

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) make long-range projections 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 and downloadable formats.

eLife assessment

This is an **important** 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. The empirical support for the main conclusions is **solid**. 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, pain, mood, and the response to stress [1–6]. The LC, translated as “blue spot” (in recognition of its characteristic presence of neuromelanin pigment), 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 [7].

Despite its prominent involvement in a number of critical brain functions and its distinctive 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. Consistent with these roles, the LC-NE system is implicated in many neurodegenerative, neuropsychiatric, and neurological disorders [4,8]. The LC is one of the earliest sites of degeneration in both 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 neurodegeneration are not well-understood. In addition to its role in neurodegenerative disorders, the LC-NE system is implicated in a number of other complex brain disorders. Noradrenergic signaling controls many cognitive functions, including sustained attention, and its dysregulation is associated with attention-deficit hyperactivity disorder (ADHD) [16,17]. Related to these findings, the NE reuptake inhibitor atomoxetine is the first non-stimulant medication that is FDA-approved for treating ADHD [18–20]. Disruption of noradrenergic signaling is also associated with anxiety, addiction, and responses to stress and trauma, and drugs that modulate noradrenergic signaling have been used in the treatment of post-traumatic stress disorder (PTSD), major depressive disorder (MDD), anxiety, and opioid withdrawal [4,21–25]. Given the wide range of functions that are modulated by the LC-NE system, improved understanding of 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 in a variety of disorders.

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 at cellular and spatial resolution [26,27]. SRT has recently been used to characterize transcriptome-wide gene expression within defined neuroanatomy of cortical regions in the postmortem human brain [27], 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 [26,28]. 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 [27]. 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 [27] and the dopaminergic substantia nigra [26].

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 [29]. We observe expression of cholinergic marker genes within NE neurons, a finding which we confirm using multiplexed single-molecule fluorescence *in situ* hybridization (smFISH) with high-resolution imaging at cellular resolution. 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 [30–32], 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 [33] and 10x Genomics Chromium snRNA-seq [34] platforms (see **Supplementary Table 1** for donor demographic details). After all quality control (QC) steps (described below), the final SRT and snRNA-seq datasets used for analyses consisted of samples from 4 and 3 donors, respectively. In each tissue sample, the LC was first visually identified by neuroanatomical landmarks and presence of 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 NE neuron marker genes [35]. Specifically, we cut 10 μm 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 [36,37] (**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.

For tissue blocks included in the study, we cut additional 10 μm tissue sections, which were used for gene expression profiling at spatial resolution using the 10x Genomics Visium SRT platform [33] (**Figure 1C**). Fresh-frozen tissue sections were placed onto each of four capture areas per

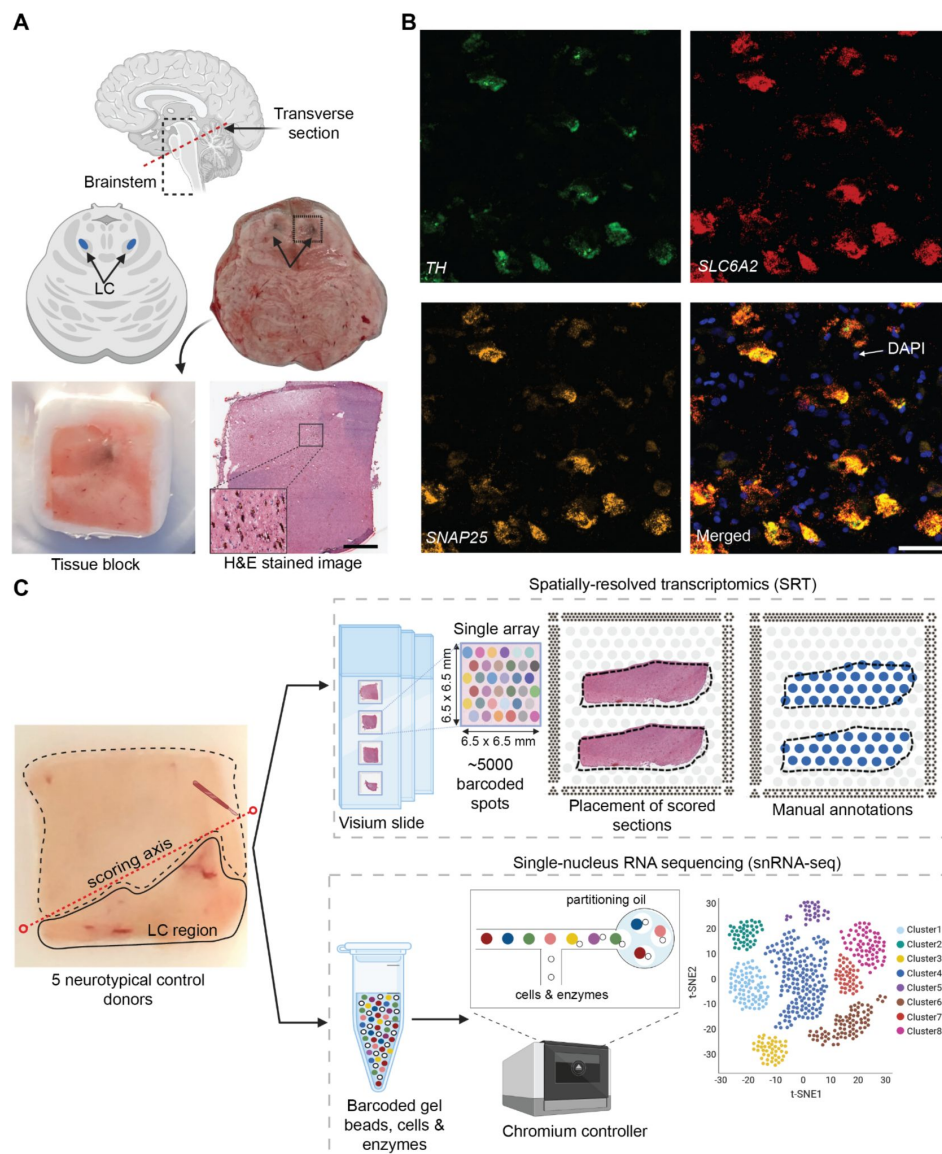


Figure 1

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 [36,37] 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. After all quality control (QC) steps, the final SRT and snRNA-seq datasets used for analyses consisted of samples from 4 and 3 donors, respectively.

Visium slide, where each capture area contains approximately 5,000 expression spots (spatial measurement locations with diameter 55 μm and 100 μm 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 [33]. 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 μm 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 [34] (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 [33], 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**). Analysis of cell segmentations of the H&E images (**Supplementary Figure 2A**) revealed that the median number of cells per spot within the LC regions ranged from 2 to 5 per sample (**Supplementary Figure 2B**). Next, we performed additional sample-level 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 3A-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 3C**), 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 [27,38,39] (Methods). Due to the large differences in read depth between samples (**Supplementary Figure 4A**, **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 4B**).

To investigate whether the LC regions could be annotated in a data-driven manner, we applied a spatially-aware unsupervised clustering algorithm (BayesSpace [40]) after applying a batch integration tool (Harmony [41]) to remove sample-specific technical variation in the molecular measurements (**Supplementary Figure 5**). The spatially-aware clustering using $k=5$ clusters (across 8 samples) 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 6A**) 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

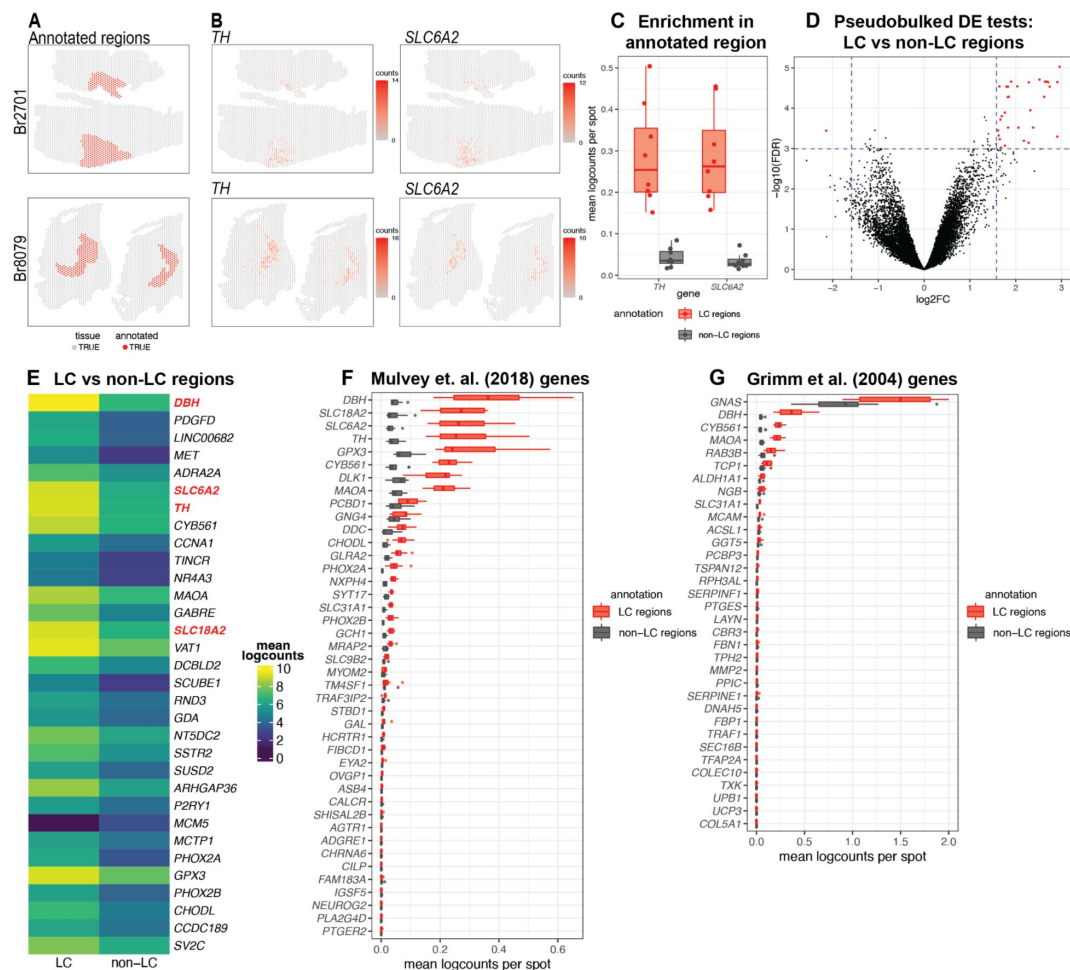


Figure 2

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, 3A-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 3C**. (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 $\log_2$ scale and vertical axis on $\log_{10}$ 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 8** 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 [30,31] 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.

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 6B**). 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 [42]. 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 7A**). 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 7B**). 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 [27]. 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 8A**). 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 FDR < 0.05 and expression FC > 2, we identified a total of 437 statistically significant genes (**Supplementary Figure 8B** 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 [43]. 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 9A–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 9D** and **Supplementary Table 3**).

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 [30]. The second study used microarrays to assess gene expression patterns from laser-capture microdissection of individual cells in tissue sections of the

rat LC [31]. 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 [30], and *DBH* and *GNAS* from [31]. 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 [34] 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 [44], 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 [38] (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 10-12**, additional details described below).

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 [28], using a two-stage clustering algorithm consisting of high-resolution *k*-means and graph-based clustering that provides sensitivity to identify rare cell populations [38]. 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*) without clear expression of excitatory or inhibitory neuronal markers, which may represent damaged neuronal nuclei and debris (Figure 3A-B and **Supplementary Figure 10**). Further evaluation of QC metrics revealed that standard QC metrics (sum of UMI counts and detected genes) for the NE neuron cluster fell within the ranges of values observed for the other neuronal and non-neuronal clusters (**Supplementary Figure 11A-B**), and that the ambiguous neuronal category included a clear subset of measurements with the overall highest mitochondrial proportions and lowest number of detected genes (likely damaged nuclei and/or debris), which remained separate from the NE neuron cluster (**Supplementary Figure 11C-D**), thus providing additional confidence that the high mitochondrial proportions observed for the NE neuron cluster were not due to mis-classified damaged nuclei and/or debris.

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 12A-B**). This supervised approach identified 332 NE neuron nuclei (173, 4, and 155 nuclei from donors

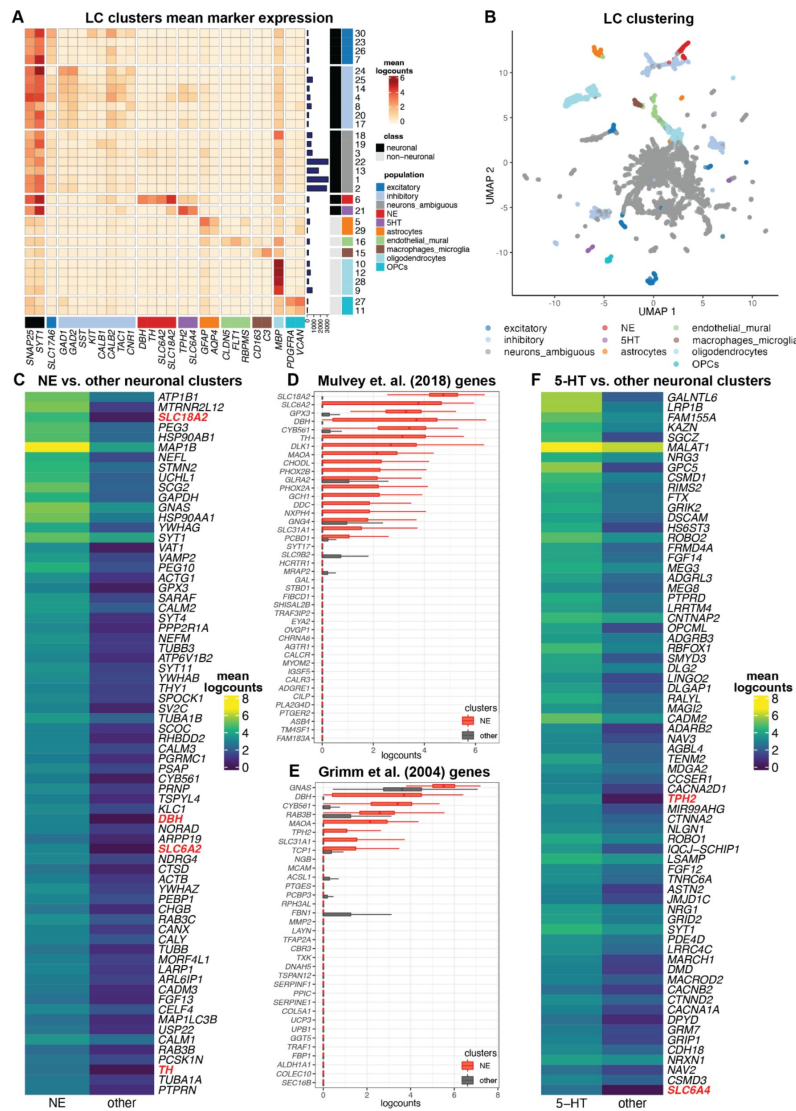


Figure 3

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. Percentages of nuclei per cluster are also shown in **Supplementary Figure 10D**. 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 (excluding ambiguous). **(D-E)** Cross-species comparison showing expression of human ortholog genes for LC-associated genes identified in the rodent LC [30,31] 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 total of 361 statistically significant genes with elevated expression in the 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 7**.

Br2701, Br6522, and Br8079, respectively), including 188 out of the 295 NE neuron nuclei identified by unsupervised clustering (**Supplementary Figure 12C**). 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 [37]) 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 13**). 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 (excluding ambiguous) 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 14A**, 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 the spatial data, we also performed DE testing between the NE neuron cluster and all other clusters (neuronal and non-neuronal, excluding ambiguous) in the snRNA-seq data, which identified 427 statistically significant genes with elevated expression in the NE neuron cluster at an FDR threshold of 0.05 and FC threshold of 2, including all of the 327 genes above (**Supplementary Figure 15** and **Supplementary Table 5**). We then compared these genes with the set of 437 statistically significant DE genes from the pseudobulked analyses (LC vs. non-LC regions) in the SRT data, which generated a list of 51 genes that were identified as statistically significant DE genes in both the SRT and snRNA-seq datasets. These 51 genes included known NE neuron marker genes (*DBH*, *TH*, *SLC6A2*, and *SLC18A2*) with high FC in both datasets (**Supplementary Figure 16** and **Supplementary Table 6**).

To compare with previous results in rodents, we evaluated the expression of the rodent LC marker genes from [30,31] 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, while the SRT data may contain reads from nuclei, cytoplasm, and cell processes from up to multiple cell populations per spot.

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 [32]. 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 17**), 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 17**).

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 potential 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*) [45]. 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 14B**, and **Supplementary Table 7**). 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 18A-B**). Similarly, we did not observe significant spatial enrichment of *TPH2* and *SLC6A4* expression within the manually annotated LC regions in the *N*=8 Visium samples that passed QC (**Supplementary Figure 18C**). Further analysis confirmed expression of additional marker genes for 5-HT neurons (*SLC18A2*, *FEV*) within the same regions as *TPH2* and *SLC6A4* in the Visium samples and within the 5-HT neuron cluster in the snRNA-seq data, but we did not observe consistent expression signals for *SLC6A18*, 5-HT autoreceptors (*HTR1A*, *HTR1B*), or previously identified marker genes for 5-HT neuron subpopulations within the dorsal and median raphe nuclei in mice [45] -- with the possible exception of the neuropeptide receptor gene *HCRT2* (**Supplementary Figures 19, 17**). To further confirm this finding, we applied RNAscope [37] 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 20**), and the 5-HT neuron marker genes (*TPH2* and *SLC6A4*) were co-expressed within individual cells (**Supplementary Figure 21**).

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 17, 22A**). In addition, similar to results from a recent publication using snRNA-seq in mice [32], we found that expression of neuropeptides *PNOC*, *TAC1*, and *PENK* varied across the individual inhibitory neuronal populations (**Supplementary Figure 17**). We also investigated a set of GABAergic neuron marker genes from this study [32] within our secondary clustering of inhibitory neurons, and observed some cluster-specific expression of several genes, including *CCK*, *PCSK1*, *PCSK2*, *PCSK1N*, *PENK*, *PNOC*, *SST*, and *TAC1* (**Supplementary Figure 22B**).

In order to integrate the single-nucleus and spatial data, we also applied a spot-level deconvolution algorithm, *cell2location* [46], 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. We found that this approach mapped cells from the NE neuron population to the manually annotated LC regions, and cells from the 5-HT neuron population to the regions where this population was previously identified based on expression of marker genes, although the overall mapping performance was relatively poor (**Supplementary Figure 23**). In particular, we observed that cells from these neuronal populations were also mapped outside the expected regions in the Visium samples, and the estimated absolute cell abundances per spot were higher than expected. We note

that these are relatively rare populations, with relatively subtle expression differences compared to other neuronal populations, and NE neurons are characterized by large size and high transcriptional activity, which may have affected performance of the algorithm. Due to the relatively low performance, we did not rely on these results for further downstream analyses.

Investigation of dopamine (DA) marker genes

TH is the rate-limiting enzyme in the synthesis of dopamine (DA), which is used as a precursor in the synthesis of NE. Hence, expression of the *TH* gene is also used as a marker for neurons that synthesize DA, and release of DA from LC projection neurons has been observed in the rodent [47–49]. To evaluate whether *TH* expression in these human LC samples labels cells that produce DA or co-express DA and NE, we also investigated expression of other genes involved in the synthesis and transport of DA. We observed minimal expression of DA neuron marker genes including *SLC6A3* (encoding the dopamine transporter), *ALDH1A1*, and *SLC26A7* within the Visium samples (Supplementary Figure 24) and within the NE neuron cluster in the snRNA-seq data (Supplementary Figure 17). To further validate this finding, we generated additional high-resolution RNAscope smFISH [37] images at 40x magnification visualizing the expression of *DBH* and *TH* (NE neuron marker genes), *TPH2* (5-HT neuron marker gene), and *SLC6A3* (DA neuron marker gene) within individual cells in regions within the LC in samples from 3 independent donors (Br8689, Br5529, and Br5426). Expression of *SLC6A3* within individual NE neurons (identified by co-expression of *DBH* and *TH*) (Supplementary Figure 25) was not apparent in these RNAscope images. Together with the RNAscope images showing co-expression of *DBH*, *TH*, and *SLC6A2* within individual NE neurons (Supplementary Figure 13), these results strengthen the conclusion that the observed *TH*⁺ cells in the LC are NE-producing neurons.

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 17). 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 [37] 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 26), 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 27).

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 [50] app for the Visium SRT data and an iSEE [51] app for the snRNA-seq data (Supplementary Figures 28–30). The data resources are also available from an R/Bioconductor ExperimentHub data package as downloadable objects in the SpatialExperiment [52] and SingleCellExperiment [38] formats, which can be loaded in an R session and contain meta-data including manual annotation labels and cluster labels.

Resource	Data	Format	Link
Shiny [50] web app	Visium SRT	Interactive web app	https://libd.shinyapps.io/locus-c_Visium/
iSEE [51] 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 [52] and SingleCellExperiment [38] formats	https://bioconductor.org/packages/WeberDivechaLCdata

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.

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 evaluated expression of dopamine (DA) marker genes to confirm that the observed *TH* expression likely maps to NE-synthesizing, as opposed to DA-synthesizing neurons. We compared our findings from the human LC to molecular profiles of LC and peri-LC neurons in rodents, and confirmed partial conservation of LC-associated genes across these species. However, we note caution in interpreting these results given differences in technological platforms and experimental approaches across studies. Finally, we validated key results with smFISH RNAscope by assessing co-localization of genes of interest with established 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]. Untargeted, transcriptome-wide 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*) [53,54]. 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*) [55–58]. We also noted enrichment of *NTSDC2* in LC, a gene which has been associated with attention-deficit hyperactivity disorder (ADHD) and regulates catecholamine synthesis *in vitro* [59,60]. 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 captured a median of 2-5 cells per measurement location within the LC regions per sample, and future studies could apply higher-resolution platforms to characterize expression at single-cell or sub-cellular spatial resolution. These estimates of cell density could also be used to estimate the expected yield of nuclei in single-nucleus data in future studies. In the single-nucleus data, we identified a relatively small number of NE neurons, which may be related to technical factors that affected the recovery of this population due to their relatively large size and fragility or contributed to damage and loss of NE neuron nuclei. For example, we opted to use fluorescence-activated nuclei sorting (FANS) to enrich for neurons based on our previous success with this approach to identify relatively rare neuronal populations in other brain regions including the nucleus accumbens and amygdala [28]. This and other 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 [61]. 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 optimal isolation of larger cell sizes, could enhance recovery of this population for future, larger-scale snRNA-seq studies in this brain region.

Since the identification of potential 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. While our dissection strategy in this initial study precluded the ability to keep track of the exact orientation of the tissue sections, we have now developed a number of specialized technical and logistical strategies for keeping track of orientation of dissections and mounting serial sections from the same tissue block onto a single array, which will make it possible to address these, and other important questions in the future. For example, interrogating spatial gradients across the rostral-caudal gradient of the LC will be important given differential association with vulnerability to neurodegeneration across this axis [62,63]. It is possible that the identified 5-HT neurons 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 20** from an independent tissue section shows that *TPH2* and *SLC6A4* expression appears to be distinct from the LC region, which is densely packed with NE cells. However, there is some evidence for expression of serotonergic markers within the LC region in rodents [31,64,65], 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 across the rostral-caudal axis of the brainstem.

Similarly, the identification of cholinergic marker gene expression, particularly the robust expression of *SLC5A7* and *ACHE* within NE neurons, was unexpected. While previous studies have identified small populations of cholinergic interneurons within or adjacent to the LC in rodents [32], 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 24**) 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 were surprised by the 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*). However, previous work suggested non-cholinergic actions of *ACHE*, particularly in other catecholaminergic neuron populations (e.g. dopaminergic neurons in the substantia nigra) [66,67], raising the possibility of a similar mechanism in the LC-NE catecholaminergic population. 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 [68] 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 (HBR) were collected with audiotaped witnessed informed consent with legal next-of-kin 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 [69,70]. 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 $10\text{ }\mu\text{m}$ tissue sections for performing SRT assays using the 10x Genomics Visium SRT platform [33]. 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 $100\text{ }\mu\text{m}$ cryosections for snRNA-seq assays using the 10x Genomics Chromium snRNA-seq platform [34]. **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 $10\text{ }\mu\text{m}$ and single-molecule fluorescent *in situ* hybridization assays were performed with RNAscope technology [37] using the Fluorescent Multiplex Kit v.2 and 4-plex Ancillary Kit (catalog no. 323100, 323120 ACD) according to the manufacturer's instructions. Briefly, $10\text{ }\mu\text{m}$ 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 13**), 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 20**), 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) [36], 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 μ m 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 [27]. 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**.

Number of cells per spot in Visium SRT data

We applied cell segmentation software (VistoSeg [71]) to the H&E stained histology images to estimate the number of cells per spot in the Visium SRT data, and calculated summary statistics for the annotated LC regions per sample, for 6 out of the 9 Visium SRT samples (**Supplementary Figure 2B**). For the remaining 3 samples, the H&E stained histology images were of insufficient quality to reliably estimate the number of cells per spot. We applied VistoSeg to count cell bodies based on the identification of nuclei using pixel-based counting (with a minimum size threshold of 100 pixels and watershed segmentation algorithm) [71].

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 μ m 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 [28]. 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 μ m 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 μ m 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) [72] 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 [38]. 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 10**), 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 4A** 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 4B**). 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 [40] 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 [73], and then used the limma package [74] 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 [75] (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 [44] 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 [38], 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 12**). 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 [38]. For the first round of clustering (results in **Figure 3A-B**, **Supplementary Figure 17**), 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 22**), 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 scan packages [39,73].

For the DE testing, we performed pairwise DE testing between all neuronal clusters, using the `findMarkers()` function from the scan package [39]. We tested for genes with log₂-fold-changes (log₂FC) 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 [46], we used the following parameters for human brain data from the Visium platform: `detection_alpha = 20`, `N_cells_per_location = 3`.

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Author Contributions

Conceptualization: KRM, KM, SCH

Data Curation: LMW, HRD, MNT, MT

Formal Analysis: LMW, MNT

Funding acquisition: LMW, KRM, KM, SCH

Investigation: HRD, MNT, SHK, AS, KDM

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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 [50] app containing Visium SRT data) and https://libd.shinyapps.io/locus-c_snRNA-seq/ (iSEE [51] 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 [52] format and snRNA-seq data in SingleCellExperiment [38] format). The R/Bioconductor data package is available in Bioconductor version 3.16 onwards. Instructions to install and access the R/Bioconductor data package are also available from GitHub at <https://github.com/lmweber/WeberDivechaLCdata>. Raw data files (FASTQ sequencing data files and high-resolution TIF image files) are available via Globus from the WeberDivecha2023_locus_coeruleus data collection from the `jhpce#globus01` Globus endpoint, which is also listed at <http://research.libd.org/globus/>. The Globus repository is not publicly accessible due to individually identifiable donor genetic variants in the FASTQ files. Approved users may request access to the Globus repository from the corresponding authors.

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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 that 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. Technical limitations currently limit the ability to integrate spatial and single-nucleus sequencing, yet the manuscript presents a valuable resource on the gene expression landscape of the human LC.

- <https://doi.org/10.7554/eLife.84628.2.sa2>

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.

- <https://doi.org/10.7554/eLife.84628.2.sa1>

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 understanding 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.

- <https://doi.org/10.7554/eLife.84628.2.sa0>

Author Response

The following is the authors' response to the original reviews.

Reviewer #1 (Recommendations for the Authors):

The authors provide their data and code via Github, and that shiny apps allow easy access to their data. However, spending a few minutes with the snRNAseq app I could not figure out how to search for individual genes (e.g. DBH) on their web interface. Some changes could help to make this app more user-friendly.

While it was not possible to easily modify the user interface of the snRNA-seq app itself, we have instead added two additional supplementary figures displaying screenshots and schematics with sequential instructions that provide a short tutorial showing how to search for individual genes and display either spatial gene expression (for the Visium SRT data) or

gene expression by cluster or population (for the snRNA-seq data) in each interactive web app (Figure 3-figure supplement 20-21). We hope this makes the apps more accessible and assists users to more easily query specific genes that they are interested in.

The first sentence of the abstract and line 70 on page 2 need to be revised for language / grammar / clarity.

We have revised these two sentences. Line 70 on page 2 contained a typo / copy-paste error. Thank you for pointing this out.

Reviewer #2 (Recommendations For The Authors):

While the efforts of the authors to identify NE neurons in the LC is appreciated, the data fall a little short of conclusively calling these neurons solely noradrenergic as there is an apparent lack of overlap between TH and SLC6A2 in the spots. Undoubtedly, some spots contain both which is consistent with the RNA scope results, but there is clearly a pattern that shows spots that don't contain both. It would be worth testing the presence of other catecholamines in some of these certain spots particularly dopamine (Kempadoo et al. 2016, Takeuchi et al., 2016, Devoto et al. 2005).

We agree this is an important point. To more rigorously investigate whether TH is co-expressed within cells that produce other catecholamines, particularly dopamine (DA) in addition to norepinephrine (NE), we have included additional analyses of the snRNA-seq and Visium data, as well as generated additional RNAscope data in the revised manuscript, as follows.

(i) We investigated the spatial expression of DA neuron marker genes besides TH, including SLC6A3 (encoding the dopamine transporter), ALDH1A1, and SLC26A7 in the Visium samples (Figure 3-figure supplement 15), which shows that these genes are not strongly expressed within the manually annotated LC regions in the Visium samples (see Figure 2-figure supplement 1).

(ii) We investigated expression of DA neuron marker genes SLC6A3, ALDH1A1, and SLC26A7 in the snRNA-seq clustering (updated heatmap in Figure 3-figure supplement 8), which shows minimal expression of these genes within the NE neuron cluster (cluster 6).

(iii) Despite the data above suggesting little expression of markers for DA neurons within the human LC, we wanted to investigate this question more thoroughly with an orthogonal method given that relatively lower coverage in the sequencing approaches may miss expression, particularly for more lowly expressed transcripts. We generated new high-resolution RNAscope smFISH images at 40x magnification for samples from 3 additional donors (Br8689, Br5529, and Br5426) showing expression of NE neuron marker genes (DBH and TH), a 5-HT neuron marker gene (TPH2), and a DA neuron marker gene (SLC6A3) within individual cells within the LC regions in these samples. Expression of SLC6A3 within individual NE neurons (identified by co-expression of DBH and TH) was not apparent in these RNAscope images (Figure 3-figure supplement 16).

Together with the previous high-magnification RNAscope images showing co-expression of NE neuron marker genes (DBH, TH, and SLC6A2) within individual NE neurons (Figure 3-figure supplement 4), these new results further strengthen the conclusion that the observed TH⁺ cells we profiled in the LC are NE-producing neurons. In our view, the lack of observed co-expression of TH and SLC6A2 within some individual Visium spots is likely due to sampling variability and relatively lower sequencing coverage in the Visium data, rather than a true lack of co-expression. We have included additional text in the Results and Discussion further discussing this issue.

Likewise, given the low throughput of RNA scope, and the fact that it was not done in a systematic manner, it does not conclusively identify the cell types in the region. It might be worth a systematic survey of the cells in the region with both NE and DA markers. Otherwise, it is suggested that the authors be more conservative with their annotations.

As discussed above, we have now generated additional high-magnification RNAscope images for 3 independent donors (Br8689, Br5529, and Br5426), visualizing expression of two NE neuron marker genes (DBH and TH), one 5-HT neuron marker gene (TPH2), and one DA neuron marker gene (SLC6A3, encoding the dopamine transporter) within individual cells within the LC region in each sample (Figure 3-figure supplement 16). Expression of the DA neuron marker gene (SLC6A3) within individual NE neuron cell bodies (identified by co-expression of DBH and TH) was not apparent in these RNAscope images. Together with our previous RNAscope images showing co-expression of DBH, TH, and SLC6A2 within individual cells (Figure 3-figure supplement 4), in our view, these results provide strong evidence that the observed TH⁺ cells in the LC are NE-producing neurons, and the data do not provide supporting evidence for the existence of DA-synthesizing neurons in the human LC.

For the manual annotation, it would be useful to include HE tissue images to better understand how the annotations were derived especially because the annotations are not well corroborated by the clustering.

We have now included the H&E stained histology images for the Visium samples in Figure 2-figure supplement 2A, which can be compared with the previous figures showing the manual annotations for the LC regions (Figure 2-figure supplement 1). The histology images can also be viewed at higher resolution through the Shiny web app (https://libd.shinyapps.io/locus-c_Visium/).

The unsupervised clustering is certainly contingent on the number of genes detected, which is in turn dependent on the quality of the material and the success of the experiment. It is unclear from the methods whether the samples were pooled for clustering. If they were pooled, the author might consider using only the samples with UMIs > 500. The low UMI may represent free-floating RNA, suggesting issues with tissue permeabilization in turn influencing the ability to confidently associate genes with spots. Sticking with the higher quality sample may improve the ability to perform unsupervised clustering.

For the spot-level unsupervised clustering using BayesSpace, our aim was to demonstrate whether it is feasible to segment the LC and non-LC regions in the Visium samples in a data-driven manner using a spatial clustering algorithm, instead of relying on manual annotations. We performed clustering across samples (i.e. pooled) -- we have included additional wording in the text and figure caption to clarify this. We agree with the reviewer there may be further optimizations possible, such as filtering out spots or samples with low UMI counts. However, filtering out low-UMI spots may also confound the clustering if low-UMI spots are associated with biological signal (e.g. preferentially located in white matter regions).

Overall, we found that applying data-driven methods such as BayesSpace to segment the LC and non-LC regions did not perform sufficiently to rely on for our downstream analyses (Figure 2-figure supplement 6), and, in our view, further incremental optimizations were unlikely to reach sufficient performance and robustness, so we chose to rely on the manual annotations instead. In addition, as noted in the Results, this avoids potentially inflated false discoveries due to issues of circularity when performing differential gene expression testing between regions defined by unsupervised clustering on the same sets of genes (Gao et al.

2022). We included the BayesSpace results (Figure 2-figure supplement 6) to provide information and ideas to method developers interested in using this dataset as a test case for further development of spatial clustering algorithms. However, further adapting or optimizing these spatial clustering algorithms ourselves was not within the scope of our current work.

It is not entirely clear why the authors used FANS, especially with the scored tissue. Do the authors think this could have negatively influenced the capture of the desired cell type since FANS can compromise the integrity of the nuclei? In other words, have the authors considered that this may have resulted in a loss rather than enrichment? The proportion of "NE" neurons in the snRNA-Seq data is less than 2% in all cases and at its lowest in sample 6522 which does not correspond well with the proportion of tissue that was manually annotated as containing NE cells, even when taken into consideration the potential size difference of cells. In the same vein, in some samples, there are more "5-HT" neurons in the region than "NE" according to the numbers.

As noted in our initial response to reviewers ("Response to Public Review Comments"), we used FANS to enrich for neurons based on our previous success with this approach to identify relatively rare neuronal 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 and low absolute number of this neuronal population (e.g. estimates of ~50K total in the entire human LC).

We do not have a definitive answer to the question of whether our use of FANS to enrich for neurons may have led to damage and contributed to the low recovery rate of LC-NE neurons (as well as the relatively increased levels of mitochondrial contamination compared to other brain regions / preparations in the human brain in our hands). Due to our limited tissue resources for this study, we did not have sufficient tissue to perform a direct comparison with non-sorted data. However, we agree with the reviewer that this is plausible, and warrants further investigation in future work. In particular, the relatively large size and fragility of LC-NE neurons, as well as our use of a standard cell straining approach (70 μ m, which may not be ideal for this population), may also be contributing factors.

Systematically optimizing the preparation to attempt to increase recovery rate (and decrease mitochondrial contamination) are important avenues for future work, and we have decided to share our data and experiences now to assist other groups performing related work. We have included additional wording in the Discussion to further highlight these issues.

The majority of the snRNA-seq remained unannotated "ambiguous" neurons. It would be highly advantageous to include an annotation for these numerous cells.

These nuclei were unidentifiable due to ambiguous marker gene expression profiles, i.e. expression of pan-neuronal marker genes without clear expression of either excitatory or inhibitory neuronal marker genes (see Figure 3A and Figure 3-figure supplement 8). Since we were not able to clearly identify these clusters, and due to our additional concerns regarding the data quality (e.g. low recovery rate of the NE neuron population of interest, potential cell damage, and mitochondrial contamination), we decided to label these neuronal clusters as "ambiguous" instead of assigning low-confidence cluster labels. We have included additional wording in the Results section to explain this issue.

The most likely explanation for identifying serotonergic neurons in these samples is the inclusion of the Raphe Nucleus within the dissection, especially since these cells do not map to the LC per se. As such, is there a way to neuroanatomically define the potential

inclusion of this region from these tissue blocks used? Or to the contrary, definitively demonstrate the exclusion of the Raphe?

As noted in our initial response to reviewers (“Response to Public Review Comments”), our dissection strategy in this initial study precluded the ability to keep track of the exact orientation of the tissue sections on the Visium arrays with respect to their location within the brainstem. Therefore, it is not possible to definitively answer the question of whether the dissections included the raphe nucleus, and if so, which portion of it, based on neuroanatomy from the tissue blocks.

However, during the course of this study and in parallel, ongoing work for other small, challenging brain regions, we 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, e.g. keeping track of the orientation of the dissections and potential inclusion of adjacent neuroanatomical structures. We have included additional details on this issue in the Discussion.

Given that one sample (Visium capture area) was excluded as it did not seem to contain a representation of the LC for the profiling of "NE" cells, does it make sense to include this sample in the analysis of 5HT cells given the authors are trying to make claims about the cell composition in and around the LC? Since there appears to be little 5HT contribution from this sample and its inclusion results in inconsistency across experiments and not any notable advantages, the authors might want to reconsider its inclusion in the results.

We identified a cluster of 5-HT neurons in the snRNA-seq data (Figure 3) and used the Visium samples to further investigate the spatial distribution of this population (Figure 3-figure supplement 9). For the enrichment analyses in the Visium data (Figure 3-figure supplement 9C), we used only the 8 Visium samples that passed quality control (QC). We included the 9th sample (which did not pass QC) in the spot plot visualizations (Figure 3-figure supplement 9A-B) for completeness, but did not base our main conclusions on this sample (in this sample, the tissue resource was likely depleted during earlier sections, so the section for the Visium sample was taken slightly past the extent of the LC within this tissue block). We have included additional wording in the Results section and figure captions to clarify this issue.

For the RNAscope images, it would be useful to include (draw) the manual annotation of the LC to facilitate interpretation. This is especially useful for demonstrating the separate populations of 5HT and "NE" cells. In general, it would be useful to keep a hashed line perimeter for all sections processed by Visium.

We have now added a dashed outline indicating the manually annotated LC region in the RNAscope image showing the full tissue section (Figure 3-figure supplement 11). The high-magnification RNAscope images (Figure 3-figure supplement 4, 16, and 17) show regions entirely within the LC regions – we have included additional wording to note this in the figure captions. For the Visium spot

plots, we either labeled spots within the annotated regions within the figures or included additional wording in the figure captions to refer to the figures showing the annotations (Figure 2-figure supplement 1).

The authors state that they successfully mapped the NE neuron population from snRNA-seq to the manually annotated regions on the Visium slides. Based on the color-coded map, these results are not very convincing since the abundance of the given transcript profile is extremely low. Here again, it would help to draw a hashed line perimeter on the slide to denote the manually annotated region. Perhaps the authors could try a different

strategy for mapping snRNA signal to the slide? However, it appears that the mapping worked better for the capture areas with higher UMI/genes counts. Perhaps the authors should consider using only the slides with high gene/UMI counts.

We agree that the performance of these analyses (Figure 3-figure supplement 14) was not clearly described in the previous version of the manuscript. We have rewritten the corresponding paragraph in the Results section to make it more clear that the mapping (spot-level deconvolution) performance was relatively poor overall, and that we did not use these results for further downstream analyses. We did however want to include these results from the cell2location algorithm to provide information and data for method developers on the challenges of these types of analyses in our dataset (e.g. due to the presence of rare populations, relatively subtle differences in expression profiles between neuronal subpopulations, and potential issues due to large nuclei size and high transcriptional activity for NE neurons). While further approaches for these types of analyses exist, and additional optimizations such as subsetting samples or spots with high UMI counts could also be investigated, in our view, these further optimizations lie outside the scope of our current work. We have also added wording in the figure caption to refer to Figure 2-figure supplement 1, which displays the corresponding annotated LC regions per sample.

It is hard to see if the RNA scope image Supplementary Figure 11 shows co-localization of SLC6A2, TH, and DBH. Having the individual image from each microscope filter along with the merged image is required to properly assess the colocalization of the signals.

We updated the multi-channel RNAscope images to show both the merged channels and individual channels in separate panels (Figure 3-figure supplement 4, 16, and 17), which makes the visualization more clear. Thank you for this suggestion. (Note that the previous Supplementary Figure 11 has been re-numbered to Figure 3-figure supplement 4.)

The heatmap showing the level of marker transcripts shows a much lower expression of specific markers, TH, DBH, SLC6A2 in NE vs other clusters looks surprisingly low (particularly TH), while the much broader marker SLC18A2 (monoamine transporter) is considerably more differential. What do the authors make of this finding?

This is correct. In the snRNA-seq data, we observed that SLC18A2 is one of the most highly differentially expressed (DE) genes in the NE neuron cluster vs. other neuronal clusters, with a high level of expression in the NE neuron cluster (Figure 3C). Note that this heatmap shows the top 70 DE genes (excluding mitochondrial genes) out of the full list of 327 statistically significant DE genes with elevated expression in the NE neuron cluster (the full list of 327 genes is provided in Supplementary File 2C). While all four of these genes (DBH, TH, SLC6A2, and SLC18A2) are identified as statistically significant DE genes, SLC18A2 is the most highly DE out of these and has an especially high level of expression in the NE neuron cluster, as noted by the reviewer (Figure 3C). This could be due to the fact that SLC18A2 transcripts are expressed at higher absolute levels in these neurons than the transcripts that are more specific to LC-NE neurons. While it is true that SLC18A2 is a “broader” marker in the sense that it is found in more cell types – e.g. cell types within brain nuclei that contain monoaminergic as well as brain nuclei that contain catecholaminergic cells – expression of SLC18A2 within the LC is highly specific to the catecholaminergic LC-NE neurons given its specialized functional role within monoamine and catecholamine neurons in packaging amine neurotransmitters into synaptic vesicles. We note that SLC18A2 plays a specialized role that is critical to the core function of LC-NE neurons, and hence we are not particularly surprised with this finding and think that one possibility is that this differential expression appears more robustly due to higher absolute levels of the marker.

While it is understandable that the authors decided to include cells/nuclei with high mitochondrial reads, further work is needed to ensure these cells are of sufficient quality to use in an unbiased way knowing that a high percentage of mitochondrial reads in nuclei sequencing is usually indicative of low-quality nuclei. This can be assessed by evaluating the quality of the nuclei with GWA, which stains an intact nuclear membrane acting as a measure of the integrity of the nuclei.

To further investigate these results, we added additional analyses evaluating quality control (QC) metrics for the NE neuron cluster in the snRNA-seq data, which had an unusually high proportion of mitochondrial reads (Figure 3-figure supplement 2, shown also below in comments for Reviewer 3) (see also related Figure 3-figure supplement 1, 3, which were included in the manuscript previously). These additional QC analyses do not show any other problematic values for this cluster, other than the high mitochondrial proportion, so we do not believe this is purely a data quality issue. We are aware that this is an unexpected result – in most cell populations, a high proportion of mitochondrial reads would be indicative of cell damage and poor data quality. However, we have recently also observed high mitochondrial proportions in other relatively rare neuronal populations characterized by large size and high metabolic demand. As discussed below for Reviewer 3, we believe that this is mitochondrial “contamination”, as there should be no mitochondrial reads per se within the nuclear compartment.

However, it may be possible that in cell populations that have abundant levels of mitochondria and high transcript expression of mitochondrial transcripts in the cell body, that the likelihood of ambient RNA capture of mitochondrial transcripts during nuclear preparation may be higher than for other cell types that have lower expression of mitochondrial transcripts. Hence, we believe that our interpretation is likely correct, i.e. that a combination of technical and biological factors contributes to the inclusion of a relatively high amount of mitochondrial RNA within the droplets for these nuclei. We agree with the reviewer that this finding warrants further investigation in future work. However, in our current study, the tissue resource is depleted for any further experimental validation of this question, so we preferred to provide our data to the community in its current form, while transparently noting this unexpected finding in our results. We have included additional text in the Results section describing the new QC analyses shown in Figure 3-figure supplement 2.

Minor comments:

Line 319-321 could be written more clearly to indicate that due to the lack of resolution in a given spot, there are "contaminating reads" that reduce the precision of the cell profile. This reduced precision is likely what results in the "lack of conservation" across species.

We have added additional wording to this sentence to clarify this point.

In the discussion, the authors write that the analyses "unbiasedly identified a number of genes enriched in human LC", however, given the manual annotation of the region for each capture area, this resulted in a biased assessment of the spots.

We have replaced this wording to refer to “untargeted, transcriptome-wide” analyses (i.e. analyses that are not based on a targeted panel of genes) instead of “unbiased”. We agree that the meaning of “unbiased” is ambiguous in this context.

Reviewer #3 (Recommendations For The Authors):

Major points:

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. Perhaps more conservative language would be appropriate (i.e. "cells that possess mRNA signatures of serotonergic neurons" or something like that). Did these cells co-express other markers one would expect in 5-HT neurons like 5-HT autoreceptors and SLC6A18? Also would be useful to compare expression profiles of these putative 5-HT neurons with any published material on bona fide dorsal raphe 5-HT neurons. For the RNAscope confirmation in the supplementary material, it would be helpful to show each marker separately as well as the overlay, and to include representative higher magnification images like were provided for the ACH markers.

Thank you for this comment. In order to further investigate the identity of these cells, we have investigated the expression of several additional genes including SLC6A18, 5-HT autoreceptor genes (HTR1A, HTR1B), marker genes for 5-HT neurons (SLC18A2, FEV), and marker genes for 5-HT neuronal subpopulations within the dorsal and median raphe nuclei from the literature (Ren et al. 2019), in both the Visium and the snRNA-seq data.

We observed some expression of SLC18A2 and FEV within the same areas as SLC6A4 and TPH2 in the Visium samples (Figure 3-figure supplement 10A-B, reproduced below; note that SLC18A2 is also a marker gene for NE neurons located within the LC regions), consistent with Ren et al. (2019). However, we did not observe a strong or consistent expression signal for the 5-HT autoreceptors (HTR1A, HTR1B) (Figure 3-figure supplement 10C-D, reproduced below), and we observed zero expression of SLC6A18 in the Visium samples. In the snRNA-seq data, within the cluster identified as 5-HT neurons, we observed some expression of SLC18A2, low expression of FEV, and almost zero expression of SLC6A18 (Figure 3-figure supplement 8, reproduced below; note that SLC6A18 is not shown since it was removed during filtering for low-expressed genes). Similarly, we observed very low expression of the 5-HT autoreceptors (HTR1A, HTR1B) and the additional marker genes for 5-HT neuronal subpopulations from Ren et al. (2019) -- with the possible exception of the neuropeptide receptor gene HCRT2, which was identified by Ren et al. (2019) within several clusters in both the dorsal and median raphe in mice (Figure 3-figure supplement 8, reproduced below).

Overall, these additional results give us some further confidence that these are likely 5-HT neurons (due to expression of SLC18A2 and FEV), while also raising further questions (due to the absence of 5-HT autoreceptor genes HTR1A, HTR1B and 5-HT neuronal subpopulation marker genes). While we believe that the most likely explanation is the inclusion of 5-HT neurons from the edges of the adjacent dorsal raphe nuclei in our samples, we acknowledge that the evidence presented is not fully conclusive and does not identify specific subpopulations of 5-HT neurons. In addition, the limited size of our dataset (number of samples and cells) and the lack of information on sample orientation precludes any definitive identification of subpopulations based on their association with specific anatomical regions within the dorsal raphe nuclei. We have updated the manuscript by (i) adjusting our language in the Results and Discussion, (ii) including the additional analyses, supplementary figures, and reference to the literature (Ren et al. 2019) discussed above, and (iii) including additional wording in the Discussion on improvements to the dissection strategy that would allow these questions to be addressed in future studies via a focused molecular profiling of the dorsal raphe nuclei across the rostral-caudal axis.

Regarding the RNAscope images, we have included additional images showing channels side-by-side and higher magnification, as suggested (and also discussed above for Reviewers 1 and 2). In addition, we have added an outline highlighting the LC region in Figure 3-figure supplement 11 (as suggested above by Reviewer 2), and included an additional high-magnification RNAscope image demonstrating co-expression of 5-HT neuron marker genes (TPH2 and SLC6A4) within individual cells (Figure 3-figure supplement 12).

Concerning the snRNA-seq experiments, why were only 3 of the 5 donors used, particularly given the low number of LC-NE nuclear transcriptomes obtained? How were the 3 donors chosen from the 5 total donors and how many 100 µm sections were used from each donor? Are the 295 nuclei obtained truly representative of the LC population or are they just the most resilient LC nuclei? How many LC nuclei would be estimated to be captured from staining the 100 µm tissue sections?

As discussed in our previous response to reviewers (“Response to Public Review Comments”), the reason we included only 3 of the 5 donors for the snRNA-seq assays was due to tissue availability on the tissue blocks. In this study, we were working with a finite tissue resource. Due to the logistics and thickness of the required tissue sections for Visium (10 µm) and snRNA-seq (100 µm), running Visium first allowed us to ensure that we could collect data from both assays – if we ran snRNA-seq first and captured no neurons, the tissue block would be depleted. Due to resource depletion, we did not have sufficient available tissue remaining on all tissue blocks to run the snRNA-seq assay for all donors. 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 have included details on the number of 100 µm sections used for each donor in Methods – this varied between 10-15 sections per donor, approximating 50-80 mg of tissue per donor.

Regarding the question about the representativeness / resilience of the LC nuclei – as discussed in our previous response to reviewers (“Response to Public Review Comments”) and above for Reviewer 2, we agree that this is a concern. As discussed above for Reviewer 2, it is plausible that our use of FANS may have contributed to cell damage and the low recovery rate of LC-NE neurons. The relatively large size and fragility of LC-NE neurons, as well as our use of a standard cell straining approach (70 µm, which may not be ideal for this population), may also be contributing factors. Due to our limited tissue resource, we did not have sufficient tissue to perform a direct comparison with non-sorted data.

Systematically optimizing the preparation to attempt to increase recovery rate is an important avenue for future work. We have included additional discussion of this issue in the Discussion.

Regarding the question about the number of expected nuclei, we have now included estimates of the number of cells per spot within the LC regions in the Visium data (see also related point below, and Figure 2-figure supplement 2B reproduced below), based on the H&E stained histology images and use of cell segmentation software (VistoSeg; Tippianni et al. 2022). While we do not have any confident estimates of the number of expected nuclei in the snRNA-seq data, these estimates of cell density from the Visium data could, together with information on additional factors such as the accuracy of the tissue scoring and the effectiveness of FANS, be used to help derive an expected number of nuclei in future studies. We have included additional wording in the Discussion to note that these estimates could be used in this manner during future studies.

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). Which part(s) of the LC was captured for the SRT and snRNAseq experiments?

As discussed in our previous response to reviewers (“Response to Public Review Comments”), 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. We have included additional details in the Discussion to further discuss this point.

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. In this specific case, can the authors estimate how many LC cells were contained in each expression spot?

We have now performed additional analyses to provide an estimate of the number of cells per spot in the Visium data (Figure 2-figure supplement 2B), based on the application of cell segmentation software (VistoSeg; Tippi et al. 2022) to identify cell bodies in the H&E stained histology images. We applied this methodology and calculated summary statistics within the annotated LC regions for 6 samples (see Methods), and found that the median number of cells per spot within the LC regions ranged from 2 to 5 per sample. We note that these estimates include both NE neurons and other cell types within the LC regions, and that applying cell segmentation software in this brain region is particularly challenging due to the wide range in cell body sizes, with NE neurons being especially large. We have included these updated estimates in the Results and Discussion, and additional details in Methods.

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 rodent 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>

Our comparisons with the mouse (Mulvey et al. 2018) and rat (Grimm et al. 2004) data showed that we observed a relatively higher overlap between the human vs. mouse data than the human vs. rat data (Figures 2F-G and 3D-E). However, we note that the substantially different technologies used (TRAP-seq in mouse vs. laser capture microdissection and microarrays in rat) make it difficult to confidently interpret the degree of overlap between the two studies, and a direct comparison of these alternative platforms (TRAP-seq vs. LCM / microarray) or species (mouse vs. rat) lies outside the scope of our study. We have included updated wording in the Results and Discussion to explain this issue and help interpret these results.

Regarding the newer mouse study using snRNA-seq (Luskin and Li et al. 2022), we have extended our analyses to perform a more in-depth comparison with this study. Specifically, we have evaluated the expression of an additional set of GABAergic neuron marker genes from this study within our secondary clustering of inhibitory neurons in the snRNA-seq data

(Figure 3-figure supplement 13B). We observe some evidence of cluster-specific expression of several genes, including CCK, PCSK1, PCSK2, PCSK1N, PENK, PNOC, SST, and TAC1. We have also included additional text describing these results in the Results section.

The finding of AChE expression in LC neurons is intriguing. Susan Greenfield has published a series of papers suggesting that AChE has functions independent of ACh metabolism that contributes to cellular vulnerability in neurodegenerative disease. This might be worth mentioning.

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 neuron populations (e.g. dopaminergic neurons in the substantia nigra) is very interesting. We have included references to this work and how it could inform interpretation of this expression (Greenfield 1991; Halliday and Greenfield 2012) in the Discussion.

High mitochondrial reads from snRNA-seq can indicate lower quality. Can the authors comment on this and explain why they are confident in the snRNA-seq data from presumptive LC-NE neurons?

As mentioned above for Reviewer 2, we have included additional analyses to further compare quality control (QC) metrics for the NE neuron cluster (which had an unusually high proportion of mitochondrial reads) against other neuronal and non-neuronal clusters and nuclei in the snRNA-seq data (Figure 3-figure supplement 2). These additional QC analyses do not show any other problematic values for this cluster. Specifically, we show that the QC metric values for sum UMIs and detected genes per droplet for the NE neuron cluster fall within the range for (A) other neurons and (B) all other nuclei (excluding droplets with ambiguous / unidentifiable neuronal signatures). In addition, we observe that the droplets with the highest mitochondrial percentages (>75%) (C-D), which also have unusually low number of detected genes (D), tend to be from the ambiguous category (droplets with ambiguous / unidentifiable neuronal signatures), suggesting that true low-quality droplets are correctly identified and included within the ambiguous category (e.g. consisting of a mixture of debris from partial damaged nuclei) instead of as NE neurons. Since our QC analyses for the NE neuron cluster do not show any problems other than the high mitochondrial percentage, we do not believe these are simply mis-classified low-quality droplets. We also note that we have recently observed high mitochondrial proportions in other relatively rare neuronal populations characterized by large size and high metabolic demand in human data. We believe that our interpretation is correct – i.e. that a combination of technical and biological factors has led to the inclusion of a relatively high amount of mitochondrial RNA within the droplets for these nuclei. We have included these additional QC analyses (Figure 3-figure supplement 2) and further discussion of this issue in the Results section.

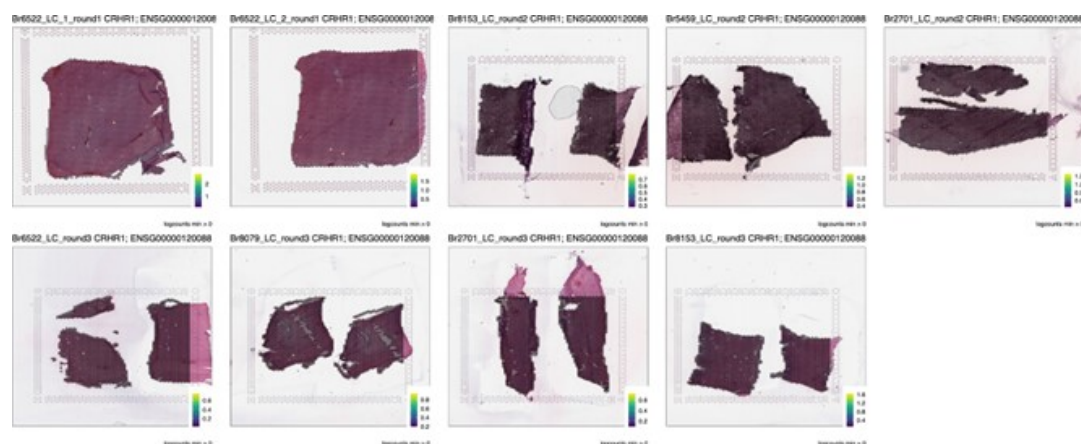
The Discussion could be expanded. Because there is a lot known and/or assumed about the LC, discussing all of it is certainly beyond the scope of this manuscript. However, perhaps the authors could pick a few more for confirmation and hypothesis generation. For example, one of the most well studied and important aspects of the LC is its regulation by neuromodulatory inputs. It would be interesting for the authors to discuss the expression of receptors for CRF, cannabinoids, orexin, galanin, 5-HT, etc, particularly when compared with the available rodent TRAP and snRNA-seq data (<https://www.biorxiv.org/content/10.1101/2022.06.30.498327v1>) contained some surprises, such as very low

expression of CRF1 in LC-NE neurons, suggesting that the powerful activation of LC cells by CRF is indirect. Does this hold up in humans?

We have expanded the Discussion to include additional discussion and references on several points, as discussed also above. Indeed these are interesting questions and these neuromodulatory systems are all of interest in the context of signaling within the LC in terms of function of the LC-NE system. We note that the manuscript serves primarily as a data resource and will be useful in many different ways depending on the different goals and interests of the readers. This is precisely why we wanted to take the time to make accessible and easy to use tools to interrogate and visualize the data. We have provided screenshots in Author response image 1-4 from the Shiny visualization app for the Visium data (https://libd.shinyapps.io/locus-c_Visium/) querying several main receptors of the neuromodulatory systems that this reviewer is particularly interested in to illustrate how the visualization apps can readily be used to query specific genes and systems of interest.

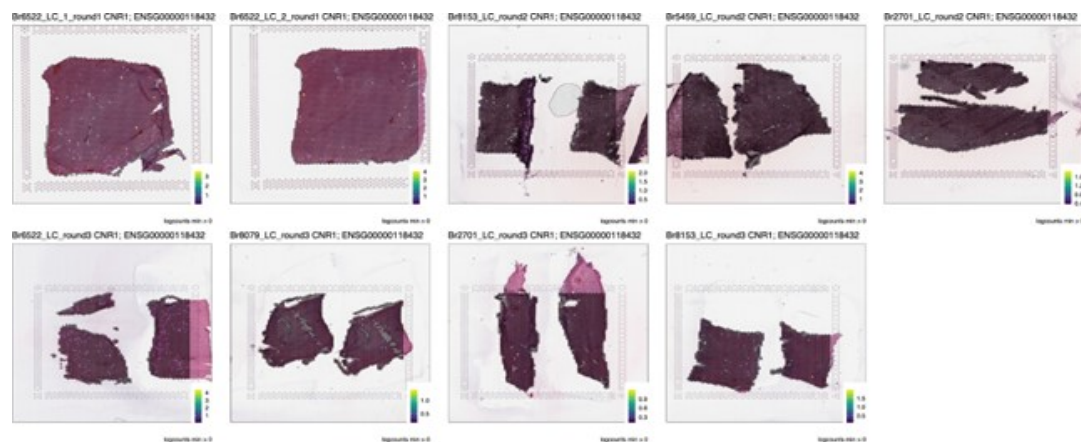
Author response image 1.

CRHR1:



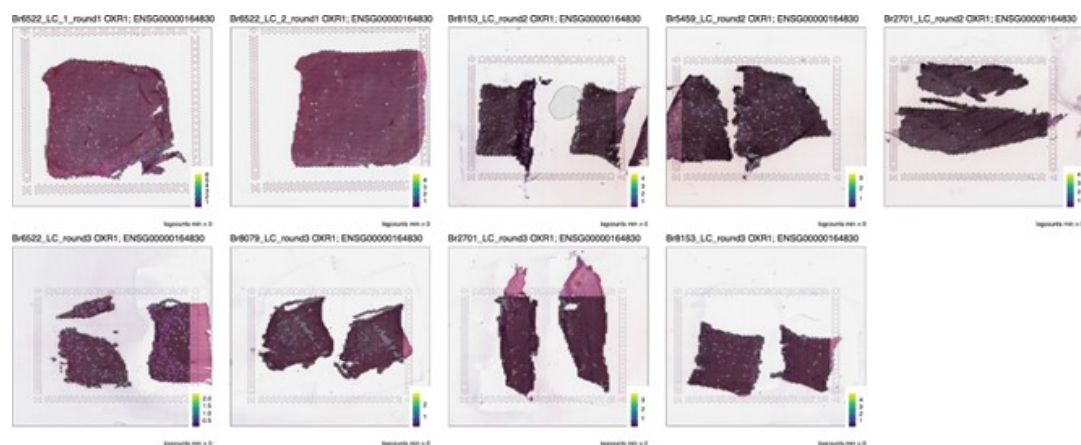
Author response image 2.

CNR1:



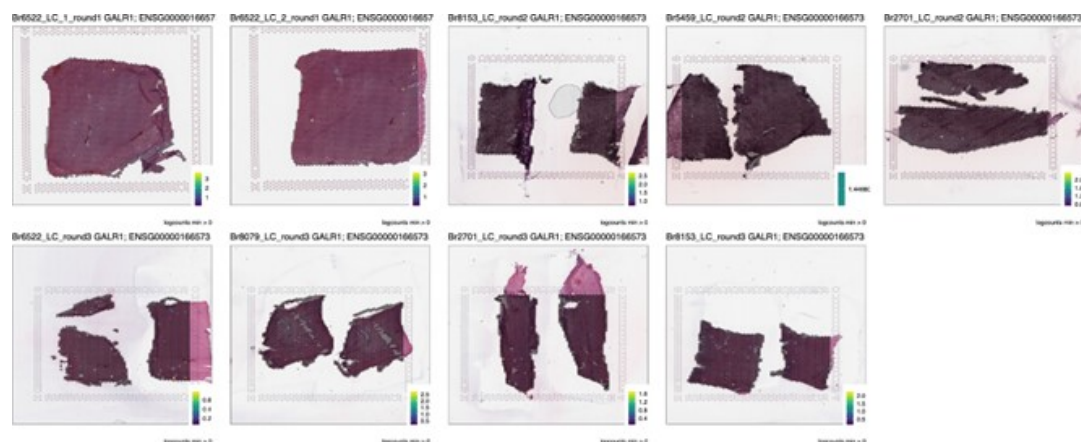
Author response image 3.

OXR1:



Author response image 4.

GALR1:



Minor points:

Line 46 add stress responses to the key functions of LC neurons

We have added this point and included additional references to support the findings.

Line 47 add that the LC was so named "blue spot" because of its signature production of neuromelanin pigment

We have added this point.

Line 49 LC's capacity to synthesize NE is not "unique" - several other brainstem/medullary nuclei also synthesize NE (e.g. A1-A7; LC is A6)

We have updated this wording.

Line 54 Although prior evidence indicated age-related LC cell loss in people without frank neurodegenerative disease, recent studies that are better powered and used unbiased stereological methods have refuted the idea that LC neurons die during normal aging (reviewed in Matchett et al., Acta Neuropathologica 141:631-50, 2021)

We have updated this part of the Introduction to focus on cell loss in the LC in neurodegenerative disease and removed the older references describing studies that suggested LC neurons die in normal aging.

Line 62 Would also be worth mentioning the role of the LC in other mood disorders where adrenergic drugs are often prescribed, such as PTSD (e.g. prazosin), opioid withdrawal (e.g. lofexidine), anxiety and depression (e.g. NE reuptake inhibitors).

We have added additional references to these disorders and their treatment with noradrenergic drugs in the Introduction.

We have also included the following updates, in response to additional reviewer comments received during the initial round of “Public Review Comments” and which are not already described in the responses to the “Recommendations for the Authors” above.

- We included updated wording in the Results section and Figure 1C caption to more clearly describe the number of donors included in the final SRT and snRNA-seq data used for analyses after all quality control (QC) steps (4 donors for SRT data, 3 donors for snRNA-seq data).
- Figure 3-figure supplement 1D (number of nuclei per cluster in unsupervised clustering of snRNA-seq data) has been updated to show percentages of nuclei per cluster.
- We have added comparisons between the lists of differentially expressed (DE) genes identified in the Visium and snRNA-seq data. To make these sets comparable, we have added (i) snRNA-seq DE testing results between the NE neuron cluster and all other clusters (instead of other neuronal clusters only, as shown in the main results in Figure 3) (excluding ambiguous neuronal) (Figure 3-figure supplement 6 and Supplementary File 2D), and (ii) calculated overlaps and comparisons between the sets of DE genes between the Visium data (pseudobulked LC vs. non-LC regions) and the snRNA-seq data (NE neuron cluster vs. all other clusters excluding ambiguous neuronal). This comparison generated a list of 51 genes that were identified as statistically significant DE genes ($FDR < 0.05$ and $FC > 2$) in both the Visium and the snRNA-seq data (Figure 3-figure supplement 7 and Supplementary File 2E).

Other additional updates:

We have added an additional data repository (Globus). Raw data files (FASTQ sequencing data files and high-resolution TIF image files) are now available via Globus from the WeberDivecha2023_locus_coeruleus data collection from the jhpce#globus01 Globus endpoint, which is also listed at <http://research.libd.org/globus/>. The Globus repository is not publicly accessible due to individually identifiable donor genetic variants in the FASTQ files. Approved users may request access from the corresponding authors. This data repository is listed in the Data Availability section.