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Glial cell expansion and intercellular signaling in the developing Medial Nucleus of the Trapezoid Body (MNTB)

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**Glial cell expansion and intercellular signaling in the developing Medial Nucleus
of the Trapezoid Body**

Ashley Nicole Brandebura

**Dissertation submitted
to the School of Medicine
at West Virginia University**

in partial fulfillment of the requirements for the degree of

**Doctor of Philosophy in
Biochemistry and Molecular Biology**

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transcriptomics, cell signaling**

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ABSTRACT

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Ashley Nicole Brandebura

Neural circuit formation is a complex process involving coordinated communication between neurons, glia and vascular-associated cells (VACs). Each cell type is responsible for a unique transcriptional and translational contribution to tissue maturation. Deciphering the intercellular signaling patterns which guide neural circuit formation during normal development is thus an essential step in understanding which components of neural circuit formation go awry in neurodevelopmental disorders. The medial nucleus of the trapezoid body (MNTB), located in the auditory brainstem, was used as a model system to study the dynamics of neural circuit formation because it contains a mostly homogenous population of postsynaptic neurons and the largest nerve terminal in the central nervous system, the calyx of Held (CH).

We conducted an extensive cell counting study using light microscopy to characterize changes in the neuronal:nonneuronal cell ratio across development of the MNTB. Using cell type-specific antibodies we obtained the relative percentages of each cell type at P3 and P6, key timepoints for CH growth and refinement to mono-innervation. Significant increases in the glial cell percentage are due mostly to an increase in percentage of oligodendrocytes. Using proliferation and mitotic markers, we demonstrated that oligodendrocytes locally divide within the boundaries of the MNTB during this timeframe. Changes in the neuronal:nonneuronal cell ratio aided in interpretation of transcriptional data obtained from a previously conducted developmental microarray study performed in the MNTB. In the microarray study, many up-regulated transcripts were known to be more highly expressed in glial cells in other brain regions. By connecting cell numbers to transcript level, we were able to determine whether a transcript highly expressed in glial cells was detected as up-regulated due to increases in cell number or due to true transcriptional up-regulation on a per cell basis. In a second study, single cell RNA-Sequencing (scRNA-Seq) allowed us to generate cell type-specific transcriptional profiles for each major cell type in the MNTB. These data added cell type-specific expression information to the developmentally regulated transcripts identified in the microarray study, many of which were previously unassigned to a specific cell type. We identified transcripts related to several major intercellular signaling pathways at P3, including FGF, Delta-Notch, TGF β and VEGF pathways. In many cases the direction of signaling between cell types could be determined based on expression patterns of transcripts encoding for ligands and their cognate receptors. Most interestingly, we were able to tie transcriptional data with structural changes that occur during MNTB tissue maturation, such as perineuronal net formation and angiogenesis. The combination of cell counting data, temporal transcriptional data at the tissue level and cell type-specific transcriptional data at the single cell level offers a broad picture of the process of neural circuit formation in the MNTB and provides a solid foundation to develop and test new mechanistic hypotheses.

Dedication:

For my grandfather: I wish you could be here to see me reach the end of this journey and start a new chapter in life. You always believed in me. I love you and know you are smiling down from above.

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List of Abbreviations

Acan: Aggrecan
Acvr11: Activin A receptor like type 1
ACSF: artificial cerebrospinal fluid
Aif1: Allograft inflammatory factor 1
Aldh1L1: Aldehyde dehydrogenase 1 family member L1
AMPA: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Ass1: Arginosuccinate synthase 1
Atoh1: Atonal 1
Atoh7: Atonal 7
Atp6v1g2: ATPase H⁺ transporting V1 subunit G2
BBB: blood-brain-barrier
Bcan: Brevican
BDNF: Brain derived neurotrophic factor
BHLH: Basic helix-loop-helix
Bmp: Bone morphogenetic protein
Bmpr: Bone morphogenetic protein receptor
BSA: Bovine serum albumin
Calb1: Calbindin1
Cd31: CD31 antigen
CF: climbing fiber
CH: calyx of Held
Cldn5: Claudin 5
CN: cochlear nucleus
Cnp: 2',3'-Cyclic nucleotide 3' phosphodiesterase
CNS: central nervous system
Cntn5: Contactin 5
CPM: counts per million
DAPI: 4',6-diamidino-2-phenylindole

Dcc: Deleted in colorectal carcinoma
DCN: dorsal cochlear nucleus
DGE: differential gene expression
Dll: Delta-like Notch canonical ligand
Dmdo: Diminuendo
Dnm1: Dynamin 1
Dnm3: Dynamin 3
E: embryonic day
EBSS: Earl's balanced salt solution
EdU: 5'-ethynyl-2'-deoxyuridine
Egr2: Early growth response 2
En1: Engrailed 1
Eng: Endoligin
EPSC: excitatory postsynaptic current
FACS: fluorescence activated cell sorting
FDR: false discovery rate
FGF: Fibroblast growth factor
Fgfr: Fibroblast growth factor receptor
Flt: FMS related tyrosine kinase 1
Fmr1: Fragile X mental retardation 1
Foxp1: Forkhead box P1
Fzd: Frizzled
Gabra5: Gamma-aminobutyric acid type A receptor alpha 5 subunit
Gdi1: GDP dissociation inhibitor 1
GFP: Green fluorescent protein
Gfap: Glial fibrillary acidic protein
Grin2a: Glutamate ionotropic receptor NMDA type subunit 2A
HA: Hemagglutinin
Hapln: Hyaluronan and proteoglycan link protein
HBSS: Hank's balanced salt solution

Hepacam: Hepatic and glial cell adhesion molecule
Hoxb1: Homeobox B1
Iba1: Ionized calcium binding adaptor molecule 1
Idh3a: Isocitrate dehydrogenase 3 (NAD(+)) alpha
IFC: integrated fluidic circuit
IPA: Ingenuity pathway analysis
IPSCs: inhibitory postsynaptic currents
ISH: *in situ* hybridization
IVR: immersive virtual reality
Jag: Jagged
Kdr: Kinase insert domain receptor
Kv3.1: Potassium voltage-gated channel subfamily c member 1
Ldhb: Lactate dehydrogenase B
LOESS: locally weighted scatter plot smoother
LM: light microscopy
LNTB: lateral nucleus of the trapezoid body
LSO: lateral superior olive
Mads: mean absolute deviations
Map: Microtubule associated protein
Mbp: Myelin basic protein
MNTB: medial nucleus of the trapezoid body
MSO: medial superior olive
nA: nanoamps
Ncan: Neurocan
NeuN: Neuronal nuclei
NG2: Neuron glial 2
NMDA: N-methyl-D-aspartate
NMJ: neuromuscular junction
NO: Nitric oxide
Ntn1: Netrin1

Olig2: Oligodendrocyte transcription factor 2
OPC: oligodendrocyte precursor cell
P: postnatal day
PBS: phosphate buffered saline
PC: purkinje cell
PCNA: Proliferating cell nuclear antigen
PDGFR α : Platelet derived growth factor receptor α
PDGFR β : Platelet derived growth factor receptor β
PFA: paraformaldehyde
Pgk1: Phosphoglycerate kinase 1
PH3: Phosphorylated histone H3
Pmca2: Plasma membrane calcium ATPase 2
Pnn: perineuronal nets
Pvalb: Parvalbumin
RFA: random forest analysis
RFP: Red fluorescent protein
RNA-Seq: RNA-Sequencing
ROI: region of interest
Rpl22: Ribosomal protein large subunit 22
S100 β : S100 calcium binding protein B
SAGE: serial analysis of gene expression
SBEM: serial blockface electron microscopy
scRNA-Seq: single cell RNA-Sequencing
SGN: spiral ganglion neuron
SICs: slow inward currents
Slc1a2: Solute carrier family 1 member 2
Slc1a3: Solute carrier family 1 member 3
Slc17a8: Solute carrier family 17 member 8
smFISH: single molecule fluorescent *in situ* hybridization
Snap25: Synaptosome associated protein 25

SOC: superior olivary complex
Sox10: SRY-box 10
SPN: superior paraolivary nucleus
Tal: T-cell acute lymphocytic leukemia protein 1
TCA: tricarboxylic acid cycle
tdTom: tdTomato
TGF β : Transforming growth factor β
Tgfbr: Transforming growth factor β receptor
Tnc: Tenascin C
Tnr: Tenascin R
TNR: Transgenic Notch reporter
VACs: vascular associated cells
VAS: ventral acoustic stria
Vcan: Versican
VCN: ventral cochlear nucleus
VEGF: Vascular endothelial growth factor
VNTB: ventral nucleus of the trapezoid body
Wnt: Wingless-type
Xgal: 5-Bromo-4-Chloro-3-Indolyl β -D-Galactopyranoside

Chapter 1: Introduction and Literature Review

The developmental maturation of neural tissue is a complex and intricate process that is precisely tuned on many regulatory levels. Neurons, glia (astrocytes, oligodendrocytes and microglia) and vascular associated cells (VAC; defined as vascular endothelial cells and pericytes) must migrate to and/or locally proliferate in their destined anatomical positions to create a coordinated cell signaling network which will ultimately regulate cell numbers, transcriptional dynamics and protein localization. During central nervous system (CNS) development, proper wiring of trillions of synaptic connections must be established. Although synaptic connections most simply include a neuron:neuron interaction, glial cells and VACs are intimately involved in regulating patterns and levels of synaptic connectivity and homeostasis of the extracellular milieu, which must contain proper levels of secreted proteins and neurotransmitters. Furthermore, glial cells regulate speed of synaptic transmission (via myelination) and VACs regulate formation of the blood-brain-barrier (BBB). The details of such a multifaceted signaling network cannot be elucidated when studying a single cell type in isolation because it is the coordinated cross-talk among neurons, glia and VACs in combination which establishes the environment in which a neural tissue can develop and mature. Thus, the key to understanding neural circuit formation is to take a whole-tissue approach to the question.

An anatomical nucleus known as the medial nucleus of the trapezoid body (MNTB), located in the auditory brainstem, is an advantageous model to study neural tissue maturation. The MNTB contains the presynaptic input to the MNTB principal neurons, the calyx of Held (CH), which is the largest nerve terminal in the mammalian brain. This review chapter will focus on the function of the MNTB in the auditory brainstem, advantages of the CH:MNTB system as a model for neural tissue development and topics related to CH and MNTB embryonic and early postnatal development. Additionally, the advantages of utilizing unbiased transcriptional screens, such as

microarrays and RNA-Sequencing (RNA-Seq), as tools for investigating the dynamics of neural circuit development will be discussed and lead into the objectives of the current work.

The auditory brainstem and MNTB

The MNTB is located in a region of the hindbrain called the ventral auditory brainstem. The auditory brainstem is the first central location to receive auditory information from the periphery and is composed of the Cochlear Nucleus (CN) and the Superior Olivary Complex (SOC), which are each subdivided into smaller cell groups. The CN is comprised of the Dorsal Cochlear Nucleus (DCN) and the Ventral Cochlear Nucleus (VCN). The DCN primarily sends projections to the Lateral Lemniscus and the Inferior Colliculus, while the VCN primarily sends projections to the nuclei of the SOC (Cant & Benson, 2003). The SOC is comprised of mainly the Lateral Superior Olive (LSO), Medial Superior Olive (MSO), Ventral Nucleus of the Trapezoid Body (VNTB), Lateral Nucleus of the Trapezoid Body (LNTB) and MNTB. The SOC, as the first site of massive binaural convergence, receives inputs from both contra- and ipsilateral CN, and is essential for many aspects of binaural auditory processing, including sound localization (reviewed by Grothe et al., 2010). The Globular Bushy Cell (GBC) in the VCN projects to the contralateral MNTB and forms the large glutamatergic CH terminal, an important auditory relay synapse which functions to encode timing and intensity differences of direct excitatory inputs from the CN to the MSO and LSO, respectively, with inhibitory inputs from the principal neurons in the MNTB to the MSO and LSO (reviewed by von Gersdorff & Borst, 2002; Grothe et al., 2010; Fig. 1A). Importantly, both the CN and the SOC mirror the tonotopic organization of the cochlea, with high-frequency to low-frequency regions traveling from the dorsal to ventral directions of the CN and high-frequency to low-frequency regions encoded by the medial to lateral portions of the nuclei in the SOC (Kandler et al., 2009; Muniak et al., 2013; Muniak & Ryugo, 2014; Fig. 1B).

Model systems for neural tissue development

In many developing neural systems, supernumerary synaptic connections are established during the early development of a neural circuit, and subsequently, these exuberant connections are removed through the selective strengthening of some synapses and removal of others (reviewed by Purves & Lichtman, 1980). Two well characterized model systems have historically been used to study the dynamics of neural circuit formation. These include the climbing fiber innervation of the Purkinje cell dendrite (CF:PC) and the motoneuron innervation of the muscle endplate, termed the neuromuscular junction (NMJ). Studies in each of these systems suggest two phases of synaptic strengthening and pruning. In the first stage, tens of inputs may project onto the postsynaptic target and most are removed while just a few are strengthened. At this point, a second round of strengthening and pruning occurs to leave just one remaining input (Balice-Gordon & Lichtman, 1993; Watanabe & Kano, 2011; Kano & Hashimoto, 2012; Tapia et al., 2012). Both the CF:PC and NMJ systems have advantages and disadvantages as model systems. For example, both the CF:PC and NMJ systems are advantageous in ease of experimental access. Slices or dissociated cell cultures containing the CF:PC and NMJ synapses can be cultured long-term *in vitro* (Tabata et al., 2000; Das et al., 2010). Furthermore, the CF:PC connection is located in the cerebellum, which due to its dorsal location, can be targeted with viral injections without causing much damage to the rest of the brain. The NMJ is located peripherally, also permitting access for *in vivo* viral manipulations. However, in both of these systems, maturation progresses slowly and refinement to mono-innervation takes around two weeks (Fig. 2). The long timeframe for development of these circuits makes it more difficult to tie rapid transcriptional and protein level changes to structural maturation of the systems.

A more recently proposed model system is the CH:MNTB connection (Hoffpauir et al., 2006; Holcomb et al., 2013), which has many advantages. The MNTB contains a mostly homogeneous population of postsynaptic neurons, the principal neurons (Banks & Smith, 1992).

A model system with predominantly one neuronal subtype is advantageous for studying cell signaling interactions and eliminates confounds of studying a heterogeneous population of neurons. Secondly, the large size of the CH terminal during early postnatal development makes it amenable to paired pre- and postsynaptic electrophysiological recordings (Forsythe, 1994; Borst & Sakmann, 1996) as well as detailed ultrastructural analysis. Also advantageous is the rapid and synchronized maturation of the CH:MNTB system. The first action potential can be evoked in the principal neurons by embryonic day (E)17 and the two phases of strengthening and pruning proceed to completion within just a few days. Between postnatal day (P)2 to P4, selected small bouton inputs grow rapidly while others are removed such that the tens of inputs are refined to 2-3 medium to large sized terminals (Kandler & Friauf, 1993; Hoffpauir et al., 2006, 2010; Holcomb et al., 2013). By P6, the majority of the principal neurons (74%) have reached a mature state of mono-innervation after a second phase of strengthening and pruning (Holcomb et al., 2013; Fig. 2). By P9, 88% of the principal neurons are mono-innervated (Fig. 2), which may reflect the mature state (our unpublished observations).

In addition, the CH and the principal neuron mature morphologically and biophysically in a synchronized manner. As the CH dramatically increases in size from an average apposed surface area of $\sim 2 \mu\text{m}^2$ at P2 to over $200 \mu\text{m}^2$ at P4 (Holcomb et al., 2013), the excitatory postsynaptic currents (EPSCs) generated increase from less than 0.5 nanoamps (nA) to over 4 nA. Concurrently, the principal neuron increases in surface area by about 32% from P0 to P4. The increase in surface area of the principal neuron contributes to the near 5-fold decrease in input resistance (about 1 gigaohm at E17 to about 200 megaohms at P6), the near 7-fold increase in current required to evoke an action potential (about 30 picoamps at E17 to over 200 picoamps at P6) and the acquisition of mature phasic firing properties (Hoffpauir et al., 2010). The synchronized maturation of synaptic partners, which occurs before the onset of exposure to airborne sound (Mikaelian et al., 1965), make the CH:MNTB system an interesting model to

study the contributions of spontaneous activity to neural circuit formation. Growth of the CH terminal occurs before opening of the ear canal, but spontaneous burst firing is detected in the spiral ganglion neurons by E14 (Marrs & Spirou, 2012), suggesting that spontaneous activity may drive CH growth. There are several studies which altered spontaneous activity in the auditory system, but these demonstrated little to no effects on CH development. Congenitally deaf mice lacking spontaneous activity in the spiral ganglion neurons (SGNs) and mice lacking the $\alpha 9$ subunit of the acetylcholine receptor resulting in disrupted spontaneous activity originating in the cochlea present with normal CH terminals (Yousoufian et al., 2005; Clause et al., 2014). However, these reports are most likely confounded by a homeostatic compensation of spontaneous activity downstream in the circuit. A recent report demonstrated that mice lacking the *Solute Carrier Family 17 Member 8 (Slc17a8)* gene, which disrupts spontaneous activity originating in the cochlea, display a homeostatic compensatory increase in spontaneous activity in the SGNs (Babola et al., 2018). These findings suggest that the role of spontaneous activity on CH development needs to be tested directly on the GBCs which form the CH terminal. The synchronized maturation of the CH terminal and the postsynaptic principal neuron make the MNTB an attractive model system to elucidate the roles of spontaneous activity on neural circuit formation.

Although the CH:MNTB system offers many advantages to studies of neural circuit development, the MNTB tissue does not grow well in culture. Recent protocols using dissociated cell culture with a wide variety of growth factors added to the media and an organotypic system which grows mostly ipsilaterally projecting, rather than contralaterally projecting CHs, offer advancements in the field because they are the first culture systems reported with a surviving CH:principal neuron connection. However, in both systems the CH terminals that grow fail to reach a physiologically relevant size and the usually rapid timeframe for growth is dramatically extended (Dimitrov et al., 2016; Kronander et al., 2017). Furthermore,

the ventral location of the MNTB in the brainstem makes it relatively inaccessible for *in vivo* viral manipulations and the scarcity of specific genetic markers for the MNTB makes it difficult to manipulate in classic Cre-mediated genetic knockout experiments. Thus, high-throughput transcriptional studies, such as those conducted in my dissertation work, are necessary to identify specific markers for cell types in the MNTB which could lead to the development of more useful genetic tools to study CH:MNTB development.

Embryonic and early postnatal development of the CH and MNTB

Embryonic development of VCN and MNTB neurons

The neurons of the VCN are born in the rhombic lip between E10-14 (Pierce, 1967). Specifically, the VCN neurons arise from rhombomeres 2-3 and are of Wingless-Type 1 (*Wnt1*) lineage (Farago et al., 2006). The neurons migrate through the Cochlear Extramural Stream to their final destination in the VCN. Neuronal migration to the VCN is dependent on expression of the Atonal 1 (*Atoh1*) transcription factor, as *Atoh1* mutant mice lack a defined VCN at E18.5 (Wang et al., 2005). MNTB neurons in the mouse are born between E11-12 (Pierce, 1973) and are derived from rhombomeres 4-5. The rhombomeric origin of MNTB neurons was determined using a combination of genetic reporter mouse lines. Staining with 5-Bromo-4-Chloro-3-Indolyl β -D-Galactopyranoside (Xgal) and anti-Green Fluorescent Protein (GFP) antibody in the *Hoxb1-Cre*, *Egr2-Cre* and *Wnt1-Cre* lines demonstrated that the MNTB neurons are of *Homeobox B1* (*Hoxb1*; expressed in rhombomere 4) and *Early Growth Response 2* (*Egr2*; expressed in rhombomeres 3 and 5) lineage, but are not of *Wingless-type 1* (*Wnt1*; expressed in rhombomeres 2-3; Maricich et al., 2009; Marrs et al., 2013; Fig. 3A-C) lineage. Proper positioning of the neurons in the MNTB nucleus is dependent on expression of the Engrailed1 (*En1*) transcription factor. *En1* knockout mice show incomplete migration of MNTB neurons and subsequent apoptosis by early postnatal ages (Altieri et al., 2015).

GBC axon extension

There are slight interspecies differences in GBC axon extension. In rats, very few GBC axons cross the midline at E15.0, but a robust increase in axonal labeling in the contralateral MNTB is observed by E17.0 (Kandler & Friauf, 1993). In mice, the axons of the GBCs leave the VCN and begin extending through the Ventral Acoustic Stria at E13.0. GBC axons rapidly cross the midline to enter the region of the eventual MNTB by E14.5 (Howell et al., 2007; Fig. 4A-B). Interestingly, the GBC axons reach the region of the MNTB before the MNTB neurons themselves. MNTB neurons can first be identified in a region termed the “presumptive SOC” at E14.5. Within the presumptive SOC the MNTB neurons are intermixed with neurons of the other SOC nuclei. The MNTB neurons then migrate laterally towards the midline to their final position in the MNTB at E17.0 (Marrs et al., 2013; Fig. 4C-D). Thus, the MNTB cannot be identified as a distinct nucleus until E17.0, shortly MNTB neurons form the first functional synaptic contacts with GBC axons at E17.5 (Hoffpauir et al., 2010).

Chemoattraction and chemorepulsion mechanisms for axon guidance have been most extensively studied in the commissural axons of the spinal cord. Long-range chemoattraction cues such as Netrin (Ntn1) ligand signaling to its receptor, Deleted in Colorectal Carcinoma (Dcc), first direct the axons towards the floorplate. Once at the floorplate, short-range chemoattraction cues through cell adhesion molecules of the immunoglobulin superfamily mediate midline crossing. Finally, a switch to chemorepulsive cues promotes the axons to extend away from the midline. The most well-characterized chemorepulsive cues in the spinal cord are mediated by the Slit ligand interaction with the Roundabout (Robo) receptors (reviewed by de Ramon Francás et al., 2017).

GBC axon extension is similar to that of the commissural axons in the spinal cord. Under normal conditions the GBC axons extend towards the ventral edge of the brainstem, cross the midline and then extend away from the midline to project onto the neurons in the contralateral MNTB.

Ntn1-Dcc signaling mediates the attractive cues towards the ventral edge of the brainstem and the midline. *In situ* hybridization (ISH) experiments showed that *Ntn1* mRNA is expressed in cells bordering the midline from E11.5 to P0. *Dcc* mRNA is detected in the VCN from E12.5 to P0. At the protein level, Dcc is detected in the axons traveling through the VAS from E12.5 to E16.5. In support of Ntn1-Dcc signaling mediating attraction towards the midline, it was demonstrated that the majority of GBC axons in *Dcc* knockout mice fail to exit the VAS. Furthermore, the GBC axons of *Ntn1* knockout mice reach the most lateral portion of the ipsilateral SOC but fail to cross the midline (Howell et al., 2007; Fig. 5A-D; A'-D').

Short-range chemoattraction of the GBC axons towards the midline was shown to be mediated by Robo3 signaling. Although Robo receptors typically transduce chemorepulsive cues, the Robo3 receptor was shown to deviate in function from the Robo1 and Robo2 receptors in the spinal cord. The Robo3.1 isoform mediates short-range chemoattraction towards the midline in the spinal cord, followed by a developmental switch to the Robo3.2 isoform after midline crossing that then mediates the chemorepulsive cues (Chen et al., 2008). *Robo3* mRNA is expressed in the VCN from E13 to E15 and at the protein level Robo3 has robust expression in the axons traveling through the VAS. The GBC axons of *Robo3* knockout mice form ipsilaterally projecting CHs and fail to cross the midline (Renier et al., 2010; Fig. 6A-B).

Similar to the spinal cord commissural axons, Slit signaling through the Robo1-2 receptors may mediate chemorepulsive cues from the midline. ISH experiments demonstrated that *Slit1* mRNA is expressed near the midline from E12.5 to P0. The receptor transcripts, *Robo1* and *Robo2*, are expressed in the VCN from E13.5 to P0 (Howell et al., 2007). However, the role of Slit-Robo signaling in chemorepulsion of GBC axons away from the midline has not yet been tested in a functional genetic knockout study.

The Ephrin ligands and their receptors, the Eph receptor tyrosine kinases, may contribute a small extent to chemorepulsion at the midline. The Eph receptor, EphB2, is expressed in the

VCN axons and its ligand, ephrin-B2, is expressed in the MNTB neurons during early postnatal development. *EphB2* knockout mice show a modest 10% increase in aberrant ipsilaterally projecting CHs compared to wildtype controls (Hsieh et al., 2010; Fig. 7). However, the incomplete phenotype suggests that other chemorepulsive mechanisms are also involved. Furthermore, EphB2 protein was not present until P0 (Hsieh et al., 2010), several days after the GBC axons initially cross the midline at E14.5 (Howell et al., 2007). The delayed expression of EphB2 suggests that Ephrin-Eph signaling might prohibit ipsilateral sprouting once the axons have already crossed the midline. The Ephrin-Eph signaling pathway is unique in that traditional forward signaling initiated from the ligand and processed through the receptor can occur as well as reverse signaling, which is initiated by the extracellular domain of the receptor and is then processed through the membrane-bound ligand. Hsieh and colleagues (2010) demonstrated that specifically reverse Ephrin-Eph signaling mediates contralateral targeting. Mice expressing an EphB2^{lacZ} fusion protein, where β -galactosidase replaced the intracellular domain of EphB2 and prevented forward signaling but not reverse signaling, developed normal contralaterally projecting CHs. However, mutant *Ephrin-B2*^{lacZ} heterozygous mice, which had normal forward but impaired reverse signaling due to the replacement of the cytoplasmic domain of Ephrin-B2 with β -galactosidase, showed a significant increase in the number of aberrant ipsilaterally projecting CHs compared to controls (Hsieh et al., 2010; Fig. 7). It is unclear whether a homozygous mutant would have a phenotype with increased severity.

Growth of the CH

Contactins

CH terminal growth in the MNTB is mediated by a number of signaling molecules, including cell adhesion proteins, kinases and growth factors. One demonstration of cell adhesion molecules mediating CH growth involves a protein in the contactin superfamily. The adhesion molecule

Contactin 5 (Ctn5) is transiently expressed in the VCN, axons of the Ventral Acoustic Stria and the MNTB between P1 and P7. *Ctn5* knockout mice present with an 8% decrease in MNTB neurons receiving a large CH terminal, 2% increase in CHs that lacked Vesicular Glutamate Transporter 1 protein and an increased number of apoptotic neurons, presumably due to lack of innervation from the GBC axons (Toyoshima et al., 2009; Fig. 8A-E). However, due to only a modest decrease in CH formation, there are likely other contactin family members, and potentially other types of cell adhesion molecules, involved in CH growth.

Sad kinases

The Serine/Threonine kinases, Sad-A and Sad-B, also regulate CH growth. Sad-A and Sad-B proteins are expressed in the CH terminal and the principal neuron in wildtype mice. Upon genetic deletion of the Sad-A and Sad-B kinases using a *Parvalbumin(Pvalb)*-Cre driver, CH occupancy of the membrane of the postsynaptic neuron decreased by 33% (Lilley et al., 2014; Figure 9A-C). However, one limitation of this report is that the *Pvalb*-Cre deleted the kinases from both the presynaptic terminal and the postsynaptic neuron. Future studies using specific presynaptic and postsynaptic Cre drivers need to be performed to interpret whether the kinases act to drive CH growth at the presynaptic side, the postsynaptic side, or both.

Bone morphogenetic proteins

Bone Morphogenetic Protein (Bmp) signaling was demonstrated to regulate CH growth and refinement to mono-innervation. ISH at P3 showed expression of the transcripts encoding for the Bmp ligand, *Bone Morphogenetic Protein 4 (Bmp4)*, and for the Bmp receptors, *Bone Morphogenetic Protein Receptor 1a (Bmpr1a)*, *Bone Morphogenetic Protein Receptor 1b (Bmpr1b)* and *Bone Morphogenetic Protein Receptor 2 (Bmpr2)*, in both the VCN and MNTB. Genetic deletion of *Bmpr1a/1b* genes using the *Egr2*-Cre driver resulted in significantly reduced CH volume and a decrease in the percentage of mono-innervated neurons. Using Serial

Blockface Electron Microscopy (SBEM), Xiao and colleagues (2013) reconstructed CH terminals and demonstrated that a wildtype CH at P8 had a volume of $\sim 780 \mu\text{m}^3$. In comparison, the largest terminal reconstructed from a *Bmpr1a/1b* knockout mouse was $\sim 100 \mu\text{m}^3$. The EPSCs of the largest input were also significantly different between the wildtype and knockout mice, with EPSCs of ~ 6 nA in wildtype mice ages P5-P6 compared to EPSCs of < 2 nA in the knockout mice at the same age. However, it is important to note that the EPSCs of the largest input reach the 6 nA threshold in knockout mice ages P14-P16, indicating that CH growth may be temporally delayed rather than completely stunted. Furthermore, SBEM analysis demonstrated that in P8 wildtype mice the principal neurons normally had one dominant large CH terminal. However, in the *Bmpr1a/1b* knockout mice a dominant CH terminal was usually absent. When the terminals were ranked by size from largest to smallest, the decrease in size between the terminals progressed in significantly shorter intervals compared to the wildtype mice, demonstrating that the knockout mice had not matured to the state of mono-innervation (Xiao et al., 2013; Figure 10A-B).

Like the *Pvalb*-Cre driver, the *Egr2*-Cre driver is expressed at the pre- and postsynaptic side, thereby limiting the ability to determine if Bmp signaling regulates CH growth and refinement to mono-innervation via anterograde signaling, retrograde signaling, or both. In a subsequent study, the same group utilized a viral injection approach to deliver Cre recombinase into the VCN at P0, resulting in genetic knockout of Bmp receptors in the presynaptic GBCs but not the postsynaptic principal neurons. A statistically significant increase in GBC axon branching was observed ($\sim 1\%$ in control vs. $\sim 14\%$ in knockout), but a reduction in CH size and refinement to mono-innervation were not recapitulated in this model (Kronander et al., 2019). The differential phenotypes observed may be due to a specific retrograde Bmp signal from the principal neurons in the MNTB to the CH terminal that mediates growth and refinement to mono-innervation. Alternatively, differences in the timing of Cre-mediated recombination could lead to

the differential phenotypes. The mouse model developed by Xiao and colleagues (2013) has early embryonic deletion of the Bmp receptors while the stereotaxic viral injection model developed by Kronander and colleagues (2019) has postnatal deletion of Bmp receptors. In the viral injection model, it is possible that the Bmp receptors are not deleted until after CH growth is mostly complete. By comparing the differential phenotypes of Bmp receptor knockouts using *Egr2*-Cre in embryonic vs. postnatal ages, Kronander and colleagues (2019) identified sequential roles for Bmp signaling at these ages. The results demonstrated that embryonic Bmp signaling regulates histogenesis of the VCN and MNTB, as embryonic deletion of Bmp receptors resulted in smaller volumes for these nuclei, whereas postnatal Bmp signaling regulates the extent of axon branching. However, the incomplete phenotypes observed in both studies suggests that other signaling pathways contribute to CH growth.

Dynamins

In support of the idea that multiple signal transduction pathways, and potentially neural activity, mediate CH growth, is the impressive disruption of CH growth by *Egr2*-Cre driven knockout of the *Dynamin1* (*Dnm1*) and *Dynamin3* (*Dnm3*) genes. The Dnm proteins mediate both synaptic vesicle recycling (Ferguson et al., 2007) and endocytosis of receptors for signal transduction pathways, such as for Bmp signaling (Paarmann et al., 2016) and Brain Derived Neurotrophic Factor (BDNF) signaling (Zheng et al., 2008). Given that the knockouts mentioned above, which all deal with disruption of one specific signaling pathway, resulted in incomplete disruptions of CH growth, it is interesting to note that *Dnm1/3* knockout resulted in a nearly complete obliteration of CH growth. Fan and colleagues (2016) demonstrated that Dnm1 and Dnm3 proteins are present in the CH terminal and the principal neuron as early as P1. Only occasional formation of large CH terminals was observed in the *Dnm1/3* knockouts at P8, but initial connectivity between GBC axons and principal neurons at P1 was unaffected. In parallel with a failure of CHs to grow, the typical expansion of MNTB nucleus size also failed to progress (Fan

et al., 2016; Fig. 11A-C). These results demonstrated that a “sledgehammer” approach led to the most dramatic reduction of CH terminal size observed to date. Deletion of the *Dnm* genes affects activity levels through inhibition of vesicle recycling as well as signal transduction through essential growth factor pathways through inhibition of endocytosis. These findings make it apparent that there is a need to experimentally separate the contributions of synaptic activity levels and signal transduction pathways in CH terminal growth.

Similar to the *Sad* kinase and *Bmp* receptor knockout studies, the pre- and postsynaptic contributions of *Dnm1/3* cannot be measured separately because the genes were deleted using the *Egr2*-Cre driver. Studies which separately manipulate the presynaptic GBCs and the postsynaptic principal neurons in conditional genetic knockout studies are sparse in the field, despite the existence of the *Atonal 7(Atoh7)*-Cre line which labels GBCs (as well as spherical bushy cells) in the VCN but not principal neurons (Saul et al., 2008) and the *En1*-Cre line (Kimmel et al., 2000) which labels principal neurons (Marrs et al., 2013; Altieri et al., 2015; Brandebura et al., 2018) but not GBCs (unpublished observations, Spirou lab). Future studies focused on signal transduction mechanisms should be carefully designed to discriminate between presynaptic and postsynaptic contributions. Furthermore, future studies should also focus on other potential growth factor pathways, such as Wnt signaling, which was demonstrated to regulate synaptic terminal maturation in other systems such as hippocampus, cerebellum and the NMJ (Hall et al., 2000; Henriquez et al., 2008; Sahores et al., 2010).

CH morphological maturation

The CH undergoes morphological refinement across development, where it transforms from a cup-like shape at P3 to P6 to a fenestrated morphology beginning at P9. The fenestration of the CH occurs differentially along the tonotopic axis of the MNTB, with CHs in the medial high-frequency encoding region reaching the fenestrated morphological state earlier than CHs residing in the lateral low-frequency encoding region. As fenestration occurs, astrocyte

processes which express glutamate transporter proteins invade the spaces between the CH processes, likely contributing to rapid glutamate clearance from the synaptic cleft (Ford et al., 2009). Interestingly, disruption of the exocyst complex in GBCs, which mediates addition of membrane at nerve terminals in *Drosophila* (Murthy et al., 2003), results in perturbation of CH morphological maturation. Schwenger & Kuner (2010) overexpressed a dominant negative form of the Exo70 protein (Exo70 Δ C), which has a C-terminal truncation and prevents membrane targeting of the Exo70 protein. Exo70 Δ C was expressed through stereotaxic viral injection into the VCN of P2 rats. Three-dimensional reconstruction of confocal images of CH terminals at P13 showed a significant decrease in CH volume compared to sham-injected mice. The volume deficit recovered by P21, but at the later age the CH terminals displayed reduced morphological complexity, as represented by a decreased degree of fenestration (Schwenger & Kuner, 2010). As such, the exocyst complex may play a role in the formation of polarized fenestrated processes at the CH terminal. The stereotaxic injection of virally transduced Exo70 Δ C was performed at P2 and thus expression of Exo70 Δ C was delayed until after CH growth was mostly complete. However, it would be interesting to evaluate earlier effects of Exo70 Δ C expression on CH terminal growth.

Growth and tonotopic organization of principal neurons

The principal neurons increase in size as the CH terminal grows and increases in synaptic strength, demonstrated by a 32% increase in surface area between P0 and P4 (Hoffpauir et al., 2010). It is therefore predicted that synaptic activity drives principal neuron growth. In support of this hypothesis, the volume of the MNTB is decreased compared to controls in *Diminuendo* (*Dmdo/Dmdo*) mice, which have a point mutation in the microRNA, miR-96. The decreased MNTB volume is due to a decrease in principal neuron size. Furthermore, two predicted targets of miR-96, are the Kv1.6 and BK potassium channels. These ion channels are significantly reduced at the protein level in *Dmdo/Dmdo* mice. Electrophysiological analysis of *Dmdo/Dmdo*

mutant mice showed significantly higher rates of short-term synaptic depression, slower kinetics of the fast α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-mediated currents and a larger contribution of the slow N-methyl-D-aspartate (NMDA)-mediated currents (Schlüter et al., 2018). The persistent presence of NMDA-mediated currents at mature ages is in stark contrast to the normal physiological switch from NMDA-mediated currents to AMPA-mediated currents which occurs around P8 in wildtype mice (Futai et al., 2001; Joshi & Wang, 2002), reflecting a more immature electrophysiological state.

Interestingly, the principal neurons are heterogeneously distributed in size along the tonotopic axis of the MNTB. The more laterally located, low-frequency encoding neurons have a larger size than the more medially located, high-frequency encoding neurons under normal conditions. In slice recordings, the lateral neurons have larger EPSCs than the medial neurons. However, *Deafwaddler* mice, which have a null mutation in the *Plasma membrane calcium ATPase 2* (*Pmca2*) gene and are deaf, present with a disruption of the tonotopic neuronal size gradient. The disruption of neuronal size gradient was recapitulated with administration of tetrodotoxin, demonstrating that disruption of synaptic activity leads to the impaired neuronal size gradient (Weatherstone et al., 2017). Additionally, principal neurons display a tonotopic gradient of Kv3.1b protein, a subunit of voltage-gated potassium channels. Under normal conditions, the medially located principal neurons express higher levels of Kv3.1b (Li et al., 2001). Tonotopic organization of Kv3.1b is disrupted by genetic deletion of the *Fragile X Mental Retardation 1* (*Fmr1*) gene, which is an RNA-binding protein that negatively regulates Kv3.1b mRNA translation in the brainstem. *Fmr1* knockouts have a reduced ability to up-regulate Kv3.1b expression in response to acoustic stimulation (Strumbos et al., 2010), suggesting impaired experience-dependent plasticity. A separate study demonstrated that *Fmr1* knockout rats have significantly smaller principal neurons (Ruby et al., 2015), suggesting that principal neuron growth may be related to up-regulation of voltage-gated potassium channels.

Glia in the MNTB

After a review of the literature above, it is clear that most studies in the MNTB have been neuron-centric. However, the CH:MNTB system does not mature in isolation. Other cell types, including glia and VACs, are present in the MNTB during early postnatal development. A thorough understanding of development of the system requires the field to focus on glial and VAC proliferation and differentiation and the mechanisms by which neurons, glia and VACs communicate with one another in the MNTB.

Glial proliferation

Astrocytes which are positive for the Aldehyde dehydrogenase 1 family member L1 (Aldh1L1) protein are present in the MNTB as early as P0; albeit few cell bodies are present at this age and the Aldh1L1 labeling mostly consists of processes beginning to infiltrate the nucleus (Dinh et al., 2014; Kolson et al., 2016). By P6 the Aldh1L1-positive astrocyte cell bodies are qualitatively more abundant and at this age there are also astrocytes positive for the S100 calcium binding protein B (S100 β ; Dinh et al., 2014). The qualitative observation of an increase in MNTB astrocyte number between P0 to P6 (Dinh et al., 2014) is supported by findings from Saliu and colleagues (2014) that demonstrated a rapid increase in the number of MNTB cells which incorporated 5'-ethynyl-2'-deoxyuridine (EdU) after injection into rats at E20, indicating cell proliferation. It was demonstrated that ~60% of the cells which incorporated EdU after injection at E20 were S100 β -positive and ~30% of EdU-positive cells were S100 β -positive after EdU injection at P1. When EdU injection was performed at P6, less than 10% of the cells which incorporated EdU were S100 β -positive, yet there was still a substantial number of EdU-positive cells (Saliu et al., 2014). These results indicate that astrocytes proliferate early in the first postnatal week and another cell type (likely oligodendrocytes and microglia; discussed in Chapter 2) begins to proliferate in the second half of the first postnatal week.

Astrocyte markers

Dinh and colleagues (2014) demonstrated that Aldh1L1-positive astrocytes are present in the MNTB at P0, but S100 β -positive astrocytes are not seen until P6 (the next age tested).

However, the two antibodies were not applied together in the same sections, so it is as of yet unclear whether the Aldh1L1-positive astrocyte population expands and the same astrocytes later become S100 β -positive, or whether there are two separate astrocyte populations.

Furthermore, another common astrocyte marker, Glial fibrillary acidic protein (Gfap), is not detected in the MNTB at P6. Gfap-positive astrocytes are not observed until P14 (the next age tested; Dinh et al., 2014). Again, it is not known whether this is a separate astrocyte population or whether the same astrocytes later become immunoreactive for Gfap.

Astrocyte contact with the CH and principal neuron

Several studies have demonstrated a close association of astrocyte processes with the developing CH either by light or electron microscopy techniques (Ford et al., 2009; Reyes-Haro et al., 2010; Dinh et al., 2014; unpublished data, Spirou lab). Ford and colleagues (2009) showed astrocyte processes immunoreactive for glutamate transporter proteins in close association with the fenestrated processes of the CH in the gerbil, in line with the well known role of astrocytes in facilitating glutamate clearance. Between P8 and P10 in the mouse, it was shown using both light and electron microscopy that astrocyte processes physically contact the CH terminal. Furthermore, spontaneous calcium responses were seen in astrocytes which temporally correlated with the detection of slow inward currents (SICs) in the principal neurons (via astrocyte glutamate release). When calcium signaling was abolished in the astrocytes using an intracellular calcium chelator, the SICs in the principal neurons were inhibited, indicating neuromodulatory communication between astrocytes and principal neurons (Reyes-Haro et al., 2010).

At earlier ages before fenestration, astrocytes contact the growing CH terminal (Dinh et al., 2014; unpublished data, Spirou lab). Using light microscopy at P6, it was demonstrated that Aldh1L1- and S100 β -positive astrocyte processes contact the nonsynaptic side of the CH terminal (Dinh et al., 2014). In SBEM reconstructions throughout the first postnatal week, astrocyte processes can be seen contacting the nonsynaptic side of the CH terminal as well as noninnervated territory on the membrane of the postsynaptic principal neuron (unpublished data, Spirou lab). Given that astrocytes are known sources of secreted growth factors (Christopherson et al., 2005; Kucukdereli et al., 2011; Allen et al., 2012; Farhy-Tselnicker et al., 2017), future studies should investigate the physiological relevance of physical contact between astrocyte processes and the growing CH terminal.

Oligodendrocytes and myelination in the MNTB

Myelin is detected in the mouse MNTB by P4 as indicated by immunostaining for Myelin basic protein (Mbp; unpublished data, Spirou lab). Sinclair and colleagues (2017) quantified the developmental increase of myelin thickness using light microscopy imaging of Mbp protein and demonstrated that the myelin thickness increases from P8 until just after P30 when it subsequently plateaus. The rate of myelination is driven by activity in the GBC axon because sensory deprivation through earplugging at hearing onset results in a significantly reduced diameter of the myelin sheath (Sinclair et al., 2017). This study indicates that activity in the GBC axon either directly or indirectly (possible via astrocytes) signals to oligodendrocytes to generate the myelin sheath. In support of an indirect signaling mechanism via astrocytes, SBEM reconstructions of early postnatal axons in the MNTB (beginning at P4) show astrocyte processes ensheathing the axons with single and multiple wraps. Furthermore, there is a developmental switch in the percentage of wraps traced back to astrocytes vs. oligodendrocytes, where more wraps originate from astrocytes at early developmental ages and this is followed by an increase in the percentage of wraps originating from oligodendrocytes

(unpublished data, Spirou lab; Fig. 12). These data raise the intriguing possibility that astrocytes serve an early function in insulation of the GBC axon and retract their processes once the oligodendrocytes have reached sufficient numbers and levels of proteins required for myelination.

Transcriptional profiling as a tool for studying neural circuit development

The studies reviewed in the sections above have provided insight into stages of development of the CH:MNTB system, including embryonic origins and migration of the GBCs and principal neurons, GBC axon guidance, initial synaptogenesis in the MNTB, growth and morphological refinement of the CH and the principal neuron, as well as what little is known about glial cell proliferation and differentiation. The cumulation of the findings from these studies have led to a well-characterized progression of structural and maturational changes that occur in the developing CH:MNTB (summarized in Fig. 13). Although some studies have touched on signaling mechanisms that regulate each of these steps, there is still very little known about intercellular communication in the MNTB in comparison to other systems such as the cortex or hippocampus. In a case such as this, high-throughput transcriptional profiling can be an invaluable tool to generate large datasets which can provide the basis for generating mechanistic hypotheses that can then be tested at the protein level.

Transcriptional studies performed at multiple developmental timepoints are useful in elucidating dynamic changes in gene expression level and how these might relate to defined maturational events in the system. For example, two studies in the retina used a Serial Analysis of Gene Expression (SAGE) approach to identify changes in gene expression from embryonic to adult ages, and these studies provided the groundwork for the elucidation of many disease-related genes (Blackshaw et al., 2001; 2004). Another group performed an impressive, dense temporal sampling of transcriptional changes in the cerebellum (eight timepoints from age E18 to P56) using microarrays and through this analysis they were able to tie differential gene expression to

specific structural changes which occur during neural circuit formation in this region (Sato et al., 2008). In the auditory system, several studies have analyzed developmental gene expression changes in the VCN, whole SOC and microdissected MNTB (Harris et al., 2005; Ehmann et al., 2013; Kolson et al., 2016). These bulk tissue analyses have helped to identify genes which may be regulated by changes in circuit activity through comparison of gene expression levels before and after the onset of hearing.

The current work builds on a microarray study performed on microdissected MNTB tissue at P0, P1, P2, P3, P4 and P6 (Kolson et al., 2016). The MNTB microarray study identified 541 genes which significantly changed expression levels between P0 to P6. The 541 genes were then grouped into eight distinct temporal profiles using k-means clustering. The temporal profiling distinguished genes that change expression levels early or late in the first postnatal week and also which genes had a unidirectional change versus those that changed bidirectionally (Fig. 14A). Some of the most interesting genes fell into Group 4 and Group 5, which increased expression levels until ~P4-P5 and then either plateaued (Group 4) or then decreased expression levels (Group 5). These genes are candidates for regulators of CH growth due to their increasing expression levels during the period of rapid terminal growth (Kolson et al., 2016).

Although quite useful in identifying transcripts with dynamically changing expression levels, the bulk tissue approach did not allow for distinction of transcript levels in different cell types (neurons, subtypes of glia and VACs). Due to the lack of relevant studies on cell type-specific transcript expression in the MNTB, attempts were made to categorize transcripts by cell type using expression data generated from the developing cortex (Cahoy et al., 2008). However, after cell type categorization it became apparent that transcripts falling into the groups with unidirectionally increasing expression profiles (Groups 1-3) were primarily glial-enriched transcripts and transcripts falling into the groups with unidirectionally decreasing expression

profiles (Groups 7-8) were primarily neuron-enriched transcripts (Fig. 14B). These results suggested that postnatal gliogenesis introduced a potential confound: increasing expression levels for transcripts enriched in glial cells could be detected as increasing due to addition of glial cells to the system or due to true transcriptional up-regulation on a per cell basis. The identification of this confound introduced the need for quantification of cell numbers across MNTB development followed by cell type-specific transcriptional profiling to identify transcripts differentially expressed in each cell type in the MNTB.

The earliest high-throughput transcriptional studies performed with cell type-specificity were performed in the cortex. Fluorescent activated cell sorting (FACS) approaches allowed for the isolation of pure populations of neurons or subtypes of glial cells using specific markers. Lovatt and colleagues (2007) were able to separately isolate cortical neurons and astrocytes using FACS and analyze differences in their gene expression profiles using microarrays, leading to the identification of unique metabolic preferences in the two cell types. A separate group used FACS and microarrays to compare gene expression profiles of cortical astrocytes and microglia in young vs. aged mice, providing a comprehensive database of gene expression changes during aging in these cell types (Orre et al., 2014).

Immunopanning techniques, which allow for the isolation of individual cell types with specific combinations of cell surface antibodies, advanced transcriptional studies of neural circuit formation even further by allowing for differential gene expression analysis across many cell types at once. In a series of studies using a combination of immunopanning, genetically-mediated fluorescent labeling, FACS, microarray and RNA-Seq technologies, specific transcriptional profiles for all major cell types in the cortex (neurons, astrocytes, oligodendrocytes, microglia and VACs) were generated at multiple developmental timepoints (Cahoy et al., 2008; Daneman et al., 2010; Zhang et al., 2014). These studies resulted in the identification of several novel cell type-specific markers as well as genes useful in the

identification of different maturational stages of oligodendrocytes (from precursor cells to immature nonmyelinating oligodendrocytes to mature myelinating oligodendrocytes) and immature vs. mature astrocytes. Furthermore, these studies resulted in large datasets of differentially expressed transcripts encoding for particular ligands and their cognate receptors across multiple cell types, providing the first network level insight into intercellular signaling patterns between neurons, glia and VACs in the brain. The datasets that were generated provided the basis to identify gene candidates which were shown in later studies to be involved in glial-mediated regulation of synaptogenesis and synaptic pruning (Kucukdereli et al., 2011; Allen et al., 2012; Chung et al., 2013; Farhy-Tselnicker et al., 2017). Important to note is that the only cell type-specific transcriptional study performed to date in the auditory brainstem was performed by Körber and colleagues (2014). The study utilized laser capture microdissection to specifically characterize the transcriptional profile of GBCs in the VCN across development. Transcripts encoding for ion channels and calcium ion binding proteins were strongly up-regulated around the onset of hearing, suggesting that opening of the ear canal and increases in circuit activity contribute to electrophysiological maturation of the CH terminal through transcriptionally-based mechanisms (Körber et al., 2014). Cell type-specific transcriptional profiling on the postsynaptic principal neurons, glial cells and VACs in the MNTB is a knowledge gap in the literature which needed to be addressed.

Single cell RNA-Seq (scRNA-Seq) technology added an additional layer of complexity to high-throughput transcriptional studies, allowing for the identification of subtypes of cells within a class (ie. subtypes of neurons). A major advantage of scRNA-Seq is that one does not have to rely on predefined genetic markers, which may not always be specific across brain regions or developmental ages, to isolate cell types for analysis. scRNA-Seq technology instead relies on semi-supervised (using a list of several defined marker genes) or unsupervised clustering (using no marker genes) methods to sort cells into transcriptionally distinct groups based upon their

gene expression profiles. scRNA-Seq studies have dramatically expanded the number of known cell types in the brain. In the retina Macosko and colleagues (2015) used scRNA-Seq to identify 39 unique cell populations and Chen and colleagues (2017) performed scRNA-Seq in the hypothalamus resulting in the identification of 11 nonneuronal cell clusters along with 34 neuronal clusters. In the auditory nerve fiber, scRNA-Seq studies resulted in the identification of three subtypes of Type I SGNs, adding additional complexity to the long-standing characterization of Type I vs. Type II SGNs (Shreshtha et al., 2018; Sun et al., 2018). Additionally, scRNA-Seq studies performed at multiple developmental timepoints allow for the construction of transcriptional regulatory networks. An impressive developmental study performed at 12 timepoints expanded the field's knowledge of transcription factor networks and cell fate decisions in the cerebellum (Carter et al., 2018). The transcriptional characterization of rare subtypes of cells, which contain unique transcriptional profiles that would otherwise be diluted in a bulk analysis, is also promoted with scRNA-Seq technology. In the cochlea, scRNA-Seq allowed for transcriptional profiling of rare subtypes of cells in the cochlear and utricular sensory epithelium (Burns et al., 2015).

The extremely low number of cells in the MNTB has hampered cell type-specific transcriptional studies in the region. Stereological estimates in the rat report only ~6,000 neurons total in the mature MNTB (Kulesza et al., 2002; Rodríguez-Contreras et al., 2006). Careful microdissection of the MNTB limits the number of cells which can be collected without contaminating the sample with cells outside the MNTB borders, making isolation of specific cell types by FACS or co-immunoprecipitation methods technically challenging. Furthermore, the low viability of MNTB cells in culture makes immunopanning methods unreliable. The advent of scRNA-Seq technology therefore opened a window of opportunity to study transcriptional profiles of individual cell types in the MNTB.

Objectives

The development of the CH:MNTB system has been well-characterized structurally and electrophysiologically. However, studies in the field have been mostly neuron-centric and little is known about intercellular signaling pathways that regulate development of the system. Based on this knowledge gap, the current work aimed to undertake two main objectives.

The first objective was to characterize changes in neuron and glial cell numbers across MNTB development. Arguably, quantification of individual cell types would provide a framework to better understand the temporal profiles of transcripts identified in the MNTB microarray study that was performed by Kolson and colleagues (2016). Using cell type-specific antibodies for neurons, astrocytes, oligodendrocytes and microglia, the percent contribution of each cell type was quantified at two timepoints during the first postnatal week in the mouse MNTB. Antibodies for proliferation and mitosis were also used to demonstrate that glial cells actively undergo cell division within the boundaries of the MNTB during the first postnatal week (Brandebura et al., 2018).

Correlation of changes in cell number with developmental gene expression changes in the MNTB was a useful first step to further refine the list of 541 genes significantly changing between P0 to P6 generated by Kolson and colleagues (2016). However, many transcripts identified in the microarray study were still unassigned to a particular cell type. Therefore, the second objective of this work was to utilize scRNA-Seq to generate cell type-specific transcriptional profiles for each major cell type in the developing MNTB. Unsupervised clustering analysis yielded four major cell clusters, which represented neurons, astrocytes, oligodendrocytes and VACs. In this study, a new genetic marker was identified for MNTB principal neurons. In addition, the analysis elucidated several candidate intercellular signaling pathways, including Fibroblast Growth Factor (FGF) and Delta-Notch signaling, which may be important in the regulation of neural circuit formation in the CH:MNTB system. The

transcriptional dataset generated in this work will provide the basis for multiple genetic perturbation experiments that will be performed in the future and the creation of new transgenic tools which will be useful to the scientific community.

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Figures

A.

B.

modified from von Gersdorff & Borst, 2002

modified from Kandler et al., 2009

Figure 1. Anatomy, circuitry and tonotopic organization of the auditory brainstem. A.) The SOC contains the MNTB, MSO and LSO, as well as several other small nuclei not depicted here. The SOC is the first site of massive binaural convergence. The MNTB receives excitatory

(+) input from the GBCs in the contralateral VCN. The MNTB neurons in turn send inhibitory (-) inputs to the LSO and MSO. The LSO and MSO (not depicted) both receive excitatory inputs from bushy cells in the ipsilateral VCN. The comparison of the intensity and timing of ipsi- and contralateral inputs is encoded in the LSO and MSO, respectively. **B.)** The auditory system, including the cochlea, CN and SOC is tonotopically organized into high-frequency (purple) and low-frequency (red) encoding regions. Panel A modified from von Gersdorff & Borst, 2002. Panel B modified from Kandler et al., 2009.

Figure 2. Timeline of maturation in several model neural systems. There are three phases of maturation characterized in model neural systems. Exuberance (red) results in the formation of supernumerary synapses. Some synapses are then strengthened while others are pruned in the second stage (blue), resulting in the strengthening of several synapses per postsynaptic target. In the final refinement to monoinnervation (green), a single synapse is strengthened while the others are removed. The Neuromuscular Junction and the Climbing Fiber:Purkinje Cell are two commonly used model systems. Maturation takes over two weeks in both cases. The Calyx of Held:MNTB is a recently proposed model neural system. Maturation at the Calyx of Held:MNTB takes a little over one week, from the first functional synaptic contact at E17 to monoinnervation at a majority (~74%) of principal neurons by P6.

Panels modified from Marrs et al., 2013

Figure 3. Lineage mapping of the MNTB. **A.)** The MNTB (arrow) is labeled by Xgal in parasagittal sections from the *Egr2-Cre;Rosa26R* line, indicating rhombomere 5 origins. Scale bar is 200 μ m. **B.)** The MNTB (arrow) is labeled with GFP in parasagittal sections from the *Hoxb1-Cre* line, indicating most MNTB neurons are of rhombomere 5 origin and a subset are of rhombomere 4 origin. **C.)** The MNTB is not labeled by Xgal in the *Wnt1-Cre;Rosa26R* line, indicating that the cells are not of rhombomere 2-3 origin. Scale bar is 200 μ m. Panels A-C are modified from Marrs et al., 2013.

Panels modified from Howell et al., 2007

Panels modified from Marrs et al., 2013

Figure 4. GBC axon pathfinding and principal migration in MNTB. A.) Lipophilic tracer injection (red) shows bushy cell axons leave the CN at E13.0. The arrowhead denotes the leading edge of labeled axons. The solid yellow line denotes the midline. Rostral (R) and Lateral (L) directions are shown. **B.)** Labeled axons cross the midline and enter the MNTB. By E14.5. The yellow arrowhead shows the leading edge of a subset of axons that turn rostrally and dorsally and extend into the lateral lemniscus. **C.)** At E14.5 the cells from different SOC nuclei are clumped together in a region denoted the presumptive SOC (pSOC) and cannot be distinguished by histological boundaries. However, labeling with the *En1-Cre* line (red) and

FoxP1 and MafB antibodies reveals that the cells which will make each SOC nucleus are regionally distributed within the pSOC. MafB-positive cells (blue) reside laterally and will make the future LSO and MSO. FoxP1-positive cells (green) reside dorsally and will make the future SPN. FoxP1/*En1*-Cre double positive cells (green and red) reside medially and will make the future MNTB. Scale bar is 200 μm . **D.)** The nuclei of the SOC are histologically separated at E17.5 and express their specific markers. The MNTB (arrowhead) is labeled by FoxP1 (green) and *En1*-Cre (red). Dashed lines in C-D denote the midline. Panels in A-B modified from Howell et al., 2007. Panels in C-D modified from Marrs et al., 2013.

Panels modified from Howell et al., 2007

Figure 5. Comparison of GBC axon migration in wildtype, *Dcc* and *Ntn1* knockout mice.

A.) Lipophilic dye injected into the VCN (asterisk). Dye labeling shows the axons leave the VCN,

extend through the ipsilateral SOC and cross the midline in the wildtype mice carrying two *Dcc* alleles. Arrow points to the 8th nerve. **A'.)** Coronal sections of the auditory brainstem show labeled axons extend into the contralateral SOC in the control mice carrying two *Dcc* alleles. **B.)** The labeled axons do not cross the midline and instead only extend into the lateral boundary of the ipsilateral SOC (arrow) in the *Dcc* knockout mice. **B'.)** Coronal sections of the auditory brainstem show the absence of labeled axons in the contralateral SOC of *Dcc* knockout mice. **C.)** Lipophilic dye was placed in the VCN (asterisk). Labeled axons leave the VCN and extend past the midline in control mice carrying two *Ntn1* alleles. **C'.)** Coronal sections confirm that labeled axons cross the midline and extend into the contralateral SOC in the control mice carrying two *Ntn1* alleles. **D.)** Labeled axons do not cross the midline and instead extend only into the lateral boundary of the ipsilateral SOC in *Ntn1* knockout mice. **D'.)** Coronal sections confirm that labeled axons do not cross the midline in *Ntn1* knockout mice. Scale bars in A-D are 200 μm . Scale bars in A'-D' are 100 μm . Solid lines denote the midline. Dorsal (D) and Medial (M) directions are labeled. Panels modified from Howell et al., 2007.

Panels modified from Renier et al., 2010

Figure 6. Comparison of GBC axon migration in wildtype and *Robo3* knockout mice. A.) Lipophilic dye was injected into the VCN. Labeled axons cross the midline (arrow) and extend into the contralateral MNTB in control mice carrying two *Robo3* alleles. **B.)** Labeled axons in *Robo3* knockout mice fail to cross the midline (arrow). Scale bars are 300 μ m. Panels modified from Renier et al., 2010.

Figure modified from Hsieh et al., 2010

Figure 7. Ephrin-Eph signaling in contralateral targeting of GBC axons. Wildtype mice with intact Ephrin-Eph signaling have normal function of the EphB2 receptor (pink) and the Ephrin-B2 ligand (blue). GBC axons in wildtype mice project to the contralateral MNTB. *EphB2* knockout mice have impaired forward and reverse signaling through the EphB2 receptor, resulting in an increase in GBC axons that project to the ipsilateral MNTB. *EphB2^{lacZ}* mutant mice have impaired forward signaling through the EphB2 receptor but have no phenotype. *Ephrin-B2^{lacZ}* mutant mice have impaired reverse signaling through the ligand, result in an increase in GBC axons which project to the ipsilateral MNTB. Figure modified from Hsieh et al., 2010.

Panels modified from Toyoshima et al., 2009

Figure 8. Comparison of CH growth and apoptosis in wildtype and *Cntn5* knockout mice.

A.) MNTB neurons (labeled by Map2 in blue) of wildtype mice at P6 have large CH terminals (labeled with synapsin1 in green) that are VGLUT1-positive (red). Scale bar is 50 μ m. **B.)** *Cntn5* knockout mice have a reduced number of CH terminals (labeled by synapsin1 in green) and an increased incidence of CH terminals which are VGLUT1-negative (red). Scale bar is 50 μ m. **C.)** The percentage of principal neurons not innervated by a CH terminal is significantly higher in the *Cntn5* knockout mice (white bar) compared to wildtype mice (black bar) at P6, but there is no significant difference at Month1 (M1). ***p $\leq$ 0.001, Student's t-test. **D.)** The percentage of CH terminals which are VGLUT1-negative is significantly higher in *Cntn5* knockout mice (white bar) compared to wildtype mice (black bar) at P6, but there is no significant difference at M1. *p $\leq$ 0.05, Student's t-test. **E.)** The percentage of MNTB neurons expressing the apoptosis marker Caspase3 is significantly higher in the *Cntn5* knockout mice (white bar) compared to the

wildtype mice (black bar) at P10 and P15. ** $p \leq 0.01$; *** $p \leq 0.001$, Student's t-test. Panels modified from Toyoshima et al., 2009.

Panels modified from Lilley et al. 2014

Figure 9. Comparison of CH growth in wildtype and *Sad-A/Sad-B* knockout mice. A.) VGLUT1 (yellow) labels a large CH terminal innervating the Parvalbumin (PV)-positive (blue) principal neuron in control mice at P14. Scale bar is 4 μ m. **B.)** The VGLUT1-positive (yellow) CH terminal appears qualitatively smaller in condition double knockout (cDKO) mice lacking Sad-A and Sad-B kinases. Scale bar is 4 μ m. **C.)** The fractional occupancy of VGLUT1 signal adjacent to PV signal is significantly reduced in cDKO mice compared to control mice. *** $p \leq 0.001$, unpaired t-test. Panels modified from Lilley et al., 2014.

Panels modified from Xiao et al., 2013

Figure 10. Comparison of CH growth in wildtype and *Bmpr1a/1b* knockout mice. A.) The front (0°) and back (180°) views of reconstructed principal neurons from SBEM are shown with their two largest inputs in control and cDKO mice lacking *Bmpr1a/1b*. The terminals are color-coded by volume (largest is green and smallest is purple). The cDKO mice have smaller terminal volumes than the control mice. Scale bar is 5 μm . **B.)** The terminal volumes are

graphed from largest to smallest in the control (black) and cDKO (red) mice. In the control mice the largest terminal is significantly larger than the largest terminal in the cDKO mice and the second largest terminal has a significantly larger drop in volume compared to the second largest terminal in the cDKO mice. Panels modified from Xiao et al., 2013.

Panels modified from Fan et al., 2016

Figure 11. Comparison of CH growth and MNTB size in wildtype and *Dnm1/3* knockout mice. **A.)** PV-25 positive principal neurons (red) are innervated by large vGlut1-positive (green) CH terminals in wildtype mice. Scale bar is 10 μm . **B.)** cDKO mice lacking *Dnm1/3* lack large CH terminals and have only small vGlut1-positive (green) punctae innervating the PV-25 positive principal neurons (red). Scale bar is 10 μm . **C.)** Wildtype mice (open squares) display an increase in MNTB area across development. cDKO mice (open circles) have significantly

smaller MNTB areas at P6, P10 and P20 compared to wildtype mice. *** $p \leq 0.005$. Panels modified from Fan et al., 2016.

Figure 12. Astrocyte vs. oligodendrocyte ensheathment of GBC axons across development. The percentage of GBC axon wrapping by glial processes is predominantly attributed to astrocytes at P4 when single/multiple astrocyte wraps (blue) account for more than 60% of total GC wrapping. At P6 the first glial wraps which could be traced back to an oligodendrocyte were found. Single/multiple oligodendrocyte wraps (orange) and compacted oligodendrocyte wraps (red) represent ~10% of glial wrapping at this age. By P9 the astrocyte wrap percentage is dramatically reduced and the glial wraps are predominantly traced back to oligodendrocytes (~85%).

Figure 13. Summary timeline of MNTB development. The first VCN-evoked action potential can be recorded in the MNTB at E17, suggesting that initial synaptogenesis has occurred by this age. Between P2 to P6 the CH terminal dramatically grows in size and by P6 most MNTB principal neurons are monoinnervated. The principal neuron grows in synchrony with the CH terminal and by P6 a switch from tonic to phasic firing occurs, temporally correlating growth of the CH and principal neuron with an acquisition of more mature electrophysiological properties. Between P0 and P9 there is substantial glial cell proliferation and differentiation. Beginning at P3 the initial stages of myelination (loose glial wrapping) can be observed. Subsequent compaction of the loose glial wraps occurs in the first postnatal week and continues until after the opening of the ear canal.

Figure 14. Temporal profiling and cell type categorization of transcripts in the MNTB. A.)

The 541 genes identified as significantly changing between P0-P6 in the MNTB microarray study (Kolson et al., 2016) were grouped into 8 distinct temporal profiles using k-means clustering. Average fold change is plotted on the $\log_2$ scale. **B.)** Significantly changing

transcripts were assigned to a specific cell type based on expression data from a microarray study in the developing cortex using a 5-fold differential expression cutoff (Cahoy et al., 2008).

Chapter 2: Glial cell expansion coincides with neural circuit formation in the developing auditory brainstem

(reprinted with permission from Developmental Neurobiology, see Appendix I)

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Conflict of Interest Statement:

George Spirou and Michael Morehead have a financial interest in syGlass visualization and analysis software used in this project.

Abstract

Neural circuit formation involves maturation of neuronal, glial and vascular cells, as well as cell proliferation and cell death. A fundamental understanding of cellular mechanisms is enhanced by quantification of cell types during key events in synapse formation and pruning and possessing qualified genetic tools for cell type-specific manipulation. Acquiring this information in turn requires validated cell markers and genetic tools. We quantified changing proportions of neurons, astrocytes, oligodendrocytes and microglia in the medial nucleus of the trapezoid body (MNTB) during neural circuit development. Cell type-specific markers, light microscopy (LM) and 3D virtual reality software, the latter developed in our laboratory, were used to count cells within distinct cell populations at postnatal days (P)3 and P6, bracketing the period of nerve terminal growth and pruning in this system. These data revealed a change from roughly equal numbers of neurons and glia at P3 to a 1.5:1 ratio of glia to neurons at P6. PCNA and PH3 labeling revealed that proliferation of oligodendrocytes contributed to the increase in glial cell number during this timeframe. We next evaluated Cre driver lines for selectivity in labeling cell populations. *En1-Cre* was specific for MNTB neurons. *PDGFR α -Cre* and *Aldh1L1-Cre*, thought to be mostly specific for oligodendrocyte lineage cells and astrocytes, respectively, both labeled significant numbers of neurons, oligodendrocytes and astrocytes and are non-specific genetic tools in this neural system.

Key words: Gliogenesis, neuron, astrocyte, oligodendrocyte, microglia

Introduction

Formation of neural circuits during brain development is increasingly appreciated as a coordinated effort among neuronal synaptic partners, glial cell types and the microvasculature. Among glial cell types, microglia have perhaps the best accepted role in synaptic pruning, and operate via the complement signaling pathway (Paolicelli et al., 2011; Schafer et al., 2012). Astrocytes can mediate synapse formation (Farhy-Tselnicker et al., 2017) and elimination (Chung et al., 2013) and affect the balance of excitatory and inhibitory synaptogenesis via temporally regulated secretion of synaptogenic and inhibitory factors (Kucukdereli et al., 2011). Oligodendrocyte precursor cells (OPCs) also can affect the number of synaptic inputs, as demonstrated for cerebellar Purkinje cells (Doretto et al., 2011). Vascular cells communicate with OPCs to mediate their attachment, migration, detachment and differentiation (Tsai et al., 2016), and likely signal also with astrocytes to function in angiogenesis and blood-brain-barrier formation (Cahoy et al., 2008; Daneman et al., 2010).

Establishing the dynamics of coordinated, intercellular communication that guides neural development is aided by quantification of the appearance and numbers of each major cell type during the formation of neural circuits. The relative proportions of neurons and glia, measured by using relatively recent high-throughput methodology (isotropic fractionation) and more classical stereological approaches (e.g., human, Pelvig et al., 2008; Azevedo et al., 2009; rat, Bandeira et al., 2009; Savchenko et al., 1997), vary across brain regions and during neural development, but typically are not reported per cell type nor with high temporal resolution. Relative numbers of cell types are also missing ingredients in most studies of whole-tissue gene expression studies across developmental age or that compare developmental with mature time points (Blackshaw et al., 2001; Harris et al., 2005; Sato et al., 2008; Brusés, 2010; Ehmann et

al., 2013; Kolson et al., 2016), yet these numbers are important to interpret temporal changes in levels of gene expression.

In order to fill this gap, we characterized and quantified neurons, astrocytes, oligodendrocytes and microglia in a developing neural circuit that forms within a precise temporal window of short duration. We utilized formation of the calyx of Held (CH), the largest nerve terminal in the mammalian brain, which grows and refines to a 1:1 innervation pattern with its postsynaptic partner, the principal neuron, in the medial nucleus of the trapezoid body (MNTB (Holcomb et al., 2013). This system exhibits expedited CH growth and pruning to the endpoint of mono-innervation for most synaptic connections between P3 to P6, in synchrony with biophysical maturation of synaptic partners (Kandler & Friauf, 1993; Hoffpauir et al., 2006, 2010; Rusu & Borst, 2011; Holcomb et al., 2013).

Previous cell counts of the MNTB were focused on neurons in rats (Kulesza et al., 2002; Rodríguez-Contreras et al., 2006). In a comprehensive study evaluating glial cells in the early postnatal MNTB, qualitative measurements found that immunofluorescent labeling of astrocytes and oligodendrocytes increases greatly during the first postnatal week, and labeling for microglia does not attain appreciable levels until after P6 (Dinh et al., 2014). A substantial number of non-neuronal cells within the MNTB exhibit EdU incorporation during this time, about 30% of which become S100 β ⁺ astrocytes (Saliu et al., 2014), but the extent to which oligodendrocytes contribute to this expansion in cell number is not known. The CH:MNTB system provides an opportunity to relate a quantitative picture of neuronal and glial cell numbers to key steps of synaptic growth and pruning. We validated that well accepted, cell type-specific immunohistochemical markers for neurons (labeled by microtubule-associated protein 2; Map2), astrocytes (labeled by aldehyde dehydrogenase family member 1 L1; Aldh1L1), microglia (labeled by ionized calcium-binding adaptor molecule 1; Iba1) and oligodendrocyte-lineage cells

(labeled by SRY-box 10; Sox10) showed non-overlapping labeling of cell populations at P3 and P6, framing the rapid growth of CH terminals and their refinement to mono-innervation (Holcomb et al., 2013). Specificity of associating Aldh1L1 labeling with particular labeled nuclei is difficult, because Aldh1L1 labels the complex cellular processes of astrocytes. We developed and employed a 3D virtual reality system, which permits faster and more accurate counting of Aldh1L1-labeled astrocytes, along with neurons and all other glial cell types.

We then tested these immunological markers as genetic tools to study tissue development, noting that cell-specific expression at more developed or mature ages may not be useful in conditional Cre lines due to potential transient, early expression in other cell types. Our lab and others showed that the *En1-Cre* mouse line labels most, if not all, MNTB neurons (Marrs et al., 2013; Altieri et al., 2015), but this has not been assessed systematically across the tonotopic and rostro-caudal axes, nor for its selective specificity only in neurons across these axes. We also investigated the utility of the *Aldh1L1-Cre* line (Tien et al., 2012) as a specific marker for astrocytes in the MNTB, and the *PDGFR α -Cre* line (Roesch et al., 2008) as a marker for oligodendrocyte-lineage cells in the MNTB. The *Aldh1L1-Cre* line is mostly specific for astrocytes in forebrain and spinal cord, albeit labeling a small percentage of neurons (~10%) (Foo & Dougherty, 2013). Lineage-labeling in the *PDGFR α -CreER* line is mostly specific for the oligodendrocyte lineage, but labels subsets of neurons (Rivers et al. 2008). Since many labs study the early postnatal timeframe, we chose to characterize the labeling patterns of non-tamoxifen-inducible lines, *Aldh1L1-Cre* (Tien et al., 2012) and *PDGFR α -Cre* (Roesch et al., 2008) in the MNTB in order to avoid issues of incomplete recombination during early development in future genetic manipulation studies. In this study, we demonstrate that lineage-labeling using common glial Cre lines is less cell type-specific in the MNTB than previously reported for other brain regions, and these glial Cre lines are not useful genetic tools for cell type-specific manipulation in studying calyceal development.

Materials and methods

All procedures involving animals were approved by the West Virginia University Institutional Animal Care and Use Committee.

Animal Breeding

Lineage mapping was performed by crossing various Cre mouse lines (heterozygous males) with a reporter line where tdTomato (tdTom) expression is Cre-dependent (Ai9; Jackson Laboratory, Bar Harbor, ME, Stock No. 007905; homozygous females) (Madisen et al., 2010). This fluorescent reporter line is referred to in this manuscript as *Ai9*. The Cre lines used were the *Engrailed1-Cre*, a generous gift from Dr. Mark Lewandoski (Kimmel et al., 2000), B6;FVB-Tg(*Aldh1L1-Cre*)JD1884Htz/J, Jackson Laboratory, Bar Harbor, ME, Stock No. 023748 (Tien et al., 2012), and C57BL/6-Tg(*PDGFR α -Cre*)1Clc/J, Jackson Laboratory, Bar Harbor, ME, Stock No. 013148 (Roesch et al., 2008). These lines are subsequently referred to as *En1-Cre*, *Aldh1L1-Cre* and *PDGFR α -Cre*, respectively. The crosses to the reporter lines are referred to here as *En1-Cre;Ai9*, *Aldh1L1-Cre;Ai9* and *PDGFR α -Cre;Ai9*. A separate reporter line, B6N.129-*Rpl22*^{tm1.1 Psam} /J, Jackson Laboratory, Bar Harbor, ME, Stock No. 011029 (Sanz et al., 2009), was used to verify the results of the Ai9 studies. This line is referred to as the *Rpl22-HA* line. The cross to the Cre line is referred to as *Aldh1L1-Cre;Rpl22-HA*. Cre-negative controls were used in both reporter lines to verify that there was no reporter activity in the absence of Cre recombinase. Wildtype C57Bl6/J mice at P3 and P6 were used for measuring Sox10⁺ proliferating cells, the co-labeling studies with Olig2, Sox10 and Aldh1L1, and all triple-labeling experiments for antibody validation. Postnatal day (P)0 is defined as the day of birth and all mice used in these experiments were P3 or P6.

Tissue Processing and Immunohistochemistry

Mice were anesthetized at P3 and P6 with an intraperitoneal injection of tribromoethanol (Avertin) at 125 mg/kg. Tissue was processed as described previously (Kolson et al., 2016). Briefly, mice were perfused transcardially with filtered 1x phosphate buffered saline (PBS) to remove blood, followed by filtered 4% paraformaldehyde solution (PFA) in PBS. Following perfusion, the brains were rapidly dissected in PBS and post-fixed in 4% PFA. Fixed brains were placed into a cryoprotectant solution of 30% sucrose in 0.01 M PBS. The cryoprotected brains were mounted onto a freezing microtome (model HM 450, Microm, Walldorf, Germany) and sectioned in the coronal plane at 40 μ m thickness.

From each brain, eight tissue sections spanning the rostro-caudal extent of the MNTB that fully contained MNTB cells throughout the entire section depth, were collected in serial order (Fig. 1A). The immunohistochemistry procedure was described previously (Kolson et al., 2016). The concentrations of primary antibodies used to label neurons (Map2), microglia (Iba1), astrocytes (Aldh1L1) and oligodendrocyte lineage cells (Sox10) in the MNTB, as well as their secondary antibodies and concentrations are listed in Supplemental Table 1. For each of the four antibody conditions, two slices were immunolabeled per brain (Fig. 1A). For a given antibody, one slice was chosen from the caudal half and one from the rostral half of the MNTB, separated by slices labeled with the other three antibodies (example of tissue processing strategy using *En1-Cre* line shown in Fig. 1A). The endogenous tdTomato expression in all slices was amplified with either anti-DsRed or anti-RFP antibody (Supplemental Table 1). Three animals from at least two different litters were processed for each Cre line. Between replicates, the section position used for each cell type-specific antibody was rotated to allow for sampling throughout the rostro-caudal axis. For the control reporter experiments with Cre-negative animals from the Ai9 reporter line and the Rpl22-HA reporter line, sections were processed with anti-DsRed and anti-HA antibodies, respectively, under imaging matched conditions to the Cre-positive animals. After application of secondary antibodies and rinsing the tissue, all sections were immersed in

300 nM 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, OR) for 20 minutes. Slices were wet-mounted in PBS (Fisher, Hampton, NH). Tiled z-stack images (1.5 μ m z-steps) containing the entire width and height of the MNTB were collected on an inverted Zeiss LSM 710 confocal microscope equipped with a motorized stage using a 20x Plan-Apochromat/0.75 NA or 40x C-Apochromat/1.2 NA objective (Zeiss Microscopy, Thornwood, NY). For all other immunostaining experiments, 2 slices per animal (wildtype C57Bl6/J mice) across 3 replicates from at least 2 different litters were evaluated.

Image Processing and Cell Counting

The tiled z-stacks were stitched together using the “Stitch” tool in the Zen 2.1 Blue software (Zeiss Microscopy, Thornwood, NY). The stitched images were then imported into FIJI as .czi files using the BioFormats Importer plug-in. The brightness and contrast for the three channel images were adjusted uniformly across the image in FIJI using the “Adjust Brightness and Contrast” tool as well as the “Stack Contrast Adjustment” plug-in. A region of interest (ROI) was drawn around the MNTB using the “polygon tool”. The dimensions of the MNTB change along the rostro-caudal axis so a new ROI was drawn to closely outline the borders in each section rather than using one standard-sized ROI. The ROI was then cropped from the section in FIJI and 15 sequential images from each z-stack, covering 22.5 μ m of tissue depth where fluorescence signal was reliably bright, were selected using the “Make Substack” tool. A composite image was created and then saved as a series of .tif files to be imported into syGlass software (see below; Pidhoryskyi et al., submitted). Adobe Photoshop and Illustrator (Adobe Systems, San Jose, CA) were used uniformly to adjust brightness and contrast of immunofluorescence images to create figures.

The image substack containing all cells in the ROI was imported into syGlass software (www.syglass.io, IstoVisio, Inc.), developed in our laboratory for 3D immersive virtual reality (IVR) visualization and analysis (Fig. 1B). This software permits rotation and translation of the

image volume under control of one hand while the other hand controls a “Counting Tool” to place cell-identifying dots within the cells in the 3D visualization environment, among other capabilities. The dot color is changed to encode the antibody combinations labeling the cell. Dot location and color are saved into a text file for analysis outside of syGlass. First, DAPI labeling was used to count the total number of cells in the MNTB. All DAPI-labeled nuclei that were sectioned on the first image of the z-stack (front face in Fig. 1B) were marked using the “Visualize First Slice” option in syGlass (white dots on left part of image in Fig. 1C; images in Fig. 1B, C captured using “Photography Tool” in syGlass). This procedure ensured that cells were counted only when their nuclei were first encountered when moving through the tissue and thereby avoided double counting of cells (colored dots in Fig. 1C). The “Visualize First Slice” mode was then turned off and the remaining DAPI-stained nuclei were classified by cell type by toggling the cell type-specific immunohistochemical marker using the “Voice Commands” feature and marked using our dot color code.

The numbers of neurons, astrocytes, oligodendrocytes and microglia were then counted based on the number of cells double-labeled with their specific markers and DAPI. These numbers were then divided by the total number of DAPI-stained nuclei in the slice to give the percent of total cells for each cell type. At P6, these counts were performed across all three Cre lines crossed to the Ai9 reporter line (n=3 for each line; *En1-Cre;Ai9*, *PDGFR α -Cre;Ai9* and *Aldh1L1-Cre;Ai9*). Given the consistency of counts across these lines (see Results) at P6, we restricted our efforts at P3 to the *En1-Cre;Ai9* line (n=3). To assess the specificity of these Cre lines for particular cell types, the numbers of neurons, astrocytes, oligodendrocytes and microglia that co-localized with tdTomato expression in each of the Cre line crosses was also counted. The number of tdTomato⁺ cells for each cell type was divided by the total number of each cell type to give the percent of tdTomato-expressing neurons, astrocytes, oligodendrocytes and microglia. One observer counted all volumes and a second observer validated a subset of volumes at

each age and from each Cre line (8/24 at P3 and 12/72 at P6). The variance between the counts from the two observers was calculated. Average variance between the counts of the two observers was under 1%, so the counts from the first observer were used.

Quantification and Statistical Analysis

The proportion of each cell type that was specifically labeled with an antibody was calculated as the percentage of total DAPI⁺ cells. Error was calculated as standard deviation. The percentage in the “Other” category was calculated by adding the percentages from the four main cell types (neurons, astrocytes, oligodendrocytes and microglia) and subtracting from 100%. A two-tailed t-test was used to calculate significance in the percentage change of neurons vs. glial cells between P3-P6. To quantify proliferation of Sox10⁺ cells, the percentage of Sox10⁺/PCNA⁺ cells out of the total Sox10⁺ cells was determined. Error was calculated as standard deviation.

Results

Neuronal and Glial Composition Assayed by Cell-Specific Markers

To provide a basis for understanding tissue dynamics during neural circuit formation, we generated a quantitative census of all major cell types in the developing MNTB. We counted cell types at ages P3, when rapid CH growth and competition begins, and P6, when most principal neurons are innervated by a single calyx (Holcomb et al., 2013), using immunofluorescence for cell-specific markers (Fig. 1). DAPI nuclear staining had to be present for a cell to be counted, and each cell-specific marker was applied to a different section in the series (green label in Fig. 2A-D; asterisks denote nuclei that were counted using each marker; DAPI channel was removed to better visualize immunolabeling). Map2 antibody, which is associated with NeuN nuclear label (data not shown), was used to mark the neurons, and clearly delineated their proximal dendrites (white arrows in Fig. 2A; Toyoshima et al., 2009). Aldh1L1 antibody was

used to label astrocytes because it marks a higher proportion of this cell type than GFAP (Cahoy et al., 2008), a marker for activated cells which was qualitatively shown to label fewer astrocytes than Aldh1L1 in early postnatal development of the MNTB (Dinh et al., 2014). Aldh1L1 fluorescence filled small processes throughout the field of view, but also revealed cell bodies by a concentration of label (white arrows in Fig. 2B) next to the nucleus or on adjacent sections (single or double asterisk, respectively, in Fig. 2B). Microglia were labeled using Iba1 (Ito et al., 1998), which provided a thin layer or small cluster of cytoplasmic label reflecting the small size of their cell body (Fig. 2C). We chose Sox10, which is a nuclear label (Fig. 2D), to mark oligodendrocytes because it is specific for this cell type and labels all oligodendrocyte-lineage cells (Rinholm et al., 2011). Olig2, another common oligodendrocyte marker, also labels MNTB astrocytes during early developmental stages (closed arrowheads in Fig. 2E, 2E'; note that Aldh1L1 is depicted in blue; Kolson et al., 2016). Sox10 (Fig 2E'') only co-localizes with Olig2 in cells that are not directly juxtaposed with Aldh1L1 labeling of the cell body (open arrowheads in Fig. 2E'''; Supplemental Video 1). Although many of these antibodies have already been validated for specificity to the target epitope in previous reports (Commo et al., 2004; Kenyon et al., 2010; Casimiro et al., 2013), we confirmed the specificity of antibodies for cell type determination by carefully examining the lack of overlap of antibody labeling. These sections were labeled for three of four markers to cover every antibody combination (Supplemental Fig. 1) and showed the antibodies used labeled distinct cell types with no overlap.

The syGlass 3D immersive visualization program was used to perform cell counts because it enables rapid and accurate assessment of sectioned DAPI-labeled nuclei at the edge of the first image plane (Fig. 1C), and it permits accurate assessment of spatial association of markers with DAPI in three dimensions. Since glial cells, especially astrocytes and microglia, have complex processes that extend radially out of the cell body in all directions, we found it easier to

associate labeling patterns with a DAPI-stained nucleus in 3D IVR (Supplemental Videos 2-3). Furthermore, 3D IVR visualization allowed for rapid counting of cells in the z-dimension without the time-consuming procedure of scrolling up and down through 2D slices to identify cells, or interpreting 3D visualization on a 2D screen. 3D IVR visualization also permitted accurate and rapid assessment, through the ability to rotate the z-stack in 360° and check dot placement on DAPI-stained nuclei, that cells were not counted twice and that closely apposed cells were not missed (Supplemental Video 4). 3D analysis in IVR was also very efficient, so we counted every cell in the image stack for each section. These procedures resulted in counting of over 29,000 DAPI-labeled cells across the nine P6 animals used in this study and over 8,000 DAPI-labeled cells in the three P3 animals and assigning over 20% of this total to a specific cell type (Supplemental Tables 3-6).

Since we could not label, and hence count, all cell types in each section, results are presented as percent of total cells for each animal. At P3 (n=3), the neurons represented $46.6 \pm 1.5\%$, astrocytes $28.7 \pm 1.0\%$, oligodendrocyte lineage cells $15.8 \pm 1.2\%$, microglia $1.1 \pm 0.1\%$ and cells denoted as “other” $7.8 \pm 1.2\%$ of the total cell population (Fig. 3A; Supplemental Table 2; raw cell counts listed in Supplemental Table 3). Most, if not all, of the cells in the “other” category are likely endothelial cells and pericytes, although these were not labeled with cell-specific antibodies. From tabulation of volume electron microscopy image volumes at these same ages (Holcomb et al. 2013), these are the only other cell types in the MNTB (manuscript in preparation). The rank order percentage of neurons, astrocytes, oligodendrocytes, “other” and microglia were the same across all animals (Supplemental Table 2).

At P6, cell counts were performed in the *En1-Cre;Ai9* (n = 3), *Aldh1L1-Cre;Ai9* (n = 3) and *PDGFR α -Cre;Ai9* (n = 3) lines for characterization of their cell type-specificity. The data for cell type-specificity in all three Cre lines is further explored below. The percentages and raw counts

of each cell type were very similar among Cre lines (Supplemental Tables 2, 4-6), and the rank order of percentages was the same across Cre lines (Supplemental Table 2) and across individual animals (data not shown). The percentages from the P6 *En1-Cre;Ai9* line quantification were used for the direct developmental comparison to the P3 *En1-Cre;Ai9* line quantification.

At P6 (n=3), neurons (Map2⁺/DAPI⁺) formed the largest percentage of the cell population, constituting $36.9 \pm 2.6\%$ of total cell number. Oligodendrocyte-lineage cells (Sox10⁺/DAPI⁺) were the second-largest cell population, constituting $29.0 \pm 2.8\%$ of total cells. The astrocyte cell population (Aldh1L1⁺/DAPI⁺) was nearly as large, constituting $25.3 \pm 1.4\%$ of total cells. Microglia (Iba1⁺/DAPI⁺) were relatively few in number, constituting $2.0 \pm 0.3\%$ of total cells. The remainder of DAPI⁺ cells, denoted as “other”, constituted $6.7 \pm 2.1\%$ of the total cell population (Fig. 3B; Supplemental Table 2; raw cell counts listed in Supplemental Table 4).

The comparison of cell percentages at P3 and P6 reflects the developmental dynamics of the system, where neurons are higher in relative fraction at P3 than P6 (46.6% vs. 36.9%; Fig. 3C; $p < 0.01$). Since neurons are not undergoing apoptosis nor proliferating during this time frame (Rodríguez-Contreras et al., 2006; Saliu et al., 2014), the change in neuronal fraction reflects an increase in the total cell population of 26.3% (46.6% of P3 total cells = 36.9% of P6 total cells, so $\text{P6 total cells} / \text{P3 total cells} = 46.6 / 36.9$, or a 26.3% increase). Oligodendrocyte-lineage cells nearly doubled in percentage from P3 to P6 (15.8 vs. 29.0), which reflected more than a doubling in the ratio of total number $\text{P6/P3: } 29.0\% \text{ of } 1.263 / 15.8\% \text{ of } 1$ (normalized P3 number) = 2.32-fold increase. Astrocytes and the cells denoted as “other” slightly decreased their percentage of total cells (astrocytes: 28.7 at P3 and 25.3 at P6; “other”: 7.8 at P3 and 6.7 at P6), reflecting an actual increase in their ratio of total number using the same calculation ($\text{P6/P3 astrocytes} = 1.11$; $\text{P6/P3 “other”} = 1.08$). The proportion of microglia is small at both

ages, but like oligodendrocyte-lineage cells, nearly doubled from 1.1 at P3 to 2.0 at P6, reflecting more than a doubling in their ratio of total number (P6/P3 microglia = 2.30; Fig. 3A-B).

Glial Cell Proliferation in the MNTB

Due to the large increase in the percentage of oligodendrocyte-lineage cells between P3 and P6, we evaluated whether this was due in part to local proliferation of oligodendrocyte-lineage cells within the MNTB. Immunohistochemical labeling with proliferating cell nuclear antigen (PCNA) demonstrated co-localization with Sox10⁺ cells both within the MNTB and ventral to the MNTB (MNTB is encompassed within dotted lines in Fig. 4A). Within the MNTB, most Sox10⁺ cells were PCNA⁺ at P3 ($94.2 \pm 3.1\%$; n=3), and some Sox10⁻ cells were PCNA⁺ (open arrows in Fig. 4A'). At P6, $87.8 \pm 1.5\%$ (n=3) of Sox10⁺ cells were PCNA⁺ (Fig. 4B). These numbers indicate that oligodendrocyte-lineage cells are highly proliferative during the first postnatal week in the MNTB and contribute to the observed doubling in the percentage of Sox10⁺ cells in the MNTB between P3-P6. However, PCNA is a long-lasting proliferation marker (Barton & Levine, 2008), so PCNA⁺ cells may proliferate ventral to but not locally within the MNTB, and then migrate into the MNTB upon differentiation. Further analysis with phosphorylated histone H3 (PH3), which is only expressed during the G2/M phase, showed that Sox10⁺ cells within the MNTB were mitotic (Fig. 4C). These results indicate that at least some proportion of Sox10⁺ cells within the MNTB are locally proliferating, but do not exclude that differentiated cells born ventral to the MNTB may also undergo dorsal migration.

***En1-Cre;Ai9* exclusively labels neurons in the MNTB**

Given the important roles for neurons and glia in early development, it is important to assess the utility of genetic tools for their selective manipulation. We assessed Cre lines reported to be relatively selective for the major cell types in the MNTB, namely neurons (*En1-Cre*),

oligodendrocytes (*PDGFR α -Cre*) and astrocytes (*Aldh1L1-Cre*). The homeobox transcription factor, Engrailed1 (*En1*), was previously shown with *in situ* hybridization and the Rosa26R and Ai9 reporter lines to label the MNTB nucleus (Marrs et al., 2013; Altieri et al., 2015). The loss of *En1* results in early postnatal neuronal cell death in the MNTB, and the *En1-Cre* crossed to a reporter line specifically labels neurons in the MNTB (Jalabi et al., 2013; Altieri et al., 2015). We expanded on these studies with a thorough and systematic counting of cells throughout the entire MNTB at P6, using our antibody markers for neurons and all major glial cell types. Nearly all neurons ($97.1 \pm 0.8\%$, $n=3$) in the MNTB expressed the *En1-Cre;Ai9* reporter at P6 (closed arrowheads point to Map2/tdTomato/DAPI-positive cells in Fig. 5A; DAPI removed for clarity; DsRed or RFP antibodies were used to amplify tdTomato signal), and no astrocytes, oligodendrocyte-lineage cells or microglia were co-labeled with tdTomato in this line (open arrowheads in Fig. 5B-D, quantification in Fig. 8A). These results demonstrate that nearly all neurons in the MNTB arise from an *En1*⁺ lineage, but it is not clear if the remaining ~3% are unlabeled due to incomplete penetrance of the Cre recombinase or if these represent a small population of non-principal neurons, as has been described in other species (Morest 1968; Zook & Casseday 1982; Helfert et al. 1989; Kuwabara & Zook 1991; Banks & Smith 1992; Schofield 1994).

***PDGFR α -Cre;Ai9* labels oligodendrocyte-lineage cells and subsets of astrocytes and neurons in the MNTB**

PDGFR α labels oligodendrocyte precursor cells (OPC) as early as E15 in brain and spinal cord (Nishiyama et al., 1996). These PDGFR α ⁺ OPCs also co-label with the other common marker of OPCs, Neuron Glial 2 (NG2), after E17 (Nishiyama et al., 1996). Therefore, the *PDGFR α -Cre* line should lineage-label oligodendrocytes of all maturational stages, from the NG2⁺/PDGFR α ⁺ OPC to the differentiated, myelinating oligodendrocyte. We chose to study the *PDGFR α -Cre* line over the *NG2-Cre* line because PDGFR α expression occurs earlier in the oligodendrocyte

lineage than NG2, and in a study of brain and spinal cord, the first postnatal week marked the height of colocalization between the two markers (Nishiyama et al., 1996). Additionally, NG2 labels a subset of pericytes (Ozerdem et al., 2001), and therefore, the *NG2-Cre* line is not specific to the OPC lineage.

We performed a co-localization study using the *PDGFR α -Cre;Ai9* line at P6 (n=3). As expected, tdTomato-expressing cells constituted $93.6 \pm 2.7\%$ of the total Sox10⁺ immunolabeled population (closed arrowheads Fig. 6C, quantification Fig. 8B). It is not clear whether the remaining 6.4% of cells was due to incomplete recombination efficiency or whether these cells are OPCs labeled with Sox10 that had not yet activated the *PDGFR α* promoter, given that Sox10 is a transcriptional switch for PDGFR α (Finzsch et al., 2008). Surprisingly, a high percentage ($70.2 \pm 3.1\%$) of neurons (Map2⁺) were also tdTomato⁺ (closed arrowheads in Fig. 6A, quantification in Fig. 8B). The *PDGFR α -Cre;Ai9* animals also exhibited a subset of labeled astrocytes ($36.3 \pm 6.9\%$ tdTomato⁺; closed arrowhead in Fig. 6B, quantification in Fig. 8B), but microglia were not labeled with tdTomato by this Cre line ($0.0 \pm 0.0\%$) (open arrowhead in Fig. 6D, quantification in Fig. 8B).

***Aldh1L1-Cre;Ai9* labels astrocytes, oligodendrocyte-lineage cells and neurons, as well as a subset of microglia in the MNTB**

A previous study found transcript for *Aldh1L1* to be highly enriched in astrocytes, and immunolabeling for Aldh1L1 protein to be both selective for astrocytes and to label more astrocytes than GFAP (Cahoy et al., 2008). We investigated the utility of the *Aldh1L1-Cre* mouse line in the MNTB, which was previously demonstrated as a suitable lineage label in spinal cord with the exception of ~10% neuronal and ~15% oligodendroglial labeling (Tien et al., 2012). As expected, the *Aldh1L1-Cre;Ai9* mice (n=3) expressed tdTomato in all astrocytes ($99.2 \pm 1.5\%$) (closed arrowheads in Fig. 7B, quantification in Fig. 8C). However, we noted generally

broad expression of tdTomato in these tissue sections and labeling in other cell types besides astrocytes was much more extensive than expected. For example, tdTomato was expressed in most neurons ($90.0 \pm 17.2\%$; closed arrowheads in Fig. 7A, quantification in Fig. 8C).

Unexpectedly, nearly all Sox10⁺ cells were tdTomato⁺ ($98.5 \pm 2.6\%$, closed arrowheads in Fig. 7C, quantification in Fig. 8C). Interestingly, although the numbers of microglia in the MNTB were small, they were nearly evenly split into populations that were tdTomato⁺ ($40.4\% \pm 15.6$) or tdTomato⁻ ($59.6 \pm 15.6\%$) (Fig. 7D-E, quantification in Fig. 8C).

This extensive labeling pattern in the *Aldh1L1-Cre;Ai9* line led us to consider whether tdTomato expression had escaped its requirement for Cre activation. The tdTomato expression was not observed in the Cre-negative littermate control labeled with DsRed antibody using imaging-matched conditions (Supplemental Fig. 2A-D), suggesting that germline activation of the Ai9 reporter had not occurred. We further confirmed results with the tdTomato reporter by using a second reporter line. *Aldh1L1-Cre* mice were crossed to the *Rpl22-HA* line, where hemagglutinin (HA) is expressed as a fusion with the endogenous Rpl22 protein upon activation by Cre (Sanz et al., 2009). The expression of the HA reporter in neurons and oligodendrocytes/astrocytes was also seen through co-localization of HA antibody with Map2 and Olig2 (Supplemental Fig. 3A-D; as noted earlier, Olig2 labels both oligodendrocytes and astrocytes in the MNTB). Again, the Cre-negative littermate control did not show any reporter (HA) labeling (Supplemental Fig. 3E-H). It is worth noting that the *Rosa26-Ai9* reporter labels more distal processes of cells than the *Rpl22-HA* reporter. This difference in labeling is because HA is fused onto the ribosomal subunit protein, Rpl22, and localizes mostly to the cell body where the ribosomes are in highest concentration (Sanz et al., 2009), whereas the tdTomato protein can freely diffuse.

Because the initial studies of the *Aldh1L1-Cre* had reported relatively little neuronal labeling (Tien et al., 2012; Foo & Dougherty, 2013), we inspected other brain regions in the *Aldh1L1-*

Cre;Ai9 line. We found, in agreement with previous reports, a relative lack of neuronal labeling in spinal cord, where the *Aldh1L1-Cre* was originally validated (Tien et al., 2012), and in the Purkinje cell layer of cerebellum, and moderate labeling in forebrain and hippocampus (Foo & Dougherty, 2013; data not shown). These results highlight the necessity to check each Cre line in the desired brain region to be studied, because there exist important differences in expression patterns, suggesting potential differences in precursor cell populations.

Discussion

This work provides a quantitative characterization of the changing cellular composition of a CNS cell group during formation of its dominant excitatory neural connection. We make a key observation of a decrease in the neuron:glia cell ratio in the developing MNTB whereby glia outnumber neurons by about 50% by P6. This relative expansion of the glial population is due in large part to proliferation of oligodendrocytes, some of which occurs locally within the MNTB. To accomplish many of these goals, we offer new tools to efficiently and accurately count cells and discriminate cellular labels in 3D image volumes using virtual reality software. Furthermore, we validate the *En1-Cre* line as a useful tool for genetic manipulation of neurons, but indicate that the *PDGFR α -Cre* and *Aldh1L1-Cre* lines are non-specific for genetic manipulations in the developing MNTB.

Cell Type Quantification in Early Postnatal Development

This work is the first systematic quantification of neurons, astrocytes, oligodendrocytes and microglia in the developing MNTB. Counting cells in our 3D virtual reality system was efficient and accurate, so we were able to count ~38,000 DAPI stained nuclei and classify ~9,000 cells across 12 animals (Supplemental Tables 3-6). These numbers reflect larger samples than are typically acquired using stereological procedures, lending greater credence to the results.

Furthermore, 3D visualization of glial cells with complex processes, such as occurs with Aldh1L1 label, allowed reliable association of cell-specific markers with DAPI-labeled nuclei, as reflected in the similarity in counts between our two observers. The demonstration that Olig2 labels astrocytes as well as oligodendrocytes in the early postnatal MNTB indicates that Sox10 is a preferred marker for quantification of oligodendrocyte-lineage cells in developing neural systems. In addition, Sox10 offers the advantage of labeling all oligodendrocyte-lineage cells, regardless of maturational state (Rinholm et al., 2011). A focus of future studies should be to quantify OPCs vs. mid-stage vs. myelinating oligodendrocytes at different developmental timepoints. One caveat to the use of Iba1, a standard immunohistochemical marker for microglia (Ito et al., 1998), is that it also labels perivascular macrophages and is not entirely specific for microglia (reviewed by Greter et al., 2015). However, given the low numbers of Iba1⁺ cells in the developing MNTB (2% at its highest proportion at P6), and the absence of perivascular macrophages from a set of volume electron microscopy image sets at these same ages (data not shown), Iba1 likely provides a good report of microglia numbers. A recent publication characterizes Siglec-H as a specific label for microglia in early development (Konishi et al., 2017), and this marker should be evaluated in the MNTB in the future.

Developmental Decrease in Neuron:Glia Ratio

Recent studies have shown that the neuron:glia ratio varies across brain region and development (reviewed by Herculano-Houzel, 2014; Bandeira et al., 2009), highlighting the diverse functions that glial cells may serve to support different types of neurons. A general observation is that neuron proliferation precedes glial proliferation, which dovetails with many studies of cellular progenitors in brain development (Malatesta et al., 2000; Noctor et al., 2001; Pinto and Götz, 2007; Kriegstein & Alvarez-Buylla, 2011; Paridaen & Huttner, 2014). We report this ratio for the first time tied closely to the key stages in synapse formation and removal of exuberant connections, which suggests roles for glial cells in tissue remodeling in the

developing MNTB. For example, the high proportion of astrocytes present between P3-P6 (this work) and their close association with the developing terminals (Reyes-Haro et al., 2010; Holcomb et al. 2013; Dinh et al., 2014) suggests their involvement in the growth and refinement of CH terminals. Astrocytes can mediate synapse elimination in the visual system (Chung et al., 2013), and chicken auditory brainstem slices cultured in astrocyte-conditioned media showed accelerated dendritic pruning (Korn et al., 2011). The low proportion of microglia (2% of total at P6) seemingly requires that these cells must be extremely active if they are to account for a significant share of synaptic pruning of calyx-forming terminals, which is largely complete by P6. As a result of the low number of microglia numbers at these ages, we propose that astrocytes or OPCs play the larger role in pruning or tagging for resorption of the supernumerary CH terminals. Furthermore, assays of gene expression often tie significance to a two-fold change in expression. Changes in cell number, as with oligodendrocyte-lineage cells and microglia in this study, may account for these increases in bulk analyses of tissue. Validation using qPCR or RNASeq, which have sufficient dynamic range, can be used to assess fractional change on a per cell basis or due to expansion or contraction of the cell population. These values should be assessed for each brain region that is studied.

The more than doubling in the number of oligodendrocyte-lineage cells between P3-P6 may have functional impacts on the CH-MNTB circuitry as well. OPCs have a known role in mediating synapse elimination at the Climbing Fiber-Purkinje cell synapse. Ablation of the OPC population results in exuberant synaptic connections (Doretto et al., 2011). Additionally, the well-known role of oligodendrocytes in myelination suggests that the observed increase in oligodendrocyte-lineage cells in the MNTB is a preparatory step for initiation of myelination, which does not begin until after P3 (unpublished observations).

Local Glial Cell Proliferation

Initial gliogenesis in rodents occurs at late embryonic ages when astrocytes and oligodendrocytes are formed from radial glial cells (reviewed by Kriegstein & Alvarez-Buylla, 2011). Gliogenesis of both astrocytes and oligodendrocytes can continue postnatally through intermediate progenitor cells. An especially prominent early postnatal wave of oligodendrocyte proliferation was noted in forebrain (Kriegstein & Alvarez-Buylla 2011; Kessaris et al., 2006). There is an early and rapid proliferation of astrocytes between E20-P1 in rat MNTB, which tapers off at later postnatal time points (Saliu et al., 2014). Our association of PCNA and PH3 labeling of oligodendrocyte-lineage cells demonstrates that glial cells proliferate within the MNTB, which is consistent with a previous cell counting study demonstrating an increase in cell number across development (Rodríguez-Contreras et al., 2006). The high percentage of PCNA⁺ oligodendrocyte-lineage cells in the MNTB between P3-P6 substantiates the observed increase in Sox10⁺ cells in the MNTB during this timeframe and suggests that oligodendrocytes contribute heavily to the previously characterized increase in MNTB cell number across development (Rodríguez-Contreras et al., 2006). PCNA⁺/Sox10⁺ cells are present in large numbers ventral to the MNTB, suggesting that in addition to local proliferation, glial cells may subsequently migrate into the MNTB.

***En1* as a lineage marker labels only neurons in the MNTB**

The homeobox transcription factor, *En1*, was previously identified to label the MNTB through *in situ* hybridization and reporter studies (Marrs et al., 2013; Altieri et al., 2015), and in a selected MNTB subvolume was confirmed to be neuron-specific (Altieri et al., 2015). This work expands upon these studies and provides a systematic analysis of reporter specificity along the rostro-caudal and medial-lateral axes of the MNTB, demonstrating Cre-dependent labeling of over 97% of the Map2⁺ neurons in the MNTB. Small numbers of non-principal neurons have been described in several species (cat, guinea pig, gerbil, bat, rat, mouse), based upon cell size and dendrite structure (Morest 1968; Zook & Casseday 1982; Helfert et al. 1989; Kuwabara & Zook

1991; Banks & Smith 1992; Schofield 1994). More complete depiction of En1-negative cell structure is necessary to resolve whether a non-principal neuron class exists within the mouse MNTB, noting that non-principal cells may also be En1-positive. The *En1-Cre* line may also be useful for genetic manipulation of neurons in other brain regions, including the superior and inferior colliculi, cerebellum, reticular formation, spinal cord (V1 interneurons), ventral nucleus of the trapezoid body, ventral nucleus of the lateral lemniscus and lateral nucleus of the trapezoid body (Saueressig et al., 1999; Marrs et al., 2013; Altieri et al., 2016). For studies of globular bushy cell innervation of MNTB, the *En1-Cre* line is a useful tool for selective manipulation of the postsynaptic neuron. Note that this promoter would also affect GBC targets in the lateral and ventral nuclei of the trapezoid body, where it also is expressed (Marrs et al. 2013).

PDGFR α and Aldh1L1 as lineage markers label multiple cell types in the MNTB

Radial glia are heterogeneous and are most studied in spinal cord and forebrain. Their ability to produce neuronal vs. glial vs. mixed progeny is spatiotemporally controlled, and these progeny can be produced either directly or indirectly through intermediate progenitor cells (Noctor et al., 2004; Pinto et al., 2008; reviewed by Kriegstein & Alvarez-Buylla, 2011). Although Aldh1L1 is generally considered to be a marker of more mature radial glia, it can be detected as early as E12.5 in spinal cord using traditional immunohistochemical methods (Anthony & Heintz, 2007). The low levels of neuronal labeling demonstrated in the *Aldh1L1-Cre* line in spinal cord (Tien et al., 2012) are reasonable because neurogenesis has already peaked before E12.5 (Nornes & Carry, 1978) when Aldh1L1 expression begins. Therefore, most spinal cord neurons would not be lineage labeled in this line. MNTB neurons are born on days E11-12 (Taber-Pierce, 1973), but may sufficiently overlap with Aldh1L1 expression in brainstem. Several studies have demonstrated that radial glia have the capacity to generate neuronal progeny as late as E18 in rodent neocortex (Noctor et al., 2001; Pinto et al., 2008), perhaps accounting for higher numbers of *Aldh1L1-Cre*-labeled neurons in these brain regions.

Secondary progenitor cells can persist into the adult brain as OPCs, which co-express NG2 and PDGFR α (Nishiyama et al., 1996). Mitotically active NG2 cells can divide into both oligodendrocytes and astrocytes at embryonic ages but not postnatally (Zhu et al., 2008, 2011; Huang et al., 2014; Kang et al., 2010). Astrocytes labeled from the *PDGFR α* promoter may arise from an OPC lineage or may transiently activate the *PDGFR α* promoter. Although the extensive labeling of neurons from the *PDGFR α* promoter was unexpected, neuronal labeling has been observed in a subset of piriform projection neurons of adult mouse brain using the tamoxifen-inducible *PDGFR α -CreER* line, suggesting that adult-born neurons in the piriform cortex arise from PDGFR α ⁺ progenitor cells (Rivers et al., 2008). The approximate 70%-30% split of labeled and unlabeled neurons in the MNTB suggests a potential for heterogeneity within the neuronal population, which could reflect origins traced to both rhombomeres 4 and 5 (Maricich et al., 2009; Marrs et al., 2013), but an incomplete penetrance of the Cre expression cannot be excluded.

The labeling of ~40% of Iba1⁺ cells in the *Aldh1L1-Cre;Ai9* line was unexpected. Due to the role of microglia as phagocytic cells, it cannot be excluded that the microglia which were tdTomato⁺ in this cross were positive due to phagocytosis of tdTomato⁺ neuron, astrocyte or oligodendrocyte processes or cell bodies. However, because ~40% of astrocytes and ~97% of oligodendrocytes were tdTomato⁺ in the *PDGFR α -Cre;Ai9* line, one would expect to see tdTomato⁺ microglia in this cross as well, but there were no instances where tdTomato and Iba1 labeling overlapped in the *PDGFR α -Cre;Ai9* line. These findings argue that Cre-mediated recombination from the *Aldh1L1-Cre* line is occurring in a subset of microglial cells in the MNTB.

Although use of the *PDGFR α -Cre* and *Aldh1L1-Cre* lines for cell type-specific genetic manipulations is not useful in the MNTB, they still serve as useful tools in other brain regions. Their utility in MNTB studies should focus on tamoxifen-inducible versions of these Cre lines.

However, it should be taken into consideration that CH growth occurs early in the first postnatal week, offering experimental challenges for tamoxifen-inducible approaches such as incomplete recombination efficiency. In general, the results of this study highlight the importance of conducting a thorough systematic analysis of each Cre line, as their expression patterns are variable across brain regions and different developmental time points.

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Figures

Figure 1. Experimental design for cell counting using 3D virtual reality. A.) Serial sections containing MNTB from Cre;Ai9 mice were stained with cell-specific antibodies for neurons (Map2), astrocytes (Aldh1L1), oligodendrocytes (Sox10) and microglia (Iba1), in an alternating manner, along with DAPI. These slices were imaged with a 40x objective and tiled. The MNTB is outlined in white in each section and the midline is indicated by a dashed white line. Scale bar

= 100 μm . **B.)** The MNTB was cropped from the larger image and imported into syGlass for cell counting in 3D virtual reality. The solid and dashed white lines demarcate cut planes that were rotated to generate the 3D view in C (arrow). Scale bar = 50 μm . **C.)** Rotated view of part of MNTB to illustrate 3D view for cell counting. Counting dots were placed onto the nuclei. White dots indicate incomplete (sectioned) nuclei in the first slice of the z-stack. These were excluded from analysis, but incomplete nuclei in the bottom slice were counted and included in the analysis. Red dots indicate cells that were counted. Note that cells throughout the entire MNTB (panel B) were counted.

Figure 2. Cell-specific antibody markers for neurons and glia. A-D.) Representative immunofluorescence images of neurons labeled with Map2 (A), astrocytes with Aldh1L1 (B), microglia with Iba1 (C) and oligodendrocytes with Sox10 (D) (DAPI channel not illustrated for clarity). Asterisks denote cells that were counted. White arrows in A denote dendrites of neurons labeled with Map2 and white arrows in B show concentrated label indicating cell bodies of astrocytes, which identified cells for counting. Double asterisks in B denote astrocytes that were counted but the characteristic concentration of Aldh1L1 label in their cell bodies was found in a different image plane. **E-E''')** Co-labeling of oligodendrocytes with Sox10 and Olig2 (open

arrowheads) show that both markers label oligodendrocytes. Co-labeling of many astrocytes (Aldh1L1) with Olig2 (closed arrowheads) indicates Olig2 is not an exclusive marker for counting oligodendrocytes. Scale bars = 20 μm .

Figure 3. Cell type composition at P3 and P6. A.) Percentage of total DAPI⁺ cells for each cell type at P3. **B.)** Percentage of total DAPI⁺ cells for each cell type at P6. Counts were made

using *En1-Cre;Ai9* mice at each age. **C.)** Bar graph demonstrating changes in the percentage of neuronal and glial cells between P3 and P6. **p < 0.01, *p < 0.05.

Figure 4. Glial cell proliferation. A.) Immunofluorescent labeling with the proliferation marker, PCNA, shows PCNA⁺/DAPI⁺ cells within and ventral to the MNTB at P3. The dashed white line indicates the borders of the MNTB. The region within the white rectangle is shown at higher magnification in A', Scale bar = 20 μ m. **A'.)** Closer view of the co-labeling demonstrates that many, but not all, PCNA⁺ cells are oligodendrocyte-lineage cells labeled with Sox10 (closed arrowheads). Open arrowhead shows a PCNA⁺ cell that is Sox10⁻. Scale bar = 20 μ m. **B.)** Quantification of Sox10⁺ cells that are also PCNA⁺ at P3 and P6. n = 3 for each age. **C.)** The mitotic marker PH3 colabels Sox10⁺ cells within the MNTB (closed arrowheads). Scale bar = 20 μ m.

Figure 5. Characterization of *En1-Cre;Ai9* line as a neuronal marker. A.) Map2 co-localization with *En1-Cre*-dependent tdTomato labeling (closed arrowheads; DsRed or RFP antibody used to amplify tdTomato signal; DAPI channel not shown for clarity). **B.)** Lack of co-localization between Aldh1L1 and tdTomato. Open arrowheads point to examples of the characteristic cytoplasmic concentration of label in astrocytes. The double asterisks denote astrocytes that were counted, but their cytoplasmic labeling is present on another z-section. **C-D.)** Lack of co-localization between Sox10 (C) and tdTomato (DsRed antibody) and Iba1 (D) and tdTomato (RFP antibody). Scale bars = 20 μ m.

Figure 6. Characterization of the *PDGFRα-Cre;Ai9* line as an oligodendrocyte lineage

marker. A.) Most Map2⁺ cells are *PDGFRα-Cre*-dependent tdTomato⁺ (closed arrowheads), but many are tdTomato⁻ (open arrowheads; DAPI channel not shown for clarity). **B.)** Examples of Aldh1L1⁺ cells that are tdTomato⁺ (closed arrowhead) or tdTomato⁻ (open arrowhead). **C.)** Most Sox10⁺ cells are tdTomato⁺ (closed arrowheads), but Sox10⁺ cells can be tdTomato⁻ (open arrowhead). **D.)** Iba1⁺ cells are tdTomato⁻ (open arrowhead). Scale bars (A-C) = 20 μm and scale bar (D) = 10 μm.

Figure 7. Characterization of the *Aldh1L1-Cre;Ai9* line as an astrocyte lineage marker. A.) Most Map2⁺ cells are *Aldh1L1-Cre*-dependent tdTomato⁺ (closed arrowheads), although a small population is tdTomato⁻ (open arrowheads; DAPI channel not shown for clarity). **B.)** Aldh1L1⁺ cells are also tdTomato⁺ (closed arrowheads). **C.)** Nearly all Sox10⁺ cells are also tdTomato⁺ (closed arrowheads). **D-E.)** Iba1⁺ cells can be tdTomato⁺ (closed arrowhead in D) or tdTomato⁻

(open arrowhead in E). This line showed the most extensive labeling of tissue in the MNTB.

Scale bars (A-C) = 20 μm and scale bars (D-E) = 10 μm .

Figure 8. Quantification of tdTomato colocalization by cell type and Cre line at P6. A.)

En1-Cre;Ai9 line shows reporter expression only in neurons. **B.)** *PDGFR α -Cre;Ai9* line shows

expression in neurons, astrocytes and most oligodendrocytes. **C.)** *Aldh1L1-Cre;Ai9* line shows extensive expression in all cell types. N = neuron; A = astrocyte; O = oligodendrocyte; M = microglia.

Supplemental Figure 1. Specificity of antibody labels for distinct cell populations. A.)

Aldh1L1, Iba1 and Sox10 antibodies label distinct cell populations of astrocytes (open

arrowheads), microglia (asterisk) and oligodendrocytes (closed arrowheads), respectively. **B.)** Map2, Aldh1L1 and Iba1 antibodies label non-overlapping cell populations of neurons (double asterisks), astrocytes (open arrowhead) and microglia (closed arrowhead), respectively. Note that Map2⁺ cells have larger nuclei than other cells and labeling can reveal proximal dendrites (Fig. 2). **C.)** Map2, Aldh1L1 and Sox10 label neurons (double asterisks), astrocytes (closed arrowheads) and oligodendrocytes (open arrowheads), respectively.

Supplemental Figure 2. *Aldh1L1-Cre;Ai9* controls. A-B.) Cre⁺ littermate shows extensive tdTomato labeling. **C-D.)** Cre⁻ littermate shows a lack of tdTomato labeling under imaging matched conditions. Scale bar = 20 μm.

Supplemental Figure 3. *Aldh1L1-Cre;Rpl22-HA* controls. A-D.) Cre⁺ littermate shows extensive co-localization of HA (red) with Map2 (arrows) and Olig2 (arrowheads). Olig 2 labels both oligodendrocytes and many astrocytes, as shown in Figure 2. **E-H.)** Cre⁻ littermate shows a lack of HA labeling under imaging matched conditions. Scale bars = 20 μ m.

Supplemental Table 1: Primary and Secondary Antibodies								
Primary	Species	Company	Cat. No.	Concentration	Secondary	Company	Cat. No.	Concentration
Map2	Chicken	Encor Biotech.	CPCA-Map2	1:2500	488 Donkey anti-chicken	Jackson Immuno Research	703-545-155	1:500
Aldh1L1	Mouse	UC Davis/NIH Neuromab Facility Antibodies Inc.	Clone N103/39	1:500	555 Donkey anti-mouse	Invitrogen	A21202	1:500
DsRed	Rabbit	Clontech	632496	1:500	555 Donkey anti-rabbit	Invitrogen	A31572	1:500
RFP	Chicken	Rockland Immunochemicals	600-901-379	1:500	555 Goat anti-chicken	Invitrogen	A21437	1:500
HA	Rat	Roche Life Science	11 867 423 001	1:500	647 Donkey anti-rat	Jackson Immuno Research	712-605-153	1:500
Iba1	Rabbit	Wako Chemicals	019-19741	1:250	647 Donkey anti-rabbit	Jackson Immuno Research	711-605-152	1:500
Olig2	Rabbit	Millipore	AB9610	1:800	647 Donkey anti-rabbit	Jackson Immuno Research	711-605-152	1:500
Sox10	Goat	Santa Cruz Biotech.	SC-17342	1:250	647 Donkey anti-goat	Jackson Immuno Research	705-605-147	1:500
PH3	Rabbit	Cell Signaling Technology	9701	1:250	555 Donkey anti-rabbit	Invitrogen	A31572	1:500
PCNA	Mouse	Dako	M0879	1:500	555 Donkey anti-mouse	Invitrogen	A31570	1:500

Supplemental Table 1. Antibody Table. Primary and secondary antibodies are listed with manufacturer and concentration.

Supplemental Table 2: Percent Total Cells by Cell Type at P3 and P6

P3: Cell Representation for En1-Cre;Ai9 Mouse Line

	Rank Order	Average percent of total cells
Map2 (Neurons) n = 3	1	46.6 ± 1.5%
Aldh1L1 (Astrocytes) n = 3	2	28.7 ± 1.0%
Sox10 (Oligodendrocyte Lineage) n = 3	3	15.8 ± 1.2%
Iba1 (Microglia) n = 3	5	1.1 ± 0.1%
Other (Nonlabeled Cells) n = 3	4	7.8 ± 1.2%

P6: Cell Representation for Each Cre;Ai9 Mouse Line

Cell Type	En1-Cre n = 3	Aldh1L1-Cre n = 3	PDGFRa-Cre n = 3	Average percent of total cells (all Cre lines) n = 9
Map2 (Neurons) n = 9	36.9 ± 2.6%	37.9 ± 4.7%	37.7 ± 2.4%	37.5 ± 3.0%
Aldh1L1 (Astrocytes) n = 9	25.3 ± 1.4%	23.0 ± 0.3%	25.4 ± 0.3%	24.6 ± 1.4%
Sox10 (Oligodendrocyte Lineage) n = 9	29.0 ± 2.8%	31.2 ± 3.7%	26.5 ± 4.2%	28.9 ± 3.7%
Iba1 (Microglia) n = 9	2.0 ± 0.3%	2.0 ± 0.5%	1.7 ± 0.7%	1.9 ± 0.5%
Other (Nonlabeled Cells) n = 9	6.7 ± 2.1%	5.8 ± 4.4%	8.6 ± 2.5%	7.0 ± 3.0%

Supplemental Table 2. Cellular composition at P3 and P6. Average percentage of total DAPI⁺ cells listed by cell type and Cre line.

Supplemental Table 3: Number of Cells Counted at P3 for En1-Cre;Ai9 Mouse Line				
(Number of Cells / Total Number of Cells by DAPI Staining)				
	Animal 1	Animal 2	Animal 3	Cell-Type Totals
Map2 (Neurons) n = 3	359/751	325/691	310/689	994
Aldh1L1 (Astrocytes) n = 3	202/727	198/664	218/764	618
Sox10 (Oligodendrocyte Lineage) n = 3	101/684	110/713	113/662	324
Iba1 (Microglia) n = 3	7/631	7/563	6/627	20
Total DAPI	2793	2631	2742	

Supplemental Table 3. Total cell numbers at P3 for *En1-Cre* line. Total cell numbers counted for each specific antibody label out of the total DAPI⁺ cells listed by individual animal replicate.

Supplemental Table 4: Number of Cells Counted at P6 for En1-Cre;Ai9 Mouse Line				
(Number of Cells / Total Number of Cells by DAPI Staining)				
	Animal 1	Animal 2	Animal 3	Cell-Type Totals
Map2 (Neurons) n = 3	261/655	293/841	248/687	802
Aldh1L1 (Astrocytes) n = 3	192/770	179/743	251/936	622
Sox10 (Oligodendrocyte Lineage) n = 3	192/743	288/939	271/887	751
Iba1 (Microglia) n = 3	16/706	14/803	16/762	46
Total DAPI	2874	3326	3272	

Supplemental Table 4. Total cell numbers at P6 for *En1-Cre* line. Total cell numbers counted for each specific antibody label out of the total DAPI⁺ cells listed by individual animal replicate.

Supplemental Table 5: Number of Cells Counted at P6 for Aldh1L1-Cre;Ai9 Mouse Line				
(Number of Cells / Total Number of Cells by DAPI Staining)				
	Animal 1	Animal 2	Animal 3	Cell-Type Totals
Map2 (Neurons) n = 3	286/795	317/731	323/936	926
Aldh1L1 (Astrocytes) n = 3	211/929	198/848	196/856	605
Sox10 (Oligodendrocyte Lineage) n = 3	351/992	284/1000	232/775	867
Iba1 (Microglia) n = 3	12/771	16/626	15/772	43
Total DAPI	3487	3205	3339	

Supplemental Table 5. Total cell numbers at P6 for *Aldh1L1-Cre* line. Total cell numbers counted for each specific antibody label out of the total DAPI⁺ cells listed by individual animal replicate.

Supplemental Table 6: Number of Cells Counted at P6 for PDGFR α -Cre;Ai9 Mouse Line				
(Number of Cells / Total Number of Cells by DAPI Staining)				
	Animal 1	Animal 2	Animal 3	Cell-Type Totals
Map2 (Neurons) n = 3	320/857	366/910	317/893	1003
Aldh1L1 (Astrocytes) n = 3	215/856	246/960	218/852	645
Sox10 (Oligodendrocyte Lineage) n = 3	227/858	166/741	264/858	657
Iba1 (Microglia) n = 3	9/872	14/783	18/747	41
Total DAPI	3443	3394	3350	

Supplemental Table 6. Total cell numbers at P6 for *PDGFR α -Cre* line. Total cell numbers counted for each specific antibody label out of the total DAPI⁺ cells listed by individual animal replicate.

Supplemental Video 1. Aldh1L1 cytoplasmic label juxtaposes nuclear Olig2 labeling, but never nuclear Sox10 labeling.

0-4 seconds: Widefield view of Aldh1L1 (cyan), Olig2 (red) and Sox10 (green) antibody labeling.

5-9 seconds: Focusing in on bottom right corner of image

10-13 seconds: Cropping region of interest, zooming in to region of interest and recentering the image

14-30 seconds: Using the syGlass “Slicing” tool to cut through the depth of the z-stack and examine co-labeling of cells. Closed arrowheads point to Aldh1L1⁺ (cyan) cells that have nuclear Olig2 (red) labeling. The Aldh1L1 labeling on these cells displays the characteristic cytoplasmic bulge, and the labeling directly juxtaposes the nuclear label. Open arrowheads point to Olig2⁺/Sox10⁺ (yellow) cells which are devoid of direct Aldh1L1 labeling around the nucleus. Note that not all Aldh1L1⁺ cells have nuclear Olig2 labeling (arrow).

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Supplemental Video 2. Microglia are easily and quickly identified in 3D virtual reality system.

0-3 seconds: Showing slice by slice through a z-stack in 2D with DAPI (blue) and Iba1 (green).

It is difficult to count microglia in 2D because they have processes that radiate out from their cell bodies and may approach DAPI-labeled nuclear staining.

Caption at 4 seconds: "Microglia quickly identified in 3D IVR"

4-17 seconds: Showing the same z-stack in 3D virtual reality system, which can be rotated to get views of cells from different angles. It is now very easy to quickly identify the microglia in 3D.

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Supplemental Video 3. Astrocytes with complicated morphology can be counted in 3D using the “Cutting” tool and viewed from different angles for accurate counting.

0-6 seconds: 3D visualization of z-stack stained with DAPI (blue), Aldh1L1 (green) and DsRed antibody against tdTomato (red) in the *En1-Cre;Ai9* line.

Caption at 5 seconds: “Complex labeling pattern of astrocytes (green) easily discerned in 3D using syGlass Cutting tool”

9-47 seconds: Counting dots are placed on astrocytes, which are easily visualized using a combination of the Cutting tool and the Rotation/Translation abilities.

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Supplemental Video 4. Manual counting and classification of cells in VR.

4 seconds: Turning on the “Visualize First Slice” mode, which turns off the 3D visualization and allows for visualization of just the top slice in the z-stack. This mode is used to place counting dots on all of the nuclei which have been cut on the top of the tissue section in order to exclude them from counting. The purpose of this step is to avoid counting nuclei twice in subsequent sections (a nucleus on the back end of one z-stack could be counted again at the front end of the next z-stack). Using this mandate of avoiding the first slice, nuclei were counted at their first appearance in the z-dimension.

7-10 seconds: Using voice commands to toggle different color channels on and off

Caption at 7 seconds: “Toggling color channels via voice commands”

13-19 seconds: Placing white dots on the cut nuclei to exclude from cell counts

Caption at 20 seconds: “Excluding (white dots) sectioned nuclei (blue DAPI stain) at leading edge of tissue”

24-28 seconds: Continuing to place white dots on the cut nuclei to exclude from cell counts

29-32 seconds: Turning off the “Visualize First Slice” mode and switching back to 3D mode

Caption at 33 seconds: “3D rotation and translation”

34-41 seconds: The z-stack is rotated on its side and backwards to demonstrate that the white dots are placed on the first slice of the z-stack and the remaining nuclei behind the first slice will be counted

42-46 seconds: Use of voice commands to toggle color channels

47-54 seconds: Changing color of counting dots to begin counting neurons (DsRed; red channel)

Caption at 55 seconds: “Marking neurons labeled with tdTomato (red) using the syGlass Counting tool”

56-63 seconds: Placing counting dots on neurons labeled with DsRed antibody against tdTomato

64-65 seconds: Using color wheel to change counting dot color to begin counting oligodendrocytes (Sox10; green)

Caption at 65 seconds: “Marking oligodendrocytes labeled with Sox10 (green) using the syGlass Counting tool”

65-82 seconds: Placing counting dots on oligodendrocytes labeled by Sox10 (green). The voice commands can be used to toggle the green channel on and off to check if the oligodendrocytes are tdTomato-positive or tdTomato-negative.

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Chapter 3: Transcriptional profiling reveals intercellular signaling patterns in the developing auditory brainstem

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The authors have no conflicts of interest to report.

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Abstract

Central nervous system (CNS) development and neural circuit formation are coordinated processes during which various cell types migrate, proliferate, differentiate and promote structural changes to construct a neural tissue. To assess temporal control of these processes we previously carried out time series analysis of gene expression in the medial nucleus of the trapezoid body (MNTB) during the critical postnatal phase of its development. While this work revealed timed execution of gene expression programs and signaling pathways, it was blind to the specific cells where gene expression changes were occurring and the directions in which cell-to-cell signals were transmitted. Here we carry out single cell gene expression profiling to determine the transcriptional programs of the major MNTB cell types, define the directional cell-to-cell signals that guide MNTB development and identify a new genetic marker for MNTB neurons. Our work paints an intricate picture of the developing MNTB where different cell types cooperate to build the perineural net (PNN) extracellular matrix and cell-to-cell signaling pathways proceed along defined axes to coordinate tissue development. Specifically, we find that: (i) Neurons signal to astrocytes via Fgf9; (ii) Delta-Notch signals connect oligodendrocytes, astrocytes and endothelial cells to activate Hes1 expression in astrocytes and endothelial cells; (iii) The Notch pathway activation in astrocytes is temporally controlled to peak around postnatal day 3; (iv) Neurons, astrocytes and oligodendrocytes signal to endothelial cells via VEGF and TGF β to guide angiogenesis. Finally, we identify the Tal1 transcription factor as a new genetic marker for MNTB principal neurons.

Key words: MNTB, transcriptomics, Tal1, perineuronal nets, Delta-Notch, Fgf9, Fgfr3, VEGF, TGF β , angiogenesis

Introduction

The medial nucleus of the trapezoid body (MNTB) in the auditory brainstem is a well-established model system to study neural circuit formation (Xiao et al., 2013; Kolson et al., 2016). Stages of maturation in the MNTB have been characterized structurally and electrophysiologically. MNTB maturation occurs on a compressed timeframe (Hoffpauir et al., 2006, 2010; Holcomb et al., 2013), which can be exploited to connect molecular, structural and electrophysiological events. Furthermore, the population of postsynaptic neurons in the rodent MNTB is mostly homogenous (Banks & Smith, 1992), thus eliminating the confounding influence of neuronal diversity that complicates studies of genetic regulation of neural circuit formation in other brain regions. The principal neurons in the MNTB receive input from the largest nerve terminal in the mammalian CNS, the calyx of Held (CH; Held, 1893). The CH grows rapidly between postnatal day (P)2 to P4 while the postsynaptic principal neuron increases in size and acquires mature biophysical properties (Kandler & Friauf, 1993; Hoffpauir et al., 2006, 2010; Rusu & Borst, 2011; Holcomb et al., 2013). Over 70% of principal neurons transition from a state of multi-innervation to mono-innervation by P6 (Holcomb et al., 2013). In synchrony with growth and synaptic refinement of the CH terminal, the glial cell population more than doubles between P3 to P6 (Brandebura et al., 2018), thereby contributing to the subsequent myelination of globular bushy cell (GBC) axons that form the CH (Saliu et al., 2014; Sinclair et al., 2017) and development of a close association of astrocyte processes with the CH terminal (Reyes-Haro et al., 2010; Dinh et al., 2014). Interestingly, growth of the CH terminal and refinement to mono-innervation occur before opening of the ear canal at P10, and as such, these maturational steps are independent of exposure to airborne sound (Mikaelian et al., 1965).

Essential steps in neural circuit formation such as axon guidance, synaptogenesis and synaptic pruning have traditionally been studied in the context of neuron-neuron signaling. More recently, evidence has accumulated suggesting that glial cells, such as astrocytes and microglia, play

fundamental roles in the regulation of these processes (reviewed by Clarke & Barres, 2013; Reemst et al., 2016). A more global definition of neural circuit formation includes extensive structural changes that occur as the neural tissue matures, such as myelination of axons, angiogenesis and blood-brain-barrier (BBB) formation. These processes are integral to neural circuit formation as they regulate the speed and fidelity of synaptic transmission and maintain neuronal homeostasis.

We previously conducted a microarray study across postnatal developmental ages with high temporal resolution on bulk microdissected MNTB tissue. The microarray study identified transcripts that dynamically changed expression levels during a period of rapid CH growth (P3-P4), synaptic refinement (P4-P6) and glial cell expansion (P3-P14; Kolson et al., 2016).

However, the bulk tissue approach did not discriminate between the different cell types present in the tissue. The present work utilizes single cell RNA-Sequencing (scRNA-Seq) to augment the temporal data with high resolution transcriptome data for each major cell type in the MNTB at P3, a timepoint coinciding with the initiation of CH growth (Holcomb et al., 2013). Analysis of the neuronal transcriptional data identified the transcription factor, *Tal1*, as a novel marker gene for the principal neurons in the MNTB, as well as neurons in several other auditory nuclei, suggesting a broader role for *Tal1* in maturation of neurons involved in auditory processing.

Single cell gene expression analysis identified directional patterns for several major intercellular signaling pathways. Building off of the work by Xiao and colleagues (2013) demonstrating that BMP signaling regulates CH growth, we now identify that all major cell types in the MNTB express BMP receptor transcripts. Since all major MNTB cell types can potentially participate in BMP signaling, the stunted growth of CH terminals observed in models of perturbed BMP signaling may be attributed to a multitude of complex signaling interactions between neurons, glia, and vascular-associated cells (VAC; includes endothelial cells and pericytes).

Furthermore, we identified transcripts related to several other pathways, including the FGF, Delta-Notch, VEGF and TGF β pathways. The differential expression patterns of transcripts

encoding for ligands and receptors suggests that these pathways guide structural and physiological maturation in the developing MNTB through neuron-glia, glia-VAC and neuron-glia-VAC signaling mechanisms. The transcriptional profiles generated in this study now connect the dynamically changing transcripts identified in the previous developmental microarray study (Kolson et al., 2016) to specific cell types and permit the correlation of transcriptional programs with known structural changes occurring in the MNTB nucleus such as with PNN formation and angiogenesis.

Results

Hierarchical clustering approach identified all major classes of cells in the MNTB

scRNA-Seq analysis was performed on dissociated cells which were isolated from microdissected MNTB tissue in P3 mice (Kolson et al., 2016) and captured using the Fluidigm C1 microfluidics platform. Cre-positive mice from the *En1-Cre;Ai9* cross were utilized so that *tdTomato* transcript levels could be used to validate the downstream clustering approach (neurons should have the highest *tdTomato* transcript levels; Altieri et al., 2015; Brandebura et al., 2018). The Fluidigm C1 protocol was used to generate scRNA-Seq libraries from 305 single cells. Libraries from 285 cells were sequenced and after quality control filters, data from 215 cells was used for subsequent analysis. Hierarchical clustering of the scRNA-Seq data revealed five major transcriptome classes in the MNTB (Figure 1; Cluster 1 n=125 cells; Cluster 2 n=38 cells; Cluster 3 n=22 cells; Cluster 4 n=19 cells; Cluster 5 n=11 cells).

Each cluster was assigned to a cell type based on the expression of known genetic markers from the literature. We highlight three marker genes per cell cluster (Figure 2A-D). Cluster 1 and Cluster 2 were identified as neuronal clusters by common neuronally expressed transcripts encoding for the GABA receptor subunit (*Gabra5*), the NMDA receptor subunit (*Grin2a*) and the

calcium ion binding protein (*Calbindin1*; *Calb1*), among others. *Calb1* protein is known to be expressed in MNTB neurons (Friauf, 1993; Figure 2A). Furthermore, Cluster 1 and Cluster 2 had the highest levels of *tdTomato* transcript, providing a validation of the cell clustering approach (Altieri et al., 2015; Brandebura et al., 2018; Supplemental Figure 10, $p \leq 0.005$, Wilcoxon Rank Sum Test). The astrocyte cluster (Cluster 3) was identified by expression of the well-known astrocyte marker *Aldehyde dehydrogenase family member 1 L1* (*Aldh1L1*; Cahoy et al. 2008). Several reports have demonstrated *Aldh1L1* protein expression in MNTB astrocytes at early postnatal ages (Dinh et al., 2014; Kolson et al., 2016; Brandebura et al., 2018). The transcripts encoding for two other common astrocyte markers, *Solute carrier family 1 member 3* (*Slc1a3*; also known as *Glial High Affinity Glutamate Transporter 3/Glast*; Rothstein et al., 1994) and *Solute carrier family 1 member 2* (*Slc1a2*; also known as *Glial High Affinity Glutamate Transporter 2/Glt1*; Rothstein et al., 1994), were also expressed at high levels in Cluster 3 (Figure 2B). Cluster 4 was identified as the oligodendrocyte cluster based on expression of *SRY-Box 10* (*Sox10*), a common oligodendrocyte marker (Rinholm et al., 2011). *Sox10* protein was previously shown to be expressed specifically in oligodendrocytes (and not astrocytes) within the MNTB at early postnatal ages (Brandebura et al., 2018). Other common oligodendrocyte-associated transcripts expressed in Cluster 4 were *2'3'-Cyclic nucleotide 3' phosphodiesterase* (*Cnp*; Braun et al., 1988) and *Myelin basic protein* (*Mbp*; Shiota et al., 1989; Figure 2C). The transcripts which identified Cluster 5 as a combination of endothelial cells and pericytes were *Claudin5* (*Cldn5*) and *FMS related tyrosine kinase 1* (*Flt1*; both demonstrated as enriched in cortical endothelial cells in Zhang et al., 2014), along with *Platelet derived growth factor receptor β* (*Pdgfr β*) for pericytes (demonstrated as enriched in cortical pericytes in Zhang et al., 2014; Figure 2D). A microglial cluster was not present because they exist in small numbers at this age (Brandebura et al., 2018). The capture plate set for 17-25 μm cell size further biased against collecting microglia cells. One potential microglial cell was identified due to high expression levels of the *Aif1* transcript (a known microglial marker; Ito et al., 1998) but it

was forced into another cluster due to the minimum cluster size of 3 cells that we used in the analysis.

Differential gene expression (DGE) analysis identified transcripts preferentially expressed in each cell cluster

To identify genes differentially expressed in each cluster beyond the known genetic markers we performed pairwise comparisons of the gene expression levels between the clusters (Supplemental Tables 1-10). Genes differentially expressed in each cluster were selected using a standard 2-fold differential expression cut-off and False Discovery Rate (FDR) ≤ 0.05 based on a previously published scRNA-Seq analysis workflow (Lun et al., 2016). In the pairwise analysis, genes could be present on multiple lists (ie. astrocytes and oligodendrocytes express many of the same genes and therefore these genes could be differentially expressed in both clusters compared to neurons and VACs). In an attempt to find more specific genes for each cluster, we also performed a second analysis that identified genes differentially expressed in each cluster compared to the average of all other clusters combined. Notably, only seven genes were differentially expressed in the pairwise analysis between the two neuronal clusters within the specified significance cutoffs (Supplemental Table 1; see below) so these clusters were combined for the subsequent analysis. The top 20 transcripts with elevated expression levels in each cluster (neurons, astrocytes, oligodendrocytes and VACs) are shown in Table 1 and the full lists are shown in Supplemental Tables 11-14. The gene sets identified for each cluster correlate well with the assigned cell types further validating our clustering approach (Table 1).

Two neuronal clusters may represent differences in maturation

In our differential gene expression analysis of the two neuronal clusters only seven transcripts had significantly different expression levels (*Gm17018*, *Tln2*, *Gstm1*, *Ednrb*, *Dbi*, *Ecpas* and *Atp1a2*; Supplemental Table 1), suggesting that the clusters are defined by the cumulative effect of relatively moderate differences in the levels of commonly expressed transcripts. To

identify the variables that were the most impactful in separating the neuronal clusters, we performed a Random Forest Analysis (RFA). There were 52 transcripts identified in the RFA with a “Mean Decrease in Gini Score” ≥ 0.1 (Figure 3A; full list in Supplemental Table 15). After plotting the transcripts in descending order of importance, 8 transcripts (*Pgk1*, *Ctsl*, *Gm17018*, *Map1b*, *Gde1*, *Atp6v1g2*, *Asns* and *Praf2*; red dots in Figure 3A) were identified as relatively more impactful in separating the two clusters based on the “elbow” in the ranking. These transcripts all display slightly lower expression levels in Cluster 2 compared to Cluster 1 (Figure 3B).

Gene Ontology (GO) analysis was performed on the genes identified that had ≥ 0.1 “Mean Decrease in Gini Score” in the RFA. Metabolism was a central theme in the GO categories, which included “nucleotide/nucleoside metabolic process”, “organophosphate metabolic process”, “dicarboxylic acid metabolic process”, “NAD metabolic process”, “tricarboxylic acid metabolic process” and “aspartate family amino acid metabolic process” (Table 2). Many genes included in these categories are related to energy (*Pgk1*, *Ldhd*, *Idh3a*, *Mdh1*, *Ass1*) and amino acid metabolism (*Asns*, *Ass1*, *Got1*). During the first postnatal week MNTB neurons significantly increase in size and the CH terminal increases in synaptic strength (Hoffpauir et al., 2010), requiring corresponding adjustment in metabolic pathways. In particular, lactate and glucose metabolism are critical to maintain high activity levels at the CH (Lucas et al., 2018). MNTB cell growth would also require increased transcription and protein catabolism, reflecting a need for an up-regulation of nucleotide and amino acid metabolic pathways. “Neuron differentiation” was also included among the top GO categories (Table 2). Transcripts related to the cytoskeleton (*Nefh*, *Map1b*) and synaptic transmission (*Nrxn1*, *Snap25* and *Akap5*) are included in the “neuron differentiation” category, which are all expressed at higher levels in Cluster 1 compared to Cluster 2. Thus, the differences in transcript expression levels identified in the RFA may

indicate heterogeneity in metabolic demand and growth rates of the maturing MNTB neurons at P3.

Tal1 identified as novel marker for MNTB neurons

The pairwise DGE analysis identified T-cell acute lymphocytic leukemia 1 (*Tal1*) transcript as having higher expression in neurons (3.3-fold higher expression compared to astrocyte cluster; $p \leq 0.003$; $FDR \leq 0.02$; Supplemental Table 2). *Tal1* is a basic helix loop helix (BHLH) transcription factor important for GABAergic neuron differentiation in midbrain areas, which is activated by its paralogue *Tal2*. Neurons lacking *Tal1* and *Tal2* up-regulate glutamatergic neuron markers (Achim et al., 2013). Although MNTB neurons are glycinergic at maturity, they release GABA (and glutamate) at early postnatal ages (Kotak et al., 1998), and thus *Tal1* may be important for physiological maturation of the MNTB neurons. *Tal1* did not make significance cut-offs for higher expression in neurons compared to the average of all other clusters (likely due to detection in the VAC cluster as well). For this reason, we utilized single molecule fluorescent *in situ* hybridization (smFISH) experiments to assay specificity of *Tal1* transcript expression in neurons. *Tal1* probe (Figure 4A) colocalized with tdTomato-positive neurons in the *En1-Cre;Ai9* cross (Figure 4B) and there was not significant probe labeling present outside of the neuronal cells (nonneuronal cells are DAPI-positive and tdTomato-negative; Figure 4C), reflecting the specificity of *Tal1* expression in the neuronal cell class in the MNTB.

Another transcription factor important for MNTB development is *En1*. Previous studies demonstrated that *En1* is also expressed in several other nuclei of the Superior Olivary Complex (SOC), including the ventral nucleus of the trapezoid body (VNTB) and the lateral nucleus of the trapezoid body (LNTB; Marrs et al., 2013; Altieri et al., 2015). In order to compare the expression pattern of *Tal1* with *En1*, smFISH experiments were performed with *Tal1* probe in the *En1-Cre;Ai9* reporter mice and the nuclei of the SOC were imaged. The results indicate that *Tal1* has a similar expression pattern as *En1*, with dense labeling in the LNTB and VNTB

and lack of expression in the medial superior olive (MSO) and LSO (Figure 5A-A'; 5B-B'; 5C-C'). Differing from *En1*, *Tal1* is also expressed in the superior paraolivary nucleus (SPN; Figure 5B-B'). These results highlight the utility of *Tal1* as a novel marker for MNTB neurons (as well as LNTB, VNTB and SPN neurons) at early postnatal ages.

Neurons and glia contribute different components of the perineuronal net (PNN)

Perineuronal nets (PNNs) are a specialized type of lattice-like extracellular matrix involved in the regulation of neural plasticity (reviewed by Sorg et al., 2016). PNN formation in many systems temporally correlates with the end of the critical period of plasticity. Degradation of PNNs can reopen a window of activity-dependent plasticity in adult animals after monocular deprivation (Pizzorusso et al., 2002) and promote axonal sprouting after denervation in spinal cord injury (Barritt et al., 2006). In the MNTB, the principal neurons are surrounded by PNNs (Schmidt et al., 2010; Blosa et al., 2013) that are established after the neurons reach a state of primarily mono-innervation at P6 (Hoffpauir et al., 2010; Holcomb et al., 2013).

Interestingly, many PNN-associated transcripts were detected as differentially expressed between the cell clusters (Figure 6 and Supplemental Tables 11-13). Among these transcripts were *Hapln1* (3.0-fold higher expression in neurons; p-value ≤ 0.001), *Bcan* (3.4-fold higher expression in astrocytes and oligodendrocytes; p-value ≤ 0.05), *Ncan* (3.2-fold higher expression in astrocytes; p-value ≤ 0.005), *Tnc* (8.0-fold higher expression in astrocytes; p-value ≤ 0.001), *Tnr* (6.8-fold higher expression in oligodendrocytes; p-value ≤ 0.001) and *Vcan* (3.5-fold higher expression in astrocytes; p ≤ 0.05). Other transcripts detected were *Acan*, *Hapln3* and *Hapln4*, where *Acan* had highest expression levels in neurons, *Hapln3* in astrocytes and VACs and *Hapln4* in neurons and astrocytes (Figure 6A). Overall our data demonstrate a coordinated production of the PNN matrix where different cell types contribute different components of the structure.

Based on our temporal gene expression data, most PNN-associated transcripts have an increasing expression profile between P0 and P6 and the increase in expression level of transcripts most highly expressed in glial cells correlates with a period of robust gliogenesis in the MNTB (Figure 6B; Saliu et al., 2014; Kolson et al., 2016; Brandebura et al., 2018). Developmental regulation of PNN-associated transcripts suggests a function for PNNs in relation to the critical period of plasticity in the MNTB. We propose a model where the establishment of PNNs stabilizes the large CH terminal projection onto the MNTB principal neurons and inhibits multi-innervation as gliogenesis proceeds (Figure 6C).

FGF signaling components are differentially expressed in neurons and astrocytes

Transcripts for five secreted FGF ligands (*Fgf1*, *Fgf2*, *Fgf9*, *Fgf10* and *Fgf18*) and three FGF receptors (*Fgfr1*, *Fgfr2* and *Fgfr3*) were detected. The *Fgf9* transcript was detected at significantly higher levels in the neuronal cluster compared to the average of the other clusters (3.3-fold higher expression; $p\text{-value} \leq 0.001$; Supplemental Table 11; Supplemental Figure 1). Strikingly, *Fgf9* has high affinity for *Fgfr3* (Hecht et al., 1995). The transcript for *Fgfr3* was expressed at the highest levels in the astrocyte cluster (6.5-fold higher expression; $p\text{-value} \leq 0.001$; Supplemental Table 12; Supplemental Figure 1). Similar to *Fgf9*, the transcript for *Fgf10* was also detected at higher levels in neurons (Supplemental Figure 1).

To validate the *Fgf9/Fgfr3* expression patterns, we performed smFISH. The probe for *Fgf9* (Figure 7A) colocalizes with tdTomato-positive neurons in the *En1-Cre;Ai9* cross (Figure 7B). *Fgf9* probe lacks significant labeling in nonneuronal cells (DAPI-positive cells that are tdTomato-negative; Figure 7C). Furthermore, the probe for *Fgfr3* (Figure 7D) colocalizes with Aldh1L1-positive astrocytes (Figure 7E) and is not present at high levels in other cells types (DAPI-positive cells that are Aldh1L1-negative; Figure 7F). The scRNA-Seq and smFISH data suggest directional signaling from neurons to astrocytes (Figure 7G).

Delta-Notch signaling components are differentially expressed in glial and endothelial cells

The *Notch1-3* receptor transcripts were expressed in the astrocyte, oligodendrocyte and VAC cell clusters, but all three transcripts had significantly higher expression in the astrocyte cluster compared to the average of all other clusters (*Notch1* 3.7-fold higher expression; p-value ≤ 0.005 , *Notch2* 4.3-fold higher expression; p-value ≤ 0.001 ; *Notch3* 3.9-fold higher expression; p-value ≤ 0.005 ; Supplemental Table 12; Supplemental Figure 2). The VAC cluster contained the highest transcript levels of the *Notch4* receptor transcript (Supplemental Figure 2). *Hes1* transcript was expressed at elevated levels in the astrocyte and VAC clusters (Supplemental Figure 2), suggesting active Notch signaling in these cell types. Transcripts for the Delta-like and Jagged ligands were detected at the highest levels in the oligodendrocyte and VAC clusters (*Dll1* in oligodendrocyte cluster, *Dll4* in VAC cluster, *Jag1* in oligodendrocyte and VAC clusters; Supplemental Figure 2). These data suggest activation of the Notch pathway primarily in astrocytes and VACs by Delta and Jag ligands presented by the oligodendrocytes and VACs. Expression of *Dll4* and *Jag1* in the VAC cluster is consistent with the previously described autocrine signaling that controls the choice between stalk and tip fate for the endothelial cells during angiogenesis (Benedito et al., 2009).

A developmental immunostaining experiment was performed in a Transgenic Notch Reporter (TNR) mouse to pinpoint which cell types participate in active Delta-Notch signaling. In this line, GFP expression reports active Notch signaling (Duncan et al., 2005). GFP homogenously colocalized with Aldh1L1 antibody labeling of astrocytes at P3 (Figure 8B). However, at P0 and P6, Notch signaling was not active in the astrocyte population (Aldh1L1-positive cells were GFP-negative; Figure 8A and Figure 8C). Although *Hes1* transcript was also expressed in the VAC cluster, GFP expression was not observed in these cell types and may be due to generally low reporter expression or silencing of the reporter transgene in this cell type. Overall, the data

indicate that MNTB astrocytes exhibit a transient burst of active Notch signal transduction mediated by Dll and Jag ligands supplied by oligodendrocytes and VACs during the first postnatal week of development (Figure 8D).

TGF β and VEGF signaling components correspond to expansion of the vascular network

TGF β signaling is mediated by a large superfamily of ligands, which includes TGF β s, BMPs, Activins, Growth and Differentiation Factors (GDFs), and Nodal, among others (reviewed by Weiss & Attisano, 2012). TGF β and related ligands signal through a complex of Type I and Type II receptors. The Type III receptor has no signaling domain and instead functions to present ligand and promote complex formation between Type I and Type II receptors (reviewed by Blobe et al., 2001). Two receptor transcripts (*Acvr11* and *Tgfb2*) and a co-receptor transcript (*Eng*) were expressed at significantly higher levels in the VAC cluster (*Acvr11* 12.1-fold higher expression, p-value ≤ 0.001 ; *Tgfb2* 11.0-fold higher expression, p-value ≤ 0.001 ; *Eng* 11.3-fold higher expression, p-value ≤ 0.001 ; Supplemental Table 14; Supplemental Figure 3A). *Tgfb3* was also expressed at the highest levels in the VAC cluster (Supplemental Figure 3A).

Transcripts for two TGF β ligands, *Tgfb2* and *Tgfb3*, were expressed by all cell clusters, with *Tgfb2* having higher expression levels (Supplemental Figure 3A). These data suggest that while all cell types are capable of transmitting TGF β signal, only VACs are set to transduce signal because this is the only cell type that expressed transcript for the Type II TGF β receptor.

Gdf11, a TGF β -related ligand, was expressed by all cell clusters along with the transcripts for its cognate receptor set (*Acvr1b*, *Tgfb1*, *Acvr2a*; Supplemental Figure 3A). Expression of *Gdf11* and its receptors across all cell types confounds interpretation of directionality in this signal. Nevertheless, it is consistent with the role of neuronal *Gdf11* autocrine signaling in inhibiting neurogenesis (Wu et al., 2003; Shi & Liu, 2011) and may contribute to maintaining steady neuronal numbers in the MNTB at postnatal ages (Rodríguez-Contreras et al., 2006; Saliu et al., 2014).

Two transcripts for VEGF ligands (*Vegfa* and *Vegfb*) were detected at moderate levels in all cell clusters and the third ligand transcript (*Vegfc*) was detected at low levels but only in the oligodendrocyte and VAC clusters (Supplemental Figure 4A). Two receptor transcripts (*Kdr* and *Flt1*) were detected at significantly higher expression levels in the VAC cluster compared to the average of all clusters combined (*Kdr* 7.9-fold higher expression; p-value ≤ 0.001 and *Flt1* 8.0-fold higher expression; p-value ≤ 0.001 ; Supplemental Table 14; Supplemental Figure 4A). The third VEGF receptor transcript (*Flt4*) was also detected at elevated levels in the VAC cluster (Supplemental Figure 4A). These data suggest that neurons and glial cells participate in secretion of VEGF ligands which signal through receptors in the VACs (Supplemental Figure 4B).

Both TGF β and VEGF signaling are well known to promote angiogenesis and vascular remodeling (Rosenstein et al., 1998; reviewed by Mancuso et al., 2008). In support of the TGF β and VEGF pathways promoting angiogenesis in the MNTB, *Nestin* transcript was expressed at significantly higher expression levels in the VAC cluster (4.7-fold higher expression; p-value ≤ 0.001 ; Supplemental Table 14). Nestin is a marker of neovascularization in endothelial cell progenitors in early postnatal development (Mokry & Nemecek, 1999). At the protein level, antibody for the common endothelial cell marker Cd31 (also known as *Pecam1*; Müller et al., 2002; *Pecam1* transcript 8.6-fold higher expression in VACS; p-value ≤ 0.001 ; Supplemental Table 14) colocalizes with Nestin antibody at P3 (arrowheads in Figure 9A). These data suggest that endothelial cell progenitors are present in the MNTB at P3. To determine if there is a developmental expansion of the vasculature, Cd31-positive staining was quantified within in the region of the MNTB at P3, P6 and P9 and normalized for size of the MNTB. Cd31 staining represents $3.0 \pm 0.2\%$ of the MNTB at P3 and $3.2 \pm 0.5\%$ at P6. However, there is a significant increase in Cd31-positive staining by P9 to $4.3 \pm 0.1\%$ of the MNTB volume (p-value ≤ 0.05 for P3 vs. P9 two-tailed t-test; Figure 9B). However, the increase in Cd31-positive staining could be

due to a developmental regulation of Cd31 protein. To verify that there is structural angiogenesis occurring, blood vessels were segmented and reconstructed from three-dimensional electron microscopy volumes at P3, P6 and P9 timepoints (Figure 9C). The percent occupancy of SBEM volume of the blood vessels was calculated. Blood vessels represented 0.6% of the SBEM volume at P3 and 0.8% of the volume at P6. Similar to the Cd31 quantification, there is a noticeable increase in blood vessel volume to 1.2% at P9 (Figure 9C). Overall the scRNA-Seq data indicates that several major signaling pathways, including the Delta-Notch, TGF β and VEGF pathways, are active in promoting structural changes related to angiogenesis during MNTB tissue maturation (Figure 10A-C).

Discussion

The MNTB was used as a model to study genetic regulation of neural tissue development and the current study is the first to report cell type-specific transcriptional profiles in the MNTB. scRNA-Seq analysis yielded four major transcriptional profiles corresponding to neurons, astrocytes, oligodendrocytes and VACs. Using DGE analysis, we identified the transcription factor, Tal1, as a novel MNTB neuron marker which is also useful in labeling neurons in several other auditory nuclei. By combining our previously collected temporal microarray data with cell type-specific transcriptional profiling, we now put forth a working model for PNN formation. Lastly, characterization of the expression patterns for components of major intercellular signaling pathways resulted in the ability to determine directional signaling patterns between cells. In the case of TGF β and VEGF signaling, we connected the transcriptional data with structural changes in the vasculature during MNTB tissue maturation. Importantly, multiple signaling pathways must exist in parallel to guide structural transformation of neural tissue.

Technical approach to investigate cellular and molecular heterogeneity in the MNTB

The scRNA-Seq approach in the MNTB was strengthened by the use of mice from the *En1-Cre;Ai9* cross. In previous studies, *En1-Cre;Ai9* mice were demonstrated to specifically label at least 97% of neurons in the MNTB (Altieri et al., 2015; Brandebura et al., 2018). In this regard, the tdTomato transcript level could be used as a control to validate the unsupervised cell clustering approach, verifying that the neuronal clusters had the highest levels of tdTomato transcript (Supplemental Figure 12).

The largest microfluidic plate size (17-25 μm) was used to capture cells because smaller plates were clogged by the large MNTB neurons. However, the large size plate biases against capture of smaller cells (glial cells and VACs). For this reason, the relative percentages of each cell type captured do not represent the actual percentages of cell types present in the MNTB at P3 (Brandebura et al., 2018). As a result of this methodological bias, transcriptional profiling was unable to be performed for microglial cells because they only represent 1% of the total cells in the MNTB and were further biased against in the microfluidics device (Brandebura et al., 2018). One potential microglial cell was identified in a post-hoc analysis based upon high expression levels of the *Aif1* transcript, a known microglial marker (Ito et al., 1998), but a microglial cell cluster could not be formed from one cell. Due to the scarcity of microglial cells in the MNTB tissue at early postnatal ages, transcriptional profiling of MNTB microglia will likely require a fluorescent reporter mouse line (such as *Aif1-GFP*) to identify the microglia followed by hand picking of single cells expressing the reporter.

Larger scale scRNA-Seq studies were successful in generating subclusters of cells representing distinct populations of astrocytes and different maturational stages of oligodendrocytes, as well as separate clusters for endothelial cells and pericytes (Zeisel et al., 2015; Chen et al., 2017). Although subtypes of astrocytes, oligodendrocytes and VACs were not generated in the current study, the main goal to transcriptionally characterize each major cell type in the developing MNTB was accomplished. The small number of cells contained in the MNTB nucleus (6,000

cells at maturity and ~3,000 at P3 based on rat cell counts; Kulesza et al., 2002; Rodríguez-Contreras et al., 2006) limits the number of cells that can be reasonably collected for sequencing. Future studies utilizing larger cell sample sizes will be useful to characterize subtypes of astrocytes, oligodendrocytes and VACs.

Distinct neuronal clusters exhibit varying levels of transcripts related to metabolism

In larger species of mammals (cat, bat and guinea pig), the MNTB contains distinct populations of principal and nonprincipal neurons (Morest, 1968; Zook & Casseday, 1982; Helfert et al., 1989). In the rat, electrophysiological and morphological characterizations support the idea that the MNTB contains a mostly homogeneous population of principal neurons and a small population of less than 5% of neurons characterized as nonprincipal cells, which were typically located along the lateral borders of the MNTB (Banks & Smith, 1992). To our knowledge, there are no reports of nonprincipal cells in the mouse MNTB. We did not include the most lateral borders of the MNTB in our microdissections to avoid contaminating the preparation with cells outside of the MNTB. Therefore, we argue it is unlikely that the two neuronal clusters represent distinct principal and nonprincipal neuronal populations or neurons from the neighboring SPN. Importantly, cells from each of the six independent cell captures were present in both neuronal clusters, ruling out batch effects due to technical variation or slight differences in age.

Rather than expressing distinct transcripts, the two neuronal clusters expressed the same repertoire of transcripts but at varying levels. Differences in expression level were noted for transcripts related to energy metabolism and overall protein catabolism, suggesting that there may be metabolic differences between the two populations in relation to synaptic activity levels or growth dynamics. There are two possibilities to explain slight metabolic differences in MNTB neurons at P3, which include position within the tonotopic landscape of the MNTB or receipt of a large vs. small terminals.

The MNTB is tonotopically organized with high-frequency encoding neurons located medially and low-frequency encoding neurons located laterally (reviewed by Kandler et al., 2009). CH membrane fenestration occurs earlier in the medial MNTB compared to the lateral MNTB (Ford et al., 2009), potentially allowing for more rapid glutamate clearance related to higher frequency of activity in the medially located neurons. Therefore, it is reasonable to argue that the more medially located principal neurons may also require an increased metabolic rate to maintain high-frequency transmission. In the principal neurons, the Kv3.1 potassium channel is tonotopically organized, with the highest immunoreactivity in the cells residing in the medial portion of the MNTB (Li et al., 2001). However, the transcript encoding for Kv3.1 (*Kcnc1*) was not identified in the top transcripts from the RFA.

A second explanation for the presence of two neuronal clusters could be receipt of a large CH vs. small inputs at P3. A previous study using SBEM analysis showed that by P6 most MNTB neurons receive a single large CH terminal, but at P3 there is a heterogeneous distribution where about 43% of principal neurons receive at least one large terminal and others receive only small-sized inputs (Holcomb et al., 2013). Although mRNA from the CH was not included in this study due to dissociation and single cell capture, it is known that the principal neurons increase in surface area and excitatory postsynaptic currents (EPSCs) from the principal neurons increase in size in temporal synchrony with growth of the CH (Hoffpauir et al., 2010). An increase in cell size paired with an increase EPSCs due to receipt of a larger terminal would certainly suggest a demand for up-regulation of transcripts related to energy metabolism. It was recently demonstrated that glucose and lactate metabolism are critical to maintain high activity levels in the MNTB (Lucas et al., 2018). The *Ass1* transcript was identified in the RFA as one of the most impactful genes in separating the neurons into two clusters. *Ass1* is a critical enzyme in the cycle which produces nitric oxide (NO; Haines et al., 2011). Interestingly, NO is produced by MNTB neurons in response to increased levels of synaptic activity (Steinert et al., 2008).

However, a definitive correlation of up-regulation of transcripts identified in the RFA with tonotopic localization and receipt of a large terminal requires alternative methods. Future studies should utilize electrophysiological recordings followed by sequencing of the patched cells to correlate level of synaptic activity, tonotopic localization and transcript expression levels. The MNTB is an ideal model for this type of study because the large size of the CH terminal allows for paired electrophysiological recordings (Forsythe, 1994; Borst & Sakmann, 1996).

Novel marker for a subset of auditory brainstem nuclei

This work identified the BHLH transcription factor, *Tal1*, as a novel marker for MNTB neurons. The specificity of *Tal1* mRNA expression in the MNTB neurons was validated using smFISH experiments. Several markers for MNTB neurons have been identified in other studies, including *Calb1* (Friauf, 1993), *Foxp1* (Marrs et al., 2013) and *En1* (Marrs et al., 2013; Altieri et al., 2015; Brandebura et al., 2018). In comparison to *Calb1*, *Tal1* has the benefit of earlier onset of expression (expressed as early as E18.5 in the Allen Developing Mouse Brain Atlas; <http://developingmouse.brain-map.org/gene/show/21110>). *Tal1* also has the added benefit of a more restricted expression pattern in the CNS compared to *Calb1* and *Foxp1*. *Calb1* is extensively expressed throughout the entire brain (Allen Developing Mouse Brain Atlas; <http://developingmouse.brain-map.org/gene/show/12092>) and *Foxp1* is expressed in the cortex and hippocampus (Allen Developing Mouse Brain Atlas; <http://developingmouse.brain-map.org/gene/show/72814>). Alternatively, *Tal1* is restricted to subpopulations of neurons in the superior colliculus, reticular formation and substantia nigra (Achim et al., 2013). The results of this study now provide information on the expression pattern of *Tal1* throughout the auditory nuclei in the SOC. The smFISH experiments demonstrated that *Tal1* is expressed in the MNTB, VNTB and LNTB in a pattern similar to *En1* (Marrs et al., 2013) and is additionally expressed in the SPN. Outside the SOC, *En1* is expressed in the superior and inferior colliculi, cerebellum, reticular formation, rhombomere 1 derived hindbrain and V1 interneurons of the spinal cord

(Saueressig et al., 1999; Zervas et al., 2004; Sgaier et al., 2005). Additional expression profiling will be necessary to determine if *Tal1* is also expressed in the cerebellum and spinal cord. Based upon the overlapping expression profiles of the *En1* and *Tal1* transcription factors within the SOC, it will be interesting to determine whether *En1* and *Tal1* are activated sequentially or in concert to regulate development of the auditory brainstem nuclei.

Tal1 expression in midbrain is activated by its earlier expressed paralogue, *Tal2*. The combination of *Tal2* and *Tal1* expression regulates GABAergic neuron differentiation, and the loss of *Tal2* and *Tal1* results in adoption of a glutamatergic cell fate (Achim et al., 2013). MNTB neurons release higher levels of GABA in comparison to glycine at early postnatal ages but switch to the predominant release of glycine after the onset of hearing (Kotak et al., 1998). Thus, *Tal1* expression may have a transient role in biophysical maturation of MNTB neurons. Examination of *Tal1* expression at later ages after the onset of hearing will be of interest to determine whether it is down-regulated upon adoption of glycinergic identity. *Tal2* was not detected in the scRNA-Seq data collected at P3, matching the report of late embryonic expression in midbrain (Achim et al., 2013). Sequencing at embryonic ages will be needed to determine if *Tal2* is expressed in MNTB neurons at earlier ages. Future work focusing on genetic deletion of *Tal1* from MNTB neurons will shed light on its role in MNTB neuron development.

PNN-associated transcripts are developmentally regulated and exhibit differential expression patterns in neurons and glia

PNNs are prominent within the auditory system and surround MNTB neurons (Schmidt et al., 2010; Blosa et al., 2013). Genetic deletion of *Bcan*, a PNN component, or enzymatic digestion of PNNs, results in significant reductions in action potential transmission speed at the CH:principal neuron synapse (Blosa et al., 2015; Balmer, 2016). In addition to a role in speed of neurotransmission, PNNs play a role in the regulation of synaptic plasticity in other systems. In

the visual cortex and spinal cord, PNN formation temporally correlates with a decrease in activity-dependent plasticity and plasticity after injury (Pizzorusso et al., 2004; Barritt et al., 2006). Within the MNTB, PNN-associated transcripts are developmentally regulated and do not form at the structural level until after P6 (Kolson et al., 2016), the age at which the majority of principal neurons have reached a state of mono-innervation (Holcomb et al., 2013). We therefore suggest that PNNs in the MNTB may promote a permanent state of mono-innervation by stabilizing the large CH terminal onto the principal neuron and inhibiting multi-innervation by tightly lining the principal neuron membrane and preventing access of nearby collaterals to innervation territory.

We previously utilized transcript and protein level analysis to characterize expression of PNN components at several developmental timepoints in the MNTB (Kolson et al., 2016). The present study now identifies cell type-specific transcript expression patterns for PNN components and connects temporal expression profiles of PNN-associated transcripts with known changes in the relative proportions of MNTB neurons and glia characterized by Brandebura and colleagues (2018). We now set forth a working model for PNN formation where *Hapln1* transcript has the earliest onset of expression, followed by expression of transcripts encoding for the tenascin and chondroitin sulfate proteoglycan (CSPG) proteins (Kolson et al., 2016). One exception was *Tnc* transcript, which has a secondary role in mediating radial glial cell interactions with the extracellular matrix during embryonic ages (Pollen et al., 2015) and could explain the early postnatal decrease in *Tnc* expression level observed in the MNTB. The scRNA-Seq data demonstrates that *Hapln1* has the highest expression levels in neurons while the *Tnr* and *Bcan* transcripts have the highest expression levels in glial cells. The developmental increase in *Tnr* and *Bcan* expression temporally correlate with increased glial cell colonization of the MNTB (Brandebura et al., 2018).

The transcriptional data match protein expression in the MNTB, where the Hapln1 linker protein was detected as early as P0, but CSPG proteins could not be detected at appreciable levels until after P6 (Kolson et al., 2016). These data suggest that PNN formation proceeds in two stages. In the first stage, neurons secrete Hapln1 linker protein, which is known to act as a binding scaffold for the tenascin and CSPG proteins (Spicer et al., 2003). In the second stage, glial cell expansion proceeds and astrocytes and oligodendrocytes subsequently secrete the tenascin and CSPG proteins to complete structural formation of the PNNs. In this model, the PNN structure encloses around the principal neurons as they transition from a state of multi- to mono-innervation (Figure 6C). The role of PNNs in relation to a permanent state of mono-innervation has not yet been appropriately addressed. Mice lacking *Bcan* still had PNNs composed of the other protein components (Blosa et al., 2015) and the enzymatic digestion experiments were performed in *ex vivo* slices which would naturally have axonal damage and could not be maintained long-term (Balmer et al., 2016). Future studies should focus on genetic deletion of *Hapln1* from MNTB neurons, which may prevent PNN formation altogether by eliminating the binding scaffold for tenascin and CSPG proteins, thereby directly addressing the role of PNNs in refinement to mono-innervation at the CH:principal neuron synapse.

Major signaling pathways

The scRNA-Seq approach allowed for the identification of transcripts related to major developmental signaling pathways in the MNTB at P3. In many cases the direction of signaling could be determined based on differential transcript expression patterns of the ligand and its cognate receptor amongst different cell types. scRNA-Seq was a strong unbiased approach to identify the most differentially expressed transcripts in each of the major cell types in the MNTB. Examples of prominent signaling pathways in the data were FGF, Delta-Notch, TGF β and VEGF signaling.

BMP signaling was previously shown to regulate CH growth and refinement to mono-innervation at the CH:principal neuron synapse (Xiao et al., 2013). Our previous microarray study of MNTB tissue identified the transcripts encoding for *Bmp4* and *Bmp5* ligands to be developmentally regulated during the first postnatal week when CH growth occurs (Kolson et al., 2016). Based on the known role of BMP signaling in MNTB development, we examined the expression patterns of transcripts related to BMP signaling in the scRNA-Seq dataset. We detected transcripts for three canonical BMP ligands, including *Bmp4*, *Bmp5* and *Bmp7* as well as the transcripts encoding for the Type I and Type II BMP receptors (*Bmpr1a*, *Bmpr1b* and *Bmpr2*). However, the ligand transcripts were detected at low levels and cell type-specificity could not be determined with confidence. The BMP receptor transcripts were detected amongst all cell clusters, indicating roles for BMP signaling in all major cell types in the MNTB (data not shown). The capability for all cell types to participate in BMP signal transduction is important for interpretation of the CH growth and mono-innervation phenotype observed by Xiao and colleagues (2013) because their conditional genetic deletions were mediated by *Egr2-Cre* activity which has not yet been fully characterized for cell type-specificity in the Ventral Cochlear Nucleus (VCN) or MNTB to our knowledge. A more recent study inhibited BMP signaling postnatally at P0 with viral administration of *Egr2-Cre* in the VCN. Postnatal perturbation of BMP signaling resulted in an increase in axon branching but growth of the CH and refinement to mono-innervation were not altered (Kronander et al., 2019). Thus, CH growth and synaptic refinement is likely mediated through intercellular BMP signaling interactions in the MNTB rather than an intrinsic program in the presynaptic neuron. One possibility is that BMP signaling in glial cells could promote the secretion of other proteins which promote terminal growth. A second possibility is that retrograde BMP signaling through the postsynaptic principal neuron stimulates growth of the presynaptic terminal.

Wnt signaling in the CNS has many functions, including activity-mediated synaptogenesis (Sahores et al., 2010) and angiogenesis (Daneman et al., 2009). Wnt7a specifically was previously shown to be involved in activity-mediated synaptogenesis in hippocampus (Sahores et al., 2010). In the MNTB the *Wnt7a* transcript is developmentally regulated during the peak of CH growth (Kolson et al., 2016), suggesting a potential role for Wnt7a in activity-mediated synaptogenesis at the CH. In the current scRNA-Seq study the astrocyte and VAC clusters had the highest expression levels of *Wnt7a* (data not shown), but the transcript expression levels were low and did not make significant cut-offs. Another study showed that Wnt7a and Wnt7b are important regulators of angiogenesis in ventral domains of the CNS (Daneman et al., 2009). The detection of *Wnt7a* and *Wnt7b* transcripts, along with the strong differential expression of the transcript encoding for the Fzd6 receptor in the VAC cluster, suggests that Wnt signaling may be transduced in VACs to promote angiogenesis.

The scRNA-Seq data identified significant differential expression of *Fgf9* in neurons and its high-affinity receptor, *Fgfr3*, in astrocytes. The smFISH experiments confirmed the specificity of expression patterns. In *Drosophila*, FGF signaling promotes the ramification of astrocyte processes towards synaptic regions (Stork et al., 2014). Given the intimate association of astrocyte processes with the growing CH terminal (Dinh et al., 2014), it is an interesting hypothesis that physical contact between astrocyte processes and the CH terminals may promote growth and regulate refinement to mono-innervation. Astrocytes are a well-known source of secreted growth factors in cortex (Christopherson et al., 2005; Kucukdereli et al., 2011; Allen et al., 2012; Farhy-Tselnicker et al., 2017) and thus may regulate CH growth through FGF-mediated contact with the CH and secretion of growth factors in close proximity to the growing terminal. Alternatively, FGF-mediated astrocyte contact with the principal neuron may define innervation territory, thereby regulating the refinement to mono-innervation observed at the CH:principal neuron synapse. This phenomenon was observed in the cerebellum where

the Bergmann glia physically ensheath Purkinje cell dendrites. Inhibition of calcium permeability through AMPA receptors in the Bergmann glia resulted in decreased physical contact between the Bergmann glia and the dendrite, leading to innervation of the dendrite by multiple climbing fibers as opposed to the physiological state of mono-innervation (Iino et al., 2001). Future studies should focus on a genetic knockout of *Fgf9* ligand from MNTB neurons and evaluate the direct effects on astrocyte morphological maturation and ramification of processes towards growing CH terminals, as well as indirect effects on CH growth and refinement to mono-innervation.

Delta-Notch signaling was another major signaling pathway identified in the scRNA-Seq data. The expression patterns for Delta-Notch signaling components suggest tripartite signaling involving oligodendrocytes, VACs and astrocytes. Differential expression of Delta-Notch signaling components in astrocytes was also identified from transcriptional data in early postnatal cortex (Cahoy et al., 2008), indicating that Delta-Notch signaling in astrocyte development may be conserved across brain regions. An interesting finding of the present study is that Notch signaling was only transiently active in the MNTB astrocytes. The TNR mouse studies demonstrated that Notch signaling was not active at P0 or P6 in MNTB astrocytes but was active at P3. The Delta-Notch pathway enhances Jak-Stat signaling through facilitation of Stat phosphorylation (Kamakura et al., 2004). Thus, Delta-Notch signaling in MNTB astrocytes may be essential for proper astrocyte maturation, due to the well-known role of the Jak-Stat pathway in astrocyte differentiation (Rajan & McKay, 1998; Hong & Song, 2014). Stat proteins activate transcription of the mature astrocyte marker, Glial fibrillary acidic protein (Gfap; Hong & Song, 2014). MNTB astrocytes begin to express Gfap protein by P9 (Dinh et al., 2014), indicating a reasonable temporal window for Delta-Notch signaling to enhance Jak-Stat signaling and initiate downstream Gfap transcription. Future studies should focus on elucidating the biological relevance of transiently active Notch signaling in MNTB astrocytes, which is

initiated and down-regulated within just three days. However, transcripts for Notch receptors were also expressed in the oligodendrocyte and VAC clusters, indicating that Delta-Notch signaling likely has a multi-faceted role in MNTB tissue maturation. Delta-Notch signaling inhibits oligodendrocyte progenitor cells from exiting the cell cycle, thereby maintaining the progenitor cell population (reviewed by Koch et al., 2013), and autocrine Delta-Notch signaling in vascular endothelial cells determines tip vs. stock cell fate to promote vascular sprouting in the tip cells and maintain vessel integrity in the stock cells (Benedito et al., 2009).

Transcripts encoding for the receptors of the TGF β and VEGF pathways were most highly expressed in the VAC cluster and the transcripts encoding for the ligands were detected in the neuronal and glial cell clusters. The involvement of TGF β and VEGF pathways in promoting angiogenesis is well-documented in the literature (Rosenstein et al., 1998; reviewed by Mancuso et al., 2008). These data support a whole-tissue contribution to angiogenesis and subsequent blood-brain-barrier formation, where neurons and glia secrete ligands to bind to receptors on the VACs. The expansion of the vasculature in the MNTB was supported by several lines of evidence. First, the VAC cluster contained the highest expression levels of *Nestin* transcript, an endothelial progenitor cell marker (Mokry & Nemecek, 1999) and Cd31-positive endothelial cells expressed Nestin protein. Quantification of Cd31 immunoreactivity across development showed a significant increase in Cd31 staining between P3 and P9, suggesting angiogenesis and vascular remodeling. Finally, SBEM analysis demonstrated an increase in the blood vessel occupancy of the MNTB across development. These results demonstrate that the scRNA-Seq approach can be used to predict cell signaling interactions that then lead to structural remodeling in the tissue.

In conclusion, the results of the present study assign cell type-specificity to transcripts encoding for ligands and receptors of major intercellular signaling pathways. The differential expression data can then be used to connect cell signaling interactions to tissue remodeling. In the future,

conditional genetic knockout studies should be designed based on the ground-work laid by the sequencing data to evaluate the functional consequences of altering particular signaling patterns.

Materials and methods

All procedures involving animals were approved by the West Virginia University Institutional Animal Care and Use Committee.

Animal breeding

Engrailed1-Cre (*En1-Cre*) heterozygous male mice (Kimmel et al., 2000), which were a generous gift from Dr. Mark Lewandoski, were crossed to a Cre-dependent tdTomato reporter line (*Ai9*; Stock No. 007905, Jackson Laboratory, Bar Harbor, ME; homozygous females; Madisen et al., 2010). Cre-positive mouse pups aged postnatal day (P)3 from the *En1-Cre;Ai9* cross were used for all scRNA-Seq and single molecule fluorescent *in situ* hybridization (smFISH) experiments. The *En1-Cre;Ai9* reporter cross was previously validated to specifically label neuronal cells in the MNTB with high efficiency (Marrs et al., 2013; Altieri et al., 2015; Brandebura et al., 2018).

Immunohistochemical experiments to confirm active Notch signaling were performed in a transgenic Notch reporter mouse line, *Tg(Cp-EGFP)25Gaia/ReyaJ* (TNR, Stock No. 018322, Jackson Laboratory; Duncan et al., 2005). Homozygous male TNR mice were crossed to female wildtype C57Bl6/J mice and the pups were collected at P0, P3 and P6. The remaining immunohistochemistry experiments were performed using wildtype C57Bl6/J mice (Jackson Laboratory, Stock No. 000664). The Serial Blockface Electron Microscopy (SBEM) volumes were generated from FVB/NJ mice. In all instances P0 is defined as the day of birth.

MNTB tissue collection and dissociation

tdTomato-positive mice from the *En1-Cre;Ai9* cross were used at P3 for the scRNA-Seq experiments. MNTB tissue from at least three mice per litter was pooled together and in total six litters were used for six separate cell captures. Microdissected MNTB tissue was collected as described previously (Kolson et al., 2016). Briefly, brains were removed from the skull in ice-cold low Ca^{2+} artificial cerebrospinal fluid (ACSF) containing 125 mM NaCl, 2.5 mM KCl, 3 mM MgCl_2 , 0.1 mM CaCl_2 , 25 mM glucose, 25 mM NaHCO_3 , 1.25 mM NaH_2PO_4 , 0.4 mM ascorbic acid, 3 mM myo-inositol, and 2 mM Na-pyruvate (all chemicals obtained from MilliporeSigma, Burlington, MA) and perfused with 95% $\text{O}_2/5\%$ CO_2 . Coronal sections of brainstem containing MNTB were sliced at 200 μm on a VF-200 compresstome (Precisionary Instruments Inc., Greenville, NC). Slices which contained MNTB on both sides were placed into ice-cold ACSF solution and the MNTB was microdissected from the surrounding tissue using a 26-gauge needle under a dissecting microscope.

Microdissected MNTB tissue was then placed in dissociation solution (equilibrated to pH 7.0 with 95% $\text{O}_2/5\%$ CO_2) composed of Earl's Balanced Salt Solution (EBSS) with papain (20 U/mL) and DNase (95 U/mL) (Papain Dissociation System, Worthington Biochemical Corporation, Lakewood, NJ). Tissue was enzymatically dissociated in a water bath at 37°C. The mixture was mechanically triturated with a fire-polished glass Pasteur pipette (original bore size 5 $\frac{3}{4}$ ", Thermo Fisher Scientific, Waltham, MA). An inhibitor solution (equilibrated to pH 7.0 with 95% $\text{O}_2/5\%$ CO_2 ; Papain Dissociation System, Worthington Biochemical Corporation) containing EBSS with bovine serum albumin (BSA; 10 mg/mL), an ovomucoid protease inhibitor (10 mg/mL) and DNase (9.5 U/mL) was added to halt further enzymatic dissociation. The solution was then centrifuged at 500 g for 10 minutes at 4°C (accuSpin Micro 17R, Thermo Fisher Scientific). The supernatant was removed and the pellet was resuspended in wash buffer containing Hank's Balanced Salt Solution (HBSS, Thermo Fisher Scientific) with 1.5 mg/mL

glucose (Thermo Fisher Scientific) and 0.1% Fraction V BSA (MilliporeSigma). Two additional rounds of washing were performed on the cell suspension alternating with centrifugation and resuspension. Before the final wash the cell suspension was passed through a 30 µm mesh filter (Sysmex Partec GmbH, Görlitz, Germany) to remove cell clumps and debris. After the final centrifugation the supernatant was removed and the pellet was resuspended in a mixture containing 60% wash buffer and 40% C1 Suspension reagent (C1 Single Cell Reagent Kit for mRNA Seq, Fluidigm, South San Francisco, CA).

Single cell microfluidics capture and preparation of cDNA

Single cells were captured and lysed using the C1 microfluidics-based system (Single Cell Auto-Prep System, Fluidigm) with the standard protocol provided by the manufacturer (PN 100-7168 K1). A total of six Integrated Fluidic Circuits (IFCs; size 17-25 µm, Fluidigm) were used to capture 215 cells from six separate litters of *En1-Cre;Ai9* mice (genotype Cre-positive) at age P3. Briefly, the C1 IFC was primed using the mRNA Seq: Prime 1773x script as provided by the manufacturer. After priming, the single cell suspension was loaded into the IFC. Additionally, a 10 µM Calcein AM (Thermo Fisher Scientific) solution in HBSS was added into Inlet 1 of the IFC to assess cell viability. Cells were captured and stained using the mRNA Seq: Cell Load & Stain 1773x script. Each capture site of the IFC was then imaged on an EVOS FI Manual microscope (Thermo Fisher Scientific) using brightfield, Texas Red (for tdTomato) and GFP filters (for Calcein). Dead cells (Calcein AM-negative), doublets and empty capture sites were marked for exclusion from library preparation in downstream workflow. tdTomato-positive cells were denoted as probable neurons based on expression of the fluorescent protein driven by Cre-mediated recombination from the *En1* promoter, which was previously demonstrated to label ~97% of neurons in the MNTB (Brandebura et al., 2018).

After imaging, the IFC was returned to the C1 to run the mRNA Seq: RT and Amp 1773x script. The workflow used the SMARTer Ultra Low RNA Kit (Clontech, Mountain View, CA) and the

Advantage 2 PCR Kit (Clontech) for lysis, reverse transcription and PCR amplification. RNA spikes (ArrayControl spikes #1/4/7, Thermo Fisher Scientific) were added in the lysis mix in a linear range of concentrations to ensure fidelity of PCR amplification. After PCR amplification (21 rounds), the product was collected from the C1 IFC and transferred into a 96-well plate with C1 DNA Dilution Reagent (C1 Single Cell Reagent Kit for mRNA Seq, Fluidigm) and stored at -20°C until library preparation.

Library preparation

The concentration of each cDNA sample was measured using a Qubit 2.0 fluorometer (Thermo Fisher Scientific) and double-stranded DNA High Sensitivity reagent (Thermo Fisher Scientific). Each library was diluted to 0.2 ng/μL optimally with C1 Harvest Reagent (C1 Single Cell Reagent Kit for mRNA Seq, Fluidigm), but libraries were used if they were at least 0.1 ng/μL. Library preparation was performed using the Nextera XT Library Preparation Kit (Illumina, San Diego, CA) with the Nextera XT 96 Index Kit v2 (Illumina) according to the manufacturer's instructions. After indexing, libraries were pooled together and purified using AMPure XP beads at 90% of total pool volume (Beckman Coulter, Pasadena, CA). The samples were eluted from the beads with C1 DNA Dilution Reagent. The concentration and library size distribution of each sample was measured on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

Sequencing

Equal volumes of each library were added to the pool for initial sequencing on an Illumina MiSeq. Alignment was performed using HiSat2 (Kim et al., 2015) with GRCm38 with spike and tdTomato transcript sequences added. The number of usable reads was calculated by subtracting the number of reads aligned to spike sequences from the total number of reads. It was noted that the probable neurons (tdTomato-positive during imaging described above) had a

lower number of reads aligning to spike sequences than the other cell types. Therefore, the MiSeq step was useful to adjust the final volumes of each library added to the final pool for deep sequencing, allowing for a more equal representation of endogenous reads in each library. Paired-end 75 base pair deep sequencing was performed at an average depth of 3.1 million reads per library on the Illumina HiSeq 4000 at the Roy J. Carver Biotechnology Center (University of Illinois at Urbana-Champaign).

Analysis of sequencing data

HiSeq data was aligned using HiSat2 (Kim et al., 2015) with GRCm38 with spike and tdTomato transcript sequences added. Features were counted using Rsubread (Liao et al., 2014). A scRNA-Seq analysis workflow similar to previously described was used as a template with slight modifications (Lun et al., 2016). The analysis utilized the scater, scran and edgeR packages in R Version 3.4.3 (Robinson et al., 2010; Lun et al., 2016; McCarthy et al., 2017). Poor quality cell libraries were removed using two metrics: number of expressed genes and percent of reads aligning to mitochondrial genes. Histograms were generated for all cell libraries for “Number of Expressed Genes” (Supplemental Figure 5A) and “Mitochondrial Proportion (%)” (Supplemental Figure 5B). A cut-off of 3 mean absolute deviations (mad; $\leq/\geq 3$ mad for number of genes expressed; ≥ 3 mad for percent of mitochondrial reads) was used to filter for outliers (Lun et al., 2016). The same metrics after filtering for outliers are displayed in Supplemental Figure 7C-D. Gene filtering was also performed. Genes with zero counts in all cells were removed. A further filtering step kept only genes that were expressed at a threshold of 10 counts detected in at least 3 cells. The threshold of 10 counts was chosen based upon the distribution of expression levels for all genes. The fourth quartile (genes falling within the top 25% expression levels) was chosen for further analysis (Supplemental Figure 6A). This resulted in an average of 8,475 genes expressed per cell library, with a range of 5,804 to 11,207 genes (Supplemental Figure 6B). Counts for all remaining genes were then normalized using a library size scaling factor

(computeSumFactors function in scan; Supplemental Figure 7) and subsequently converted to $\log_2$ counts per million (CPM) values, which were then used for downstream analysis.

Cells were then clustered based on similarities in their gene expression profiles using an unsupervised hierarchical clustering workflow (Lun et al., 2016). The tdTomato transcript was removed prior to clustering. First, the mean variance was fitted to the mean log expression using LOESS (locally weighted scatter plot smoother) regression. Genes with variance deviating more than 2-fold from the fit with a False Discovery Rate (FDR) of less than 5% were selected (Lun et al., 2016; Supplemental Figure 8). These genes were then additionally filtered using a Spearman's Correlation test with $FDR \leq 0.001$ and absolute value of $\rho > 0.4$ to remove noisy genes and allow for selection of highly variable genes which correlate in expression values across cells (Lun et al., 2016). This subset of genes was used for initial hierarchical clustering analysis using Ward's Distance (Lun et al., 2016) with a minimum cell cluster size of 5 cells. Each gene's expression value is displayed as the $\log_2$ -transformed CPM expression value subtracted from the median CPM for all cells for that gene (Supplemental Figure 9). Using the initial cell clusters, pairwise orthogonal contrasts were initialized to identify genes whose expression level was significantly different between the two clusters in comparison (≥ 2 -fold difference between any two clusters with $FDR \leq 0.05$). An additional criterion was placed that the gene must be detected at ≥ 5 CPM in at least 20% of the cells within a cluster to avoid clustering on noisy genes that may be outliers within subsets of cells within a cluster. The capture site images corresponding to cells expressing genes from two cell clusters at this stage were then double-checked for doublet captures and some cells were removed from the analysis based on doublet capture that was originally overlooked. Genes identified as differentially expressed between clusters were then used to cluster the cells again in a two-step iterative process, at which point the composition of the clusters no longer changed (Figure 1). The tdTomato CPM value was plotted for each cell as a control for the clustering approach (highest

tdTomato CPM values should be expressed in cells within the neuronal clusters based upon the known specificity of the *En1-Cre;Ai9* line for labeling neurons in the MNTB; Altieri et al., 2015; Brandebura et al., 2018). The Wilcoxon Rank Sum test with an adjusted p-value of ≤ 0.005 was used to indicate that the neuronal clusters had significantly higher tdTomato CPM values than the nonneuronal clusters (Supplemental Figure 10).

Differential Gene Expression analysis

Transcripts were identified as preferentially expressed within a particular cluster using two separate methods. First, pairwise orthogonal contrasts were used to detect transcripts differentially expressed in one cluster compared to every other cluster as described above. The cutoff was ≥ 2 -fold differential expression with an FDR ≤ 0.05 (Supplemental Tables 1-10). This method identifies any transcript which has a significant difference in expression level between the two clusters in comparison. The benefit of this analysis is that transcripts can be identified in certain pairwise comparisons which would not be identified compared to the average of all clusters (for example if a transcript is differentially expressed in two clusters, such as oligodendrocytes and astrocytes). For the second method, non-pairwise orthogonal clusters were constructed to identify differentially expressed transcripts between one cluster and the average of the other clusters. In this case, the two identified neuronal clusters were combined into one cluster due to their similarity in the pairwise analysis (Supplemental Table 1). Again, a cutoff of ≥ 2 -fold differential expression with an FDR ≤ 0.05 was used (Supplemental Tables 11-14).

Random Forest and Gene Ontology analysis

To detect slight differences in transcript expression levels between the neuronal clusters, a Random Forest Analysis was used to highlight transcripts which pushed the two clusters apart. This was done using the randomForest package in R and simulated with a 50-50 split over

1,000 iterations. A Gene Ontology analysis was performed in R on the top transcripts identified in the Random Forest Analysis (transcripts with ≥ 0.1 Mean Decrease in Gini Score, $n = 52$ transcripts) using the GO.db package with Molecular Function and Biological Process categories selected.

Single molecule fluorescent *in situ* hybridization (smFISH)/Immunohistochemistry

smFISH experiments were performed on Cre-positive mice from the *En1-Cre;Ai9* cross at age P3. Mice were anesthetized with an intraperitoneal injection of 2,2,2-tribromoethyl alcohol (125 mg/kg, MilliporeSigma). The mice were transcardially perfused first with filtered phosphate buffered saline (PBS, MilliporeSigma) at room temperature, followed by filtered 4% paraformaldehyde (PFA, Thermo Fisher Scientific) at room temperature. Brains were rapidly dissected from the skull in ice-cold PBS and post-fixed overnight at 4°C before cryoprotection overnight in 30% sucrose (Thermo Fisher Scientific). Brains were then embedded in Tissue Freezing Medium (General Data Inc., Cincinnati, OH) and frozen at -80°C until sectioning at 16 μm on a cryostat (Model CM3050S, Leica Biosystems, Wetzlar, Germany).

The smFISH experiments were performed using the RNAScope Protocol according to the manufacturer's instructions (#320293-UM, Advanced Cell Diagnostics, Newark, CA). A heat-induced epitope retrieval step was performed (10 mM citric acid, pH 6.0) for 15 minutes at 95°C. Slides were then washed twice in PBS and subsequently in 100% ethanol (Pharmco-Aaper, Brookfield, CT). Protease III (Advanced Cell Diagnostics, Newark, CA) was added to the slides which were placed in a hybridization oven (Model No. 241000, Boeckel Scientific, Feasterville Trevose, PA) for 30 minutes at 40°C. Slides were then washed twice in PBS. Probe was added to the slides and hybridized for 2 hours at 40°C followed by two washes in Wash Buffer (Advanced Cell Diagnostics). Slides were then placed in the Amp1-4 reagents (Cat. No. 320851, Advanced Cell Diagnostics) for 30 minutes, 15 minutes, 30 minutes, 15 minutes, respectively, with washing in between each amplification step in Wash Buffer. In all cases

probes were conjugated to Alexa 488 in the last amplification step. The probes used were Tal1 (Cat. No. 428221, Advanced Cell Diagnostics), Fgf9 (Cat. No. 499811, Advanced Cell Diagnostics) and Fgfr3 (Cat. No. 440771, Advanced Cell Diagnostics).

Slides were then processed through an immunohistochemistry protocol with cell type-specific antibodies. Blocking and application of primary and secondary antibodies was performed as previously described (Kolson et al., 2016). The antibodies used were DsRed (1:500, Cat. No. 632496, Clontech) for neurons in the *En1-Cre;Ai9* cross and Aldh1L1 (1:500, Cat. No. 75-140, Antibodies Inc., Davis, CA) for astrocytes (Cahoy et al., 2008; Brandebura et al., 2018). DsRed antibody was used in combination with 555 Donkey anti-rabbit (1:500, Cat. No. A31572, Thermo Fisher Scientific) and Aldh1L1 antibody was used in combination with 647 Donkey anti-mouse (1:500, Cat. No. 715-605-151, Jackson ImmunoResearch Laboratories Inc., West Grove, PA). 4',6-diamidino-2-phenylindole (DAPI, Advanced Cell Diagnostics) was added to the slides before mounting with Fluoromount-G (SouthernBiotech, Birmingham, AL). Three replicates from at least two different litters were performed for all smFISH experiments.

Immunohistochemistry

Tissue for immunohistochemistry was prepared using transcardial perfusion and post-fixation at 4°C overnight as described above. Brains were then cryoprotected in 30% sucrose overnight and sliced at 40 µm thickness in the coronal plane on a freezing microtome (Model HM 450, Microm, Walldorf, Germany). Free-floating sections were subjected to the immunohistochemistry protocol as described above.

Antibodies used in various immunohistochemistry experiments on *En1-Cre;Ai9*, TNR and C57Bl6/J mice include Map2 (1:2,500, Cat. No. CPCA-MAP2, Encor Biotechnology Inc., Gainesville, FL), DsRed (1:500, Cat. No. 632496, Clontech), anti-GFP (1:500, Cat. No. A11122, Thermo Fisher Scientific), Aldh1L1 (1:500, Cat. No. 75-140, Antibodies Inc.), Cd31 (1:250, Cat.

No. AF3628, R&D Systems, Minneapolis, MN), and Nestin (1:500, Cat. No. 611658, BD Biosciences, San Jose, CA). Appropriate secondary antibodies were used (Jackson Immuno Research Laboratories, Inc.). DAPI (Thermo Fisher Scientific) was applied for 20 minutes followed by a PBS wash. Slices were then wet-mounted in PBS. Three replicates from at least two different litters were performed for all immunohistochemistry experiments.

Segmentation and reconstruction of blood vessels from SBEM volumes

The preparation and acquisition of SBEM image volumes from FVB mouse pups at postnatal days 3, 6, and 9 has been detailed previously (Holcomb et al., 2013). Briefly, FVB mouse pups were transcardially perfused with cacodylate buffer (0.15 M) containing formaldehyde (2%), glutaraldehyde (2.5%), and calcium chloride (2 mM). The brains were removed from the skull and post-fixed in the same solution as original fixation and later resectioned at 200 μ m. MNTB was identified using a dissecting microscope and a 1.5 mm x 1.5 mm area including the MNTB was excised. Excised sections were then processed for electron microscopy using potassium ferrocyanide (3%) and aqueous osmium tetroxide (4%) at room temperature, followed sequentially by thiocarbohydrazide (1%), osmium (2%), and uranyl acetate (1%) overnight. Finally, tissue was baked at 60°C for 30 minutes in a lead aspartate solution. Tissue was then shipped to the National Center for Microscopy and Imaging Research (NCMIR) at University of California, San Diego, for SBEM imaging. Tissue was mounted on aluminum specimen pins, trimmed, grounded with silver paint, and sputter coated with gold/palladium for enhanced conductivity. Images were then acquired using either a Quanta field emission gun scanning electron microscope (FEI) or a Merlin scanning electron microscope (Zeiss Microscopy).

Segmentation of blood vessels from SBEM image volumes was accomplished using the Seg3D software (Scientific Computing Institute, University of Utah). Acquired image volumes for the three ages (P3, P6, and P9) were converted from TIFF image stacks into a pyramid tiff structure using the Seg3D executable "CreateLargeVolume.exe". Image data was then opened in the

Seg3D software and downsampled by a factor of 32 to enhance speed and accuracy of segmentation. Blood vessels were identified by their regular circularity, light color, and presence of surrounding endothelial cells. A high-pass filter was applied to the data using the Threshold tool to extract high intensity pixels, and blood vessels were further extracted by manually seeding each vessel and extracting it using the “Connected Components” tool. The resulting segmentation was checked for errors, corrected using the “Paint Brush” and “Fill Holes” tools, rendered in 3D using the “Isosurface” (ISO) tool, and exported in Visualization Toolkit (VTK) format. A custom python script was used to convert the VTK models to OBJ format. Blood vessel reconstructions were then imported into the Rhinoceros CAD software (RhinoCAD, McNeel, Seattle, WA), scaled using their respective pixel dimensions (see Holcomb et al., 2013) and their volumes were measured using the “Volume” command. Percentage of total volume was then calculated by dividing the blood vessel volume by the total image volume and multiplying by 100.

Light microscopy imaging, image analysis and figure generation

Imaging was performed on an inverted Zeiss LSM 710 confocal microscope equipped with a motorized stage. 20x Plan-Apochromat/0.75 NA or 40x C-Apochromat/1.2 NA objectives (Zeiss Microscopy, Oberkochen, Germany) were used. Z-stacks were collected at 0.3 μm steps. In some instances, the entire ventral edge of the coronal slice was imaged to include all anatomical nuclei in the Superior Olivary Complex (SOC). These images were acquired at 40x and multiple image tiles were stitched together using the Zen Black 2.1 software (Zeiss Microscopy).

Time series experiments were performed on littermate mice and imaging conditions were matched between slices. For the Cd31 quantification, two slices were analyzed for each replicate at P3, P6 and P9. Z-stacks were collapsed and a region of interest (ROI) was drawn

around the MNTB in FIJI using the “Freehand” tool. The ROI was determined on each individual slice based on counterstaining with Map2 to label the neurons. The number of pixels in each ROI was determined using the “Measure” tool. The Cd31 pixels were converted to Black & White scale and selected using the “Threshold” tool. The images were then converted to “Binary” and the “Fill Holes” and “Despeckle” tools were used. The number of Cd31-positive pixels in the ROI was determined using the “Histogram” tool. The percentage Cd31-positive pixels was calculated by dividing the number of Cd31-positive pixels by the total number of pixels in the ROI and multiplying by 100. A two-tailed t-test was used to determine statistical significance.

Figures were generated using Zen Black 2.1 software (Zeiss Microscopy), Adobe Photoshop and Adobe Illustrator (Adobe Inc., San Jose, CA).

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Figures

Figure 1. Hierarchical clustering analysis on cells after sequencing. Heatmap shows expression levels of genes selected for clustering (blue to red = low to high expression). Expression values are depicted as the difference from the median (in Counts Per Million) for any particular gene and is displayed on the $\log_2$ fold change scale. The dendrogram across the top columns represents Ward's distance for each cell ($n = 215$) and the dendrogram along the side represents Ward's distance for each gene ($n = 1,103$). The clustering analysis yielded 14 gene clusters (right side) and 5 cell clusters (bottom).

Figure 2. Identification of cell clusters based on known marker genes. A.) Cluster 1 and Cluster 2 were identified as neuronal clusters based on the expression of Calb1, Gabra5 and

Grin2a. **B.)** Cluster 3 was identified as the astrocyte cluster based on the expression of Aldh1l1, Slc1a2 and Slc1a3. **C.)** Cluster 4 was identified as the oligodendrocyte cluster based on the expression of Cnp, Mbp and Sox10. **D.)** Cluster 5 was identified as the VAC cluster based on the expression of Cldn5, Flt2 and Pdgfrb. Expression values are displayed as the $\log_2$ transformed CPM. N1 = Neuron Cluster 1; N2 = Neuron Cluster 2; A = Astrocyte; O = Oligodendrocyte; V = Vascular Associated Cell.

Top 20 Differentially Expressed Genes Per Cell Cluster							
Neuron				Astrocyte			
Gene Name	Fold Change (log2)	P-Value	FDR	Gene Name	Fold Change (log2)	P-Value	FDR
Gm14204	5.33296256	2.49E-10	1.14E-08	Tnc	8.017817973	1.09E-12	1.04E-09
Grin2a	5.118059505	4.49E-09	1.59E-07	Fgfr3	6.548101937	7.53E-09	3.61E-06
C230085N15Rik	5.123636104	2.61E-08	7.58E-07	9330159F19Rik	4.796441151	8.43E-07	0.000202
Gm42616	4.672227628	5.25E-08	1.48E-06	Gm3764	4.197875058	1.24E-06	0.000227
Ass1	4.429968247	1.12E-07	3.00E-06	Aqp4	5.028716493	1.77E-06	0.000227
Ankrd45	4.786700477	1.23E-07	3.19E-06	Slc6a11	4.902113994	1.83E-06	0.000227
Tmem59l	4.211614186	1.34E-07	3.37E-06	Tmem229a	5.756433817	1.90E-06	0.000227
Cda	5.111879642	2.05E-07	5.05E-06	Agt	5.847760162	3.58E-06	0.000344
Syng3	3.918222427	3.36E-07	7.74E-06	Slc1a3	3.934042358	1.28E-05	0.000883
Lamp5	3.460966213	6.15E-07	1.31E-05	Fads2	5.311119667	1.33E-05	0.000883
Ramp3	4.315890349	1.75E-06	3.29E-05	Al464131	5.607022332	1.38E-05	0.000883
Epb41l4b	4.18621685	5.01E-06	8.43E-05	Pla2g7	4.47221024	1.78E-05	0.001055
Cds1	3.919713688	6.92E-06	0.000113	Slc1a2	4.414266397	1.87E-05	0.001055
Rasgrf1	4.483980388	8.11E-06	0.000127	Acot1	5.849711356	2.17E-05	0.001097
Gabra5	3.625585091	8.65E-06	0.00013	Kxd1	4.752519748	2.56E-05	0.00119
Car10	3.366233111	1.01E-05	0.000146	Igsf11	5.470171059	2.61E-05	0.00119
Fsd1	4.038406282	1.03E-05	0.000147	Hepacam	4.459636719	3.70E-05	0.001611
Kcnk1	4.130664812	1.47E-05	0.000201	Tulp4	3.887830737	3.98E-05	0.001615
Hapln1	3.000500493	1.57E-05	0.000212	Aldh1l1	4.864451285	4.04E-05	0.001615
Prepl	4.138461882	1.62E-05	0.000214	Adgrg1	4.192390822	6.52E-05	0.002404

Oligodendrocyte				Vascular Associated Cells			
Gene Name	Fold Change (log2)	P-Value	FDR	Gene Name	Fold Change (log2)	P-Value	FDR
Bcas1	9.01998098	2.81E-12	2.70E-09	Acvrl1	12.12671347	1.51E-44	1.45E-41
Ralgps2	6.175908141	5.59E-10	2.68E-07	Il2rg	11.16868551	2.99E-38	1.43E-35
Ugt8a	8.307817085	1.70E-09	5.15E-07	Ifitm3	11.40188055	8.59E-36	2.75E-33
Shisa1	8.173344462	2.15E-09	5.15E-07	Cldn5	12.48972892	3.30E-33	7.91E-31
Tnr	6.822185459	1.17E-08	2.23E-06	Ctla2a	12.23968331	3.05E-32	5.84E-30
Gpr17	6.601437261	6.72E-08	1.07E-05	Srgn	11.77975643	2.48E-31	3.97E-29
Plp1	6.093060749	1.14E-07	1.56E-05	Icam2	11.29527127	5.48E-31	7.51E-29
Scrg1	6.8161038	1.56E-07	1.78E-05	Eng	11.3198578	3.40E-29	4.08E-27
Mbp	6.154865308	1.79E-07	1.78E-05	Grap	11.5176317	6.68E-27	7.12E-25
Sox10	7.346628967	1.86E-07	1.78E-05	Robo4	10.80166765	1.10E-26	1.05E-24
Cyfp2	4.748608293	4.00E-07	3.48E-05	Pltp	11.60600608	2.60E-22	2.26E-20
Olig2	6.146375621	8.75E-07	6.99E-05	Slc38a5	11.24018164	1.17E-21	9.39E-20
Cnp	5.582313424	1.09E-06	8.08E-05	Cd34	10.46470466	5.04E-21	3.72E-19
Dscam	4.81675982	1.38E-06	9.45E-05	Tgfb2	11.03182231	5.63E-21	3.86E-19
Sema5a	5.471186835	1.96E-05	0.000938	Fzd6	9.567143296	1.80E-20	1.15E-18
Sox6	5.359888942	2.16E-05	0.000957	Tek	10.16177766	7.58E-20	4.54E-18
Nfasc	4.14092051	4.54E-05	0.001675	Adgrl4	10.57482412	1.13E-19	6.36E-18
Rnd2	4.288526473	5.52E-05	0.001825	Lama4	8.99109189	2.21E-19	1.18E-17
Opcml	4.597896573	6.05E-05	0.001871	Cgnl1	10.20153391	6.60E-19	3.33E-17
Omg	4.855301525	0.000138	0.003403	Adgre5	9.866715788	2.65E-18	1.27E-16

Table 1. Top 20 differentially expressed genes per cell cluster. The top 20 genes differentially expressed in the Neuron (N1 and N2 combined) cluster, Astrocyte cluster,

Oligodendrocyte cluster and VAC cluster are listed in order sorted by lowest FDR. The gene name with fold change ($\log_2$), p-value and FDR are listed. The calculations of differential gene expression are based on the average CPM values of one cluster compared to the average CPM values of all other clusters. $\text{FDR} \leq 0.05$ with ≥ 2 -fold change.

A.

N1 and N2 Random Forest Analysis

B.

Figure 3. Random Forest analysis identified gene variables that separate N1 and N2

clusters. A.) The graph displays the genes identified from a Random Forest analysis on N1 and N2 neuronal clusters which had “Mean Decrease in Gini Scores” ≥ 0.1 . The genes are displayed in descending order of importance. The top 8 genes (red) are the most impactful as shown to

the right of the “elbow” in the ranking. **B.)** Violin plots display the range and distribution of $\log_2$ -transformed CPM values for the top 8 transcripts identified in the Random Forest Analysis for the cells in the two neuronal clusters.

Gene Ontology	Category	Total Genes	DE Genes	Adjusted p-value	Genes
regulation of cellular localization	BP	759	12	2.39E-05	<i>Slc25a5, Cryab, Gdi1, Map1b, Nrnx1, Pin1, Vsnl1, Bcap31, Rab3b, Inpp5f, Ndr4, Akap5</i>
regulation of protein kinase A signaling	BP	19	3	5.03E-05	<i>Aip, Nrnx1, Akap5</i>
nucleotide metabolic process	BP	466	9	6.41E-05	<i>Hprt, Ldhb, Mdh1, Pgk1, Rit2, Idh3a, Atp5g2, Cda, Akap5</i>
nucleoside phosphate metabolic process	BP	473	9	7.19E-05	<i>Hprt, Ldhb, Mdh1, Pgk1, Rit2, Idh3a, Atp5g2, Cda, Akap5</i>
protein kinase A signaling	BP	24	3	1.04E-04	<i>Aip, Nrnx1, Akap5</i>
nucleobase-containing small molecule metabolic process	BP	501	9	1.12E-04	<i>Hprt, Ldhb, Mdh1, Pgk1, Rit2, Idh3a, Atp5g2, Cda, Akap5</i>
organophosphate metabolic process	BP	766	11	1.32E-04	<i>Hprt, Ldhb, Mdh1, Pgk1, Rit2, Gde1, Idh3a, Atp5g2, Cda, Inpp5f, Akap5</i>
dicarboxylic acid metabolic process	BP	70	4	1.50E-04	<i>Ass1, Got1, Mdh1, Idh3a</i>
NAD metabolic process	BP	30	3	2.04E-04	<i>Ldhb, Mdh1, Idh3a</i>
neuron projection development	BP	833	11	2.75E-04	<i>Gdi1, Hprt, Map1b, Nefl, Nrnx1, Rit2, Snap25, Inpp5f, B4gat1, Ndr4, Akap5</i>
regulation of intracellular transport	BP	445	8	2.79E-04	<i>Slc25a5, Cryab, Gdi1, Map1b, Bcap31, Inpp5f, Ndr4, Akap5</i>
tricarboxylic acid metabolic process	BP	34	3	2.98E-04	<i>Ass1, Mdh1, Idh3a</i>
aspartate family amino acid metabolic process	BP	35	3	3.25E-04	<i>Ass1, Got1, Asns</i>
regulation of plasma membrane bounded cell projection organization	BP	586	9	3.60E-04	<i>Gdi1, Map1b, Nefl, Nrnx1, Rit2, Snap25, Inpp5f, Ndr4, Akap5</i>
regulation of neuron projection development	BP	464	8	3.69E-04	<i>Gdi1, Map1b, Nefl, Rit2, Snap25, Inpp5f, Ndr4, Akap5</i>
regulation of cell projection organization	BP	589	9	3.74E-04	<i>Gdi1, Map1b, Nefl, Nrnx1, Rit2, Snap25, Inpp5f, Ndr4, Akap5</i>
positive regulation of protein kinase A signaling	BP	8	2	4.03E-04	<i>Nrnx1, Akap5</i>
regulation of endocytic recycling	BP	9	2	5.17E-04	<i>Inpp5f, Ndr4</i>

aspartate metabolic process	BP	9	2	5.17E-04	<i>Ass1,Got1</i>
ribonucleoside catabolic process	BP	9	2	5.17E-04	<i>Hprt,Cda</i>
regulation of transport	BP	1383	14	5.61E-04	<i>Slc25a5,Cryab,Gdi1,Map1b,Nrxn1,Rit2,Snap25,Vsnl1,Bcap31,Caly,Rab3b,Inpp5f,Ndrg4,Akap5</i>
negative regulation of intracellular transport	BP	100	4	5.88E-04	<i>Slc25a5,Cryab,Gdi1,Map1b,Gdi1,Hprt,Map1b,Nefl,Nrxn1,Rit2,Snap25,Inpp5f,B4gat1,Ndrg4,Akap5</i>
neuron development	BP	916	11	6.17E-04	<i>Gdi1,Hprt,Map1b,Nefl,Nrxn1,Rit2,Snap25,Inpp5f,B4gat1,Ndrg4,Akap5</i>
nucleoside catabolic process	BP	10	2	6.44E-04	<i>Hprt,Cda</i>
cellular localization	BP	2128	18	6.65E-04	<i>Aip,Slc25a5,Cryab,Gdi1,Map1b,Nefl,Nrxn1,Rit2,Snap25,Pin1,Vsnl1,Bcap31,Caly,Rab3b,Kxd1,Inpp5f,Ndrg4,Akap5</i>
neuron differentiation	BP	1081	12	6.79E-04	<i>Gdi1,Hprt,Map1b,Nefl,Nrxn1,Rit2,Snap25,Pin1,Inpp5f,B4gat1,Ndrg4,Akap5</i>
positive regulation of neuron projection development	BP	284	6	7.50E-04	<i>Gdi1,Map1b,Nefl,Rit2,Ndrg4,Akap5</i>
glutamine family amino acid metabolic process	BP	47	3	7.80E-04	<i>Ass1,Got1,Asns</i>
grooming behavior	BP	11	2	7.86E-04	<i>Hprt,Nrxn1</i>
establishment of localization in cell	BP	1608	15	8.05E-04	<i>Aip,Slc25a5,Cryab,Gdi1,Map1b,Nefl,Nrxn1,Snap25,Vsnl1,Bcap31,Caly,Rab3b,Inpp5f,Ndrg4,Akap5</i>
positive regulation of signaling	BP	1111	12	8.66E-04	<i>Calb1,Nrxn1,Rit2,Snap25,Pin1,Vsnl1,Bcap31,Rab3b,M</i>
nicotinamide nucleotide metabolic process	BP	112	4	9.00E-04	<i>Ldhd,Mdh1,Pgk1,ldh3a</i>
oxaloacetate metabolic process	BP	12	2	9.40E-04	<i>Got1,Mdh1</i>
pyridine nucleotide metabolic process	BP	114	4	9.62E-04	<i>Ldhd,Mdh1,Pgk1,ldh3a</i>
alpha-amino acid biosynthetic process	BP	52	3	1.05E-03	<i>Ass1,Got1,Asns</i>
pyridine-containing compound metabolic process	BP	117	4	1.06E-03	<i>Ldhd,Mdh1,Pgk1,ldh3a</i>

phosphate-containing compound metabolic process	BP	2218	18	1.10E-03	<i>Cryab,Hprt,Ldhb,Mdh1,Nrxn1,Pgk1,Prkar1b,Rit2,Pin1,Gde1,ldh3a,Ppa1,Atp5g2,Cda,Maged1,Inpp5f,Ndrg4,Akap5</i>
learning or memory	BP	204	5	1.10E-03	<i>Calb1,Nrxn1,Prkar1b,Snap25,Ndrg4</i>
NAD binding	MF	53	3	1.11E-03	<i>Ldhb,Mdh1,ldh3a</i>
small molecule metabolic process	BP	1315	13	1.16E-03	<i>Ass1,Got1,Hprt,Ldhb,Mdh1,Pgk1,Rit2,Asns,Gde1,ldh3</i>
cellular amino acid biosynthetic process	BP	54	3	1.17E-03	<i>Ass1,Got1,Asns</i>
establishment of mitochondrion localization, microtubule-mediated	BP	14	2	1.29E-03	<i>Map1b,Nefl</i>
mitochondrion transport along microtubule	BP	14	2	1.29E-03	<i>Map1b,Nefl</i>
phosphorus metabolic process	BP	2261	18	1.38E-03	<i>Cryab,Hprt,Ldhb,Mdh1,Nrxn1,Pgk1,Prkar1b,Rit2,Pin1,Gde1,ldh3a,Ppa1,Atp5g2,Cda,Maged1,Inpp5f,Ndrg4,Akap5</i>

Table 2. Gene Ontology analysis for neuronal clusters. Gene Ontology analysis was performed on genes identified in the Random Forest analysis with “Mean Decrease in Gini Score” ≥ 0.1 .

Figure 4. Tal1 is a novel marker for MNTB neurons. A.) Probe for Tal1 (green) colocalizes with **B.)** tdTomato-positive neurons (red) in the *Engrailed1-Cre;Ai9* cross. **C.)** DAPI labeling

(blue) demonstrates that nonneuronal cells (DAPI-positive and tdtomato-negative) lack labeling for Tal1 probe. Scale bar is 20 μm . 40x image at 1.0 zoom.

Figure 5. Tal1 expression pattern in SOC. A-A'.) Tal1 mRNA (green) is expressed in the tdTomato-positive neurons (red) in the VNTB (right of white solid line) and LNTB (left of white solid line). Image taken at 40x magnification. Scale bar is 20 μ m. Tal1 mRNA labeling within the region surrounded by the white box is shown in A'. **B-B'.)** Tal1 mRNA (green) is present in the tdTomato-negative neurons (red) in the SPN. Image taken at 40x magnification. Scale bar is 20 μ m. Tal1 mRNA labeling within the region surrounded by the white box is shown in B'. **C-C'.)** Tal1 mRNA (green) is not expressed in the tdTomato-negative neurons (red) in the MSO. Image taken at 40x magnification. Scale bar is 20 μ m. The lack of Tal1 mRNA expression in the region of interest shown within the white box is shown in C'. **D-D'.)** Tal1 mRNA (green) is not expressed in the tdTomato-negative neurons (red) in the LSO. Image taken at 40x magnification. Scale bar is 20 μ m. The lack of Tal1 mRNA expression in the region of interest shown within the white box is shown in D'.

Figure 6. Transcripts encoding for PNN components are distributed amongst neurons and glial cells. A.) The transcript for the Hapln1 linker protein is expressed at significantly higher levels in the N1 and N2 clusters. The transcript for Bcan is expressed at significantly higher levels in the astrocyte and oligodendrocyte clusters. Ncan, Tnc and Vcan transcripts are expressed at significantly higher levels in the astrocyte cluster. Tnr is expressed at significantly

higher levels in the oligodendrocyte cluster. Acan, Hapln3 and Hapln4 transcripts are detected but not differentially expressed in any cluster. Expression values are displayed as the $\log_2$ transformed CPM. Asterisks denote genes significantly differentially expressed in a cell cluster. *** p-value ≤ 0.001 ; ** p-value ≤ 0.005 ; * p-value ≤ 0.05 . DGE values were calculated as preferentially expressed in one cluster compared to the average of all other clusters, where N1 and N2 clusters were combined. N1 = Neuron Cluster 1; N2 = Neuron Cluster 2; A = Astrocyte; O = Oligodendrocyte; V = Vascular Associated Cell. **B.)** PNN-associated transcripts were identified as significantly changing in expression levels during the first week of postnatal development in the MNTB, during the height of CH growth and synaptic refinement. The expression levels are graphed as $\log_2$ fold change by postnatal day. Connecting the temporal data with known changes in the neuron:glia ratio and cell type-specific expression allows for a full picture of PNN formation. **C.)** Schematic of PNN formation in the MNTB. Neurons (N) secrete the Hapln1 linker protein early in development while multiple terminals project onto the neuron. As gliogenesis and refinement to mono-innervation proceeds (gradient boxes), astrocytes (A) and oligodendrocytes (O) secrete tenascins and chondroitin sulfate proteoglycans (Cspgs) that bind to the neuronally-secreted Hapln1 scaffold to form the lattice-like PNN around the MNTB neuron. PNNs may stabilize innervation of the principal neuron by the CH terminal and prevent multi-innervation.

G FGF Signaling

Figure 7. Fgf9 ligand is expressed in MNTB neurons and Fgfr3 is expressed in MNTB astrocytes. A.) Probe for Fgf9 (green) is colocalized with **B.)** tdTomato-positive neurons (red) in the *Engrailed1-Cre;Ai9* cross. **C.)** Probe for Fgf9 (green) is not present in nonneuronal cells which are DAPI-positive (blue) but tdTomato-negative (red). **D.)** Probe for Fgfr3 (green) is

colocalized with **E.)** Aldh1L1-positive astrocytes (cyan). **F.)** Probe for Fgfr3 (green) is not present in other cells types (DAPI-positive but Aldh1L1-negative; blue). Scale bars are 10 μ m. 40x images at 2.0 zoom. **G.)** Schematic of expression patterns for transcripts encoding for FGF ligands and receptors demonstrates that neurons (N) contain significantly higher levels of the Fgf9 ligand transcript and astrocytes (A) contain significantly higher levels of the receptor transcript, Fgfr3. The black arrows indicate the direction of signaling from neurons to astrocytes.

Figure 8. Delta-Notch signaling is transiently activated in MNTB astrocytes. **A.)** At P0 Aldh1L1-positive astrocytes (red) in the MNTB are GFP-negative (green; GFP transgene designed to report active Notch signaling). **B.)** P3 astrocytes (Aldh1L1-positive cells in red) are also GFP-positive (green), demonstrating active Delta-Notch signaling. **C.)** At P6 astrocytes

(Aldh1L1-positive; red) have down-regulated Notch signaling (GFP-negative; green). Scale bars are 20 μm . $n = 3$ at each age. **D.)** Schematic of expression patterns for transcripts encoding for Dll and Jag ligands and Notch receptors demonstrates that oligodendrocytes (O) and VACs (V) express the Dll and Jag ligands while astrocytes (A) contain significantly higher levels of the Notch receptor and Hes transcripts. The black arrows indicate the direction of signaling from oligodendrocytes and VACs to astrocytes. Active Delta-Notch signaling in astrocytes is confirmed by the GFP reporter experiments shown in Panel B.

Figure 9. Angiogenesis in MNTB. A.) The endothelial cell progenitor marker Nestin (Nes; green) colocalizes with the endothelial cell tight junction protein Cd31 (red) at P3 (shown with

arrowheads). Scale bar is 20 μm . **B.)** The percentage of Cd31-positive pixels out of total pixels in the MNTB significantly increases from P3 to P9. *p-value ≤ 0.05 . n = 3 at each age. **C.)** The blood vessels from the P3 (purple), P6 (blue) and P9 (red) SBEM volumes were segmented and reconstructed as 3D objects (P3 dimensions: x = 96.0 μm , y = 84.0 μm , z = 50.2 μm ; P6 dimensions: x = 98.0 μm , y = 78.4 μm , z = 68.0 μm ; P9 dimensions: x = 153.6 μm , y = 115.2 μm , z = 59.2 μm). **D.)** The percentage of electron microscopy tissue volume occupied by blood vessels increases across development from 0.6% at P3 to 0.8% at P6 to 1.2% at P9. n = 1 at each age.

Figure 10. Summary of pathways involved in angiogenesis. A.) VACs express the Dll4, Jag1, Notch4 and Hes1 transcripts. Dll4 and Jag1 levels regulate tip vs. stock cell fate. Jag1

binding to Notch promotes angiogenesis and vascular sprouting in tip cells (+). Dll4 binding to Notch inhibits angiogenesis and maintains integrity of the vascular structure (-). **B.)** Neurons, astrocytes and oligodendrocytes express transcripts for TGF β ligands and VACs express significantly higher levels of the TGF β and activin receptor transcripts. The TGF β signaling pathway promotes angiogenesis. **C.)** Neurons, astrocytes and oligodendrocytes express the VEGF ligand transcripts and VACs contain significantly higher levels of the VEGF receptor transcripts. VEGF signaling promotes angiogenesis.

Supplemental Figure 1. FGF signaling pathway. Violin plots show expression levels of transcripts which are part of the FGF signaling pathway within each cell cluster. Expression values are displayed as the log₂ transformed CPM. Asterisks denote genes differentially expressed in a cell cluster. *** p-value ≤ 0.001. DGE values were calculated as preferentially expressed in one cluster compared to the average of all other clusters, where N1 and N2 clusters were combined. N1 = Neuron Cluster 1; N2 = Neuron Cluster 2; A = Astrocyte; O = Oligodendrocyte; V = Vascular Associated Cell.

Supplemental Figure 2. Delta-Notch signaling pathway. Violin plots show expression levels of transcripts which are part of the Delta-Notch signaling pathway within each cell cluster. Expression values are displayed as the log₂ transformed CPM. Asterisks denote genes differentially expressed in a cell cluster. *** p-value ≤ 0.001; ** p-value ≤ 0.005. DGE values were calculated as preferentially expressed in one cluster compared to the average of all other clusters, where N1 and N2 clusters were combined. N1 = Neuron Cluster 1; N2 = Neuron Cluster 2; A = Astrocyte; O = Oligodendrocyte; V = Vascular Associated Cell.

Supplemental Figure 3. TGF β signaling pathway. A.) Violin plots show expression levels of transcripts which are part of the TGF β signaling pathway within each cell cluster. Expression values are displayed as the log₂ transformed CPM. Asterisks denote genes differentially expressed in a cell cluster. *** p-value ≤ 0.001 . DGE values were calculated as preferentially expressed in one cluster compared to the average of all other clusters, where N1 and N2 clusters were combined. N1 = Neuron Cluster 1; N2 = Neuron Cluster 2; A = Astrocyte; O = Oligodendrocyte; V = Vascular Associated Cell. **B.)** Schematic of expression patterns for transcripts encoding for TGF β ligands and receptors demonstrates that all cell types express the TGF β and Gdf ligand transcripts while the VACs (V) contain significantly higher expression

levels of the TGF β and activin receptor transcripts. Neurons also express TGF β and activin receptor transcripts. The straight black arrows indicate signaling from neurons (N), oligodendrocytes (O) and astrocytes (A) to VACs and the curved black arrow indicates neuronal Gdf11 autocrine signaling.

Supplemental Figure 4. VEGF signaling pathway. A.) Violin plots show expression levels of transcripts which are part of the VEGF signaling pathway within each cell cluster. Expression values are displayed as the log₂ transformed CPM. Asterisks denote genes differentially expressed in a cell cluster. *** p-value ≤ 0.001. DGE values were calculated as preferentially expressed in one cluster compared to the average of all other clusters, where N1 and N2 clusters were combined. N1 = Neuron Cluster 1; N2 = Neuron Cluster 2; A = Astrocyte; O = Oligodendrocyte; V = Vascular Associated Cell. **B.)** Schematic of expression patterns for transcripts encoding for VEGF ligands and receptors demonstrates that all cell types express

the VEGF ligand transcripts while the VACs (V) express significantly higher levels of the VEGF receptor transcripts. The black arrows indicate the direction of signaling from neurons (N), oligodendrocytes (O) and astrocytes (A) to VACs.

Supplemental Figure 5. Quality control for cells. A.) Histogram displaying number of expressed genes per cell library before filtering out cells detected as outliers. **B.)** Histogram displaying percentage of reads mapping to mitochondrial transcripts before filtering out cells detected as outliers. **C.)** Histogram displaying number of expressed genes per cell library after filtering out cells detected as outliers. **D.)** Histogram displaying percentage of reads mapping to mitochondrial transcripts after filtering out cells detected as outliers. Cell were detected as outliers if ≤ 3 mad's outside of distribution for number of genes expressed or ≥ 3 mad's outside of distribution for percentage of reads mapping to mitochondrial transcripts.

Supplemental Figure 6. Gene filtering metrics. A.) Boxplot displaying the distribution of gene counts. Counts range from 0-99,969 counts, but the plot was set to 50 on y-axis to clearly see the median and 3rd quartile. The 3rd quartile value was 9.8 and thus the limit for gene detection was set to 10 in order to utilize the genes expressed in the top 25% of expression range for clustering. **B.)** Histogram displaying the number of expressed genes per cell library after gene filtering.

Supplemental Figure 7. Library size normalization. Individual cell libraries were assigned a Size Factor to normalize for differences in library size.

Supplemental Figure 8. Identification of Highly Variable Genes (HVGs). Genes were graphed by mean log-expression (x-axis) and variance of log-expression (y-axis). A best-fit curve was generated to fit the data and HVGs were identified as being ≥ 2 -fold outside of the fit with a $\text{FDR} \leq 0.05$. HVGs were then used for initial round of cell clustering.

Supplemental Figure 9. Hierarchical clustering of cells using HVGs. Heatmap shows expression levels of HVGs selected for initial clustering (blue to red = low to high expression). Expression values are depicted as the difference from the median (in CPM) for any particular gene and is displayed on the $\log_2$ fold-change scale. The dendrogram across the top columns represents Ward's distance for each cell ($n = 215$) and the dendrogram along the side represents Ward's distance for each gene ($n = 1,515$). The clustering analysis yielded 8 genes clusters (right side) and 5 cell clusters (bottom).

Supplemental Figure 10. tdTomato transcript level by cluster. Boxplots of tdTomato transcript CPM values for each cluster. The neuronal clusters (N1 and N2) have significantly higher tdTomato CPM than the nonneuronal clusters (A, O and V). N1 = Neuron Cluster 1; N2 = Neuron Cluster 2; A = Astrocyte; O = Oligodendrocyte; V = Vascular Associated Cell. * p-value ≤ 0.005 ; ** p-value ≤ 0.001 .

Neuron Cluster 1			
Gene Symbol	Fold Change (log2)	P-Value	FDR
Gm17018	3.650873436	6.33E-06	0.006783

Neuron Cluster 2			
Gene Symbol	Fold Change (log2)	P-Value	FDR
Tln2	-4.405777974	8.36E-08	0.000269
Gstm1	-4.064574652	5.16E-06	0.006783
Ednrb	-3.756123269	1.93E-05	0.01551
Dbi	-3.248412246	3.52E-05	0.02263
Ecpas	-3.470224745	6.19E-05	0.033167
Atp1a2	-3.556669965	0.000108	0.049442

Supplemental Table 1. Differentially expressed genes in N1 to N2 pairwise comparison.

Genes differentially expressed in N1 compared to N2 (left) and genes differentially expressed in N2 compared to N1 (right) with fold change (log₂), p-value and FDR. FDR ≤ 0.05 with ≥ 2-fold change.

Neuron Cluster 1			
Gene Symbol	Fold Change (log2)	P-Value	FDR
Resp18	6.544034452	1.76E-19	2.94E-17
Atp2b2	4.665926252	9.90E-17	1.27E-14
Cntnap2	4.850485624	4.97E-16	5.37E-14
Hapln1	4.292786611	1.37E-15	1.35E-13
Syng3	5.945085851	5.52E-15	5.13E-13
Nipsnap1	6.318830845	1.44E-13	1.15E-11
Tubb3	4.502441852	2.05E-13	1.60E-11
Gabrg2	5.561616231	2.51E-13	1.91E-11
Tmem59l	5.01713107	1.08E-12	7.87E-11
Crmp1	4.147543117	1.29E-12	8.97E-11
Syt1	3.75727013	1.84E-12	1.23E-10
Churc1	5.378640377	1.99E-12	1.30E-10
Calb1	5.334922465	2.91E-12	1.84E-10
Cish	6.823060156	3.25E-12	1.99E-10
Glr3	5.329547166	3.38E-12	2.02E-10
Cdk5	5.439016971	4.79E-12	2.76E-10
B3galt2	4.06541177	4.93E-12	2.80E-10
Nsf	4.645888781	5.32E-12	2.97E-10
Arl4c	5.272873183	6.00E-12	3.30E-10
Hprt	4.999116579	9.90E-12	5.35E-10
Cd99l2	6.674444564	1.54E-11	7.83E-10
Ciapi1	6.058564163	2.96E-11	1.48E-09

Astrocyte Cluster 3			
Gene Symbol	Fold Change (log2)	P-Value	FDR
Tnc	-9.04205474	2.62E-48	8.76E-45
Ntn1	-7.208853176	6.41E-38	1.07E-34
Sema6d	-8.17945117	1.56E-37	1.74E-34
Sall3	-7.168199421	3.82E-33	3.19E-30
Tmem229a	-8.092933848	1.01E-29	6.74E-27
Abca1	-7.236118164	2.80E-28	1.57E-25
Rgma	-5.706298563	1.08E-27	5.18E-25
Ednrb	-7.097618616	3.71E-25	1.55E-22
Ptprz1	-6.184844777	1.43E-24	5.32E-22
Hepacam	-6.385717898	7.38E-24	2.47E-21
Ptn	-5.946919758	1.23E-22	3.75E-20
Slc4a4	-6.748608937	9.33E-22	2.60E-19
Dbi	-4.772252867	3.57E-21	9.21E-19
Acot1	-6.444729423	1.04E-20	2.50E-18
Slc6a11	-6.266014664	1.33E-20	2.97E-18
Fgfr3	-7.198988467	2.65E-20	5.54E-18
Fabp7	-5.184925435	9.31E-20	1.83E-17
Aqp4	-6.577062937	1.53E-19	2.84E-17
Ncan	-4.413991009	1.69E-19	2.94E-17
Pdpr	-6.320677903	1.02E-18	1.63E-16
Agt	-6.666632356	1.75E-18	2.66E-16
Gja1	-6.393003009	1.06E-17	1.55E-15

Hpcal1	4.785717229	3.54E-11	1.69E-09	Slc1a2	-6.098752762	5.03E-17	7.02E-15
Dync1i1	5.807922159	3.90E-11	1.81E-09	Dock1	-6.734922474	5.25E-17	7.03E-15
Sirt3	6.313064079	7.93E-11	3.54E-09	Gstm2-ps1	-5.038287299	1.41E-16	1.75E-14
Atp6ap2	4.29385147	8.29E-11	3.65E-09	Gm3764	-5.053495781	1.72E-16	2.06E-14
Kctd13	6.423057721	1.07E-10	4.61E-09	Slc1a3	-5.358791112	2.87E-16	3.31E-14
Tagap1	6.472561693	1.12E-10	4.73E-09	Id3	-6.368697348	4.81E-16	5.37E-14
Kcnk1	6.525804161	1.21E-10	5.06E-09	Tril	-6.349875198	7.20E-16	7.53E-14
Grb14	6.077482245	1.64E-10	6.46E-09	Spry2	-4.500095792	9.60E-16	9.74E-14
Gas6	5.71850965	1.91E-10	7.36E-09	Serpine2	-5.45923003	3.54E-15	3.39E-13
Sgpp2	5.645765047	2.44E-10	9.30E-09	Atp1a2	-5.537001368	1.55E-14	1.40E-12
Nrxn3	3.599531077	2.64E-10	9.82E-09	Sox9	-5.769645625	1.67E-14	1.47E-12
Arhgap44	5.359093251	3.54E-10	1.27E-08	S100a1	-5.612691153	3.06E-14	2.63E-12
Ly6e	5.908918391	3.58E-10	1.28E-08	Pla2g7	-5.546274188	6.47E-14	5.42E-12
Ndr4	3.401041217	8.56E-10	2.96E-08	Apoe	-5.456096035	7.68E-14	6.27E-12
Nceh1	4.751155356	8.69E-10	2.97E-08	Car2	-5.650468486	1.07E-12	7.87E-11
Rit2	6.40789128	8.86E-10	2.97E-08	Al464131	-5.913896332	1.12E-12	7.95E-11
Pcdh8	4.584436083	8.95E-10	2.97E-08	Notch2	-5.577457955	1.49E-12	1.02E-10
Ass1	4.972677654	1.29E-09	4.18E-08	Ctnna1	-5.426370418	2.37E-12	1.53E-10
Nap1l2	5.370402672	1.41E-09	4.54E-08	Gm44645	-4.962563807	3.28E-12	1.99E-10
Atp1b1	3.480095195	1.53E-09	4.83E-08	Ttyh2	-5.348050984	4.40E-12	2.59E-10
Fam169a	5.171190939	2.43E-09	7.53E-08	Marcks	-3.511209644	1.14E-11	6.08E-10
Gm14204	5.443346122	2.58E-09	7.93E-08	Mgll	-5.372425529	1.19E-11	6.23E-10
Adgrl2	4.525667553	2.96E-09	8.93E-08	Selenop	-5.106469249	1.37E-11	7.04E-10

Mtftp1	5.469037855	3.90E-09	1.15E-07	Fabp5	-3.690149613	3.32E-11	1.64E-09
Cda	5.412358183	4.62E-09	1.36E-07	Aldh1l1	-5.563749896	3.43E-11	1.66E-09
Acsl4	3.888738438	5.78E-09	1.65E-07	Ttyh1	-4.743817652	3.75E-11	1.77E-09
Brinp1	5.155626452	6.14E-09	1.74E-07	Npas3	-5.226784376	5.74E-11	2.63E-09
Zfp385b	4.853282597	6.47E-09	1.82E-07	Mlc1	-5.541002909	6.84E-11	3.09E-09
Rian	4.933141573	7.60E-09	2.10E-07	Gatm	-5.227163637	8.67E-11	3.77E-09
Snhg11	3.866482099	8.05E-09	2.21E-07	Gstm1	-5.168567469	1.24E-10	5.13E-09
Hspa12a	4.386608367	8.79E-09	2.39E-07	Lxn	-5.448072846	1.34E-10	5.47E-09
Hs6st2	4.145492032	9.46E-09	2.56E-07	Vcam1	-5.372613471	1.57E-10	6.35E-09
B4gat1	5.756549879	1.12E-08	2.99E-07	Timp4	-5.388987108	1.60E-10	6.39E-09
Slc1a1	5.477037086	1.25E-08	3.30E-07	Scd2	-3.901874672	1.80E-10	7.02E-09
Sipa1l2	5.548708298	1.52E-08	3.97E-07	Kcnj10	-5.052100255	2.64E-10	9.82E-09
Tex264	5.562971994	1.80E-08	4.67E-07	Etv5	-5.251051752	2.74E-10	1.01E-08
Gad1	6.042859871	2.36E-08	6.00E-07	Arhgef26	-5.59860492	3.22E-10	1.17E-08
Tspyl5	4.547379993	2.36E-08	6.00E-07	Qk	-4.351320771	4.88E-10	1.72E-08
Tcaf1	3.87164493	3.22E-08	8.10E-07	Slc25a18	-5.42997634	8.54E-10	2.96E-08
L1cam	4.423335045	3.24E-08	8.11E-07	Slc6a1	-5.239509202	8.86E-10	2.97E-08
Ankrd45	5.825304028	3.30E-08	8.19E-07	Mmd2	-5.023969399	1.06E-09	3.47E-08
Slc6a5	3.440451191	3.47E-08	8.56E-07	Tcf4	-4.406789364	1.44E-09	4.60E-08
Cxadr	4.467553789	5.22E-08	1.26E-06	Igsf11	-5.008244492	1.93E-09	6.04E-08
Slitrk1	5.349547013	5.26E-08	1.26E-06	Plpp3	-4.270793683	2.82E-09	8.60E-08
Zc2hc1a	4.688318378	5.64E-08	1.34E-06	S100a16	-5.151150131	3.06E-09	9.16E-08
Tmeff1	4.473462927	6.30E-08	1.48E-06	Plat	-5.419590861	5.36E-09	1.56E-07

Prkar1b	5.076680765	6.58E-08	1.53E-06	Paqr8	-5.047817014	5.41E-09	1.56E-07
Ankrd29	5.664783032	7.94E-08	1.82E-06	Olig1	-4.83516067	7.06E-09	1.97E-07
Lamp5	3.730336658	7.97E-08	1.82E-06	Lrig1	-5.337182078	1.18E-08	3.14E-07
Kcnn1	5.089557946	8.72E-08	1.95E-06	Mt2	-4.029355691	2.07E-08	5.34E-07
Rcan2	4.058982349	8.83E-08	1.96E-06	Cst3	-3.105211104	3.85E-08	9.40E-07
Atl1	5.167989807	1.32E-07	2.86E-06	Gm2a	-3.792831284	4.89E-08	1.19E-06
Slc32a1	4.219705355	1.49E-07	3.21E-06	Zbtb20	-4.850208333	5.83E-08	1.37E-06
Gm43175	5.710902258	1.51E-07	3.22E-06	Aldoc	-4.593220023	7.87E-08	1.82E-06
Ptprn	5.394638649	1.72E-07	3.62E-06	Slc39a1	-3.459730657	8.25E-08	1.87E-06
Gdpd1	4.011260723	1.72E-07	3.62E-06	Mt3	-3.21849587	8.37E-08	1.88E-06
Scn1a	3.518283452	2.08E-07	4.33E-06	Ccnd2	-4.952346992	1.01E-07	2.22E-06
St8sia3	4.84183733	2.11E-07	4.36E-06	Sdc4	-5.00895305	1.17E-07	2.57E-06
Clstn3	5.138025994	2.16E-07	4.44E-06	Cyp26b1	-4.655005367	1.29E-07	2.81E-06
Aig1	4.842991912	2.32E-07	4.73E-06	Sparc	-4.551095944	1.90E-07	3.98E-06
Psd3	4.359218173	2.43E-07	4.94E-06	Mfge8	-5.021958041	3.28E-07	6.47E-06
Uchl5	4.89931639	2.47E-07	4.99E-06	Acsbg1	-4.694863591	8.24E-07	1.53E-05
Msra	4.870886531	2.69E-07	5.40E-06	Tcf7l2	-4.460986089	2.39E-06	4.08E-05
Dpysl5	4.917171393	2.97E-07	5.92E-06	Insig1	-3.466801232	2.87E-06	4.88E-05
Tro	3.898952538	3.16E-07	6.27E-06	Slc1a4	-4.406363687	4.66E-06	7.69E-05
Reep1	4.871310585	3.56E-07	6.97E-06	Slc38a3	-4.332529566	8.34E-06	0.000131
Retreg1	5.443955004	3.65E-07	7.12E-06	Rhoq	-4.292617061	8.81E-06	0.000137
Ncdn	4.466301854	4.00E-07	7.74E-06	Glud1	-3.720442347	1.11E-05	0.000169
Gm44509	5.041196498	4.95E-07	9.53E-06	Egr1	-4.012863655	1.25E-05	0.000187

Napg	4.048252218	5.41E-07	1.04E-05	Sfxn5	-3.280703771	1.46E-05	0.000214
Pcbp3	5.061053033	5.94E-07	1.13E-05	Pea15a	-3.858750217	1.64E-05	0.000234
Ppp2r2c	4.024617196	6.05E-07	1.14E-05	Spon1	-4.307837562	1.77E-05	0.000252
Hdac9	4.619392159	7.61E-07	1.43E-05	Ndrg2	-3.80913131	2.12E-05	0.000297
Atp6v1g2	3.400936988	7.93E-07	1.48E-05	Nhsl1	-3.901962709	2.39E-05	0.000327
Syt2	3.749562805	8.21E-07	1.53E-05	Bcan	-3.709847029	2.79E-05	0.000374
H2-D1	5.019966043	9.45E-07	1.74E-05	Gpm6b	-3.254408832	4.54E-05	0.00058
Kcnh7	4.053618926	9.80E-07	1.79E-05	Ttyh3	-3.564320656	5.97E-05	0.000741
Strbp	3.610148898	1.03E-06	1.87E-05	Rab8b	-4.046126361	7.28E-05	0.000894
Dnm1	3.94834943	1.11E-06	2.01E-05	Myo10	-3.937090896	7.65E-05	0.000936
Cacnb4	4.19620316	1.35E-06	2.43E-05	Gnai2	-3.791903292	8.43E-05	0.001026
Ano4	5.024511935	1.44E-06	2.58E-05	Pcdh17	-3.761051709	9.61E-05	0.001145
Gdap1l1	4.371394444	1.48E-06	2.63E-05	Usp19	-3.75671077	9.93E-05	0.001179
Ccdc184	4.148684125	1.50E-06	2.66E-05	Ramp1	-3.593007365	0.000105	0.001235
Fbxo7	5.074489537	1.52E-06	2.68E-05	Cd63	-3.479481675	0.000134	0.001543
Eif5a2	5.021348341	2.00E-06	3.50E-05	Cyb5a	-3.594730131	0.000192	0.002136
App	3.207172695	2.23E-06	3.89E-05	2-Sep	-2.961584287	0.000215	0.002353
Fsd1	4.571559085	2.25E-06	3.90E-05	Bmpr1a	-3.805220284	0.000242	0.002602
Dnajc6	4.11615745	2.26E-06	3.90E-05	Nr2f1	-3.531068381	0.000266	0.002815
Actl6b	4.726023075	2.31E-06	3.96E-05	Nr2f2	-3.643729054	0.000271	0.002841
Slc38a1	3.490810017	3.02E-06	5.10E-05	Myo6	-3.708145949	0.000279	0.00292
Slc12a5	4.225597666	3.32E-06	5.59E-05	Gng12	-3.799803337	0.000305	0.00317
Afap1	4.67613356	3.50E-06	5.85E-05	Adcyap1r1	-3.744312174	0.000331	0.003425

Ramp3	4.408979605	3.85E-06	6.41E-05	Gm10800	-3.626751823	0.000346	0.003534
Cacna1e	3.967901794	4.46E-06	7.39E-05	Hsd12	-3.332990929	0.000384	0.003829
Gm42616	5.058754474	4.84E-06	7.95E-05	Emp2	-3.831779512	0.000481	0.00472
Tacc2	4.75336825	5.30E-06	8.65E-05	Specc1	-3.686850555	0.000552	0.005376
Abca5	3.9165959	6.05E-06	9.81E-05	Mt1	-2.611213049	0.000776	0.007256
Nrip3	3.928975779	6.06E-06	9.81E-05	Chpt1	-3.603030221	0.000794	0.007405
Cx3cl1	4.171333683	6.14E-06	9.89E-05	Hadha	-3.424735314	0.001179	0.010363
Vopp1	4.865598847	6.25E-06	0.0001	Atp1b2	-2.919970646	0.001196	0.010462
Sdr39u1	4.385556049	6.33E-06	0.000101	Grm5	-3.278223107	0.001287	0.011221
C230071H17Rik	4.791466585	7.54E-06	0.000119	Reep3	-3.341189744	0.001392	0.01208
Tmem246	4.073886921	7.54E-06	0.000119	Plxnb1	-3.349884826	0.00194	0.015967
Gm42372	4.993395111	7.67E-06	0.000121	Cdk4	-3.247165419	0.00198	0.016148
Trim67	4.85423383	8.87E-06	0.000138	Rps27l	-3.132293816	0.001995	0.016215
Crtac1	3.581439708	9.38E-06	0.000145	Adgrg1	-3.264543732	0.002138	0.016973
Map7d2	3.511143466	9.90E-06	0.000152	Baz1b	-3.418674219	0.002139	0.016973
Epb41l4b	4.482625477	1.09E-05	0.000167	Vcan	-3.378591555	0.002264	0.017842
Grin2a	4.878175742	1.15E-05	0.000174	Pcdh10	-3.314742189	0.002484	0.01939
Lrfn5	3.817092131	1.17E-05	0.000177	Asrgl1	-3.26569863	0.002631	0.020259
Atp1a3	3.107849164	1.22E-05	0.000184	Per3	-3.457258133	0.003334	0.024981
Gprasp2	4.160771374	1.33E-05	0.000198	Acot2	-3.171558747	0.003444	0.025613
Edil3	3.607933665	1.38E-05	0.000205	Mbp	-3.372476132	0.003597	0.026591
Dbn1	4.252958923	1.40E-05	0.000207	Kctd5	-3.263083447	0.004705	0.033957
Tmem130	3.459281886	1.41E-05	0.000208	Rcn2	-2.944263989	0.00476	0.034279

Snap25	2.955152007	1.50E-05	0.000219
Kcnk3	3.976297325	1.53E-05	0.000222
Rgs10	4.182894848	1.58E-05	0.000228
Gap43	2.982125439	1.61E-05	0.000232
Trim32	4.299751303	1.98E-05	0.000281
Syp	3.32088227	1.99E-05	0.000282
Trnt1	4.438828822	2.06E-05	0.00029
Rufy3	3.497698374	2.14E-05	0.000299
Camk2n2	3.941990801	2.21E-05	0.000307
Apbb2	3.481948148	2.25E-05	0.000311
Ppa1	3.828026869	2.29E-05	0.000315
Slc4a10	4.066228815	2.33E-05	0.00032
Rapgef6	4.414860721	2.50E-05	0.00034
Tfrc	4.074532001	2.50E-05	0.00034
Sez6l2	3.708920301	2.58E-05	0.000349
Dpysl4	4.168498933	2.72E-05	0.000366
Ncoa7	4.398945423	3.03E-05	0.000403
Cds1	4.242036419	3.04E-05	0.000403
Rims2	4.165436917	3.04E-05	0.000403
Sptbn2	4.198543374	3.16E-05	0.000416
Glr2	3.950500892	3.25E-05	0.000426
Esrrb	4.128743782	3.26E-05	0.000426
Reep2	4.012766778	3.56E-05	0.000464

Tsc22d4	-3.089806907	0.00614	0.043107
Cdkn1b	-2.878354214	0.006217	0.04356
Itgb1	-3.236471833	0.006244	0.043657
1810037117Rik	-2.729953417	0.006729	0.046951

Tyro3	4.275005262	3.62E-05	0.00047
Car10	3.495038423	4.18E-05	0.00054
Map9	3.678302623	4.21E-05	0.000542
Ndel1	4.222066297	4.47E-05	0.000573
Map1b	2.958866802	4.62E-05	0.000588
B830012L14Rik	4.6833953	4.64E-05	0.000589
Gnai1	3.853088705	4.77E-05	0.000603
Gdap1	3.829584311	4.90E-05	0.000617
Cers6	4.175221367	5.30E-05	0.000665
Ipo11	4.212893936	5.67E-05	0.000709
Mtx2	3.935022311	5.90E-05	0.000735
Rgs8	3.559536089	6.07E-05	0.00075
Ddx25	4.204473115	6.39E-05	0.000787
Thy1	3.265615095	8.66E-05	0.001051
Mab21l1	3.622015844	8.88E-05	0.001074
Snx16	3.612075584	9.22E-05	0.00111
Kndc1	3.830266579	9.27E-05	0.001113
Kcnb2	3.378872649	9.57E-05	0.001144
Zfyve9	3.768690064	0.000101	0.0012
1500004A13Rik	3.742277214	0.000107	0.001255
Pdhx	4.037071626	0.000107	0.001255
Ache	4.098129953	0.000117	0.001366
Camk1d	3.338951878	0.000122	0.001419

Stac2	4.041271265	0.000 132	0.001 527
Al593442	4.348517237	0.000 143	0.001 647
Disp2	3.2638965	0.000 144	0.001 647
Kcnc1	3.587815131	0.000 144	0.001 649
Ncs1	3.839380422	0.000 145	0.001 649
Igfbp3	4.151954238	0.000 153	0.001 741
Retreg2	3.923760086	0.000 156	0.001 77
Gm37899	4.048189276	0.000 167	0.001 879
Pik3r3	3.826981637	0.000 169	0.001 904
Kcnh2	3.816986187	0.000 174	0.001 954
Zfpm2	3.631467405	0.000 175	0.001 956
Emc4	3.284829372	0.000 201	0.002 225
Lin7a	3.795562296	0.000 201	0.002 225
Kif3b	3.817849305	0.000 203	0.002 236
Ckmt1	3.346062311	0.000 214	0.002 352
Cend1	3.215009056	0.000 216	0.002 353
Mapre3	3.688827102	0.000 226	0.002 456
Cntn5	2.97122853	0.000 232	0.002 507
Trpc3	3.644751492	0.000 232	0.002 507
Got1	3.234229843	0.000 235	0.002 533
Fam189a1	3.988393659	0.000 247	0.002 638
Gria1	3.211557159	0.000 248	0.002 64
Sacs	3.414196786	0.000 253	0.002 694

Chd5	3.790841859	0.000 265	0.002 808
Ube2d1	3.469869557	0.000 27	0.002 841
Kitl	3.540251485	0.000 297	0.003 102
Chordc1	3.846708207	0.000 316	0.003 278
Gm26782	3.375440371	0.000 334	0.003 446
Scrt2	3.201681221	0.000 337	0.003 457
Pak1	3.41952708	0.000 345	0.003 532
Map6	3.48910363	0.000 351	0.003 57
Smpd3	3.850172896	0.000 354	0.003 589
Cav2	3.751100052	0.000 359	0.003 631
Tmem181b -ps	3.170949855	0.000 362	0.003 649
Acd	3.783243852	0.000 371	0.003 736
Mgat5	3.524760504	0.000 373	0.003 736
Lrrc49	3.136408862	0.000 377	0.003 772
Fastk	3.945737088	0.000 411	0.004 083
Arhgef7	3.41530662	0.000 426	0.004 22
Scg5	3.02642568	0.000 447	0.004 414
Gm9911	4.197369025	0.000 456	0.004 495
Lingo1	3.636908632	0.000 527	0.005 158
Adarb2	3.682957245	0.000 541	0.005 278
Ppif	3.922337967	0.000 558	0.005 419
Fkbp1b	3.382811895	0.000 565	0.005 467
Atp6v1h	3.066539555	0.000 583	0.005 612

Eno2	3.001358861	0.000 583	0.005 612
Arl6ip4	3.612053196	0.000 588	0.005 641
Cox10	3.741193211	0.000 64	0.006 122
Nt5c3b	3.557751629	0.000 652	0.006 222
Nefl	2.820647976	0.000 683	0.006 498
Lsm1	3.388689479	0.000 703	0.006 673
Me3	3.447309242	0.000 714	0.006 752
Cdk5r1	3.130485885	0.000 718	0.006 776
5031439G0 7Rik	3.688359206	0.000 724	0.006 81
Necap1	3.473531485	0.000 761	0.007 138
Rnf152	3.268296638	0.000 798	0.007 425
Ralgapa1	3.294644299	0.000 807	0.007 489
Kcnma1	3.652954487	0.000 81	0.007 497
Khdrbs2	3.554393566	0.000 817	0.007 541
Gm12709	3.030380054	0.000 872	0.008 023
Sez6	3.370141148	0.000 897	0.008 231
Slc25a36	3.305099704	0.000 919	0.008 405
Inpp4a	2.810133683	0.000 936	0.008 542
Ica1	3.64344525	0.001 019	0.009 253
Dcun1d4	3.68647149	0.001 02	0.009 253
Usp5	3.732437299	0.001 028	0.009 286
Eif4a2	2.809361101	0.001 029	0.009 286
Rab11fip4	3.520337001	0.001 037	0.009 337

Coro1c	3.726019024	0.001 067	0.009 581
Nedd4l	3.491970445	0.001 086	0.009 722
Meg3	2.871298517	0.001 1	0.009 821
Myh10	3.132202822	0.001 103	0.009 821
Tmx4	3.017392251	0.001 112	0.009 869
Mapk8ip2	3.388168412	0.001 114	0.009 869
Fstl1	3.368576804	0.001 118	0.009 88
Pnma2	3.667751022	0.001 121	0.009 88
Cep170b	3.279807015	0.001 193	0.010 456
Camta1	2.842545736	0.001 382	0.012 018
Tmem135	3.64326069	0.001 492	0.012 915
Gde1	3.127406107	0.001 501	0.012 953
Snap91	2.852403282	0.001 535	0.013 214
Ccdc85a	3.114259782	0.001 573	0.013 509
Ube3c	3.418415914	0.001 584	0.013 568
Tmem25	3.587809446	0.001 641	0.014 016
Got2	3.111329011	0.001 665	0.014 185
Pclo	2.908169178	0.001 675	0.014 241
Rgs2	3.354532692	0.001 691	0.014 341
Napb	2.839489546	0.001 704	0.014 411
Wnk2	3.322658026	0.001 72	0.014 511
Cadm3	3.236825833	0.001 729	0.014 546
Atp6ap1	3.05802605	0.001 734	0.014 553

Zfp804a	3.376017294	0.001 826	0.015 274
Mllt11	2.788855922	0.001 829	0.015 274
Gsg1l	3.49325754	0.001 882	0.015 677
Lmo3	2.98926097	0.001 907	0.015 846
Ap1p1	2.944660392	0.001 918	0.015 861
Ppfia4	3.550192018	0.001 922	0.015 861
Dip2c	3.446768156	0.001 923	0.015 861
Pcdh9	2.762112643	0.001 972	0.016 148
Hcn1	2.911845911	0.001 977	0.016 148
Cntnap1	3.169007856	0.001 982	0.016 148
Rnf187	2.966561812	0.002 005	0.016 259
Slitrk4	3.413370425	0.002 023	0.016 368
Rab3b	3.16812523	0.002 056	0.016 591
Mturn	3.329636983	0.002 071	0.016 673
Esrrg	3.109540803	0.002 08	0.016 69
Lhfpl4	3.399225942	0.002 083	0.016 69
Tal1	3.29709272	0.002 127	0.016 973
Vmp1	3.164106479	0.002 136	0.016 973
Epb41l3	2.845627772	0.002 173	0.017 202
Rnf227	3.165655956	0.002 227	0.017 594
Plppr3	3.212624437	0.002 296	0.018 047
Unc13a	3.192697597	0.002 332	0.018 288
Tmem229b	3.219561487	0.002 381	0.018 632

Gabra5	3.141907504	0.002 538	0.019 763
Nxph1	3.143986144	0.002 546	0.019 766
Coro2b	3.402795531	0.002 55	0.019 766
Efr3b	3.173596459	0.002 59	0.020 03
Endod1	3.453060133	0.002 619	0.020 208
Igsf3	2.92976466	0.002 654	0.020 387
Sae1	3.327788579	0.002 747	0.021 055
Mn1	3.230207145	0.002 874	0.021 976
Ttbk2	2.946514481	0.003 015	0.022 933
Rusc1	3.288994452	0.003 019	0.022 933
Ly6h	2.969110801	0.003 02	0.022 933
Ube3a	2.672602235	0.003 043	0.023 06
Unc5d	2.809626572	0.003 134	0.023 691
Elavl3	2.798690467	0.003 217	0.024 262
Arf3	2.936236711	0.003 265	0.024 573
Nap1l3	3.261907697	0.003 304	0.024 809
Phf24	3.036288799	0.003 349	0.025 034
Stx1b	3.175216089	0.003 365	0.025 095
Synj1	2.980942342	0.003 449	0.025 613
Gm32710	2.612343112	0.003 499	0.025 924
A230103L1 5Rik	2.912999243	0.003 625	0.026 739
Scn2a	2.792782303	0.003 635	0.026 753
Ift81	3.302111388	0.004 105	0.030 145

Cacna2d3	2.747656192	0.004 129	0.030 256
Nsg2	2.878689927	0.004 193	0.030 663
Mroh1	3.421598002	0.004 259	0.031 075
Gnas	2.700471649	0.004 313	0.031 399
Grb2	3.096181437	0.004 383	0.031 841
Camk4	2.961111852	0.004 464	0.032 358
Plppr4	3.064596226	0.004 487	0.032 458
Slc27a4	3.33421134	0.004 829	0.034 704
Armcx3	3.066015682	0.005 029	0.036 064
Prkar2b	2.948181756	0.005 174	0.037 023
Tenm4	3.305470222	0.005 226	0.037 316
Prkce	3.022324627	0.005 283	0.037 643
Eml5	3.0149997	0.005 673	0.040 337
Rb1cc1	3.075158692	0.005 744	0.040 758
Ogdh	2.916065132	0.005 775	0.040 89
Epb41l1	2.828020415	0.005 905	0.041 722
Slc35f1	2.867582374	0.006 08	0.042 87
Tspyl4	2.812771943	0.006 118	0.043 046
Pitpnc1	2.527453055	0.006 756	0.047 036
Arhgap33	3.055176329	0.006 876	0.047 777
Purg	2.903000415	0.007 022	0.048 666
Krt222	3.219903932	0.007 036	0.048 666
Caly	2.84930941	0.007 048	0.048 666

Amn	3.05954395	0.007 225	0.049 788
Dusp8	2.953457898	0.007 263	0.049 943

Supplemental Table 2. Differentially expressed genes in N1 to Astrocyte pairwise

comparison. Genes differentially expressed in N1 compared to Astrocytes (left) and genes differentially expressed in Astrocytes compared to N1 (right) with fold change ($\log_2$), p-value and FDR. FDR ≤ 0.05 with ≥ 2 -fold change.

Neuron Cluster 1			
Gene Symbol	Fold Change (log2)	P-Value	FDR
Syngn3	4.594740344	1.31E-08	1.75E-06
Rab3a	3.688859158	3.22E-08	3.35E-06
Ass1	4.582212148	1.18E-07	1.09E-05
Fabp3	3.384982082	1.65E-07	1.48E-05
Gm14204	4.833667079	1.08E-06	8.63E-05
Socs2	3.701552458	1.09E-06	8.63E-05
Fastk	5.49760206	1.16E-06	9.00E-05
Golga7b	4.893935645	1.19E-06	9.05E-05
Pip5k1c	4.767458987	3.10E-06	0.000225
Hras	5.501836199	3.79E-06	0.000263
Cish	4.690756698	4.63E-06	0.000315
Vsnl1	3.435267527	5.47E-06	0.000357
Ankrd45	5.062709492	6.40E-06	0.000402
Grin2a	5.189587153	6.63E-06	0.000409
Tuba4a	4.002069553	9.58E-06	0.00057
Ache	4.607772068	1.76E-05	0.000993
Hapln1	3.055621102	2.16E-05	0.001201
Lamp5	3.242202025	2.98E-05	0.00163
Tsc22d3	3.987973907	4.44E-05	0.002288
Aldh5a1	4.552717129	5.34E-05	0.002624
Ccdc184	3.763189323	5.69E-05	0.002748
Paxx	4.084115342	9.21E-05	0.004092

Oligodendrocyte Cluster 4			
Gene Symbol	Fold Change (log2)	P-Value	FDR
Tnr	-8.630857797	4.90E-40	1.63E-36
Gpr17	-8.65512874	9.57E-31	1.59E-27
Bcas1	-8.106964645	1.06E-26	1.18E-23
Kank1	-6.28788161	2.53E-20	2.11E-17
Ugt8a	-7.447962016	2.08E-19	1.39E-16
Sox6	-6.717839682	7.65E-19	4.25E-16
Mbp	-6.99836871	6.49E-17	3.09E-14
Olig2	-5.973396453	1.29E-16	5.39E-14
S100a1	-5.883976629	2.49E-15	9.21E-13
Pcdh17	-6.292319107	3.44E-15	1.15E-12
Cnp	-6.18187759	4.86E-14	1.47E-11
Sirt2	-5.237816303	4.37E-13	1.21E-10
Ptprz1	-4.984394391	8.96E-13	2.30E-10
Shisa1	-6.118775253	1.96E-12	4.66E-10
Ttyh2	-5.7330036	3.14E-12	6.99E-10
Gatm	-5.611990116	6.10E-12	1.27E-09
Tcf7l2	-5.758073294	1.17E-11	2.29E-09
Sox10	-5.856086113	1.36E-11	2.51E-09
Zeb2	-5.68898445	1.43E-11	2.52E-09
Serpine2	-4.90171974	1.97E-10	3.28E-08
Qk	-4.481138735	8.30E-10	1.32E-07
Marcks	-3.455558939	2.88E-09	4.36E-07

Snap25	2.759412427	0.000 126	0.005 466
Atp1a3	2.811703023	0.000 126	0.005 466
Zmat4	4.183092967	0.000 168	0.006 977
Tubb2a	2.690029254	0.000 237	0.009 767
Tmeff1	3.438177605	0.000 243	0.009 863
Ldhb	2.747718543	0.000 259	0.010 266
Stac2	3.937652736	0.000 344	0.013 471
Nbl1	3.746665751	0.000 374	0.014 499
Hspa12a	3.233812922	0.000 397	0.015 221
Rasgrf1	3.839463586	0.000 444	0.016 607
Atp6v1g2	2.835961411	0.000 529	0.019 082
Ncs1	3.659484606	0.000 546	0.019 352
Ppp1r1a	3.173350778	0.000 799	0.027 644
Mfn2	3.578728418	0.000 874	0.029 423
Gm42616	4.003991093	0.001 004	0.033 451
Kifc2	3.51804728	0.001 042	0.034 381
1110008P1 4Rik	3.023371263	0.001 435	0.045 981
Gm17018	3.368011681	0.001 474	0.046 767
Hpcal1	3.04079711	0.001 488	0.046 767

Ptn	-4.45354544	3.17E- 09	4.59E- 07
Insig1	-4.029158712	9.00E- 09	1.25E- 06
Gpbp1l1	-4.578717903	1.74E- 08	2.23E- 06
Gja1	-5.088538259	1.91E- 08	2.35E- 06
Aqp4	-4.985770278	2.15E- 08	2.56E- 06
Tulp4	-4.381031174	2.23E- 08	2.56E- 06
Myo6	-5.044000891	2.49E- 08	2.76E- 06
Tcf4	-4.277484326	2.88E- 08	3.09E- 06
Slc6a11	-4.55302168	3.66E- 08	3.70E- 06
Ednrb	-4.612880053	4.28E- 08	4.20E- 06
Ddr1	-5.142946068	7.27E- 08	6.92E- 06
Bcan	-4.25230323	2.30E- 07	2.01E- 05
Gm3764	-3.911678946	7.43E- 07	6.35E- 05
Tril	-4.56694362	7.80E- 07	6.50E- 05
Slc1a3	-4.056714129	2.09E- 06	0.000 154
Slc4a4	-4.299349895	3.18E- 06	0.000 225
Gnai2	-4.294330193	5.44E- 06	0.000 357
Zcrb1	-3.542851062	6.36E- 06	0.000 402
Vcan	-4.338811591	6.81E- 06	0.000 412
Sobp	-3.948366042	1.14E- 05	0.000 666
Mgll	-4.131672763	1.62E- 05	0.000 93
Sh3bgrl3	-3.575364969	3.05E- 05	0.001 638
9330159F1 9Rik	-3.766027744	3.83E- 05	0.002 026

Zcchc24	-4.180288189	4.46E-05	0.002288
Fyn	-3.491812017	5.34E-05	0.002624
Nfasc	-3.56077207	5.36E-05	0.002624
Slc1a2	-3.965887357	6.04E-05	0.002873
Slc39a1	-3.148348638	6.12E-05	0.002873
Kxd1	-3.934276168	6.62E-05	0.003064
Arid1a	-3.580047807	8.03E-05	0.003667
Samd4b	-3.874737649	8.38E-05	0.003775
Gmfb	-3.603958532	0.000153	0.006544
Sema5a	-4.055542836	0.000161	0.00677
Gng12	-3.926829633	0.000251	0.010095
Gm2a	-3.119991597	0.000431	0.01631
Epb41l2	-3.561177377	0.000494	0.018285
Gm18194	-2.933940562	0.000521	0.019065
Scd2	-3.004583136	0.000533	0.019082
Enpp2	-3.808887084	0.000759	0.026633
Usp31	-3.562601293	0.000805	0.027644
Gramd1a	-3.487949627	0.000853	0.029003
Reep3	-3.38284206	0.001289	0.042121
Tcf12	-3.621302766	0.001366	0.044203

Supplemental Table 3. Differentially expressed genes in N1 to Oligodendrocyte pairwise comparison. Genes differentially expressed in N1 compared to Oligodendrocytes (left) and

genes differentially expressed in Oligodendrocytes compared to N1 (right) with fold change ($\log_2$), p-value and FDR. FDR ≤ 0.05 with ≥ 2 -fold change.

Neuron Cluster 1			
Gene Symbol	Fold Change (log2)	P-Value	FDR
Tmem59l	5.599303116	6.57E-11	2.04E-09
Mllt11	4.368498624	5.26E-09	1.55E-07
Inpp5f	4.870235276	2.52E-08	7.24E-07
Prune2	4.612497851	3.80E-08	1.08E-06
Fsd1	6.791372636	4.27E-08	1.20E-06
Arxes1	6.005581519	7.69E-08	2.10E-06
Adap1	5.811156836	1.22E-07	3.25E-06
Tubb3	4.009616706	1.66E-07	4.39E-06
Tspan7	4.020403236	2.06E-07	5.37E-06
Got1	4.558729241	3.03E-07	7.80E-06
Map1b	3.845779847	3.31E-07	8.47E-06
Gabra5	5.325645545	5.32E-07	1.33E-05
Ldhb	3.741795371	5.67E-07	1.39E-05
Car10	4.785299107	1.18E-06	2.72E-05
Caly	4.641751504	1.44E-06	3.32E-05
B3galt1	4.486965631	1.84E-06	4.21E-05
Atp6v0e2	3.850260079	2.31E-06	5.14E-05
Slc39a6	6.906233716	2.89E-06	6.38E-05
Mir670hg	5.432553193	2.90E-06	6.38E-05
Snrpn	3.357453067	3.25E-06	7.06E-05
Tmx2	3.754838185	3.37E-06	7.30E-05
Apba2	5.208317892	3.88E-06	8.31E-05

Vascular Associated Cell Cluster 5			
Gene Symbol	Fold Change (log2)	P-Value	FDR
Fn1	-11.33212315	2.50E-87	9.41E-84
Fli1	-10.71639885	1.84E-84	3.47E-81
Cldn5	-10.42164389	3.91E-79	4.91E-76
Kdr	-10.34452238	1.41E-74	1.32E-71
Ctla2a	-10.92888852	3.00E-74	2.26E-71
Hmgcs2	-10.51114157	5.04E-72	2.93E-69
Tek	-10.04171761	5.45E-72	2.93E-69
Flt1	-10.5867715	1.36E-70	6.39E-68
Srgn	-9.601569063	2.79E-70	1.17E-67
Ecscr	-9.516796856	3.62E-70	1.36E-67
Pecam1	-9.257881378	4.64E-70	1.59E-67
Cgnl1	-9.52838136	6.47E-70	2.03E-67
Fzd6	-8.866592458	9.78E-70	2.83E-67
Grap	-9.968419384	3.63E-69	9.75E-67
Abcb1a	-8.894294627	2.49E-68	6.24E-66
Eng	-9.00459811	3.01E-68	7.08E-66
Icam2	-8.483951898	3.78E-68	8.37E-66
Ifitm3	-8.936782651	1.05E-67	2.20E-65
Adgre5	-9.282418067	3.27E-67	6.48E-65
Adgrf5	-9.644694209	5.84E-67	1.10E-64
Acvrl1	-9.388916724	2.27E-65	4.07E-63
Robo4	-8.359730488	4.26E-65	7.30E-63

Ndrg3	4.527651992	4.59E-06	9.70E-05	Tgfbr2	-9.279463295	3.29E-64	5.38E-62
Dusp26	5.92834805	5.57E-06	0.000116	Il2rg	-8.782610056	7.65E-64	1.20E-61
Fam81a	4.546349016	5.84E-06	0.000121	Adgrl4	-10.40481925	3.66E-63	5.51E-61
Gabrg2	4.650109066	6.75E-06	0.000138	Gbp7	-9.561955839	1.04E-62	1.51E-60
Cds1	5.675541665	7.38E-06	0.000149	AU021092	-8.729840349	1.49E-62	2.08E-60
Mcts1	4.505305335	7.75E-06	0.000156	Cd93	-10.95741735	1.85E-62	2.48E-60
Gng3	3.488134162	8.76E-06	0.000175	Tmem204	-9.112562814	1.45E-61	1.88E-59
Stmn3	3.383550796	9.79E-06	0.000194	Tmem123	-9.616448326	1.70E-60	2.13E-58
Tagln3	4.309827619	9.83E-06	0.000194	Htra3	-7.857839867	2.05E-60	2.49E-58
Calb1	4.398372676	9.84E-06	0.000194	Arhgap29	-8.954664011	2.88E-60	3.38E-58
Scg5	3.871662937	1.01E-05	0.000198	Arhgdib	-8.388451731	3.55E-60	4.05E-58
Ramp3	5.394010533	1.18E-05	0.000229	Pdgfrb	-9.819856292	4.26E-59	4.72E-57
Scn8a	4.560707398	1.25E-05	0.00024	Slc38a5	-10.70986191	5.24E-59	5.63E-57
Unc5d	4.089490189	1.25E-05	0.00024	Apcdd1	-7.585064032	5.68E-59	5.94E-57
Ndrg4	3.164020295	1.31E-05	0.00025	Uaca	-7.307691824	8.76E-59	8.91E-57
Asns	4.928840074	1.56E-05	0.000296	Cd34	-9.990844591	2.13E-58	2.11E-56
Brinp1	4.911803417	1.61E-05	0.000305	Lama4	-8.952957971	5.93E-58	5.73E-56
Epb41l4b	5.784560607	1.79E-05	0.000336	Rasgrp3	-9.312432934	6.74E-58	6.34E-56
Glrbl	4.433560748	1.99E-05	0.000368	Foxf2	-8.441308787	1.43E-57	1.31E-55
Lamp5	3.673621507	2.13E-05	0.000389	Ucp2	-9.222731698	4.93E-56	4.42E-54
Atp6v1g2	3.532300851	2.17E-05	0.000393	Cyyr1	-8.612114776	7.62E-56	6.67E-54
Mab21l1	4.5456071	2.27E-05	0.000409	Cobll1	-9.090767417	1.49E-55	1.27E-53
Crtac1	3.930110977	2.28E-05	0.000409	Rgs5	-11.09406764	6.70E-55	5.60E-53

Arhgap33	5.269818849	2.47E-05	0.000438	Vwf	-8.241472911	1.57E-54	1.28E-52
Rab3b	4.584222426	2.79E-05	0.000488	Slc39a8	-9.857368996	1.98E-54	1.58E-52
Mapk10	4.172128043	3.14E-05	0.000545	Slc46a3	-7.847238348	2.30E-52	1.80E-50
Pld3	4.492652598	3.17E-05	0.000548	Pltp	-9.725069741	3.02E-51	2.32E-49
Hcn1	3.946467212	3.40E-05	0.000584	Slco2b1	-8.143614836	7.78E-51	5.85E-49
Ccl27a	3.947899535	3.65E-05	0.000624	Podxl	-8.519859446	1.09E-50	8.08E-49
Slc6a15	6.325493447	3.71E-05	0.00063	Ly6c1	-10.25176867	2.49E-50	1.80E-48
Snap25	3.237407771	3.84E-05	0.000649	Arap3	-7.354617764	4.12E-50	2.92E-48
Alg2	4.486705622	4.06E-05	0.000679	Slco1c1	-10.09528211	1.69E-49	1.18E-47
Slc6a5	3.223157808	4.15E-05	0.000691	Ptprb	-9.574318429	2.19E-49	1.50E-47
Cda	4.985104013	4.22E-05	0.000701	Arpc1b	-6.822931449	8.86E-47	5.95E-45
Slc22a17	3.815269816	4.28E-05	0.000707	Egfl7	-9.214994439	1.17E-45	7.74E-44
Grin2a	5.850058499	4.70E-05	0.000768	Nid1	-9.932129718	5.69E-45	3.69E-43
Chpf	5.267172502	4.83E-05	0.000787	Slc22a8	-9.845342813	1.90E-44	1.21E-42
Sez6l	5.132597168	4.89E-05	0.000793	Cdh5	-8.056205648	5.16E-42	3.24E-40
Gm9911	6.879259749	4.98E-05	0.000805	Dab2	-8.259932484	7.99E-42	4.93E-40
Nrxn1	3.421378921	5.38E-05	0.000861	Itm2a	-10.22860283	7.57E-38	4.60E-36
Srxn1	5.764429415	5.70E-05	0.000909	Zfp36l1	-7.122270628	1.35E-37	8.08E-36
Rab33a	5.396076483	5.72E-05	0.000909	Mecom	-8.523218287	3.20E-37	1.88E-35
Parp6	4.82289022	6.43E-05	0.001009	Jup	-7.95497868	5.43E-34	3.14E-32
Fam57b	4.555860363	6.64E-05	0.001036	Nostrin	-6.423617791	6.81E-34	3.88E-32
Satb1	3.345113899	6.66E-05	0.001036	Gng11	-7.762237487	7.76E-33	4.36E-31
Gap43	3.269264454	6.88E-05	0.00106	Emcn	-9.449221556	8.17E-33	4.52E-31

Lrrc4c	5.46256907	6.90E-05	0.00106	Calcr1	-8.548655035	9.02E-33	4.92E-31
Grin3a	5.564561458	7.14E-05	0.001092	Adamts12	-8.574955162	1.09E-32	5.86E-31
Fam20c	4.667569173	7.38E-05	0.001124	Notch1	-7.249303802	1.34E-31	7.12E-30
Ankrd45	5.541889929	7.69E-05	0.001165	Mfsd2a	-8.720709755	2.23E-31	1.17E-29
Ddx25	5.216591619	7.71E-05	0.001165	BC028528	-8.805478094	2.71E-31	1.40E-29
Osbpl6	4.752941125	8.41E-05	0.001267	Abca1	-8.017023185	1.57E-30	8.00E-29
Scn2a	3.753015598	8.64E-05	0.001295	Anxa3	-8.280431519	3.51E-30	1.76E-28
Yars	4.990015919	8.88E-05	0.001327	Epas1	-8.573081963	9.84E-30	4.87E-28
Ccdc85a	4.165336714	9.39E-05	0.001397	Wwtr1	-7.798608837	2.00E-28	9.80E-27
Hs3st5	4.492158495	9.99E-05	0.00148	Jcad	-8.151805313	1.12E-26	5.43E-25
Chga	3.870392529	0.000102	0.001505	Tdrp	-7.726654526	1.89E-26	9.02E-25
Maged1	3.396756048	0.000103	0.001513	Angptl4	-7.016157825	1.31E-25	6.16E-24
Fgf9	4.153766753	0.000104	0.001517	Tram2	-7.796341669	6.51E-25	3.02E-23
9330159F19Rik	4.470921035	0.000104	0.00152	Pglyrp1	-7.948480245	8.40E-25	3.86E-23
Kcnip1	4.836388018	0.000107	0.001553	Prex2	-7.190484105	1.09E-24	4.92E-23
Ralgps2	5.169878697	0.000108	0.001558	Mfap2	-7.173024992	6.38E-23	2.86E-21
Syp	3.421773626	0.000108	0.001559	Lef1	-6.973035064	3.57E-22	1.58E-20
Fbxo44	4.829012001	0.000116	0.001664	Serpinh1	-6.696234076	8.39E-22	3.67E-20
Tmem246	4.42515327	0.000127	0.001812	Tmem98	-6.181072097	1.58E-21	6.84E-20
Cacna2d3	3.443850713	0.000128	0.001821	Slc40a1	-8.031676985	4.38E-21	1.88E-19
Adk	4.796202366	0.000139	0.001958	S1pr1	-7.000948671	5.86E-21	2.48E-19
Shtn1	4.252538328	0.000149	0.002084	Thsd1	-6.129252211	9.84E-20	4.12E-18
Ass1	4.02378179	0.000156	0.00217	Ocln	-7.377508727	1.96E-19	8.11E-18

Gprasp1	3.732495439	0.000 156	0.002 17	Sema6d	-6.261569931	9.80E- 19	4.01E- 17
Mdh1	2.944299269	0.000 157	0.002 171	Ramp2	-7.150890595	1.18E- 18	4.77E- 17
Pitpnc1	3.097994937	0.000 161	0.002 214	Ttyh2	-7.137354101	3.18E- 18	1.27E- 16
Ap3b2	4.557605303	0.000 165	0.002 26	Palmd	-6.429202743	1.30E- 17	5.14E- 16
Stau2	4.445723833	0.000 165	0.002 265	Anxa2	-7.312674598	1.72E- 17	6.73E- 16
Usp29	4.863970518	0.000 167	0.002 275	Hes1	-5.111452957	1.87E- 17	7.24E- 16
Fam169a	4.394448261	0.000 192	0.002 593	Pon2	-6.891379603	2.11E- 17	8.12E- 16
Nap1l5	3.574240818	0.000 207	0.002 798	Tagln2	-7.35265608	2.21E- 17	8.42E- 16
Atp2b2	3.304871056	0.000 21	0.002 826	S100a11	-6.13551489	4.45E- 17	1.67E- 15
Gm14204	4.450383214	0.000 228	0.003 023	Myh9	-5.048474143	6.70E- 16	2.50E- 14
Gdi1	3.401987342	0.000 229	0.003 028	Cpt1a	-6.07402619	8.66E- 16	3.20E- 14
Serpini1	4.025438832	0.000 243	0.003 203	Ppic	-6.700744987	6.10E- 15	2.23E- 13
Slc25a11	4.413702625	0.000 264	0.003 428	Fxyd5	-6.537617411	1.41E- 14	5.12E- 13
Elavl4	3.106314628	0.000 264	0.003 428	Jam2	-6.563762589	1.79E- 14	6.43E- 13
Gm42616	5.425554999	0.000 265	0.003 428	Ctnna1	-6.307020457	2.36E- 14	8.37E- 13
Scn1a	3.361753148	0.000 266	0.003 428	Nr3c1	-6.677540529	4.11E- 14	1.44E- 12
Akap5	4.262803575	0.000 27	0.003 466	Cnn2	-6.629123069	1.35E- 13	4.70E- 12
Timm9	4.361430937	0.000 277	0.003 546	Sparc	-6.450906214	1.50E- 13	5.18E- 12
Snhg11	3.49520279	0.000 281	0.003 586	Rest	-5.930148428	4.18E- 13	1.43E- 11
Chchd6	4.28693416	0.000 296	0.003 759	Nes	-6.1973349	4.55E- 13	1.54E- 11
Chgb	3.834094635	0.000 312	0.003 931	Ets1	-7.095162034	4.61E- 13	1.55E- 11
Praf2	3.933393629	0.000 318	0.003 996	Wasf2	-5.615054507	5.37E- 13	1.79E- 11
Hspbp1	4.229928554	0.000 345	0.004 284	Selenop	-5.918721756	3.75E- 12	1.24E- 10

Ctnnd2	3.138165297	0.000 346	0.004 284	Gm24447	-4.572290917	9.55E- 12	3.13E- 10
Serf1	3.686020075	0.000 347	0.004 284	Cavin1	-6.518127967	9.82E- 12	3.19E- 10
Sv2c	3.371760895	0.000 359	0.004 416	Myo1b	-6.222899662	1.07E- 11	3.45E- 10
Mapre3	4.420146826	0.000 369	0.004 473	Col4a2	-5.861092625	1.34E- 11	4.28E- 10
Meis2	3.249251801	0.000 37	0.004 473	Zc3hav1	-6.370749315	1.74E- 11	5.50E- 10
Rsrc1	4.426989622	0.000 37	0.004 473	Prcp	-6.487878924	3.01E- 11	9.45E- 10
Fhl1	4.101729263	0.000 37	0.004 473	S100a13	-5.184665355	4.71E- 10	1.45E- 08
Ext1	4.900396839	0.000 374	0.004 5	Slc2a1	-6.014399125	7.06E- 10	2.16E- 08
Tro	3.66248872	0.000 388	0.004 655	Vamp8	-5.384445269	1.29E- 09	3.91E- 08
Grik3	4.741181595	0.000 407	0.004 864	Gatm	-5.611075116	2.43E- 09	7.33E- 08
Kcnma1	4.737213563	0.000 417	0.004 965	Cald1	-5.570122526	4.62E- 09	1.38E- 07
Shank2	4.391075943	0.000 421	0.004 976	Slc16a1	-5.433977697	4.95E- 09	1.47E- 07
Slc1a1	4.45291551	0.000 421	0.004 976	Serinc5	-5.295171835	1.27E- 08	3.71E- 07
Csdc2	4.019223997	0.000 428	0.005 03	Utrn	-5.466197429	1.68E- 08	4.88E- 07
Fxyd7	3.596615374	0.000 442	0.005 189	Ccdc141	-5.686962974	3.01E- 08	8.58E- 07
Stx1b	4.286345699	0.000 455	0.005 322	Slc7a1	-4.901489452	6.19E- 08	1.73E- 06
Plppr1	4.397673529	0.000 459	0.005 35	Eogt	-5.38104111	7.03E- 08	1.94E- 06
Sgip1	3.263442016	0.000 482	0.005 597	Tpp1	-5.374379088	7.35E- 08	2.02E- 06
Acsl4	3.211887882	0.000 492	0.005 685	Cmtm6	-5.208634599	9.49E- 08	2.57E- 06
Fkbp3	2.945444251	0.000 501	0.005 737	Lgals9	-5.599400641	1.01E- 07	2.72E- 06
Srgap3	3.585506031	0.000 501	0.005 737	Jag1	-5.26795222	1.82E- 07	4.79E- 06
Astn1	4.191499832	0.000 514	0.005 86	Heg1	-5.409723975	2.61E- 07	6.78E- 06
Gde1	3.646438055	0.000 519	0.005 9	Vwa1	-5.342108631	3.65E- 07	9.28E- 06

Rims1	3.934865567	0.000 544	0.006 171	Esam	-5.468742678	3.74E- 07	9.46E- 06
Lrp11	4.886021042	0.000 552	0.006 238	Rhoc	-4.837828551	4.73E- 07	1.19E- 05
St3gal5	4.016674935	0.000 554	0.006 248	Clic4	-4.900724142	5.38E- 07	1.33E- 05
Ptprn	4.741123803	0.000 564	0.006 32	Crip2	-4.994125094	5.60E- 07	1.38E- 05
Lgr4	3.827370214	0.000 564	0.006 32	Gjc1	-4.974524831	7.30E- 07	1.77E- 05
Strbp	3.375629293	0.000 637	0.007 117	Ctdsp2	-4.9327567	7.44E- 07	1.79E- 05
Tuba4a	3.768887335	0.000 642	0.007 149	Dock1	-5.014104498	7.55E- 07	1.81E- 05
Gm37176	4.881912321	0.000 645	0.007 156	Col4a1	-4.884498961	7.70E- 07	1.83E- 05
Spats2l	4.500570831	0.000 65	0.007 201	Pmepa1	-4.792829123	7.80E- 07	1.85E- 05
Ctnna2	4.382385365	0.000 658	0.007 263	Tspan14	-5.191976053	8.69E- 07	2.04E- 05
Zc2hc1a	3.938832495	0.000 671	0.007 384	Msn	-5.136451873	9.28E- 07	2.17E- 05
Rtn1	2.923660699	0.000 674	0.007 396	Ildr2	-5.269040635	9.95E- 07	2.31E- 05
Cyfp2	4.043176165	0.000 681	0.007 451	Ptprg	-5.046531814	1.96E- 06	4.45E- 05
Lrfn5	3.853835976	0.000 684	0.007 459	Fth1	-3.053069175	2.13E- 06	4.80E- 05
Apba1	3.805061955	0.000 687	0.007 476	Abhd2	-3.639064894	2.18E- 06	4.87E- 05
Nsg1	3.223223695	0.000 69	0.007 485	Ctsc	-4.473038261	2.96E- 06	6.47E- 05
Lmo3	3.501099232	0.000 712	0.007 679	Gnai2	-4.699280131	3.57E- 06	7.69E- 05
Cep170b	3.953451703	0.000 73	0.007 848	Tpm4	-4.95694217	4.56E- 06	9.69E- 05
Elavl2	3.572070897	0.000 759	0.008 123	Gm13597	-2.884041017	5.05E- 06	0.000 106
Ppa1	3.802614749	0.000 76	0.008 123	Id3	-4.780357646	5.16E- 06	0.000 108
Pgm2l1	3.326438947	0.000 784	0.008 355	S100a16	-4.850101968	6.39E- 06	0.000 131
Eno2	3.216268085	0.000 79	0.008 401	Rras	-4.210803796	6.78E- 06	0.000 138
Sgpp2	4.042809816	0.000 793	0.008 405	Myl12a	-4.766224053	1.13E- 05	0.000 22

Clip3	3.52965083	0.000 808	0.008 546	Nfkbia	-4.485821831	1.68E- 05	0.000 317
Hs6st2	3.343565932	0.000 816	0.008 592	Ostf1	-4.527670129	1.90E- 05	0.000 354
Mgat5	3.909011687	0.000 817	0.008 592	Slc38a3	-4.797017819	1.91E- 05	0.000 354
Esrrg	3.754144152	0.000 831	0.008 711	Mkl2	-4.866985184	2.04E- 05	0.000 374
Rab3a	3.080248161	0.000 837	0.008 752	Tax1bp3	-4.568447675	2.17E- 05	0.000 393
Phyh	3.502030043	0.000 881	0.009 155	Myo10	-4.600328233	2.46E- 05	0.000 438
Mier3	4.522726869	0.000 936	0.009 706	Cdkn1c	-4.4146074	2.49E- 05	0.000 44
1500009L1 6Rik	3.948624323	0.000 972	0.010 024	Bsg	-3.139706484	2.68E- 05	0.000 472
Tusc2	3.999913457	0.001 037	0.010 661	Nampt	-4.479771251	3.10E- 05	0.000 54
Ppp2r2b	3.644663359	0.001 048	0.010 721	Slc7a5	-4.396207686	3.67E- 05	0.000 625
Pin1	3.566054298	0.001 048	0.010 721	Lmo2	-4.262636963	4.04E- 05	0.000 679
Rufy3	3.461548669	0.001 068	0.010 898	10-Sep	-4.282483836	4.39E- 05	0.000 722
Ly6h	3.587457359	0.001 102	0.011 21	Adam10	-4.060348052	5.24E- 05	0.000 843
Vamp2	3.185382942	0.001 111	0.011 271	Lrp8	-4.505313585	5.79E- 05	0.000 916
Ryr2	4.047412229	0.001 136	0.011 495	Csrp2	-4.371712475	6.42E- 05	0.001 009
Cntn1	2.988782413	0.001 165	0.011 754	Sgms1	-4.644267869	6.87E- 05	0.001 06
Arxes2	3.648853883	0.001 178	0.011 844	Ushbp1	-4.383380896	0.000 109	0.001 559
Dscam	4.172131726	0.001 18	0.011 844	Lamc1	-4.417970405	0.000 133	0.001 885
Syt2	3.316909006	0.001 218	0.012 157	B4galt1	-4.321471466	0.000 141	0.001 979
Tox3	3.950815996	0.001 239	0.012 336	Tcf4	-3.773369614	0.000 184	0.002 505
Gabra3	4.420727562	0.001 272	0.012 632	Ece1	-4.49538458	0.000 216	0.002 896
Cnrip1	3.86522719	0.001 297	0.012 85	Rgs12	-4.573491828	0.000 217	0.002 896
Akap6	4.006746468	0.001 308	0.012 918	Igfbp4	-4.029893583	0.000 219	0.002 906

NA	4.354567992	0.001 4	0.013 795	Gja1	-4.239884444	0.000 25	0.003 281
Aplp1	3.331214489	0.001 514	0.014 883	Plat	-4.473683947	0.000 253	0.003 311
Amer1	4.383220037	0.001 559	0.015 133	Clic1	-4.32398899	0.000 298	0.003 781
Gaa	4.153661259	0.001 56	0.015 133	Slc1a4	-4.265201035	0.000 305	0.003 847
Idh3a	3.088327574	0.001 654	0.016 004	Gimap6	-4.415107084	0.000 333	0.004 168
Dusp8	3.62342471	0.001 721	0.016 613	C130074G 19Rik	-4.130601962	0.000 347	0.004 284
Stmn2	2.726473678	0.001 83	0.017 62	Hdac7	-4.161722102	0.000 368	0.004 473
Slc2a3	4.04337072	0.001 854	0.017 799	Igfbp7	-4.039555913	0.000 371	0.004 473
Ttbk2	3.427264839	0.001 88	0.018 009	Slc39a10	-3.999857068	0.000 422	0.004 976
Atp1a3	2.856256955	0.001 89	0.018 034	Arl4a	-3.777147433	0.000 484	0.005 601
Cend1	3.199278496	0.001 893	0.018 034	Vamp3	-4.298243377	0.000 498	0.005 736
Ncs1	3.916217901	0.001 902	0.018 08	Fcho2	-4.130500606	0.000 698	0.007 547
Ppp1r1a	3.361858284	0.001 91	0.018 107	Itpr2	-3.963974806	0.000 851	0.008 874
Rnd2	3.921975141	0.002 009	0.018 952	Rel1	-3.96490914	0.000 966	0.009 994
Npdc1	3.493036036	0.002 022	0.019 024	Atp6v0e	-3.800241773	0.001 186	0.011 87
Atl1	4.004047503	0.002 027	0.019 024	Klf4	-3.965383252	0.001 526	0.014 955
Tox2	4.074740095	0.002 088	0.019 55	Zfhx3	-3.987390415	0.001 53	0.014 955
Cpeb4	3.727613835	0.002 142	0.020 01	2-Sep	-2.975351128	0.001 55	0.015 119
Rit2	4.215810997	0.002 158	0.020 11	Abcg2	-3.906570825	0.001 928	0.018 229
Map2k4	4.08613074	0.002 207	0.020 458	Mxd4	-4.115918251	0.002 176	0.020 228
Dync1i1	3.701869224	0.002 226	0.020 587	Vim	-3.711914562	0.002 272	0.020 861
Cadm2	3.205029022	0.002 258	0.020 832	Ginm1	-3.791698405	0.002 347	0.021 445
Fabp3	2.884700347	0.002 27	0.020 861	Lxn	-3.832881616	0.002 36	0.021 506

Ncoa7	4.066485593	0.002 317	0.021 218
Oxct1	2.744197597	0.002 437	0.022 124
Pcdh7	3.264638408	0.002 44	0.022 124
Amn	3.886744892	0.002 445	0.022 124
Ptpre	4.051412063	0.002 495	0.022 519
Mrps6	3.456852606	0.002 505	0.022 56
Tmem38a	4.342520403	0.002 521	0.022 62
Atp6v1a	3.028428604	0.002 524	0.022 62
5730409E0 4Rik	3.81985688	0.002 541	0.022 719
Tnik	3.358925514	0.002 618	0.023 349
Paqr4	4.175501718	0.002 624	0.023 349
Tyro3	4.010180829	0.002 658	0.023 593
Gdap1	3.70139944	0.002 68	0.023 736
Tmod2	3.11519763	0.002 694	0.023 807
Clu	3.484633896	0.002 721	0.023 987
Cask	3.317365207	0.002 779	0.024 44
Nlgn3	3.361742573	0.002 923	0.025 65
Fam49b	3.56933347	0.002 942	0.025 752
Nceh1	3.385004555	0.002 962	0.025 864
Camta1	2.890140539	0.002 996	0.026 092
Crmp1	2.87947521	0.003 01	0.026 103
Amph	3.803434201	0.003 018	0.026 113
Cdh10	3.866637526	0.003 053	0.026 357

Sdcbp	-3.670307489	0.003 002	0.026 092
Hadh	-3.666283334	0.003 437	0.028 879
Tiam1	-3.499665888	0.004 229	0.034 231
Reep3	-3.463432518	0.004 238	0.034 231
Rhoj	-3.696783596	0.005 266	0.040 928
Cd164	-3.657295487	0.006 116	0.046 107
4931406P1 6Rik	-3.331716379	0.006 127	0.046 107
Cd2ap	-3.719421649	0.006 644	0.048 936

Dgkg	4.099837472	0.003 075	0.026 487
Kcnq3	2.803725379	0.003 141	0.026 996
Igsf3	3.248606305	0.003 172	0.027 2
Ppargc1a	2.850683106	0.003 24	0.027 719
Sptbn2	3.827119284	0.003 258	0.027 809
Mkrn1	3.697745982	0.003 269	0.027 835
Snhg14	2.956630767	0.003 297	0.028 015
Kcnn1	3.798177142	0.003 316	0.028 11
Chd5	3.876310364	0.003 335	0.028 208
Akap7	3.183299484	0.003 354	0.028 306
Rnf187	3.127342376	0.003 372	0.028 396
Tenm2	2.800266772	0.003 452	0.028 942
Pcp4	2.984881265	0.003 483	0.029 131
Cntnap2	3.019208688	0.003 495	0.029 171
Nptn	2.858208691	0.003 509	0.029 224
2610002M 06Rik	4.173585519	0.003 646	0.030 296
Cadps	3.083978833	0.003 675	0.030 469
Cux2	2.9458994	0.003 701	0.030 617
Map7d2	3.14248764	0.003 723	0.030 728
Acot7	3.091886093	0.003 759	0.030 961
Jakmip2	3.655569826	0.003 818	0.031 379
Ppfia2	3.673921362	0.003 877	0.031 797
Ank3	2.841888449	0.003 916	0.032 046

Dnajb4	3.831536822	0.004 038	0.032 968
Nap1l2	3.525081989	0.004 092	0.033 341
Dpysl4	3.681758141	0.004 132	0.033 59
Pfdn2	3.459704279	0.004 157	0.033 724
Ncam2	3.863091256	0.004 265	0.034 361
Nxph1	3.455035464	0.004 272	0.034 361
Rgs17	2.89661907	0.004 331	0.034 644
Agtpbp1	3.71474919	0.004 337	0.034 644
Plppr3	3.591491199	0.004 343	0.034 644
Fam171b	3.585513306	0.004 344	0.034 644
Fbxo9	3.281678181	0.004 488	0.035 714
Syngn3	3.184813372	0.004 577	0.036 232
Pfkm	3.643629459	0.004 579	0.036 232
Map6	3.421004561	0.004 582	0.036 232
Prkaa2	4.016659719	0.004 609	0.036 369
Rian	3.454077665	0.004 64	0.036 541
Zfp804a	3.70482842	0.005 026	0.039 495
Bex3	2.788544579	0.005 077	0.039 813
Ndn	3.197894501	0.005 168	0.040 437
Hapln1	2.716739207	0.005 178	0.040 437
Plekhb2	3.139668356	0.005 23	0.040 756
Pnma2	3.816974266	0.005 276	0.040 928
Grina	3.113787179	0.005 285	0.040 928

Gata3	3.233945282	0.005 295	0.040 928
Celsr2	3.226640697	0.005 345	0.041 227
E430014B0 2Rik	3.692958011	0.005 416	0.041 661
Mfn2	3.603415192	0.005 423	0.041 661
Atp2a2	2.771930176	0.005 594	0.042 884
Kcnk3	3.440822629	0.005 78	0.044 222
Nefl	2.750410558	0.005 793	0.044 227
290001100 8Rik	3.22951101	0.005 93	0.045 185
Rdh13	2.925626915	0.005 965	0.045 276
Atat1	3.341745625	0.005 966	0.045 276
Wnk2	3.563329675	0.005 99	0.045 364
Myo16	3.090523841	0.006 049	0.045 716
Khdrbs2	3.640711641	0.006 137	0.046 107
Lin7b	3.567979382	0.006 189	0.046 403
Ank2	2.702691908	0.006 286	0.047 04
Bri3bp	3.488225441	0.006 321	0.047 064
Gm5499	2.904986299	0.006 324	0.047 064
Kndc1	3.51612506	0.006 327	0.047 064
Slc25a4	2.43919167	0.006 369	0.047 287
Lsm3	3.323015952	0.006 387	0.047 322
Gng2	3.307757746	0.006 518	0.048 201
Pantr1	3.391313219	0.006 586	0.048 608

Supplemental Table 4. *Differentially expressed genes in N1 to VAC pairwise comparison.*

Genes differentially expressed in N1 compared to VACs (left) and genes differentially expressed

in VACs compared to N1 (right) with fold change ($\log_2$), p-value and FDR. $\text{FDR} \leq 0.05$ with ≥ 2 -fold change.

Neuron Cluster 2			
Gene Symbol	Fold Change (log2)	P-Value	FDR
Snhg11	4.296183814	2.76E-10	2.04E-07
Meg3	4.614605541	5.71E-10	2.57E-07
Cntnap2	5.330781893	6.95E-10	2.57E-07
Trank1	7.137809498	9.97E-09	2.77E-06
Carmil3	6.203447009	2.89E-08	6.42E-06
Nrxn3	4.467430816	6.44E-08	1.19E-05
Ptprn	6.671859285	9.52E-08	1.56E-05
Syt1	4.106195151	1.03E-07	1.56E-05
Atp2b2	4.616467014	1.05E-07	1.56E-05
Arl4c	5.616414195	1.52E-07	2.11E-05
Gas6	5.944253748	1.76E-07	2.30E-05
Mtftp1	6.162111125	2.17E-07	2.67E-05
Rian	4.690206426	3.61E-07	3.82E-05
Pcdh8	5.352208873	3.62E-07	3.82E-05
Esrrb	6.082598805	3.86E-07	3.90E-05
Vwa5b2	6.55781345	4.26E-07	4.11E-05
Thy1	5.064711435	4.54E-07	4.20E-05
Slc1a1	5.577050598	7.69E-07	6.83E-05
Cep170b	5.750238211	1.21E-06	0.000104
Slitrk1	5.642202936	1.28E-06	0.000104
Clstn3	5.346069362	1.31E-06	0.000104
Gm43175	5.282695594	1.52E-06	0.000116

Astrocyte Cluster 3			
Gene Symbol	Fold Change (log2)	P-Value	FDR
Agt	-8.616940645	2.72E-18	6.04E-15
Tnc	-7.763022092	7.20E-13	7.99E-10
Fgfr3	-6.636264451	6.72E-10	2.57E-07
Plat	-6.676554591	4.54E-09	1.44E-06
Pdpn	-5.919052904	1.62E-08	4.00E-06
Sema6d	-5.932191442	3.72E-08	7.51E-06
Etv5	-5.263966654	3.02E-07	3.53E-05
Notch2	-5.12427709	1.85E-06	0.000126
Al464131	-5.46247433	1.87E-06	0.000126
Sdc4	-5.251815753	3.72E-06	0.000206
Mfge8	-5.185084869	1.29E-05	0.000486
Rhoq	-4.865342082	1.31E-05	0.000486
Hepacam	-4.363714349	8.37E-05	0.002008
Gm44645	-4.080352963	0.000126	0.002637
Gja1	-4.123485288	0.000179	0.003516
Slc1a3	-3.607628439	0.000234	0.004408
Kcnj10	-4.052777241	0.000358	0.0062
Pigs	-4.004984869	0.000361	0.006214
Kcnd2	-4.068507549	0.000373	0.006342
Serpine2	-3.891735927	0.000374	0.006342
Ccnd2	-4.241744878	0.000386	0.006359
Rab31	-4.176730132	0.000645	0.00974

Tmem181b-ps	4.50138138	1.71E-06	0.000126	Slc6a1	-4.027805084	0.000803	0.011876
Rims2	5.425925462	1.83E-06	0.000126	Abca1	-4.085728684	0.000834	0.012181
Slc12a5	5.365993824	2.11E-06	0.000136	Adcyap1r1	-3.99186802	0.000884	0.012579
Bin1	5.943545623	2.14E-06	0.000136	Car2	-3.958617842	0.001203	0.015987
Gm44562	4.95172092	2.97E-06	0.000183	Tpm3	-3.917408866	0.001428	0.017699
Adgrl2	4.415018963	3.10E-06	0.000186	Ctsl	-3.767006238	0.002302	0.026192
Syng3	5.436029676	3.18E-06	0.000186	Aqp4	-3.596211208	0.002838	0.031176
Dnm1	4.705483029	3.53E-06	0.000201	Slc4a4	-3.601313063	0.003528	0.037638
Zfp385b	5.284302618	4.02E-06	0.000217	Cd9	-3.572308681	0.00398	0.041466
Epb41l4b	5.486035173	4.42E-06	0.000234	Ptn	-3.280347554	0.004223	0.043543
Nceh1	5.002682519	5.96E-06	0.000307	Timp4	-3.563344363	0.004493	0.045109
Gprasp2	5.378955686	6.78E-06	0.000342				
9330162G02Rik	3.944030056	7.52E-06	0.000368				
Atp1b1	3.77320927	7.64E-06	0.000368				
Hpcal1	5.931715361	8.23E-06	0.000384				
Gm37363	5.344895763	8.31E-06	0.000384				
Rapgef6	5.187019713	8.65E-06	0.000392				
L1cam	4.824531248	8.83E-06	0.000392				
Miat	4.918860227	1.06E-05	0.000462				
Hapln1	3.997691726	1.16E-05	0.000486				
Cxadr	4.816104935	1.18E-05	0.000486				
Emi5	4.793499839	1.21E-05	0.000486				
Gabrg2	5.018771673	1.22E-05	0.000486				

Reep1	5.139834803	1.25E-05	0.000486
Slc6a5	3.716288331	1.30E-05	0.000486
Abca5	4.690288818	1.31E-05	0.000486
Scn1a	4.129914917	1.35E-05	0.000493
Chd5	4.356690549	1.54E-05	0.000549
Fam189a1	4.510613165	1.83E-05	0.000631
Gm32710	4.027748588	1.85E-05	0.000631
Glr3	4.73779651	1.90E-05	0.000631
Lrfr5	4.61502555	1.90E-05	0.000631
Cntnap1	4.59967245	1.91E-05	0.000631
Srcin1	4.817846311	2.09E-05	0.000683
Afap1	5.229717178	2.31E-05	0.000733
Psd3	4.637977351	2.34E-05	0.000733
Kcnc1	4.715440023	2.35E-05	0.000733
Adarb2	5.264267925	2.66E-05	0.00082
App	3.914287104	2.90E-05	0.00088
Epb41l1	5.041756348	3.65E-05	0.001095
Nsf	4.106813737	3.82E-05	0.001131
Ppp2r2c	4.70768816	3.93E-05	0.001148
Crmp1	4.155205884	4.06E-05	0.001169
Kcnh7	4.271697048	4.11E-05	0.001171
Rgs8	4.616219355	4.32E-05	0.001213
Stum	4.962186064	4.64E-05	0.001287

Sacs	4.435937992	4.75E-05	0.001301
Syt2	4.068125242	5.06E-05	0.001369
Setd5	4.949030697	5.21E-05	0.001392
Unc13a	4.081026289	6.89E-05	0.001798
Ppfia4	4.911430116	6.89E-05	0.001798
C230085N15Rik	4.474176655	7.04E-05	0.001815
Tln2	6.431577575	7.38E-05	0.001882
Kcnk3	4.432882615	7.58E-05	0.001893
Tubb3	4.257285716	7.67E-05	0.001893
B3galt2	4.000592208	7.68E-05	0.001893
Tro	4.167014443	7.79E-05	0.0019
Kcnb2	4.041867996	8.42E-05	0.002008
Clmp	4.497415904	8.63E-05	0.002038
Cadm3	4.344175866	9.05E-05	0.002113
Arhgef7	4.471642434	9.17E-05	0.002121
Strbp	3.6546092	9.45E-05	0.002132
A230103L15Rik	4.073626929	9.48E-05	0.002132
C230071H17Rik	4.444051535	9.51E-05	0.002132
Smpd3	4.316834349	0.000101	0.002237
Gm44830	4.280612796	0.000107	0.002338
Dlat	5.294448842	0.000107	0.002338
Sox1ot	4.671719367	0.000113	0.002438
Cacna1e	3.988951814	0.000117	0.002493

Sgpp2	4.072337877	0.000 124	0.002 63
Hspa12a	4.146870191	0.000 153	0.003 164
Ankrd45	4.436503812	0.000 156	0.003 211
Cacna1a	3.971303255	0.000 162	0.003 289
Grb2	4.440295762	0.000 165	0.003 319
Cacnb4	4.276941472	0.000 167	0.003 341
Lhfpl4	4.48387796	0.000 179	0.003 516
Kcnq1ot1	3.724932833	0.000 2	0.003 901
Napg	3.950561374	0.000 222	0.004 265
Kcna1	4.12729677	0.000 223	0.004 265
Calb1	4.538323326	0.000 229	0.004 342
Tenm4	4.166108451	0.000 247	0.004 615
Dip2c	4.469160425	0.000 259	0.004 786
Tcaf1	4.046462228	0.000 275	0.005 048
Sez6l2	4.230365808	0.000 324	0.005 887
Hs6st2	3.948520609	0.000 34	0.006 083
C2cd5	4.746818066	0.000 34	0.006 083
Zc2hc1a	4.511777949	0.000 345	0.006 087
1500004A1 3Rik	4.214297537	0.000 346	0.006 087
Rbfox3	4.131493469	0.000 349	0.006 093
Ecpas	4.911117243	0.000 378	0.006 359
Bsn	4.632298609	0.000 384	0.006 359
Atp6ap2	4.158967429	0.000 387	0.006 359

Stac2	4.454830083	0.000 407	0.006 633
Scrt2	3.693961217	0.000 41	0.006 633
Gm42616	3.880159855	0.000 415	0.006 668
Gdap1	4.28872407	0.000 477	0.007 61
Tshz2	3.619133407	0.000 511	0.008 093
Pclo	3.589113426	0.000 521	0.008 205
Ube3a	3.302460061	0.000 585	0.009 145
Tspyl5	4.195204381	0.000 614	0.009 526
Pygo1	4.42277363	0.000 625	0.009 635
Trpc3	3.926982201	0.000 634	0.009 699
Mdga2	4.057597575	0.000 638	0.009 699
Cds1	4.161359908	0.000 688	0.010 319
Slitrk4	4.1676028	0.000 775	0.011 537
Ralgapa1	3.757314222	0.000 834	0.012 181
Kcnq2	4.440110643	0.000 844	0.012 248
Acsl4	3.776919454	0.000 877	0.012 579
Tfrc	3.838815585	0.000 882	0.012 579
Lpgat1	3.843068344	0.000 913	0.012 901
Rnf152	4.065980641	0.000 942	0.013 236
Syngap1	3.266976477	0.001 015	0.014 154
Tmem130	3.76837414	0.001 027	0.014 154
Tmem181a	3.696828751	0.001 027	0.014 154
Efr3b	3.944242819	0.001 046	0.014 334

Got2	4.057541232	0.001 111	0.015 122
Kcnn2	3.78053214	0.001 149	0.015 547
Map6	3.808964793	0.001 167	0.015 694
5730409E0 4Rik	4.064847481	0.001 178	0.015 752
Plppr4	4.077384581	0.001 212	0.016 014
Hspg2	3.608806173	0.001 238	0.016 197
Rcan2	3.556852026	0.001 241	0.016 197
Lynx1	3.915878785	0.001 279	0.016 531
Igsf3	3.474596642	0.001 281	0.016 531
Rab11fip4	3.848720831	0.001 328	0.016 984
Zfpm2	3.930093345	0.001 332	0.016 984
Gria1	3.691824542	0.001 341	0.016 999
Prkce	3.697364175	0.001 391	0.017 533
Celsr2	3.754984347	0.001 401	0.017 569
Map7d2	3.747218274	0.001 427	0.017 699
Myh10	3.550773187	0.001 465	0.018 06
Madd	3.575156936	0.001 609	0.019 73
Cntn5	3.277749307	0.001 685	0.020 542
B830012L1 4Rik	3.891809123	0.001 748	0.021 126
Grin2a	3.840565824	0.001 752	0.021 126
Eml4	3.897756704	0.001 77	0.021 233
Cdk5r1	3.683019491	0.001 783	0.021 266
Ablim1	3.735945376	0.001 799	0.021 266

Map9	3.579672516	0.001 802	0.021 266
Edil3	3.731363442	0.001 829	0.021 472
Grin1	3.735747159	0.001 841	0.021 498
Gm37899	3.714984052	0.002 075	0.024 101
Disp2	3.480078818	0.002 112	0.024 411
Atp11b	3.805692611	0.002 256	0.025 935
Arf3	3.591675775	0.002 297	0.026 192
Snhg14	3.094100999	0.002 376	0.026 904
Slc38a1	3.337029998	0.002 447	0.027 558
Gnai1	3.76332076	0.002 614	0.029 292
Col11a1	3.665267043	0.002 733	0.030 478
Ap1p1	3.740191974	0.002 787	0.030 926
Eno2	3.410980962	0.002 838	0.031 176
Hcn1	3.385493607	0.002 859	0.031 255
Unc80	3.702644897	0.003 297	0.035 745
Ttn	3.714587733	0.003 302	0.035 745
Slc4a3	3.472853455	0.003 361	0.036 207
Mroh1	3.701315072	0.003 514	0.037 638
Mylk	3.737410365	0.003 762	0.039 904
Rb1cc1	3.416669418	0.003 776	0.039 904
Ddx1	3.94246273	0.003 888	0.040 883
Gramd1b	3.657877775	0.003 948	0.041 319
Napb	3.32184016	0.004 185	0.043 396

Gls	3.379587723	0.004 246	0.043 543
Me3	3.377138275	0.004 258	0.043 543
Phf24	3.500917755	0.004 413	0.044 924
B4galt6	3.447083014	0.004 464	0.045 047
Snap91	3.153018775	0.004 466	0.045 047
Nav2	3.274110895	0.004 616	0.046 138
Stmn4	3.255589011	0.004 892	0.048 68
Crtac1	3.301240083	0.004 929	0.048 827

Supplemental Table 5. Differentially expressed genes in N2 to Astrocyte pairwise

comparison. Genes differentially expressed in N2 compared to Astrocytes (left) and genes differentially expressed in Astrocytes compared to N2 (right) with fold change ($\log_2$), p-value and FDR. FDR ≤ 0.05 with ≥ 2 -fold change.

Neuron Cluster 2			
Gene Symbol	Fold Change (log2)	P-Value	FDR
Kifc2	4.491280047	0.000138	0.01649
Esrrb	4.467129523	0.000298	0.033921

Oligodendrocyte Cluster 4			
Gene Symbol	Fold Change (log2)	P-Value	FDR
Olig2	-6.034225524	4.82E-10	1.10E-06
Tnr	-6.335006345	4.76E-09	5.42E-06
Sox6	-5.891205846	8.77E-09	6.66E-06
Sema5a	-5.928749626	5.85E-08	3.33E-05
Ugt8a	-6.274771093	1.48E-07	5.92E-05
Bcas1	-6.026996289	1.56E-07	5.92E-05
Gpr17	-5.943571788	3.61E-07	0.000117
Ddr1	-6.021937222	6.98E-07	0.000199
Ece1	-5.334902076	8.23E-07	0.000208
Samd4b	-5.047214633	1.08E-06	0.000246
Shisa1	-5.130281799	2.48E-05	0.004945
Sc5d	-4.790692622	2.61E-05	0.004945
Tulp4	-4.850238537	3.28E-05	0.005704
Sox10	-4.855592664	3.51E-05	0.005704
Cnp	-4.826352381	4.11E-05	0.006239
Gmfb	-4.895692071	4.61E-05	0.006558
Zeb2	-4.665976656	5.25E-05	0.007027
Mbp	-4.934406131	6.86E-05	0.008682
Cyfp2	-4.026816872	0.000339	0.036707

Supplemental Table 6. Differentially expressed genes in N2 to Oligodendrocyte pairwise comparison. Genes differentially expressed in N2 compared to Oligodendrocytes (left) and

genes differentially expressed in Oligodendrocytes compared to N2 (right) with fold change ($\log_2$), p-value and FDR. FDR ≤ 0.05 with ≥ 2 -fold change.

Neuron Cluster 2			
Gene Symbol	Fold Change (log2)	P-Value	FDR
Scn8a	6.142686992	3.87E-06	9.66E-05
Adap1	6.434754267	2.73E-05	0.000633
Miat	5.946934841	4.11E-05	0.00093
Cep170b	6.424328933	4.58E-05	0.001019
Snhg11	3.924897203	5.98E-05	0.001302
Zdbf2	6.267130365	7.00E-05	0.001512
Epb41l4b	6.77729255	7.11E-05	0.001525
Srcin1	5.55003283	8.88E-05	0.001889
C230085N15Rik	5.858057947	0.000119	0.002508
Chga	5.981806462	0.000182	0.003742
Grik3	6.341046227	0.000215	0.004381
Apba2	5.367299329	0.000256	0.005183
Cds1	5.601258752	0.000263	0.005278
Snhg14	3.918505057	0.000321	0.006365
Sez6l	4.929580473	0.000395	0.007716
Gabra5	4.973545127	0.000425	0.008195
Srgap3	4.546206283	0.000449	0.008548
Hcn1	4.419909343	0.000481	0.009092
Ptprn	6.011492439	0.00051	0.009584
Kcna2	4.538404645	0.00054	0.010066
Hs3st5	5.346620146	0.000543	0.010066
Car10	4.493309111	0.000559	0.010308

Vascular Associated Cell Cluster 5			
Gene Symbol	Fold Change (log2)	P-Value	FDR
Ctla2a	-10.57378817	4.35E-24	1.26E-20
Cldn5	-9.8661296	2.26E-23	3.27E-20
Lgals9	-9.711773512	2.24E-22	2.16E-19
Cd34	-9.806773865	1.66E-21	1.20E-18
Fzd6	-8.244316284	2.29E-19	9.65E-17
Tdrp	-8.997420303	2.31E-19	9.65E-17
Eng	-8.42405665	2.33E-19	9.65E-17
Gng11	-8.215535301	2.77E-19	1.00E-16
Pltp	-9.537188816	4.64E-19	1.49E-16
Ecscr	-8.95114337	5.20E-19	1.51E-16
Emcn	-9.682827111	5.90E-19	1.55E-16
Grap	-9.264423738	8.57E-19	2.07E-16
Adgrl4	-9.411261686	1.04E-18	2.24E-16
Srgn	-8.967906593	1.14E-18	2.24E-16
Calcr1	-9.066562669	1.16E-18	2.24E-16
Icam2	-7.935294731	1.65E-18	2.99E-16
Ifitm3	-8.348399583	6.12E-18	1.04E-15
Adgrf5	-8.934754292	7.12E-18	1.14E-15
Mfap2	-8.304527874	1.81E-17	2.76E-15
Acvrl1	-8.763942558	3.26E-17	4.72E-15
Prcp	-9.469527387	3.51E-17	4.83E-15
Slc39a8	-9.536598548	5.27E-17	6.93E-15

Ryr2	4.790165333	0.000 586	0.010 729	Il2rg	-8.189492669	5.75E- 17	7.24E- 15
Slc6a5	3.499370947	0.000 677	0.012 192	Foxf2	-8.002332075	1.89E- 16	2.28E- 14
Inpp5f	5.014625333	0.000 678	0.012 192	BC028528	-8.864696949	5.30E- 16	6.14E- 14
Slc22a17	4.414150302	0.000 736	0.013 041	Tgfbr2	-8.398016441	6.12E- 16	6.81E- 14
Agap2	4.735665719	0.000 739	0.013 041	Cdh5	-7.748572728	7.27E- 16	7.79E- 14
Grin2a	4.820795044	0.000 8	0.013 766	Tek	-8.745907884	8.26E- 16	8.54E- 14
Chd5	4.443572694	0.000 887	0.014 879	Fxyd5	-7.924408902	1.07E- 15	1.05E- 13
Meis2	4.027300846	0.000 894	0.014 879	Arhgap29	-8.040006217	1.11E- 15	1.05E- 13
Atp2a2	3.998087247	0.000 896	0.014 879	Cavin1	-8.706653965	1.13E- 15	1.05E- 13
Usp29	5.35931338	0.000 9	0.014 879	Pglyrp1	-8.452082198	1.36E- 15	1.23E- 13
Lrfn5	4.648679855	0.001 072	0.017 243	Pecam1	-8.059888777	2.36E- 15	2.07E- 13
Stmn3	3.67612084	0.001 164	0.018 428	Lama4	-8.329790761	2.89E- 15	2.39E- 13
9330162G0 2Rik	3.665667528	0.001 165	0.018 428	Ostf1	-7.603224631	2.89E- 15	2.39E- 13
Meg3	3.542588037	0.001 181	0.018 573	Abcb1a	-7.644833605	4.94E- 15	3.97E- 13
Ank2	3.617062123	0.001 216	0.018 927	Prex2	-7.794700052	6.15E- 15	4.81E- 13
Syngap1	4.024444632	0.001 235	0.019 106	Kdr	-8.455245067	6.75E- 15	5.14E- 13
Fam49b	5.149590483	0.001 32	0.020 1	Slc38a5	-8.946758807	9.19E- 15	6.82E- 13
Gm44830	4.585096414	0.001 327	0.020 1	Anxa2	-8.247660317	1.55E- 14	1.12E- 12
Scn1a	3.974709617	0.001 402	0.021 018	Cgnl1	-8.036703026	1.90E- 14	1.34E- 12
Mdga2	4.64961017	0.001 442	0.021 518	Podxl	-7.71422476	3.44E- 14	2.37E- 12
Satb1	3.578669203	0.001 672	0.024 556	Adgre5	-7.894376285	4.38E- 14	2.95E- 12
Fam81a	5.288131241	0.001 687	0.024 655	Arap3	-6.702291379	4.78E- 14	3.15E- 12
Gm32710	3.947172688	0.001 727	0.025 11	Lef1	-7.316107718	5.74E- 14	3.69E- 12

Gabra3	4.96233285	0.001 752	0.025 349	Cd93	-8.616637098	6.18E- 14	3.89E- 12
B4galt6	4.295446261	0.001 79	0.025 774	Robo4	-7.239142544	7.71E- 14	4.75E- 12
5730409E0 4Rik	4.876586004	0.001 804	0.025 843	Fli1	-8.260288812	1.18E- 13	7.11E- 12
Gm37363	4.966757437	0.001 832	0.026 111	Tram2	-7.901453022	1.22E- 13	7.19E- 12
Dscam	4.797209026	0.001 908	0.027 071	Heg1	-8.0540311	1.32E- 13	7.53E- 12
Dynll2	4.271007625	0.001 951	0.027 469	Myo1b	-7.758809958	1.33E- 13	7.53E- 12
Rims2	4.763047322	0.001 955	0.027 469	Apcdd1	-6.663709674	1.76E- 13	9.82E- 12
Fam189a1	4.171270388	0.002 087	0.029 065	Jcad	-8.140482877	2.15E- 13	1.17E- 11
Esrrb	4.664138271	0.002 089	0.029 065	Nr3c1	-8.049318253	2.52E- 13	1.35E- 11
Dzip1	4.040529422	0.002 159	0.029 891	Nes	-7.291812847	2.61E- 13	1.37E- 11
Celsr2	4.31727838	0.002 263	0.030 605	Ucp2	-7.705875077	7.01E- 13	3.62E- 11
Tnik	4.244843863	0.002 268	0.030 605	Fn1	-8.148068522	7.58E- 13	3.85E- 11
Bcl7a	5.388917393	0.002 282	0.030 605	Dap	-6.753789311	7.99E- 13	3.99E- 11
Gramd1b	4.721952051	0.002 292	0.030 605	Gbp7	-8.068697081	9.28E- 13	4.55E- 11
Slc1a1	4.560240275	0.002 295	0.030 605	Slco2b1	-7.124058233	1.22E- 12	5.83E- 11
Col11a1	4.507094464	0.002 331	0.030 941	Arhgdib	-7.139014232	1.23E- 12	5.83E- 11
9330159F1 9Rik	4.717395341	0.002 401	0.031 213	Uaca	-6.193290716	1.45E- 12	6.78E- 11
Eml5	4.250794175	0.002 405	0.031 213	Esam	-8.057165993	1.55E- 12	7.13E- 11
Rbfox3	4.267441525	0.002 439	0.031 508	Nid1	-8.273288405	1.75E- 12	7.92E- 11
Lhfpl4	4.438544413	0.002 486	0.031 973	Slc40a1	-8.292777787	1.84E- 12	8.18E- 11
Igsf3	3.793828382	0.002 669	0.033 878	Ctsc	-5.860677599	3.06E- 12	1.34E- 10
Cdk16	4.910836819	0.002 783	0.035 013	S100a11	-6.292873581	9.61E- 12	4.15E- 10
Gm42616	4.238015787	0.002 801	0.035 041	AU021092	-7.15855195	1.28E- 11	5.43E- 10

Shtn1	4.668880654	0.002 817	0.035 041	Gimap6	-7.065247872	3.09E- 11	1.30E- 09
Ttc3	3.148439176	0.002 821	0.035 041	Lamc1	-6.486262748	3.88E- 11	1.60E- 09
Abcc5	3.928437066	0.002 834	0.035 043	Rras	-5.281438314	6.70E- 11	2.73E- 09
Slc2a3	4.68229204	0.003 016	0.037 145	Mxd4	-7.110190982	9.81E- 11	3.94E- 09
Npdc1	4.480001218	0.003 04	0.037 197	Vwf	-6.543737149	1.59E- 10	6.32E- 09
Ptprs	2.998721054	0.003 046	0.037 197	Adamtsl2	-7.503258825	1.69E- 10	6.61E- 09
Stum	4.663575206	0.003 43	0.041 532	Htra3	-6.198657911	1.83E- 10	7.06E- 09
Unc13a	3.887076626	0.003 46	0.041 723	Clic1	-6.591251356	1.86E- 10	7.07E- 09
Ncam1	3.330731153	0.003 665	0.043 83	Eogt	-6.84816556	2.09E- 10	7.86E- 09
Cux2	3.874962261	0.003 665	0.043 83	Mecom	-7.214702906	2.13E- 10	7.90E- 09
Sv2c	3.483917911	0.003 738	0.044 514	Rasgrp3	-7.064888778	4.24E- 10	1.55E- 08
Shank2	4.08847039	0.003 763	0.044 63	Ets1	-7.417457446	4.31E- 10	1.56E- 08
Thy1	3.9814652	0.003 81	0.045 01	Ptprb	-7.248746794	5.35E- 10	1.91E- 08
Gprasp1	3.906441505	0.003 855	0.045 303	Itm2a	-7.541019288	9.23E- 10	3.26E- 08
Lrrc4b	3.74733634	0.003 867	0.045 303	Jup	-6.649105366	1.49E- 09	5.20E- 08
Apba1	4.100470553	0.004 13	0.048 193	Slc16a1	-6.932348282	1.81E- 09	6.23E- 08
Strbp	3.42108325	0.004 229	0.049 151	Tpm4	-6.862865863	3.27E- 09	1.11E- 07
				Igfbp7	-5.904709933	3.99E- 09	1.34E- 07
				Clic4	-6.114571436	4.52E- 09	1.50E- 07
				Myl12a	-6.414436867	5.98E- 09	1.97E- 07
				Ly6c1	-7.316184884	6.19E- 09	2.01E- 07
				Tmem204	-6.489558853	9.86E- 09	3.17E- 07
				Serpinh1	-5.956308325	1.15E- 08	3.66E- 07

Cdk6	-5.896371854	1.23E-08	3.87E-07
Ece1	-6.203851267	1.26E-08	3.91E-07
Flt1	-6.825427148	1.59E-08	4.89E-07
Ccdc141	-6.235865611	1.88E-08	5.74E-07
Hbp1	-6.019383436	2.33E-08	7.03E-07
Anxa3	-6.718626289	3.07E-08	9.15E-07
Dab2	-6.061296321	4.01E-08	1.18E-06
Lbr	-6.227506769	4.62E-08	1.35E-06
Pdgfrb	-6.788198757	5.41E-08	1.57E-06
Nostrin	-5.042129509	9.91E-08	2.84E-06
Rhoj	-5.581556739	1.34E-07	3.79E-06
Amotl1	-5.760702634	1.83E-07	5.10E-06
Slc22a8	-6.790631726	1.83E-07	5.10E-06
Slc2a1	-5.930369967	8.59E-07	2.37E-05
Arpc1b	-4.95189422	1.43E-06	3.91E-05
Tspan14	-5.705111235	1.47E-06	3.96E-05
Zfp36l1	-5.113935441	1.75E-06	4.65E-05
Plat	-5.730473123	1.75E-06	4.65E-05
Ocln	-5.994944407	2.00E-06	5.25E-05
Mcc	-5.344014515	2.07E-06	5.40E-05
Gjc1	-5.425284893	2.12E-06	5.47E-05
Cyyr1	-5.636693885	2.19E-06	5.60E-05
Jag1	-5.759197285	2.86E-06	7.25E-05

Zfhx3	-5.435950303	3.75E-06	9.44E-05
Mkl2	-5.568271684	6.38E-06	0.000158
Trp53	-5.275682421	6.84E-06	0.000168
Sypl	-5.251484669	6.93E-06	0.000169
Egfl7	-5.69221471	9.19E-06	0.000222
Rgs5	-5.985991311	1.09E-05	0.00026
Cald1	-5.234515234	2.12E-05	0.000503
Fcho2	-5.109692613	2.24E-05	0.000528
Tpp1	-5.221547443	2.32E-05	0.000541
C130074G19Rik	-4.698592817	2.81E-05	0.000645
Palmd	-4.957520747	3.50E-05	0.000798
Lpp	-4.820802798	4.49E-05	0.001008
Ushbp1	-4.730698235	5.35E-05	0.001183
Map3k20	-4.881252443	5.43E-05	0.001191
Abca1	-4.87322271	0.000124	0.002601
Slco1c1	-5.320473055	0.000141	0.002937
Slc38a2	-4.551621987	0.000162	0.003342
Rest	-4.531733825	0.000312	0.006231
Becn1	-4.427138803	0.000365	0.007177
Dram2	-4.636571937	0.000397	0.007716
Ttyh2	-4.779623522	0.000438	0.008393
S1pr1	-4.56518392	0.000641	0.011675
Pkig	-4.398164511	0.000689	0.012302

Nampt	-4.596339761	0.000 752	0.013 184
Col4a2	-4.307198595	0.000 783	0.013 652
Bcap31	-4.212400918	0.000 805	0.013 766
Abcg2	-4.511886641	0.000 807	0.013 766
Epas1	-4.640828818	0.000 809	0.013 766
Lrp8	-4.341427431	0.000 848	0.014 352
Hmgcs2	-4.800466996	0.000 929	0.015 279
Ifngr1	-3.892190759	0.000 991	0.016 208
Jam2	-4.459850422	0.001 026	0.016 68
Sparc	-4.214592643	0.001 043	0.016 861
Slc7a5	-4.143944372	0.001 154	0.018 428
Ppm1f	-4.266628946	0.001 201	0.018 79
Hes1	-3.583735856	0.001 284	0.019 671
Bsg	-3.594659098	0.001 285	0.019 671
Slc46a3	-4.267006703	0.001 389	0.020 931
Ginm1	-4.196299876	0.001 473	0.021 858
Ctnna1	-4.251068406	0.001 495	0.022 075
Lims1	-4.106349506	0.002 169	0.029 891
Tagln2	-4.481767564	0.002 251	0.030 605
Stx6	-4.136873509	0.002 295	0.030 605
Tmem98	-3.87152468	0.002 363	0.031 107
Sema6d	-4.013919804	0.002 365	0.031 107
Rgs12	-4.232160098	0.002 381	0.031 178

4931406P1 6Rik	-3.750383388	0.002 594	0.033 222
Arl4a	-4.11180888	0.002 611	0.033 293
Cnn2	-4.32379606	0.002 707	0.034 206
Ptprg	-4.142774165	0.003 343	0.040 648

Supplemental Table 7. Differentially expressed genes in N2 to VAC pairwise comparison.

Genes differentially expressed in N2 compared to VACs (left) and genes differentially expressed in VACs compared to N2 (right) with fold change ($\log_2$), p-value and FDR. $\text{FDR} \leq 0.05$ with ≥ 2 -fold change.

Astrocyte Cluster 3				Oligodendrocyte Cluster 4			
Gene Symbol	Fold Change (log2)	P-Value	FDR	Gene Symbol	Fold Change (log2)	P-Value	FDR
Slc25a18	8.287821184	4.09E-09	9.08E-06	Ugt8a	-7.059987084	2.60E-07	0.000288
Lxn	4.619117351	0.00023	0.033498	Sema5a	-5.855173895	2.01E-06	0.001488
Mt3	3.905139266	0.000296	0.036183	Cdk5	-5.015258375	7.02E-06	0.003897
Aldh5a1	4.571635139	0.00031	0.036183	Slc1a1	-4.777736985	1.24E-05	0.00474
				Sobp	-6.027377806	1.28E-05	0.00474
				Dscam	-5.198965678	2.43E-05	0.006894
				Bcas1	-5.44092796	2.48E-05	0.006894
				Zcrb1	-5.17787517	7.28E-05	0.017969
				Zzef1	-4.8744469	9.18E-05	0.019554
				Pdhx	-4.846335236	9.68E-05	0.019554
				Pcmt1d1	-5.018988185	0.000145	0.026731
				C230071H17Rik	-4.595634766	0.000156	0.026731
				Pik3r3	-4.431013441	0.000178	0.028285
				Psd3	-4.246255268	0.000241	0.033498
				Rgs8	-4.802368261	0.000259	0.03379

Supplemental Table 8. *Differentially expressed genes in Astrocyte to Oligodendrocyte pairwise comparison.* Genes differentially expressed in Astrocytes compared to Oligodendrocytes (left) and genes differentially expressed in Oligodendrocytes compared to Astrocytes (right) with fold change (log₂), p-value and FDR. FDR ≤ 0.05 with ≥ 2-fold change.

Astrocyte Cluster 3			
Gene Symbol	Fold Change (log2)	P-Value	FDR
Ncan	4.897178594	6.40E-09	2.50E-07
Fgfr3	9.488545206	2.52E-08	9.09E-07
Aqp4	6.092944575	4.58E-08	1.61E-06
Fabp7	4.388455865	8.56E-08	2.87E-06
Slc6a11	6.487666615	4.94E-07	1.53E-05
Adcyap1r1	7.619056806	6.98E-07	2.04E-05
Acot1	8.876797529	9.29E-07	2.61E-05
Al464131	9.089142446	1.45E-06	3.92E-05
Igsf11	7.6226321	2.83E-06	7.31E-05
Pla2g7	5.556318141	3.32E-06	8.49E-05
Tnc	8.446662373	5.20E-06	0.00013
Sall2	8.099784274	5.88E-06	0.000144
Slc1a3	4.39540078	6.60E-06	0.00016
Agt	7.507052143	1.24E-05	0.000293
Spry2	4.172037706	1.92E-05	0.000449
Etv5	7.074663405	2.05E-05	0.000475
Sall3	7.203242969	2.53E-05	0.000583
Bcan	6.117270864	3.96E-05	0.000884
Grin3a	7.397132991	6.39E-05	0.001341
Opcml	6.879197683	8.13E-05	0.00162
Pou3f3	6.725856385	8.99E-05	0.00178
Mmd2	4.447137093	9.36E-05	0.001828

Vascular Associated Cell Cluster 5			
Gene Symbol	Fold Change (log2)	P-Value	FDR
Fn1	-10.42591866	1.39E-17	3.91E-14
Cldn5	-10.29357399	3.65E-17	5.13E-14
Ctla2a	-10.3528197	4.33E-16	4.06E-13
Mfsd2a	-10.11711508	7.01E-15	3.83E-12
Slc38a5	-10.41572661	7.17E-15	3.83E-12
Ly6c1	-10.84362277	8.18E-15	3.83E-12
Emcn	-10.37234906	1.91E-14	7.69E-12
Lmo2	-9.023763526	2.44E-14	8.57E-12
Igfbp7	-8.04577265	3.01E-14	9.42E-12
Fzd6	-8.363654264	4.67E-14	1.31E-11
Fxyd5	-8.774512294	9.51E-14	2.43E-11
Eng	-8.740518957	1.20E-13	2.82E-11
Tek	-9.873238206	1.55E-13	3.34E-11
Cgnl1	-9.312568842	1.88E-13	3.75E-11
Slc40a1	-9.418227694	2.00E-13	3.75E-11
Icam2	-8.336453632	2.25E-13	3.95E-11
Fli1	-9.351483613	2.42E-13	4.00E-11
Esam	-9.462698284	4.60E-13	7.18E-11
Tdrp	-9.183275212	5.41E-13	8.01E-11
Ecsr	-9.227655816	5.92E-13	8.01E-11
Ocln	-9.772316413	6.22E-13	8.01E-11
Srgn	-9.363795178	6.27E-13	8.01E-11

Adgrg1	5.523611334	9.47E-05	0.001836	Flt1	-8.988366108	8.96E-13	1.10E-10
Ednrb	4.071980401	1.00E-04	0.001912	Pltp	-9.776055058	1.06E-12	1.25E-10
Aldh1l1	6.465222869	0.000103	0.001952	Cnn2	-9.401182457	1.12E-12	1.26E-10
Scd2	3.720700601	0.000107	0.002018	Cdh5	-8.375100521	1.45E-12	1.57E-10
Meis1	7.479207026	0.000108	0.002033	Robo4	-8.134254598	2.41E-12	2.51E-10
Apoe	3.640574844	0.000119	0.002221	Ifitm3	-8.618905567	2.90E-12	2.91E-10
Pantr1	5.929218122	0.00014	0.00255	Myo1b	-8.750887753	4.12E-12	3.99E-10
Plpp3	4.246236339	0.00017	0.00306	Vwa1	-8.705771208	4.56E-12	4.28E-10
Npas3	5.066325459	0.00018	0.003226	Pglyrp1	-8.724006154	4.88E-12	4.42E-10
Tmem229a	7.091725158	0.000183	0.003253	Rgs5	-9.551535488	6.34E-12	5.57E-10
Ckb	3.871924813	0.000188	0.003281	Uaca	-6.865259684	8.20E-12	6.99E-10
Vcan	6.247885987	0.000188	0.003281	Il2rg	-8.574462748	9.06E-12	7.28E-10
Ptprz1	4.505619976	0.000218	0.003754	Grap	-9.21331714	9.26E-12	7.28E-10
Gm3764	5.187886534	0.000251	0.004231	Ly6e	-8.350759476	9.32E-12	7.28E-10
Ptn	3.492515059	0.000256	0.004277	Acvrl1	-9.149022509	1.27E-11	9.67E-10
Ttyh1	4.419885776	0.000273	0.004512	Gimap6	-8.713867904	1.57E-11	1.16E-09
Gpm6b	4.153463624	0.000314	0.005131	Tfrc	-7.11327573	1.81E-11	1.30E-09
Trim9	5.18586828	0.00032	0.005199	Pecam1	-8.21740041	1.91E-11	1.34E-09
Acsbg1	5.507415068	0.000345	0.005547	Adgre5	-8.514696869	2.31E-11	1.59E-09
Sez6l	5.189250748	0.000361	0.00573	Abcb1a	-8.101828992	2.61E-11	1.75E-09
Slc4a4	4.692147787	0.000393	0.006209	Tgfbr2	-8.977932311	2.84E-11	1.85E-09
Grm5	5.081149929	0.000467	0.007218	Arhgdib	-8.084486754	3.95E-11	2.52E-09
Hipk2	5.852086372	0.000489	0.007475	Adgrf5	-8.609072479	4.37E-11	2.73E-09

Clu	5.254597988	0.000 506	0.007 689	Arap3	-7.25386953	4.82E- 11	2.95E- 09
Hmgcs1	4.374104062	0.000 57	0.008 565	C130074G 19Rik	-7.093260647	5.07E- 11	3.03E- 09
Hepacam	4.621699995	0.000 589	0.008 763	Kdr	-8.114546577	5.69E- 11	3.33E- 09
Dbi	3.324705877	0.000 628	0.009 216	AU021092	-8.029148678	5.94E- 11	3.41E- 09
Sox9	4.207763668	0.000 629	0.009 216	Igfbp4	-6.327190962	6.52E- 11	3.67E- 09
Fabp5	3.625140448	0.000 67	0.009 66	Adgrl4	-8.529327072	6.88E- 11	3.79E- 09
Slc25a18	5.938086541	0.000 704	0.010 103	Thsd1	-7.259844405	7.46E- 11	4.03E- 09
Ctnnd2	4.009217739	0.000 708	0.010 104	Slc9a3r2	-7.179667906	9.37E- 11	4.97E- 09
Mt3	3.825154556	0.000 748	0.010 63	Ptprb	-8.104442949	1.04E- 10	5.39E- 09
Grm3	5.0603867	0.000 868	0.012 2	Vwf	-7.764132537	1.09E- 10	5.55E- 09
Apba2	5.30180365	0.001 016	0.014 068	Cd34	-8.392865138	1.11E- 10	5.55E- 09
Aldoc	4.279701056	0.001 054	0.014 452	Isyna1	-7.336388303	1.92E- 10	9.49E- 09
Mt1	3.412097778	0.001 324	0.017 819	Slc46a3	-7.654295799	2.00E- 10	9.69E- 09
Rhoq	5.180514087	0.001 498	0.019 966	Palmd	-7.292309471	3.15E- 10	1.50E- 08
Slitrk2	5.338003444	0.001 515	0.020 093	Mkl2	-8.258745287	3.66E- 10	1.72E- 08
Lrp1	4.983809552	0.001 562	0.020 623	Hmgcs2	-8.646555398	4.54E- 10	2.09E- 08
Cpe	3.418139099	0.001 588	0.020 77	Anxa3	-8.495026955	7.76E- 10	3.52E- 08
Mtss1l	4.715290781	0.001 727	0.022 383	Ushbp1	-6.99480637	1.00E- 09	4.47E- 08
Carmil1	5.593194493	0.001 823	0.023 52	Slc22a8	-8.724600697	1.19E- 09	5.23E- 08
Kcnd2	5.073858683	0.001 937	0.024 871	Tmem204	-7.812529098	1.30E- 09	5.63E- 08
Pdpm	4.814736543	0.002 156	0.027 188	Gjc1	-6.96739573	3.21E- 09	1.37E- 07
Cmip	5.134700339	0.002 455	0.030 545	Cd93	-7.689818601	3.33E- 09	1.40E- 07
Slc25a5	3.02405866	0.002 638	0.032 399	Adamtsl2	-8.359059424	3.40E- 09	1.40E- 07

Gm44645	4.634287964	0.002 758	0.033 576
Asrgl1	4.913011621	0.002 794	0.033 87
Zdhhc2	4.492423444	0.002 816	0.033 98
Sema4b	4.055823275	0.002 9	0.034 696
Ntm	3.94686962	0.002 984	0.035 559
Ddhd1	5.032631152	0.003 364	0.039 915
Bmpr1a	4.883155632	0.003 601	0.042 367
Astn1	4.58611897	0.003 644	0.042 698
Ndrg2	4.031977047	0.003 668	0.042 803
Nrxn1	3.697200482	0.003 87	0.044 605
Pygb	4.369160754	0.003 934	0.045 049
Sesn3	4.580224833	0.004 081	0.046 27
Spred1	4.394519338	0.004 135	0.046 54
Ncam1	3.490865661	0.004 301	0.048 144
Tril	4.619697187	0.004 335	0.048 144

Amotl1	-7.232093917	3.96E- 09	1.61E- 07
Pdgfrb	-8.780415837	4.79E- 09	1.93E- 07
Nampt	-8.111396846	4.98E- 09	1.97E- 07
Tes	-6.904169573	8.85E- 09	3.41E- 07
Itm2a	-7.159125934	9.42E- 09	3.58E- 07
Htra3	-6.593077876	1.49E- 08	5.60E- 07
Cyyr1	-6.875610619	1.75E- 08	6.48E- 07
Akap12	-6.498188037	1.88E- 08	6.87E- 07
Myl12a	-7.056657459	2.72E- 08	9.67E- 07
Jcad	-7.001589362	5.31E- 08	1.84E- 06
Rasgrp3	-7.135290329	5.57E- 08	1.91E- 06
Foxf2	-6.746189775	8.31E- 08	2.82E- 06
Slc39a8	-7.759941676	9.10E- 08	3.01E- 06
H2-D1	-6.616799177	1.04E- 07	3.41E- 06
Col4a1	-6.520908718	1.27E- 07	4.12E- 06
Slco1c1	-6.946207352	1.71E- 07	5.46E- 06
Slc16a1	-7.009206189	2.70E- 07	8.53E- 06
Slco2b1	-6.242261686	3.21E- 07	1.00E- 05
4-Sep	-6.489816047	5.35E- 07	1.64E- 05
Sipa1	-5.01763471	5.52E- 07	1.66E- 05
Itpr1	-5.514058535	5.54E- 07	1.66E- 05
Nomo1	-5.99725618	6.27E- 07	1.86E- 05
Ablim1	-5.608099381	7.50E- 07	2.18E- 05

St8sia4	-5.912207965	7.98E-07	2.29E-05
Cavin1	-6.450806654	8.79E-07	2.50E-05
Pdlim7	-5.114016185	1.07E-06	2.97E-05
Lrch3	-5.930989445	1.27E-06	3.51E-05
Cdkn1a	-6.02422393	1.44E-06	3.92E-05
Slc7a8	-6.063556034	1.57E-06	4.18E-05
Gbp7	-7.067713696	1.57E-06	4.18E-05
F11r	-5.294533509	1.72E-06	4.53E-05
Prickle1	-5.427943727	1.80E-06	4.68E-05
Ets1	-6.458869933	3.37E-06	8.55E-05
Egfl7	-6.194855239	4.64E-06	0.000117
Mpzl1	-5.824485212	5.26E-06	0.00013
Vamp8	-5.289166503	8.33E-06	0.0002
Col4a2	-5.800505415	8.69E-06	0.000207
Arhgap29	-5.675806801	2.82E-05	0.000645
Lrp8	-5.398324521	3.06E-05	0.000693
Nostrin	-4.804484604	3.34E-05	0.000752
Rell1	-5.382379658	4.06E-05	0.000898
Pls3	-5.025966818	4.19E-05	0.00092
Lef1	-5.366161496	4.98E-05	0.001087
Frmd4b	-4.82941921	5.19E-05	0.001115
4931406P1 6Rik	-4.82442019	5.19E-05	0.001115
Nid1	-5.794979653	5.33E-05	0.001136

Far2	-5.213993242	5.82E-05	0.001231
Ramp2	-5.641359016	7.05E-05	0.001468
Tagln2	-5.684548785	7.49E-05	0.001547
Virma	-5.292816587	7.54E-05	0.001547
Cbfa2t3	-4.60682799	7.70E-05	0.001569
Prcp	-6.0024741	7.85E-05	0.001581
Coro1b	-5.327965313	7.87E-05	0.001581
Abcg1	-5.005727429	9.17E-05	0.001803
Uchl5	-4.980785029	9.97E-05	0.001912
Hdac7	-5.086033104	0.000124	0.002287
Ppm1f	-5.197170818	0.000139	0.00255
Prex2	-5.196110941	0.00016	0.002911
Hprt	-4.490542743	0.000188	0.003281
Eogt	-5.141417227	0.000204	0.003545
Apbb2	-4.371258142	0.000219	0.003754
Lypla1	-4.855823176	0.000248	0.004221
Lmna	-4.923022025	0.000251	0.004231
BC028528	-5.426469536	0.000268	0.00446
Mfap1a	-4.403400284	0.000306	0.005032
Vopp1	-4.252345791	0.000341	0.005505
Reep1	-4.654491117	0.000359	0.00573
Slc7a1	-4.54444925	0.0004	0.006285
Myh10	-4.107856974	0.000419	0.006538

Rapgef6	-4.051313456	0.000 435	0.006 756
Git2	-4.339251522	0.000 477	0.007 336
Myh9	-3.813011594	0.000 563	0.008 506
Ipo11	-4.466305224	0.000 582	0.008 699
Plekha1	-4.718996913	0.000 607	0.008 99
Lama4	-5.143188172	0.000 636	0.009 267
Srpr	-4.256027728	0.000 658	0.009 532
Setd5	-4.113242501	0.000 834	0.011 789
Jup	-4.804084321	0.000 897	0.012 543
Tsc22d1	-3.898525805	0.000 947	0.013 187
Slc6a6	-4.283158253	0.001 039	0.014 329
Ifngr1	-4.309412454	0.001 095	0.014 944
Arhgap31	-3.973631661	0.001 205	0.016 376
Nr2c2	-4.154801757	0.001 265	0.017 102
Nop53	-4.071198811	0.001 457	0.019 507
Sike1	-4.317819771	0.001 58	0.020 762
Gng11	-4.465136691	0.001 672	0.021 771
Vamp3	-4.256173735	0.001 952	0.024 956
Pmepa1	-4.297982292	0.002 078	0.026 441
Klf4	-4.396354243	0.002 136	0.027 061
Znrf2	-3.689474654	0.002 269	0.028 484
Podxl	-4.590491525	0.002 387	0.029 832
Tcaf1	-3.935201313	0.002 49	0.030 846

Lgals9	-4.617894611	0.002 626	0.032 387
Mtus1	-4.063606375	0.002 654	0.032 451
Coro2b	-3.907329113	0.002 86	0.034 367
Esyt2	-3.782944272	0.003 473	0.041 032
Bsg	-3.800869662	0.003 711	0.043 122
Fcho2	-3.957778804	0.003 795	0.043 913
Efr3b	-3.649847907	0.003 941	0.045 049
Heg1	-4.452876428	0.004 029	0.045 864
Churc1	-3.356937206	0.004 138	0.046 54
Ucp2	-4.339071236	0.004 317	0.048 144
Rhoj	-4.032734807	0.004 349	0.048 144

Supplemental Table 9. Differentially expressed genes in Astrocyte to VAC pairwise

comparison. Genes differentially expressed in Astrocytes compared to VACs (left) and genes differentially expressed in VACs compared to Astrocytes (right) with fold change ($\log_2$), p-value and FDR. FDR ≤ 0.05 with ≥ 2 -fold change.

Oligodendrocyte Cluster 4			
Gene Symbol	Fold Change (log2)	P-Value	FDR
Bcas1	11.58870747	9.76E-10	7.47E-08
Sox10	9.990003422	1.47E-08	8.26E-07
Ugt8a	10.79279822	2.33E-08	1.21E-06
Shisa1	10.81137548	3.01E-08	1.51E-06
Omg	8.928069273	5.18E-08	2.47E-06
Tnr	9.099432025	9.64E-08	4.30E-06
Olig2	8.619587505	1.68E-07	7.09E-06
Opcml	8.317889206	1.89E-07	7.82E-06
Bcan	6.660114669	4.94E-06	0.00017
Hipk2	6.1298404	1.35E-05	0.000439
Vcan	7.208125468	1.96E-05	0.000609
Gpr17	7.278580066	2.35E-05	0.000713
Cyfip2	6.822999401	2.45E-05	0.000728
Dixdc1	5.763965178	2.81E-05	0.000827
Ddr1	8.677406613	3.06E-05	0.000884
Slc24a3	6.026334352	5.93E-05	0.001614
Sox6	6.348937811	6.84E-05	0.001846
Grin3a	5.973467102	8.69E-05	0.002257
Rnd2	6.280090189	9.23E-05	0.002376
Sez6l	5.952211279	0.000101	0.002549
Dscam	6.59605061	0.000127	0.003164
Mbp	7.155607002	0.000145	0.003583

Vascular Associated Cell Cluster 5			
Gene Symbol	Fold Change (log2)	P-Value	FDR
Ctla2a	-10.75564003	2.61E-15	5.07E-12
Adgrl4	-10.37466998	4.38E-15	5.07E-12
Slc38a5	-11.05644738	6.01E-15	5.07E-12
Nid1	-10.82042862	7.09E-15	5.07E-12
Cldn5	-9.992959519	1.17E-14	6.72E-12
Cd34	-10.21159967	2.32E-14	1.11E-11
Ly6c1	-10.69928867	3.01E-13	1.23E-10
BC028528	-9.644867707	3.86E-13	1.38E-10
Emcn	-10.21816399	6.52E-13	2.07E-10
Slc40a1	-9.615457708	7.25E-13	2.07E-10
Slc2a1	-8.721350332	1.84E-12	4.77E-10
Pltp	-9.894215766	5.68E-12	1.35E-09
Slc22a8	-9.907621965	8.59E-12	1.89E-09
Gng11	-8.34820986	9.24E-12	1.89E-09
Rasgrp3	-8.907198689	1.69E-11	3.21E-09
Eng	-8.603784751	2.10E-11	3.75E-09
Grap	-9.434016879	2.99E-11	5.02E-09
Tdrp	-8.903677915	4.65E-11	7.38E-09
Heg1	-8.93885274	4.98E-11	7.48E-09
Srgn	-9.084705465	5.40E-11	7.71E-09
Adamtsl2	-9.524388806	5.97E-11	7.89E-09
Cdh5	-8.354182623	6.07E-11	7.89E-09

Pcdh17	6.651407129	0.000 161	0.003 899	Icam2	-8.044291043	8.37E- 11	1.04E- 08
Cnp	6.191809185	0.000 199	0.004 709	Wwtr1	-8.219730681	9.69E- 11	1.15E- 08
Slitrk2	5.93450114	0.000 247	0.005 689	Cnn2	-9.108747208	1.01E- 10	1.16E- 08
Nfasc	5.581716313	0.000 414	0.009 17	Ocln	-9.053438579	2.40E- 10	2.64E- 08
Scn8a	5.113452244	0.000 66	0.014 283	Ifitm3	-8.43959066	2.68E- 10	2.77E- 08
Pou3f3	5.282539199	0.000 75	0.015 765	Ppic	-9.034629374	2.83E- 10	2.77E- 08
Ctnna2	4.852814326	0.000 785	0.016 378	Cyrr1	-8.035732266	2.88E- 10	2.77E- 08
Sema5a	5.252524612	0.000 815	0.016 886	Gimap6	-8.569261804	2.91E- 10	2.77E- 08
Gadd45g	5.976844591	0.000 899	0.018 358	Tmem123	-9.266535118	3.78E- 10	3.49E- 08
Zcrb1	5.25802676	0.001 265	0.020 265	Slc39a8	-9.51832975	4.34E- 10	3.88E- 08
Nova1	5.083609671	0.001 066	0.021 454	Robo4	-7.842824598	5.33E- 10	4.62E- 08
Ptpro	4.546632211	0.001 733	0.033 46	Gjc1	-7.890969574	5.74E- 10	4.82E- 08
S100a1	4.604785058	0.001 95	0.037 4	Adgre5	-8.450240677	7.50E- 10	6.12E- 08
Slc6a11	4.774185766	0.002 116	0.039 53	Acvrl1	-8.854235047	8.30E- 10	6.59E- 08
Cpeb4	5.323362593	0.002 204	0.040 387	Il2rg	-8.281176683	9.93E- 10	7.47E- 08
Sirt2	4.336552806	0.002 273	0.041 374	Cd93	-8.032436707	1.20E- 09	8.76E- 08
Ncam1	3.664148332	0.002 304	0.041 678	Lgals9	-8.075771802	1.46E- 09	1.05E- 07
Sf3b3	4.978080125	0.002 602	0.046 196	Esam	-8.385699976	1.74E- 09	1.21E- 07
Tulp4	5.620191343	0.002 75	0.048 255	Isyna1	-7.388763523	2.03E- 09	1.37E- 07
Csmd1	4.369268064	0.002 805	0.048 887	Htra3	-7.332958026	2.05E- 09	1.37E- 07
				Podxl	-8.340725967	2.35E- 09	1.53E- 07
				Tek	-8.428869277	2.85E- 09	1.81E- 07
				Jup	-7.57959888	4.00E- 09	2.49E- 07

Palmd	-7.215338334	4.93E-09	3.00E-07
Ildr2	-7.850566422	7.05E-09	4.20E-07
Egfl7	-7.521324696	1.02E-08	5.94E-07
Slco1c1	-7.555941342	1.16E-08	6.61E-07
Serpinh1	-6.656472364	1.63E-08	8.94E-07
Cgnl1	-7.648042552	1.75E-08	9.42E-07
Rgs5	-8.036121527	1.87E-08	9.92E-07
Prex2	-7.397595472	2.81E-08	1.43E-06
Thsd1	-6.749926615	3.62E-08	1.78E-06
Mecom	-7.857221466	4.02E-08	1.95E-06
S100a11	-6.215508092	6.03E-08	2.82E-06
Epas1	-7.278329262	6.55E-08	3.00E-06
Tgfbr2	-7.702512897	6.62E-08	3.00E-06
Lama4	-7.725885863	1.17E-07	5.14E-06
C130074G 19Rik	-6.324897249	1.56E-07	6.74E-06
Klf4	-6.703193731	1.69E-07	7.09E-06
Fzd6	-6.588051915	1.92E-07	7.82E-06
Nostrin	-5.693525468	3.67E-07	1.48E-05
Fxyd5	-6.528885858	4.13E-07	1.64E-05
Cavin1	-6.646163424	4.63E-07	1.81E-05
Vwf	-6.548597311	4.77E-07	1.84E-05
Slc16a1	-6.27919797	7.80E-07	2.97E-05
Angptl4	-6.489129883	1.15E-06	4.32E-05

Cobll1	-6.41475474	1.20E-06	4.47E-05
Crip2	-6.070308225	1.45E-06	5.32E-05
Ushbp1	-5.936626621	1.97E-06	7.13E-05
Lmo2	-5.990591279	2.87E-06	0.000102
4-Sep	-6.03514419	4.48E-06	0.000158
Itm2a	-5.631225909	4.82E-06	0.000168
Tmem98	-5.629937896	5.65E-06	0.000192
Mansc1	-5.214699509	8.10E-06	0.000272
Pecam1	-6.019524591	9.31E-06	0.000309
Ecscr	-6.059678851	1.26E-05	0.000415
Nampt	-5.869012554	1.37E-05	0.000441
Gm24447	-4.571731423	1.57E-05	0.000499
Pdgfrb	-6.542131408	1.75E-05	0.00055
Anxa2	-6.006817867	2.05E-05	0.000631
Ablim1	-5.131753256	2.39E-05	0.00072
Pop5	-5.595325223	2.84E-05	0.000828
Gbp7	-6.290136428	3.12E-05	0.000892
Nr3c1	-5.798724498	3.81E-05	0.001072
Tfric	-5.076073712	3.83E-05	0.001072
Bsg	-4.539672782	4.83E-05	0.00134
Flt1	-5.546911565	5.56E-05	0.001527
Apccdd1	-5.03956105	7.10E-05	0.001896
Col4a1	-5.218927694	8.28E-05	0.002191

Adgrf5	-5.559756031	8.56E-05	0.002245
Fli1	-5.590110579	9.53E-05	0.002432
Tiam1	-4.984893794	0.000122	0.003055
Ccdc141	-5.122932872	0.000155	0.003789
Slc7a1	-4.529891018	0.000191	0.004595
Myo1b	-5.244163607	0.000198	0.004709
Tes	-5.011394937	0.000221	0.005167
Eogt	-5.170450651	0.000241	0.005611
Slc7a5	-4.489220202	0.000301	0.006887
Zc3hav1	-5.229578143	0.00034	0.007709
Csrp2	-4.839778279	0.000372	0.008361
Ptprb	-4.9817922	0.000407	0.00909
Slc46a3	-4.909710367	0.000456	0.010014
Slc39a10	-4.990212427	0.000511	0.011151
Fn1	-4.967927532	0.000669	0.014367
Anxa5	-4.746227592	0.000674	0.014372
Col4a2	-4.504309002	0.000708	0.014991
St8sia4	-4.513212012	0.000881	0.018124
Cdkn1a	-4.490903084	0.001155	0.02309
Tmem204	-4.774475767	0.001224	0.024292
Tspan14	-4.554757621	0.001242	0.024481
Cbfa2t3	-3.978479964	0.001397	0.027344
Kdr	-4.462277105	0.001723	0.03346

Slc43a2	-4.294760643	0.002 038	0.038 831
Tram2	-4.659878722	0.002 067	0.039 125
Rhoc	-4.174113614	0.002 116	0.039 53
Nes	-4.371105349	0.002 154	0.039 975
Nomo1	-4.174378841	0.002 182	0.040 241
Stx6	-4.353816136	0.002 42	0.043 5
Jcad	-4.385523336	0.002 447	0.043 717
Pglyrp1	-4.457205212	0.002 752	0.048 255

Supplemental Table 10. Differentially expressed genes in Oligodendrocyte to VAC

pairwise comparison. Genes differentially expressed in Oligodendrocytes compared to VACs (left) and genes differentially expressed in VACs compared to Oligodendrocytes (right) with fold change ($\log_2$), p-value and FDR. FDR ≤ 0.05 with ≥ 2 -fold change.

Differentially Expressed Genes in Neurons			
Gene Symbol	Fold Change (log2)	P-Value	FDR
Gm14204	5.33296256	2.49E-10	1.14E-08
Grin2a	5.118059505	4.49E-09	1.59E-07
C230085N15Rik	5.123636104	2.61E-08	7.58E-07
Gm42616	4.672227628	5.25E-08	1.48E-06
Ass1	4.429968247	1.12E-07	3.00E-06
Ankrd45	4.786700477	1.23E-07	3.19E-06
Tmem59l	4.211614186	1.34E-07	3.37E-06
Cda	5.111879642	2.05E-07	5.05E-06
Syngn3	3.918222427	3.36E-07	7.74E-06
Lamp5	3.460966213	6.15E-07	1.31E-05
Ramp3	4.315890349	1.75E-06	3.29E-05
Epb41l4b	4.18621685	5.01E-06	8.43E-05
Cds1	3.919713688	6.92E-06	0.000113
Rasgrf1	4.483980388	8.11E-06	0.000127
Gabra5	3.625585091	8.65E-06	0.00013
Car10	3.366233111	1.01E-05	0.000146
Fsd1	4.038406282	1.03E-05	0.000147
Kcnk1	4.130664812	1.47E-05	0.000201
Hapln1	3.000500493	1.57E-05	0.000212
Prepl	4.138461882	1.62E-05	0.000214
Unc5d	3.279458053	1.65E-05	0.000214
Mllt11	3.163705792	1.73E-05	0.000222
Ccdc85a	3.341420867	2.42E-05	0.00029
Sgpp2	3.61144935	2.91E-05	0.000342
Slitrk1	3.799226587	3.36E-05	0.000384
Tmem246	3.539129382	3.47E-05	0.000385
Calb1	3.496268069	3.49E-05	0.000385
Hivep3	3.967895868	3.91E-05	0.000426
Mir670hg	3.825978898	4.27E-05	0.000459
Golga7b	3.734662972	5.44E-05	0.000567
Ache	3.827761699	6.33E-05	0.000646
Fgf9	3.319081132	7.29E-05	0.000713
Snhg11	2.911269813	8.44E-05	0.000809
Ccdc184	3.354574764	9.45E-05	0.000889
Strbp	2.941481814	9.59E-05	0.000893
Lrfr5	3.308586818	0.000107	0.000971
Atp6v1g2	3.075859607	0.000107	0.000971
Gdap1	3.476385366	0.000117	0.001045
Kcnn1	3.585753077	0.000119	0.00106
Hs6st2	3.092030475	0.000121	0.001068

Mab211l	3.44031747	0.000133	0.001147
Atp2b2	2.893720617	0.000152	0.001254
Cacna2d3	2.997179031	0.000172	0.001394
Plppr3	3.491287323	0.000173	0.001394
B830012L14Rik	3.702058932	0.00018	0.001412
Cep170b	3.496939304	0.000184	0.001438
Fastk	3.919119494	0.000193	0.00149
Isyna1	4.365490593	0.000195	0.001496
Actl6b	3.787703915	0.000199	0.001512
Esrrb	3.552784688	0.000219	0.00165
Tubb3	2.993806461	0.00023	0.001725
Got1	3.156556715	0.000257	0.001894
Gm43175	3.463434992	0.000302	0.002209
Cish	3.431746951	0.000322	0.002321
Slc4a10	3.304096257	0.000324	0.002321
Crtac1	2.945002658	0.000342	0.002431
Slc6a5	2.692374508	0.000356	0.002511
Atl1	3.39688733	0.000365	0.002553
Gm9911	3.763010111	0.000383	0.002659
Snap25	2.689279398	0.000393	0.002714
Scn1a	2.840564042	0.000437	0.00295
Tmeff1	3.153941763	0.000453	0.003039
Hspa12a	2.990427505	0.000501	0.00334
Zdbf2	3.47639216	0.000514	0.003399
Sv2c	2.829354958	0.000521	0.003399
Gm44509	3.649384875	0.000556	0.003605
Caly	3.235990999	0.000604	0.003835
Mtx2	3.235342961	0.000696	0.004392
Ddx25	3.470614437	0.000701	0.004393
Pld3	3.174910367	0.000724	0.004506
Stac2	3.337883085	0.000758	0.004689
Zfp385b	3.177253739	0.000847	0.005172
Cntnap2	2.832029546	0.000895	0.005421
Gm45652	3.867130272	0.000916	0.005488
Slc39a6	3.437845902	0.000932	0.005545
Arhgdib	4.221216984	0.000943	0.005545
Atp1a3	2.647891796	0.000983	0.005716
Adap1	3.135133311	0.001074	0.006167
Gm37176	3.337289753	0.001111	0.006266
Esrrg	3.050207414	0.001113	0.006266
Cend1	2.88875894	0.001117	0.006266
Stum	3.306047329	0.001128	0.006291
Zfp804a	3.397082552	0.00115	0.006374

Slc1a1	3.137858428	0.00117	0.006446
Rbfox3	3.06017679	0.001263	0.006864
Arl4c	2.969374428	0.001267	0.006864
Eif5a2	3.365777921	0.001277	0.006878
Ncdn	3.099519511	0.001411	0.007403
1500009L16Rik	3.248922653	0.001413	0.007403
Nipsnap1	3.115109274	0.001454	0.00754
Spats2l	3.152215687	0.001505	0.007614
Dync1i1	3.095182967	0.001507	0.007614
Igsf3	2.796692747	0.001523	0.007614
Pnma2	3.336238647	0.001531	0.007614
Krt222	3.28468139	0.001553	0.007679
Trank1	3.395014787	0.001635	0.008001
Ndr4	2.549505483	0.001672	0.008114
Fabp3	2.743188773	0.001677	0.008114
Chga	2.98713178	0.001693	0.008114
Rian	2.88070421	0.001697	0.008114
Rcan2	2.781819712	0.001701	0.008114
Gabrg2	2.977874872	0.001715	0.008144
Dgkg	3.249879043	0.001725	0.008149
Stx1b	3.043324685	0.001777	0.008352
AI593442	3.228574198	0.001852	0.008663
Pcbp3	3.183314627	0.002157	0.009896
Rit2	3.322313355	0.002488	0.011308
Ccl27a	3.001753689	0.002713	0.012156
Napb	2.718781908	0.002831	0.012571
Celsr2	2.833763944	0.003223	0.014113
Snap91	2.61954641	0.00342	0.01484
Serpini1	2.903726242	0.00346	0.014923
Ldhd	2.53198142	0.003507	0.015016
Pfkm	3.057059445	0.003536	0.015072
Sez6	2.852080941	0.003559	0.015102
Lin7a	2.966535548	0.003629	0.015333
Ryr2	2.899532037	0.003651	0.015358
Syt7	2.693105483	0.003982	0.016533
Kifc2	2.952652081	0.004584	0.018708
Rims1	2.794468586	0.004753	0.019314
Shtn1	2.933247796	0.004906	0.019769
Tox2	3.068177691	0.005078	0.020375
Hpcal1	2.812901919	0.005149	0.0205
Tspyl5	2.851306428	0.005152	0.0205
Parp6	3.013276995	0.005196	0.02059
Dpysl5	2.948586475	0.005224	0.020617

Pgm2l1	2.609518382	0.005755	0.02262
Syp	2.628267823	0.005799	0.022701
Phyh	2.720232586	0.005871	0.022887
Stmn2	2.430519381	0.005983	0.023231
Dhps	3.095891216	0.006035	0.023338
Lrp11	3.015086658	0.00621	0.02366
Ncs1	2.870489559	0.006215	0.02366
Fam20c	2.948117096	0.006217	0.02366
Meis2	2.530503877	0.006319	0.023951
Nceh1	2.736172833	0.006432	0.024211
Raly1	3.037263701	0.006463	0.024211
Elavl2	2.678168324	0.006689	0.024962
Amn	2.98354841	0.00683	0.025387
Alg2	2.809941612	0.007459	0.027246
Glrb	2.714188898	0.007689	0.02772
Cx3cl1	2.822686767	0.007986	0.028685
Rgs10	3.042032325	0.008431	0.030036
Hcn1	2.603992801	0.008457	0.030036
Nyap2	2.878477399	0.008555	0.030275
Camk1d	2.594427252	0.008622	0.030399
Wnk2	2.811198315	0.008864	0.030936
Dlat	2.93564833	0.008871	0.030936
Mapk10	2.693632983	0.009322	0.032274
Ptprn	2.940382526	0.009371	0.032328
Nap1l2	2.809517921	0.009729	0.03336
Map7d2	2.624372022	0.00974	0.03336
Sipa1l2	2.860530664	0.009798	0.033438
Usp29	2.816917599	0.009862	0.033438
Vsnl1	2.614473662	0.009868	0.033438
Kctd13	2.976060059	0.009978	0.033693
Agtppb1	2.817739065	0.0104	0.034995
Crmp1	2.490295771	0.010649	0.035582
B3galt2	2.50857661	0.011009	0.036533
Scg5	2.579429912	0.011063	0.036584
Nrxn3	2.458616855	0.011403	0.037581
Tln2	3.088316841	0.011478	0.037696
Lmo3	2.587325192	0.01272	0.040969
A230103L15Rik	2.589137175	0.012731	0.040969
Ppif	2.943803518	0.012903	0.041383
Pitpnc1	2.378211447	0.013298	0.042408
Srxn1	2.928160296	0.01331	0.042408
Fam189a1	2.815049649	0.013384	0.0425
Kcnq2	2.907480281	0.013542	0.042859

Olfm1	2.692622435	0.01386	0.043527
Sgip1	2.477970257	0.014093	0.043935
Prune2	2.56589638	0.014136	0.043935
Rab3a	2.53363378	0.014166	0.043935
Msra	2.778401131	0.014202	0.043935
Ppa1	2.746832287	0.014913	0.045692
Retreg1	2.871422603	0.015095	0.046103
Fkbp1b	2.687926789	0.015834	0.047907
Pcp4	2.58045591	0.015836	0.047907
Hras	2.937985117	0.016484	0.04971

Supplemental Table 11. Differentially expressed genes in neurons. Genes differentially expressed in neurons (N1 and N2 combined) compared to the average of all nonneuronal clusters (astrocytes, oligodendrocytes and VACs) with fold change ($\log_2$), p-value and FDR. FDR ≤ 0.05 with ≥ 2 -fold change.

Differentially Expressed Genes in Astrocytes			
Gene Symbol	Fold Change (log2)	P-Value	FDR
Tnc	8.017817973	1.09E-12	1.04E-09
Fgfr3	6.548101937	7.53E-09	3.61E-06
9330159F19Rik	4.796441151	8.43E-07	0.000202
Gm3764	4.197875058	1.24E-06	0.000227
Aqp4	5.028716493	1.77E-06	0.000227
Slc6a11	4.902113994	1.83E-06	0.000227
Tmem229a	5.756433817	1.90E-06	0.000227
Agt	5.847760162	3.58E-06	0.000344
Slc1a3	3.934042358	1.28E-05	0.000883
Fads2	5.311119667	1.33E-05	0.000883
AI464131	5.607022332	1.38E-05	0.000883
Pla2g7	4.47221024	1.78E-05	0.001055
Slc1a2	4.414266397	1.87E-05	0.001055
Acot1	5.849711356	2.17E-05	0.001097
Kxd1	4.752519748	2.56E-05	0.00119
Igsf11	5.470171059	2.61E-05	0.00119
Hepacam	4.459636719	3.70E-05	0.001611
Tulp4	3.887830737	3.98E-05	0.001615
Aldh1l1	4.864451285	4.04E-05	0.001615
Adgrg1	4.192390822	6.52E-05	0.002404
Clu	3.672794778	7.35E-05	0.002513
Ddr1	5.175709671	7.60E-05	0.002513
Rorb	5.022954585	9.86E-05	0.002803
Sall2	4.435758874	9.87E-05	0.002803
Scrg1	4.64742121	0.00017	0.004403
Olig2	4.503141133	0.000179	0.004521
Ncan	3.246471252	0.000197	0.004835
Ptprz1	3.631223747	0.00021	0.005042
Tmem100	4.540538103	0.000289	0.006607
Ttyh1	3.653222628	0.000371	0.007894
Arap2	4.414456902	0.000401	0.008174
Hes1	4.05425967	0.000455	0.008898
Sobp	3.674896982	0.000507	0.009343
Slc7a11	4.081146127	0.000539	0.009753
Pdpm	4.151569222	0.000595	0.010563
Trim9	3.643452437	0.000637	0.010929
Notch2	4.331733687	0.000641	0.010929
Slc4a4	3.990775783	0.000662	0.01095
Ptn	3.417754263	0.000936	0.015078
Spry2	3.099044027	0.000943	0.015078

Fabp7	3.31045524	0.000981	0.015419
Rgma	4.215376676	0.001001	0.015482
Adcyap1r1	3.868381006	0.001095	0.01616
Sema6d	3.773768883	0.001153	0.01676
Gja1	3.811670146	0.001184	0.016945
Tram2	4.057144833	0.001255	0.017702
Bcan	3.423042117	0.001309	0.018194
Per3	4.075893749	0.001697	0.02199
Notch1	3.73300179	0.001793	0.022097
Npas3	3.678203161	0.001797	0.022097
Usp31	3.675661691	0.001865	0.022639
Wwtr1	4.278526567	0.001897	0.022745
Zcrb1	3.23074395	0.002013	0.023832
Notch3	3.933433059	0.002238	0.025857
Angptl4	4.20116672	0.002362	0.026411
Tmem44	3.764099667	0.002482	0.027087
Grm5	3.445404393	0.002486	0.027087
Car2	3.530409014	0.002677	0.028845
Dhps	3.492188866	0.003824	0.039011
Omg	3.64763482	0.004431	0.043803
Igfbp7	3.870865709	0.004573	0.044751
Vcan	3.482906113	0.004694	0.04547
Apcdd1	3.13890445	0.0048	0.046031
Sox9	3.513161957	0.004983	0.047167
Ednrb	3.427452427	0.005276	0.048256

Supplemental Table 12. Differentially expressed genes in astrocytes. Genes differentially expressed in astrocytes compared to the average of all other clusters (N1 and N2, oligodendrocytes and VACs) with fold change ($\log_2$), p-value and FDR. FDR ≤ 0.05 with ≥ 2 -fold change.

Differentially Expressed Genes in Oligodendrocytes			
Gene Symbol	Fold Change (log2)	P-Value	FDR
Bcas1	9.01998098	2.81E-12	2.70E-09
Ralgps2	6.175908141	5.59E-10	2.68E-07
Ugt8a	8.307817085	1.70E-09	5.15E-07
Shisal1	8.173344462	2.15E-09	5.15E-07
Tnr	6.822185459	1.17E-08	2.23E-06
Gpr17	6.601437261	6.72E-08	1.07E-05
Plp1	6.093060749	1.14E-07	1.56E-05
Scrg1	6.8161038	1.56E-07	1.78E-05
Mbp	6.154865308	1.79E-07	1.78E-05
Sox10	7.346628967	1.86E-07	1.78E-05
Cyfip2	4.748608293	4.00E-07	3.48E-05
Olig2	6.146375621	8.75E-07	6.99E-05
Cnp	5.582313424	1.09E-06	8.08E-05
Dscam	4.81675982	1.38E-06	9.45E-05
Sema5a	5.471186835	1.96E-05	0.000938
Sox6	5.359888942	2.16E-05	0.000957
Nfasc	4.14092051	4.54E-05	0.001675
Rnd2	4.288526473	5.52E-05	0.001825
Opcml	4.597896573	6.05E-05	0.001871
Omg	4.855301525	0.000138	0.003403
Vxn	4.786884263	0.000168	0.004028
Mpzl1	4.574383085	0.000197	0.004606
Cmtm5	5.040035218	0.00029	0.006179
Lhfpl3	4.809026528	0.00031	0.006346
Dpysl4	4.029729909	0.000328	0.006399
Sirt2	3.976638264	0.000402	0.007554
Fcho2	4.563638764	0.000486	0.008499
S100a6	4.747998661	0.000712	0.011007
Gm18194	3.395855188	0.000845	0.012088
Clstn3	3.795820714	0.001003	0.013738
Fyn	3.569196786	0.001108	0.01476
Ppfibp1	4.678490493	0.001221	0.016046
Sf3b3	4.050716201	0.001485	0.019147
Slitrk2	3.871479811	0.001504	0.019147
Mgat5	3.519623119	0.0021	0.024555
Rhoq	4.192698476	0.002151	0.024859
Ext1	3.76107431	0.002372	0.026533
S100a13	4.1297539	0.002649	0.028229
Abcb1a	3.704567529	0.002902	0.030253
Map2k4	3.704301928	0.002954	0.030462

Fam169a	3.453726708	0.003026	0.030869
Kcnj10	4.088937093	0.003138	0.031346
Tubb4a	3.323534211	0.003347	0.032423
Mien1	3.505768621	0.003431	0.032899
Nol11	4.021775164	0.003715	0.035035
Bcan	3.385838478	0.00443	0.039336
Itpr2	3.940113022	0.005251	0.044172
S100a1	3.777826467	0.005468	0.045202

Supplemental Table 13. Differentially expressed genes in oligodendrocytes. Genes differentially expressed in oligodendrocytes compared to the average of all other clusters (N1 and N2, astrocytes and VACs) with fold change ($\log_2$), p-value and FDR. FDR ≤ 0.05 with ≥ 2 -fold change.

Differentially Expressed Genes in VACs			
Gene Symbol	Fold Change (log2)	P-Value	FDR
Acvrl1	12.12671347	1.51E-44	1.45E-41
Il2rg	11.16868551	2.99E-38	1.43E-35
Ifitm3	11.40188055	8.59E-36	2.75E-33
Cldn5	12.48972892	3.30E-33	7.91E-31
Ctla2a	12.23968331	3.05E-32	5.84E-30
Srgn	11.77975643	2.48E-31	3.97E-29
Icam2	11.29527127	5.48E-31	7.51E-29
Eng	11.3198578	3.40E-29	4.08E-27
Grap	11.5176317	6.68E-27	7.12E-25
Robo4	10.80166765	1.10E-26	1.05E-24
Pltp	11.60600608	2.60E-22	2.26E-20
Slc38a5	11.24018164	1.17E-21	9.39E-20
Cd34	10.46470466	5.04E-21	3.72E-19
Tgfb2	11.03182231	5.63E-21	3.86E-19
Fzd6	9.567143296	1.80E-20	1.15E-18
Tek	10.16177766	7.58E-20	4.54E-18
Adgrl4	10.57482412	1.13E-19	6.36E-18
Lama4	8.99109189	2.21E-19	1.18E-17
Cgnl1	10.20153391	6.60E-19	3.33E-17
Adgre5	9.866715788	2.65E-18	1.27E-16
Ly6c1	11.59705095	1.10E-17	5.05E-16
Emcn	11.18887187	6.07E-17	2.65E-15
Slc39a8	9.565226795	6.36E-17	2.65E-15
Adgrf5	8.831886044	1.12E-16	4.48E-15
Cdh5	9.598874461	3.81E-16	1.46E-14
Cd93	9.162322675	5.11E-16	1.88E-14
Pecam1	8.623022427	5.92E-16	2.10E-14
Htra3	9.107755317	6.67E-16	2.29E-14
Slc40a1	9.699494221	1.05E-14	3.46E-13
Tdrp	9.579957698	2.62E-14	8.38E-13
Podxl	8.767175023	3.22E-14	9.95E-13
Ecscr	9.923747216	6.45E-14	1.93E-12
Nid1	9.36444904	7.61E-14	2.21E-12
Adamtsl2	9.694050871	5.61E-13	1.58E-11
Ocln	9.94469107	1.03E-12	2.81E-11
Foxf2	7.221848747	1.52E-12	4.05E-11
Slc22a8	9.300565311	2.42E-12	6.28E-11
AU021092	7.857286543	5.20E-12	1.30E-10
Kdr	7.851177194	5.27E-12	1.30E-10
Fli1	9.616441119	6.34E-12	1.52E-10

Gbp7	9.485043362	6.71E-12	1.57E-10
Rasgrp3	8.641642523	6.91E-12	1.58E-10
Vwf	7.790249135	1.45E-11	3.22E-10
Fn1	7.695777067	1.64E-11	3.57E-10
Apcdd1	6.816307697	2.49E-11	5.31E-10
Gng11	8.146981547	2.57E-11	5.36E-10
Cnn2	9.759703409	3.10E-11	6.33E-10
Arap3	8.540307547	4.51E-11	9.02E-10
BC028528	8.309464764	5.34E-11	1.04E-09
Rgs5	8.815608282	5.96E-11	1.14E-09
Flt1	8.032772007	1.64E-10	3.09E-09
Ptprb	7.784581763	2.00E-10	3.69E-09
Esam	8.37105065	2.08E-10	3.76E-09
Abcb1a	6.881325639	2.55E-10	4.54E-09
Cyyr1	7.863410803	6.05E-10	1.06E-08
Thsd1	7.995614054	7.93E-10	1.36E-08
Slco2b1	6.844114888	1.17E-09	1.97E-08
Gjc1	8.032966811	1.31E-09	2.16E-08
Cavin1	7.51401446	1.45E-09	2.36E-08
Egfl7	8.181059297	1.63E-09	2.60E-08
Tram2	7.617747503	3.65E-09	5.74E-08
Itm2a	6.959655918	3.86E-09	5.97E-08
Pdgfrb	7.793896921	4.67E-09	7.10E-08
Fxyd5	7.712176263	5.79E-09	8.67E-08
Gimap6	8.133269976	7.24E-09	1.07E-07
Slc16a1	6.111894959	1.70E-08	2.47E-07
Mecom	7.074907871	1.80E-08	2.58E-07
Tmem204	7.03358269	1.90E-08	2.68E-07
Slco1c1	7.47065211	2.79E-08	3.88E-07
Arhgap29	6.258479125	3.67E-08	5.03E-07
Prex2	6.863957834	4.12E-08	5.57E-07
Jcad	6.925136853	4.18E-08	5.57E-07
Pglyrp1	7.109102765	4.61E-08	6.06E-07
Lgals9	7.585726498	5.64E-08	7.31E-07
Jup	6.857516791	6.06E-08	7.75E-07
Anxa3	7.968519717	7.00E-08	8.84E-07
Palmd	7.106231332	8.43E-08	1.05E-06
Slc46a3	6.83563274	9.51E-08	1.17E-06
Arhgdib	8.086244478	1.08E-07	1.31E-06
Nostrin	5.983325268	1.34E-07	1.59E-06
Prcp	7.281784682	1.34E-07	1.59E-06
Heg1	7.117780438	1.44E-07	1.69E-06
Tmem123	7.427304682	3.15E-07	3.63E-06

Uaca	5.56994883	3.29E-07	3.76E-06
C130074G19Rik	6.710566289	6.92E-07	7.81E-06
Nampt	5.666890682	1.14E-06	1.27E-05
Myo1b	6.502681536	1.26E-06	1.39E-05
Epas1	6.441349716	1.54E-06	1.67E-05
Tfrc	4.619205171	1.58E-06	1.70E-05
Isyna1	6.024155688	3.71E-06	3.96E-05
Eogt	5.926941382	5.11E-06	5.38E-05
Serpinh1	5.533953751	7.25E-06	7.56E-05
Ushbp1	5.999827367	1.82E-05	0.000184
Lef1	5.609005153	1.98E-05	0.000196
Col4a2	5.222411023	1.99E-05	0.000196
Slc2a1	5.688252469	2.99E-05	0.000287
Col4a1	5.228572256	4.09E-05	0.000388
Ets1	5.333911992	5.13E-05	0.000473
Ppic	5.785777436	5.94E-05	0.000537
Bsg	3.622337536	6.35E-05	0.000564
Ablim1	4.099207831	8.24E-05	0.000719
Lmo2	5.085665162	0.000104	0.000891
Ly6e	4.299568666	0.000123	0.001026
Anxa2	5.172088809	0.000136	0.001128
Vwa1	5.428602212	0.000158	0.001287
Hmgcs2	5.544165957	0.00016	0.00129
4-Sep	4.853012412	0.00017	0.00133
Nr3c1	5.005591385	0.000172	0.00133
Cobll1	5.323013116	0.000304	0.00226
Ildr2	5.179414599	0.000401	0.002889
Mfsd2a	5.158612277	0.000469	0.003323
Klf4	5.04625551	0.000583	0.003882
Arpc1b	4.507265321	0.000624	0.004113
Tes	4.866374147	0.000648	0.00423
Csrp2	4.786787695	0.000655	0.004247
Slc7a1	4.293925557	0.000736	0.004708
Hdac7	4.7105889	0.000893	0.005408
Crip2	4.376028943	0.000993	0.00588
Nes	4.697632454	0.001022	0.006011
Wwtr1	4.759360761	0.001147	0.006588
Mkl2	4.538439147	0.001308	0.007335
S100a11	4.519384735	0.00141	0.007859
Gm24447	3.967760729	0.001427	0.007895
Myh9	3.414624167	0.001818	0.009685
Cdkn1a	4.426628512	0.002286	0.01198
Slc7a5	3.895513348	0.002696	0.013536

Mansc1	4.339787681	0.002825	0.013873
Angptl4	4.461613911	0.00283	0.013873
Slc7a8	4.356824833	0.003322	0.015696
Abca1	4.29288681	0.003885	0.017739
Ramp2	4.411960576	0.003914	0.017788
Tagln2	4.0930612	0.00506	0.022058
Nomo1	3.723938566	0.005457	0.023362
4931406P16Rik	3.600351097	0.005925	0.024671
Tiam1	4.029868546	0.005966	0.024671
Ccdc141	3.979344716	0.005968	0.024671
Sparc	3.723953355	0.00718	0.028452
Lrp8	3.698968874	0.008637	0.033532
Dab2	3.804463804	0.009619	0.036317
S1pr1	3.647716021	0.010087	0.037363
Calcr1	3.643118262	0.010748	0.039492
Clic1	4.096741445	0.011282	0.041288
Ppm1f	3.93842286	0.011367	0.041291
Rgs12	4.002361459	0.013201	0.045538
St8sia4	3.663336851	0.01344	0.046033
Tpm4	3.656702853	0.013647	0.046487
Ucp2	4.154911611	0.013836	0.046557

Supplemental Table 14. Differentially expressed genes in VACs. Genes differentially expressed in VACs compared to the average of all other clusters (N1 and N2, astrocytes and oligodendrocytes) with fold change ($\log_2$), p-value and FDR. FDR ≤ 0.05 with ≥ 2 -fold change.

Gene	MeanDecrease Gini
Pgk1	0.343458374
Ctsl	0.28742111
Gm17018	0.285888148
Map1b	0.260475673
Gde1	0.259621238
Atp6v1g2	0.235286636
Asns	0.216310193
Praf2	0.197207893
Cda	0.184123729
Gdi1	0.182431933
Fsd1	0.181127995
Vsnl1	0.177197162
Aip	0.173727387
Mrps6	0.17332148
Ass1	0.16706586
Ldhb	0.162077186
Idh3a	0.157925742
Snap25	0.155667352
Lmo3	0.149253325
Bcap31	0.144271054
Slc25a5	0.141111445
Tagln3	0.14035993
Mab21l1	0.138746011
Mcts1	0.134925886
Nrxn1	0.133960001
Caly	0.130964696
Ppa1	0.127370656
Tuba4a	0.122878662
Mdh1	0.12240058
Tmx2	0.122215978
Kxd1	0.121333058
Inpp5f	0.119934911
Akap5	0.117968156
Lamp5	0.113987672
Atp5g2	0.112688696
Maged1	0.111205918
Fkbp3	0.10905891
Sh3bgrl3	0.108523413
Chchd6	0.107176142
B4gat1	0.107067069

Gene	MeanDecrease Gini
Elavl2	0.009637099
Arl4c	0.009623122
Dbi	0.009570686
Cdkn1b	0.009551082
Sipa1l2	0.009545804
Camk1d	0.009503236
Gm3764	0.009371559
Ramp1	0.0093426
Ptprz1	0.009270914
Rab33a	0.009267634
Tcf4	0.009264357
Kcnh2	0.009243915
Aldh5a1	0.008925158
Zfyve9	0.008902198
Pdlim7	0.008838858
Sirt2	0.00880418
Slc6a11	0.008799065
Epb41l1	0.008795205
Paqr4	0.008784592
Sall2	0.008743377
Atg2b	0.008686691
Mapk8ip3	0.008683492
Slc4a10	0.008655463
Dynll2	0.0085955
Gm43175	0.00856569
Grin2a	0.008548474
Gm44562	0.008548337
Rufy3	0.008528568
Clip3	0.008503573
Gdap1	0.00850015
Pik3r3	0.008477887
Lbr	0.008463594
Disp2	0.008442365
Mfn2	0.008387206
Cxadr	0.008329353
Crip2	0.008316343
Ttn	0.008315949
Usp5	0.008299691
Sdcbp	0.008238582
Zfp804a	0.00823849

Gene	MeanDecrease Gini
Akap12	0.003425613
Zdhhc2	0.003406763
Esam	0.003391775
Atp6v0e	0.003372841
Mmd2	0.003368464
Id3	0.003348511
Myl12a	0.00333886
Carmil1	0.003338175
Sox10	0.003336907
Esyt2	0.003334198
Egr1	0.003329796
Slc4a4	0.003329556
Kcnq3	0.003319469
Pla2g7	0.003300184
Igsf3	0.003297188
Prcp	0.003291163
Git2	0.003286667
Ece1	0.003245495
Pygo1	0.003231362
Smpd3	0.003228659
Ctsc	0.003206801
Ppfia4	0.003195367
S100a1	0.00318884
Gm24447	0.003188301
Trim9	0.003182545
Nomo1	0.003146048
Jag1	0.003143405
Spred1	0.003135045
Gramd1b	0.003124344
Ttyh2	0.003122766
Pygb	0.00312197
Paqr8	0.003120012
B4galt1	0.003101678
Myo6	0.003094544
Npas3	0.003094367
Timp4	0.00309431
Ryr2	0.00309327
Ube3a	0.003086488
Emp2	0.00307989
Lingo1	0.003075842

Calb1	0.106025204	Gm37176	0.00821511	Per3	0.003066423
Hprt	0.102984418	Coro1b	0.008193761	Slc43a2	0.003061479
Cryab	0.102592799	Marcks	0.008191174	Zbtb20	0.003044566
Ndr4	0.10116501	App	0.008173885	Vamp3	0.003044014
Prkar1b	0.099518848	Car10	0.008145321	Ank3	0.002974537
Rgs10	0.096895303	Ube3c	0.008099711	Gpr17	0.002957186
Rab3b	0.096343324	Mturn	0.008069867	Cadm3	0.002950588
Pin1	0.09237049	Tacc2	0.008008061	Spon1	0.002933371
Got1	0.091578184	Got2	0.008002202	Klf4	0.002925712
Nefl	0.091275269	Gabra5	0.007970826	Lpp	0.00291898
Clec2l	0.090952991	Rasgrf1	0.00788627	Zeb2	0.002906998
Rit2	0.090286042	Slitrk4	0.007870197	Nr2c2	0.002850882
Tspan7	0.088515058	Usp29	0.007835969	Lrp8	0.002828895
Lgr4	0.085383841	Gm2a	0.007812165	Emc1	0.002824047
B3galt1	0.082351348	Nav2	0.00765973	Vim	0.002816595
Tsc22d3	0.082342053	A230103L15		Aldh1l1	0.002810875
Cask	0.081904112	Rik	0.007623442	Plekha1	0.002808601
Gdpd1	0.081457706	Dnajb4	0.007595106	Gatm	0.002801821
Tubb3	0.080372659	Coro1c	0.007592561	Slc7a8	0.002798956
Tubg1	0.078747995	Tox3	0.007544737	Sypl	0.002781565
Arl6ip4	0.077312035	Plppr3	0.007484343	St8sia4	0.002779438
Rab3a	0.076830497	Sobp	0.007469295	Ccnd2	0.00277122
Snrpn	0.076151257	Omg	0.007465501	Ddr1	0.00277108
Uchl5	0.075347566	Pls3	0.00743963	Nfkb1a	0.002767587
Serf1	0.075088796	Nceh1	0.007424645	Specc1	0.002738162
Bex3	0.074564113	Rnf187	0.007420933	Bmpr1a	0.00273003
Nrip3	0.073666729	Fabp7	0.007336091	Phldb1	0.002716226
Tubb2a	0.073365483	Sgip1	0.007313885	Olig1	0.002713765
Fabp3	0.072532482	Cyb5a	0.007266879	Pcdh10	0.002713308
Prune2	0.071847134	Prkaa2	0.007262845	Car2	0.002711593
Msra	0.070788244	Spats2l	0.007244358	Ptpro	0.002708632
Hmgcs1	0.070624077	Trim67	0.007205434	Sdc4	0.002599253
Mllt11	0.069148327	Napg	0.00720172	Mkl2	0.002580684
Zc2hc1a	0.06731531	Kcnk1	0.007172293	Rab8b	0.002577405
Cav2	0.067059326	Abcg2	0.00708728	Tes	0.002555324
Plekha2	0.064722875	Tfrc	0.007064633	Hadha	0.002537342
Pkig	0.06439301	Cds1	0.007062952	Chpt1	0.002532045
Becn1	0.063653061	Map2k4	0.007056139	Csrp2	0.002518302
Lrrc4b	0.063008789	Grin1	0.007054899	Ginm1	0.002515014
Eif4a2	0.061799743	Epb41l3	0.007031679	Nr2f2	0.002485344
Mn1	0.061618225	Rgs17	0.007027976	Itpr2	0.002459746
Clu	0.060959552	Slitrk2	0.007017865	Sipa1	0.002428551
		Adap1	0.00700769		

Pld3	0.059026066	Slc4a3	0.007005262	Abca1	0.002425897
Fgf9	0.058274754	Bmpr2	0.007002135	Far2	0.002394828
Syng3	0.057693965	L1cam	0.006970776	Slc6a1	0.002390957
Hpcal1	0.057410492	Plppr4	0.006968508	Ptprg	0.002386119
Stau2	0.056683231	Atp1a2	0.006950629	Clic4	0.002382802
Gm9385	0.056593293	Retreg2	0.006905812	Col4a2	0.0023782
Mapre2	0.056419467	Sez6l	0.006904116	Vwa1	0.002377549
Cnrip1	0.056365616	Slc38a1	0.006902321	Sgms1	0.002371985
Ppfia2	0.056231031	Meis2	0.006847915	Slc39a8	0.002350833
Ccl27a	0.055781456	Slc22a17	0.0068347	Mxd4	0.00234048
Atp6ap2	0.054881327	Tpm4	0.006831263	Slc1a2	0.00233666
Mier3	0.054809764	Aldoc	0.006825386	Cavin1	0.002331734
Golga7b	0.0548011	Psd3	0.006798578	10-Sep	0.002331678
Gadd45g	0.052652196	Aig1	0.00679691	Lamc1	0.002328585
Paxx	0.051803725	Elavl3	0.006793982	Itpr1	0.002313473
Nt5c3b	0.051531081	Atp1b2	0.006770667	F11r	0.002313136
Slc25a11	0.051407217	Scn1a	0.006758815	Acsbg1	0.00228932
Pak1	0.051214569	Rtn1	0.006734811	Myo10	0.002288767
St8sia3	0.050914398	Kndc1	0.006723877	Mlc1	0.002288188
Atp6v0e2	0.050714336	Kifc2	0.006710337	Mgll	0.002275692
Necap1	0.05060316	Trank1	0.006705184	Lrig1	0.002266684
Slc25a36	0.050462755	Sez6l2	0.0066702	S100a13	0.00225818
Cdk5	0.049873205	Ech1	0.00666727	Tiam1	0.002254456
Hspbp1	0.048933714	Cpe	0.006660115	Tmem229a	0.002226426
Cish	0.048735031	Gaa	0.006658112	Msn	0.00221992
Resp18	0.048520345	Asrgl1	0.006652556	Tril	0.002217867
Mapk10	0.047582537	Slc7a1	0.006643915	Tpp1	0.00221627
Chordc1	0.047474282	Ctnna2	0.006626428	Eogt	0.002191104
Atp6v1h	0.047398809	Gprasp1	0.006554047	Itgb1	0.002188178
Kitl	0.046121879	Napb	0.006548892	Hepacam	0.002172101
Ly6e	0.045758966	Agtpbp1	0.006540894	Amotl1	0.002152678
Tagap1	0.045207193	Prkar2b	0.006537933	Cd2ap	0.002147948
Tpm3	0.045089312	Carmil3	0.006522771	Zfhx3	0.002145848
Lypla1	0.044549745	Gm44509	0.006514539	Ifngr1	0.002136968
Atp1a3	0.043818325	Camk4	0.006510438	Gm44645	0.002123342
Acot7	0.043608316	Dgkd	0.006492896	Rhoj	0.002070165
Tspyl5	0.043091452	Zfp385b	0.006492549	Rell1	0.002064542
Eif5a2	0.042920019	Pabpn1	0.006481929	Slc1a4	0.002063398
Nlgn3	0.042529052	Fam171b	0.006478818	Vasp	0.002058831
Mir670hg	0.042453452	Stmn3	0.006398133	Notch2	0.00205684
Dusp26	0.042450792	Pfkip	0.006360962	Mfge8	0.002047821

Fstl1	0.042344963	Cers6	0.006357793	Wwtr1	0.002046542
Cdh10	0.04186374	Gm42616	0.006341389	Virma	0.0020389
Churc1	0.041840507	Kctd5	0.006325212	Enpp2	0.002038027
Gng3	0.041341603	Mylk	0.006310012	Mansc1	0.00202587
Alg2	0.040309567	Gm32710	0.006300858	Wasf2	0.002024918
Dync1i1	0.040254555	Prkce	0.006277888	Tcf12	0.002024846
Tmem24 6	0.039974521	Ednrb	0.006270446	Mecom	0.002013689
Scg5	0.039765812	Dlat	0.006268034	Kcnj10	0.002012049
Fam81a	0.039693499	B830012L14 Rik	0.006263885	Rhoq	0.001958072
Myh9	0.03963198	E430014B02 Rik	0.00625734	Hdac7	0.001923562
Gm2678 2	0.03947594	Gm37899	0.006255501	Pde8a	0.001916748
Plppr1	0.038846363	Tnik	0.006253683	Serinc5	0.001914825
Hapln1	0.038789721	Kcnt1	0.00625044	S100a16	0.001909203
Slc38a2	0.038676876	Tmem130	0.00620785	Ctdsp2	0.001907812
Mkrn1	0.038524056	Gm42372	0.006192739	Serpinh1	0.001902555
Amph	0.038368289	Gstm1	0.006191767	Tnc	0.001886919
Timm9	0.038242162	Arhgap31	0.006188814	Igsf11	0.001882263
Sae1	0.037955842	Ptpre	0.006135238	Tns3	0.001879478
Ppp1r1a	0.037704295	Zdbf2	0.006127055	Slc38a3	0.001855991
Gmfb	0.037704126	Heg1	0.006103619	Tsc22d4	0.001849681
Rab31	0.037140667	Dbn1	0.006062574	Plat	0.00184769
Gap43	0.036796678	Cd164	0.006059774	Anxa2	0.001827493
Kcnip1	0.036680907	Strbp	0.006055049	Gimap6	0.001821371
Cend1	0.036294116	Scn8a	0.006051743	Pcdh17	0.001809308
Ndufs8	0.035945347	Gprasp2	0.006041472	Ppm1f	0.001797062
Grina	0.035767154	Tex2	0.006038503	Gjc1	0.001793769
Crmp1	0.034884016	Mfap1a	0.005997444	Itm2a	0.001785562
Grb14	0.034690661	Lmna	0.005990208	Tmem98	0.001775678
Ramp3	0.034527858	Arhgap33	0.005957654	Cbfa2t3	0.001769664
Atp6ap1	0.034477195	Hs3st5	0.005934435	Mfsd2a	0.001763867
Bsg	0.034336747	Apba1	0.005933894	Tspan14	0.001753734
Nkiras2	0.034334614	Ntm	0.005912787	Sema6d	0.001740825
Acd	0.034310427	Col4a1	0.005910668	Ctla2a	0.00173719
Abcg1	0.034248926	Cyp26b1	0.00587447	Rest	0.001736752
Lrrc4c	0.034239903	Arf3	0.005868072	Shisa1	0.001733129
Chgb	0.033990783	Ttbk2	0.00586753	Emcn	0.001728976
Cd63	0.03332204	Kcnk3	0.005845117	Rgs12	0.001726746
Jakmip2	0.033161351	Dnm1	0.005839224	Arhgef26	0.001712257
Arhgap4 4	0.032795068	Dscam	0.005817503	Slc25a18	0.001707533

Tmeff1	0.032622536	Camk2n2	0.005809276	Ets1	0.001695402
Ciapi1	0.032428859	Eml5	0.005809108	Cdkn1a	0.001671542
Tox2	0.03240889	Phf24	0.005763639	Pon2	0.001659567
Csdc2	0.032302218	Afap1	0.005751906	Calcr1	0.00165903
Nipsnap1	0.032098034	Lgals9	0.005751616	Ramp2	0.00165883
Ndr3	0.031874889	Mdga2	0.005738845	Dab2	0.001643194
Tmem59l	0.031753142	Mtus1	0.005726261	Lxn	0.001610873
Yars	0.031727549	Slc32a1	0.005707703	Cdkn1c	0.00161047
Tsnax	0.030961863	Rgs8	0.005704283	Jam2	0.001610116
Pcp4	0.030890159	Adgrl2	0.005680069	C130074G19	
Reep2	0.03061444	Hsdl2	0.005666017	Rik	0.001585666
Meg3	0.030488295	Lrp1	0.005649037	Cald1	0.00157981
Gabrg2	0.030228035	Dzip1	0.005617884	Ugt8a	0.001571516
Rgs2	0.029841145	Shank2	0.00561337	Notch1	0.001568165
Arl4a	0.029530318	Fam155a	0.005596144	Vamp8	0.001563244
Ica1	0.029242189	Akap11	0.00559562	Kank1	0.001551245
Apbb2	0.029220738	Ttc3	0.005588056	Dap	0.001549927
Ube2d1	0.029140151	Srpr	0.00558554	Olig2	0.001548853
Phyh	0.028872508	C230071H17		Myo1b	0.00151892
Tcaf1	0.028750666	Rik	0.005577069	Sall3	0.001514908
Map1lc3		Gm44831	0.005573263	Tram2	0.001495189
a	0.028565106	Dgkg	0.00552189	Epas1	0.001490342
Ddx25	0.028295958	Rusc1	0.005519141	Tax1bp3	0.001483096
Nap1l2	0.027971743	Khdrbs2	0.00546469	Ntn1	0.00147764
Cntn1	0.027729224	Slc1a3	0.005464267	Slc2a1	0.001460875
Syngap1	0.027728477	Nova1	0.005459402	Rgs5	0.001460758
Ccdc184	0.027591223	Ank2	0.005414105	Lef1	0.001456823
Gad1	0.027225724	Cdk5r1	0.005381358	Ildr2	0.001438972
Atp6v1a	0.027117227	Rbfox3	0.005336101	Mfap2	0.001427791
Sgpp2	0.026937875	Nostrin	0.005335956	Pmepa1	0.001416027
Anxa5	0.026633299	Me3	0.00533146	Zc3hav1	0.001408012
Cacna1a	0.026515896	Arhgef7	0.005326955	Ppic	0.001398648
Tmod2	0.026433917	Gja1	0.005284182	Slco1c1	0.001392979
Gdap1l1	0.026334389	Mroh1	0.005281924	Slco2b1	0.00138432
Kif3b	0.025885371	Pip5k1c	0.005279215	Zcchc24	0.001368175
Adk	0.025806866	Syt2	0.005266928	Hes1	0.001366002
Lsm1	0.025782602	Gramd1a	0.005244269	Vwf	0.001365562
Kctd13	0.025649267	Pclo	0.005241736	S1pr1	0.001331141
Rdh13	0.025079656	Vamp2	0.005228223	Arhgap29	0.001320296
Arxes2	0.02506446	Apoe	0.005211235	Ctnna1	0.001313693
Lin7a	0.025027673	Usp19	0.005210658	Nr3c1	0.001305894

Ly6h	0.024909462	Esrrb	0.005189218	Cmtm6	0.001302106
Sike1	0.024643389	Igfbp7	0.005187894	Slc38a5	0.001297899
Trim32	0.024641642	Gpm6b	0.005185083	Fn1	0.001296632
Stmn2	0.024588321	Cdk16	0.005179862	Tagln2	0.001285636
B3galt2	0.02451765	Rb1cc1	0.005140841	Al464131	0.001273551
Sirt3	0.024341244	Atp2a2	0.00513512	Adamtsl2	0.0012691
Acsl4	0.024146118	Atp11b	0.00512535	Rhoc	0.001254984
Sult4a1	0.024135221	Syt1	0.005118835	Fxyd5	0.001242725
Unc5d	0.023996623	Tenm4	0.005105252	Pdpn	0.001240762
Pgm2l1	0.023954578	Gria1	0.00507962	Clic1	0.001239435
Kcnma1	0.023946759	Insig1	0.005072441	Vcam1	0.001234454
Pfkm	0.02390788	Efr3b	0.00506069	Slc40a1	0.001218826
Sfxn5	0.023882572	Dixdc1	0.005053886	Cd93	0.001216259
Agap2	0.023418942	Lynx1	0.005052503	Ocln	0.001213748
Dnajc6	0.023357266	Slc1a1	0.005030859	Palmd	0.001191273
Lmo2	0.023318165	Pcdh8	0.00503084	Agt	0.001174772
Ppif	0.023019292	Sema5a	0.005029807	Ptprb	0.001170139
Glrbl	0.022850307	Pcdh9	0.005015691	Slc22a8	0.001140571
Nap1l5	0.022807413	Ptprn	0.005011924	Cd34	0.001140225
Kcnd2	0.022765455	Hdac9	0.005002676	Ushbp1	0.001130606
Al593442	0.022696138	Fosl2	0.004997968	Cpt1a	0.001117245
Mgat5	0.022358316	Stac2	0.004967708	Tgfbr2	0.001110432
Ndn	0.022126284	Slc2a3	0.004956487	Utrn	0.001108348
Ctnnd2	0.021957946	Hivep3	0.004951665	Adgrf5	0.001075045
Fkbp1b	0.021931861	Mtftp1	0.004911576	S100a11	0.001067598
Gm9911	0.021918383	Abhd2	0.004877653	Zfp361l	0.001067154
P4hb	0.021656926	Ppargc1a	0.004870295	Acot1	0.00105052
Actl6b	0.02157212	Fam49b	0.004854614	Angptl4	0.001044727
Vmp1	0.02143736	Ralgapa1	0.004809704	Jcad	0.00101915
Rnf227	0.02110901	Gabra3	0.004809526	Nid1	0.001010815
Fth1	0.021029581	Ecpas	0.004809136	Cldn5	0.000987355
Tro	0.020949215	Nr2f1	0.004807946	Gng11	0.000969703
Tusc2	0.020924402	Gm12709	0.004794837	Cyyr1	0.000967368
Map7d2	0.020830701	Slc9a3r2	0.00479445	Arap3	0.000964178
Hras	0.020433975	Myo16	0.0047887	Rgma	0.000961408
Hs6st2	0.01989728	Plxnb1	0.004784827	Tmem123	0.000944461
Lrrc49	0.019865653	Usp31	0.004783854	BC028528	0.000917007
Gng4	0.019621023	Pigs	0.004771184	Grap	0.000916463
Ddx1	0.019538279	Mt1	0.00475928	Prex2	0.000901412
St3gal5	0.019419021	Hadh	0.004742508	Jup	0.000900318
Shtn1	0.019399214	Rapgef6	0.004733547	Pglyrp1	0.00089454
Arxes1	0.019394065	Npdcc1	0.004706672	Flt1	0.000879374
Stx1b	0.019339585	Cmip	0.004705819	Fli1	0.00087932

Rims1	0.019248365	Sema4b	0.004686824	Kdr	0.000861563
Trnt1	0.019177502	Slc35f1	0.004684811	AU021092	0.000857765
Cyfip2	0.019096538	Tcf7l2	0.004667641	Podxl	0.00084342
Tln2	0.018958963	Lrfrn5	0.00466742	Anxa3	0.000838802
Dpysl4	0.018739738	Tenm2	0.004667256	Ly6c1	0.000835517
Fam169a	0.018698002	Aqp4	0.004665512	Rasgrp3	0.000832669
Glr2	0.018643272	Gnai2	0.004661107	Gbp7	0.000817792
Nap1l3	0.018630565	Slc24a3	0.004651274	Pecam1	0.000811489
Elavl4	0.018601692	Chga	0.004650637	Thsd1	0.000760502
Kcnn1	0.01851321	Scrt2	0.004648546	Arhgdib	0.000722367
Scn2a	0.018423164	Bsn	0.004646594	Cobll1	0.000719858
Parp6	0.018241713	Grm3	0.004638482	Pltp	0.000707411
Endod1	0.018154867	Celsr2	0.004624729	Lama4	0.000699447
Emc4	0.018094503	Col11a1	0.004617707	Nes	0.000695976
Slc16a1	0.018035193	Rab11fip4	0.004610498	Apcdd1	0.000689223
Nsf	0.017955889	Hcn1	0.004572281	Tmem204	0.000688286
Inpp4a	0.017901359	Slc6a5	0.004561123	Gm13597	0.000686233
Appl1	0.017871196	Igfbp3	0.004552511	Adgre5	0.00067878
Zmat4	0.017631594	Sv2c	0.00455022	Ecscr	0.000673182
Fbxo44	0.017554709	Kcna1	0.0045457	Srgn	0.000664164
Map9	0.017417527	Pou3f3	0.00450319	Hmgcs2	0.000663993
Rps27l	0.017402635	Isyna1	0.0044914	Pdgfrb	0.000661261
Miat	0.017374893	Map6	0.004490357	Fzd6	0.000656835
Ckmt1	0.017301022	Snap91	0.004483268	Abcb1a	0.000645785
Slc39a1	0.01726466	Adcyap1r1	0.004473473	Igfbp4	0.000627099
Esrrg	0.017254914	C2cd5	0.004463656	Foxf2	0.000604713
Fam57b	0.017092207	Sv2a	0.004459033	Cnn2	0.000600533
Ache	0.01704121	Kcnh7	0.004448207	Egfl7	0.000586693
Armxc3	0.017021667	Cep170b	0.004444451	Cdh5	0.000545138
Sdr39u1	0.01700422	Kcnq2	0.004432617	Slc46a3	0.000521698
Nbl1	0.016934298	Ttyh3	0.004431188	Tek	0.000506144
Ppp2r2b	0.016909761	Chm	0.004429204	Adgrl4	0.000488704
Atat1	0.01686562	Tshz2	0.004427012	Htra3	0.000484674
Fabp5	0.016825995	Adgrg1	0.00442508	Cgnl1	0.000478584
Cacna2d3	0.016792593	Tyro3	0.004423422	Icam2	0.000463074
Mt2	0.016781642	Kcna2	0.004412194	Uaca	0.000442749
Ankrd45	0.016755821	Grik3	0.004404115	Ucp2	0.000435857
Nsg2	0.016746825	Rims2	0.004388011	Arpc1b	0.000384613
Ckb	0.016696654	Astn1	0.004371127	Acvrl1	0.000330082
Fastk	0.016548815	Unc80	0.004351675	Il2rg	0.000319177
Atl1	0.016548533	Ccdc141	0.004336798	Rras	0.000316068
Sptbn2	0.016531055	Clstn3	0.004334568	Ifitm3	0.000297995

Ogdh	0.016505731	C230085N15	Rik	0.004333212	Eng	0.000254859
Tsc22d1	0.016315121	Satb1	Satb1	0.004307704	Robo4	0.000152908
Apba2	0.016196781	Prickle1	Prickle1	0.004303594		
Cacnb4	0.016127031	Ncan	Ncan	0.004301127		
Mgst3	0.01603868	Slc39a10	Slc39a10	0.004300714		
Ift81	0.016009874	Amn	Amn	0.004274829		
Ankrd29	0.015923489	Kcnb2	Kcnb2	0.004274217		
Nedd4l	0.015913408	Cntn5	Cntn5	0.004268451		
Nop53	0.015884077	Rian	Rian	0.004233523		
Pantr1	0.015876102	Unc13a	Unc13a	0.004214042		
Fxyd7	0.015727412	Slitrk1	Slitrk1	0.004211021		
Gnai1	0.015720737	Ablim1	Ablim1	0.004203632		
Pfdn2	0.015634876	Qk	Qk	0.00420237		
Sox6	0.015516105	Bin1	Bin1	0.004199067		
Fbxo7	0.015496099	Chpf	Chpf	0.004197519		
Cadm2	0.015438493	Kif1a	Kif1a	0.004196166		
Akap7	0.015415331	Ncam1	Ncam1	0.004189301		
Pcbp3	0.01531239	Lhfpl4	Lhfpl4	0.004187066		
Ncs1	0.015235101	Fam189a1	Fam189a1	0.004186648		
Ext1	0.015230643	Tmem181a	Tmem181a	0.004184525		
Trp53	0.015156822	Fbxo9	Fbxo9	0.00417661		
2-Sep	0.015052981	Frmd4b	Frmd4b	0.004171993		
Strn	0.014995052	Stum	Stum	0.004152743		
Ncoa7	0.014971812	Snhg14	Snhg14	0.00413683		
Atp2b2	0.014889142	Gng12	Gng12	0.004130004		
Gm1080 0	0.01487807	Ndrp2	Ndrp2	0.004127264		
Lamtor3	0.014807751	Slc6a6	Slc6a6	0.004121577		
Tulp4	0.014693824	Trpc3	Trpc3	0.004117783		
Kcnq1ot1	0.014686489	Akap8l	Akap8l	0.004117715		
Tex264	0.014648501	Cux2	Cux2	0.004114776		
Slc39a6	0.014578757	Hspg2	Hspg2	0.004112728		
Rnd2	0.014567907	Syt11	Syt11	0.004112214		
Syp	0.014543663	Glud1	Glud1	0.004109464		
Tmem25	0.014526703	Cntnap2	Cntnap2	0.004101481		
Tmem38 a	0.014260785	Scd2	Scd2	0.00409461		
Snx16	0.014200798	Sox9	Sox9	0.004092421		
Raly1	0.014065119	Nrxn3	Nrxn3	0.004089944		
Socs2	0.013994646	Ptn	Ptn	0.004089666		
Lrp11	0.01387575	Meis1	Meis1	0.004076783		
Gm1819 4	0.013758554	Vwa5b2	Vwa5b2	0.004072264		

Ap3b2	0.013694587	Ano4	0.004045931
Cst3	0.013674847	Sorbs2	0.004042081
Rsrc1	0.013573517	Ralgps2	0.004039795
Pnma2	0.013522997	Slc12a5	0.004015765
Ppp2r2c	0.013367119	Osbpl6	0.004001993
Sc5d	0.013233249	Lrch3	0.003977765
Cadps	0.013211616	Decr2	0.003970406
Mpzl1	0.013165686	Fgfr3	0.00396826
Vopp1	0.013146747	Kcnc1	0.003941625
Rnf152	0.013080328	Plpp3	0.003924951
Zcrb1	0.013045791	Epb41l4b	0.003924429
Mt3	0.012940893	Grm5	0.003921393
Tmx4	0.012917907	Zzef1	0.003897388
Crtac1	0.012901454	Ostf1	0.003896869
Gm1420 4	0.0128993	Cnp	0.003889714
Oxct1	0.012821585	Mcc	0.003880729
Nptn	0.01266619	Nfasc	0.003874829
Fhl1	0.012634631	Ncam2	0.003859399
Tmem22 9b	0.012550594	Dip2c	0.003856963
Pop5	0.012530263	Lpgat1	0.003855972
Pitpnc1	0.012424082	Grm7	0.003855012
Retreg1	0.012401668	Gm37363	0.003854124
Zfpm2	0.012396638	Sf3b3	0.003842282
Mapk8ip 2	0.012372866	Opcml	0.003838112
Gm4483 0	0.012347377	Kcnn2	0.00382455
Dcun1d4	0.012271113	Reep1	0.00382222
Map3k20	0.012270914	Plekha6	0.003818524
Cd99l2	0.012256877	Sez6	0.003818334
Cntnap1	0.012093626	Slc27a4	0.00380592
Nsg1	0.012038726	Mbp	0.003795838
Pcdh7	0.01200947	Sacs	0.003773369
Akap6	0.012007582	Grin3a	0.00376426
Gas6	0.011897485	Tal1	0.00375866
Cx3cl1	0.01189407	Abca5	0.003753825
Lin7b	0.011891686	Fcho2	0.003753509
Coro2b	0.011557417	Ttyh1	0.00374855
Adam10	0.011460971	Epb41l2	0.00374544
Nampt	0.011397822	Pea15a	0.003729375
Hspa12a	0.01137798	Chd5	0.003726313
Rcn2	0.011348633	Atp1b1	0.003723889

Thy1	0.011263652	Myh10	0.003716436
Ncdn	0.011249464	Znrf2	0.003715147
Aplp1	0.011230101	Mtss1l	0.003700647
Fyn	0.011226027	Snhg11	0.003694205
Mtx2	0.011176148	Cdk6	0.003688489
Synj1	0.011154174	Clmp	0.003666909
Dpysl5	0.011105747	Srgap3	0.003666782
Pdhx	0.01094847	Gbbp1l1	0.00365096
Fam20c	0.010934437	Cacna1e	0.003630482
Dram2	0.010900763	Ddhd1	0.003630365
Wnk2	0.010879688	Vcan	0.003629608
Ndel1	0.010877443	Sparc	0.00362837
Spry2	0.010829204	Etv5	0.003574576
Mapre3	0.010794318	Adarb2	0.003567091
Arid1a	0.010765441	Sesn3	0.003557102
Cox10	0.010690836	Tdrp	0.003556686
Srcin1	0.010622934	Setd5	0.003549899
Brinp1	0.010602637	Serpine2	0.003544991
Abcc5	0.010572323	Tnr	0.00353667
Pcmdt1	0.01054576	Bcan	0.003528484
Nxph1	0.010493156	Slc7a5	0.003527306
Rcan2	0.010492064	Gsg1l	0.003518893
Edil3	0.01048128	Sox1ot	0.003513698
Dusp8	0.010364485	Selenop	0.003511868
Cpeb4	0.010304203	Bcas1	0.00349811
Ccdc85a	0.010139142	Dock1	0.003486756
Ptpsr	0.010100375	Jarid2	0.003480847
Serpini1	0.010083507	Baz1b	0.003477863
Srxn1	0.00989014	Grb2	0.00347294
Camta1	0.009783135	Samd4b	0.003450585
4-Sep	0.00977416	Madd	0.00343859
Hbp1	0.009718318	Eno2	0.003435611
lpo11	0.009681272	Hipk2	0.003426633

Supplemental Table 15. Random Forest analysis. Genes used for clustering were analyzed with the Random Forest algorithm to determine variables that separate N1 and N2 clusters. Genes are listed from highest importance (highest Mean Decrease in Gini Score) to lowest importance (lowest Mean Decrease in Gini Score).

Chapter 4: Discussion and Future Directions

The work described in Chapter 2 and Chapter 3 adds meaningful information to the literature regarding cellular composition of the MNTB across development and cell type-specific transcriptional regulation during MNTB maturation. The major findings of this work include characterization of a period of rapid glial cell proliferation and identification of major intercellular signaling pathways that are likely important in regulating MNTB tissue maturation. These results will now be discussed in the broader context of what is known about neural circuit development in the MNTB and remaining questions to be addressed.

Connecting temporal gene expression profiles to changes in cell number

In a developmental microarray study performed on microdissected MNTB tissue, Kolson and colleagues (2016) reported a list of 541 genes which were developmentally regulated between P0 and P6. The list of genes contained a variety of cell adhesion molecules, ion channels, growth factors and calcium ion binding proteins, providing insight into transcriptional regulation of the structural changes which occur during neural circuit formation in the MNTB. A major strength of the MNTB microdissection approach was that it allowed for a highly specific isolation of cells in the MNTB, without contamination from cells residing in nearby auditory nuclei. This study was the first report of developmental transcriptional regulation specifically in the MNTB nucleus. However, the bulk tissue analysis did not allow for cell type-specific assignment of transcript expression. An attempt was made to assign developmentally regulated transcripts to a specific cell type based upon reports of cell type-specific transcript expression in the developing cortex (Cahoy et al., 2008). In this analysis, transcripts were assigned to “neuron”, “astrocyte”, “oligodendrocyte” or “unspecified”. Microglia, vascular endothelial cells and pericytes were not included in the cortical dataset and thus were not included as options for cell type assignment in the MNTB dataset (Kolson et al., 2016). Upon examination of the temporal profiles of

developmentally regulated transcripts in relation to cell type-specific expression classification, it became apparent that the majority of up-regulated transcripts were enriched in glial cells (astrocytes or oligodendrocytes) and the majority of down-regulated transcripts were enriched in neurons. This finding immediately raised the question of whether up-regulation of glial-enriched transcripts was due to an increase in glial cell number across development (postnatal gliogenesis) or represented true up-regulation of these transcripts on the level of single cells. A lack of knowledge of the cellular composition of the MNTB across development impeded comprehensive interpretation of the results from Kolson and colleagues (2016), necessitating a developmental cell counting study to be performed.

Brandebura and colleagues (2018) used cell type-specific antibodies for neurons, astrocytes, oligodendrocytes and microglia at P3 and P6 to report the percentage of each cell type at those ages. The results indicated a decrease in the neuron:glia ratio between P3 and P6, with a near doubling in the percentage of oligodendrocytes. The microglia were scarce in number, but the microglia also doubled in their percentage. The astrocyte percentage remained relatively constant (Brandebura et al., 2018). Because the neurons in the MNTB do not proliferate or undergo apoptosis during the first postnatal week (Rodríguez-Contreras et al., 2006; Saliu et al., 2014), the increase in glial cell percentage indicates an increase in the total number of glial cells in the MNTB across development, either through migration or proliferation. Thus, the up-regulation of glial-enriched transcripts identified in the study by Kolson and colleagues (2016) requires careful interpretation of the extent of up-regulation compared to the increase in cell number across development. Assuming a constant neuronal number (no proliferation or apoptosis as previously stated), a decrease in the neuronal percentage from 46.6% of total cells at P3 to 36.9% of total cells at P6 indicates a 26.3% increase in total cell number between these developmental timepoints. The relative percentages of astrocytes, oligodendrocytes and microglia at P3 and P6 could then be used to calculate a fold-change in their cell numbers.

These results indicate a 1.1-fold change in astrocytes, 2.3-fold change in oligodendrocytes and 2.3-fold change in microglia (Brandebura et al., 2018). The significance of these findings is that changes in cell numbers can then be used to better interpret whether up-regulation of glial-enriched transcripts in the dataset from Kolson and colleagues (2016) was solely due to an increase in cell number or due to true up-regulation of the transcript. For example, a transcript known to be enriched in oligodendrocytes which only increased 2-fold from P3 to P6 would be purely due to a 2.3-fold increase in oligodendrocyte cell number, but an oligodendrocyte-enriched transcript which increased more than 2-fold would be due to up-regulation of the transcript on a single cell level. Thus, the connection of changing cell numbers to developmental regulation of glial-enriched transcripts was a valuable contribution to the field.

Assignment of developmentally regulated transcripts to cell types in the MNTB

As mentioned above, the developmental cell counting study performed by Brandebura and colleagues (2018) aided in interpretation of temporal gene expression profiles. The connection of cell type-specific transcript expression from the Cahoy (2008) dataset in cortex to changing cell numbers in the MNTB was useful when a transcript of interest was known to be differentially expressed within a specific cell type. However, many of the developmentally regulated transcripts identified in the Kolson (2016) dataset were listed as “unspecified” due to lack of cell-specific expression as reported by Cahoy and colleagues (2008). In the cases of developmentally regulated transcripts categorized as “unspecified” there existed a need to determine cell type-specific expression in the MNTB. Furthermore, it was not known whether the cell type-specific expression data from the cortex accurately reflected cell type-specific expression patterns in the MNTB. Thus, a scRNA-Seq study was performed on microdissected MNTB tissue at P3, allowing for the generation of cell type-specific transcriptional profiles within the MNTB.

A hierarchical clustering approach yielded transcriptional profiles for neurons, astrocytes, oligodendrocytes and VACs (vascular endothelial cells and pericytes combined). These profiles were useful in determining cell type-specific expression patterns for developmentally regulated transcripts identified in the MNTB. One major group of transcripts identified as developmentally regulated in the microarray study included transcripts involved in the formation of perineuronal nets, a specialized type of extracellular matrix known to surround neurons in the MNTB (Schmidt et al., 2010; Blosa et al., 2013). Importantly, the cellular specificity of these transcripts in the MNTB was not known. The scRNA-Seq study identified neuronal expression of *Hapln1* transcript and glial (astrocyte and oligodendrocyte) expression of transcripts encoding for the tenascin and chondroitin sulfate proteoglycan components of PNNs. The addition of cell type-specific expression patterns for PNN-associated transcripts led to the generation of a new working model for PNN formation in the MNTB. By combining cell counts, temporal gene expression profiles and cell type-specific expression data, we propose that neurons initiate secretion of the Hapln1 linker protein binding scaffold, which is detected at the protein level by P0 (Kolson et al., 2016). Glial-mediated secretion of the tenascin and chondroitin sulfate proteoglycan proteins is delayed by several days, as indicated by the temporal expression profiles obtained in the microarray study (Kolson et al., 2016) and matching the increase in glial cell numbers observed in the cell counting study (Brandebura et al., 2018). Knowledge of cell type-specific expression patterns for PNN-associated transcripts now allows for conditional genetic knockout studies to be performed, which will be useful in evaluating the phenotypic consequences of genetically deleting individual PNN components and understanding each cell type's role in PNN formation.

Heterogeneity in cell type-specific gene expression across brain regions

It is a reasonable assumption that transcriptional programs may need to vary between brain regions to accomplish maturational changes that are specific for that region. Along those lines,

cell type-specific transcript expression may vary between brain region, and thus it is of emerging importance to perform cell type-specific transcriptional profiling in different brain regions and identify differences between them. Interestingly, one PNN-associated transcript, *Hapln1*, was differentially expressed between the cortex dataset generated by Cahoy and colleagues (2008) and the MNTB dataset. In the cortex *Hapln1* is most highly expressed in astrocytes (Cahoy et al., 2008) and in the MNTB *Hapln1* is most highly expressed in neurons (Chapter 3; Figure 6). These results highlight the importance of evaluating regional differences in cell type-specific transcriptional profiles. The difference in cell type-specific expression of *Hapln1* transcript in cortex vs. MNTB may reflect regional differences in the process of PNN formation. Alternatively, there could be differences in the cell type-specific expression of *Hapln1* transcript across developmental ages because the Cahoy dataset (2008) was generated from P7 cortex and the MNTB dataset was generated from P3 MNTB. Whether a switch in the cell type-specific expression pattern of *Hapln1* transcript from neurons to astrocytes occurs across development remains to be determined. An expansion of the scRNA-Seq dataset to multiple developmental timepoints will be necessary to evaluate which transcripts remain constant in their cell type-specific expression patterns across development and which transcripts may switch expression patterns.

Transcriptional profiling of subtypes of cells

As mentioned above, the scRNA-Seq study resulted in transcriptional profiles for neurons, astrocytes, oligodendrocytes and VACs. Two clusters were generated for MNTB neurons, which have historically been characterized as a mostly homogeneous cell population in the mouse (Banks & Smith, 1992). A RFA identified the top transcripts responsible for pushing the captured neurons into two clusters. However, antibody labeling with several of these candidates (Prpf2, Rgs10, Rit2) revealed homogeneous labeling in the MNTB neurons (data not shown). It is unclear at this time if the two neuronal clusters obtained in the scRNA-Seq study represent

distinct neuronal populations or instead represent slight differences in transcript expression level which may be related to maturational state, tonotopic position, cell size or level of synaptic activity. Future studies utilizing methods like Patch-Seq (electrophysiological analysis followed by transcriptional analysis) will need to be performed for correlation of transcript expression level in the neurons with the tonotopic positional identity of the neuron and level of synaptic activity.

An expansion of the scRNA-Seq dataset to include a larger number of cells would allow for the generation of additional transcriptional profiles, representing subtypes of cells. For example, the VAC cluster included both endothelial cells and pericytes. The cluster contained too few cells to split this group into two separate clusters. Likewise, a larger number of cells may allow for dividing the oligodendrocyte and astrocyte cell clusters into smaller subdivisions which could reflect different developmental states of oligodendrocytes and astrocytes. Microglia, which are scarce in number at P3, were not captured at a high enough frequency to generate a transcriptional profile for this cell type, but a larger sample size and alternative experimental methods would allow for generation of a transcriptional profile for microglia, as well as evaluation of differences between activated and resting microglia.

Gliogenesis in the MNTB

A study performed by Saliu and colleagues (2014) reported a peak in the number of MNTB cells that incorporated EdU at P2 that remained relatively constant until P12, after which the numbers of EdU-labeled cells rapidly decreased. S100 β co-staining was then used at 3 and 7 days post-injection to determine differences in the percentage of EdU-labeled cells which undertook an astrocyte cell fate when EdU was injected at ages E20, P1 and P6. The results indicated that the majority of astrocyte proliferation occurs at late embryonic and early postnatal ages because when EdU was injected at E20 over half of the cells (~59%) were also S100 β -positive. When EdU was injected at P1, ~30% of EdU-labeled cells became S100 β -positive. However, when

EdU was injected at P6, less than 10% of EdU-labeled cells adopted an astrocyte fate, indicating that astrocytes proliferate in a late embryonic to early postnatal wave. It is still unclear at this point whether all MNTB astrocytes become S100 β -positive or whether S100 β -positive astrocytes are a specific subpopulation. If S100 β -positive astrocytes represent a specific subpopulation, the numbers obtained in the study performed by Saliu and colleagues (2014) may be an underestimate.

The peak levels of EdU incorporation do not decline until after P12 and the S100 β co-staining experiments demonstrated that less than 10% of the EdU-labeled cells undertook an astrocyte cell fate when EdU was injected at P6. Furthermore, there were no observations of neurons (labeled with NeuN) that incorporated EdU at any of the ages tested. Together these results indicate that the majority of EdU-labeled cells at later postnatal ages are neither neurons nor astrocytes. Because the microglia are scarce in number (1% of total cell population at P3 and 2% of total cell population at P6; Brandebura et al., 2018), the proliferating cells in the MNTB up until P12 most likely undertake an oligodendrocyte cell fate. In support of this idea, it was demonstrated that over 85% of Sox10-positive oligodendrocyte lineage cells in the MNTB were co-labeled with the proliferation marker PCNA at ages P3 and P6 (Brandebura et al., 2018). One remaining question to be answered is the contribution of vascular endothelial cell and pericyte proliferation in the MNTB during these timepoints. The cell counts performed by Brandebura and colleagues (2018) did not include a category for vascular endothelial cells and pericytes. However, blood vessel reconstructions from SBEM volumes indicate an increase in percentage of MNTB volume occupied by blood vessels from 0.6% at P3 to 0.8% at P6 and 1.2% at P9 (Chapter 3, Figure 7C). The increase in blood vessel occupancy in the MNTB across development suggests that VACs may also be represented in the EdU-labeled cells observed in the study by Saliu and colleagues (2014). Oligodendrocyte lineage cells in the MNTB were co-labeled with the mitotic marker PH3 (Brandebura et al., 2018), indicating that at least some

proportion of oligodendrocyte lineage cells locally divide within the boundaries of the MNTB. Another interesting follow-up study would be to quantify the extent of local proliferation vs. migration into the MNTB for astrocytes, oligodendrocytes, microglia and VACs.

Unanswered questions regarding lineage of cells in the MNTB

Aldh1L1 is used as a marker for astrocytes (Cahoy et al., 2008) and *PDGFR α* is used as a marker for oligodendrocytes (Hall et al., 1996). Within the MNTB very little is known about glial cell lineages, so it was deemed necessary to characterize the labeling patterns of the *Aldh1L1*-Cre (Tien et al., 2012) and *PDGFR α* -Cre lines (Roesch et al., 2008). The characterization of these lines in the MNTB demonstrated that the *Aldh1L1*-Cre and *PDGFR α* -Cre lines have limited utility as genetic tools to specifically label or manipulate astrocytes and oligodendrocytes in the MNTB. In stark contrast to the mostly restricted labeling of astrocytes using the *Aldh1L1*-Cre line in forebrain and spinal cord (Tien et al., 2012), the *Aldh1L1*-Cre line in the MNTB labeled ~90% of neurons, ~98% of oligodendrocytes and ~40% of microglia in the MNTB. Similarly, the *PDGFR α* -Cre line was not specific to the oligodendrocyte lineage in the MNTB. This line labeled ~70% of neurons and ~35% of astrocytes in the MNTB. Future experiments should be performed to determine if the broad labeling patterns exhibited by these transgenic lines are due to true lineage effects or simply due to transient activation of the promoter.

The nearly complete labeling of the MNTB by the *Aldh1L1*-Cre line was striking in comparison to the reported ~10% neuronal labeling in spinal cord using the *Aldh1L1*-Cre (Tien et al., 2012). These results raise questions about the lineage origin of MNTB neurons. As mentioned previously, MNTB neurons are born between E11 and E12 (Pierce, 1973), but their anatomical birthplace is unknown. It was previously demonstrated that radial glial cells in the subventricular zone, subgranular zone and spinal cord express *Aldh1L1* (Anthony & Heintz, 2007; Foo & Dougherty, 2013), thereby raising the possibility that MNTB neurons may arise from an *Aldh1L1*-positive neural stem cell lineage. Interestingly, a subset of neurons in the piriform

cortex were demonstrated to arise from PDGFR α -positive stem cells (Rivers et al., 2008).

Although PDGFR α is not typically known as a radial glial cell marker, the extensive labeling of MNTB neurons in the *PDGFR α -Cre* line may suggest that these neurons arise from a lineage of neural stem cells that express PDGFR α .

It is also interesting that the *PDGFR α -Cre* line labeled ~35% of astrocytes, while the remaining 65% of astrocytes were unlabeled. These results suggest that a subset of astrocytes may arise from PDGFR α -positive OPCs. The cell fate of mitotically active OPCs is spatiotemporally regulated. Under some circumstances OPCs divide symmetrically to form oligodendrocyte lineage cells, while in some instances the OPCs divide asymmetrically to form one oligodendrocyte lineage cell and one astrocyte lineage cell. Using tamoxifen-inducible Cre lines which label OPCs, it was generally determined that OPCs can generate astrocytes during embryonic ages, but not postnatally. Furthermore, it was determined that more gray matter astrocytes arise from an OPC lineage compared to white matter astrocytes (Zhu et al., 2008, 2011; Huang et al., 2014). The ~35% of MNTB astrocytes which are labeled by the *PDGFR α -Cre* line may represent embryonically born cells which arise from an OPC lineage and subsequently differentiate into astrocytes. However, properly designed experiments using tamoxifen-inducible Cre lines will need to be performed to investigate this possibility. Overall, the Cre line characterizations performed by Brandebura and colleagues (2018) offer the first insight in the field regarding genetic lineage of glial cells in the MNTB.

Identification of directional intercellular signaling patterns

Neural circuit formation is a complex process involving intercellular signaling interactions between neurons, glia and VACs. The transcriptional profiles generated in the scRNA-Seq study allowed for cell type-specific expression analysis of transcripts encoding for ligands and receptors of major intercellular signaling pathways. Connecting the expression patterns of related ligands and receptors allowed for the identification of directional signaling patterns.

One prominent signaling pathway identified in the scRNA-Seq study was Delta-Notch signaling. The data suggest that Delta-Notch signaling in the MNTB is a tripartite signaling process, involving oligodendrocytes, VACs and astrocytes. The Delta and Jagged ligands were detected at low levels overall, but oligodendrocytes had the highest levels of *Dll1* transcript and VACs had the highest levels of *Dll4* and *Jag1* transcripts. The Notch receptors were differentially expressed in the astrocytes. Analysis of GFP reporter mouse (Duncan et al., 2005) for active Delta-Notch signaling demonstrated that astrocytes in the MNTB were engaging in Delta-Notch signaling. Interestingly, astrocyte Delta-Notch signaling occurred in a transient burst because Delta-Notch signaling was not active in the MNTB astrocytes at P0 or P6. The biological function of tripartite Delta-Notch signaling remains to be determined. Future conditional genetic knockout or dominant negative studies will need to be performed to determine the role of Delta-Notch signaling in MNTB tissue maturation.

The TGF β and VEGF pathways were also prominent within the scRNA-Seq dataset. Transcripts encoding for the TGF β and VEGF receptors were differentially expressed in the VAC cluster, while the ligands were detected in neuronal and glial cell clusters. The TGF β and VEGF pathways are implicated in angiogenesis and vascular remodeling (Rosenstein et al., 1998; reviewed by Mancuso et al., 2008). The scRNA-Seq data therefore suggests that establishment of the vasculature in the MNTB is a process which requires signaling between all major cell types in the tissue. The transcriptional data was tied to structural expansion of the vasculature in the MNTB. Between P3 and P9 there is an increase in the presence of the endothelial cell adhesion molecule, Cd31, as well as an increase in the blood vessel volume in the MNTB (Chapter 3, Figure 7). The ability to relate transcriptional data to actual structural changes which occur across MNTB development highlights the utility of the scRNA-Seq dataset in predicting signaling processes involved in neural tissue maturation. Future studies should focus on

conditional genetic knockout approaches to evaluate each cell type's contribution to expansion of the vasculature and acquisition of a tight blood-brain-barrier with low permeability.

Conclusion

The rapid growth of the CH terminal and refinement to mono-innervation of the principal neuron, along with the presence of a mostly homogeneous postsynaptic neuronal population, make the MNTB an attractive model system to study transcriptional regulation of neural circuit formation. Importantly, neural circuits do not form in isolation, and the contributions of glial cell types and VACs in tissue maturation in the MNTB, as well as in other brain regions, are largely unknown. This work provides cellular and transcriptional level information on the neuronal, glial and VAC populations across MNTB development. Findings from the MNTB as a model system can then likely be generalized to other regions of the brain.

A developmental cell counting study provided information on changes of the cellular composition of the MNTB at P3 and P6. There is a decrease in the neuron:glia ratio which is due to a rapid period of glial cell proliferation. The expansion of the glial cell numbers is mostly due to proliferation of oligodendrocyte lineage cells at these ages.

Cell type-specific transcriptional profiling provided a large dataset to analyze directional signaling patterns between neurons, glia and VACs. Through follow-up experiments, the temporal dynamics of signaling could be determined and transcriptional data could be tied to structural changes which occur across MNTB tissue maturation.

The contribution of an extensive characterization of changes in cell number and cell type-specific transcriptional profiles in the MNTB has vast implications for the studies of neural circuit formation across the brain. The directional signaling patterns identified in the scRNA-Seq dataset from MNTB can be used for hypothesis generation for future mechanistic perturbation

studies in the MNTB and elsewhere throughout the CNS. This work serves to highlight the importance of taking a whole-tissue approach to studies of neural circuit formation.

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