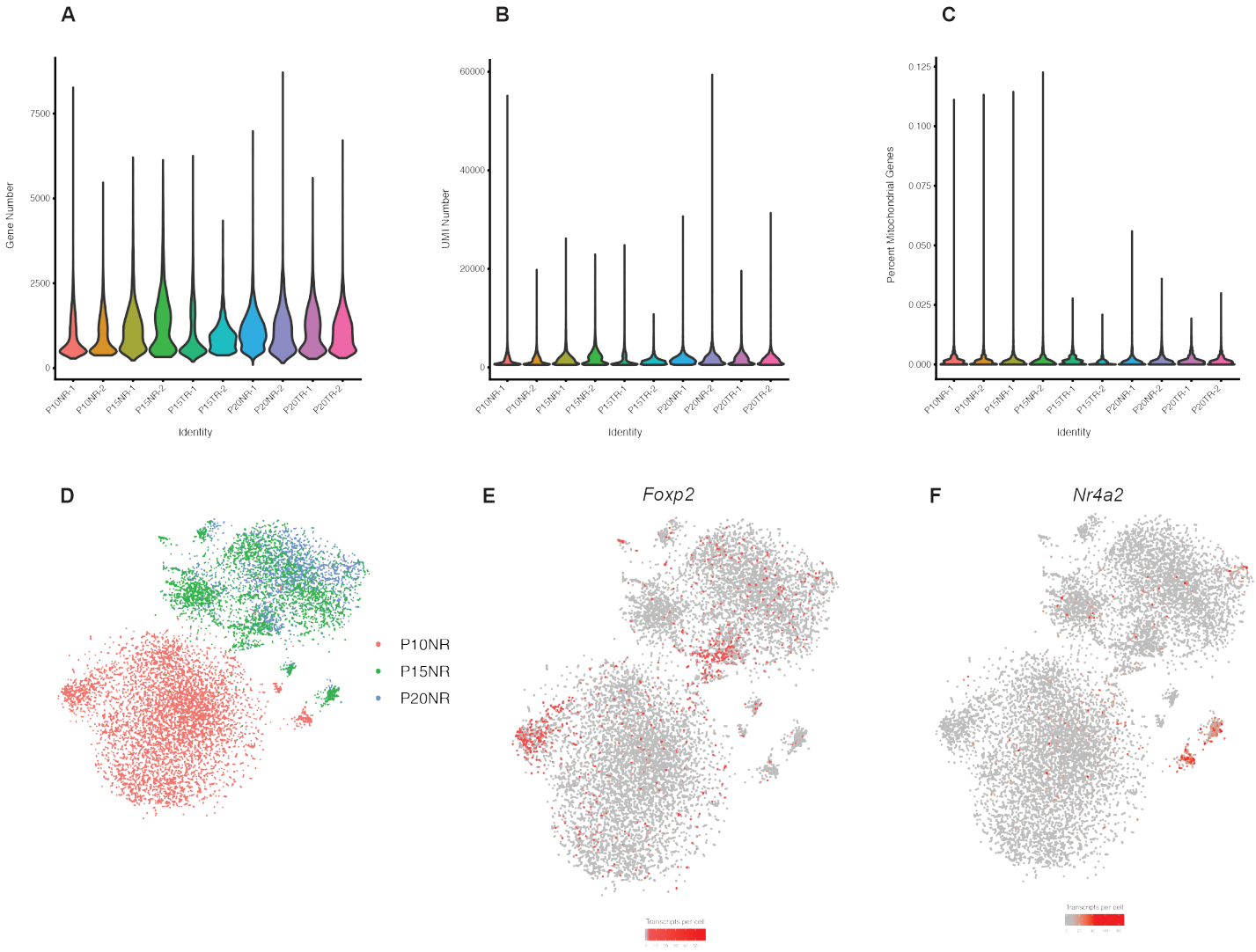
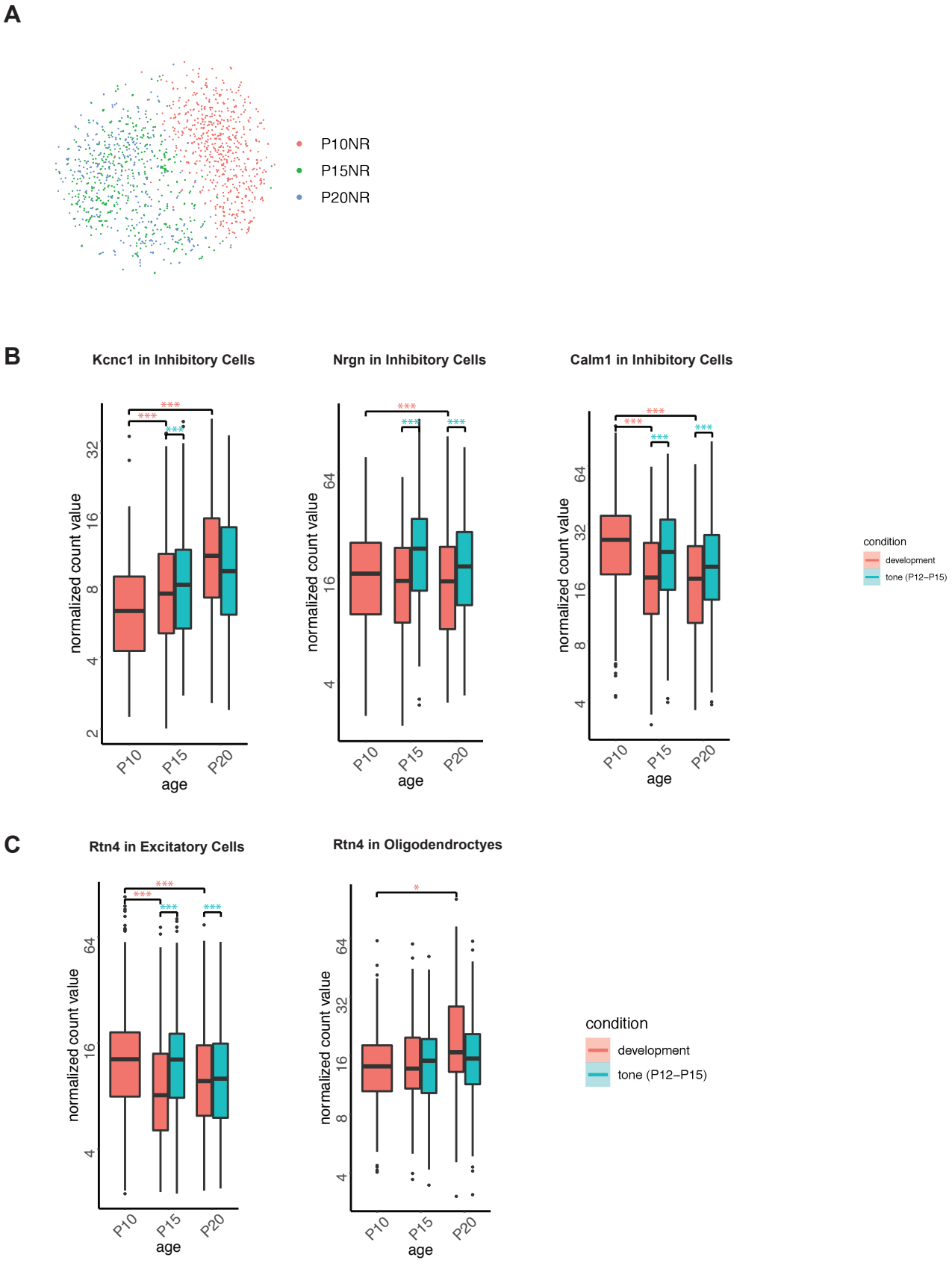
Supplemental Figure 1



Supplemental Figure 1
(A) Gene number detected in each replicate across all conditions.
(B) Unique molecular identifier (UMI) number detected in each replicate across all conditions.
(C) Percent mitochondrial genes in each replicate across all conditions
(D) t-SNE plot of excitatory neurons from P10, P15, and P20 normally reared mice, highlighting the clustering by developmental time point
(E) t-SNE plot of Foxp2 expression in excitatory neurons from P10, P15, and P20 normally reared mice. Red cells indicate Foxp2 expression.
(F) t-SNE plot of Nr4a2 expression in excitatory neurons from P10, P15, and P20 normally reared mice. Red cells indicate Nr4a2 expression.

Supplemental Figure 2



Supplemental Figure 2

(A) t-SNE plot of inhibitory neurons from P10, P15, and P20 normally reared mice, highlighting the clustering by developmental time point

(B) Boxplots of the trajectory of average gene expression for Kcnc1, Nrgn, and Calm1 in inhibitory cells from P10 to P20 under conditions of normal development and tone rearing from P12-P15. Box ranges represent the 25^{th} and 75^{th} percentiles, while the box whiskers indicate the 95% confidence interval. Mean normalized gene expression is indicated. Pairwise gene expression change significance indicated by * (* FDR<0.05, ** FDR<0.01, ***FDR<0.001).

(C) Boxplots of the trajectory of average gene expression for *Rtn4* in excitatory cells and oligodendrocytes from P10 to P20 under conditions of normal development and tone rearing from P12-P15. Box ranges represent the 25^{th} and 75^{th} percentiles, while the box whiskers indicate the 95% confidence interval. Mean normalized gene expression is indicated. Pairwise gene expression change significance indicated by * (* FDR<0.05, ** FDR<0.01, ***FDR<0.001).

Extended Experimental Procedures

Animals

All experiments were conducted in accordance with procedures approved by the Institutional Animal Care and Use Committees of Harvard University. Single-cell sequencing was performed on tissue from wild-type C57Bl6/J mice obtained from Jackson Labs.

Tone rearing

Tone reared litters and their mothers were placed in their normal cage with lid removed in a sound-attenuating chamber (IAC) with exposure to 7-kHz tone pips (100 ms at 5 Hz for 1 s, followed by 2 s of silence) at 80 dB sound pressure level (SPL). Sound was delivered by two speakers at corners of the chamber. The chamber was otherwise identical to normal rearing conditions, such as 12:12-h light:dark cycle and ad libitum access to food and water. Tone rearing was performed between postnatal days 12-15, and litters were moved back to standard housing on postnatal day 16.

Tissue collection

Appropriately aged (See Fig 1A) C57BL/6J mice were anesthetized using isoflurane until unresponsive to toe pinch. Animals were rapidly decapitated and the brain was dissected and placed on ice cold petri dish. Using a scalpel, a 1 mm square piece of tissue was dissected from the brain at coordinates described in Fig 1B with care taken to remove the underlying white matter. Both left and right cortex tissue sections were immediately placed in 1.5 mL Eppendorf tube and flash frozen in liquid

nitrogen. Tubes were then transferred to a -80 degree freezer before preparation for single cell sequencing.

Generation of single-nuclei suspensions

Mice were euthanized on postnatal days 5, 10, 16, and 21 and A1 was flash frozen at -80C. Frozen tissue was thawed in 500 uL Buffer HB (0.25M sucrose, 25mM KCl, 5mM $MgCl_2$, 20mM Tricine-KOH pH 7.8, 0.15mM spermine tetrahydrochloride, 0.5mM spermidine trihydrochloride, 1mM DTT). The tissue was transferred to a 2 mL dounce. 500ul 5% IGEPAL CA-630 (Sigma) and 1 mL HB were added to the tissue and the tissue was homogenized with a tight pestle 10-15 times. The sample was transferred to a 15 mL tube and total solution brought to 9 mL with HB. In a Corex tube (Fisher Scientific), 1 mL 30% iodixanol layered on top of 1 mL 40% iodixanol. The 9 mL sample was layered on top of the iodixanol cushion. The sample was spun at 10,000 xg for 18 minutes. 1 mL of sample at the 30-40% iodixanol interface was collected. After counting nuclear with a hemocytometer, the sample was diluted to 100,000 nuclear/mL with 30% iodixanol (with RNAsin) and subjected to single nuclear droplet encapsulation with Indrops.

Single-nucleus RNA sequencing using inDrops

Individual nuclei were captured and barcoded using the inDrops platform as previously described.[1] Briefly, single-cell suspensions were fed into a microfluidic device that packaged the cells with barcoded hydrogel microspheres and reverse transcriptase/lysis reagents. After cell encapsulation, primers were photo-released by

UV exposure. Two libraries of approximately 3000 cells each were collected for each sample. Indexed libraries were pooled and sequenced on a Nextseq 500 (Illumina).

Sequencing data processing

Sequencing data were aligned to the genome and processed according to a previously published pipeline (https://github.com/indrops/indrops). Briefly, this pipeline was used to build a custom transcriptome from Ensembl GRCm38 genome and GRCm38.84 annotation using Bowtie 1.1.1, after filtering the annoatation gtf file ((gencode.v17.annotation.gtf filtered for feature_type=”gene”, gene_type="protein_coding" and gene_status="KNOWN"). Unique molecular identifiers (UMIs) were used to link sequence reads back to individual captured molecules. All steps of the pipeline were run using default parameters unless explicitly stated.

Quality control, dimensionality reduction, and clustering of cells

All cells were combined into a single dataset. All mitochondrial genes were removed from the dataset. Cells with fewer than 500 or more than 15,000 UMI counts were excluded. Cells were then clustered using the Seurat R package. The data were log normalized and scaled to 10,000 transcripts per cell. Variable genes were identified using the following paramenters: x.low.cutoff = 0.0125, x.high.cutoff = 3, y.cutoff = 0.5. We limited the analysis to the top 30 principal components (PCs). Clustering resolution was set to 0.6. Clusters containing fewer than 100 cells were discarded. The expression of known marker genes was used to assign each cluster to one of the main cell types. Due to the developmental regulation of neuronal markers, a combination of Snap25, *Slc17a6*, and *Stmn2* was used to identify excitatory neurons. Gad1, Olig1, Aqp4, Cldn5,

Vtn, Cx3cr1, and Mrc1 were used to identify inhibitory neurons, oligodendrocytes, astrocytes, endothelial and smooth muscle cells, pericytes, microglia, and macrophages, respectively. Clusters with significant expression of two or more markers were removed, as they represented doublet clusters resulting from simultaneous capture of two or more cells in a single droplet. Clusters from each cell type were combined and the Seurat-based clustering repeated to characterize subtype diversity. The Seurat FindMarkers function was used to identify genetic markers of cellular subtypes.

Identification of differentially expressed genes

To identify differentially expressed genes by cell type in A1, we performed a differential gene expression analysis using Monocle2. The analysis was conducted on each cell type, comparing each consecutive pair of time points as well as between normally reared and tone reared samples. The data were modeled and normalized using a negative binomial distribution. Genes whose false discovery rate (FDR) was less than 5% were considered statistically significant.

Acute Auditory Thalamocortical Slice Preparation

C57BL/6J (Jackson Lab, Bar Harbor, ME), *GAD65*$^{-/-}$ (c/o Dr. K. Obata, RIKEN BSI) or *NgR*$^{-/-}$ mice (P8-P20; c/o Dr. Z. He, Boston Children's Hospital) were rapidly decapitated without anesthesia and placed in ice-cold ACSF. Slices were sectioned on a vibratome (Microm HM 650V, Thermo scientific, Walldorf, Germany) according to the method of Cruikshank et al. (Cruikshank et al., 2002). Briefly, 600 μm thick horizontal slices with the lateral end raised 15°, were cut in ice-cold ACSF before returning to room

temperature. One left hemisphere slice was used per animal. The ACSF contained (in mM) 125 NaCl, 25 glucose, 25 $NaHCO_3$, 2.5 KCl, 2 $CaCl_2$, 1.25 NaH_2PO_4, 1 $MgCl_2$ (315-328 mOsm).

Voltage-Sensitive Dye Imaging

Slices were stained, imaged and the recordings analysed as described by Barkat et al.[2] Briefly, slices were incubated in Di-4-ANEPPS (5 μg/l, Invitrogen, cat# D-1199) for at least 90 min before transfer to an ACSF (room temperature) recording chamber. They were imaged using an Olympus MVX10 microscope and a MiCam Ultima CMOS based camera (SciMedia, Tokyo, Japan). Fluorescent changes (1 ms frame rate for 512 ms periods) were averaged across 10 trials and integrated across different regions of interest by spatial averaging and across different trials using MiCam Ultima analysis software. Fluorescence change was normalized to resting fluorescence ($\Delta F/F_0$). Discrete sites in the MGBv were activated with an ACSF-filled patch pipette (0.5 mA, 1 ms pulse) at 100 ms after recording onset. The stimulating electrode was positioned on the rostral MGBv at six discrete positions spaced at 100 μm intervals along the medio-lateral axis.

Regions of interest (5x5 pixels, 125x125 μm) were chosen along the rostro-caudal axis of A1 at constant depth from the pia, corresponding to upper L4 based on Nissl stains, covering a total distance of 2250 μm. Response amplitude was defined as maximum fluorescence change ($\Delta F/F$) per trial at a given region of interest. Peak amplitude was defined as maximum response amplitude across all L4 locations. Variations in daily preparation were normalized by slice: all signals were divided by the maximum change in fluorescence measured in a set of experiments (Norm. $\Delta F/F$ =

(ΔF/F)/max(ΔF/F)). Response latency was defined as the time elapsed from stimulus pulse to half-maximal signal amplitude.

Diazepam Treatment

Benzodiazepine-treated mice were prepared by intraperitoneal Diazepam, DZ (cat# D0899, Sigma, St-Louis, MO) injection (dilution 2 mg DZ/ml in saline; 0.01 ml/g) every 24 hours for three days (P8-P11, P12-P15 or P16-P19).

Immunohistochemistry

Standard protocols were used to label thalamocortical slices. Briefly, 80 µm slices were kept in 4% paraformaldehyde overnight at room temperature, then washed in PBS, solubilized and blocked in triton X-100 (0.8%), BSA (20%) in PBS (4°C overnight), incubated with mouse monoclonal anti-MBP (cat# MAB384, Millipore, Billerica, MA; 1:200 dilution), rabbit anti-PV (cat# PV25 , Swant, Marly, Switzerland; 1:500 dilution) or Fluorescein Wisteria Floribunda Lectin for PNN (Vector, cat#FL-1351, Vector, Burlingame, CA; 1:200 dilution) in 5% BSA in PBS (4°C overnight) followed by a fluorescent secondary antibody. For myelin and PNN, eight regions of interest covering 125x125 µm were chosen along the medio-lateral A1 axis orthogonal to the pia. For comparison across age, normalization relative to fluorescence in upper layer (first region of interest) was performed. For PV analysis, regions of interest covering 650 x 650 µm across cortical layers 3 to 5 were chosen.

Fluorescence in situ hybridization (FISH)

Brains were immediately frozen on dry ice in tissue freezing medium. Brains were sliced on a cryostat (Leica CM 1950) into 20 μm sections, adhered to SuperFrost Plus slides (VWR), and immediately stored at −80C until use. Samples were processed according to the ACD RNAscope Fluorescent Multiplex Assay manual. Images of FISH labelled tissue were taken using a ZEISS AxioScan.Z1 with 20x/0.8 objective and single band pass filters matched for DAPI (96), GFP (38), DsRed (43) and Cy5 (50). Stitched tiled images were aligned and A1 was designated manually based on cortex curvature and hippocampal anatomy. Nuclei were segmented and mean intensity was measured using QuPath automated segmentation and measurement.[3] Tubb3+ cells were selected as having mean intensity higher than 1.2x the population mean. Results were compared using Kruskal-Wallis rank sum test with a post hoc Dunn Test for pair-wise comparisons.

Statistical Analyses

Results are reported as mean±s.e.m and compared across groups using a two-tailed, unpaired Student's t-test or a two-way ANOVA test (Fig 1B, 3 and 4D) with statistical significance at $P<0.05$. n represents number of animals or number of slices (Fig 4D).

References

1. Klein, A. M. *et al.* Droplet barcoding for single-cell transcriptomics applied to embryonic stem cells. *Cell* **161**, 1187–1201 (2015).
2. Barkat, T. R., Polley, D. B. & Hensch, T. K. A critical period for auditory thalamocortical connectivity. *Nat. Neurosci.* **14**, 1189–1194 (2011).

3. Bankhead, P. *et al.* QuPath: Open source software for digital pathology image analysis. *Sci. Rep.* **7**, 16878 (2017).