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Temporal Patterns of Gene Expression During Calyx of Held Development

D. R. Kolson^{1,2}, J. Wan⁵, J. Wu^{1,2}, M. Dehoff^{1,2}, A. N. Brandebura^{1,2}, J. Qian⁵, P. H. Mathers^{1,2,3,4,*}, and G. A. Spirou^{1,2,3,*}

¹Sensory Neuroscience Research Center, West Virginia University School of Medicine, Morgantown, WV

²Center for Neuroscience, West Virginia University School of Medicine, Morgantown, WV

³Otolaryngology HNS, West Virginia University School of Medicine, Morgantown, WV

⁴Biochemistry, West Virginia University School of Medicine, Morgantown, WV

⁵Wilmer Eye Institute, Johns Hopkins University School of Medicine, Baltimore, MD

Abstract

Relating changes in gene expression to discrete developmental events remains an elusive challenge in neuroscience, in part because most neural territories are comprised of multiple cell types that mature over extended periods of time. The medial nucleus of the trapezoid body (MNTB) is an attractive vertebrate model system that contains a nearly homogeneous population of neurons, which are innervated by large glutamatergic nerve terminals called calyces of Held (CH). Key steps in maturation of CHs and MNTB neurons, including CH growth and competition, occur very quickly for most cells between postnatal days (P)2 and P6. Therefore, we characterized genome-wide changes in this system, with dense temporal sampling during the first postnatal week. We identified 541 genes whose expression changed significantly between P0–6 and clustered them into eight groups based on temporal expression profiles. Candidate genes from each of the eight profile groups were validated in separate samples by qPCR. Our tissue sample permitted comparison of known glial and neuronal transcripts and revealed that monotonically increasing or decreasing expression profiles tended to be associated with glia and neurons, respectively. Gene ontology revealed enrichment of genes involved in axon pathfinding, cell differentiation, cell adhesion and extracellular matrix. The latter category included elements of perineuronal nets, a prominent feature of MNTB neurons that is morphologically distinct by P6, when CH growth and competition are resolved onto nearly all MNTB neurons. These results provide a genetic framework for investigation of general mechanisms responsible for nerve terminal growth and maturation.

*Co-corresponding authors: Peter H. Mathers, West Virginia University School of Medicine, PO Box 9303 Health Sciences Center, One Medical Center Drive, Morgantown, WV 26506-9303, pmathers@hsc.wvu.edu, George A. Spirou, West Virginia University School of Medicine, PO Box 9303 Health Sciences Center, One Medical Center Drive, Morgantown, WV 26506-9303, gspirou@hsc.wvu.edu.

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INTRODUCTION

Understanding cellular mechanisms controlling synaptic development and maturation remains one of the more elusive challenges in neuroscience. Synaptic growth and refinement are controlled by precise genetic programs that are executed in unison by neurons and glia; these cell types all interact dynamically adding additional levels of intricacy to the finely tuned and coordinated events (Clarke and Barres, 2013). To gain a better understanding of these complex events, it is advantageous to remove as many confounding issues as possible. Innervation of the medial nucleus of the trapezoid body (MNTB), in the auditory brainstem, by the calyx of Held (CH) offers several advantages over other brain regions to study gene expression during neural circuit development: (1) the MNTB is comprised of predominantly one neuronal subtype, the principal cell; (2) the CH is a large nerve terminal that originates from a homogeneous cell type (globular bushy cell) in the ventral cochlear nucleus (Fig 1a) that is easily visualized and that establishes mono-innervation of the MNTB cell; and (3) structural and functional maturation of these synaptic partners occurs over just a few days between postnatal day 2 (P2) and P6 (Kandler and Friauf, 1993; Hoffpauir et al., 2006; Rodriguez-Contreras et al., 2006; Hoffpauir et al., 2010; Rusu and Borst, 2011; Holcomb et al., 2013). Notably, these events occur before the ear canals open, when animals are exposed to environmental sounds and exhibit experience-dependent plasticity (Mikaelian et al., 1965; Ehret, 1976; Borst and Soria van Hoeve, 2012). These discrete events allow for correlation between *in vivo* gene expression in a specific brain nucleus and known developmental and environmental events that can affect levels of expression.

Previous genome-wide studies on the auditory brainstem focused on developmental profiles of normal and cochlea-ablated cochlear nuclei (Harris *et al.*, 2005), globular bushy cells within the developing cochlear nucleus which innervate the MNTB via the CH (Korber *et al.*, 2014), the entire developing superior olivary complex, using both SAGE and microarray studies (Koehl *et al.*, 2004; Ehmann *et al.*, 2013) and, more specifically, developmental comparisons of the lateral superior olive to the MNTB (Xiao *et al.*, 2013). To characterize the transcriptome of a developing neural circuit, we performed a detailed microarray study of the early postnatal MNTB that utilized dense temporal sampling before, during, and after CH formation. Our study identified 541 genes differentially expressed between P0 and P6 that were clustered into eight groups based upon their temporal profiles. More than 20 candidate genes, covering every profile group, were validated with qPCR. Gene ontology analysis identified genes involved in axon pathfinding, cell differentiation, cell adhesion, and extracellular matrix among other categories. A significant fraction of the genes were categorized as expressed in either astrocytes or oligodendrocytes.

METHODS

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

Tissue Collection and RNA Extraction

FVB mice (NCI; Frederick, MD and Jackson Laboratory; Bar Harbor, ME) were used in all experiments. Brains were quickly dissected in ice-cold, low Ca^{2+} artificial cerebral spinal fluid (ACSF) at postnatal day 0 (P0), P1, P2, P3, P4, P6, and P14. P0 was defined as being between 6 and 12 hours after birth. Littermate collections were started on P0 and then taken on subsequent days at the same time of day (± 2 hours). The low Ca^{2+} ACSF contained the following: 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. Each of the reagents was obtained from Sigma (St. Louis, MO). The ACSF solution was saturated with 95% O_2 /5% CO_2 prior to use.

Coronal tissue slices were cut from the dissected brains using a VF-200 tissue slicer (Precisionary Instruments Inc., Greenville, NC) at a thickness of approximately 200 μm and immediately placed into ice-cold, low Ca^{2+} ACSF. Only slices with discernible MNTB on both faces of the tissue were used for the experiments (Fig. 1A). The MNTB regions within the brain slice were carefully removed using a 26-gauge needle under a dissecting scope to obtain an enriched population of MNTB cells (Fig. 1A'). The dissected tissue was transferred directly into either lysis solution (microarrays; Stratagene, La Jolla, CA) or TRIzol reagent (qPCR; Invitrogen, Carlsbad, CA) and frozen at -80°C . The RNA extraction and DNase treatment were later performed using either the Absolutely RNA Nanoprep kit (microarrays; Stratagene) or the RNeasy Micro Kit (qPCR; Qiagen, Valencia, CA). An Agilent 2100 Bioanalyzer (Santa Clara, CA) was used to measure the concentration and assess the quality of the RNA.

RNA Amplification and Microarray Analysis

In order to follow a single litter across developmental ages and still obtain sufficient quantities of cDNA from a single mouse pup for hybridization to the microarrays, RNA obtained in the previous steps was amplified using the WT-OvationTM Pico RNA Amplification System (NuGEN Technologies, San Carlos, CA). Only samples with an RNA integrity number (RIN) (Schroeder *et al.*, 2006) of 5.0 or higher, as measured on the Agilent 2100 Bioanalyzer, were considered acceptable for amplification. We used 5 μg of the resulting cDNA as a template to create sense strand cDNA with the WT-Ovation Exon Module (NuGEN). This was followed by fragmentation and biotin-labeling using the FL-Ovation cDNA Biotin Module V2 (NuGEN) and hybridization to mouse GeneChip 1.0 ST Exon Arrays (Affymetrix, Santa Clara, CA). The microarrays were scanned with a GeneChip Scanner 3000 7G (Affymetrix, Santa Clara, CA).

The microarray data were normalized using Genomics Suite software version 6.3 (Partek Inc., St. Louis, MO). We used GC pre-background adjustment, RMA background normalization, and quantile normalization between samples. Our analysis was limited to the

core-level probes/genes supported by RefSeq transcripts or full-length mRNAs. Before continuing with our analyses, we removed some genes from further analysis where expression levels from all samples were within the lowest 25%, because such intensities are very close to background levels. We then used Significance Analysis of Microarrays (SAM) (<http://www-stat.stanford.edu/~tibs/SAM/>) (Tusher *et al.*, 2001) to identify the genes showing differential expression at any stage(s) from P0 to P6. P14 data was only used for clustering and profile analysis. With a one-way analysis of variance and a false discovery rate (FDR) of 0.1%, we achieved a list of 541 differentially expressed genes (DEGs) between the ages from P0 to P6. These significantly changing genes were then clustered using *k*-means with pairwise correlation metrics and *k* = 8. In order to increase weighting for the later ages, which have unsampled intervals of 2 days (P4–6) and 8 days (P6–14), only the time points of P0, P2, P4, P6, and P14 were used in the *k*-means clustering analysis. An additional *k*-means clustering analysis was performed using 15 points, which included extrapolated time points for every age between P0 and P14; this analysis yielded very similar results to the 5 point *k*-means clustering (data not shown). As a final filter, all 541 genes were graphed and compared visually within group to ensure accurate profile clustering by our algorithm. Based upon these graphs, 3 of the genes were moved into different groups that captured their profiles more accurately (*Calb1* from group 4 to 3, *Cd24a* from group 1 to 6, *Hmgcr* from group 1 to 6). Gene Ontology (GO) analysis was performed by first counting the number of genes for each GO term in our 541 DEG list and all background genes, which includes every gene determined to be expressed on the microarray. We then obtained the enrichment ratio for each GO category by comparing the relative occurrence in the DEG group to that in the background genes. The statistical significance was calculated using a hypergeometric model, adjusted with the Bonferroni multiple-test correction, and a p-value cutoff of <0.05 to identify the most highly enriched GO categories in our 541 gene list. The resulting list was manually filtered to yield a final list containing the most informative and least redundant categories (Table 3).

Quantitative Real-time PCR Analysis

Pooled RNA was extracted as described above from the MNTB of 4–6 littermates and reverse-transcribed into cDNA using SuperScript III Reverse Transcriptase (Invitrogen) and random priming. We emphasize that different animals were used for validation than were used for microarray studies. Primers (Table 1) were designed using NCBI Primer-Blast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) to cross introns and detect all known splice variants, when possible, and were synthesized by Integrated DNA Technologies (Coralville, IA). The PCR amplification efficiencies for each primer set were confirmed to be between 90% and 110%, and each set of primers was confirmed to amplify a single product by obtaining a single band on a 2% agarose gel. Quantitative real-time PCR was performed using Brilliant II SYBR Green qPCR Master Mix (Stratagene) with 250 nM of each primer and 0.2 ng of cDNA in each reaction. Each sample was analyzed in triplicate and each time point was sampled at least three times using a Stratagene MX3005P instrument (Santa Clara, CA) with cycling conditions of 95°C for 10 minutes followed by 40 cycles of 95°C for 30 seconds, 60°C for 1 minute, and 72°C for 1 minute. A melting analysis was performed at the conclusion of the run to further verify the amplification of a single product.

To identify reference gene candidates for use in the qPCR studies, we first examined the microarray data to find the most stably expressed genes between P0 and P14 followed by validation of the four top candidates (see Results). The stability of each reference gene was assessed by comparing its expression to the geometric mean of all four candidates. The relative expression levels of the significantly changing genes chosen for validation were quantified by first calculating a normalization factor for each sample. The normalization factor is calculated by taking the geometric mean of the four reference genes (Vandesompele *et al.*, 2002). This factor is then used to correct for sample input amount and allows for relative comparisons between the quantification cycles for each gene. In order to quantify the mRNA levels in these experiments, a method based on the standard C_t calculation was used with the inclusion of the primer efficiencies for the genes of interest and reference genes (Pfaffl, 2001). The resulting values for each gene were then converted to fold-change values relative to a specific age, usually P0, and shown as a temporal profile across time. Average values at each age were plotted using the curved line interpolation feature, which utilizes cubic splines, in Microsoft Excel (Figs. 4–7). We also calculated the Pearson correlation coefficient (r) between the qPCR and microarray data for each gene.

Immunofluorescence

FVB mice at P0, P3, P6 and P14 were anesthetized with ketamine/xylazine (100/10 mg/kg) in addition to hypothermia at P0, P3, and P6. Animals were perfused transcardially with phosphate-buffered saline (PBS) followed by a solution of 4% paraformaldehyde (PFA) in PBS. The brain was removed from the skull and post-fixed overnight at 4°C in 4% PFA in PBS. The brain was transferred to cryoprotectant (30% sucrose in 0.075 M sodium phosphate buffer, pH 7.4) at 4°C for 1–2 days prior to freezing and sectioning. Coronal sections of the brainstem were cut at 40 μ m thickness using a freezing microtome (model HM 450, Microm, Waltham, MA).

First, the sections were placed in 10mM citric acid, pH 6.0, at 95°C for 30 mins to perform epitope retrieval. The sections were moved into blocking solution (3% goat serum in PBS containing 0.1% Triton X-100) for 1 hour. Next, the sections were incubated overnight at 4°C with agitation using combinations of the following primary antibodies diluted in 3% goat serum in PBS: chicken polyclonal anti-MAP2 (EnCor Biotechnology, Gainesville, FL at 1:5,000); guinea pig polyclonal anti-VGluT 1&2 (EMD Millipore, Billerica, MA at 1:2500 each); rabbit polyclonal anti-parvalbumin (Swant, Marly, Switzerland at 1:250); mouse monoclonal anti-Aldh1L1 (clone N103/39, Antibodies Inc., Davis, CA at 1:250); rabbit anti-Iba1 (Wako Chemicals USA, Richmond, VA at 1:250); rabbit anti-Olig2 (EMD Millipore at 1:800); rabbit anti-aggrecan (EMD Millipore at 1:200); mouse anti-brevican (BD Biosciences, San Jose, CA at 1:1000); and goat anti-Hapln1 (R&D Systems, Minneapolis, MN at 1:400). After washing in PBS, sections were placed in the appropriate secondary antibodies diluted in blocking solution at 1:500 for two hours at room temperature with agitation (Molecular Probes, Grand Island, NY; Jackson ImmunoResearch Laboratories, West Grove, PA). The sections were again washed with PBS before imaging using a confocal microscope (model LSM-510 Meta and LSM-710, Zeiss, Thornbridge, NY). Scanning parameters were optimized for one time point (usually the age of highest expression) and left unchanged for the other three time points. Brightness and contrast of the

resulting images were optimized for maximum clarity using Zeiss LSM Image Browser. The exceptions to this optimization protocol are parvalbumin, aggrecan, brevican, and Hapln1, which were gain-matched across ages, using identical scanning and optimization parameters at each age allowing for direct comparison.

RESULTS

Microarray Analysis of Gene Expression in MNTB

In order to characterize the transcriptome of the developing MNTB, we performed a detailed microarray analysis spanning the first two weeks of postnatal development (Fig. 1). During this time frame, MNTB neurons pass through several developmental milestones, including (1) rapid growth of the large calyx of Held (CH) terminal onto principal cell bodies between postnatal day (P) 2 and P6; (2) coordinated maturation of biophysical properties of principal neurons during the same time frame (Hoffpauir et al., 2006; Hoffpauir et al., 2010; Rusu and Borst, 2011); (3) topographic refinement of axonal projections of MNTB neurons to the lateral superior olive between P4–P8 (Kim and Kandler, 2003; Noh et al., 2010); and (4) the onset of sensitivity to airborne sounds between P8–P12 (Mikaelian et al., 1965; Ehret, 1976; Borst and Soria van Hoeve, 2012). We employed the densest temporal sampling of neonatal vertebrate brain of which we are aware (daily P0–4, P6, P14) to investigate the changing transcript levels associated with these events. Since our focus for this report is on expression changes associated with CH formation, which is complete on most MNTB neurons in mouse by P6 (Hoffpauir et al., 2006; Hoffpauir et al., 2010), we identified genes whose expression changed significantly between P0–6. Given the increasingly appreciated roles for glia in neural circuit assembly (Kucukdereli et al., 2011)(reviewed by Allen, 2013), we utilized a preparation that contained all cell types present within the developing MNTB.

Clustering of Genes by Expression Profiles

Using a one-way analysis of variance with a false discovery rate of 0.1%, we identified 541 genes changing significantly between P0 and P6, which could precede or correlate with milestones #1–3 above. A *k*-means clustering analysis was used to group the genes with similar temporal profiles (Fig. 2A). Four groups containing 299 genes had monotonically increasing expression levels (groups 1–4), two groups containing 54 genes had complex profiles displaying intervals of both increasing and decreasing expression levels (groups 5–6), and two groups containing 188 genes had monotonically decreasing expression levels (groups 7–8). The major differences among groups that share a common direction of change (i.e. groups 1–4 and 7–8) are the age associated with the inflection point in each profile and the slope of the profile between P6 and P14, which indicates gene expression changes that occur after the majority of calyces have formed and encompassing the opening of the ear canals. A heat map representation of the 541 genes arranged by group shows the average normalized signal intensity and illustrates most of the expression changes (Fig. 2B).

We first looked for genes that exhibited significant expression changes in 2-day increments (P0–2, P2–4, P4–6) as an indicator of a focused period of gene expression. The *k*-means groups themselves offer certain predictions about these temporal profiles, with group 1 showing increased expression late whereas group 4 increases expression quite early. Groups

4–7 reach maxima, minima or inflection points by P6 and may exhibit significant changes over restricted temporal intervals. A large number of the 541 genes exhibited significant changes in a single 2-day period (284/541 or 52%; (t-test, $p < 0.01$)). Each *k*-means group had substantial representation of these genes, ranging from 40–70%, with groups 4–6 containing the highest proportions (62–70%). Of these genes, 55% exhibited changes during P0–2, 20% during P2–4 and 25% during P4–6. As expected, some groups had a bias (>75% of qualifying genes) for a particular 2-day time period: groups 4–6 for P0–2 and group 1 for P4–6. To characterize the most rapidly-changing genes, we also looked for genes that exhibited at least 80% of their fold-change during a single 2-day period. We found 28 genes whose change was restricted to one of the 2-day time periods. Of these, 16 had changes restricted to P0–2, 3 restricted to P2–4 and 9 restricted to P4–6. In keeping with the analysis presented in the preceding paragraph, all group 4–6 genes (9 genes) were in the P0–2 time period and all group 1 genes were in the P4–6 time period.

We next examined cell-specific expression of the 541 genes based upon the transcriptome database provided by Cahoy *et al.* (2008) for developing neurons and glia in the neocortex. When cross-referencing cell-specific expression with the enrichment values obtained from supplemental tables (Cahoy *et al.*, 2008), we used a 5-fold enrichment cutoff to produce lists that included 72 genes enriched in neurons, 51 genes enriched in astrocytes, 69 genes enriched in oligodendrocytes, and 349 genes with no cell-type specific enrichment (Table 2). Interestingly, there is a strong association between expression group and cell type. More than 75% of the astrocyte-enriched genes (40 of 51) and 95% of the oligodendrocyte-enriched genes (68 of 69) display increasing expression profiles (groups 1–4). Conversely, more than 70% of the neuron-enriched genes (53 of 72) display decreasing expression profiles (groups 7 and 8).

Since our tissue dissection included glial cells, we performed a series of immunofluorescence experiments using some well-known markers for specific cell types to assay their presence across the time period in question in the developing MNTB (Figs. 3 and 4). Substantial numbers of both neurons and astrocytes, marked by MAP2- and Aldh1L1-immunoreactivity, respectively, are visible at all of the ages examined between P0 and P14 (Fig. 3A). The neurons are clumped closer together at P0, but begin spreading apart by P3, as revealed in serial section electron microscopy images (Holcomb *et al.*, 2013). Few Aldh1L1-positive astrocytic processes are apparent at P0, but these form a dense network by P14. Microglia (Iba1-positive cells) are evident at ages ranging from P0 to P14, although they appear less numerous than astrocytes or neurons. In order to label both immature and mature oligodendrocytes, we chose Olig2 to use as a marker, but Olig2 can also label some motor neurons and astrocytes (Masahira *et al.*, 2006). Therefore, we used a combination of markers to show that oligodendrocytes (Olig2-positive cells that are Aldh1L1 and MAP-2 negative) are also present from P0 through P14 (arrows in Fig. 3B). These data confirm the presence of at least four different cell types and support our classification of genes as listed in Table 2.

Validation of Gene Expression Profiles

Confirmation of the expression level changes for a subset of genes was performed using quantitative real-time PCR (qPCR) and included at least two representatives from each group. In order to address the recurring challenge of normalizing our qPCR data to appropriate reference genes (Gutierrez *et al.*, 2008), notably more difficult across developmental age, we utilized published data and our microarray data to identify potential reference gene candidates (Bahr *et al.*, 2009). Our requirements were that reference gene candidates are stably expressed across the developmental time window of P0 to P14, have “housekeeping” functions consistent with their constant expression, and have varied expression levels across the set of reference genes to better represent the range seen in the list of 541 differentially expressed genes. Four reference genes from this candidate list were validated. Two reference genes with high expression levels on the microarray were chosen from previously identified lists (*Atp5b* and *Ywhaz*) and are found in the geNorm mouse reference gene kits, (<http://medgen.ugent.be/~jvdesomp/genorm/>). Two additional reference genes with mid-range expression levels were chosen from our microarray data (*Helz* and *Etf1*). The measured amounts for each of these gene products showed minimal variability across the ages that were examined (Fig. 4A). The two genes identified from our own data set exhibited the greatest stability, supporting the validity of this approach to reference gene selection (Bahr *et al.*, 2009).

To begin our validation of microarray expression data, we chose the *parvalbumin* (*Pvalb*) gene that encodes a well-studied calcium-binding protein. This protein has been localized to the principal neurons of the MNTB in previous studies (Hartig *et al.*, 2001; Felmy and Schneggenburger, 2004). The expression profiles for the microarray and the qPCR demonstrate excellent correlation, especially considering that different animals were used for each technique (Fig. 4B). The qPCR data exhibit a larger fold-change than the microarray data, reflecting the larger dynamic range afforded by qPCR (Chang *et al.*, 2000; Mutch *et al.*, 2002). The protein levels for *Pvalb* were assessed across the P0–14 age range by immunofluorescence. *Pvalb* is co-localized with MAP2-positive cell bodies and dendrites indicating that, in the MNTB, it is expressed predominantly, perhaps exclusively, in neurons (P14 column in Fig. 4C). *Pvalb* protein became detectable at P6 in MNTB cells and increased in level greatly by P14. We first detected *Pvalb* in growing calyces via co-localization with VGluT1 and 2 and in axons at P3, also with increasing levels through P14 (white arrowheads in P3, P6, and P14 columns in Fig. 4C). These data are consistent with previous findings (Lohmann and Friauf, 1996; Felmy and Schneggenburger, 2004).

We applied qPCR to systematically validate at least two genes from each of the eight *k*-means clustering groups. Group 1, consisting of 52 genes, begins its increase later than any of the other monotonically increasing groups, followed by a sharp increase through P14. Two gene candidates, *Mog* and *Sema4d*, were validated from this group. The qPCR temporal profiles correlate very well with the age of initial increase in expression (P6 for *Mog*; P2 for *Sema4d*) and capture the large differences in the magnitudes of expression changes between these genes at P14 (Fig. 5). Groups 2, 3, and 4 consist of 70, 106, and 71 genes, respectively, and exhibit profiles that are increasing in a mostly linear fashion from P0 through P6. The largest differences among these group profiles occur between P6 and

P14 where group 2 shows a continuing linear increase, group 3 increases less sharply, and group 4 plateaus in expression. Representative genes, *Aldoc* and *Cdh20* from group 2, *Calb1* and *Sox10* from group 3, and *Bmp5* and *Mitf* from group 4, were validated (Fig. 5). The qPCR results capture the temporal profiles that are evident in the microarray data with remarkable fidelity. The genes from these groups cover a wide range of functions as discussed below.

Groups 5 and 6 contain the smallest number of genes, but they display the most dynamic profiles. Group 5 contains 34 genes and has an expression profile that increases early, followed by a decrease through P14. Group 6 contains 20 genes and has a mirror image expression profile to group 5, decreasing early followed by an increase through P14. Based on these temporal profiles, the genes from these two groups represent very interesting prospects to mediate specific developmental and maturational roles during the time of rapid calyx growth. Three members from group 5, *Ednrb*, *Mlip*, and *Ramp3*, were validated (Fig. 6). As with groups 3 and 4, overall patterns along with many of the subtle daily changes that are apparent in the microarray data were confirmed by qPCR. We confirmed *Cd24a* and *Dact1* as the two representatives from group 6 (Fig. 6), with *Cd24a* having its highest expression at P14. These differential features of their expression were validated with the qPCR data.

The monotonically decreasing groups 7 and 8 contain 95 genes and 93 genes, respectively. The profiles for these groups differ slightly in their slopes, with group 7 decreasing sharply between P0 and P4 then leveling out by P14 while group 8 has a less rapid initial decrease and continuing decline through P14. Genes chosen for validation from group 7 are *Slc17a8* (also known as *VGluT3*) and *Tnc* and from group 8 are *Cbln1* and *Dcx* (Fig. 7). Again, we found excellent correlation between these validation profiles and the microarray data. Across all groups, our procedures yielded strong correlation between the microarray profiles and the qPCR profiles, with Pearson correlation coefficients greater than 0.9 for each of the validated genes.

Gene Ontology

We performed Gene Ontology (GO) analysis (see Methods) to identify the most highly enriched and informative GO categories in our 541 gene list. We considered multiple GO categories to be non-specific (such as developmental process), so we highlight the most informative GO categories and list them according to enrichment (p-value) in Table 3. These categories span a range of cellular functions and processes. Seven of the 11 GO categories are involved in regulating biological processes including regulation of neurogenesis (the highest p-value of 5.62E-11), cell adhesion, regulation of neuron projection development, regulation of cell differentiation, axon ensheathment, axon guidance, and lipid metabolic process. The remaining categories are synapse and extracellular matrix, which fall under the broad category of cellular component, and calcium ion binding and ion transmembrane transporter activity, which fall under the broad category of molecular function. Axon guidance, synapse, cell adhesion, and extracellular matrix are notable GO categories for their relevance to neural developmental. Several of these categories are presented below in further detail.

The axon guidance, regulation of cell differentiation, and synapse categories are likely important for both CH formation and innervation of MNTB targets in the lateral superior olive. The GO category for axon guidance contained 18 members, including 3 semaphorins (*Sema3a*, *Sema3f*, *Sema5a*), 1 plexin (*Plxna3*), 2 ephs (*Ephb1*, *Ephb2*), *L1cam*, *Dcc*, *Unc5a*, and *Robo2*. Most of these genes (13 of 18) decrease in expression across P0–14 (group 7 or 8). This distribution of expression profiles matches known involvement of these genes in the establishment of specific neuronal connectivity. For example, these secreted semaphorins signal through plexins and are implicated in growth cone expansion or collapse, dendrite growth and limitation in the formation of excitatory synapses (Fournier *et al.*, 2000; Bouzioukh *et al.*, 2006; Tran *et al.*, 2007; Shelly *et al.*, 2011). *Dcc*, *Robo2*, and *Unc5a* are membrane-associated extracellular signaling proteins involved in growth cone attraction and repulsion. *Ephb1* (group 6), *Ptprm* (group 4), *Nfasc*, *Nr4a3*, and *Sema5a* (all in profile group 3) are the only genes within the axon guidance category that do not have decreasing expression profiles, suggesting an ongoing involvement in MNTB maturation and function. The category of regulation of cell differentiation contained several growth factors (*Tgfb3*, *Bmp4*, *Bmp5*, and *Wnt7a*) and *Dact1*. Bmp's are part of the larger Tgfb-superfamily and act through the Tgfb/SMAD signaling pathway. Both *Bmp*'s are increasing (*Bmp4*, group 3; *Bmp5*, group 4) during early postnatal development, whereas *Tgfb3* (group 7) is decreasing. *Wnt7a* shows increasing expression (group 4). *Dact1* (group 6) protein interacts with dishevelled, a central element of the Wnt signaling pathway. The GO category of synapse contained 43 genes in our list, split between increasing (25 genes) and decreasing (18 genes) profile groups. Seven genes (*Cplx1*, *Lgi3*, *Slc17a8*, *Syt2*, *Syt5*, *Unc13b*, and *Vamp1*) in this GO category are associated with synaptic vesicles and their release. An additional 7 genes (*Chrm3*, *Gabra2*, *Gla1*, *Glr1b*, *Gria3*, *Grin2b* and *Lin7c*) encode proteins that are most commonly localized to the postsynaptic membrane, 6 of which are receptor subunits (all except *Lin7c*). Two of the genes from this category encode proteins that are commonly associated with the presynaptic membrane (*Atp1a2* and *Erc2*). Synapse-associated genes were distributed across groups, although some were associated with known aspects of functional maturation of MNTB neurons (*Slc17a8* (*VGLUT3*) decreasing (group 7); *Gria3* increasing (group 3) and *Grin2b* decreasing (group 8), consistent with increased AMPAR-mediated and decreased NMDAR-mediated current (Futai *et al.*, 2001; Joshi and Wang, 2002; Hoffpauir *et al.*, 2006).

The GO category for cell adhesion was the second largest and has 60 members from the 541 gene list. Cell adhesion molecules have diverse functions within the nervous system, including organization of three-dimensional tissue structure, bidirectional cell signaling, and formation and maturation of synapses (Redies *et al.*, 2011; Bukalo and Dityatev, 2012). The cell adhesion molecules identified in our study originate from all eight profile groups. Seven members from the cadherin superfamily (*Cdh2*, *Cdh4*, *Cdh20*, *Cdh22*, *Dsg2*, *Pcdh15*, and *Celsr3*), named for their Ca²⁺-dependent function, along with one catenin, *Ctnna2*, are present on the 541 list. One of the best-studied members from this diverse group of adhesion molecules is *Cdh2*, also known as N-cadherin. *Cdh2* is associated with multiple synaptogenic events including the accumulation of AMPA receptors and dendritic spine stabilization and growth (Malinverno *et al.*, 2010)(reviewed by Brigidi and Bamji, 2011). Cadherins are required for the initial stages of synapse assembly in young neurons but

become dispensable at older ages (Togashi *et al.*, 2002; Bozdagi *et al.*, 2004). Three of the four vertebrate members of the L1-CAM family of adhesion molecules (*L1cam*, *Chl1*, and *Nfasc*) and another immunoglobulin superfamily member (*Igsf11*) are also listed within this gene ontology group. The L1-CAM family members are broadly expressed in the developing nervous system and play important roles in axon outgrowth and fasciculation, axon guidance (also listed in that GO category), and synaptic plasticity (Barry *et al.*, 2010; Demyanenko *et al.*, 2011)(reviewed by Maness and Schachner, 2007). Three different proteoglycans (*Acan*, *Bcan*, and *Hspg2*) are found in this GO category and all belong to profile group 3. Aggrecan and brevican are associated with perineuronal nets (PNNs), which are dense mesh-like extracellular structures that surround the cell bodies and proximal dendrites of select neurons in the central nervous system (see below). Other cell adhesion members include three integrins (*Itga2*, *Itga6*, and *Itga8*), two tenascins (*Tnc* and *Tnr*), and a contactin (*Cntn4*). Taken together, the mixture of both increasing and decreasing expression patterns for genes associated with cell adhesion suggests that some of these protein families undergo subtype switching during CH formation and associated MNTB cell maturation.

A key factor in cellular maturation is the developmental expression of genes that underlie establishment of ionic gradients and conduction of ions across membranes. For example, intracellular chloride concentrations in SOC neurons decrease during the first postnatal week (reviewed by Friauf *et al.*, 2011), and MNTB neurons become less excitable and establish their transient firing properties during the time frame of CH growth and competition (Hoffpauir *et al.*, 2010). Comparison of SOC-wide ion channel expression between P4 and P25 revealed changes in Na⁺, K⁺ and Ca²⁺ channel expression (Ehmann *et al.*, 2013). Our GO category of ion transmembrane transporter activity contains genes for ion channels, including delayed and inward rectifier K⁺ channels (*Kcna2*, *Kcnab3*, and *Kcnj10*), the Na⁺ channel, *Scn1a*, multiple Ca²⁺ channel subunits (*Cacna1b*, *Cacna1e*, *Cacna1g*, and *Cacnb3*) and Ca²⁺-activated Cl⁻ channels (*Clca1* and *Clca2*). K⁺- and Na⁺-channel genes showed increased expression (groups 2–4), consistent with functional changes in physiological phenotype and Ca²⁺-channel genes decreased in expression (groups 7 and 8). This list also contains a number of P-type ATPases, most of which are cation transporters, including the Na⁺/K⁺ ATPase (*Atp1a2*, *Atp1b1*, and *Atp1b2*), and smooth endoplasmic reticulum and plasma membrane Ca²⁺ pumps (*Atp2a2*, *Atp2b4*, and *Atp13a4*); nearly all genes increased in expression (groups 2–4; *Atp2b4* decreasing in group 7).

Perineuronal Nets Evident in the MNTB at P6

Given the presence of several genes for perineuronal net (PNN) components, the prevalence of PNNs in the MNTB (Blosa *et al.*, 2013) and the roles for PNNs in synaptic plasticity (reviewed by Wang and Fawcett, 2012), we examined developmental changes in protein levels for several PNN glycoproteins. We performed immunofluorescence labeling for two chondroitin sulfate proteoglycans from group 3 (aggrecan and brevican) and a hyaluronan and proteoglycan link protein from group 5 (Hapln1) between the ages of P0 and P14 and compared their protein expression profiles to the temporal profiles from the microarray (Fig. 8). We found that both aggrecan and brevican exhibit very little labeling at P0 and P3, consistent with the microarray profiles. At P6, brevican begins to show weak labeling around the neurons, and both aggrecan and brevican show strong ring-like labeling patterns

around the MNTB neurons at P14 (Fig. 8A, B). Hapln1 shows moderate but diffuse labeling at the earliest postnatal ages (P0 and P3). A ring-like pattern begins to emerge for Hapln1 at P3, and by P14, the labeling is strong and very similar to aggrecan and brevican (Fig. 8C). Unlike the profiles for aggrecan and brevican, the Hapln1 microarray and protein expression profiles do not correlate well, although this might be expected given the dynamic pattern of Hapln1 mRNA expression (group 5) and the extracellular localization of the protein, where turnover does not occur rapidly. Because of the similarity in the distribution of these proteins, we performed a co-localization experiment at P14 to compare their expression patterns. Aggrecan and brevican label concentric circles with some overlap, with brevican often forming the inner ring adjacent to the neurons surrounded by an outer ring of aggrecan labeling (Fig. 8D). This pattern is similar to the localization seen in the MNTB of adult mice (Blosa *et al.*, 2013). Hapln1 expression almost completely overlaps both aggrecan and brevican, while also exhibiting slightly more extensive labeling than either of them (blue label in Fig. 8E–F).

As reported in the study of overall gene expression in the superior olivary complex (Ehmann *et al.*, 2013), multiple genes associated with cochlear deafness were identified in our study. Seven of the 26 genes identified in that study appeared on our 541 list, which included *Ednrb*, *Sox10*, *Aqp4*, *Slc17a8*, *Slc12a2*, *Mitf*, and *Eps8*. We identified an additional eight deafness genes (*Pcdh15*, *Otof*, *Whrn*, *Smpx*, *Cldn11*, *Celsr3*, *Kcnj10*, and *Tnc*) in our 541 gene list that were not reported in their study. Many of these genes were in groups 4 and 5, and nearly all genes increased in expression (exceptions were *Slc17a8*, *Celsr3*, *Otof*, and *Tnc*). These findings indicate that cochlear deafness can have associated central effects that are masked by the peripheral deficits. Potential therapies for cochlear deafness that mitigate only peripheral anomalies may be insufficient, then, to restore hearing to normal levels.

DISCUSSION

We employed a study design to densely sample gene expression in the MNTB at 24-hour intervals over the first five postnatal days (P0–P4), then at P6 and P14 to overlap growth of the CH and bracket the onset of hearing in mice at ~P10 (Ehret, 1976). This age range was chosen to capture key events in postsynaptic neurons during growth and competition among CH nerve terminals, which begins at P2 and resolves during a rapid time frame onto most MNTB neurons by P6, as described by high resolution 3D imaging (Hoffpauir *et al.*, 2006; Holcomb *et al.*, 2013). Our analysis identified 541 significantly changing genes between P0 and P6, and these genes were assigned to eight different groups based upon their temporal expression profiles. The sample tissue included neurons and glia, which increasingly are appreciated as interacting partners during development. Our focus on the MNTB is complementary to recent developmental studies of the SOC in its entirety (Ehmann *et al.*, 2013), comparison of the MNTB with the LSO (Xiao *et al.*, 2013), or of ventral cochlear nucleus neurons (primarily globular bushy cells) (Korber *et al.*, 2014). The time series for these studies partially overlapped our time frame, but did not extend to as young as P0–2.

Reliability of Microarray Data

The qPCR confirmation of more than 20 candidate genes showed excellent agreement with the microarray results, which has several important implications for the data set. The microarray data used individual littermate animals and RNA amplification; the qPCR data used different animals and pooled RNA samples from 4–6 littermates without amplification of the resulting RNA. Given the similarity in temporal profiles, RNA amplification appears to have accurately preserved the relative quantities of the transcripts across a wide range of expression levels. The main difference between the two data sets was a lesser magnitude of microarray expression level changes, probably due its limited dynamic range relative to qPCR (Chang *et al.*, 2000; Mutch *et al.*, 2002). We used 4–5 microarray replicates for each time point, which added statistical power to the results. Furthermore, the consistently high correlation of microarray data to qPCR results lends confidence to the entire microarray data set, including genes that have not been individually confirmed. Another factor that may have contributed to the reproducibility of the gene expression results was the use of four reference genes that were chosen for their stability in our developing system. The use of multiple reference genes is extremely important in calculating accurate results for qPCR (Vandesompele *et al.*, 2002; Huggett *et al.*, 2005). Reference genes for developmental time points can be quite difficult to identify because of the large changes that occur in the entire transcriptome during these time periods, so we have added two genes from our microarray results (*Helz* and *Etf1*) to be considered for use in other developmental studies. The approach of choosing our reference genes from the most stably expressed genes detected on the microarrays (Bahr *et al.*, 2009) permitted rapid identification of viable candidates in the developing MNTB.

Combined Analysis of MNTB Neurons and Glia

Our microdissection technique yielded the transcriptome of the entire developing system as opposed to cell type-specific information that could have been obtained through strategies utilizing cell sorting. We took this technical approach to permit rapid isolation of RNA soon after sacrificing the animal, and this holistic approach to ensure that both neuronal and glial transcripts were identified. In recent years it has become apparent that glia play crucial roles in development, including the control of synapse formation, synapse elimination, and modulation of synaptic transmission (reviewed by Faissner *et al.*, 2010 and Clarke and Barres, 2013). We have observed at least four types of cells in the developing MNTB (neurons, astrocytes, oligodendrocytes, and microglia), and we expect that there should also be some contribution from oligodendrocyte precursor cells, vascular endothelial cells and pericytes.

The pre-synaptic CH projection is also a potential source of transcripts observed in our analysis of the MNTB. Recent observations across biological systems support a general mechanism of local translation for cells to adapt within cellular microenvironments (reviewed by Jung *et al.*, 2014). Local translation has long been known to support LTP in dendrites (Steward and Levy, 1982; Kang and Schuman, 1996). More recently, navigation of growth cones has been shown to rely on local protein synthesis (Campbell and Holt, 2001; Yao *et al.*, 2006; Tcherkezian *et al.*, 2010). Selective profiling of gene expression in growth cones reveals transcripts for cytoskeletal, cell surface and secreted proteins that could play

roles in synapse formation (Zivraj *et al.*, 2010). Some of these transcripts overlap with cytoskeletal and signaling protein categories in our list. Estimating the abundance of mRNA in presynaptic terminals for local translation has been challenging (reviewed by Willis and Twiss, 2010), but ~2500 different mRNA transcripts are present in embryonic and mature dorsal root ganglion axons (Gumy *et al.*, 2011). In embryonic axons, the most highly expressed proteins fall into protein synthesis and mitochondrion GO categories, which were not prevalent in our list. Protein synthetic capacity in axons has been estimated to be much less than in somas (Lee and Hollenbeck, 2003). Several proteins related to glutamatergic neurotransmission (Slc17a7 (VGluT1) and Slc17a6 (VGluT2)) and a Ca²⁺-binding protein (calretinin) are expressed in GBC's but not in MNTB neurons (Fig. 4 and (Felmy and Schneggenburger, 2004)). These transcripts were present at low, near detection levels and were not changing during P0–6. Therefore, the contribution of presynaptic elements (axons and terminals) to our results is potentially measurable but likely minimal. This issue certainly merits further investigation.

Extracellular Matrix and Perineuronal Nets

Our study identified 26 genes associated with the extracellular matrix (ECM), exhibiting a wide range of expression profiles spanning all 8 groups. Among these genes are structural components, metalloproteinases, cell adhesion molecules, and growth factors. The ECM achieves a high degree of organization around many cell types in the CNS as perineuronal nets (PNNs). Many neurons that exhibit PNNs are inhibitory and immunoreactive for calbindin and PV (Hartig *et al.*, 1995). MNTB neurons meet these criteria and exhibit prominent PNNs in adults (Hartig *et al.*, 2001; Blosa *et al.*, 2013).

Structural components of PNNs that appear on our 541 gene list include the chondroitin sulfate proteoglycans aggrecan and brevican, tenascin-R, and the link proteins Hapln1 and Hapln4 (reviewed by Wang and Fawcett, 2012). Although aggrecan is considered to be synthesized by neurons, other PNN elements likely have mixed neuronal and glial origins (Carulli *et al.*, 2006; Giamanco and Matthews, 2012). These observations further validated collecting all MNTB cells for analysis. Based upon our immunofluorescence data (Fig. 8), it appears that PNNs are established during the first postnatal week and become well-organized after P6 (yet before P14), which follows resolution of mono-innervation onto most MNTB neurons (Holcomb *et al.*, 2013) and is in general agreement with previous descriptions of rodent MNTB (Lurie *et al.*, 1997; Friauf, 2000; Myers *et al.*, 2012). This time course is consistent with roles for PNNs in limiting plasticity and closing critical periods (Pizzorusso *et al.*, 2002; Carulli *et al.*, 2010). The regions of non-overlap between aggrecan and brevican apparently continues into adulthood (Blosa *et al.*, 2013) and may reflect their association with different link proteins and the proposed existence of microdomains within the PNN (Bekku *et al.*, 2012; Giamanco and Matthews, 2012). We report an early presence of Hapln1 immunolabeling by P0, the earliest indication of PNN assembly in MNTB, consistent with embryonic expression of PNN genes (Milev *et al.*, 1998). This early latticework may be sufficient to bind secreted growth factors, such as semaphorins, which have demonstrated association with PNNs (Vo *et al.*, 2013) and are present in our microarray. Notably, *Hapln1* showed the largest differential expression level between MNTB (high expression) and lateral superior olive (low expression), a different cell group in the

superior olivary complex whose cells do not receive calyceal terminals (Xiao *et al.*, 2013). Regulation of PNN structure is mediated by synthesizing and degrading enzymes. The Adamts family of metalloproteinases is capable of cleaving proteoglycans (Stanton *et al.*, 2011), and thus could play a role in regulating CH formation. Five of 19 family members (*Adamts1*, *Adamts4*, *Adamts16*, *Adamts18*, and *Adamts20*) were identified on our 541 list, and, like the PNN structural elements, were mostly members of increasing expression groups. Another metalloproteinase, *Mmp17* (Group 7), is required for the proteolytic activation of *Adamts1* (Gao *et al.*, 2004). The inhibition of these metalloproteinases by *Timp4* (Group 4) could be an important mediator of PNN formation in the MNTB by promoting proteoglycan stabilization.

Genes with Demonstrated Roles in CH Formation

Although VCN axons can drive activity in MNTB neurons from E17 (Hoffpauir *et al.*, 2010), most synaptic contacts prior to calyx growth are located on dendrites (Holcomb *et al.*, 2013). Calyceal axons may shift contacts from dendrites to soma or extend growth cones as part of a final phase of elongation during the period of calyx formation and competition. Axon guidance molecules could serve traditional roles or additional roles in synapse formation and competition during brain development (reviewed by (Vanderhaeghen and Cheng, 2010)). Signaling systems for commissural axon attraction and crossing (netrin/*Dcc*; slit/*Robo*) have been verified for VCN axons (Howell *et al.*, 2007; Renier *et al.*, 2010; Michalski *et al.*, 2013). Of these, *Dcc* exhibits declining levels in MNTB (group 8), which may reflect its expression in embryonic MNTB neurons (D. Howell and P. Mathers, unpublished observations). Ephrins and their receptors (*EphA4*, *EphB1*, *EphB2*) inhibit ipsilateral innervation, especially after lesion of the contralateral inputs (Hsieh and Cramer, 2006; Hsieh *et al.*, 2007; Hsieh *et al.*, 2010). *EphB2* shows decreasing expression on our microarray, yet the critical period for ipsilateral sprouting in mice closes by P10, so other factors likely prevent ipsilateral calyx formation (Nakamura and Cramer, 2013). Both *EphA6* (group 4) and *EphB1* (group 6) increase expression at later ages and could contribute to this process. Knockout of *Cntn5*, a member of the contactin set of adhesion molecules, results in loss of about 8% of calyces (Toyoshima *et al.*, 2009). *Cntn4* (group 8) and *Cntnap1* (group 3) have as yet unexplored roles in auditory brainstem neural circuit formation, although *Cntn4* is important for glomerular targeting of olfactory receptor neurons (Kaneko-Goto *et al.*, 2008). Manipulation of growth factors via conditional knockout of Bmp receptors in VCN and MNTB leads to delayed competition and attenuated growth of CHs, with persistent presynaptic deficits (Xiao *et al.*, 2013). Except for the severe phenotypes in *netrin1* and *Dcc* KO animals (Howell *et al.*, 2007), these gene knockouts permit calyx growth and monoinnervation of MNTB neurons, sometimes following an extended delay. These observations underscore the likely multifactorial nature of synaptic targeting in the MNTB.

Comparison with Other Gene Expression Studies

Calyceal terminals are also found in chick ciliary ganglion, where they mono-innervate their target and grow rapidly over several days, much like the CH. A comparison of gene expression prior to and during early stages of calyx formation revealed 51 genes classified as synaptogenic (Bruses, 2010), six of which were on our 541 list. Four of these genes (*Igsf11*,

L1cam, *Kirrel3* and *Col14a1*) encode cell adhesion molecules and one encodes an adhesion molecule associated protein (*Cntnap1*); the remaining gene (*Lrrtm3*) encodes a membrane spanning protein. Gene expression profiles of globular bushy cells between P3 and P8 identified 22 genes that code for synapse-associated proteins (Korber *et al.*, 2014). Three of these genes (*Scn1a*, *Pvalb*, *Syt2*) were on our 541 list and have known expression (*Pvalb*) or likely have functions in MNTB neurons. In contrast to the previously mentioned studies, comparison of gene expression between MNTB and whole SOC (Ehmann *et al.*, 2013) identified many genes found in our study, despite the different analyses (changing temporal expression within the MNTB P0–6 (this study) and comparison of SOC to whole brain at P4 and P25 or SOC pre- and post ear canal opening (Ehmann *et al.*, 2013) Most genes common to both lists are likely expressed in multiple SOC nuclei, since selective expression in MNTB likely would be diluted in the larger sample. For example, two of the most significantly enriched genes in the Ehmann *et al.* (2013) study (*Gpr37* and *Gpr37II*), in comparison to whole brain, were also found on our 541 list. In the same study, two of the top eight genes enriched in P4 vs P25 SOC (*Dcx* and *Dpysl3*) also showed decreasing expression on our 541 list (group 8). *Dpysl3* is involved in semaphorin signaling, which is well represented in our data set (6 semaphorins and 3 plexins). Five of the top 7 genes enriched at P25 vs P4 SOC (*Apod*, *Mal*, *Mobp*, *Mog*, *Trf*) also showed increasing expression on our 541 gene list (groups 1 and 3). Several of these genes are involved in myelin formation and lipid transport (*Apod*, *Mal*, *Mobp*, *Mog*) and likely reflect primarily glial expression. Unique genes in our study can reflect MNTB-specific expression or the denser temporal sampling prior to the onset of sensitivity to airborne sound. Since few genes changed expression between P16–25 (Ehmann *et al.*, 2013), analysis of changes between P6–14 in our study could identify many genes common to mice and rats associated with experience-dependent refinement of form and function.

The range of GO categories and cell types associated with gene expression patterns indicates a complex interaction among tissue elements to sculpt neural circuits. Dense sampling permitted identification of eight distinct temporal expression profiles that imply coordinated genetic networks. Notably, the gene expression changes in this system prior to P6 reveal events that precede ear canal opening and so are relatively independent of environmental influences. About two-thirds of the genes in groups 4–6 are associated with rapid changes in expression level and most of these occur during P0–2; rapid changes in group 1 gene expression occurred later during P4–6. Therefore, some genes in groups 4–6 may have roles in initiating CH growth, competition, and MNTB maturation. Further analysis of gene expression networks is complicated by requirements for additional information, such as protein levels, and the need to establish cellular locations for particular genes. These are topics for future study. The presence of a range of growth and guidance factors, elements of synapse structure and assembly, and scaffolding of perineuronal nets on the 541 gene list suggests the interplay of multiple factors in choreographing tissue reorganization to achieve proper neuronal connectivity. The homogeneity of pre- and postsynaptic cell types and the highly organized extracellular matrix, combined with the temporal dynamics of gene expression, render the CH:MNTB neural connection a useful model to sort out the complexity of forming and stabilizing topographic projections between brain regions.

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Figure 1. Tissue harvesting and experimental design

A, Example of a freshly prepared brainstem slice of ~200 μm thickness at P3 showing auditory cell groups and landmarks. Neurons in the VCN generate CHs in the contralateral MNTB, and inhibitory MNTB projections innervate the ipsilateral LSO and SPN. These connections are illustrated in red. MNTB, medial nucleus of the trapezoid body; SPN, superior paraolivary nucleus; LSO, lateral superior olive; DCN, dorsal cochlear nucleus; VCN, ventral cochlear nucleus; 7N, seventh cranial nerve; 8N, eighth cranial nerve; D, dorsal; L, lateral. **A'**, Dashed lines show cuts made to extract MNTB tissue for RNA

collection. **B**, Timeline illustrating major events in early MNTB development, designated by colored blocks. Large calyces are found on nearly all MNTB cells by P4, and most growth is complete by P6 (Holcomb et al. 2013). Samples were collected at time points marked with black diamonds. Scale bars: 500 μ m.

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Figure 2. Significantly changing genes grouped into eight temporal expression clusters

A, Group profiles after performing *k*-means clustering analysis ($k = 8$) with correlation metrics. Four groups showed an increase in expression, two groups showed a decrease in expression, and two groups (5 and 6) showed more complex temporal dynamics. **B**, Heat map of the 541 genes representing the average signal intensity for each gene at a given age using the scale located at the bottom. The colors correlate to normalized microarray signal intensity values from 4 (blue) through 12 (red). Genes are arranged by clustering group (Group 1 at top) and by increasing microarray signal intensity at P0 within each group. The

total number of genes is designated on the left and the number of genes belonging to each group is shown on the associated graph.

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Figure 3. Neurons, astrocytes, microglia, and oligodendrocytes are present in the neonatal MNTB

A, Neurons are labeled with anti-MAP-2 (green), astrocytes are labeled with anti-aldehyde dehydrogenase 1 family, member L1 (Aldh1L1; red), and microglia are labeled with anti-ionized calcium binding adaptor molecule 1 (Iba1; blue). There are few microglia detected in the area of the MNTB, but they are visible at all ages. Some examples of microglia are marked with white arrowheads at each age. There is no co-localization between any of the markers. **B**, Oligodendrocytes (white arrowheads) are found within the MNTB across all

ages. They are identified by labeling of their nuclei with anti-oligodendrocyte transcription factor 2 (Olig2; blue) while not exhibiting cytoplasm labeled by either MAP-2 (neurons; green) or Aldh1L1 (astrocytes; red). Scale bars: 20 μm .

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Figure 4. Parvalbumin expression validated by qPCR and confirmed with protein expression visualized by immunofluorescence

A, Reference genes (*Atp5b*, *Ywhaz*, *Helz*, and *Etf1*) were selected from the least significantly changing transcripts in the microarray data, because they showed the greatest stability across the age range studied (P0 - P14). Each gene was normalized to the geometric mean of expression for the reference gene group (see methods for details). Abbreviations: *Atp5b*- ATP synthase, H⁺ transporting mitochondrial F1 complex, beta subunit; *Etf1*- eukaryotic translation termination factor 1; *Helz*- helicase with zinc finger domain; *Ywhaz*- tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide. **B**, *Parvalbumin* (*Pvalb*) microarray and qPCR results exhibit similar temporal profiles and differ primarily in dynamic range of changing expression levels. Data were normalized to the geometric mean of the four reference genes and shown relative to values at P0. In the microarray plot, littermates share the same colored symbol across ages and different colored symbols distinguish different litters. Data points from the qPCR experiments were generated from pooled animal samples (4–6 littermates/sample); each

symbol represents a different litter. Pearson correlation coefficient (r) for the comparison of the qPCR and microarray data is 0.97. **C**, Anti-Pvalb (red) immunofluorescence increases in MNTB neurons between P0 and P14. Pvalb is not detected at P0. At P3 and P6, Pvalb is mostly located in axons (weakly at P3) and growing calyces of Held (CH; white arrowheads). CHs are indicated by co-localization with anti-vesicular glutamate transporter (VGluT) 1 and 2 (blue), which label the growing and mature CH. At P6, low levels of Pvalb are evident in MNTB neurons, which are marked by anti-microtubule-associated protein 2 (MAP2; green) expression at all ages. By P14, strong Pvalb expression is observed in the MNTB neurons and CHs (with associated axons). Scale bars: 10 μ m.

Figure 5. Confirmation of microarray results for selected genes showing increasing expression with age

Gene expression profiles from groups 1–4 were assayed by microarray and validated by qPCR. Two representative genes from each group are shown. Plot format is the same as in Figure 4B, C. Note that the fold-change scale is generally larger for qPCR than for microarray data, especially for large changes. Abbreviations: *Mog*- myelin oligodendrocyte glycoprotein, $r = 0.99$; *Sema4d*- semaphorin 4d, $r = 0.96$; *Aldoc*- aldolase C, fructose-biphosphate, $r = 0.98$; *Cdh20*- cadherin 20, $r = 0.99$; *Calb1*- calbindin 1, $r = 0.98$; *Sox10*-

SRY-box containing gene 10, $r = 0.99$; *Bmp5*- bone morphogenetic protein 5, $r = 0.96$; *Mitf*- microphthalmia-associated transcription factor, $r = 0.98$.

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Figure 6. Confirmation of microarray results for selected genes showing complex temporal expression profiles

Gene expression profiles from groups 5 (increasing, then decreasing) and 6 (decreasing, then increasing) were assayed by microarray and validated by qPCR. Representative genes from both groups are shown. Plot format is the same as in Figures 4–5. Abbreviations: *Ednrb*- endothelin receptor type B, $r = 0.96$; *Mlip*- muscular LMNA-interacting protein, $r = 0.97$; *Ramp3*- receptor (calcitonin) activity modifying protein 3, $r = 0.92$; *Cd24a*- Cd24a antigen, $r = 0.99$; *Dact1*- dapper homolog 1, antagonist of beta-catenin (Xenopus), $r = 0.99$.

Figure 7. Confirmation of microarray results for selected genes showing decreasing expression with age

Gene expression profiles from groups 7 and 8 were assayed by microarray and validated by qPCR. Two representative genes from each group are shown. Plot format is the same as in Figures 4–6. Note that different fold-change scale is generally larger for qPCR.

Abbreviations: *Slc17a8*- solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 8 (*VGluT3*), $r = 0.92$; *Tnc*- tenascin C, $r = 0.99$; *Cbln1*- cerebellin 1 precursor protein, $r = 0.98$; *Dcx*- doublecortin, $r = 0.99$.

Figure 8. Perineuronal net components are evident from birth but coalesce around MNTB principal cells after CH growth begins

A, Gene expression profiles for aggrecan (Acan), brevican (Bcan), and hyaluronan and proteoglycan link protein 1 (Hapln1) were assayed by microarray. Plot format is the same as in Figures 4–7. **B–D**, Aggrecan, brevican, and Hapln1 are labeled and shown in red at P0, P3, P6, and P14. A merged panel showing the neurons marked with MAP2 (green) and the presynaptic terminals marked with VGluT 1 and 2 (blue) at P14 is also displayed. **B**, Aggrecan labeling is not seen at P0, very weak at P3 and P6, and strong at P14. **C**, Brevican

labeling is not seen at P0 or P3, weak but visible at P6 (several neurons marked with white asterisks), and strong at P14. **D**, Hapln1 labeling is moderate at P0, nearly encircles MNTB neurons by P6 and becomes prominent by P14. **E–G**, Co-labeling of aggrecan, brevican, and Hapln1 is shown at P14. **E**, Aggrecan and brevican show some co-localization, but brevican forms an inner ring surrounded by aggrecan on many neurons (green and red arrowheads). **F–G**, Hapln1 (blue) exhibits more extensive labeling than either aggrecan or brevican and co-localizes almost entirely with their combined expression patterns. Scale bars: 20 μ m.

Table 1

Primers for real-time PCR experiments

Gene Symbol	Primers (5'-3')	Amplicon Size
Reference Genes		
Atp5b	ATG CAG GAA AGG ATC ACC ACC ACC GAT GCC CAA CTC AGC AAT AGC CCG	159
Etf1	TGC CGA TAG GAA CGT GGA GAT CTG G GCC ACT CGT GAA ATC TGG TCT TTG GG	126
Helz	TCC ACT GAG AGC CAT CAC ACA GCC TGG TGC TCA CTC CAC TGC TGT AGC	177
Ywhaz	GTT GTA GGA GCC CGT AGG TCA TCG GCT TTC TGG TTG CGA AGC ATT GGG	192
Validated Genes		
Aldoc	TCG TCC GCA CCA TCC AGG ATA AGG ACA GCG CCA TTT GGC AAA ATC AGC	170
Bmp5	TCA CTG TGA CCA GCA ACC ACT GGG AGG CCC ATG TCT TCC CAC AAG ACC	128
Calb1	GGC CAG GTT ACT ACC AGT GCA GGA TCT TTC AGC AAA GCA TCC AGC TCA	150
Cbln1	CAG AAC GCA GCA CTT TCA TCG CCC GTC ACC GGC GAA GGC TGA AAT CAC	140
Mlip	TCC AAG AGC AGC TGG TCG AGA AAC C AAA CAG GCC ACT GTT GTC TTC AAG G	196
Cd24a	TGC TTC TGG CAC TGC TCC TAC CC AGC GTT ACT TGG ATT TGG GGA AGC	107
Cdh20	TCT GCT CCC AGT CAG CTT GAG TCG TGT AGG GTT GTT TGC GAT GTC GCC	119
Dact1	AGA ACA TCT TGC TGC TGC GAA GGC CCA GGT GCT CTT CAG ATG TCT TCT CC	138
Dex	TGA CGG ATC CAG GAA GAT TGG AAG C CTG TTG CTG CTA GCC AAG GAC TGG	180
Ednrb	GAA CAA GTG CAT GCG CAA TGG TCC GGG CAC CAG CTT ACA CAT CTC AGC	151
Mitf	GAC ATG CGG TGG AAC AAG GGA ACC GAA AGT CCA TGC GCT CTA GCC TGC	182
Mog	GGC TAC ACC TGC TTC TTC AGA GAC C GGC ACA AGT GCG ATG AGA GTC AGC	119

Gene Symbol	Primers (5'-3')								Amplicon Size
Pvalb	GCA	GGA	TGT	CGA	TGA	CAG	ACG	TGC	130
	TCC	GGG	TTC	TTT	TTC	TTC	AGG	CCC	
Ramp3	CTG	GAG	CTG	TGG	CTT	GTT	TAG	CCC	115
	GAA	CCC	TTG	AGC	ACA	CTC	AGT	GCG	
Sema4d	ATG	TGT	GTG	GGA	CCA	ATG	CGT	TCC	107
	GTC	GAA	GGG	GCA	TCT	TCC	TTT	GCC	
Slc17a8 (Vglut3)	CCT	TCC	TGG	TGC	TTG	CTG	TAG	GAT	187
	CAC	TCC	TCC	CGG	GTC	TTG	TGC	TTA	
Sox10	ACT	ACA	AGA	GTG	CCC	ACC	TGG	ACC	127
	TCT	GTC	TTT	GGG	GTG	GTT	GGA	GGG	
Tnc	CAG	GGT	TGC	CAC	CTA	TTT	GCC	TGC	196
	AGT	TTG	GCG	GTA	GGA	GGT	CTC	TGG	

Table 2

Group distribution and cell-specific expression of 0.1% FDR gene list

Group 1	Astrocyte	Neuron	Bace2	Gsn	Mog	Trf	Cmya5	Fryl	Nipa4	Sorbs3
	Chil1	none	Cldn11	Hhip	Myo1d	Tubb4a	Cox8b	Gira1	Pla2g16	Sor1
	Dio2	Oligodendrocyte	Cpox	Lgi3	Pla2g4a	Unspecified	Csrp1	Hspb8	Plekhh1	Spp1
	Hsd11b1	Adamts4	Erbp3	Mal	Slc12a2	Acs1	Efh1	Khl2	Plekhh3	Tppp
	Tpbp	Apod	Fbxo32	Map7	St18	Ankrd33b	Elovl5	Lpar1	Sema4d	
		Arhgef10	Gpr37	Mcam	Tmem63a	Cdk18	Ernm	Mob3b	Slco1a4	
Group 2	Astrocyte	Gprc5b	Neuron	Gje1	Tspan2	Auh	Fmn1	Kcnj10	Pip4k2a	Trmp1
	Aldh1a1	Papss2	none	Gltp	Wipf1	Bbox1	Fn3k	Klf9	Qk	Ttyh1
	Aldoc	Pde4b	Oligodendrocyte	Man1a	Zfp365	Cd81	Gabra2	Lynx1	S1pr1	Tyrobp
	Aqp4	Pygm	Cryab	Sema3d	Unspecified	Cdh20	Gatm	Mctp2	Scd2	Zeb2
	Atp1a2	Slc1a2	Ctss	Slc44a1	Abcg1	Efcab14	Gramd3	Myadml2	Smarca2	
	Atp1b2	Sparg	Eliz	Srpk3	Agpat9	Elmo1	Hhatl	Nipal2	Smyd1	
	Cd38	Tlr3	Enpp2	Sytl2	Arsb	Etla	Igf11	Omg	Tcf4	
	F3		Fa2h	Tmeff2	Asah2	Fam102a	Inpp5j	Ostf1	Tmbim1	
Group 3	Astrocyte	Neuron	Oligodendrocyte	Mbp	Tmem2	Chpt1	Gria3	Nexn	Rnd2	Trpm3
	Agt	Calb1	Alox5	Mobp	Ugt8a	Cntnap1	Hapln4	Nptn	Rora	Tuba4a
	Bcar3	Cd1	Arcp1b	Nfasc	Unspecified	Cpxm2	Hipk2	Nr1d1	S100b	Ubi3
	Clu	Nefh	Bmp4	Pilp	9130019P16Rik	Dtd2	Hlf	Nr4a3	Sgk1	Vamp1
	Gpc5	Rasgrf1	Chn2	Pip1	Acan	Dynl2	Hrasls	Paqr6	Sgpp2	Whrn
	Htra1	Rassf5	Cnksr3	Pkrcq	Adamts1	Flt3	Hspg2	Parm1	Six4	Zfp750
	Igf1	Rgs4	Cnp	Rasgef1b	Anxa5	Girb	Inpp1	Pcp4l1	Slc6a5	
	Ndrp2	Zmat4	Ddc	Rnf122	Arhgef6	Gm2a	Itga8	Plcg2	Sncx18	
	Slc13a3		Elovl7	Sema5a	Atp1b1	Gng12	Kank1	Pls3	Sox8	
	Slc14a1		Enpp6	Sirt2	Bcan	Gpr123	Kcna2	Prune2	Sytl2	
	Slc39a12		Lims2	Sox10	Casr	Gpr37l1	Mbnl2	Pvalb	Tenn4	
	Slc7a10		Mag	Tmem163	Cast	Gpx6	Neu4	Rgl1	Tnr	
Group 4	Astrocyte	Rftn2	Rit2	Unspecified	Bmp5	Ec1	Hsd12	Nceh1	S100a1	Tcf7l2
	Apoe	Slc4a4	Scn1a	Abhd3	Brinp3	Enep	Hspa12a	Nfatc2	S100a13	Timp4
	Atp13a4	Tril	Oligodendrocyte	Ampd3	Cd47	Epb4.1l2	Itpr2	Pcdh15	S100a6	Zfp488
	Cst3	Wnt7a	Bcas1	Arhgap24	Chrm3	Epha6	Kcnab3	Ptprm	Sema6d	
	Cyp2j9		Gpr17	Arhgap31	Cldn10	Eps8	Lmcd1	Rab6b	Serpina3n	
	Dtna	Cplx1	Mitf	Aarg1	Coro6	Fbn2	Luzp2	Ramp1	Serpine2	
	Pla2g7	Fosl2	Tmem159	Atp2a2	D630028G08Rik	Fyn	Lypd6	Rap2a	Sod3	
	Ppp1r3c	Kltl		BC002189	Dbpht2	Galnt13	Mreg	Rreb1	Sptsxb	
Group 5	Astrocyte	Neuron	Oligodendrocyte	Unspecified	Dck3	Hrc	Slc12a8	Sphk1		
	Ednrb	Dgk	none	1700025G04Rik	Epcam	Itga2	Smpx	Tmem215		
	Gdpd2	Lrrtm3		Adamts16	Galnt14	Lrrfip1	Sntg1	Trpc3		
	Hapln1	Mlip		Akap7	Gipc2	Mme	Sox6			
	Il33	Ramp3		Alox8	Gucy2f	Parp8				
	Slc6a11	Slc6a17		Angpt1	Hcrt2	Rrad				
Group 6	Astrocyte	Neuron	Oligodendrocyte	Unspecified	Cnnm4	Ephb1	Hopx	Rhpn2	Tmem200a	
	Pla2g3	Cd24a	none	Adamts20	Dact1	Gm4951	Lrrc8d	Rps6ka5	Txnrd3	
	Psd2	Uchl1		Cdh22	Dok4	Hmgcr	Phlda1	Slco2a1		
Group 7	Astrocyte	Cnr1	St8sia4	Apba2	Cla1	Fbln2	Mapk11	Plcb1	Sept3	Vash2
	Itga6	Coro1a	Tmtc4	Arhgap33	Cla2	Fbxl7	Mapt	Plxna1	Sik1	Vim
	Masp1	Dpyr5	Trhde	Atp2b4	Col14a1	Fmnl1	Myrcn	Plxna3	Slc17a8	Vstm2a
	Sdl	Jph4	Oligodendrocyte	Crabp2	B4gal2	Fxyd6	Naip1	Plxnb2	Sncg	Vstm2b
	Tnc	Mmp17	none	BC068157	Ctnna2	Gmpip	Negr1	Ppp1r13b	Tacr1	Wnk2
	Neuron	Mturn	Unspecified	Camsap3	Ctnn1	Igf3	Nol4l	Psd	Tbc1d16	Zfp503
	Act16b	Pde10a	2310067E19Rik	Ccdc116	D930015E06Rik	Kdm2b	Nuak1	Ptpru	Tgfb3	Zfp57
	Camkv	Pde2a	4931428F04Rik	Ccser1	Dnmt1	Klf26b	Otof	Rbfox2	Ttll1	Zfp704
	Ccdc120	Sst	Adamts18	Cdh4	Ezr	Kirrel3	Ovgp1	Rufy3	Ubxn11	Zfp811
	Celsr3	St8sia2	Adcy5	Cidea	Fads3	Mad2l2	Pak7	Sema3f	Unc5a	Zmyrn3
Group 8	Astrocyte	Cntn4	Frmf3	Prkar2b	Oligodendrocyte	Celf3	Fbxl19	Mcm6	Pnck	Stc1
	none	Crmp1	Fstl5	Rab3b	Hn1	Chl1	Grin2b	Mdga2	Ptpn5	Stk32b
	Neuron	Cxadr	Gap43	Rasgrp2	Unspecified	Dapk1	Hdac9	Meis1	Ptprt	Sub1
	Abiln3	D11Bwg0517e	Gng2	Sema3a	4930506M07Rik	Dcc	Khdrbs2	Meis2	Rimkb	Syt5
	Cacna1b	Dcx	Hook1	Sez6	Acvr2a	Dcll1	Klf21b	Msr	Robo2	Tenn3
	Cacna1e	Dpyr3	Llcam	Spint2	Adarb2	Dock7	Klfh23	Myk	Samsd5	Tmem145
	Cacna1g	Enc1	Lmo3	Stmn2	B3gnt5	Dig2	Lin7c	Nln	Satb1	Tubb5
	Cbln1	Erc2	Myt1l	Thsd7b	Cacnb3	Ebf2	Lppr1	Nop58	Sh3yl1	Tusc3
	Celf2	Frbp1l	Olfm1	Zcchc12	Ccdc3	Ephb2	Lrrn3	Pde1c	Spats2l	Unc13b
	Cnih2	Foxp1	Oprf1		Cdh2	Fam19a4	March6	Phip	Stac	

Table 3

Gene Ontology Analysis

GO term	Ontology	Count	Enrichment score	p-value	Genes associated with the GO term
Regulation of neurogenesis	BP	58	3.1	5.62E-11	4930506M07Rik, Apoe, Bmp4, Bmp5, Brinp3, Cd24a, Cdh2, Cdh4, Cnr1, Cntn4, Dec, Dock7, Dpysl3, Ednrb, Enc1, Ephb2, Fyn, Gpr37l1, Gprc5b, Inpp5j, Inpp11, Kank1, Lpar1, Lpar1, Mapt, Meis1, Mycn, Negrl1, Olfr1, Omg, Plxna3, Plxnb2, Rap2a, Rnd2, Robo2, Ruffy3, Sema3a, Sema3f, Sema4d, Sema5a, Serpine2, Sez6, Sgk1, Sirt2, Slc39a12, Sox10, Sox8, Sphk1, Stmn2, Tcf4, Tcf7l2, Tenm3, Tenm4, Thr, Trf, Vim, Vwc2l, Wnt7a, Zfp488
Cell Adhesion	BP	60	2.8	1.95E-09	Acan, Agt, Angpt1, Atplb1, Atplb2, Bcan, Camsap3, Cbln1, Cd24a, Cd47, Cdh2, Cdh20, Cdh22, Cdh4, Celsr3, Chl1, Cldn11, Ctnn4, Ctnnap1, Col14a1, Corola, Cpxm2, Csrp1, Ctnn2, Cxadr, Dsg2, Ephb1, Hapln1, Hapln4, Igsf11, Inpp11, Itga6, Itga8, Kitl, L1cam, Lims2, Mag, Mcam, Mog, Mycn, Negrl1, Nfasc, Nptn, Nuak1, Omg, Pcdh15, Plcb1, Ptprn, Ptpru, Robo2, Sema4d, Sorbs3, Spp1, Tenm3, Tnc, Tnr, Ttyh1, Wnt7a
Regulation of neuron projection development	BP	40	3.7	3.02E-09	Apoe, Bmp4, Bmp5, Cd24a, Cdh2, Cdh4, Cnr1, Dec, Dpysl3, Enc1, Ephb2, Fyn, Gprc5b, Inpp5j, Inpp11, Kank1, Lpar1, Mapt, Negrl1, Omg, Plxna3, Plxnb2, Rap2a, Rnd2, Robo2, Ruffy3, Sema3a, Sema3f, Sema4d, Sema5a, Serpine2, Sez6, Sgk1, Slc39a12, Sphk1, Stmn2, Tenm3, Tnr, Vim, Wnt7a
Regulation of cell differentiation	BP	85	2.1	1.55E-07	4930506M07Rik, Abcg1, Acvr2a, Adamts20, Alox8, Apoe, Bmp4, Bmp5, Brinp3, Cd24a, Cdh2, Cdh4, Clu, Cnr1, Cntn4, Col14a1, Dact1, Dec, Dock7, Dpysl3, Ednrb, Enc1, Ephb2, Fbn2, Flt3, Foxp1, Fyn, Gpr37l1, Gprc5b, Hdac9, Hoxp, Inpp5j, Inpp11, Kank1, Kitl, Lpar1, Mad2l2, Mapt, Meis1, Meis2, Mitf, Mycn, Negrl1, Nr1d1, Olfr1, Omg, Plcb1, Plxna3, Plxnb2, Rap2a, Rbfox2, Rnd2, Robo2, Rora, Ruffy3, S100b, Sema3a, Sema3f, Sema4d, Sema5a, Serpine2, Sez6, Sgk1, Sirt2, Slc39a12, Smyd1, Sox10, Sox6, Sox8, Sphk1, Stmn2, Tcf4, Tcf7l2, Tenm3, Tenm4, Tgfb3, Tnr, Trf, Tyrobp, Vim, Vwc2l, Zeb2, Zfp488
Synapse	CC	43	2.6	3.69E-05	Atpl1a2, Bcan, Calb1, Cbln1, Cdh2, Chn2, Chrm3, Cnih2, Cplx1, Cxadr, Dact1, Dna, Ephb2, Eps8, Erc2, Gabra2, Gap43, Glra1, Glrb, Gria3, Grin2b, Lgi3, Lin7c, Lrrtm3, Mmne, Olfr1, Otof, Pcdh15, Prkce, Rasgrp2, Sep3, Serpine2, Sez6, Slc17a8, Slc1a2, Slc6a17, Syt2, Syt5, Tmem163, Unc13b, Vamp1, Vwc2l, Whrn
Calcium ion binding	MF	39	2.6	2.68E-04	Acan, Alox8, Anxa5, Atp2a2, Cacna1b, Cacna1e, Calb1, Casr, Cdh2, Cdh20, Cdh22, Cdh4, Celsr3, Dgkg, Dsg2, Ehd1, Enpp2, Fbn2, Fbn2, Fstl5, Ipr2, Man1a, Masp1, Mep2, Mmp17, Otof, Pcdh15, Pla2g4a, Plcb1, Pk8, Pvalb, Rasgrp2, S100a1, S100a13, S100a6, S100b, Sparc, Syt2, Ttyh1
Extracellular matrix	CC	30	2.9	7.06E-04	Acan, Adamts1, Adamts16, Adamts18, Adamts20, Adamts4, Apoe, Bcan, Bmp4, Chl1, Clu, Col14a1, Cpxm2, F3, Fbn2, Fbn2, Gpc5, Hapln1, Hapln4, Hspg2, Htra1, Lmcd1, Mmp17, Serpine2, Sod3, Sparc, Tgfb3, Tnc, Tnr, Wnt7a
Axon ensheathment	BP	13	5.7	1.42E-03	Arhgef10, Cldn11, Fyn, Kcnj10, Mal, Mbp, Nfasc, Omg, Plp1, Qk, Sirt2, Tspan2, Ugr8a
Ion transmembrane transporter activity	MF	46	2.1	4.65E-03	Atpl3a4, Atpl1a2, Atpl1b1, Atpl1b2, Atp2a2, Atp2b4, Cacna1b, Cacna1e, Cacna1g, Cacnb3, Cc1a1, Cc1a2, Cox8b, Cpxx, Fxyd6, Gabra2, Glra1, Glrb, Gria3, Grin2b, Ipr2, Kcna2, Kcnab3, Kcnj10, Nipal2, Nipal4, S100a6, Scn1a, Slc12a2, Slc13a3, Slc17a8, Slc1a2, Slc39a12, Slc44a1, Slc4a4, Slc6a11, Slc6a17, Slc6a5, Slc7a10, Slc1a4, Slc2a1, Trf, Trpc3, Trpm3, Tyhl1, Tusc3
Axon guidance	BP	18	3.6	9.87E-03	Cdh4, Chl1, Dec, Dpysl5, Ephb1, Ephb2, Foxp1, Gap43, L1cam, Nfasc, Nr4a3, Plxna3, Ptprn, Robo2, Sema3a, Sema3f, Sema5a, Unc5a
Lipid metabolic process	BP	52	1.9	1.31E-02	Abcg1, Acs13, Agpat9, Aldh1a1, Alox5, Alox8, Apod, Apoe, Asah2, B3gn5, Cd81, Cds1, Chpt1, Cidea, Crabp2, Eccl1, Elovl5, Elovl7, Enpp2, Enpp6, F2h, Fads3, Gdtd2, Gm2a, Hmger, Hras, Hsd11b1, Inpp5j, Inpp11, Map7, Nceh1, Neu4, Pip4k2a, Pla2g16, Pla2g3, Pla2g4a, Pla2g7, Plcb1, Plcg2, Plp1, Prkar2b, Qk, Scd2, Sgpp2, Sh3y11, Sirt2, Sor11, Sphk1, Spssb, St8sia2, St8sia4, Ugr8a

* italicized genes also belong to the subcategory of regulation of neuron differentiation. BP, biological process; CC, cellular component; MF, molecular function