $N=8$ Visium samples from 4 donors. The integrated measurements were subsequently used as the input for spatially-aware clustering using BayesSpace (35). **(A)** Principal component analysis (PCA) (top 2 PCs) calculated on molecular expression measurements, with spots labeled (left to right) by donor ID, round ID, and sample ID, without applying any batch integration. **(B)** Harmony embeddings (top 2 Harmony embedding dimensions) after applying Harmony batch integration on sample IDs, with spots labeled (left to right) by donor ID, round ID, and sample ID, demonstrating that the technical variation has been reduced.

A BayesSpace clustering



B Spatially-aware clustering performance



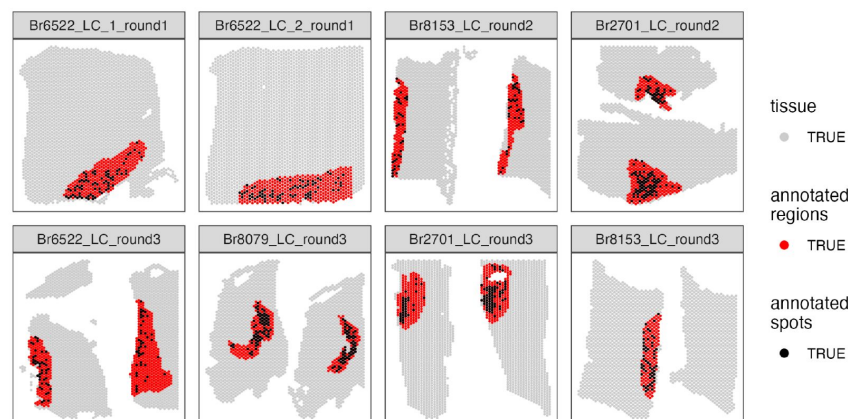
Rand index (ARI) (see **Methods** for definitions).

Supplementary Figure 5:

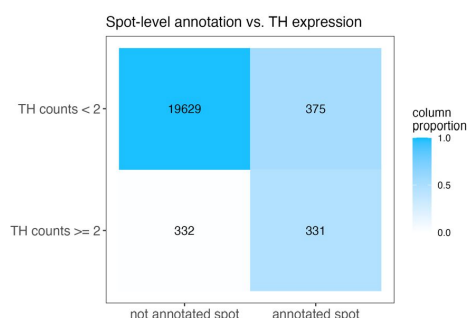
Identifying LC and non-LC regions in a data-driven manner by spatially-aware unsupervised clustering in SRT data.

We applied a spatially-aware unsupervised clustering algorithm (BayesSpace (35)) to investigate whether the LC and non-LC regions in each Visium sample could be annotated in a data-driven manner. **(A)** Using BayesSpace with $k=5$ clusters, we clustered spots from the $N=8$ Visium samples using the Harmony batch-integrated molecular measurements. Cluster 4 (red) corresponds most closely to the manually annotated LC regions. **(B)** BayesSpace clustering performance evaluated in terms of concordance between cluster 4 (red) and the manually annotated LC region in each sample. Clustering performance was evaluated in terms of precision, recall, F1 score, and adjusted

A Annotations



B

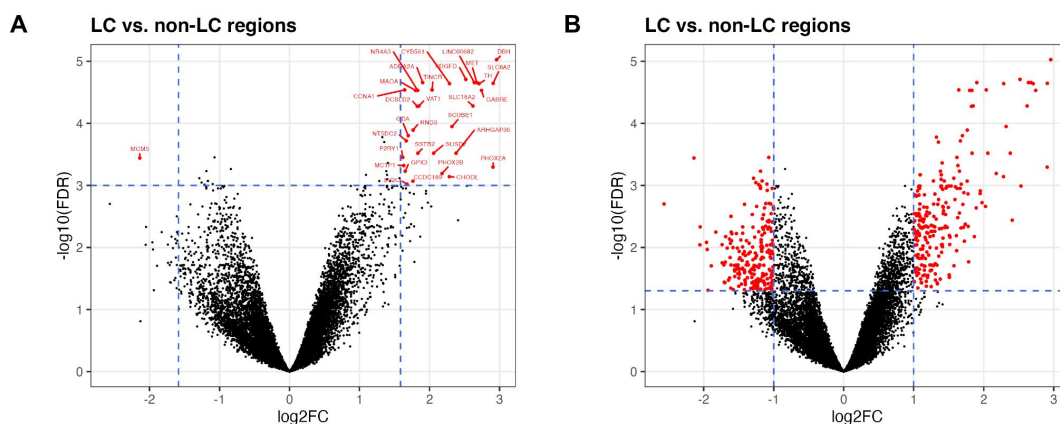


Supplementary Figure 6:

Comparison of spot-level and region-level manual annotations in SRT data.

(A) We manually annotated individual Visium spots (black) overlapping with NE neuron cell bodies within the previously manually annotated LC regions (red), based on pigmentation, cell size, and morphology from the H&E stained histology images, in the $N=8$ Visium samples. (B) We observed relatively low overlap between spots with expression of the NE neuron marker gene *TH* (≥ 2 observed UMI counts per spot) and the set of annotated individual spots. The differences included both false positives (annotated spots that were not *TH+*) and false negatives (*TH+* spots that were not annotated).

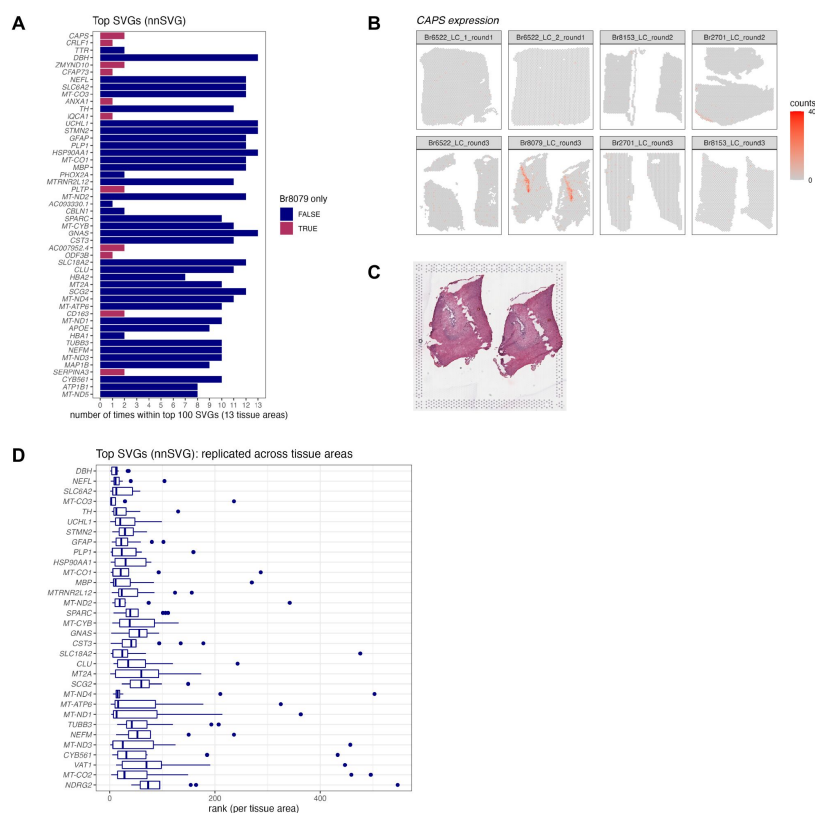
Therefore, we did not use the spot-level annotations for subsequent analyses, and instead used the LC region-level annotations for all further analyses.



Supplementary Figure 7:

Results from differential expression (DE) analysis to identify expressed genes associated with LC regions in SRT data.

We performed DE testing between the manually annotated LC and non-LC regions by pseudobulking spots, defined as aggregating UMI counts from the combined set of spots, within the annotated LC and non-LC regions in each sample. **(A)** Using a false discovery rate (FDR) significance threshold of 10^{-3} and an expression fold-change (FC) threshold of 3 (dashed blue lines), we identified 32 highly significant genes (red points). **(B)** Using standard significance thresholds of $FDR < 0.05$ and expression $FC > 2$, we identified 437 significant genes (red). Vertical axes are on reversed log10 scale, and horizontal axes are on log2 scale. Additional details are provided in **Supplementary Table 2**.

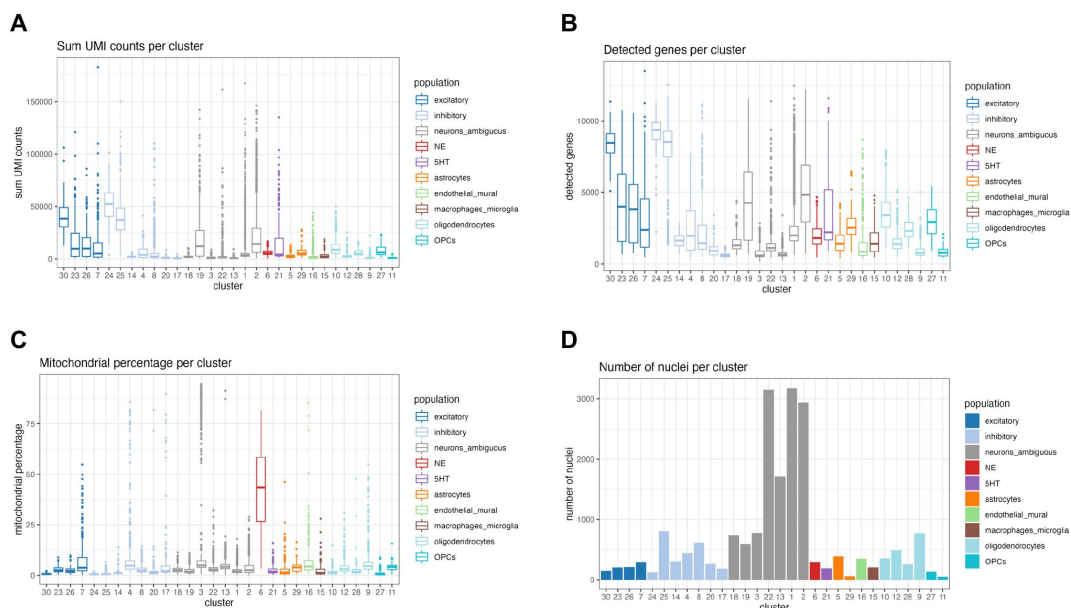


Supplementary Figure 8:

Results from applying nnSVG to identify spatially variable genes (SVGs) in SRT data.

We applied nnSVG (38), a method to identify spatially variable genes (SVGs), in the Visium SRT samples. We ran nnSVG within each contiguous tissue area containing a manually annotated LC region (13 tissue areas in the $N=8$ Visium samples) and calculated an overall ranking of top SVGs by averaging the ranks per gene from each tissue area. **(A)** The top 50 ranked SVGs from this analysis included a subset (11 out of 50) of genes that were highly ranked in samples from only one donor (Br8079, genes highlighted in maroon). We determined that this was due to the inclusion of a section of the choroid plexus adjacent to the LC for this donor. Bars show the number of times (out of 13 tissue areas) each gene was included within the top 100 SVGs. Rows are ordered

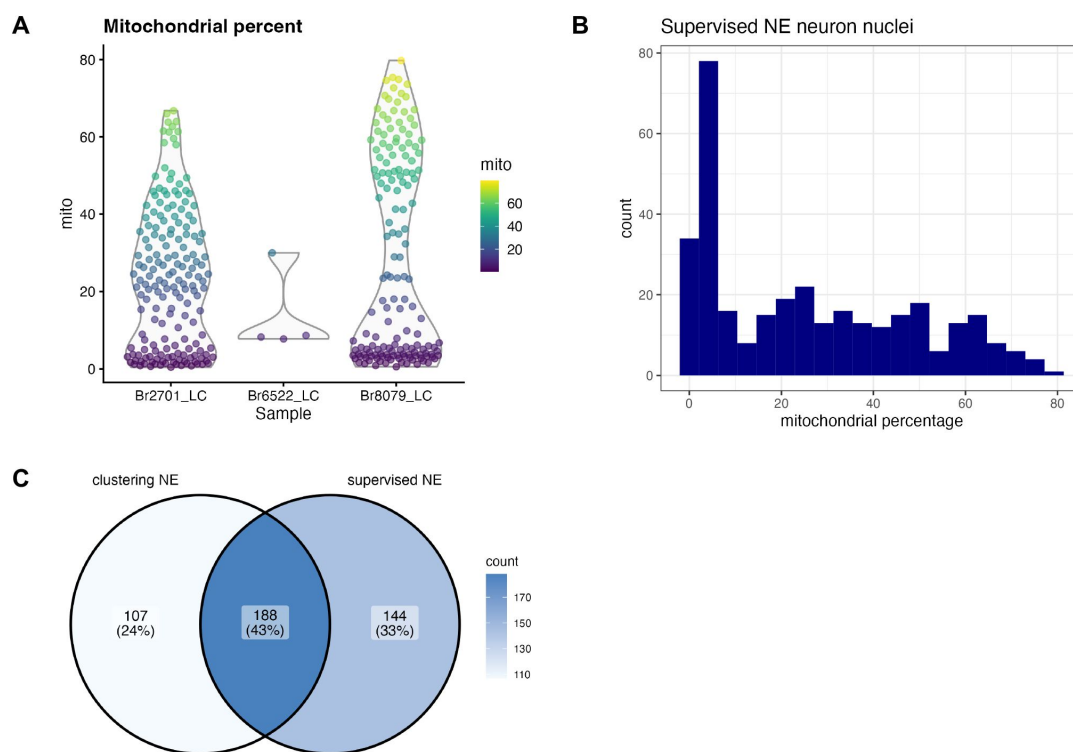
by overall average ranking in descending order. **(B)** Spatial expression of *CAPS*, a choroid plexus marker gene, in the $N=8$ Visium samples. **(C)** Histology image showing the two tissue areas for sample Br8079_LC_round3. **(D)** In order to focus on LC-associated SVGs, we calculated an overall average ranking of SVGs that were each included within the top 100 SVGs in at least 10 out of the 13 tissue areas, which identified 32 highly-ranked, replicated LC-associated SVGs. Boxplots show the ranks in each tissue area. Rows are ordered by the overall average ranking in descending order.



Supplementary Figure 9:

Distribution of nucleus-level quality control (QC) metrics across unsupervised clusters in snRNA-seq data.

(A) Sum of UMI counts per nucleus and cluster, (B) total number of detected genes per nucleus and cluster, (C) percentage of mitochondrial reads per nucleus and cluster, and (D) number of nuclei per cluster. We observed an unexpectedly high percentage of mitochondrial reads in the NE neuron cluster (cluster 6, red, C). Since NE neurons were of particular interest for analysis, we did not remove nuclei with a high percentage of mitochondrial reads during QC filtering.

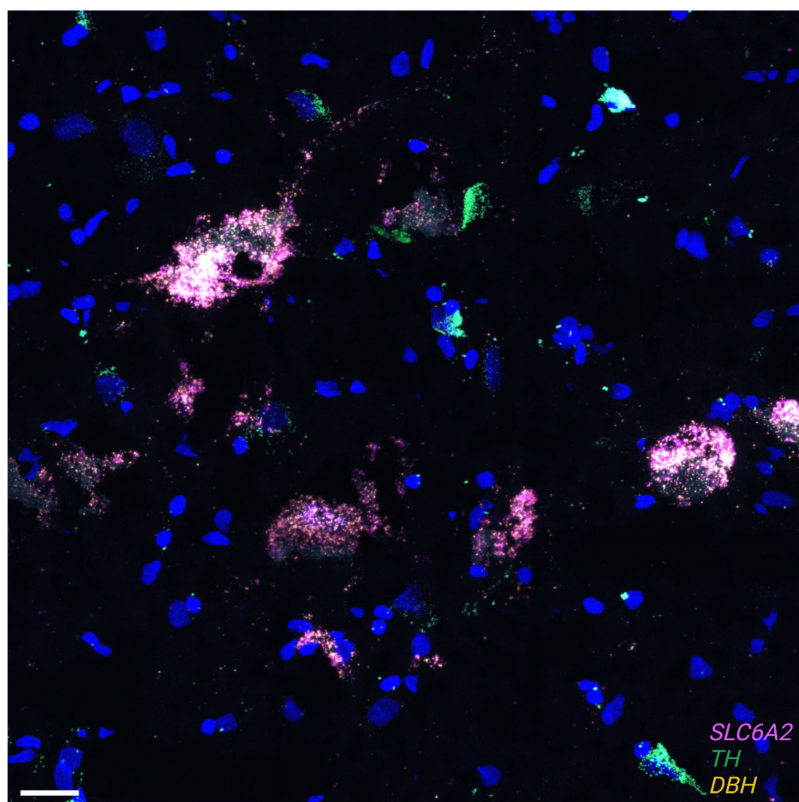


Supplementary Figure 10:

Supervised identification of NE neuron nuclei by thresholding on expression of NE neuron marker genes in snRNA-seq data.

We applied a supervised strategy to identify NE neuron nuclei by simply thresholding on expression of NE neuron marker genes (selecting

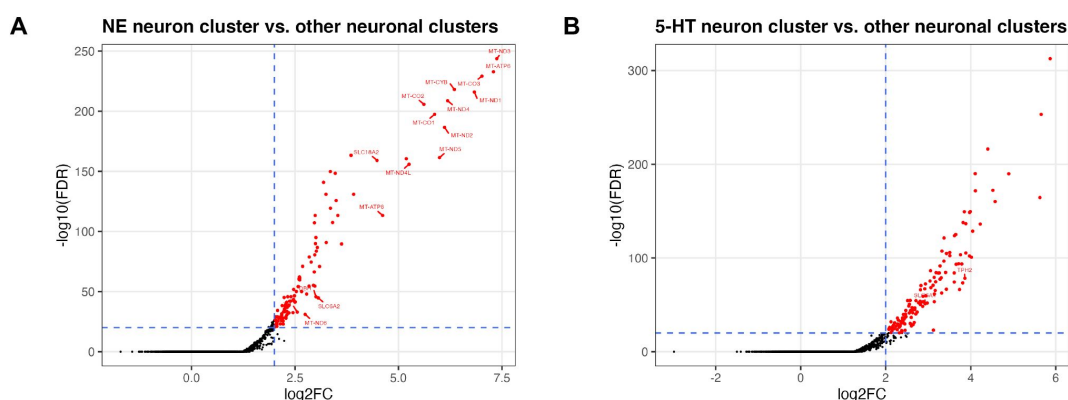
nuclei with ≥ 1 UMI counts of both *DBH* and *TH*). We observed a higher than expected proportion of mitochondrial reads within this set of nuclei, and did not filter on this parameter during QC processing, in order to retain these nuclei. **(A)** Percentage of mitochondrial reads within the supervised set of nuclei by donor (Br2701, Br6522, and Br8079). **(B)** Histogram showing percentage of mitochondrial reads within the supervised set of nuclei across all donors. **(C)** Venn diagram showing overlap between NE neuron cluster identified by unsupervised clustering (left) and NE neuron population identified by supervised thresholding (right). Values display number of nuclei.



Supplementary Figure 11:

Expression of NE neuron marker genes in individual cells using RNAscope and high-magnification confocal imaging.

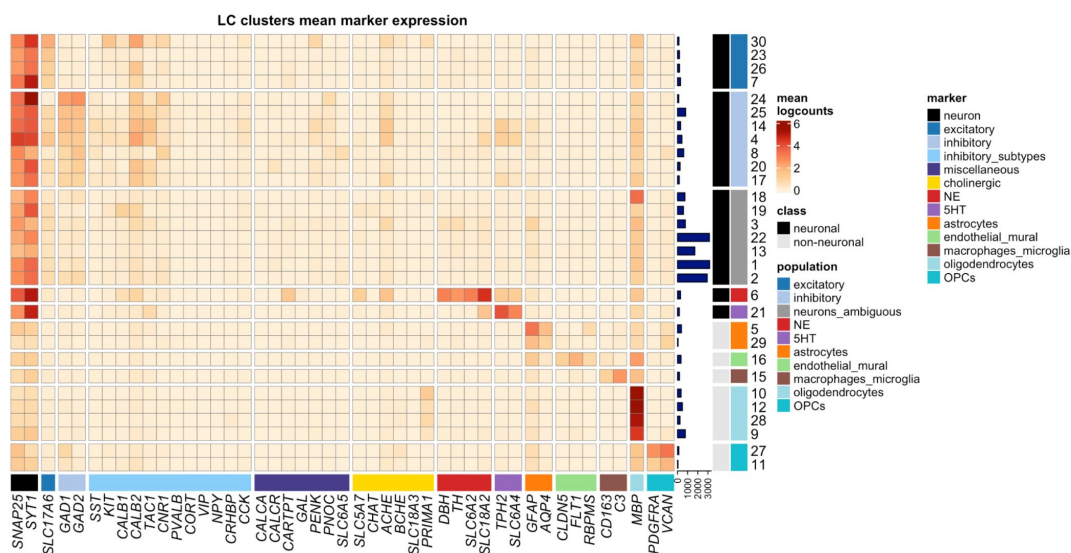
We applied RNAscope (32) and high-magnification confocal imaging to visualize expression of NE neuron marker genes (*DBH* in yellow, *TH* in green, and *SLC6A2* in pink, with white representing all three colors overlapping) and DAPI stain for nuclei (blue) on additional tissue sections from an additional independent donor, Br8689. The figure displays a region from a single tissue section, demonstrating clear co-localization of expression of the three NE neuron marker genes (white points) within individual cells. Scale bar: 20 μm .



Supplementary Figure 12:

DE testing results between neuronal clusters in the LC and surrounding region in snRNA-seq data.

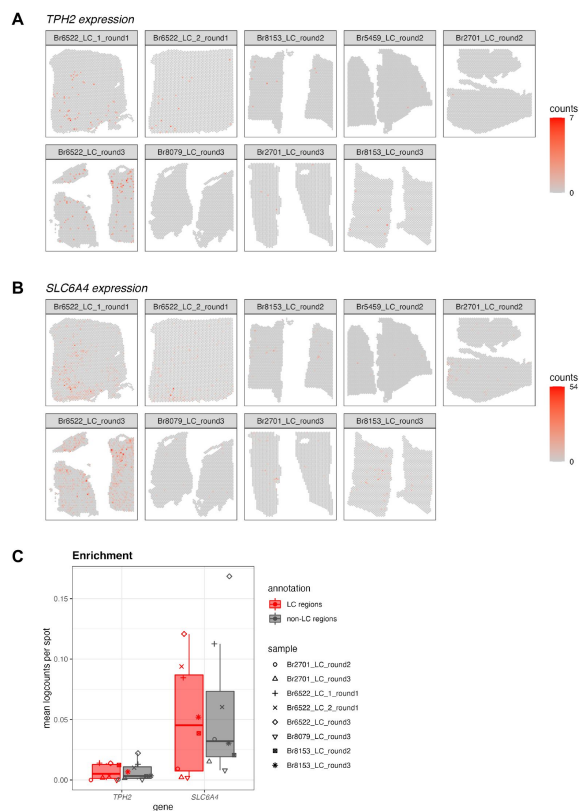
(A) Volcano plot showing 327 statistically significant DE genes ($\text{FDR} < 0.05$ and $\text{FC} > 2$) elevated in expression within the NE neuron cluster compared to all other neuronal clusters captured in this region. The significant DE genes include known NE neuron marker genes (*DBH*, *TH*, *SLC6A2*, and *SLC18A2*) and mitochondrial genes. (B) Volcano plot showing 361 statistically significant DE genes ($\text{FDR} < 0.05$ and $\text{FC} > 2$) elevated in expression within the 5-HT neuron cluster compared to all other neuronal clusters captured in this region. The significant DE genes include known 5-HT neuron marker genes (*TPH2* and *SLC6A4*). Vertical axes are on reversed log10 scale, and horizontal axes are on log2 scale. Additional details are provided in **Supplementary Tables 4, 5**.



Supplementary Figure 13:

Unsupervised clustering results showing additional inhibitory neuronal, miscellaneous, and cholinergic marker genes in snRNA-seq data.

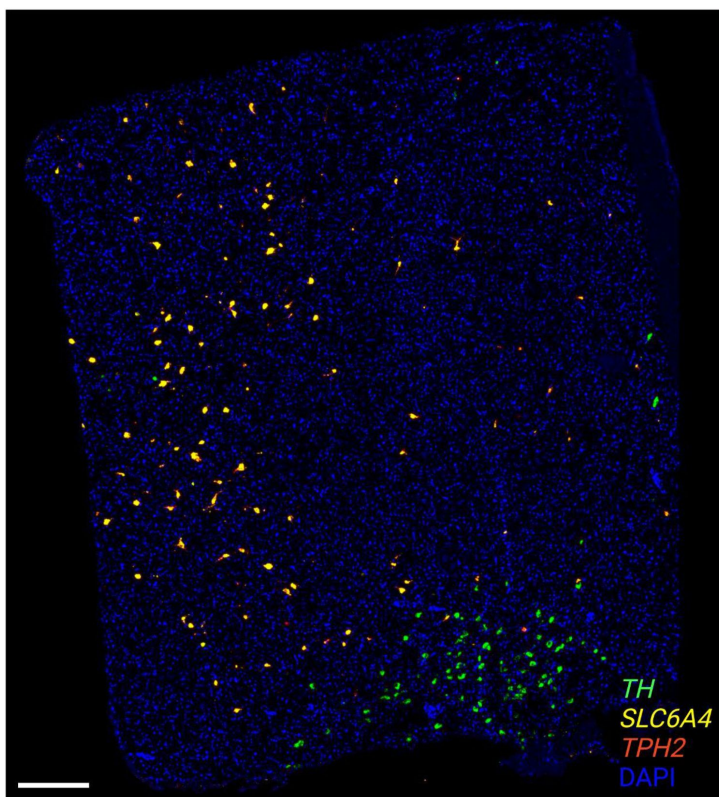
Extended form of heatmap displayed in [Figure 3A](#), showing additional inhibitory neuronal marker genes (light blue), miscellaneous marker genes including neuropeptides and receptors included for comparison with (27) (dark blue-purple), and cholinergic marker genes (yellow). We observed diversity in expression of inhibitory neuronal marker genes across inhibitory neuronal subpopulations (additional results in [Supplementary Figure 16](#)), and we observed expression of cholinergic marker genes within NE neurons (additional results in [Supplementary Figure 18](#)).



Supplementary Figure 14:

Spatial expression and enrichment analysis of 5-HT neuron marker genes in Visium SRT samples.

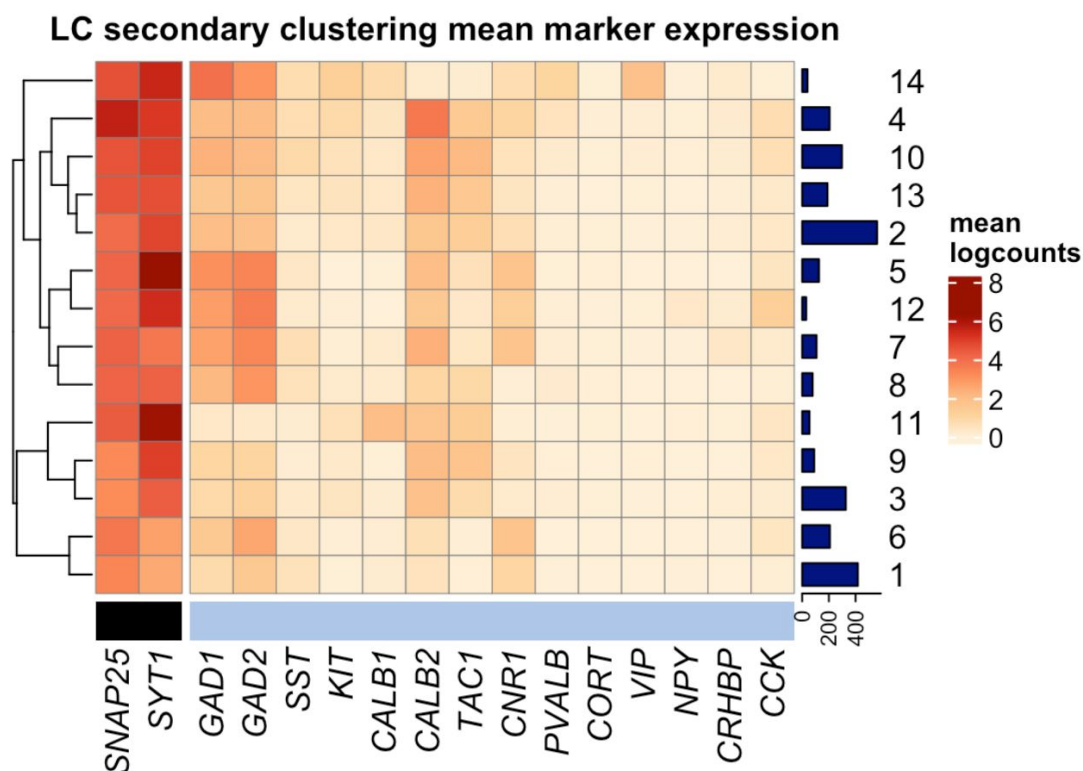
(A-B) We visualized the spatial expression of 5-HT (5-hydroxytryptamine or serotonin) neuron marker genes (*TPH2* and *SLC6A4*) in the $N=9$ initial Visium SRT samples, which showed that the population of 5-HT neurons was distributed across both the LC and non-LC regions. (C) Enrichment of 5-HT neuron marker gene expression (*TPH2* and *SLC6A4*) within manually annotated LC regions compared to non-LC regions in the $N=8$ Visium SRT samples. Boxplots show values as mean log-transformed normalized counts (logcounts) per spot within each region per sample, with samples represented by shapes.



Supplementary Figure 15:

Expression of NE neuron and 5-HT neuron marker genes using RNAscope.

We applied RNAscope (32) to visualize expression of an NE neuron marker gene (*TH*) as well as 5-HT neuron marker genes (*TPH2* and *SLC6A4*) within an additional tissue section from donor Br6522, demonstrating that the NE and 5-HT marker genes were expressed within distinct cells and that the NE and 5-HT neuron populations were not localized within the same regions. Scale bar: 500 μ m.

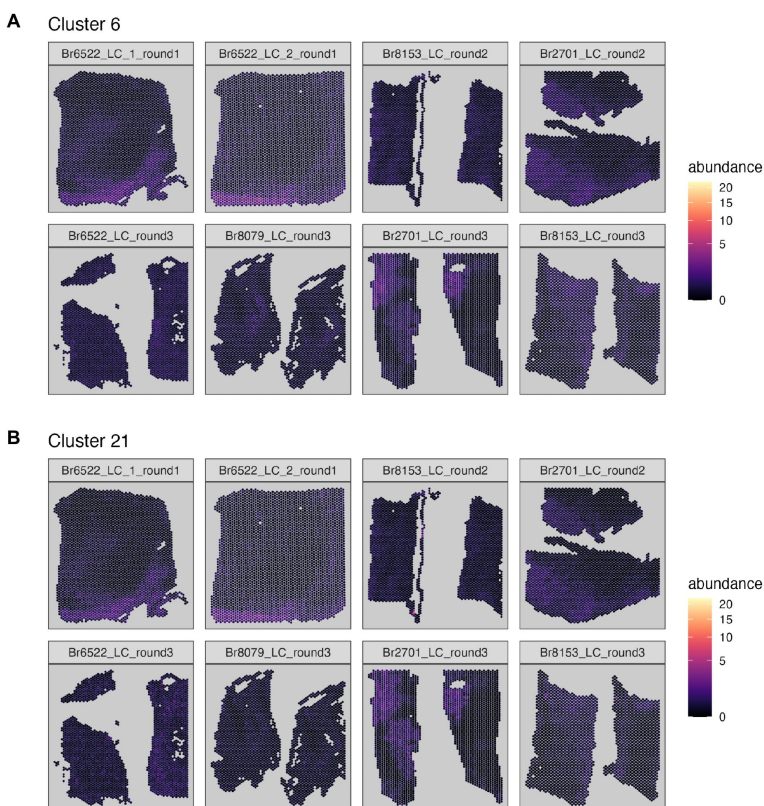


**Supplementary
Figure 16:**

**Inhibitory
neuronal
subpopulations
identified by
secondary
unsupervised
clustering on
inhibitory
neurons in
snRNA-seq
data.**

We applied a secondary round of unsupervised clustering to the inhibitory neuron nuclei identified in the first round of clustering. This identified 14

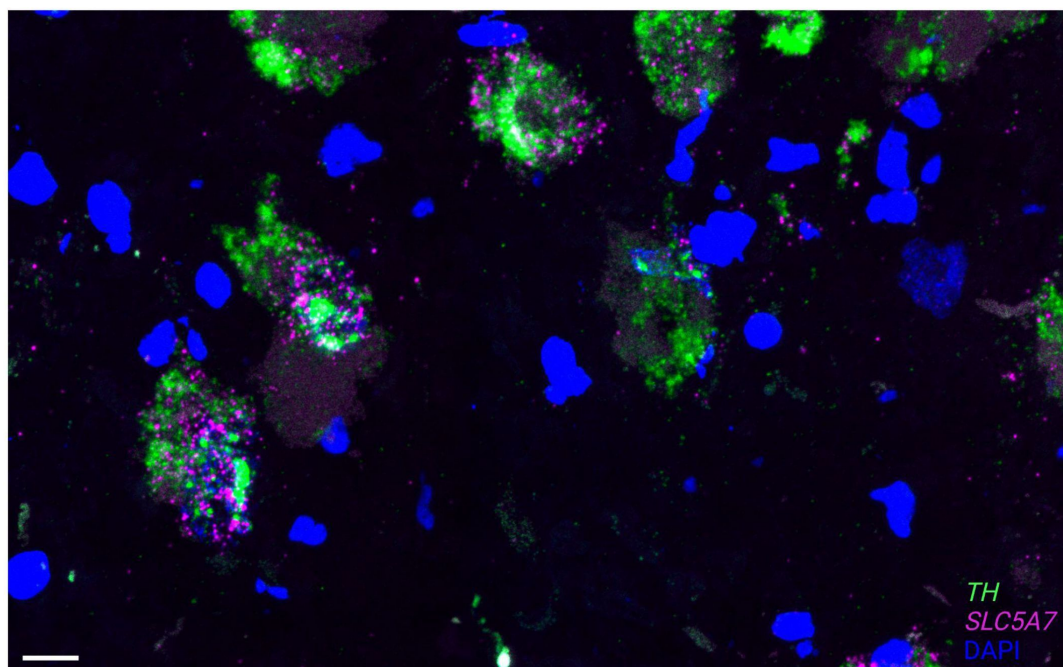
clusters representing inhibitory neuronal subpopulations. Heatmap displays expression of neuronal marker genes (black) and inhibitory neuron marker genes (light blue) (columns) in the 14 clusters (rows). Cluster IDs are shown in labels on the right, and numbers of nuclei per cluster are shown in horizontal bars on the right. Heatmap values represent mean log-transformed normalized counts (logcounts) per cluster.



Supplementary Figure 17:

Spot-level deconvolution to map the spatial coordinates of snRNA-seq populations within the Visium SRT samples.

We applied a spot-level deconvolution algorithm (`cell2location` (41)) to integrate the snRNA-seq and SRT data by estimating the cell abundance of the snRNA-seq populations, which are used as reference populations, at each spatial location (spot) in the Visium SRT samples. This correctly mapped **(A)** NE neurons (cluster 6) and **(B)** 5-HT neurons (cluster 21) to the spatial regions where these populations were previously identified based on expression of marker genes (**Supplementary Figures 2 and 14**). However, the estimated absolute cell abundance of these populations per spot was higher than expected.



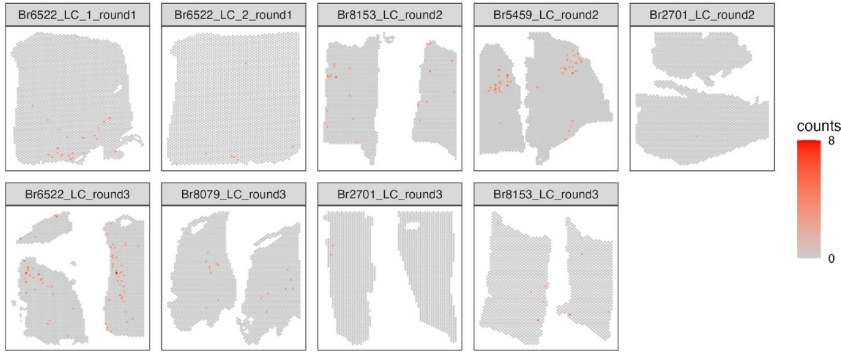
Supplementary Figure 18:

High-resolution images demonstrating co-expression of cholinergic marker gene within NE neurons.

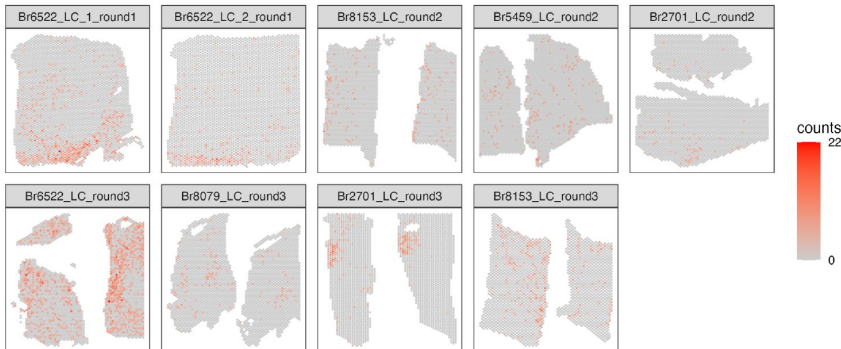
We applied RNAscope (32) and high-resolution imaging at 63x magnification to visualize expression of *SLC5A7* (cholinergic marker gene

encoding the high affinity choline transporter, shown in pink) and *TH* (NE neuron marker gene encoding tyrosine hydroxylase, shown in green), and DAPI stain for nuclei (blue), in a tissue section from donor Br8079. This confirmed co-expression of *SLC5A7* and *TH* within individual cells. Scale bar: 25 μ m.

A *SLC5A7* expression



B *ACHE* expression



Supplementary Figure 19:

Spatial expression of cholinergic marker genes in Visium SRT samples.

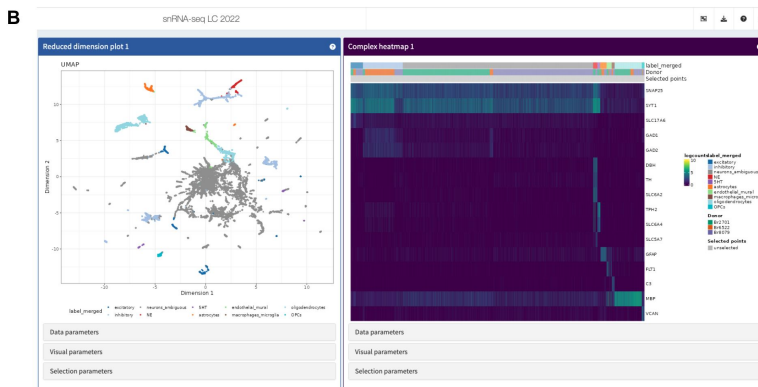
We visualized the spatial expression of cholinergic marker genes **(A)***SLC5A7* and **(B)***ACHE* in the $N=9$ initial Visium SRT samples, which showed that these genes were expressed both within and outside the annotated LC regions. Color scale shows UMI counts per spot.



Supplementary Figure 20:

Interactive web-accessible data resources.

All datasets described in this manuscript are freely accessible via interactive web apps and downloadable R/Bioconductor objects (see [Table 1](#) for details). **(A)** Screenshot of Shiny (42) web app providing interactive access to Visium SRT data. **(B)** Screenshot of iSEE (43) web app providing interactive access to snRNA-seq data.



Back Matter

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Formal Analysis: LMW, MNT

Funding acquisition: LMW, KRM, KM, SCH

Investigation: HRD, MNT, SHK, AS, KDM

Methodology: HRD, SHK, MNT, KRM

Project Administration: KRM, KM, SCH

Resources: RB, JEK, TMH

Software: LMW, HRD, LCT

Supervision: SCP, KRM, KM, SCH

Validation: HRD

Visualization: LMW, HRD, MT

Writing – original draft: LMW, HRD, MNT, KM, SCH

Writing – review & editing: LMW, HRD, MNT, KRM, KM, SCH

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Competing Interests

The authors declare that they have no competing interests. Matthew N. Tran (MNT) is now a full-time employee at 23andMe and whose current work is unrelated to the contents of this manuscript. His contributions to this manuscript were made while previously employed at the Lieber Institute for Brain Development (LIBD).

Code Availability

Code scripts to reproduce all analyses and figures in this manuscript, including the computational analysis workflows for the snRNA-seq and SRT data, are available from

GitHub at <https://github.com/lmweber/locus-c>. We used R version 4.2 and Bioconductor version 3.15 packages for analyses in R.

Data Availability

The datasets described in this manuscript are freely accessible in web-based formats from https://libd.shinyapps.io/locus-c_Visium/ (Shiny (42) app containing Visium SRT data) and https://libd.shinyapps.io/locus-c_snRNA-seq/ (iSEE (43) app containing snRNA-seq data), and in R/Bioconductor formats from <https://bioconductor.org/packages/WeberDivechaLCdata> (R/Bioconductor ExperimentHub data package containing Visium SRT data in SpatialExperiment (44) format and snRNA-seq data in SingleCellExperiment (33) format). The R/Bioconductor data package is available in Bioconductor release version 3.16 from Nov 2, 2022 onwards. Instructions to install and access the R/Bioconductor data package are also available from GitHub at <https://github.com/lmweber/WeberDivechaLCdata>. Raw data including FASTQ sequence data files and raw image files will be made available from a Globus endpoint.

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Reviewer #1 (Public Review):

Weber et al. collect locus coeruleus (LC) tissue blocks from 5 neurotypical European men, dissect the dorsal pons around the LC and prepare 2-3 tissue sections from each donor on a slide for 10X spatial transcriptomics. From three of these donors, they also prepared an additional section for 10x single nucleus sequencing. Overall, the results validate well-known marker genes for the LC (e.g. DBH, TH, SLC6A2), and generate a useful resource that lists genes which are enriched in LC neurons in humans, with either of these two techniques. A comparison with publicly available mouse and rat datasets identifies genes that show reliable LC-enrichment across species. Their analyses also support recent rodent studies that have identified subgroups of interneurons in the region surrounding the LC, which show enrichment for different neuropeptides. In addition, the authors claim that some LC neurons co-express cholinergic markers, and that a population of serotonin (5-HT) neurons is located within or near the LC. These last two claims must be taken with great caution, as several technological limitations restrict the interpretation of these results. Overall, there is limited integration between the spatial and single-nucleus sequencing, thus the data does not yet provide a conclusive list of bona fide LC-specific genes. The authors transparently present limitations of their work in the discussion, but some points discussed below warrant further attention.

Specific comments:

1. snRNAseq:

a. Major concerns with the snRNAseq dataset are A) the low recovery rate of putative LC-neurons in the snRNAseq dataset, B) the fact that the LC neuron cluster is contaminated with mitochondrial RNA, and C) that a large fraction of the nuclei cannot be assigned to a clear cell type (presumably due to contamination or damaged nuclei). The authors chose to enrich for neurons using NeuN antibody staining and FACS. But it is difficult to assess the efficacy of this enrichment without images of the nuclear suspension obtained before FACS, and of the FACS results. As this field is in its infancy, more detail on preliminary experiments would help the reader to understand why the authors processed the tissue the way they did. It would be nice to know whether omitting the FACS procedure might in fact result in higher relative recovery of LC-neurons, or if the authors tried this and discovered other technical issues that prompted them to use FACS.

b. It is unclear what percentage of cells that make up each cluster.

c. The number of subjects used in each analysis was not always clear. Only 3 subjects were used for snRNAseq, and one of them only yielded 4 LC-nuclei. This means the results are essentially based on $n=2$. The authors report these numbers in the corresponding section, but the first sentence of the results section (and Figure 1C specifically!) create the impression that $n=5$ for all analyses. Even for spatial transcriptomics, if I understood it correctly, 1 sample had to be excluded ($n=4$).

2. Spatial transcriptomics:

a. It is not clear to me what the spatial transcriptomics provides beyond what can be shown with snRNAseq, nor how these two sets of results compare to each other. It would be more intuitive to start the story with snRNAseq and then try to provide spatial detail using spatial transcriptomics. The LC is not a homogeneous structure but can be divided into ensembles based on projection specificity. Spatial transcriptomics could - in theory - offer much-needed insights into the spatial variation of mRNA profiles across different ensembles, or as a first step across the spatial (rostral/caudal, ventral/dorsal) extent of the LC. The current analyses, however, cannot address this issue, as the orientation of the LC cannot be deduced from the slices analyzed.

b. Unfortunately, spatial transcriptomics itself is plagued by sampling variability to a point where the RNAscope analyses the authors performed prove more powerful in addressing direct questions about gene expression patterns. Given that the authors compare their results to published datasets from rodent studies, it is surprising that a direct comparison of genes identified with spatial transcriptomics vs snRNAseq is lacking (unless this reviewer missed this comparison). Supplementary Figure 17 seems to be a first step in that direction, but this is not a gene-by-gene comparison of which analysis identifies which LC-enriched genes. Such an analysis should not compare numbers of enriched genes using artificial cutoffs for significance/fold-change, but rather use correlations to get a feeling for which genes appear to be enriched in the LC using both methods. This would result in one list of genes that can serve as a reference point for future work.

c. Maybe the spatial transcriptomics could be useful to look at the peri-LC region, which has generated some excitement in rodent work recently, but remains largely unexplored in humans.

3. The comparison of snRNAseq data to published literature is laudable. Although the authors mention considerable methodological differences between the chosen rodent work and their own analyses, this needs to be further explained. The mouse dataset uses TRAPseq, which looks at translating mRNAs associated with ribosomes, very different from the nuclear RNA pool analyzed in the current work. The rat dataset used single-cell LC laser microdissection followed by microarray analyses, leading to major technical differences in terms of tissue processing and downstream analyses. The authors mention and reference a recent 10x mouse LC dataset (Luskin et al, 2022), however they only pick some neuropeptides from this study for their analysis of interneuron subtypes (Figure S13). Although this is a very interesting part of the manuscript, a more in-depth analysis of these two datasets would be very useful. It would likely allow for a better comparison between mouse and human, given that the technical approach is more similar (albeit without FACS), and Luskin et al have indicated that they are willing to share their data.

4. Statements in the manuscript about the unexpected identification of a 5-HT (serotonin) cell-cluster seem somewhat contradictory. Figure S14 suggests that 5-HT markers are expressed in the LC-regions just as much as anywhere else, but the RNAscope image in Figure S15 suggests spatial separation between these two

populations. And Figure S17 again suggests almost perfect overlap between the LC and 5HT clusters. Maybe I misunderstood, in which case the authors should better clarify/explain these results.

Reviewer #2 (Public Review):

The data generated for this paper provides an important resource for the neuroscience community. The locus coeruleus (LC) is the known seed of noradrenergic cells in the brain. Due to its location and size, it remains scarcely profiled in humans. Despite the physically minute structure containing these cells, its impact is wide-reaching due to the known neuromodulatory function of norepinephrine (NE) in processes like attention and mood. As such, profiling NE cells has important implications for most neurological and neuropsychiatric disorders. This paper generates transcriptomic profiles that are not only cell-specific but which also maintain their spatial context, providing the field with a map for the cells within the region.

Strengths:

Using spatial transcriptomics in a morphologically distinct region is a very attractive way to generate a map. Overlaying macroscopic information, i.e. a region with greater pigmentation, with its corresponding molecular profile in an unbiased manner is an extremely powerful way to understand the specific cellular and molecular composition of that brain structure.

The technologies were used with an astute awareness of their limitations, as such, multiple technologies were leveraged to paint a more complete and resolved picture of the cellular composition of the region. For example, the lack of resolution in the spatial transcriptomic platform was compensated by complementary snRNA-seq and single molecule FISH.

This work has been made publicly available and accessible through a user-friendly application such that any interested researcher can investigate the level of expression of their gene of interest within this region.

Two important implications from this work are 1) the potential that the gene regulatory profiles of these cells are only partially conserved across species, humans, and rodents, and 2) that there may be other neuromodulatory cell types within the region that were otherwise not previously localized to the LC

Weaknesses:

Given that the markers used to identify cells are not as specific as they need to be to definitively qualify the desired cell type, the results may be over-interpreted. Specifically, TH is the primary marker used to qualify cells as noradrenergic, however, TH catalyzes the synthesis of L-DOPA, a precursor to dopamine, which in turn is a precursor for epinephrine and norepinephrine suggesting some of the cells in the region may be dopaminergic and not NE cells. Indeed, there are publications to support the presence of dopaminergic cells in the LC (see Kempadoo et al. 2016, Takeuchi et al., 2016, Devoto et al. 2005). This discrepancy is further highlighted by the apparent lack of overlap per given Visium spots with TH, SCL6A2, or DBH. While the single-nucleus FISH confirms that some of the cells in the region are noradrenergic, others very possibly represent a different catecholamine. As such it is suggested that the nomenclature for the cells be reconsidered.

The authors are unable to successfully implement unsupervised clustering with the spatial data, this greatly reduces the impact of the spatial technology as it implies that the transcriptomic data generated in the study did not have enough resolution to identify individual cell types.

The sample contribution to the results is highly unbalanced, which consequently, may result in ungeneralizable findings in terms of regional cellular composition, limiting the usefulness of the publicly available data.

This study aimed to deeply profile the LC in humans and provide a resource to the community. The combination of data types (snRNA-seq, SRT, smFISH) does in fact represent this resource for the community. However, due to the limitations, of which, some were described in the manuscript, we should be cautious in the use of the data for secondary analysis. For example, some of the cellular annotations may lack precision, the cellular composition also may not reflect the general population, and the presence of unexpected cell types may represent the accidental inclusion of adjacent regions, in this case, serotonergic cells from the Raphe nucleus.

Nonetheless having a well-developed app to query and visualize these data will be an enormous asset to the community especially given the lack of information regarding the region in general.

Reviewer #3 (Public Review):

In this study, the authors present the first comprehensive transcriptome map of the human locus coeruleus using two independent but complementary approaches, spatial transcriptomics and single nucleus RNA sequencing. Several canonical features of locus coeruleus neurons that have been described in rodents were conserved, but potentially important species differences were also identified. This work lays the foundation for future descriptive and experimental approaches to understand the contribution of the locus coeruleus to healthy brain function and disease.

This study has many strengths. It is the first reported comprehensive map of the human LC transcriptome, and uses two independent but complementary approaches (spatial transcriptomics and snRNA-seq). Some of the key findings confirmed what has been described in the rodent LC, as well as some intriguing potential genes and modules identified that may be unique to humans and have the potential to explain LC-related disease states. The main limitations of the study were acknowledged by the authors and include the spatial resolution probably not being at the single cell level and the relatively small number of samples (and questionable quality) for the snRNA-seq data. Overall, the strengths greatly outweigh the limitations. This dataset will be a valuable resource for the neuroscience community, both in terms of methodology development and results that will no doubt enable important comparisons and follow-up studies.

Major comments:

Overall, the discovery of some cells in the LC region that express serotonergic markers is intriguing. However, no evidence is presented that these neurons actually produce 5-HT.

Concerning the snRNA-seq experiments, it is unclear why only 3 of the 5 donors were used, particularly given the low number of LC-NE nuclear transcriptomes obtained, why those 3 were chosen, and how many 100 μ m sections were used from each donor. It is also unclear if the 295 nuclei obtained truly representative of the LC population or whether they are just the most "resilient" LC nuclei that survive the process.

The LC displays rostral/caudal and dorsal/ventral differences, including where they project, which functions they regulate, and which parts are vulnerable in neurodegenerative disease (e.g. Loughlin et al., *Neuroscience* 18:291-306, 1986; Dahl et al., *Nat Hum Behav* 3:1203-14, 2019; Beardmore et al., *J Alzheimer's Dis* 83:5-22, 2021; Gilvesy et al., *Acta Neuropathol* 144:651-76, 2022; Madelung et al., *Mov Disord* 37:479-89, 2022). It was not clear which part(s) of the LC was captured for the SRT and snRNAseq experiments.

The authors mention that in other human SRT studies, there are typically between 1-10 cells per expression spot. I imagine that this depends heavily on the part of the brain being studied and neuronal density, but it was unclear how many LC cells were contained in each expression spot.

Regarding comparison of human LC-associated genes with rat or mouse LC-associated genes (Fig. 2D-F), the authors speculate that the modest degree of overlap may be due to species differences between rodents and human and/or methodological differences (SRT vs microarray vs TRAP). Was there greater overlap between mouse and rat than between mouse/rat and human? If so, that is evidence for the former. If not, that is evidence for the latter. Also would be useful for more in-depth comparison with snRNA-seq data from mouse LC: <https://www.biorxiv.org/content/10.1101/2022.06.30.498327v1>.

The finding of ACHE expression in LC neurons is intriguing, especially in light of work from Susan Greenfield suggesting that ACHE has functions independent of ACH metabolism that contributes to cellular vulnerability in neurodegenerative disease.

High mitochondrial reads from snRNA-seq can indicate lower quality. It was not clear why, given the mitochondrial read count, the authors are confident in the snRNA-seq data from presumptive LC-NE neurons.

Author Response:

eLife assessment

This is a valuable initial study of cell type and spatially resolved gene expression in and around the locus coeruleus, the primary source of the neuromodulator norepinephrine in the human brain. The data are generated with cutting-edge techniques, and the work lays the foundation for future descriptive and experimental approaches to understand the contribution of the locus coeruleus to healthy brain function and disease. However, due to small sample size and the need for additional confirmatory data, the data only incompletely support the main conclusions presented here. With the strengthening of the analyses, this paper, and the associated web application, will be of great interest to neuroscientists working on arousal-based behaviors and neurological and neuropsychiatric phenotypes.

Thank you for the assessment and comments. Overall, the majority of the issues raised by the reviewers relate either directly or indirectly to limitations of the sample size that precluded further optimization of protocols and expansion of the dataset. We fully acknowledge the limited sample size in this dataset and aim to be transparent about the limitations of the study. This is the first report of snRNA-seq and spatially-resolved transcriptomics in the human locus coeruleus (LC). The LC is a very small nucleus, located deep within the brainstem, which is extremely challenging to study due to its small size, difficult to access location, and the very small number of norepinephrine (NE) neurons located within the nucleus, which were of prime interest for this study. We note that this study represents our initial attempt to molecularly and spatially characterize cell types within the human LC. We note that we did not have significant, established funding from extramural sources dedicated to this study, and tissue resources for the LC are difficult to ascertain, contributing to the small sample size in this initial study. We acknowledge that there are limitations in sample size as well as data quality. Findings from this study will be used to inform, improve, and optimize future and ongoing experimental design, as well as technical and analytical workflows for larger-scale studies. As brought up by one of the reviewers, this field is still in its infancy -- pilot experimentation in new brain regions is labor-intensive and these sequencing approaches remain costly. Moreover, due to the small size and difficulties in dissecting, tissue resources from the human brain in this area

are a highly limited resource. Hence, notwithstanding limitations, in our view it is important to release the data for community access at this time. Specific responses to the reviewers' comments are provided point-by-point in the following sections.

Reviewer #1 (Public Review):

Weber et al. collect locus coeruleus (LC) tissue blocks from 5 neurotypical European men, dissect the dorsal pons around the LC and prepare 2-3 tissue sections from each donor on a slide for 10X spatial transcriptomics. [...] The authors transparently present limitations of their work in the discussion, but some points discussed below warrant further attention.

Specific comments:

1. snRNAseq:

a. Major concerns with the snRNAseq dataset are A) the low recovery rate of putative LC-neurons in the snRNAseq dataset, B) the fact that the LC neuron cluster is contaminated with mitochondrial RNA, and C) that a large fraction of the nuclei cannot be assigned to a clear cell type (presumably due to contamination or damaged nuclei). The authors chose to enrich for neurons using NeuN antibody staining and FACS. But it is difficult to assess the efficacy of this enrichment without images of the nuclear suspension obtained before FACS, and of the FACS results. As this field is in its infancy, more detail on preliminary experiments would help the reader to understand why the authors processed the tissue the way they did. It would be nice to know whether omitting the FACS procedure might in fact result in higher relative recovery of LC-neurons, or if the authors tried this and discovered other technical issues that prompted them to use FACS.

Thank you for these comments. We agree these are valid concerns in assessing the data quality and validity of the findings from the snRNA-seq dataset. We will respond to these concerns here to the best of our ability, but in some cases, we do not have definitive answers since comparison data are not yet available for this region. In particular, we were limited in resources for this initial study -- some of the results of the study and issues that we identified in attempting to molecularly profile cells in the human LC were surprising to us, and we intend to generate additional samples and troubleshoot these issues to improve data quality and increase recovery in future work. However, these experiments are (i) expensive, (ii) time- and labor-intensive, and (iii) the tissue for this region is limited and difficult to ascertain. Given the extremely small size of the LC, the tissue resource is quickly depleted. For this study, we had fixed resources and made best-guess decisions on how to proceed with the experimental design, based on our experience with snRNA-seq in other human brain regions (Tran and Maynard et al. 2021). However, the LC is a unique region, and our experiences with this dataset will guide us to make technical adjustments in future studies. Due to the limitations in the tissue resources and the lack of data currently available to the community, we wanted to share these results immediately while acknowledging the limitations of the study as we work to increase our resource availability to expand molecular and spatial profiling studies in this region of the human brain.

Regarding the reviewer's concern that our choice to use FACS to enrich for neurons could have potentially led to more damage and contributed to the low recovery rate of LC-NE neurons and the mitochondrial contamination -- we do not have a definitive answer to this question, since we did not perform a direct comparison with non-sorted data. As noted above, our limited tissue resource dictated that we could not do both. We made the decision to enrich for neurons based on our previous experience with identifying relatively rare populations in other brain regions (e.g. nucleus accumbens and amygdala; Tran and Maynard et al. 2021). Based on this previous work, our rationale was that without neuronal enrichment, we could potentially miss the LC-NE population, given the relative scarcity of this neuronal population. The low recovery rate and relatively lower quality / contamination issues may be due to technical issues that lead to LC-NE neurons being more susceptible to

damage during nuclear preparation and sorting. We agree that directly comparing to data prepared without NeuN labeling and sorting is reasonable, as the additional perturbations may indeed contribute to cell damage. As mentioned in the discussion, we do not have a definitive answer to the reasons for increased mitochondrial contamination and we suspect that multiple technical factors may contribute -- including the relatively large size and increased fragility of LC-NE neurons. We agree that systematically optimizing the preparation to attempt to increase recovery rate and decrease mitochondrial contamination are important avenues for future work.

b. It is unclear what percentage of cells that make up each cluster.

We will add this information in the clustering heatmaps or as a supplementary plot in a revised version of the manuscript.

c. The number of subjects used in each analysis was not always clear. Only 3 subjects were used for snRNAseq, and one of them only yielded 4 LC-nuclei. This means the results are essentially based on n=2. The authors report these numbers in the corresponding section, but the first sentence of the results section (and Figure 1C specifically!) create the impression that n=5 for all analyses. Even for spatial transcriptomics, if I understood it correctly, 1 sample had to be excluded (n=4).

This is correct. We will update the figures and text in a revised version of the manuscript to make this limitation (small sample size) more clear, and to further emphasize that the intention of this study is to provide initial data to help determine next steps and best practices for a larger scale and more comprehensive study on this region, especially given the limited availability of tissue resources and currently limited data resources available for this region.

2. Spatial transcriptomics:

a. It is not clear to me what the spatial transcriptomics provides beyond what can be shown with snRNAseq, nor how these two sets of results compare to each other. It would be more intuitive to start the story with snRNAseq and then try to provide spatial detail using spatial transcriptomics. The LC is not a homogeneous structure but can be divided into ensembles based on projection specificity. Spatial transcriptomics could - in theory - offer much-needed insights into the spatial variation of mRNA profiles across different ensembles, or as a first step across the spatial (rostral/caudal, ventral/dorsal) extent of the LC. The current analyses, however, cannot address this issue, as the orientation of the LC cannot be deduced from the slices analyzed.

We understand the point of the reviewer. However, we structured the manuscript in this format due to our aims of creating a data resource for the community as well as being transparent about the limitations of our study. Our experiments began with the spatial experiments on the tissue blocks because this (i) helped orient ourselves to the region, and (ii) provided guidance for how best to score the tissue blocks for the snRNA-seq experiments to maximize recovery of LC-NE neurons. Therefore, we also decided to present the results in this sequence.

The spatial data also provides more information in that the measurements are from nuclei, cytoplasm, and cell processes (instead of nuclei only). This is one of the main differences / advantages between the platforms at this level of spatial resolution. As noted above, we were also working with a finite tissue resource -- if we ran snRNA-seq first and captured no neurons, the tissue block would be depleted. Due to the logistics / thickness of the required tissue sections for Visium and snRNA-seq respectively, running Visium first allowed us to ensure that we could collect data from both assays.

Regarding a point raised below on why we only ran snRNA-seq on a subset of the donors -- this was due to resource depletion and not enough available tissue remaining on the tissue blocks to run the assay. We have conducted extensive piloting in other brain regions on the amount (mg) of tissue that is needed from various sized cryosections, and the LC is particularly difficult since these are small tissue blocks and the extent of the structure is small. Hence, in some of the subjects, we did not have sufficient tissue available for the snRNA-seq assay.

We agree with the reviewer that spatial studies could, in future work, offer needed and important information about expression profiles across the spatial axes (rostral/caudal, ventral/dorsal) of the LC. Our study provides us with insight about optimizing the dissections for spatial assays, as well as bringing to light a number of technical and logistical issues that we had not initially foreseen. For example, during the course of this study and parallel, ongoing work in other small, challenging brain regions, we have now developed a number of specialized technical and logistical strategies for keeping track of orientation and mounting serial sections from the same tissue block onto a single spatial array, which is extremely technically challenging. We are now well-prepared for addressing these issues in future studies with larger numbers of donors and samples, e.g. spaced serial sections across the extent of the LC to make these types of insights. Due to the rarity of the tissue, limited availability of information in this region, and high expense of conducting these studies, we want to share this initial data with the community immediately. We also note that in addition to the 10x Genomics Visium platform, which lacks cellular and sub-cellular resolution, many new and exciting spatial platforms are entering the market, which may be able to address questions in very small regions such as the LC at higher spatial resolution.

b. Unfortunately, spatial transcriptomics itself is plagued by sampling variability to a point where the RNAscope analyses the authors performed prove more powerful in addressing direct questions about gene expression patterns. Given that the authors compare their results to published datasets from rodent studies, it is surprising that a direct comparison of genes identified with spatial transcriptomics vs snRNAseq is lacking (unless this reviewer missed this comparison). Supplementary Figure 17 seems to be a first step in that direction, but this is not a gene-by-gene comparison of which analysis identifies which LC-enriched genes. Such an analysis should not compare numbers of enriched genes using artificial cutoffs for significance/fold-change, but rather use correlations to get a feeling for which genes appear to be enriched in the LC using both methods. This would result in one list of genes that can serve as a reference point for future work.

We agree this is a good suggestion, and will add additional computational analyses to address this point in a revised version of the manuscript.

c. Maybe the spatial transcriptomics could be useful to look at the peri-LC region, which has generated some excitement in rodent work recently, but remains largely unexplored in humans.

We agree this is an excellent suggestion -- assessing cross-species comparisons related to convergence, especially, of GABAergic cell populations in the human LC is of high interest. We note that these types of extensions are exactly the reason why we have provided the publicly accessible web app (R/Shiny app, which includes the ability to annotate regions). We hope that others will use these apps for specialized topics they are interested in. As discussed above, we note that our initial dissections precluded the ability to keep track of the exact orientation of our tissue sections on the Visium arrays with respect to their location within the brainstem, so definitive localization of this region across subjects is difficult in our current study. However, it is possible, for example, to investigate whether there is a putative peri-LC region that is densely GABAergic that is homologous with the GABAergic peri-LC region in rodents. We also raise attention to a recent preprint by Luskin

and Li et al. (2022), who apply snRNA-seq and spatially-resolved transcriptomics to molecularly define both LC and peri-LC cell types in mice -- in a revised version of our manuscript, we will extend our computational analyses of inhibitory neuronal subtypes in our data (Supplementary Figures 13, 16) to directly compare with those identified in this study in more detail. As noted above, we have now developed a number of specialized technical and logistical strategies for keeping track of orientation of sections from the tissue block onto a single spatial array, and we feel that combined with optimized dissection strategies for this region and the guide of RNAscope for GABAergic markers on serial sections, that annotating the peri-LC region on spatial arrays in future studies will be possible.

3. The comparison of snRNAseq data to published literature is laudable. Although the authors mention considerable methodological differences between the chosen rodent work and their own analyses, this needs to be further explained. The mouse dataset uses TRAPseq, which looks at translating mRNAs associated with ribosomes, very different from the nuclear RNA pool analyzed in the current work. The rat dataset used single-cell LC laser microdissection followed by microarray analyses, leading to major technical differences in terms of tissue processing and downstream analyses. The authors mention and reference a recent 10x mouse LC dataset (Luskin et al, 2022), however they only pick some neuropeptides from this study for their analysis of interneuron subtypes (Figure S13). Although this is a very interesting part of the manuscript, a more in-depth analysis of these two datasets would be very useful. It would likely allow for a better comparison between mouse and human, given that the technical approach is more similar (albeit without FACS), and Luskin et al have indicated that they are willing to share their data.

As noted above, we plan to extend our comparisons with the dataset from Luskin and Li et al. (2022) in a revised version of the manuscript, which will provide a more in-depth cross-species comparison. In addition, we also note that there are some additional recent studies using TRAPseq of LC-NE neurons in a functional context, i.e. treatment vs. control experiments or in model systems (e.g. Iannitelli et al. 2023), which provide new opportunities for understanding disease context using in-depth cross-species comparisons. By providing our dataset and reproducible code, we will enable others to adapt and extend these types of comparisons (i.e. TRAPseq of LC-NE neurons or LC snRNA-seq following functional manipulations or in the context of disease or behavioral models) in the future.

4. Statements in the manuscript about the unexpected identification of a 5-HT (serotonin) cell-cluster seem somewhat contradictory. Figure S14 suggests that 5-HT markers are expressed in the LC-regions just as much as anywhere else, but the RNAscope image in Figure S15 suggests spatial separation between these two populations. And Figure S17 again suggests almost perfect overlap between the LC and 5HT clusters. Maybe I misunderstood, in which case the authors should better clarify/explain these results.

In our view, the most likely scenario is that the 5-HT neurons come from contamination from the dorsal raphe nucleus based on spatial separation from the RNAscope images, which we agree are more definitive. As mentioned above, since we do not have definitive documentation for the tissue sections in terms of orientation, it is difficult to say with clarity that the regions are the dorsal raphe and which sub-portion of the dorsal raphe they are. This initial study has now allowed us to optimize and improve our dissection strategy and approaches for retaining documentation of the orientation of the tissue sections from their intact position within the brainstem as they move from cryosection to placement on the array, which will enable us to better annotate regions with definitive anatomical information with respect to the rostral/caudal and dorsal/ventral axes in future experiments. Given that there are reports in the rodent that 5-HT markers have been identified in LC-NE neurons (Iijima 1993; Iijima 1989), and taking into account the technical

limitations in our study, we felt that it was premature to definitively conclude in the manuscript that we were sure these signals arose from the dorsal raphe. We will update this language in a revised version of the manuscript to ensure that these limitations are clear (referring to Supplementary Figures S14-15, S17).

Reviewer #2 (Public Review):

The data generated for this paper provides an important resource for the neuroscience community. The locus coeruleus (LC) is the known seed of noradrenergic cells in the brain. Due to its location and size, it remains scarcely profiled in humans. Despite the physically minute structure containing these cells, its impact is wide-reaching due to the known neuromodulatory function of norepinephrine (NE) in processes like attention and mood. As such, profiling NE cells has important implications for most neurological and neuropsychiatric disorders. This paper generates transcriptomic profiles that are not only cell-specific but which also maintain their spatial context, providing the field with a map for the cells within the region.

Strengths:

Using spatial transcriptomics in a morphologically distinct region is a very attractive way to generate a map. Overlaying macroscopic information, i.e. a region with greater pigmentation, with its corresponding molecular profile in an unbiased manner is an extremely powerful way to understand the specific cellular and molecular composition of that brain structure.

The technologies were used with an astute awareness of their limitations, as such, multiple technologies were leveraged to paint a more complete and resolved picture of the cellular composition of the region. For example, the lack of resolution in the spatial transcriptomic platform was compensated by complementary snRNA-seq and single molecule FISH.

This work has been made publicly available and accessible through a user-friendly application such that any interested researcher can investigate the level of expression of their gene of interest within this region.

Two important implications from this work are 1) the potential that the gene regulatory profiles of these cells are only partially conserved across species, humans, and rodents, and 2) that there may be other neuromodulatory cell types within the region that were otherwise not previously localized to the LC

Weaknesses:

Given that the markers used to identify cells are not as specific as they need to be to definitively qualify the desired cell type, the results may be over-interpreted. Specifically, TH is the primary marker used to qualify cells as noradrenergic, however, TH catalyzes the synthesis of L-DOPA, a precursor to dopamine, which in turn is a precursor for epinephrine and norepinephrine suggesting some of the cells in the region may be dopaminergic and not NE cells. Indeed, there are publications to support the presence of dopaminergic cells in the LC (see Kempadoo et al. 2016, Takeuchi et al., 2016, Devoto et al. 2005). This discrepancy is further highlighted by the apparent lack of overlap per given Visium spots with TH, SCL6A2, or DBH. While the single-nucleus FISH confirms that some of the cells in the region are noradrenergic, others very possibly represent a different catecholamine. As such it is suggested that the nomenclature for the cells be reconsidered.

We appreciate the reviewer's comment, and are aware of the reports suggesting the potential presence of dopaminergic cells in the LC. We initially had the same thought as the reviewer when we observed Visium spots in the spatial data with lack of overlap between TH, SCL6A2, and DBH as well as single nuclei in the snRNA-seq data with lack of overlap between TH, SCL6A2, and DBH. This surprising result was exactly why we performed the

smFISH/RNAscope experiment with these three marker genes. Given known issues with read depth and coverage in the 10x Genomics assays, we wanted to better understand if this was a technical limitation in the sequencing coverage, or rather a true biological finding. The RNAscope data showed very clearly that nearly every cell body we looked at had co-localization of these three marker genes. We included an image from a single capture array of one tissue section in Supplementary Figure 11, but could, in a revised version of the manuscript, provide additional examples to illustrate how conclusive the images were by visualization. As such, we were quite convinced that the lack of overlap on Visium spots and in single nuclei in the snRNA-seq data was more likely related to technical issues with sequencing coverage, rather than a biological finding. We also note that we checked for the presence of the dopamine transporter, *SLC6A3*, and as can be appreciated in the iSEE web app for the snRNA-seq data or the R/Shiny web app for the Visium data, there is virtually no expression of *SLC6A3* in the dataset, which in our view provides additional evidence against the possibility that there are substantial quantities of dopaminergic cells in this human LC dataset. We will include supplementary plots showing the lack of *SLC6A3* expression in a revised version of the manuscript.

The authors are unable to successfully implement unsupervised clustering with the spatial data, this greatly reduces the impact of the spatial technology as it implies that the transcriptomic data generated in the study did not have enough resolution to identify individual cell types.

The reviewer is correct -- this is a fundamental limitation of the 10x Genomics Visium platform, i.e. the spatial resolution captures multiple cells per spot (e.g. around 1-10 cells per spot in human brain tissue). We note that new spatial platforms now provide cellular resolution (e.g. Vizgen MERSCOPE, 10x Genomics Xenium, 10x Genomics Visium HD), which will help address this in future work. However, many of these cellular-resolution in situ sequencing platforms have the limitation that they do not quantify genome-wide expression, and instead require users to select *a priori* gene panels to investigate. This is a problem if no genome-wide reference datasets are available. Hence, despite the limited spatial resolution of the Visium platform, this dataset is useful precisely for helping investigators choose gene panels for higher-resolution platforms or higher-order smFISH multiplexing.

We also applied spatial clustering (using BayesSpace; Zhao et al. 2021) to attempt to segment the LC regions within the Visium samples in a data-driven manner as an alternative to the manual annotations, which was unsuccessful (and hence we relied on the manually annotated regions for downstream analyses) (Supplementary Figure S5). However, this is a different application of unsupervised clustering, which is separate from the task of identifying cell types.

The sample contribution to the results is highly unbalanced, which consequently, may result in ungeneralizable findings in terms of regional cellular composition, limiting the usefulness of the publicly available data.

We acknowledge the limitations of the work due to the small/unbalanced sample sizes. As mentioned above for Reviewer 1, this was an initial study in this region -- results of which will inform our (and hopefully others') experimental design and approach to molecular profiling in this difficult to access brain region. Overall, this study was executed with finite tissue and financial resources and was intended to uncover limitations and help develop best practices and design workflows for future studies with larger numbers of donors and samples. Given the limited data availability for this brain region, we wanted to make this dataset available for the research community immediately. In addition, we note that making this genome-wide dataset available will help inform targeted gene panel design for higher-resolution platforms (e.g. 10x Genomics Xenium).

This study aimed to deeply profile the LC in humans and provide a resource to the community. The combination of data types (snRNA-seq, SRT, smFISH) does in fact represent this resource for the community. However, due to the limitations, of which, some were described in the manuscript, we should be cautious in the use of the data for secondary analysis. For example, some of the cellular annotations may lack precision, the cellular composition also may not reflect the general population, and the presence of unexpected cell types may represent the accidental inclusion of adjacent regions, in this case, serotonergic cells from the Raphe nucleus.

We agree, and have attempted to explain these limitations in the manuscript. We will clarify the language regarding the interpretation of the annotated cell populations and unexpected cell types, and the limited sample sizes, in a revised version of the manuscript.

Nonetheless having a well-developed app to query and visualize these data will be an enormous asset to the community especially given the lack of information regarding the region in general.

Reviewer #3 (Public Review):

[...] This study has many strengths. It is the first reported comprehensive map of the human LC transcriptome, and uses two independent but complementary approaches (spatial transcriptomics and snRNA-seq). Some of the key findings confirmed what has been described in the rodent LC, as well as some intriguing potential genes and modules identified that may be unique to humans and have the potential to explain LC-related disease states. The main limitations of the study were acknowledged by the authors and include the spatial resolution probably not being at the single cell level and the relatively small number of samples (and questionable quality) for the snRNA-seq data. Overall, the strengths greatly outweigh the limitations. This dataset will be a valuable resource for the neuroscience community, both in terms of methodology development and results that will no doubt enable important comparisons and follow-up studies.

Major comments:

Overall, the discovery of some cells in the LC region that express serotonergic markers is intriguing. However, no evidence is presented that these neurons actually produce 5-HT.

The reviewer is correct that we did not provide any additional evidence to show that these neurons actually produce 5-HT. As noted above in the response to Reviewer 1, in our view, the most likely explanation is that these neurons are from dorsal raphe contamination on the tissue section. However, due to technical and logistical limitations in this study, we could not definitively say this because we did not clearly track the orientation of the tissue sections, and we did not have remaining tissue sections from all donor tissue blocks to repeat RNAscope experiments. For some of the donors, where we had remaining tissue sections to go back to repeat RNAscope experiments after completion of the snRNA-seq and Visium assays, we could see clear separation of the LC region / LC-NE neuron core from where putative 5-HT neurons were located (Supplementary Figure 15). However, we did not have sufficient tissue resources to map this definitively in all donors, and the orientation and anatomy of each tissue block were not fully annotated.

Due to the lack of clarity, and the fact that there have been reports that LC-NE neurons express serotonergic markers (Iijima 1993; Iijima 1989), we felt that it was premature to definitively declare that these putative 5-HT neurons that we identified were definitively from the raphe. We will clarify the language around this discrepancy in a revised version of the manuscript to ensure that these limitations are clearly described.

Concerning the snRNA-seq experiments, it is unclear why only 3 of the 5 donors were used, particularly given the low number of LC-NE nuclear transcriptomes obtained, why those 3

were chosen, and how many 100 µm sections were used from each donor. It is also unclear if the 295 nuclei obtained truly representative of the LC population or whether they are just the most "resilient" LC nuclei that survive the process.

As discussed above for Reviewer 1, the reason we included only 3 of the 5 donors for the snRNA-seq assays was due to the tissue availability on the tissue blocks. We will clarify the language in a revised version of the manuscript to make this limitation more clear. We will also include additional details in the Methods section on the number of 100 µm sections used for each donor (which varied between 10-15, approximating 60-80 mg of tissue).

The LC displays rostral/caudal and dorsal/ventral differences, including where they project, which functions they regulate, and which parts are vulnerable in neurodegenerative disease (e.g. Loughlin et al., Neuroscience 18:291-306, 1986; Dahl et al., Nat Hum Behav 3:1203-14, 2019; Beardmore et al., J Alzheimer's Dis 83:5-22, 2021; Gilvesy et al., Acta Neuropathol 144:651-76, 2022; Madelung et al., Mov Disord 37:479-89, 2022). It was not clear which part(s) of the LC was captured for the SRT and snRNAseq experiments.

As discussed above for Reviewer 1, a limitation of this study was that we did not record the orientation of the anatomy of the tissue sections, precluding our ability to annotate the tissue sections with the rostral/caudal and dorsal/ventral axis labels. We agree with the reviewer that additional spatial studies, in future work, could offer needed and important information about expression profiles across the spatial axes (rostral/caudal, ventral/dorsal) of the LC. Our study provides us with insight about optimizing the dissections for spatial assays, as well as bringing to light a number of technical and logistical issues that we had not initially foreseen. For example, during the course of this study and parallel, ongoing work in other, small, challenging regions, we have now developed a number of specialized technical and logistical strategies for keeping track of orientation and mounting serial sections from the same tissue block onto a single spatial array, which is extremely technically challenging. We are now well-prepared for addressing these issues in future studies with larger numbers of donors and samples in order to make these types of insights.

The authors mention that in other human SRT studies, there are typically between 1-10 cells per expression spot. I imagine that this depends heavily on the part of the brain being studied and neuronal density, but it was unclear how many LC cells were contained in each expression spot.

The reviewer is correct that we did not include this information in the manuscript. We attempted to apply a computational method to count nuclei contained in each gene expression spot based on analyzing the histological H&E images (*VistoSeg*; Tippani et al. 2022), which we have developed and previously applied in data from the dorsolateral prefrontal cortex (DLPFC) (Maynard and Collado-Torres et al. 2021). Based on the segmentation using this workflow we observe that the counts in this region are similar to what we observed in the DLPFC, i.e., typically between 1-10 LC cells per expression spot, with approximately 1-2 LC-NE neurons (which are characterized by their large size) per expression spot. However, these analyses had several technical issues related to the images themselves, the relatively large size and pigmentation of LC-NE neurons, and parameter settings that had been optimized for different brain regions. We are currently optimizing this analysis workflow for these images to provide more accurate estimates of cell counts per spot to give readers additional context on the number of nuclei per spot in the annotated LC regions and outside the LC regions in a revised version of the manuscript.

Regarding comparison of human LC-associated genes with rat or mouse LC-associated genes (Fig. 2D-F), the authors speculate that the modest degree of overlap may be due to species differences between rodents and human and/or methodological differences (SRT vs microarray vs TRAP). Was there greater overlap between mouse and rat than between mouse/rat and human? If so, that is evidence for the former. If not, that is evidence for the

latter. Also would be useful for more in-depth comparison with snRNA-seq data from mouse LC: <https://www.biorxiv.org/content/10.1101/2022.06.30.498327v1>.

We will investigate this question and discuss this in updated results in a revised version of the manuscript.

The finding of ACHE expression in LC neurons is intriguing, especially in light of work from Susan Greenfield suggesting that ACHE has functions independent of ACH metabolism that contributes to cellular vulnerability in neurodegenerative disease.

We thank the reviewer for pointing this out. We were very surprised too by the observed expression of *SLC5A7* and *ACHE* in the LC regions (Visium data) and within the LC-NE neuron cluster (snRNA-seq data), coupled with absence of other typical cholinergic marker genes (e.g. *CHAT*, *SLC18A3*), and we do not have a compelling explanation or theory for this. Hence, the work of Susan Greenfield and colleagues suggesting non-cholinergic actions of *ACHE*, particularly in other catecholaminergic neurons (e.g. dopaminergic neurons in the substantia nigra) is very interesting. We will include references to this work and how it could inform interpretation of this expression in a revised version of the manuscript (Greenfield 1991; Halliday and Greenfield 2012).

High mitochondrial reads from snRNA-seq can indicate lower quality. It was not clear why, given the mitochondrial read count, the authors are confident in the snRNA-seq data from presumptive LC-NE neurons.

We will include additional analyses to further investigate and/or confirm this finding (e.g. comparing sum of UMI counts / number of detected genes and mitochondrial percentage per nucleus for this population to confirm data quality) in additional supplementary figures in a revised version of the manuscript.

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