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Differential Expression of Cytoskeletal Genes in the Cochlear Nucleus

David R. Friedland, Paul Popper, Rebecca Eernisse, Benjamin Ringger, and Joseph A. Cioffi

Department of Otolaryngology & Communication Sciences Medical College of Wisconsin, Milwaukee, WI

Abstract

The relationship between structure and function is clearly illustrated by emerging evidence demonstrating the role of the neuronal cytoskeleton in physiological processes. For example, alterations in axonal caliber, a feature of the cytoskeleton, have been shown to affect reflex arc latencies and are prominent features of several neuropathological disorders. Even in the non-pathologic situation, however, axonal diameter may be a crucial element for the normal function of specialized auditory neurons. To investigate this relationship we used serial analysis of gene expression (SAGE) and microarray analyses to characterize the expression of cytoskeletal genes in the central auditory system. These data, confirmed by real time RT-PCR, identified differential expression of intermediate neurofilament transcripts (i.e., Nefh, Nef3 and Nfl) among the subdivisions of the cochlear nucleus. *In situ* hybridization was used to identify specific classes of neurons within the cochlear nucleus expressing these neurofilament genes. Robust neurofilament expression was seen in bushy cells and cochlear nerve root neurons suggesting an association between cytoskeletal structure and rapid conduction velocities. Gene expression data were also identified for other classes of cytoskeletal and structural genes important in neuronal function. These results may help to explain some causes of hearing loss in hereditary neuropathies and provide an anatomic basis for understanding normal neuronal function in the central auditory system.

Keywords

cochlear nucleus; gene expression; cytoskeleton; microarray; SAGE; neurofilament

The cochlear nucleus receives all primary afferent input from the auditory nerve and processes such information for delivery to higher brain stem regions. The nucleus is divided into subdivisions, each with dominant classes of neurons and with particular roles in signal processing (see review (Cant and Benson 2003)). The anterior ventral cochlear nucleus (AVCN) has large populations of spherical bushy cells which send timing information to the olivary complex for sound localization. The posterior ventral cochlear nucleus (PVCN) contains similar classes of neurons, the globular bushy cells, but also has many octopus and multipolar cells with unique electrophysiological properties (Oertel et al. 1990). The dorsal cochlear nucleus (DCN) shows very little similarity to the ventral divisions and has

Correspondence: David R. Friedland, MD, PhD Medical College of Wisconsin Dept of Otolaryngology and Communication Sciences 9200 W Wisconsin Ave Milwaukee, WI 53226 414-805-5626 (office) 414-805-7936 (fax) dfriedla@mcw.edu.

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morphologically and physiologically distinct cell types (Ostapoff et al. 1994). This parsing of auditory processing roles and general segregation of cell types suggests that each subdivision can be distinguished on a structural and functional level. Furthermore, there may be genetic determinants of structure and function that can be identified by molecular biological methods and explain the various responses of cochlear nucleus neurons to auditory nerve stimuli.

Signal processing is most conspicuously a function of neurotransmitter and ion channel kinetics. However, a stronger role for the cytoskeleton in regulation of neuronal function has emerged (Al-Chalabi and Miller 2003, Fitzpatrick et al. 1998, Fuchs and Cleveland 1998, Lalonde and Strazielle 2003). The morphological features that the cytoskeleton imparts upon neurons, such as axon caliber and dendritic spine shape, affect the physiological properties of electrical conduction and interactions with surrounding neurons (Hoffman et al. 1987, Kriz et al. 2000). Recent studies have demonstrated cytoskeletal gene differences in the hippocampus of behaviorally disparate animals and there are implications of cytoskeletal abnormalities in some psychiatric disorders (Benitez-King et al. 2004, Feldker et al. 2003). Additional evidence as to the importance of the cytoskeleton in neuronal function has been the identification of cytoskeletal gene mutations underlying some hereditary neuropathies and neuro-degenerative disorders (Bruijn et al. 2004, De Jonghe et al. 2001, Lalonde and Strazielle 2003, Lupski 2000, Mendonca et al. 2005, Skvortsova et al. 2004).

This investigation hypothesizes that the cytoskeleton of cochlear nucleus neurons plays a role in determining morphological and physiological properties important for auditory processing. As such, this study uses high throughput genetic methods to characterize normal cytoskeletal gene expression patterns in the three major subdivisions of the cochlear nucleus. These data were correlated with morphologically and physiologically distinct cell classes to help understand some of the molecular mechanisms that make auditory neurons unique. These data also provide a template for understanding changes in the central auditory system that occur with sensorineural hearing loss and in association with many neurological disorders.

Cytoskeletal Components

The cellular cytoskeleton is composed of microtubules, intermediate filaments and microfilaments. Microtubules, the thickest of the cytoskeletal core structures, are principally involved in maintenance of cell body shape and the movement of organelles. Microtubules are composed of alpha- and beta- tubulin molecules and interact with an assortment of microtubule-associated proteins (MAPs). The smallest cytoskeletal structures are the microfilaments which primarily consist of actin fibers and lie just under the cell membrane anchoring and organizing membrane proteins. Intermediate in size between the microtubules and microfilaments are the aptly named intermediate filaments. The neuron associated intermediate filaments include internexin, peripherin and three forms of neurofilaments. The cytoskeletal classification can be organized using the gene ontology consortium hierarchy for cellular components (Figure 1).

Light, Medium and Heavy Chain Neurofilaments

Neurofilaments are members of the neuron specific Type IV intermediate filament group, and named for the molecular masses of their proteins as determined by gel electrophoresis. The light chain is about 68 kDa and is encoded by the human NEFL and rat Nfl genes. The medium chain is 150 kDa and a product of the NEF3 gene in humans and Nef3 gene in rats. The heavy chain ranges between 190 and 210 kDa and is a product of the human NEFH and rat Nefh genes. Each protein consists of a head, rod and tail. The rod regions are highly conserved and consist of an α -helical region which intertwine to form coiled-coil dimers. In

vivo, these dimers contain at least one Nfl coiled with either a Nef3 or Nefh. These obligate heterodimers then align in anti-parallel fashion (head-to-tail and tail-to-head) to form tetramers which link to form protofilaments and then organize into the 10nm neurofilaments.

The neurofilaments are the main components of the axonal cytoskeleton and are the principle determinants of axonal caliber. Axonal caliber is thought to be regulated by the phosphorylation of the neurofilaments, principally the heavy chain, which repels neighboring filaments and increases spacing between the fibers (Cleveland et al. 1991, Garcia et al. 2003). In addition, levels of medium and heavy chain neurofilament and their specific interaction with the light chain, appear to regulate axon thickness (Xu et al. 1996). Additional studies have shown that the stoichiometric relationship between the neurofilament subtypes is also an important determinant of structure with effects on dendritic arborization also noted (Kong et al. 1998). Axonal caliber is important for neuronal function as there is a positive correlation with conduction velocity (Hoffman et al. 1987, Kriz et al. 2000). In fact, mice with altered neurofilament levels had significant axonal atrophy and an almost 50% reduction in conduction velocity (Kriz et al. 2000).

Such findings demonstrate that the neurofilament cytoskeleton has a significant effect on neuronal transmission and may play an important role in establishing normal electrophysiological properties. This may be even more critical in sensory systems, such as the auditory system, which is dependent on rapid and high fidelity responses to stimuli. Cytoskeletal protein abnormalities also underlie many neuropathological disorders which have associated signs and symptoms of hearing loss and central auditory neuropathy.

METHODS

This study employed two different methods of high throughput transcriptome analysis to identify cytoskeletal genes and their differential expression among the divisions of the cochlear nucleus. We utilized serial analysis of gene expression (SAGE) due to its potential to detect any expressed transcript and for the strength of statistical comparisons among libraries of similar size. We also used microarrays (i.e. genechips) for their ease of use, consistency and ability to provide quantitative measures of gene expression. The specific protocols for each of these methodologies have been well reviewed in the literature.

Animals

Six-week-old Brown Norway rats (n=40) were used for isolation of RNA from the cochlear nucleus. The rats were anesthetized with Nembutal (50mg/kg IP), tested for loss of paw-pinch reflex, and decapitated. Brains were removed and the cochlear nuclei dissected off the surface of the brain stems on RNase-free Petri dishes. The nuclei were grossly divided into the three dominant subdivisions: AVCN, PVCN and DCN. Tissue from 40 rats (i.e., 80 cochlear nuclei) was pooled to minimize bias in RNA sampling due to slight variations in dissection of the cochlear nucleus subdivisions. RNA was extracted from the dissected tissue using the TRIzol protocol (Invitrogen, Carlsbad, CA). The RNA was analyzed by gel electrophoresis for integrity and spectrophotometry for purity and concentration.

Sage Library Generation

A commercial kit is available for SAGE library generation (Invitrogen, Carlsbad, CA) and the general protocol and our methods are available in the existing literature (Halum et al. 2004, Velculescu et al. 1995). The foundation of SAGE is that a 10 base-pair portion of an mRNA transcript located near the 3' terminus, referred to as a SAGE tag, is sufficiently unique to be able to identify that gene exclusively. In practice, a SAGE tag can occasionally be found in more than one transcript and thus represent more than one gene. Also, a single

gene may be large enough to be represented by more than one tag. Overall, however, the general principles have been confirmed and SAGE can provide a consistent gene expression profile of the tissue of interest (Lee et al. 2002, Velculescu et al. 1995). The final data consist of a list of SAGE tags and the frequency with which they have been found in the library. As tags are pulled at random from the library, the frequency is a relative measure of expression level. Tags are subsequently matched to known genes or transcribed sequences using a regularly updated public database (SAGEMap: <http://www.ncbi.nlm.nih.gov/SAGE/>). Theoretically, continuing to sequence a SAGE library will identify all the tags in that tissue and thus potentially every gene that is transcribed. We generated SAGE libraries from AVCN, PVCN and DCN that contain about 34,000 total tags representing approximately 3,000 known genes in each region. Statistical analyses of differential expression between libraries was performed using eSAGE which uses the statistic of Audic and Claverie (1997) and has been shown to be equivalent to the Fisher's Exact test and Z test (Audic and Claverie 1997, Margulies and Innis 2000).

Microarray Experiments

Genechips differ from SAGE in basic principles but represent an economical and well-established means of probing the transcriptome. Microarray studies start with a finite set of genes of interest. These genes are represented by oligonucleotide probes which are aligned and fixed to a glass slide. Microarray experiments detect the hybridization of tissue mRNA (specifically, cDNA made from the mRNA) to the probes on the slide by fluorescence to determine the presence and level of expression of a given transcript. For this study microarray experiments were performed commercially (GenUs Biosystems, Chicago, Ill.). The CodeLink Rat Whole Genome Bioarray (GE Healthcare) with over 35,000 probes was used to analyze the expression in each of the cochlear nucleus subdivisions. Fluorescence levels were compared to local background to determine whether each hybridization represented a "good call" and thus a valid representation of expression. Hybridization intensity was normalized to the mean of the chip with GeneSpring software (Silicon Genetics, Redwood, CA). Each subdivision was run on two chips and the average normalized intensities utilized to determine expression levels for each probe.

Real Time RT-PCR—Real-time reverse transcriptase PCR (real-time RT-PCR) was used to determine the relative expression levels of the three neurofilament genes. The housekeeping gene hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as a reference because of its consistent expression levels throughout the cochlear nucleus in both the SAGE and microarray experiments. RNA was treated with DNase I, Amp Grade (Invitrogen, Carlsbad, CA) and then reverse transcribed using oligo-dT primers (Invitrogen, Carlsbad, CA) and the Omniscript® Reverse Transcription kit (Qiagen Inc., Valencia, CA).

Real-time PCR was performed using Taqman® Gene Expression Assays (Applied Biosystems, Foster City, CA) for Nef3 (ABI#: Rn00566763_m1), Nfl (ABI#: Rn00582365_m1), Nefh (ABI#: Rn00709325_m1), Peripherin (ABI#: Rn00561807_m1), Vimentin (ABI#: Rn00579738_m1) and HPRT (ABI#: Rn01527838_g1). The reactions were run on the iCycler iQ Multicolor Real-Time Detection System (Bio Rad Laboratories, Hercules, CA). The thermal cycling conditions were as follows: 50°C hold for 2 min, 95°C hold for 10 min, followed by two-step PCR for 40 cycles of 95°C for 15 sec and 60°C for 1min. Negative controls, in which reverse transcriptase was omitted from the reaction, were run for each sample and all samples were performed in triplicate. Threshold cycle (Ct) values of HPRT and the gene of interest were then used to calculate the relative mean normalized expression in each subdivision (Muller et al. 2002). Each experiment was run in triplicate and the relative fold increase was averaged for the three runs. The data were

normalized to the subdivision with the lowest level to determine a relative fold increase in the other regions.

In situ hybridization—Brown Norway rats (n=3 per probe) were perfused transcardially with ice-cold 4% paraformaldehyde in 0.1M PBS, pH=7.2. Brain stems were dissected out, post-fixed for 1 hour in the same fixative and cryoprotected in 20% sucrose in RNase-free 0.1M PBS until sectioning. Cryostat sections were taken transversely through the brainstem at 20µm and collected on SuperfrosPlus™ slides (Fisher Scientific, Tustin, CA). The *in situ* hybridization protocol is well-documented and a brief description with probe specific information is provided below.

Reverse transcriptase polymerase chain reaction (RT-PCR) was used to generate amplicons to be used as probes for *in situ* hybridization. Authenticity of the probes was confirmed by DNA sequencing and the probes were subcloned into an RNA expression vector: pCR®II-TOPO® (Invitrogen, Carlsbad, CA). The following PCR primer sets were used: Nef3: Forward 5'-AGAGCGCAAAGACTACCTGAAGA, Reverse 5'-GGATATTGTGACTGAGGGCTGTC; Nfl: Forward 5'-TCTGAAGGAGAAGCAGAAGAGGA, Reverse 5'-ACATTGCCGTAGATCCTGAAGTC; and NefH: Forward 5'-CTCTGCCCAAGAGGAGATAACTG, Reverse 5'-CCCTCTTCTGCCTCTTCTTCTTC.

Digoxigenin-labeled single stranded cRNA probes were transcribed from linearized templates in 20µl reaction mixture: 1µg linearized plasmid, 2µl DIG RNA labeling mix (10 × concentration, Roche Applied Science, Penzberg, Germany), 1µl RNasin (40 U/µl), 10mM DTT, 4µl of 5X transcription buffer, and 20 units of Sp6 or T7 RNA polymerase (all from Promega, Madison, WI). Slide-mounted sections were sequentially treated and pre-digested with proteinase K, washed in DEPC ddH₂O, rinsed in 0.1 triethanolamine (pH 8.0), rinsed in 2XSSC (1XSSC = 0.15 M NaCl/0.04 M NaCitrate, pH 7.2), dehydrated in graded ethanols and air-dried. Slides were covered with 200µl/slide of hybridization mixture and incubated at 55°C for 16 hours. Sections were exposed to RNase (100µg/ml in 0.5 M NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0) for 30 min at 37°C, washed in decreasing concentrations of SSC with the final rinse done in 0.1XSSC at 55°C for 30 min. Following a final wash in Tris buffered saline, the slides were incubated with alkaline phosphatase conjugated sheep anti-digoxigenin antibody (Roche; 1:500 in TBS containing 0.3 % TRITON × 100 and 1 % blocking reagent) for 16 - 24 hours at 4C. Sections were washed and subsequently incubated in NBT/BCIP (Roche; NBT: nitroblue tetrazodium; BCIP: 5-bromo-4-chloro-3-indoyl-phosphate, 4 toluidine salt; buffer: 0.1M Tris, 0.1M NaCl, 0.05M MgCl₂, pH9.5) for 16 - 24 hours. The color reaction was frequently checked and upon reaching the desired intensity, slides were washed in distilled H₂O, air dried and mounted with Gel/Mount (Biomed, Foster City, CA). Negative control sections were treated with RNase (100 µg/ml) for 30 minutes at 37C prior to the proteinase K treatment.

RESULTS

Data analysis focused specifically on those genes mapping to the cytoskeleton node of the cellular component ontology from the gene ontology consortium (www.geneontology.org). SAGE data were mapped onto the cytoskeleton gene ontology and visualized in a treemap to identify highly expressed cytoskeletal classes (for example see Figure 2). As expected in neuronal populations, the microtubule component was very highly represented and had many genes mapping to the kinesin complex. Among the microfilament cytoskeletal components, the dynein complex, myosins and actin filaments had good representation. The intermediate filament class had genes found in all its subcategories including the neurofilaments, keratins and lamins. The microarray data were similarly analyzed and

expression levels for the individual genes was determined (for treemap example see Figure 3). These analyses identified high levels of tubulin in the cochlear nuclei as well as elevated levels of Nef3 and Nfl.

The transcripts of known cytoskeletal genes and their SAGE tag frequencies are listed in Table 1. There were 111 genes found by SAGE and the majority of these were identified in each cochlear nucleus subdivision. Only 29 of the 111 genes (26%) were expressed uniquely in a single subdivision and, of these, 22 were represented by a single tag. Of the remaining 7 transcripts found in a lone subdivision none were represented by more than 3 tags. Three tags were found for peripherin 1 (Prph1) and profilin 2 (Pfn2), which were only found in the AVCN, and another 3 tags for synaptonemal complex protein 3 (Sycp3) which was found only in the PVCN. For those represented by 2 tags, the majority were in the DCN (i.e., growth arrest specific 7: Gas7, Janus kinase 3: Jak3, and striatin: Strn) with the other being found only in the PVCN (vesicle associated membrane protein 3: Vamp3). None of the genes found in only one subdivision were expressed in sufficient tag numbers to have statistically significant differences in expression.

The genes mapping to the cytoskeleton ontology in the microarray experiments were identified with their respective hybridization intensities (Table 2). Only those genes with average intensities greater than the normalized median in at least one sample were included, as probes with overall lower expression levels did not demonstrate reliable reproducibility. There were 105 microarray probes with cytoskeletal roles among the three cochlear nucleus subdivisions. A liberal comparison based upon a two-fold change in intensity with at least one transcript expressed higher than the median showed that only myosin 5b was expressed in the AVCN higher than the PVCN. No genes meeting these criteria were found with higher expression in the PVCN than the AVCN. There were 5 genes with higher expression in the AVCN than DCN and these included the light and medium chain neurofilaments, Nef3 and Nfl. The other 3 genes were myosin 5b, activity regulated cytoskeletal associated protein (Arc) and an uncharacterized clone. Nef3, Nfl and myosin 5b were also expressed at similarly elevated levels in the PVCN as compared to the DCN. Several genes were found to be higher in the DCN than the AVCN including serine/threonine kinase 12, tektin1, troponin 1 type 3, sperm antigen 4 and keratin 8. No additional genes meeting the above criteria for differential expression were found between the DCN and PVCN.

The *intermediate* filament class was specifically analyzed due to the functional implications of these structural proteins. There were 10 genes found by SAGE and 13 genes present on the microarray that fell into this cytoskeletal sub-category (Tables 3 and 4). The results of SAGE showed that only two, peripherin1 and lamin B1, were expressed in a single subdivision. Both of these were found exclusively in the AVCN and peripherin1 expression was above baseline levels and represented by 3 tags. A single tag for the intermediate filament gene vimentin was found in PVCN and DCN and not in AVCN. The remaining intermediate filament genes were represented by at least one tag in all subdivisions of the cochlear nucleus. In comparing subdivisions, the neurofilament genes Nef3, Nefh and Nfl were all highly expressed in the AVCN and PVCN as compared to the DCN. The differences in frequency between each ventral division and the DCN for these tags were statistically significant ($p < 0.05$). Statistically significant differential expression was also seen for the abundance of internexin in the AVCN as compared to the two other subdivisions.

Analysis of microarray data similarly noted an approximately 3-fold greater expression level of Nef3 and Nfl in the AVCN and PVCN as compared to the DCN. Nefh was not represented on the microarray and no expression levels were obtained. The other primary intermediate filament, internexin, was also not on the microarray although the SAGE

experiment showed differential expression of this gene as well. The microarray had representations for intermediate filament genes that were not found by SAGE. These included Bfsb1, Krt21, Krt9, Dlgap2 and Rsn. None of these had expression levels significantly greater than the median and were therefore not considered in further analyses.

The notable difference between the SAGE and microarray data was in their representative levels of peripherin1 and vimentin. SAGE identified relatively few tags for these transcripts but they both had robust hybridization levels in all three subdivisions in the microarray experiments. To investigate this further, real time RT-PCR was performed using probes specific for vimentin and peripherin1. We inferred relative abundance by comparing the real time PCR cycle number at the point the threshold crossed background (C_T) to our housekeeping gene, HPRT, and to other genes we have studied. Both these transcripts appeared at cycle numbers relative to our housekeeping gene that were consistent with having moderate expression levels in all three subdivisions of the cochlear nucleus. Their actual levels of expression thus appear to be more accurately represented by the microarray hybridization intensities. Real-time RT-PCR showed that vimentin expression in the PVCN and DCN were between 1.5 and 1.8 fold higher than the AVCN. This result is consistent with SAGE, as tags for this gene were found only in the PVCN and DCN. Results consistent with SAGE were also found for peripherin1 which showed a 10.9 fold higher expression level in AVCN than in DCN and 5 fold higher expression compared to PVCN by real-time RT-PCR. Recall, this transcript was represented by 3 tags in AVCN and no tags were found in the other regions.

Further characterization of the differential expression of the intermediate filaments focused on the three neurofilament genes (i.e. Nef3, Nefh and Nfl) as these have been most frequently implicated in neuropathological processes and in normal neuronal function. In order to accurately determine their relative expression levels, real-time RT-PCR was performed on RNA extracted from each of the cochlear nucleus subdivisions. The data were normalized to the subdivision with the lowest level to determine a relative fold increase in the other divisions (Figure 4). Nef3 was found to be least expressed in the DCN with the AVCN and PVCN showing 6.4 and 4.0 fold higher expression, respectively. Similarly, Nfl was between 4.4 and 4.7 fold more highly expressed in AVCN and PVCN than in DCN. Nefh was also found to have higher levels of transcript in the VCN than the DCN: 4.4 fold higher in the AVCN and 2.2 fold higher in the PVCN. Thus, all the neurofilament cytoskeletal genes were more highly expressed in the ventral subdivisions of the rat cochlear nucleus than in the DCN. There was no significant differential expression between the AVCN and PVCN.

Localization of the transcripts for the neurofilament genes within each subdivision of the cochlear nucleus was investigated using *in situ* hybridization. The neurons expressing each of the neurofilament subtypes were analyzed with regards to location within each subdivision and the relative size and morphology of the cells. In general, Nef3, Nefh and Nfl appeared to localize to similar, but restricted classes of cells, and had notable expression in the ventral divisions. These findings are shown in figures 5 to 7 and specifically addressed below.

Strong hybridization with the Nef3 probe was observed in neurons of the AVCN and PVCN (Figure 5). This staining was most pronounced in the rostral AVCN, the ventral PVCN, the dorsomedial PVCN and around the nerve entry zone. These locations corresponded to regions known to have large populations of spherical bushy cell, globular bushy cell, octopus cell and cochlear nerve root neurons, respectively. There were some populations of small cells in these ventral nuclei that also seemed to be expressing the Nef3 transcript and these were in the marginal cell regions. In contrast, the DCN showed sparse staining. The

few positively staining cells were large with round to elongated shapes, located in the middle and deep layers of the nucleus. These neurons are discussed further below as similar staining patterns in the DCN were seen with Nefh.

The pattern of Nfl expression was similar to that observed with Nef3 (Figures 6). Staining was seen in predominantly large cells of the AVCN and PVCN. The cells around the cochlear nerve root were most obvious due to their intensity of staining in all specimens. Large Nfl expressing neurons were also regularly seen in the PVCN near the nerve root in globular bushy cell regions and extending dorsomedially toward octopus cell domains. In the rostral AVCN large round cells were readily apparent in the spherical bushy cell regions. Irregular and variably sized neurons within the mid-portions of the VCN were seen in tissues with higher background staining and based on location and morphology most likely represent the various classes of multipolar cells. Hybridization in the DCN was not observed with Nfl except in a few sections with very high background. In these sections, the majority of cells were small and diffusely distributed throughout the nucleus. These cells could not be definitively identified as having hybridized probe.

Nefh expression also showed similar patterns to Nef3 but variably sized and shaped neurons in the AVCN and PVCN were more readily apparent (figure 7). Their somatic features and distribution are consistent with the multipolar cell populations. As with the other neurofilament probes, expression was also pronounced in the nerve root, where globular bushy cells and octopus cells reside. In contrast to Nfl, but similar to that seen with Nef3, expression was seen in large cells in the DCN. The few positive cells found with the Nefh probe were in the deep layers of the DCN and did not seem to extend as superficial as some of the Nef3 positive cells.

Further analysis of DCN positive neurons employed morphological measurements of maximal cell body length and width with calculations of a roundness factor (width:length ratio). This was performed to investigate whether these cells could be classified into categories of DCN neurons as has been previously described (Kane et al. 1981, Ryugo and Willard 1985). Measurements of Nefh cells (n=7) showed an average maximal diameter of $30.56 \pm 4.93\mu\text{m}$ (range: $26.41\mu\text{m}$ to $40.46\mu\text{m}$) and an average roundness value of 0.65 (range: 0.44 to 0.84). The Nef3 neurons (n=9) had a similar average maximal diameter of $27.42 \pm 4.06\mu\text{m}$ (range: $23.31\mu\text{m}$ to $36.44\mu\text{m}$) with an average r-value of 0.71 (range: 0.56 to 0.83). The use of *in situ* hybridization stained tissue to perform such morphological measures has not been compared to the traditional histological preparations previously used in characterizing these cell types and these results should be interpreted with caution.

DISCUSSION

Cytoskeletal proteins provide the structural framework within neurons but also serve to define some of the electrophysiological properties of these cells. This study sought to provide an initial characterization of the expression patterns of specific cytoskeletal genes among the subdivisions of the cochlear nucleus. The patterns of expression were analyzed with respect to known morphological and physiological properties of the cochlear nucleus subdivisions and their neurons. These results may provide a molecular basis for further understanding normal and pathological auditory processing.

The principal finding in this investigation is that the neurofilament cytoskeletal genes appear to be highly expressed in the ventral subdivisions of the cochlear nucleus as compared to the DCN. These differential expression patterns were confirmed using real-time RT-PCR. The basis for this differential expression was further explored using *in situ* hybridization which identified high levels of transcript expression in several neuronal populations of the AVCN

and PVCN. This observation contrasted with the expression patterns in the DCN where only a few large cells in the deeper layers demonstrated appreciable levels of staining. Further analysis of these data examined specific ventral cochlear nucleus neurons and their morphological and physiological properties that may correlate with high levels of neurofilament expression.

Nef3 and Nfl transcript appeared to co-localize throughout the ventral cochlear nucleus by *in situ* hybridization and showed similar patterns of differential expression in the SAGE, microarray and real-time RT-PCR experiments. This co-localization is not unexpected as neurofilaments form as obligatory heteropolymers with Nfl promoting polymerization (Petzold 2005). The regions of co-localization for the Nef3 and Nfl neurofilament transcripts in the VCN included spherical and globular bushy cell domains, nerve root neurons and potentially octopus cell regions.

The spherical and globular bushy cells of the AVCN and PVCN are considered important in functions of sound localization (Cant and Benson 2003, Manis and Marx 1991, Wang et al. 1998). These cells have been well-studied as to the morphological and electrophysiological properties that may sub-serve this role. The most conspicuous specialized feature associated with the spherical bushy cell is the large auditory nerve synapse engulfing each neuron: the endbulb of Held (Ryugo and Fekete 1982, Wang et al. 1998). This large synapse ensures faithful transmission of peripheral auditory stimuli to the central nervous system. Globular bushy cells lack the endbulb-type contacts but receive a large number of auditory nerve terminals. These neurons project to the superior olivary complex (SOC) where interaural differences are further processed. Bushy cell axonal morphology has been studied as potentially contributing to the timing differences reaching the SOC (Beckius et al. 1999, Smith et al. 1993). In a study by Beckius and colleagues bushy cell axonal caliber was noted to be quite “substantial” near the main trunk and consistently about 2µm in diameter along the midportion (Beckius et al. 1999). Based upon their electrophysiological model, this thickness would provide very high conduction rates as is seen experimentally. They found little variability in axon caliber suggesting that regulation of axon thickness to control transmission velocity was not likely and that a major factor in establishing interaural timing cues was fiber length (Beckius et al. 1999). Thus, holding conduction velocity at a high and consistent rate among all the bushy neurons projecting to the SOC would “standardize” the initial stimulation allowing for the anatomy of the projection, rather than individual cellular electrophysiology, to influence interaural timing cues. As axon caliber is directly related to conduction velocity, similarly sized axons would control for this variable. In addition, a large caliber axon would maximize the conduction velocity thus facilitating the rapid localization of the sound stimulus (Higashimori et al. 2005, Kriz et al. 2000, Yagi et al. 1977).

Morphologically, the spherical and globular bushy cells are characterized by having large axons that do not branch within the cochlear nucleus prior to leaving via the trapezoid body (Cant and Benson 2003, Smith and Rhode 1987). While both classes have prominent axons, globular bushy cell projections appear to have the largest axon calibers within the cochlear nucleus (Spirou et al. 1990, Tolbert and Morest 1982, Tolbert et al. 1982). A primary determinant of axonal caliber is the neurofilament cytoskeleton and thus, on anatomical terms, high levels of neurofilament transcript would be expected to support this scaffold. Further, the endings of the globular bushy cells in the MSO (i.e., the calyces of Held) are among the largest in the mammalian brain (Guinan and Li 1990, Smith et al. 1991). While transcript level can not always be equated to levels of protein, simple maintenance of this extensive cytoskeleton may account for the high levels of neurofilament mRNA identified in these cells. The intermediate filament network, however, has been shown to be in a constant

dynamic state and ongoing gene transcription would be expected to maintain the robust cytoskeleton in these large neurons (Vikstrom et al. 1992).

Another cochlear nucleus region found to express high levels of neurofilament transcript was in the mid-to-posterior PVCN where octopus cells have been reported (Kane 1973, Osen 1969). This region may also contain globular bushy cells and definitive distinction between these cell types could not be obtained with this histochemical method. The octopus cells, named because of their prominent unidirectional dendritic projections, fire at the onset of an acoustic stimulus with great precision (Oertel et al. 2000, Oertel et al. 1990). They project to the ventral nucleus of the lateral lemniscus (VNLL) where they end in moderately sized endbulb-like synapses and to the superior paraolivary nucleus where they terminate in large boutons (Schofield 1995). Large terminals arising from these axons may be indicative of a robust cytoskeleton and account for increased levels of neurofilament expression in the region of the PVCN where these cell bodies reside.

Consistent neurofilament expression was also observed in the region of the cochlear root neurons. These cells have very short latencies and project to the reticular formation and areas important in startle and reflex control (Lee et al. 1996, Lopez et al. 1999, Scott et al. 1999). This class of neuron does not appear to process auditory information but rather functions as a warning system. The root neurons have remarkably large axons which, like the previously discussed bushy cells, do not branch until leaving the cochlear nucleus (Lopez et al. 1999). The lack of early branching and large caliber axons may require specialized or augmented structural support by the neurofilaments and help account for the elevated expression levels identified in this study.

The cochlear root neurons and globular bushy cells co-localize in the ventral VCN which represented the most heavily and consistently staining region for the neurofilament genes. These cells have similar light microscopic appearances consisting of an ovoid shape with eccentric nucleus but have been distinguished by morphometric measures (Merchan et al. 1988). Given the relative abundance of bushy cells we considered that some of our more ventral populations may not represent such nerve root neurons. However, we consistently observed these cells in the body of the auditory nerve and oriented, as described by Merchan and colleagues, with their long axes parallel to the nerve fibers (Figure 5C, 6A, 6C). Measurements of these cells, in these preparations, showed those cells within the nerve fibers had maximum diameters of >30um while the ovoid cells at the entry zone had diameters between 25-30um. This dimorphism is consistent with previously reported measures of these two cell populations under light microscopy (Merchan et al. 1988). The highest overall neurofilament expression, by *in situ* hybridization, thus appears to localize to the two VCN cell populations with the largest reported axon calibers and functional dependence on rapid conduction velocity. As these cells are unique to the VCN, this would also account for the high levels of neurofilament gene expression found in this region by SAGE, microarray and real-time RT-PCR.

Nefh expression patterns differed from that seen with Nef3 with the observation of staining in neurons of various morphologies distributed throughout the ventral cochlear nucleus. Given their location and appearance these likely represent multipolar cells of the VCN. Multipolar cells are described in detail in this issue and likely consist of many subtypes based upon anatomical and physiological criteria (Doucet and Ryugo 2006). They project within and between the cochlear nuclei and to other auditory brainstem centers without demonstrating the large, morphologically specialized endings seen with bushy cells. Multipolar axons are thin and can branch widely within the cochlear nucleus itself (Doucet and Ryugo 1997, Oertel et al. 1990, Palmer et al. 2003). The multipolar cells exhibit response properties that consist of very regular firing intervals in relation to the stimulation

and are quite distinct from the patterns of response in bushy or octopus cells (see review (Doucet and Ryugo 2006)). It may be these anatomical and physiological differences between multipolar cells and those neurons previously discussed that accounts for the apparent abundance of Nefh in the former as compared to Nef3.

These observed differences in neurofilament gene expression may represent a stoichiometric shift among the cell populations in the relative combinations of Nef3, Nfl and Nefh forming the intermediate filament skeleton. This, in turn, may affect the structural anatomy of the axon and ultimately physiological response properties. Experimental over-incorporation of Nefh chain into existing neurofilament networks results in a reduction of axonal caliber which is seen pathologically and may also be critical for normal physiological mechanisms (Gotow 2000, Straube-West et al. 1996)). Analysis of rat brain shows dynamic shifts in the relative ratios of Nefh to Nef3 and Nfl during development. Nefh appears later and in a lower relative level than Nef3 or Nfl (Scott et al. 1985). This observation is consistent with recent knock-out genetic experiments for the individual neurofilaments which have shown that Nef3 is more important than Nefh in determining axonal growth (Lariviere and Julien 2004). Thus, shifts in neurofilament ratios have morphological implications with an association between Nef3 expression and large axonal calibers. The contrasting correlate is that relatively high Nefh expression may be associated with thinner axons under normal physiological conditions. Thus, multipolar cells may have a unique proportion of Nefh which accounts for the differential gene expression seen in these experiments. Such a stoichiometric shift in Nefh abundance may then underlie the morphological differences between multipolar and bushy cells and some of their physiologically distinct properties.

Expression of all the neurofilament genes was lower in the DCN and this finding was mirrored by the paucity of staining in this region by *in situ* hybridization. Nef3 and Nefh were seen in large cells of the middle and deep layers and morphometric analysis suggests they may represent a combination of fusiform and giant cells. Two cells had diameters greater than 35µm which places them in previously reported ranges for giant cells (Brawer et al. 1974, Osen 1969) but the majority were between 25µm-35µm and consistent with fusiform cell sizes (Brawer et al. 1974). Width to length ratios were examined in an attempt narrow down the cell identification but the range of ratios indicated that such cells may represent elongate, ovoid and spherical classes of giant cell (Kane et al. 1981). The few cells with width:length ratios less than 0.5 may represent fusiform cells but their location in the deep DCN is also consistent with elongate giant cells (Kane et al. 1981). Measurements of greater numbers of cells and ultrastructural analyses would be necessary to better distinguish these cell forms.

Comparison of Neurofilament Expression to Other Brain Regions

The central auditory nuclei are part of a highly specialized system and may be expected to have unique gene expression patterns as compared to non-auditory centers. In addition, there may be differences between different brain stem auditory nuclei as has been shown among the subdivisions of the cochlear nucleus. The use of SAGE for the investigation of gene expression allows comparison of transcript frequency to existing libraries without requiring simultaneous performance of experiments. We compared the frequencies for the principal SAGE tags used to assess levels of Nef3, Nefh and Nfl in the cochlear nucleus to existing libraries for the normal rat SOC and hippocampus (Datson et al. 2001, Koehl et al. 2004). The tag frequencies have been normalized to counts per million to account for differences in library size (Table 5). Nfl is the basic neurofilament polymer and is well represented in all regions by two different SAGE tags without obvious differential expression to SOC or hippocampus. The majority of the differential expression of Nfl between DCN and the rest of the nucleus in this study appears to arise from one specific tag (i.e., GAAAAATAGT).

Such a finding may indicate the presence of a splice variant of Nfl that is differentially expressed and may impart functionally distinct properties on these cells.

There was only a single principal tag for Nef3 which was found in high numbers in the AVCN and SOC in contrast to low levels in the DCN and hippocampus. Similarities between the SOC and AVCN would include the presence of large Endbulb synapses and there may be unique features of the neuronal targets of these endings that Nef3 imparts; potentially high fidelity response and rapid transmission rates. The principal Nefh tag was high in AVCN and PVCN but relatively low in other regions. This, as discussed above, may reflect the contribution of multipolar cells to the pool of RNA transcript in the ventral nucleus and again suggests a unique role for Nefh in these cell types. Further investigation of such differential transcript expression throughout the auditory brainstem and on a cellular level may help characterize these genetic differences.

Intermediate Filament Mutations and Neuropathological Disease

The interdependence between structure and function is clearly illustrated by the number of neuropathological conditions associated with mutations and abnormalities of the intermediate filament cytoskeletal genes. These associations may be manifest as pathological accumulations of neurofilament proteins as markers of disease or by direct mutations of the cytoskeletal gene which become the causative agent of disease. Pathological accumulations of neurofilaments are seen in many neurodegenerative disorders including amyotrophic lateral sclerosis, Parkinson disease, Alzheimer's disease and various other neuropathies. These accumulations have traditionally been thought to be a by-product of neuronal dysfunction but it appears that such cytoskeletal disorganization can have its own functional sequelae.

Amyotrophic lateral sclerosis (ALS) is an adult onset neurodegenerative disorder leading to the loss of motor neurons in the spinal cord and brain. About 10% of these cases are associated with a congenital mutation in the SOD1 gene but the majority are sporadic without identifiable cause. A common feature in this disorder is an accumulation of neurofilaments in the soma and proximal axons. Even though there is neurofilament accumulation, studies have demonstrated up to a 60% reduction in Nfl expression in ALS motor neurons (Bergeron et al. 1994). In contrast, transgenic mice with over-production of Nefh develop a progressive ALS-like neuropathy (Cote et al. 1993, Julien et al. 1995). In humans, there does indeed appear to be increased immunoreactivity for phosphorylated Nefh in ALS (Petzold 2005). These seemingly contradictory findings may reflect a stoichiometric imbalance in the normal neurofilament ratios which may disrupt axonal transport and lead to heavy chain accumulations near the site of translation. In addition to accumulations of Nefh, multiple reports have found deletions and insertions in the tail region in a small subset of sporadic ALS cases (Lariviere and Julien 2004). This region contains the specific amino acid repeats important for phosphorylation.

Increased staining for phosphorylated Nefh is also seen in multiple sclerosis and Alzheimer's disease (Petzold 2005). Such staining may reflect a response to injury by the primary underlying pathology but there is some evidence that the axons with high Nefh staining are the ones that degenerate (Morrison et al. 1987). Similar patterns of staining have also been seen in diabetic neuropathy, glaucoma and stroke.

Mutations in the light chain neurofilament gene have also been associated with neuropathological diseases. Charcot-Marie-Tooth (CMT) is a progressive neurological disorder with two general phenotypes: a primary demyelinating form that results in conduction delays (CMT1) and a neuropathy with primary axonal loss that has normal conduction velocity for remaining axons but reduced spike amplitudes (CMT2). The latter is

further divided into several forms and CMT2E has been linked to mutations in the Nfl gene (Mersiyanova et al. 2000, Zuchner et al. 2004). A CMT pedigree with Nfl mutation included a member with deafness but the relationship to the discovered mutation is not clear (Zuchner et al. 2004). Other severe forms of CMT, typically associated with PMP22 (peripheral myelin protein) mutations, have also been associated with sensorineural hearing loss (Joo et al. 2004, Sambuughin et al. 2003).

The Cytoskeleton and Auditory Function

Many of the neurodegenerative disorders discussed have associations with auditory disturbance. For example, degeneration in the auditory system and/or auditory processing abnormalities have been described in Alzheimer's disease and ALS (Gil et al. 1995, Iliadou and Kaprinis 2003, Ohm and Braak 1989). Also, a subset of phenotypes of Charcot-Marie-Tooth (CMT) is clearly associated with sensorineural hearing loss. Further, audiometric studies of patients with hereditary motor and sensory neuropathy (HMSN), which CMT is a form of, demonstrated abnormal central auditory function (Alcin et al. 2000, Musiek et al. 1982, Papadakis et al. 2003). A notable feature of some of these patients are discrimination deficits out of proportion to their pure tone averages which may suggest a central processing disorder. Indeed, ABR testing showed an absence of the central wave responses suggesting that the pathology began proximal to the cochlear nerve and not in the peripheral cochlea. Overall, the association between auditory dysfunction and global neuropathies is not unexpected as normal auditory processing is highly dependent upon timing and high fidelity transmission of the initial stimulus. Given the association of many of the neuropathies with neurofilament abnormalities, the deficits in auditory function thus appear to arise from alterations in the cytoskeleton.

A neurofilament deficient quail (Quv) has provided a model for studying auditory function in cytoskeletal abnormalities. Auditory evoked potential testing was performed on Quv quails and, although electrocochleography remained normal, there was abnormal latency for the brain stem responses (Sheykholeslami et al. 2001). Further electrophysiological studies showed that higher brainstem auditory pathways also had significant conduction delays. This study demonstrated normal peripheral function but central auditory pathology. Electron microscopic evaluation of the cochlear nerve showed normal myelin but reduced axonal diameters due to an absence of neurofilaments (Sheykholeslami et al. 2001). The reduced fiber diameter correlated well with current understanding of the relationship between axon caliber and conduction.

Conclusion

These clinical and experimental data suggest that cytoskeletal genes function as more than just a scaffold and instead play significant roles in cellular electrophysiology. The current investigation into the cochlear nucleus demonstrates differential expression of cytoskeletal genes among the subdivisions of the cochlear nucleus, and between brain regions, which reflect the underlying neuroanatomical and electrophysiological properties. These findings help to establish a profile of normal cytoskeletal gene expression in the first central auditory center which, in turn, can be used as a template for assessing changes in hearing that may occur with neuropathologic disease and other central processing disorders. Further investigations, such as the development of specific neurofilament knockout animals, can better characterize the functional deficits that may arise from abnormalities of the auditory neuron cytoskeleton.

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Figure 1. Treemap representation of the cytoskeleton node of the gene ontology. This nested diagram demonstrates the components of the cytoskeleton and their sub-categories. The intermediate filaments, which are most critical in neurons, represent only a small fraction of the overall cellular scaffold. Within this category are the neuron-specific neurofilaments.

Figure 2.

Treemap representation of SAGE data from the cochlear nucleus incorporated into the cytoskeleton node of the Gene Ontology. The treemap has been filtered to show the major categories of cytoskeletal protein and a list of all genes found by SAGE which fit this category (for details of gene names see Table 1). The colors reflect the relative frequencies of the SAGE tags with brighter green representing the most abundant. In the broad categories it is apparent that although the intermediate filaments are a small component of the ontology they are the most highly expressed class in these tissues (i.e., brighter green).

Figure 3.

Treemap representation of microarray data from the cochlear nucleus incorporated into the cytoskeleton node of the Gene Ontology. The treemap has been filtered to show the major categories of cytoskeletal protein and a list of all genes found by microarray which fit this category (for details on gene names see Table 2). Similar to Figure 2, the brighter color represents higher hybridization intensity and the intermediate filament category is very highly represented. This treemap has been secondarily filtered to represent hybridization intensity as box size for the individual genes so that those below the median expression level do not interfere with the analysis. Probes for uncharacterized genes have been left in this view, in contrast to the gene list in Table 2, and these may present future targets for genetic analyses in the cochlear nucleus.

Figure 4.

Graphic representation of the relative expression levels of the neurofilament genes in each of the cochlear nucleus sub-divisions as determined by real-time RT-PCR. All the neurofilaments were more highly expressed in AVCN and PVCN than in DCN by at least a factor of 2-fold. As all expression levels are normalized to a standard, these data reflect differential gene expression by cochlear nucleus neurons rather than methodological issues of tissue sampling, cell number or cell density. These data confirm the findings by SAGE and microarray and were further investigated using *in situ* hybridization (Figures 5-6).

Figure 5.

Photomicrographs of *in situ* hybridization for Nef3 in the cochlear nucleus. An overview of the cochlear nucleus (A) shows darkly labeled cells in the PVCN and AVCN with little staining in the DCN. Higher power views provide better localization. Panel (B) shows the PVCN in which Nef3 expressing cells are seen at the nerve root and dorsally near octopus cell domains. A more rostral cut through the PVCN (C) show positive cells in globular bushy cell regions. Further sections through the nucleus (D) show staining in the ventral regions of the AVCN in spherical bushy cell areas. This panel also demonstrates the relative paucity of staining in the DCN (scale bar = 200um for B, C and D). Panel (E) and (F) depict Nef3 expressing neurons in the AVCN and PVCN, respectively. These cells are fairly

homogeneous, round to ovoid, and consistent with bushy cell appearances in traditional light microscopy. Some of the cells in the PVCN appear to have prominent axon hillocks which would indicate large caliber axons important for rapid conduction (see text). Examination of the DCN (G) shows few strong staining cells and those that are present are in the middle to deep layer of the nucleus. These likely represent fusiform and giant cells (scale bar = 50um for E, F and G).

Figure 6.

Photomicrographs of *in situ* hybridization for Nfl in the cochlear nucleus. Panel (A) shows the PVCN in which Nfl is heavily expressed in neurons around the nerve root, ventral portion of the nucleus and dorsomedially. These cell populations may represent nerve root neurons, globular bushy cells and octopus cells, respectively (scale bar = 200um). A section through the mid-portion of the ventral cochlear nucleus (B) show positive cells diffusely distributed throughout the subdivision. These cells are polymorphic and may include several types of multipolar cells (scale bar = 100um). Panel (C) shows a higher magnification view of some of the Nfl expressing neurons within the cochlear nerve root. These cells are thought to be important in startle reflexes and thus would have large axons allowing for

rapid signal conduction (scale bar = 50um). Cells in the ventral regions of the PVCN (D) and AVCN (E) that were strongly positive for Nfl had generally ovoid appearances with occasional prominent axon hillocks. These cells are likely globular bushy and spherical bushy cells, respectively. Other cells in the central core of the VCN had irregular shapes consistent with multipolar cells (F). These cells populations included both large and small neurons suggesting more than one class of multipolar cell is expressing this neurofilament (scale bar = 20um for D, E and F).

Figure 7.

Photomicrographs of *in situ* hybridization for *Nefh* in the cochlear nucleus. An overview of the VCN shows *Nefh* expressing cells throughout the nucleus with a prominent heavily staining cluster of cells near the ventral-rostral pole (A). These latter cells may be spherical bushy cells while the remainder may include classes of multipolar cell (scale bar = 100um). Panels (B) and (C) shows *Nefh* expressing cells in the DCN. Only a few cells demonstrated such gene expression and were predominantly localized to the deeper layers of the nucleus. These may thus represent one or more class of giant cell (scale bar = 200um in B; 50um in C). Panels (D) and (E) show the polymorphic cells expressing *Nefh* found throughout the ventral cochlear nucleus. This was in contrast to the more discrete expression of *Nef3*. The

various sizes and shapes of these cells suggest they belong to the multipolar class. We postulate that there is a different stoichiometric ratio of the neurofilament subunits in these cells that distinguishes them morphologically and physiologically from bushy cells (scale bar = 50um in D; 20um in E).

Table 1
Frequency of SAGE Tags for Cytoskeletal Genes in the Cochlear Nucleus Subdivisions

Frequency of SAGE tags representing cytoskeletal or cytoskeletal-associated transcripts in each of the subdivisions of the cochlear nucleus. The tag frequency for a given gene represents the sum of tags present in the library for that species. The genes found in this study represent microtubule, intermediate filament and microfilament classes of cytoskeletal protein. Analysis of the neuronally important intermediate filament group showed differential expression for tags for Nefh, Nef3, Nfl and α -internexin (all within the top 25 most abundant cytoskeletal tags in AVCN). The differences between expression of these genes in AVCN and PVCN as compared to expression in the DCN were significant at $p < 0.05$. Several genes were found in only one subdivision but their tag frequencies were too low to assess significance (see text for detail)

Genes	Description	AVCN	PVCN	DCN
Tuba1	alpha-tubulin	322	394	475
Cfl1	cofilin 1	61	91	88
Snap25	synaptosomal-associated protein	78	45	41
Csrp1	cysteine and glycine-rich protein 1	40	56	51
Nefh	neurofilament, heavy polypeptide	54	66	26
Myo5b	myosin 5B	42	54	40
Tmsb4x	thymosin beta-4	50	43	26
Nef3	neurofilament 3, medium	57	34	10
Gfap	glial fibrillary acidic protein	49	21	18
Stmn3	stathmin-like 3	40	16	9
Actb	actin, beta	16	20	21
Stmn4	stathmin-like 4	14	17	24
Olfm1	olfactomedin related ER localized protein	8	16	21
Nfl	neurofilament, light polypeptide	22	14	4
Mapt	microtubule-associated protein tau	13	14	10
Vamp2	vesicle-associated membrane protein 2	13	11	13
Spna2	alpha-spectrin 2	18	13	4
Map1b	microtubule-associated protein 1b	14	8	8
Mtap1a	microtubule-associated protein 1 A	12	11	7
Fez1	protein kinase C-binding protein Zeta1	16	9	4
Inexa	internexin, alpha	19	6	3
Tmsb10	thymosin, beta 10	8	5	14
Dncic2	dynein, cytoplasmic, intermediate polypeptide 2	6	7	10
Ptk2	protein tyrosine kinase 2	3	12	6
Dctn4	dynactin 4	6	7	6
Dncli2	LIC-2 dynein light intermediate chain 53/55	6	9	4
Vapa	vesicle-associated membrane protein, associated protein a	5	4	10
Cyln2	cytoplasmic linker 2	4	10	5
Plec1	plectin	3	6	10
Klc1	kinesin light chain 1	11	3	3
Stmn1	stathmin 1	6	4	7
Pfn1	profilin	5	9	3

Genes	Description	AVCN	PVCN	DCN
Bin1	myc box dependent interacting protein 1	6	3	6
Stx7	syntaxin 7	1	10	4
Gabarap	gamma-aminobutyric acid receptor associated protein	7	5	2
Stx5a	syntaxin 5a	4	3	5
Stmn2	stathmin-like 2	2	5	4
Cabp1	calcium binding protein 1	3	6	1
Kif1b	kinesin family member 1B	2	6	2
Tpm1	tropomyosin 1, alpha	5	2	2
Tubb5	tubulin, beta 5	3	3	3
Pin	dynein, cytoplasmic, light chain 1	5	1	2
Cnn3	calponin 3, acidic	3	3	2
Dctn1	dynactin 1	1	4	3
Lmna	lamin A	1	4	2
Egfr	epidermal growth factor receptor	5	0	1
Myh6	myosin heavy chain, polypeptide 6	3	1	2
Nme1	expressed in non-metastatic cells 1	2	3	1
Stk39	serine threonine kinase 39 (STE20/SPS1 homolog, yeast)	2	4	0
Krt2-8	keratin complex 2, basic, gene 8	1	4	1
Olfm3	olfactomedin 3	1	1	4
Usp2	ubiquitin specific protease 2	1	3	2
Vapb	vesicle-associated membrane protein, associated protein B and C	1	3	2
Aurkb	aurora kinase B	0	3	3
Add1	adducin 1, alpha	3	2	0
Csnk1d	casein kinase 1, delta	2	1	2
Waspip	Wiskott-Aldrich syndrome protein interacting protein	2	1	2
Ap1g1	adaptor protein complex AP-1, gamma 1 subunit	1	2	2
Myh9	myosin, heavy polypeptide 9	1	0	4
Vdp	vesicle docking protein, 115 kDa	1	2	2
Stx4a	syntaxin 4	0	3	2
Gabarapl2	GABA(A) receptor-associated protein like 2	1	1	2
Bet1	blocked early in transport 1 homolog (S.cerevisiae)	0	1	3
Sh3kbp1	SH3-domain kinase binding protein 1	0	2	2
Pfn2	profilin II	3	0	0
Prph1	peripherin 1	3	0	0
Acta1	actin alpha 1	2	1	0
Als2cr3	amyotrophic lateral sclerosis 2 chromosome region, candidate-3	2	1	0
Coro1b	coronin, actin-binding protein, 1B	1	1	1
Gosr2	golgi SNAP receptor complex member 2	1	2	0
Mtap6	microtubule-associated protein 6	1	2	0
Ptk2b	protein tyrosine kinase 2 beta	1	1	1
Stx6	syntaxin 6	1	0	2
Gpr51	G protein-coupled receptor 51	0	1	2

Genes	Description	AVCN	PVCN	DCN
Myo1e	myosin IE	0	2	1
Sycp3	synaptonemal complex protein 3	0	3	0
Cspg6	chondroitin sulfate proteoglycan 6	1	0	1
Jak1	Janus kinase 1	1	1	0
Lamb2	laminin, beta 2	1	0	1
Msn	moesin	1	0	1
Tekt1	tektin 1	1	0	1
Tmod2	tropomodulin 2	1	1	0
Fez2	fasciculation and elongation protein zeta 2 (zygin II)	0	1	1
Gas7	growth arrest specific 7	0	0	2
Jak3	Janus kinase 3	0	0	2
Rhoip3	Rho interacting protein 3	0	1	1
Strn	striatin	0	0	2
Vamp3	vesicle-associated membrane protein 3	0	2	0
Vim	vimentin	0	1	1
Hspb7	heat shock 27kD protein family, member 7 (cardiovascular)	1	0	0
Lmnbl	lamin B1	1	0	0
Tctex1	t-complex testis expressed 1	1	0	0
Actg2	actin, gamma 2	0	1	0
Add3	adducin 3, gamma	0	0	1
Arpc1b	actin related protein 2/3 complex, subunit 1B	0	1	0
Arhgap4	Rho GTPase activating protein 4	0	0	1
Cetn2	centrin 2	0	1	0
Clasp2	CLIP-associating protein 2	0	1	0
Cutl1	cut (Drosophila)-like 1	0	1	0
Dmd	dystrophin	0	0	1
Epim	epimorphin	0	0	1
Kif3c	kinesin family member 3C	0	0	1
Mlc3	fast myosin alkali light chain	0	1	0
Myo1c	myosin IC	0	1	0
Nup62	nuclear pore glycoprotein 62	0	1	0
Pacsin2	protein kinase C and casein kinase substrate in neurons 2	0	1	0
Pscd3	pleckstrin homology, Sec7 and coiled/coil domains 3	0	0	1
Stx3a	syntaxin 3	0	1	0
Sycp2	synaptonemal complex protein 2	0	0	1
Tmod1	tropomodulin 1	0	0	1
Vil2	villin 2 (ezrin p81)	0	0	1

Table 2
Normalized Microarray Hybridization Intensities of Cytoskeletal Genes in the Cochlear Nucleus

Hybridization intensities for cytoskeletal and cytoskeletal-associated probes on the GE Codelink Rat Whole Genome Bioarray. The hybridization intensities were normalized to the median for the array and each experiment was run in duplicate. Many genes in common with the SAGE data, particularly among the highly expressed transcripts, are noted and represent all classes of cytoskeletal protein. As in the SAGE data (Table 1), the neurofilaments Nef3 and Nfl show greater expression levels in the two VCN subdivisions than in the DCN. Nefh was not represented on the microarray.

Gene	Description	AVCN	PVCN	DCN
Klc1	kinesin light chain C mRNA	281.75	309.69	204.81
Nef3	neurofilament 3, medium	152.95	169.63	47.30
Pin	dynein, cytoplasmic, light chain 1	123.30	128.84	108.44
Nfl	neurofilament, light polypeptide	140.10	151.03	54.57
Fez1	protein kinase C-binding protein Zeta 1	122.65	121.66	95.58
Olfm1	olfactomedin related ER localized protein	97.74	99.56	89.46
Fez1	protein kinase C-binding protein Zeta 1	102.50	97.48	80.31
Mtap1a	microtubule-associated protein 1 A	100.64	105.34	64.35
Pfn2	profilin II	104.94	88.20	73.50
Gfap	glial fibrillary acidic protein	84.82	100.50	62.74
Dncli2	LIC-2 dynein light intermediate chain 53/55	79.98	75.74	63.84
Cfl1	cofilin 1	71.35	74.63	71.56
Spna2	alpha-spectrin 2	69.08	71.57	53.54
Dctn1	dynactin 1	67.94	65.70	57.93
Cnn3	calponin 3, acidic	62.19	59.81	64.98
Tuba1	alpha-tubulin	61.78	62.91	52.06
Prph1	peripherin 1	61.15	62.71	44.69
Vamp3	vesicle-associated membrane protein 3	55.66	59.35	53.44
Dncic2	dynein, cytoplasmic, intermediate polypeptide 2	57.12	56.17	49.42
Tubb5	tubulin, beta 5	54.90	55.66	44.93
Stmn2	stathmin-like 2	53.41	50.70	45.40
Cabp1	caldendrin	54.09	50.66	42.79
Mtap6	microtubule-associated protein 6	52.66	52.50	40.65
Vim	vimentin	32.40	42.03	59.74
Mapt	microtubule-associated protein tau	48.73	43.56	39.02
Gpr51	G protein-coupled receptor 51	39.91	36.17	48.26
Clasp2	CLIP-associating protein 2	41.73	46.22	35.70
Map1b	microtubule associated protein IB, mRNA	44.65	46.88	31.51
Stk39	serine threonine kinase 39	43.61	44.29	34.50
Bin1	myc box dependent interacting protein 1	39.28	46.09	36.34
Stmn3	stathmin-like 3	40.30	31.67	27.19
Trim3	tripartite motif protein 3	34.39	34.26	27.17
Gabarap	gamma-aminobutyric acid receptor associated protein	32.96	32.00	29.42

Gene	Description	AVCN	PVCN	DCN
Nme1	expressed in non-metastatic cells 1	28.24	28.48	27.94
Olfm3	olfactomedin 3	24.65	24.22	35.02
Errp	epidermal growth factor receptor related protein, mRNA	27.07	28.19	20.85
Stmn2	stathmin-like 2	28.54	20.99	24.93
TMBR-3	brain alpha-tropomyosin, mRNA, 3' end	25.17	23.57	23.57
Bet1	blocked early in transport 1 homolog, <i>S. cerevisiae</i>	21.52	19.78	19.89
Zrp3	zygin-related protein type III, mRNA	22.03	19.01	16.81
Pfn1	profilin	19.12	20.16	16.33
Vdp	vesicle docking protein, 115 kDa	19.43	16.23	18.03
Zrp3	zygin-related protein type III, mRNA	19.99	16.56	14.53
Actg2	actin, gamma 2	15.65	16.57	16.31
Stx5a	syntaxin 5a	15.78	16.44	16.25
Pacsin2	protein kinase C / casein kinase substrate in neurons 2	16.98	15.42	15.22
Stx4a	syntaxin 4	15.79	15.35	13.58
Cnn3	calponin 3, acidic	14.37	12.52	15.30
Ptk2	protein tyrosine kinase 2	11.69	12.44	12.33
Gphn	gephyrin	12.50	11.14	12.64
Tctex1	t-complex testis expressed 1	13.01	11.37	11.50
Inexa	Internexin, alpha	13.58	9.75	7.02
Sh3kbp1	SH3-domain kinase binding protein 1	10.58	12.80	10.01
Snx16	sorting nexin 16	12.12	9.71	11.26
Vil2	<i>Rattus norvegicus</i> mRNA for ezrin p81	8.00	8.84	13.54
Vamp2	vesicle-associated membrane protein 2	7.59	10.16	12.26
Coro1b	coronin, actin-binding protein, 1B	10.88	9.44	9.18
Trim3	tripartite motif protein 3	10.17	9.18	9.27
Plec1	plectin	8.47	8.60	11.33
Ubp45	deubiquitinating enzyme Ubp45, mRNA	9.58	8.70	8.43
Tubb5	tubulin, beta 5	8.96	9.20	8.53
Stk12	serine/threonine kinase 12	3.93	3.10	19.61
Usp2	ubiquitin specific protease 2	9.26	7.94	8.20
Tekt1	tektin 1	5.60	5.38	14.01
Cspg6	chondroitin sulfate proteoglycan 6	6.73	6.33	7.24
Olfm1	olfactomedin related ER localized protein	6.56	6.39	7.19
Cabp1	calcium binding protein 1	7.63	6.03	5.58
Cyln2	cytoplasmic linker 2	7.09	6.21	5.81
Lmna	lamin A	5.81	6.99	5.50
Apc	adenomatosis polyposis coli	5.84	5.50	6.15
Vac14	hydin	6.21	5.13	5.57
Bin1	myc box dependent interacting protein 1	5.54	5.68	5.28
Myl3	myosin, light polypeptide 3	5.52	5.98	4.89
Lamb2	laminin, beta 2	4.55	5.87	5.27
Stk39	serine threonine kinase 39	5.02	5.11	5.01

Gene	Description	AVCN	PVCN	DCN
Acta1	actin alpha 1	4.65	5.36	5.08
Arc	activity regulated cytoskeletal-associated protein	6.80	4.36	2.79
Myr8	myosin heavy chain	5.52	4.13	3.98
Jak3	Janus kinase 3	3.71	5.55	3.81
Myr5	Unconventional myosin from rat 3	4.01	4.24	3.77
Tmod1	tropomodulin 1	4.82	3.28	3.77
Stx7	syntaxin 7	4.10	3.25	3.90
Jak3	Janus kinase 3	2.87	4.15	2.94
Mtap6	microtubule-associated protein 6	3.44	2.63	2.61
Stx1a	syntaxin 1a	2.17	2.26	2.99
Myo5b	myosin 5B	4.15	2.06	0.64
Vamp2b	vesicle associated membrane protein 2B	2.20	2.29	1.80
Gphn	gephyrin	2.26	1.87	2.16
Dctn4	dynactin 4	2.14	1.93	2.17
Tnni3	troponin 1, type 3	0.70	0.66	4.57
Add3	adducin 3, gamma	1.59	1.39	1.64
Myo7a	myosin VIIA	1.32	1.21	1.89
Csnk1d	casein kinase 1, delta	1.01	1.18	1.42
Tnnt2	troponin T2	1.06	1.57	0.91
Sycp2	synaptonemal complex protein 2	0.97	1.03	1.39
Kif3c	kinesin family member 3C	1.14	1.00	1.10
Rsn	restin	1.02	1.03	1.12
Pscd2	pleckstrin homology, Sec7 and coiled/coil domains 2	1.16	0.84	1.05
Vapb	vesicle-associated membrane protein, associated protein B	1.05	1.06	0.88
Dlgap2	discs, large, Drosophila, homolog-associated protein 2	1.12	0.95	0.89
Arpc1b	actin related protein 2/3 complex, subunit 1B	0.98	1.13	0.84
Spag4	sperm antigen 4	0.64	0.64	1.49
Myl2	myosin, light polypeptide 2	1.10	0.88	0.77
Mefv	Mediterranean fever	0.98	0.94	0.83
Krt8	keratin 8	0.51	0.40	1.61

Table 3
Frequency of SAGE Tags for Intermediate Filament Genes

Frequency of SAGE tags for the intermediate filament genes. The intermediate filament class contains the neurofilaments which are the principal components of the axonal cytoskeleton. The high levels of Gfap are contributions from supporting cells in the nucleus but the neurofilament tags are neuron specific and thus represent expression by cochlear nucleus neurons. The differences between AVCN and DCN for each of the 5 most abundant tags was significant at $p < 0.05$. Interestingly, peripherin and vimentin were poorly represented in the SAGE library but follow-up analysis by real-time RT-PCR showed then to be present in more robust levels. Peripherin was found to be more highly expressed in the AVCN which is consistent with these data that show peripherin tags only in the AVCN library.

Gene	Description	AVCN	PVCN	DCN
Nef3	neurofilament 3, medium	57	34	10
Nefh	neurofilament, heavy polypeptide	54	66	13
Gfap	glial fibrillary acidic protein	49	21	18
Nfl	neurofilament, light polypeptide	22	14	4
Inexa	internexin, alpha	19	6	3
Prph1	peripherin 1	3	0	0
Krt2-8	keratin complex 2, basic, gene 8	1	4	1
Lmna	lamin A	1	4	2
Lmnb1	lamin B1	1	0	0
Vim	vimentin	0	1	1

Table 4
Microarray Hybridization Intensities for Intermediate Filament Genes

Hybridization intensities for the intermediate filament genes found on the GE Codelink Rat Whole Genome Bioarray. This class of cytoskeletal component is dominated by the neurofilaments (Nefh was not on the microarray) and the ratios of expression between the subdivisions is similar to that noted with SAGE. Many of the genes found by microarray which were not detected by SAGE were expressed below the median level for the chip and thus are likely to be present in very low levels. In contrast to the SAGE analysis, peripherin1 and vimentin were moderately expressed which correlates well with the real-time RT-PCR results. While the real-time PCR indicated that peripherin1 was differentially expressed between AVCN and DCN this level of difference was not as apparent on the array.

Gene	Description	AVCN	PVCN	DCN
Nef3	neurofilament 3, medium	152.95	169.63	47.30
Nfl	neurofilament, light polypeptide	140.10	151.03	54.57
Gfap	glial fibrillary acidic protein	84.82	100.50	62.74
Prph1	peripherin 1	61.15	62.71	44.69
Vim	vimentin	32.40	42.03	59.74
Inexa	Internexin, alpha	13.58	9.75	7.02
Lmna	lamin A	5.81	6.99	5.50
Dlgap2	discs, large (Drosophila) homolog-associated protein 2	1.12	0.95	0.89
Rsn	restin	1.02	1.03	1.12
Lmnb1	lamin B1	0.58	0.59	0.71
Krt8	keratin 8	0.51	0.40	1.61
Bfsp1	lens fiber cell beaded-filament structureprotein	0.49	0.64	0.43
Krt21	cytokeratin 21	0.13	0.13	0.10
Krt9	keratin 9	0.02	0.05	0.06

Table 5
Relative SAGE Tag Frequency for Neurofilament Genes in the Rat Brain

Relative SAGE tag frequencies for the neurofilament genes in the rat brain. The tag counts for the neurofilament genes in the AVCN, PVCN and DCN were compared to SAGE libraries generated from normal rat superior olivary complex (SOC) and hippocampus. All counts were normalized to “tags per million” to account for differences in library size. High levels of Nef3 were noted in AVCN and SOC which may represent expression in neurons receiving large endbulb terminals. Similarly, the high levels of Nefh in the AVCN and PVCN as compared to other regions may arise from the contribution of multipolar cell transcript to the analyzed RNA. Nfl was widely distributed in all regions which is expected as this is the most abundant neurofilament and serves as the initial scaffold and catalyst for assembly.

Neurofilament	SAGE Tag	AVCN	PVCN	DCN	SOC	Hippocampus
Nef3	GGAGAACCTT	1667	901	295	1367	78
Nefh	AAGGAGGAGG	1579	1859	767	162	198
Nfl	AGTTGATGCA	322	113	177	260	377
Nfl	GAAAAATAGT	585	366	89	293	169

* tag counts are normalized to tags per million