

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Confirmation of specific labeling of sensory and sympathetic innervation of LN with Nav1.8^{Cre} and TH

Related to Figure 1

A. 3D reconstruction of a representative confocal image of whole-mount popLNs from *ChAT^{BAC}-eGFP* animals, where cholinergic cells are genetically labeled with GFP, stained for β 3-Tubulin (red), GFP (green). **B.** 3D reconstruction of a representative confocal image of whole-mount iLNs from tamoxifen-treated *Bmx-CreER^{T2} Rosa26^{LSL-eYFP/+}* animals, where arterial endothelial cells are genetically labeled with YFP, stained for β 3-Tubulin (red), YFP (green), and smooth muscle actin (SMA, blue), demonstrating preferential association between nerves and arterial vessels supplying LNs. **C.** The schematic for fluorescent wheat-germ agglutinin (WGA) -based retrograde tracing of iLN-innervating sensory neurons. **D** and **E.** Representative confocal images of sections from DRGs and SGs containing WGA-AF488⁺ retrogradely-labeled iLN-innervating neurons of *Nav1.8^{Cre/+}; Rosa26^{LSL-tdTomato/+}* animals with endogenous fluorescence from tdTomato and WGA-AF488 shown in red and green, respectively. **F** and **G.** Representative confocal images of sections from DRGs and SGs containing WGA-AF488⁺ retrogradely-labeled iLN-innervating neurons of *Nav1.8^{Cre/+}; Rosa26^{LSL-tdTomato/+}* animals, with TH stained in red and endogenous fluorescence from WGA-AF488 shown in green. **H.** Quantification of the percentage of WGA-AF488⁺ retrogradely-labeled LN-innervating DRG or SG neurons that are genetically labeled with Nav1.8^{Cre} or are TH⁺. Significance assessed by Welch's t-test, $p=0.0003$ (***), $p<0.001$ (***), based on a total of 60 WGA-AF488⁺ DRG neurons and 167 WGA-AF488⁺ SG neurons from 3 mice.

Figure S2. Anatomical characterization of sensory innervation of different LN subdomains

Related to Figure 2

A. A representative confocal section of whole-mount iLNs from *Nav1.8^{Cre/+}; Rosa26^{tdTomato/+}; Prox1-EGFP* animals, stained for tdTomato (red), GFP (green), CD45 (blue) and CD169 (white), allowing identification of LN subdomains, including T cell zone labeled as (T), B follicles labeled as (B) and the medulla labeled as (M). Note that the outermost layer of GFP⁺ LECs (green) delineates LN boundary. **B.** A representative confocal section of whole-mount iLNs from *Nav1.8^{Cre/+}; Rosa26^{LSL-tdTomato/LSL-tdTomato}* animals, stained for tdTomato (red), PNAd (green) and SMA (blue), demonstrating a general lack of contact between tdTomato⁺ sensory fibers (arrowhead) and PNAd⁺ high endothelial venules (HEVs). **C.** 3D view of a representative confocal substack of whole-mount iLNs from *Nav1.8^{Cre/+}; Rosa26^{LSL-tdTomato/LSL-tdTomato}* animals, stained for tdTomato (red) and CD31 (green), illustrating the perivascular origin of capsular/subcapsular fibers (arrowhead). **D** and **E.** Section view of an *intravital* two-photon micrograph of representative capsular/subcapsular tdTomato⁺ sensory fibers (red) in relation to *in vivo* antibody labeled CD169⁺ SCS macrophages (green) and

collagen fibers (blue) detected by second-harmonic generation microscopy in popliteal LNs from *Nav1.8^{Cre/+}; Rosa26^{LSL-tdTomato/+}* animals, demonstrating the presence of capsular (D) and subcapsular (E) fibers.

Figure S3. Evaluation of the specificity and efficiency of the viral-based retrograde labeling
Related to Figure 3

A. 3D reconstruction of a representative confocal image of whole-mount iLNs targeted for intranodal injection with AAV-Cre from *Rosa26^{LSL-tdTomato/LSL-tdTomato}* animals, stained for tdTomato (red) to show the extent of labeling at the site of injection. **B.** Representative epifluorescence image of tdTomato⁺ DRG neurons retrogradely labeled from perinodal injection in a whole-mount spinal cord-DRG preparation without antibody amplification. **C.** Quantification of the number of retrograde-labeled neurons from intranodal and perinodal injection. Intranodal: 16.25 (mean) $\pm$ 2.394 (SEM); perinodal: 1.500 (mean) $\pm$ 0.6455 (SEM), $p=0.0064$ (**) by Welch's t-test, $n=4$ per group. **D.** 3D reconstruction of a representative confocal image of whole-mount iLNs targeted for intranodal injection with AAV-Flex-tdTomato from *Nav1.8^{Cre/+}; Rosa26^{LSL-eYFP/+}* animals, stained for tdTomato (red) and CD31 (green) to visualize the axonal projections of retrogradely-labeled neurons. **E.** Representative epifluorescence image of tdTomato⁺ DRG neurons retrogradely labeled from the skin overlying the iLN in a whole-mount spinal cord-DRG preparation without antibody amplification. **F.** 3D reconstruction of a representative confocal image of whole-mount skin targeted for intradermal injection with AAV-Flex-eGFP from *Nav1.8^{Cre/+}* animals, stained for GFP (green) and DAPI (blue) to demonstrate the specificity of labeling from the skin. **G.** Soma size distribution of retrogradely-labeled iLN-innervating and randomly sampled CGRP⁺ neurons from the same DRGs following intranodal injection of AAV-Cre into *Rosa26^{LSL-tdTomato/LSL-tdTomato}* animals based on 49 tdTomato⁺ iLN-innervating and 114 CGRP⁺ DRG neurons from 3 and 2 mice, respectively. **H.** 3D reconstruction of a representative confocal image of whole-mount iLNs from *Nav1.8^{Cre/+}; Rosa26^{LSL-tdTomato/LSL-tdTomato}* animals, stained for tdTomato (red), NFH (green) and TH (blue) to visualize co-innervation of LNs by myelinated and unmyelinated sensory fibers.

Figure S4. Expression of neuronal functional pathways by LN- and skin-innervating sensory neurons and evaluation of specificity and sensitivity of select genes as markers of LN-innervating sensory neurons

Related to Figure 5

A. and **B.** Expression of Prokr2 (A) and Ptgir (B) by innervation target and Neuron Type. Significance assessed by Mann-Whitney-Wilcoxon test * $p<0.05$, *** $p<0.001$. **C-E** Heatmaps of gene lists curated in the Usoskin et al. Sensory Neuron Atlas (Usoskin et al., 2015). Blue bar represents skin-innervating neurons, yellow bar represents LN-innervating neurons. **F-I.** Representative confocal images of RNAscope analysis for indicated target (green) on DRG

sections containing tdTomato⁺ retrogradely-labeled neurons (red) (**F**, **H**: LN-innervating, **G**, **I**: skin-innervating).

Figure S5. A single-cell transcriptomic atlas of inguinal LNs (iLN) at steady state

Related to Figure 6

A. tSNE of 9,662 single cells from an initial clustering analysis. * indicates clusters that required additional subclustering to resolve all constituent cell types. **B.** tSNE of T cell parent cluster. **C.** Dot plot of the top DE genes between Tregs and remaining non-Treg T cells. **D.** tSNE of additional subclustering of non-Treg T cells from **B.** **E.** Dot plot of the top DE genes between CD4 T cells and CD8 T cells. **F.** tSNE of parent macrophage and dendritic cell cluster. **G.** Dot plot of the top DE genes between Macrophages and cDC 2 cells. **H.** tSNE of parent blood endothelial cell cluster. **I.** Dot plot of the top DE genes between BEC and HEC. **J.** tSNE of parent NK cells. **K.** Dot plot of the top DE genes between mitotic NK cells and non-mitotic NK cells ("NK cells"). **L.** tSNE of parent Neutrophil cluster. **M.** Dot plot of the top DE genes between Neutrophils 1 and Neutrophils 2. **N.** tSNE of parent Lymphatic Endothelial Cell (LEC) cluster. **O.** Dot plot of the top DE genes between LEC 1 and LEC 2. For all dot plots, circle diameters reflect the percent of cells expressing a given marker within that cell type, circle colors reflect relative expression abundance within that cell type; light grey: low, black: high. **P.** Heatmap of gene-normalized, scaled DGE of the top 10 cell type-specific genes for each identified cell type (yellow: high relative expression; purple: low relative expression). Cell types reflect final clustering after subclustering, cell type-specific genes identified by comparing one cell type to all other cells in the dataset. Cell types are subsampled to 25 cells per cell type for heatmap (see **Table S3**).

Figure S6. Evaluation of methods to determine neuron-LN cell interaction potentials

Related to Figure 6

A. Schematic of method for identifying cognate neuron-LN-cell pairs. **B.** Pearson correlation of Interaction Potential (calculated as in **Figure 6G**) with cell quality (measured as median UMI/cell for each cell type). **C** and **D.** Comparison of cell type summary statistics to calculate Interaction Potential (**C**: average expression, **D**: percent expression) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, dashed lines represent 99% confidence interval from permutation test (see **STAR Methods**). **E.** Comparison of Interaction Potential after downsampling to equivalent cell numbers (using the scaled average Interaction Potential, as in **Figure 6G**). **F.** Left: Heatmap of top expressed neuropeptides in LN-innervating sensory neurons; Right: Dotplot of respective cognate receptor expression (denoted by arrows) in LN cells at steady state (circle diameter reflects the percent of cells expressing a given marker within that cell type, circle color reflects relative expression abundance within that cell type; light grey: low, black: high). **G.** and **H.** Top candidate interaction molecules driving high Interaction Potential in **G.** Non-Endothelial Stromal, **H.** BEC.

Figure S7. scRNA-seq of iLNs following optogenetic neuronal stimulation

Related to Figure 7

A. 3D reconstruction of representative confocal sections of whole-mount iLNs following intranodal injection of AAV-Flex-tdTomato into *Nav1.8^{Cre/+}; Rosa26^{LSL-eYFP/+}* animals, stained for tdTomato (red) and CD31 (green) to reveal the axonal trajectory of iLN-innervating neurons in relation to the site of illumination (arrow). **B.** Abundance of light-induced differentially expressed genes (left: downregulated with light stimulation; right: upregulated with light stimulation) among 2 ChR2+ animals, representing preliminary analysis of optogenetic-based neuronal triggering. **C.** Schematic of mouse cohort and study design. **D.** Correlation between the pseudopopulation average gene expression profile of each cell type from the steady state iLN atlas (**Figure 6**, rows) and the light-stimulated iLN atlas (**Figure 7**, columns). **E.** Quantification of alterations in cell composition by photostimulation, represented as fold change in fractional abundance of each cell type between matched light-exposed and light-unexposed sides within each mouse. Blue circles: ChR2+ mice, n=4; black squares: ChR2- mice, n=3. **F.** Cellularity of light-exposed and light-unexposed iLNs from ChR2- and ChR2+ animals at the end of photostimulation. ChR2-: p= 0.2470 (ns); ChR2: p= 0.6755 (ns) by 2-way ANOVA with Sidak's multiple comparisons test; 5 mice per genotype. **G.** Correlation between abundance of DE genes and interaction potential over multiple of DE gene cutoff parameters.

SUPPLEMENTARY TABLES

Table S1. Metadata-annotated neuron scRNA-Seq data

Related to Figures 3 and 4

Table S2. Differentially expressed genes by Neuron Type and between LN-innervating and skin-innervating neurons, gene ontology lists.

Related to Figures 4 and 5

Table S3. Metadata-annotated steady state LN cell atlas UMI vs. cell matrix

Related to Figure 6

Table S4. Steady state LN cell atlas: Cell type cluster defining genes and subcluster defining genes.

Related to Figure 6

Table S5. Differentially expressed genes following neuronal stimulation by cell type and genotype.

Related to Figure 7

SUPPLEMENTARY MOVIES

Movie 1. 3D animation of Figure 1A.

Movie 2. 3D animation of Figure 1B.

Movie 3. 3D animation of Figure 2A.

Movie 4. Slice view of Figure 2C.

Movie 5. 3D animation of Figure 2E.

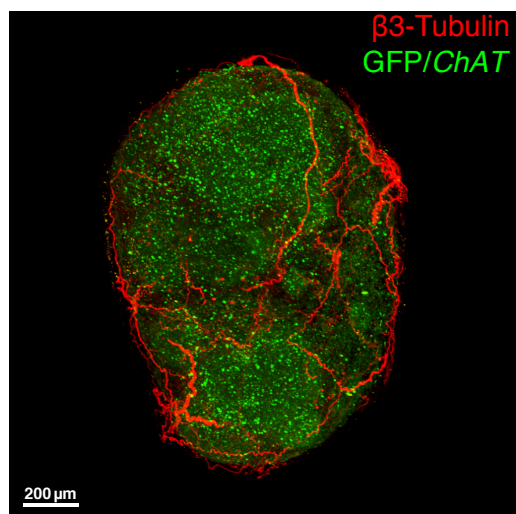
Movie 6. 3D animation of surface rendering of Figure S2B.

Movie 7. 3D animation of Figure 2F.

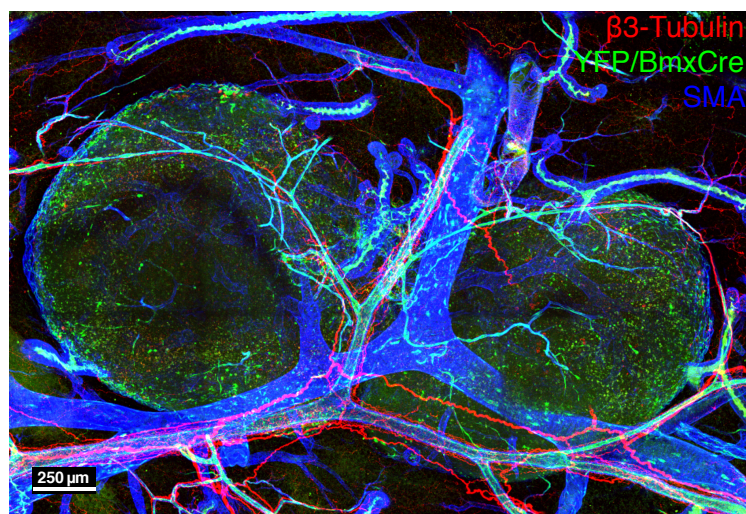
Movie 8. 3D animation of Figure S3D.

Figure S1

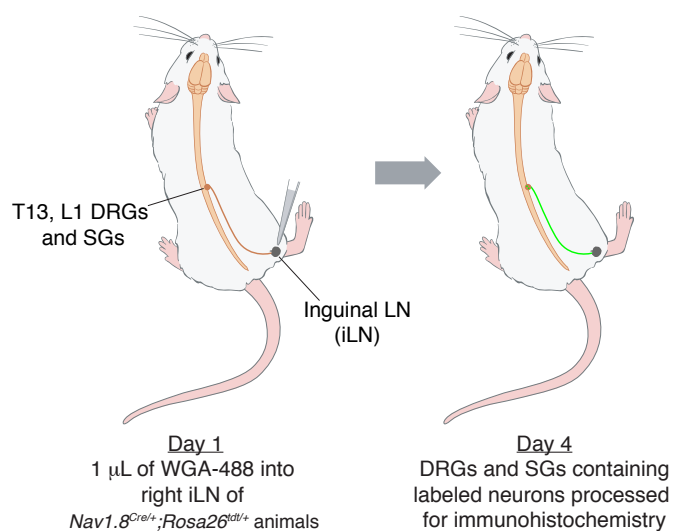
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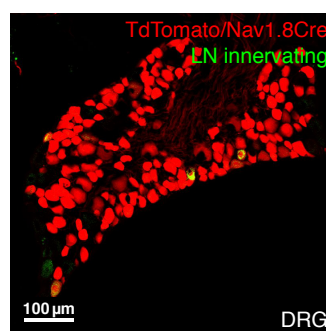
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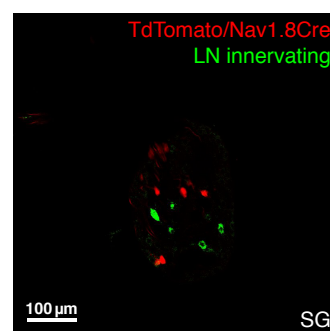
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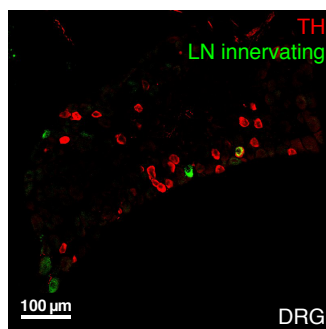
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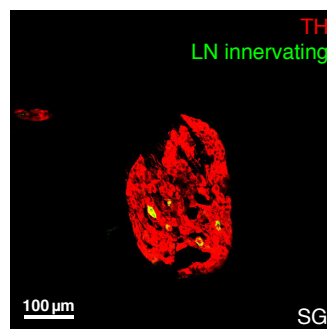
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F



G



H

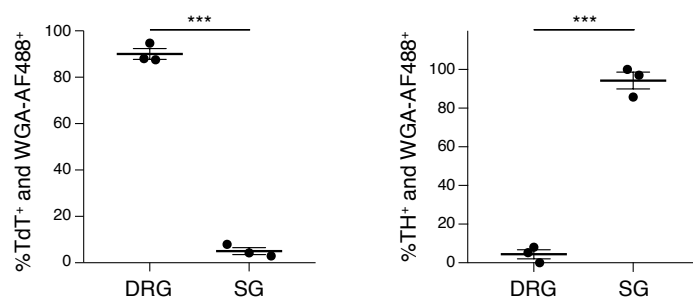
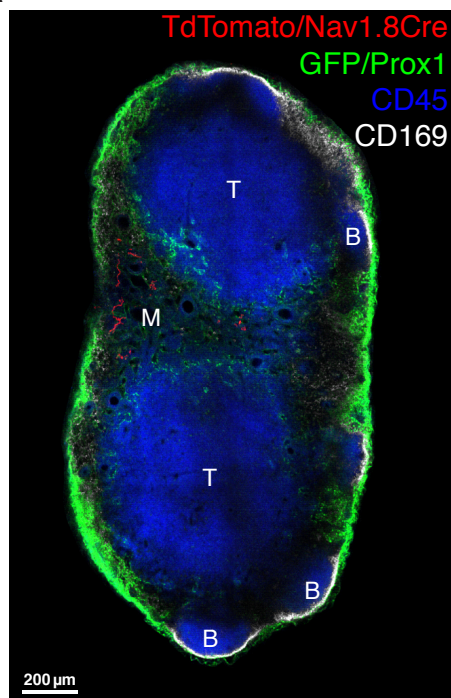
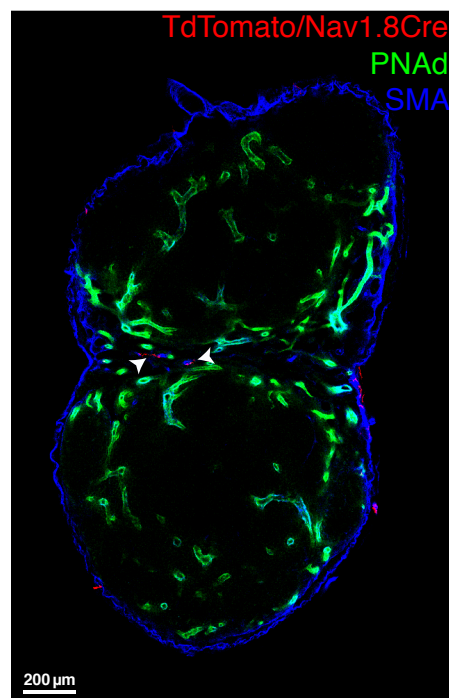


Figure S2

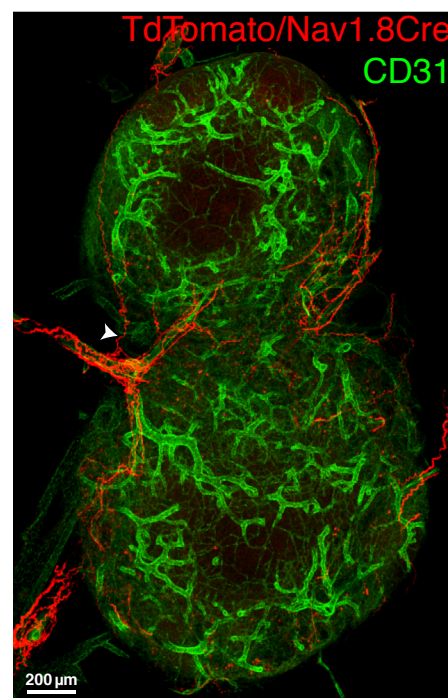
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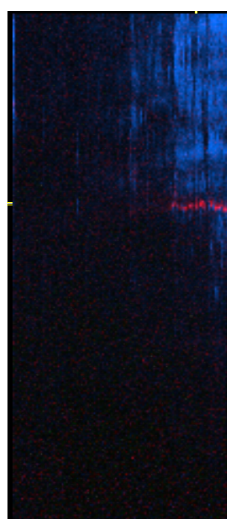
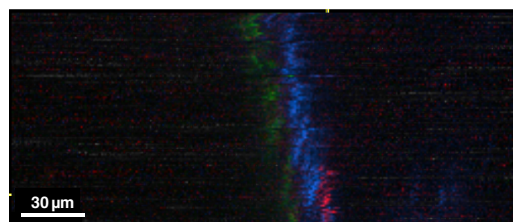
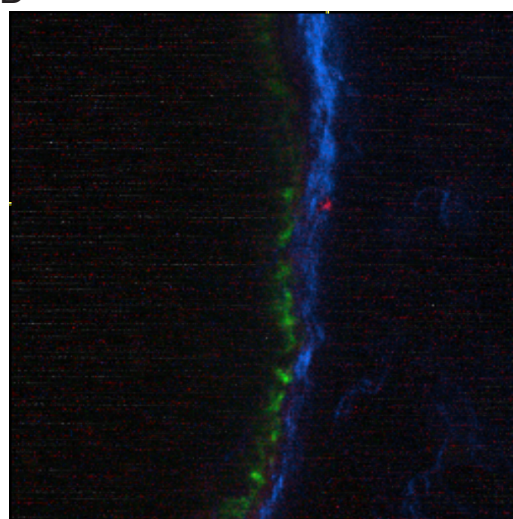
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C

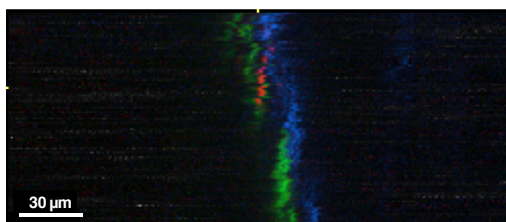
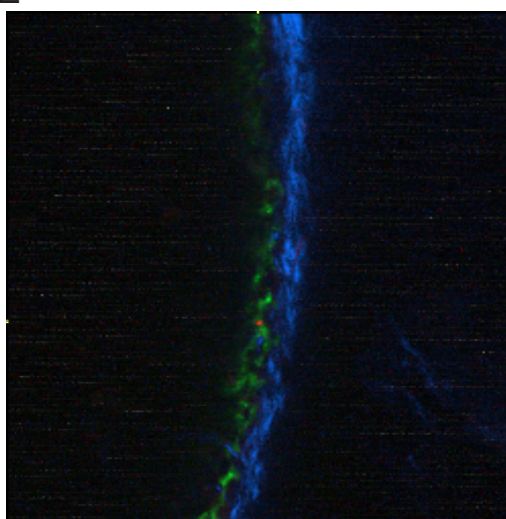


D



TdTomato/
Nav1.8Cre
CD169
Collagen

E



TdTomato/
Nav1.8Cre
CD169
Collagen

Figure S3

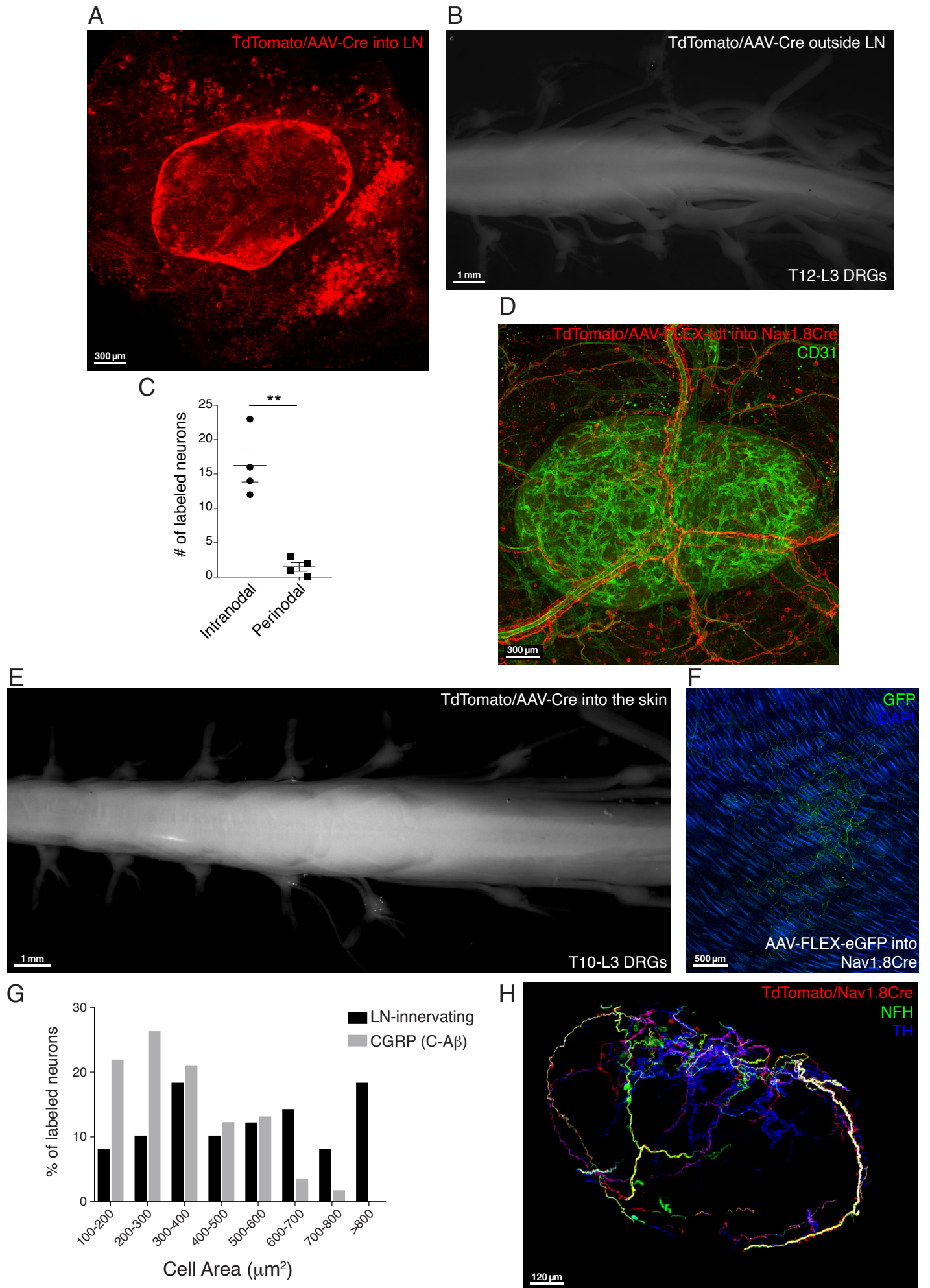


Figure S4

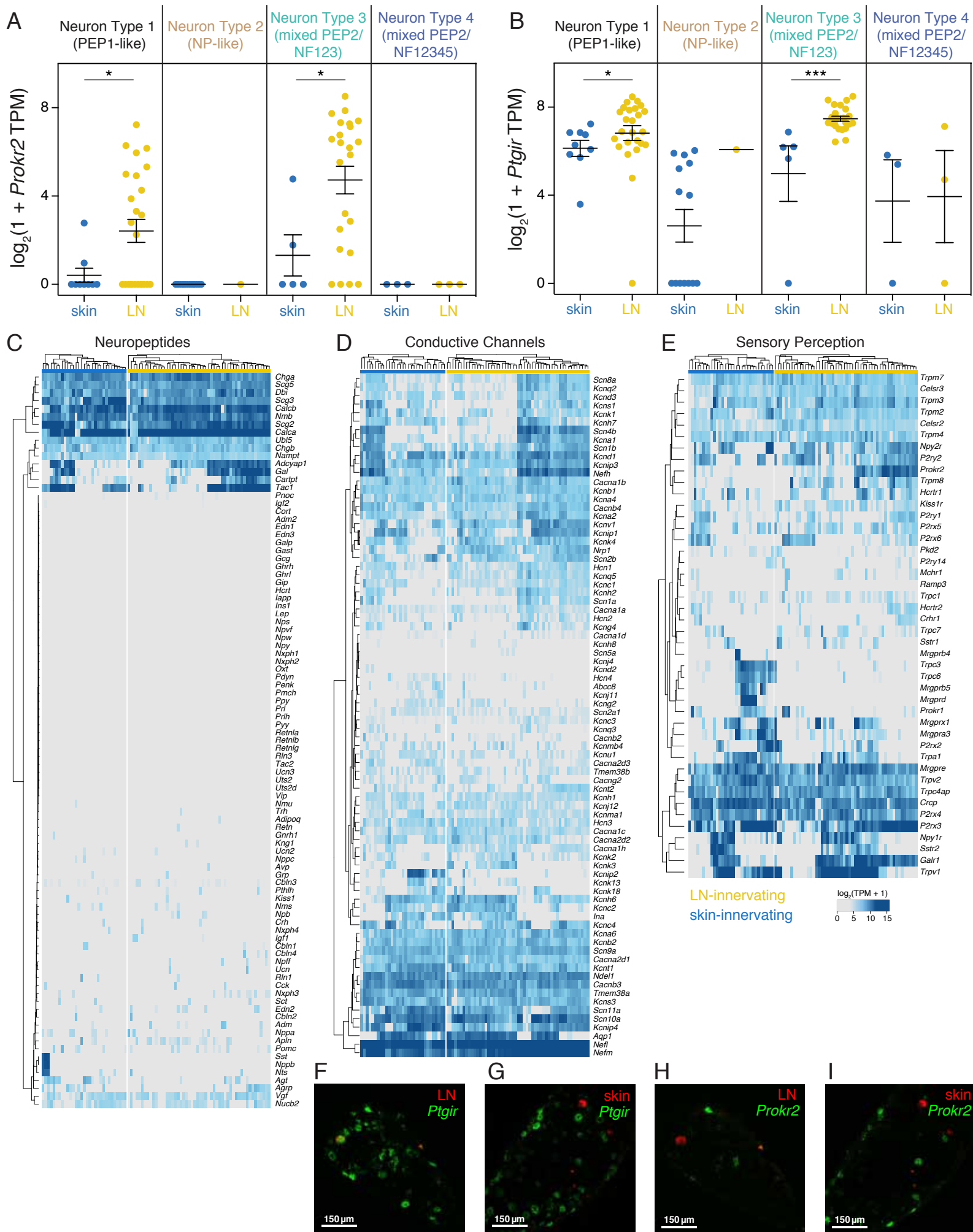


Figure S5

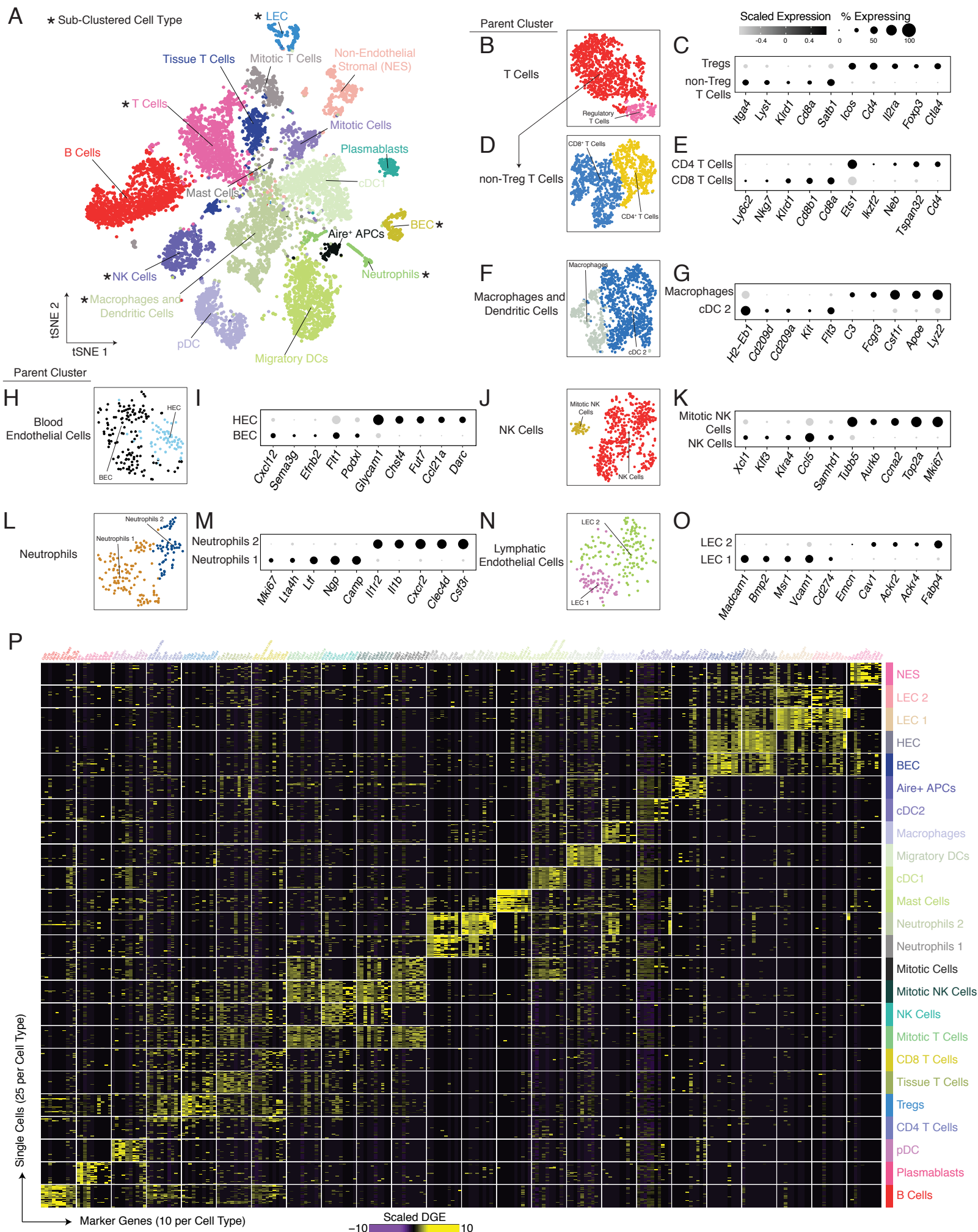


Figure S6

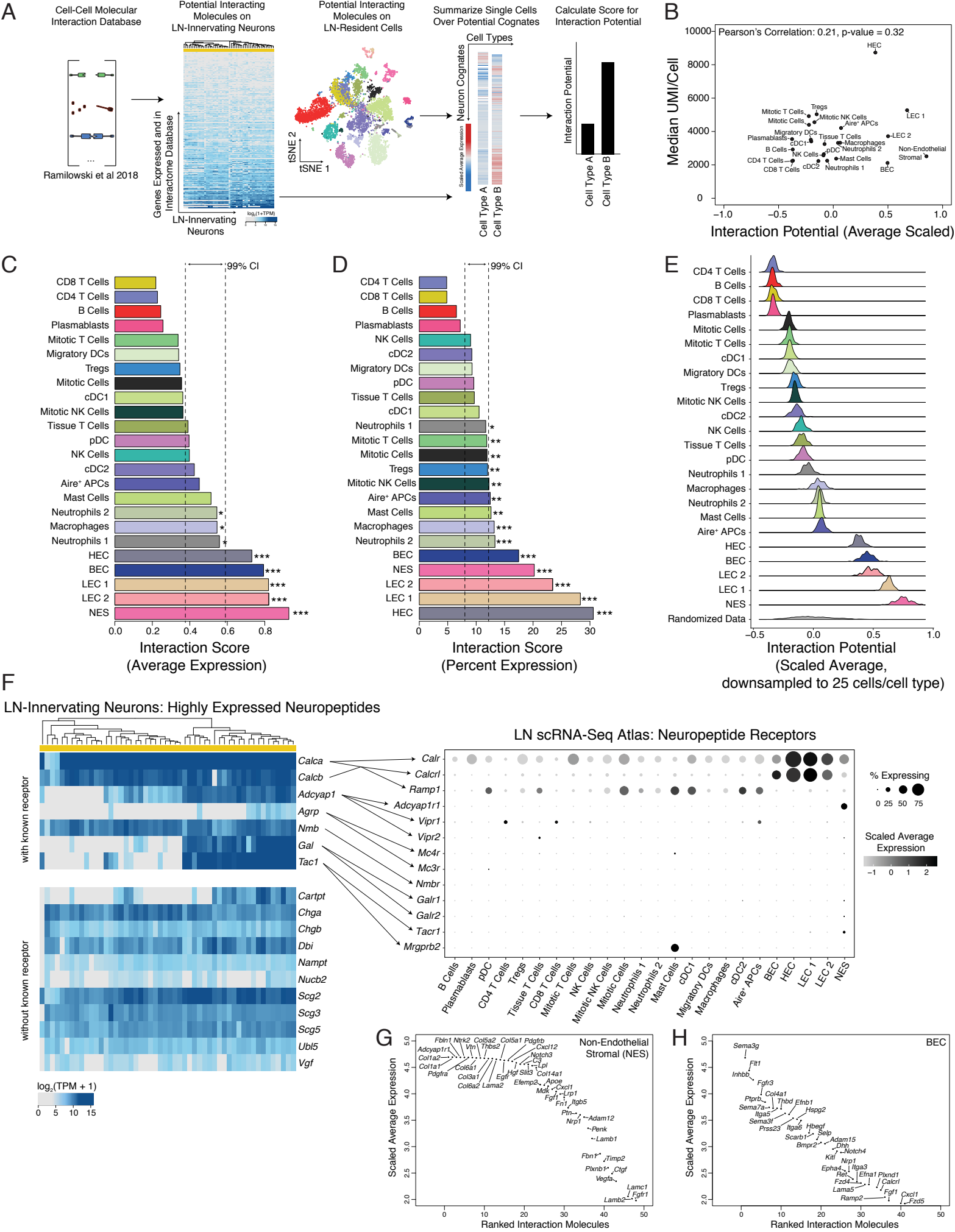


Figure S7

