ABSTRACT

Bruce E. Mock. FUNCTIONAL AGING OF THE INNER EAR SENSORY SYSTEMS IN MOUSE MODELS OF AGE-RELATED HEARING LOSS (under the direction of Sherri M. Jones, Ph.D.) Department of Communication Sciences and Disorders; September 2008.

Age related structural and functional change in the cochlea have been well described and predisposing factors including genetic background, gender, and environmental factors have been identified. To date, nine genetic loci contributing to age related hearing loss (ARHL) have been identified and auditory function has been described in mouse strains carrying these mutations. The effect of these ARHL mutations on the other inner ear modality (vestibular) is poorly understood. The objective of the current study was to characterize and compare age related change in auditory and vestibular function (more specifically macular function) in three strains carrying ARHL mutations (C57BL/6J, CE/J, and NOD NON-H2^{nb1}/LtJ) and one control strain with no known mutations (CBA/CaJ). Auditory brainstem response (ABR) and distortion product otoacoustic emissions (DPOAE) were used to assess cochlear function and vestibular evoked potential (VsEP) was used to assess macular function. The macular organs from young, mid-life, and old animals were harvested, prepared, and imaged using scanning electron microscopy (SEM) and a qualitative comparison of the number and distribution of macular otoconia was made.

ABR and VsEP thresholds, peak latencies, and peak to peak amplitudes were quantified. Linear regression, student's T-test, and ANOVA were used to describe and compare auditory and macular function between genders, within strain, and between strains. DPOAE amplitude was plotted as a function of geometric mean frequency for 11 age groups. No statistical analysis was performed on DPOAE data.

No significant gender difference was found for auditory or vestibular function in any strain so genders were pooled for further analysis. The intra-strain comparison of auditory and macular sensitivity reveals a significantly different rate of change in the two modalities in all three strains ($p<0.0001$). Inter-strain comparison of change in macular sensitivity shows that the CBA/CaJ and CE/J strains lose sensitivity at a significantly faster rate than the C57 strain. A comparison of SEM images from young and old mice revealed no apparent qualitative difference in macular otoconia that would explain the observed declines in macular sensitivity.

These results suggest 1) there are age related declines in auditory and macular sensitivity and aging alone affects the two inner ear sensory systems at a similar rate; 2) that a genetic disposition for age related functional decline in one inner ear modality does not obligate or predict age related decline in the other inner ear modality. Genetic background is an important factor in age related hearing loss and vestibular dysfunction and will likely play an increasingly important role in diagnosis and treatment of these disorders.

FUNCTIONAL AGING OF THE INNER EAR SENSORY SYSTEMS IN MOUSE MODELS OF AGE-RELATED HEARING LOSS

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Models of Age-Related Hearing Loss

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LIST OF SYMBOLS AND ABBREVIATIONS

Ahl	Designator for the *Cdh23*^{753A} mutation
ANOVA	Analysis of Variance
ARHL	Age Related Hearing Loss
B & K	Brüel & Kjær
Ca^{+}	Calcium
Cdh23	Cadherin23
CDP	Computerized Dynamic Posturography
DNA	Deoxyribonucleic Acid
DPOAE	Distortion Product Otoacoustic Emission
EAM	External Auditory Meatus
EP	Endolymphatic Potential
FFT	Fast Fourier Transform
GM	Geometric Mean
Hz	Hertz
IHC	Inner Hair Cell
K^{+}	Potassium
kHz	Kilohertz
ml	Milliliter
ms	millisecond
mtDNA	Mitochondrial Deoxyribonucleic Acid

mV	Millivolt
µsec	Microsecond
µV	Microvolt
OHC	Outer Hair Cell
OAE	Otoacoustic Emission
OKN	Optokinetic Nystagmus
OVAR	Off Vertical Axis Rotation
p	Probability
pe SPL	Peak Equivalent Sound Pressure Level
PTC	Probe tube Calibration
ROM	Reactive Oxygen Metabolites
SCC	Semi-Circular Canal
S	Second
SEM	Scanning Electron Microscopy
SG	Spiral Ganglion Cells
SNHL	Sensorineural Hearing Loss
SPL	Sound Pressure Level
TDT	Tucker Davis Technologies
TM	Tympanic Membrane
TTS	Temporary Threshold Shift
VOR	Vestibulo-Ocular Reflex
VsEP	Vestibular Evoked Potential

<	Less than
>	Greater than
°C	Degrees Celsius

CHAPTER I: REVIEW OF THE LITERATURE

Introduction

The mammalian inner ear contains the peripheral organs for two sensory systems: the auditory system and the vestibular system. The organ of Corti in the cochlea transduces sound into neural impulses and the maculae of the vestibule and the cristae of the semicircular canals transduce linear and angular motion of the head, respectively. Deficits in these sensory systems or their neural connections can lead to hearing impairment and/or balance disorders. Age related hearing loss (ARHL), also known as presbycusis, is the most common type of hearing impairment in humans affecting 50% of the population by age 80 (Gorlin, Toriello, & Cohen, 1995; Morton, 1991). Factors such as disease, ototoxic drugs, genetic background, noise exposure, and even gender may influence ARHL. ARHL is a progressive disorder, so as the average age of the population increases the prevalence of ARHL also increases. Due to changing demographics in the U.S., it is estimated that by 2050, 20.7% of the population will be 75 or older (http://www.census.gov). ARHL is one of the most common chronic health problems in the elderly and is considered a major public health issue due to its adverse effects on quality of life and the economic costs that result from this communication disorder. A 1999 study by the National Council on Aging (http://www.ncoa.org/attachments/UntreatedHearingLossReport.pdf) reported that those with untreated hearing loss were more likely to report depression, anxiety, and paranoia and were less likely to participate in organized social activities. Decreases in auditory sensitivity with aging have been widely investigated and explained by structural and

physiological changes in the outer, middle, inner ear and central pathways (e.g. Schuknecht, et al., 1974; Wiley, Cruickshanks, Nondahl, & Tweed, 1999; Ingham, Thornton, & Withington, 1998). While many studies have examined the prevalence (50% of the population by age 80 (Gorlin, et al., 1995)) and impact of presbycusis on society (e.g., decreased potential earnings, increased social isolation, cost of treatment) (Mulrow, Aguilar, & Endicott, 1990; Lutman, 1990; Hinchcliffe, 1990; Stach, Spretnjak, & Jerger, 1990; Gates & Cooper, 1991, Mohr, et al., 2000, Strawbridge, et al., 2000) relatively little has been done to investigate the effects of vestibular dysfunction on society or public health. Studies by the National Institute on Deafness and other Communication Disorders estimated that 3.4% of the U.S. adult population (6.2 million people) suffers from chronic dizziness and/or imbalance (Hoffman & Sklare, 2003). It is estimated that as many as 7 million people per year seek care for disequilibrium and/or vertigo and that 30% of the population experiences episodes of dizziness before age 65 (Roydhouse, 1974). The increased probability of falls and injury in the elderly has become accepted as a fact of life. In 1992, the 75 and older age group, while comprising only 5.2% of the population accounted for 58.8 % of deaths due to falling (National Safety Council, 1992).

A Short Primer on Cochlear Anatomy & Physiology

There are several sources that describe general cochlear function (see Jahn & Santos-Sacchi, 2001). The cochlea is the sensory organ for audition and the organ of Corti within the cochlea houses the sensory cells for hearing. The cochlea is a spiral shaped organ, divided into three partitions: the scala vestibuli, the scala media, and the scala tympani, with an average of two and three quarter turns around a finely perforated

portion of the temporal bone. A bony ridge, the spiral lamina, extends out from the central portion of the cochlea and provides structural support for the organ of Corti. Within the spiral lamina a tube shaped cavity, known as the modiolus, houses the cell bodies of the axons of the auditory nerve. This collection of neural cell bodies forms a helical shape that parallels the scala of the cochlea and is known as the spiral ganglion (SG). The scala media is separated from the scala vestibuli and the scala tympani by Reisner's membrane and the basilar membrane, respectively. The scala tympani and scala vestibuli are filled with a fluid called perilymph and the scala media is filled with a fluid called endolymph. The endolymph of the scala media has a resting potential of about +80 mV, known as the endolymphatic potential (EP). This positive charge is a result of potassium ions (K+) being pumped into the endolymph by the stria vascularis (Pickles, 1988), a structure located on the lateral wall of the cochlear duct. The positive charge of the endolymph, in conjunction with the negative intracellular potential of the hair cells (approximately -70mV), results in a large electrical gradient, which contributes to sensory transduction by the inner hair cells. Pathologies of the stria vascularis, often in the form of reduced blood supply and atrophy have been shown to play a role in ARHL in humans and animals (Gratton, Schmiedt, & Schulte, 1996; Gratton & Schulte, 1995: Wu & Marcus, 2003).

Sound energy in the medium of air is transferred into mechanical energy by the tympanic membrane and bones (ossicles) of the middle ear. This mechanical energy is introduced to the cochlea when the stapes, the most medial of the ossicles, moves in and out of the oval window of the cochlea and creates a pressure wave in the fluids of the

cochlea and a traveling wave along the basilar membrane. The traveling wave progresses from the basal region of the cochlea towards the apical region of the cochlea.

There are two types of hair cells present in the organ of Corti, inner hair cells (IHC) and outer hair cells (OHC). The inner hair cells are supported by the border cells of the inner sulcus and the outer hair cells are supported by Deiter's cells. The pillar cells provide support for the IHC and OHC on their medial aspect. The upper portions of the hair cells are embedded in a cellular matrix known as the reticular lamina, through which stereocilia project into the endolymph. OHC stereocilia are embedded in the tectorial membrane while those of the IHC approach the tectorial membrane but are not believed to have mechanical attachment like the OHCs. There is one row of IHC, stretching from cochlear base to apex, numbering approximately 3500. Innervation to the IHC consists of primarily type I afferent fibers, which are large, myelinated fibers and each hair cell receives many of these fibers. This "many to one" connection is known as convergence. The IHCs are stimulated when their stereocilia are moved or sheared by the traveling wave. The IHCs convert the mechanical energy of the traveling wave into an electrochemical impulse that is transmitted as an action potential by the eighth cranial nerve to the brainstem and ultimately to the cortex where perception occurs. The organ of Corti also contains an average of 12000 OHCs, each cell topped by approximately 150 stereocilia aligned in a "v" or "w" shaped pattern. There are three rows of outer hair cells, broadening to four in the apical region. Innervation of the OHCs is comprised of SG that contain primarily efferent fibers and each OHC shares innervation with many other OHCs. This "one to many" type of innervation is known as divergence. The efferent

fibers have been shown to play a role in inhibiting or modulating afferent output (Bonfils, Remond, & Pujol, 1986). The outer hair cells and their stereocilia have been shown to contain motor proteins (prestin in particular) that produce muscle like contractions. Significant evidence exists to suggest that the OHC are motile, changing in length and stiffness with stimulation (Zheng, Madison, Oliver, Fakler, & Dallos, 2002; Zenner, 1986; Santos-Sacchi & Dilger, 1988). This motility influences the mechanical coupling of the basilar and tectorial membranes and the manner in which inner hair cells shearing occurs. The complex interaction among the OHC, IHC, basilar membrane and tectorial membrane is known as cochlear micromechanics. It is believed that the motility of the OHC serves as an amplifier for basilar membrane motion and fine tunes the traveling wave passing through the cochlear partition. This "cochlear amplifier" is a primary contributor to the exceptional frequency resolution and sensitivity of the "normal" cochlea (Ryan, Dallos, & McGee, 1979).

The cochlea, the auditory nerve, brainstem relays, and cortex are tonotopically organized, meaning that each frequency corresponds to a specific anatomical location. In the cochlea, basal regions are most sensitive to high frequencies with a progression to low frequencies in the apical regions. The cochlea is innervated by cranial nerve VIII, the vestibulocochlear nerve, which also innervates the vestibular organs.

A Short Primer on Vestibular Anatomy & Physiology

The vestibular labyrinth of the inner ear is comprised of a membranous labyrinth enclosed within the osseous labyrinth. The membranous labyrinth is filled with endolymph and the space between the membranous and osseous labyrinth is filled with

perilymph. Within the vestibular labyrinth there are five sensory organs: the macular organs (utricle and saccule) and three semicircular canals (SCC) (posterior, superior, and horizontal). These five vestibular end organs are responsible for the detection and sensory transduction of gravitational forces, linear, and angular accelerations.

The utricle and saccule are elliptical sacs located in the posterior portion of the vestibule and contain the sensory neuroepithelium for the transduction of gravitational forces and linear accelerations. The macular organs contain hair cells (types I and II) and their stereociliary bundles are embedded in a gelatinous layer (statoconial membrane) containing calcium carbonate crystals (otoconia). The otoconia impart density to the statoconial membrane that is greater than that of the endolymph. As a result of the high density of the otoconia, the gelatinous layer moves in response to gravity or linear acceleration. When the head is tilted or linear accelerations act on the head, the weight of the gelatinous membrane results in movement of the gelatinous layer relative to the sensory epithelium. In linear accelerations, the gelatinous layer lags behind head movement due to inertia. This movement causes a shearing motion of the stereociliary bundles and generates an excitatory (depolarizing) or inhibitory (hyperpolarizing) response in the hair cells depending on the direction of shearing. Vestibular stereociliary bundles are comprised of one kinocilium and multiple stereocilia, all of which protrude from the apical surface of the hair cell. Shearing motion of the stereociliary bundle toward the kinocilium results in a depolarization of the hair cells and creation of an excitatory action potential in afferent neurons. Movement of the stereociliary bundle away from the kinocilium results in hyperpolarization of hair cells and inhibits firing of

their corresponding neurons. The saccule is oriented in a predominantly vertical position, while the sensory area of the utricle is predominately horizontal in orientation. The sensory areas of the utricle and saccule both contain a distinctive curved central zone known as the striola. The stereociliary bundles on the two sides of the striola are oriented so that their kinocilia point in opposite directions. The result of this complex spatial orientation is that displacement of the gelatinous membrane in one direction results in excitation in one portion of the hair cells and inhibitory response in another portion. Furthermore, due to the curvature of the striola, hair cells are oriented at different angles making the end organ sensitive to and capable of encoding movement in a variety of directions. When the head is in the upright, neutral position, utricular and saccular hair cells are most sensitive to movement in the horizontal and vertical planes, respectively.

The three SCC, posterior, anterior, and horizontal, are membranous ducts housed within the enclosed channels of the osseous labyrinth. When the head is in a neutral upright position, the anterior and posterior canals are oriented vertically at approximately 45º angles to each other and the horizontal canal is oriented horizontally at approximately 30º above the horizontal plane. Contained within the ampulla of each semicircular canal is a neuroepithilial area known as the crista ampullaris which contains hair cells with stereocilia/kinocilia that are sensitive to angular accelerations of the head. The crista ampullaris is coupled to a gelatinous membrane, known as the cupula, which projects into the endolymph and spans the ampulla. The cupula is deflected by movement of the endolymph within the canal in response to angular movements of the head. The stereociliary bundles within each crista are oriented such that the kinocilia face the same

direction. In the anterior and posterior canals, kinocilia are oriented toward the canal side of the ampulla. This is in contrast to the horizontal canal in which the kinocilia are oriented toward the utricle. In the anterior and posterior canals, movement of endolymph away from the utricle results in depolarization of the hair cells and increases neural firing while movement towards the utricle results in hyperpolarization and decreased neural firing. In the horizontal canal, movement of endolymph towards the utricle results in depolarization and increased neural firing while movement away from the utricle results in hyperpolarization and decreased neural firing. The multi-plane orientation of the SCC is the basis for the directional sensitivity of the SCC system.

In summary, the peripheral vestibular organs consist of two macular organs (utricle and saccule) and three semicircular canal organs (posterior, anterior, and horizontal). The macular organs are responsible for sensory transduction of gravitational forces and linear acceleration. The SCC are responsible for sensory transduction of angular acceleration. The five organs contain type I and II hair cells and their corresponding stereocilia and kinocilia, which are stimulated by the mechanical movement of overlying membranes (the cupula of the SCC and statoconial membrane of the macula) in response to head movement.

Background

Auditory Structural Change with Aging

Structural changes in the aging auditory system have been studied in depth and changes have been identified in all parts of the auditory system from the outer ear to the cortex. In the outer and middle ear, alterations include: decreased elasticity and increased pinna size, changes in ear canal epithelium resulting in flaccidity of canal walls, excess cerumen accumulation (Ballachanda, 1995), thickening of the tympanic membrane (Etholm & Belal, 1974) and arthritic alterations in the ossicular chain (Rosenwasser, 1964). Small age related changes in middle ear tympanic membrane velocity are reported in mice (Rosowski, Brinsko, Tempel, and Kujawa, 2003).

In the inner ear, the cochlea is most susceptible to age related structural change. Studies examining the pathology of age related changes in the cochlea have utilized human as well as animal models of presbycusis. Cochlear pathologies associated with ARHL include: reduction in the number and integrity of sensory cells (IHC & OHC) (Willott, Parham, & Hunter, 1991; Willott, Chisholm, & Lister, 2001; Mizuta, Nozawa, Morita, & Hoshino, 1993; Spongr, Flood, Frisina, & Salvi, 1997; Francis, Ryugo, Golelikow, Prosen, & May, 2003; Li & Hulcrantz, 1994; Felder & Schrott-Fischer, 1995), damaged OHC stereocilia (Li & Hulcrantz, 1994), reduction in the number and integrity of neurons innervating the organ of Corti (Felder & Schrott-Fischer, 1995; Ohlemiller & Gagnon, 2004;Willott & Erway, 1998; Felix, 2002; Otte, Schunknecht, & Kerr, 1978; Johnsson & Hawkins, 1972), ruptures of the reticular lamina (Li & Hulcrantz, 1994), development of giant IHC cilia (Li & Hulcrantz, 1994, Johnsson,

Felix, Gleeson, & Pollak, 1990), elevation of the head of pillar cells (Li & Hulcrantz, 1994), accumulation of lipofuscin in the OHC (Schuknecht & Gacek, 1993; Li & Hulcrantz, 1994; Ishii, 1977), collapse of the organ of Corti and replacement by a single epithelial layer (Bartolomé, del López, Carricondo, Poch-Broto, & Gil-Loyzaga, 2002; Li & Hulcrantz, 1994; Schuknecht & Gacek, 1993), changes in vascular supply (Gratton & Schulte, 1995; Brown, Miller, & Nuttall, 1995; Nakashima, Miller, & Nuttall, 1995) reduced efficiency of metabolic processes (Saitoh et al., 1995; Spicer & Schulte, 2005; Brown et al., 1995; Hequembourg & Liberman, 2001), and increased thickness of the basilar membrane (Nadol, 1979; Hequembourg & Liberman, 2001). Typically, the most basal and apical regions of the cochlea are affected first with pathology progressing to include more central portions of the cochlea.

Schuknecht and Gacek (1993) classified age-related pathologic changes in the cochlea into four forms of presbycusis: 1) sensory - involving the loss of IHC and OHC in the organ of Corti, sterociliary bundles, and their supporting structures; 2) neural - involving the neural fibers innervating the cochlea (focus has primarily been on afferent innervation); 3) vascular or metabolic - involving blood supply to the cochlea and maintenance of endolymphatic homeostasis and cochlear potentials; and 4) structural or mechanical - involving the spiral ligament and structural integrity of the organ or Corti.

Sensory Presbycusis

Sensory presbycusis is characterized by pathologic changes affecting the hair cells in the organ of Corti. Damaged or missing hair cells are a common finding in both animal models of presbycusis and in human temporal bone studies. Morphological studies of

human temporal bones from subjects diagnosed with presbycusis have reported reduced hair cell counts that vary by location in the cochlea. OHCs are reduced in number more than IHCs and the basal and apical regions show a greater loss of both types of hair cells compared to more central regions (Wright, Davis, Bredberg, Ulehlova, & Spencer, 1987). Felder and Schrott-Fischer (1995) reported an approximately 80% reduction of OHC in the apical portions of the cochlea when compared to normal hearing middle-aged persons, and very little change in the number of IHCs. Wright et al., (1987) reported similar findings of exacerbated loss of OHCs in apical and basal regions but also found deceases in IHC densities. The reduction in IHCs was less than that of the OHC and was accentuated in the basal regions of the cochlea. Animal studies investigating age related changes in cochlear hair cells have varied in their findings depending on the species and genetic strain examined.

The inner ear anatomy of the C57BL/6 strain has been studied extensively because it is a widely accepted model for precocious presbycusis. This mouse strain develops a progressive, sensorineural hearing loss reaching profound levels with onset occurring in the high frequencies as early as three to six months of age (Henry & McGinn, 1991, Henry, 2004). Quantitative measures of hair cell loss in this strain have described the time course and extent of hair cell loss over the entire lifespan. Spongr et al. (1997) examined temporal bones from mice ranging from 1 to 26 months of age. At one month of age only a few OHCs were missing at the extreme basal and apical regions. By three months of age there was an 88% loss of OHCs and 55% loss of IHCs at the base of the cochlea. Measures at eight months of age revealed a loss of 100% of OHCs in basal

regions and 87% loss of IHCs in basal regions. The loss of sensory hair cells in the cochlea is a primary morphological change that contributes to ARHL.

Neural Presbycusis

Neural presbycusis has typically been investigated by measuring the density of SG that innervate the cochlea. Numerous studies have shown that a loss of SG is one of the defining characteristics of presbycusis (Schuknecht et al., 1974; Johnsson & Hawkins, 1972; Willott, et al., 2001; Willott, et al., 1991). The loss of SG has been shown to vary as a function of location within the cochlea. Post mortem microscopic study of humans diagnosed with presbycusis reveals a pattern of progressive neural degeneration. Otte et al. (1978) counted neurons in 100 subjects spanning nine decades of life and reported a range from 37,000 neurons at the end of the first decade of life to 18,000 neurons in the ninth decade. This amounts to an average loss of around 2,100 neurons per decade. The literature suggests that the loss of nerve fibers occurs throughout the cochlea and that reduction in neurons is typically more severe in the basal regions compared to the rest of the cochlea (Johnsson et al., 1990). Ohlemiller and Gagnon (2004) reported a strong correlation between abnormalities in pillar cells and Reissner's membrane and neuronal loss in four strains of mice. This finding indicates that neural presbycusis may be proceeded by or concomitant with changes in non-sensory structures. In addition, Ohlemiller and Gagnon (2004) described an apical to basal progression for pathology of non-sensory structures which is in contrast to most reports of age related cochlear change (i.e. basal to apical progression). Spongr et al. (1997) for example, describes a pronounced base to apex gradient of SG in the C57BL/6J strain of mice, which

progresses to a severe loss by 30 months of age. Felix et al. (1990) performed quantitative analysis of primary afferents at three locations in the inner ear: the osseous spiral lamina, the modiolus, and the internal auditory canal. In young individuals they found that the primary afferents are almost identical at the three sites yet in normal hearing individuals over 60 years of age they found that the loss of peripheral nerve processes in the spiral lamina was consistently more severe than the loss in more central locations (i.e. internal acoustic meatus). This suggests that dendrites of primary afferents degenerate at a faster rate than axons projecting to the brainstem.

Metabolic Presbycusis

Reduced blood supply to the cochlea, and more specifically to the stria vascularis, is widely reported in histological studies of age related cochlear change (Gratton et al., 1996; Schmiedt, 1996; Wu & Marcus, 2003; Gratton & Schulte, 1995; Saitoh et al., 1995). The stria vascularis, with it's dense layer of blood vessels and specialized cells, provides metabolic energy to the cochlea and is the primary source of potassium recycling required to maintain the endolymphatic potential (Bosher & Warren, 1971). Historically the spiral ligament was thought to simply provide mechanical support for the organ of Corti as a suspension point for the basilar membrane on the lateral wall of the cochlea. Spicer and Schulte (1991), in a detailed study of spiral ligament cytoarchitecture, classified four types of fibrocytes (types I - IV) and characterized the ion transport properties of these structures. This work uncovered a network of cell membrane transporters and gap junctions among cell groups within the spiral ligament and surrounding structures, principally the stria vascularis. This suggests that the spiral

ligament likely plays an important role in potassium recycling between the organ of Corti and stria vascularis (Spicer & Schulte, 1996). Studies of the EP and the cochlear structures responsible for the ion pumping necessary to maintain the EP (Gratton & Schulte, 1995; Gratton, et al., 1996; Gratton & Schulte, 2003; Hequembourg & Liberman, 2001; Lang, Schulte, & Schmiedt, 2003; Ohlemiller, Lett & Gagnon, 2006) have identified degenerative changes in the stria vascularis and spiral ligament. Hequembourg and Liberman (2001) found "significant and widespread degeneration in the spiral ligament in the aging C57BL/6 mouse" and proposed that a widespread degeneration of fibrocytes of the spiral ligament precedes the loss of hair cells and afferent neurons. Pauler, Schuknecht, and White (1988) using human subjects, compared histology of the stria vascularis with behavioral pure-tone thresholds and showed a direct correlation between cell losses of the stria vascularis and hearing loss. Gratton and Schulte (1995) examined age-related changes in the stria vascularis and its microvasculature and found a progressive degeneration of capillaries, beginning at the extreme base and apex of the cochlea and progressing towards middle regions with age. Kusunoki et al., (2004) examined human temporal bones ranging from 1 day of age to 86 years of age and found a significant loss of stria vascularis area in the basal turns of the cochlea in the elderly group versus infants. Wright and Schuknecht (1972) studied temporal bones of human ears with known high frequency hearing loss and found severe spiral ligament atrophy, manifested as a patchy loss of fibrocytes. Interestingly, they also found patchy loss of spiral ligament fibrocytes in ears with no reported hearing loss.

There is abundant evidence that age-related degenerative changes occur in the stria vascularis and spiral ligament; however the results concerning the EP itself (measured *in situ* in animal models) are mixed. The EP is inherently difficult to measure and varies by measurement site, species, and genetic strain and their findings concerning the EP itself vary depending on the animal model and genetic strain utilized. Gerbils and BALB/cJ mice show a reduced EP as a function of age (Gratton et al., 1996) while the C57 and CBA/CaJ mouse strains do not (Lang, et al., 2002; Ohlemiller, et al., 2006; Wu & Marcus, 2003).

In summary, there is strong evidence from animal models and humans temporal bone studies that degeneration of the stria vascularis and spiral lamina occur as a result of aging. These structural changes would suggest decreased potassium recycling and a reduction in the EP leading to ARHL. *In vivo*, measurement of the EP is currently only possible in animal models. These studies show mixed age-related change in the EP results (see below). The role of stria vascularis/spiral ligament degeneration and reductions in the EP in ARHL have not been definitively determined.

Mechanical Presbycusis

Schuknecht and others (Covell & Rogers, 1957; Nomura, 1970; Nadol, 1979) have suggested that stiffening of the basilar membrane and resulting change in the resonant characteristics of the cochlea could explain a fourth type of presbycusis deemed mechanical presbycusis. This hypothesis is based on findings of animal and human temporal bone studies that have identified increased basilar membrane thickness with age in the basal region of the cochlea. Nadol (1979) examined a limited number of human

temporal bones and found a "marked" thickening of the basilar membrane. Ishii, Murofushi, and Takeuchi (1994) performed light and electron microscopy of the temporal bones of aged rats and reported a reduced number of collagen fibers and a thickening of the basilar membrane at the base due to an increase in a homogeneous ground substance. Bhatt, Liberman, and Nadol (2001) quantified age related changes in the basilar membrane and correlated the finding with audiometric patterns. They measured basilar membrane thickness in four regions of the cochlea in normal subjects and those with hearing loss. While finding age related thickening of the basilar membrane in both groups, they found no significant increase in basilar membrane thickness between controls and those with hearing loss. They concluded that while basilar membrane thickening could conceivably contribute to ARHL, the most affected areas of the cochlea correspond to frequencies higher than those typically tested audiometrically (i.e. >8000 Hz). The existing evidence suggests that there are pathologic changes in the basilar membrane but the role such changes play in ARHL remains speculative.

Auditory Functional Change with Aging

Age-related functional changes in the auditory system are influenced by individual differences in terms of noise exposure, exposure to ototoxins, ear disease (e.g., see review by Gratton & Vazquez, 2003), the central auditory system (Willott, et al., 2001), lifestyle (diet, exercise, etc.) (Willott et al., 2001) and genetic make up (Johnson, Erway, Cook, Willott, & Zheng, 1997; Li, 1992; Fransen, Lemkens, Laer, & Van Camp, 2003). These are potential confounding variables to studies which attempt to investigate the effect of age on auditory structures and function. These factors, along

with the inherent genetic variability of humans, make the genetic study of age-related hearing loss in humans especially problematic. These factors are difficult to control for in aging studies in humans, so while there is a wealth of research describing ARHL in humans, it is difficult to separate hearing loss due to age alone and hearing loss due to environmental, genetic, or environmental and genetic interactions. It is for this reason, that much of the literature is focused on describing functional changes measured behaviorally and using physiological measures in animal models of ARHL. The review that follows will discuss behavioral measurement of hearing sensitivity, physiological tests that indicate cochlear function, and reports in the literature of age related change in these measures in humans and animal models.

Longitudinal studies in humans, using pure tone audiometry (the gold standard measurement of auditory sensitivity) to characterize ARHL, report a progressive decline in auditory sensitivity beginning as early as age 30. The loss of auditory sensitivity typically begins in the high frequencies and progresses to lower frequencies with age (Pearson et al., 1995). Behavioral measures indicate that the rate of decline in hearing sensitivity is age and frequency dependent with rates of 3 dB per decade reported for those under 55 years of age and 9 dB per decade reported for those over 55 years of age (Davis, Ostri, & Parving, 1991). Objective measures of peripheral auditory status; ABR, DPOAE, and electrocochleography (ECochG), indicate that the primary site of lesion for presbycusis is the cochlea.

Auditory Brainstem Response (ABR)

ABR is an electrophysiological measure which can be measured in humans as well as animals, and provides an objective indication of auditory peripheral sensitivity, allows differentiation between cochlear and retro-cochlear pathology, and assesses the neurological integrity of the auditory pathway in the brainstem. The ABR response is evoked with the presentation of transient stimuli such as tone bursts/pips or clicks, and is measured at some distance from the neural generators with electrodes placed on the scalp (known as a far field response). Filtering, amplification, and signal averaging techniques are used to resolve the response from internal and external sources of electrical noise. The normal human response consists of a waveform with five to seven peaks occurring around 1 to 7 milliseconds (ms) following stimulus presentation. Mouse ABR waveforms generally show 4 to 6 peaks (see Figure 1).

The ABR represents the depolarization of cochlear hair cells, synchronous firing of neurons in the auditory portion of cranial nerve VIII and auditory relays of the brainstem. In the elderly, prolonged absolute peak latencies and reduced response amplitude are reported when compared to young normal subjects (Rosenhall, Bjorkman, Pederson, & Kall, 1985; Boettcher, 2002). The effect of aging on ABR interpeak latencies is disputed in the literature. Numerous studies indicate that inter-peak latencies are unaffected by age (Beagley & Sheldrake, 1978; Harkins, 1981; Costa, Benna, Bianco, Ferrero, & Bergamasco, 1990). While others suggest that aging results in increased interpeak latencies (Oku & Hasegawa, 1997; Rosenhall et al., 1985). Studies investigating the effect of age on ABR amplitudes are in agreement that ABR amplitudes

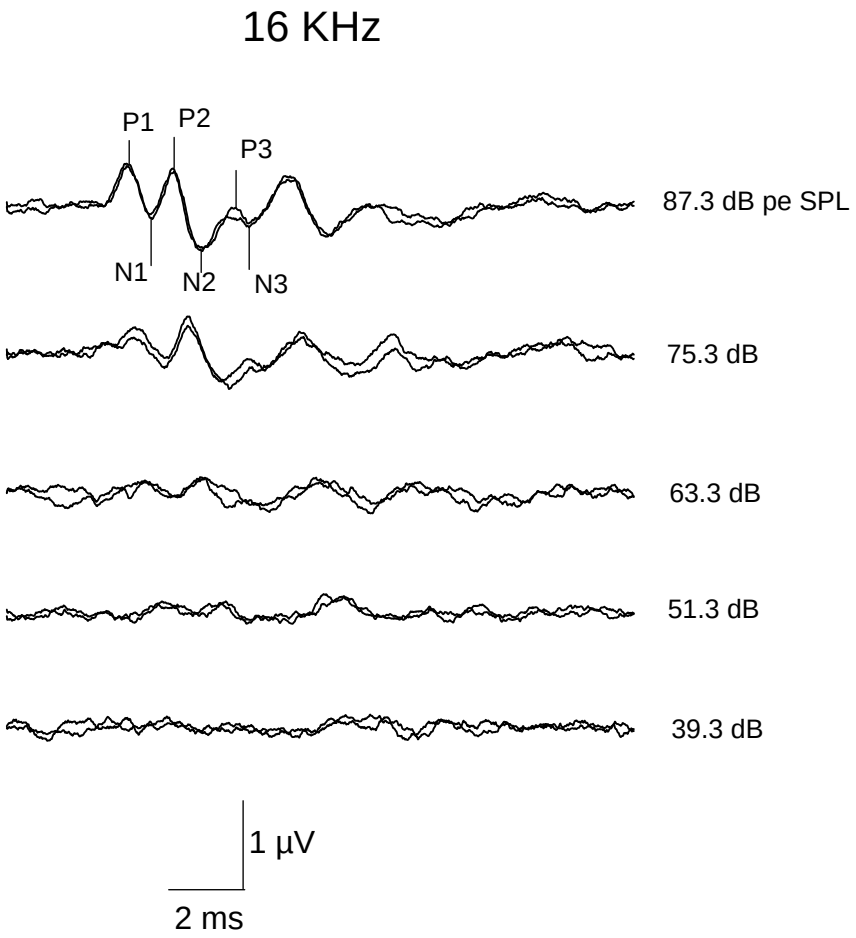


Figure 1. Representative ABR waveforms for one mouse. Stimuli used to obtain these waveforms were 16 kHz tone bursts presented in decreasing stimulus intensity (12 dB steps). P1, P2, P3, N1, N2, and N3 peaks are labeled. Threshold for this intensity series was designated as 45.3 dB pe SPL.

decrease with age, even in individuals who show no loss of sensitivity on behavioral measures or when loss of auditory sensitivity is accounted for (Sand, 1991; Psatta & Matei, 1988; Oku & Hasegewa, 1997; Jerger & Hall, 1980; Boettcher, Mills, Norton, & Schmiedt, 1993; Beagley & Sheldrake, 1978; Harkins, 1981; Costa et al., 1990). Wave I amplitude is typically affected more by aging than wave V amplitude (Costa et al., 1990; Psatta & Matei, 1988; Sand, 1990). Boettcher et al. (1993) reported that as ABR thresholds increased in gerbils, ABR peak to peak amplitudes decrease. In summary, ABR amplitudes are reduced with decreases in auditory sensitivity but have also been shown to decrease independently of age related changes in auditory sensitivity.

The latency of an auditory evoked potential is defined as the amount of time that passes from the onset of the stimulus to occurrence of each response peak. The latency of auditory evoked potentials is influenced by characteristics of the stimulus, the integrity of the cochlea, cranial nerve VIII, and auditory brainstem structures. The ABR response consists of 5 to 7 peaks with each peak corresponding to an anatomic location where the response is generated. More medial or rostral generators produce longer latency responses. The ABR response originates in the primary afferents of the cochlea, progresses to cranial nerve VIII, travels through the auditory relays of the brainstem and ultimately to the auditory cortex. The generation of ABR peaks, especially in more central structures, likely arises from multiple sites. Peak 1 is generated by the peripheral portion of cranial nerve VIII (Wada & Starr, 1983; Anchor & Starr, 1980; Hashimoto, Ishiyama, Yoshimoto, & Nemoto, 1981). Peak 2 is likely generated in the central or intercranial portion of cranial nerve VIII (Hashimoto, et al., 1981; Pratt et al., 1992;

Moller & Jannetta, 1982). Peak 3 is believed to originate in the cochlear nucleus (Moller & Jannetta, 1982). The generator for peak 4 is believed to be the superior olivary complex (SOC) (Moller & Jannetta, 1982; Moller, Jho, Yokoto & Janetta, 1994). Peak 5 is likely generated in neural tracts of the SOC and lateral lemniscus (Hashimoto et al., 1981; Moller & Janetta, 1982).

Interpeak latencies are a measure of neural conduction time. The latency of ABR peaks are influenced by the rate of onset and spectral characteristics of the stimulus. The ABR response is primarily an onset response (Suzuki & Horiuchi, 1981). The basilar membrane is tonotopically organized with basal portions most sensitive to high frequencies and a progression to apical portions which are most sensitive to low frequencies. As a result of the tonotopic organization of the cochlea, the latency for high frequency stimuli is shorter than that for low frequency stimuli. Latency of ABR peaks is influenced not only by the point of maximal displacement on the basilar membrane but also by the EP and synchrony of firing of the population of neurons that innervate the area being stimulated.

A threshold is defined as the point that must be exceeded to begin producing a given effect or result or to elicit a response. Threshold is by definition a measure of sensitivity. Age-related hearing loss is characterized by a loss of sensitivity beginning with the high frequencies and progressing with age to affect lower frequencies. This loss of sensitivity is reflected in ABR measures by increased thresholds beginning in the high frequencies progressing to lower frequencies with increased age. In humans, ABR peak 5 is the most robust and is typically the last detectable peak as intensity goes to sub-

threshold levels (Worthington & Peters, 1980). In this author's experience, mouse peaks P1 and P2 are typically the most robust and are the last detectable peaks before sub-threshold levels are reached.

Otoacoustic Emissions (OAE)

Kemp (1978) was the first to describe acoustic events measured in the external auditory meatus (EAM) which occur spontaneously and in response to acoustic stimuli presented to the EAM. These evoked acoustic responses have become known as otoacoustic emissions (OAE) and are a valuable clinic tool for assessment of cochlear function and more specifically outer hair cell (OHC) function. Animal models were a valuable tool in investigating the source of OAEs and provided strong evidence that OAEs are critically dependent on OHC function (Whitehead, Lonsbury-Martin, & Martin, 1992, Horner, Lenoir, & Bock, 1985; Schrott, Puel, Rebillard, 1991).

OAEs can be divided into two broad categories: spontaneous and evoked. Spontaneous emissions are measured in the EAM in the absence of an evoking stimulus. Spontaneous emissions are measurable in normal ears and pathological ears, although to a lesser degree, making them of little use clinically.

Evoked emissions are measured using acoustic stimuli and the two most commonly used OAEs in clinical settings are transient (TEOAE) and distortion product evoked emissions (DPOAE). DPOAEs occur following the introduction of two distinct acoustic signals (primary frequencies known as f1 and f2). The non-linear properties of the cochlear amplifier (OHC) result in a third distinct acoustic signal measurable in the EAM. DPOAEs occur at many frequencies but research has shown that the most robust

DPOAE response in humans occurs at the cubic difference tone (2f1-f2) when the ratio of the frequencies of the primaries is 1.2 (f2/f1 = 1.2). Due to the robustness of the 2f1-f2 emission, it is the most commonly utilized response both clinically and in research.

The amplitude of the DPOAE response is dependent on stimulus parameters such as frequency spacing and amplitude as well as properties of and the status of the EAM, the tympanic membrane, the middle ear ossicles, and cochlear function, as well as the level of the primaries. DPOAE response amplitude (in dB SPL) is generally plotted as a function of geometric mean frequency and is plotted along with the noise floor. The noise floor is generally measured by averaging sound pressure levels in two to three noise bins on either side of the 2f1-f2 frequency. If the sound pressure measured at 2f1-f2 is more intense (by some predetermined criteria usually 3 to 6 dB SPL) than the average sound pressure level in the surrounding bins, then that is considered a DPOAE response. The resulting plot of DPOAE amplitude and noise floor is known as a DP Gram (see Figure 2). Kemp (1986) reported the presence of the evoked acoustic response in all normal ears tested and the absence of the response in ears with cochlear deafness. OAEs are typically present in ears with normal pure tone sensitivity and mild hearing loss and generally absent in ears with peripheral hearing loss greater than 50 dB HL (Gorga et al., 1997). Aging studies utilizing distortion product otoacoustic emissions (DPOAE) have shown significant interaction between age, frequency, and threshold. As age and frequency increase, DPOAE amplitude decreases (Dorn et al., 1998). However, when hearing loss is controlled for, there is no direct effect of age on DPOAE amplitude (Dorn et al., 1998;

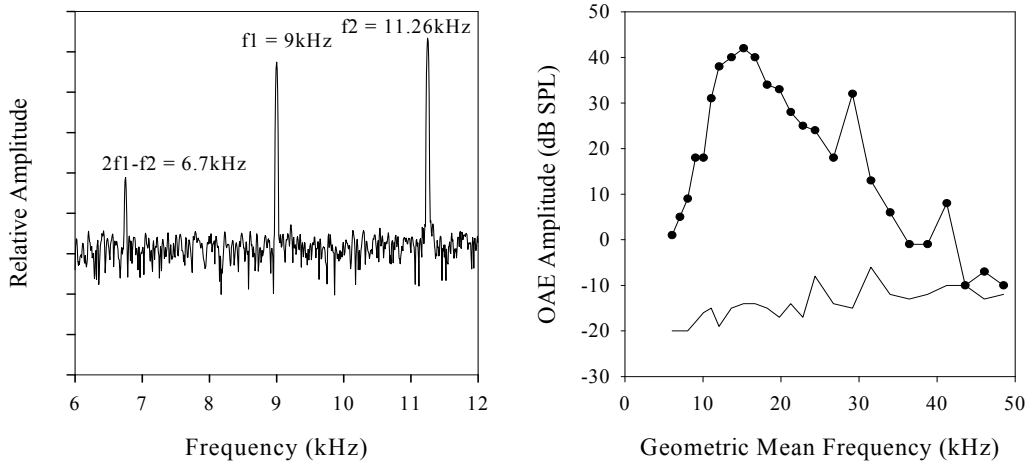


Figure 2. DPOAEs for one mouse. The left panel depicts the Fast Fourier Transform (FFT) for one frequency pair (f1 and f2 primaries) and the 2f1-f2 response. The DPOAE at 2f1-f2 is clearly visible. The right panel shows the DP Gram (DPOAE amplitude as a function of geometric mean frequency) for this same mouse. The solid line represents the noise floor amplitude (i.e., amplitude of frequency bins surrounding the 2f1-f2 frequency bin) and points represent DPOAE amplitude as a function of geometric mean frequency.

Strouse, Ochs, & Hall, 1996; Oeken, Lenk, & Bootz, 2000). Jimenez et al. (1999) measured DPOAEs in the CBA/CaJ, a strain with no known genetic mutations, to the age of 15 months in order to assess the effect of age alone on DPOAE threshold and amplitude. They reported robust and unchanged DPOAEs for all test frequencies through the oldest ages. Parham, Sun, & Kim (1999) measured DPOAEs to 25 months of age in the CBA/JNia strain, a related strain with no known genetic mutation. They found small, frequency dependent, age related changes in DPOAE amplitude and threshold levels at the advanced age of 17 months, and moderate changes at the very advanced age of 25 months. These results are in agreement with ABR findings (a slow progressive loss of auditory sensitivity beginning at advanced age) for this strain and confirm cochlear pathology as the origin of hearing loss.

DPOAE measures in the C57BL/6J mouse strain (a strain widely accepted as a precocious model for presbycusis) have revealed age and frequency dependent changes in DPOAE threshold and amplitude (Jimenez et al., 1999; Martin et al., 2007; Parham, 1997). Parham (1997) reported increased DPOAE threshold levels and reduced response amplitudes, effecting higher frequencies first and progressing to include lower frequencies, with changes beginning between 2 and 8 months of age. Jimenez, Stagner, & Martin (1999) tested the C57 strain up to 15 months of age, at monthly intervals, and showed similar results; DPOAE amplitude decreased and threshold increased in an age and frequency dependent manner. Jiminez et al. (1999) tested at monthly intervals which allowed a more detailed assessment of the onset of cochlear functional change. Their

results show age related reduction in DPOAE amplitudes after 2 months of age, beginning in the high frequencies and progressing with age to affect all frequencies.

DPOAE provide an objective measure of OHC function and provide important information concerning the site of lesion for age related pathology. In summary, the literature on mouse models of ARHL supports the idea that age related changes in DPOAE threshold and amplitude are strain and frequency specific and that OHC pathology is one component of ARHL.

Gender Differences in Age Related Hearing Loss

Gender differences in auditory function, including ARHL, are reported in animal models as well as humans and include reports that young women generally have better hearing than young men (Corso, 1963; Rosen, Plester, & El-Mofty, 1964; Northern, Downs, Rudmose, Glorig, & Fletcher, 1972; Surjan, 1973), ARHL progresses faster in men than women (Corso, 1963; Pederson, Rosenhall, & Moller, 1989; Wallhagen, Strawbridge, Cohen, & Kaplan, 1997; McFadden & Pasanen, 1998) and that female mice lose hearing earlier than males in mouse models of early onset ARHL (C57 strain) (Henry, 2004).

Some researchers have hypothesized that the female hormone estrogen may inhibit ARHL. Kilicdag et al. (2004) measured auditory sensitivity in postmenopausal women using estrogen therapy, hormone replacement therapy (a mixture of estrogen and progesterone), and a control group receiving no therapy. Their results show that the estrogen therapy group had better hearing at all frequencies than the hormone replacement group and the control group and concluded that estrogen therapy may slow

the rate of ARHL. Guimaraes et al., (2006) conducted a similar study that compared pure tone sensitivity, DPOAE, TEOAE, and hearing in noise in post menopausal women treated with estrogen and progestin, estrogen alone, and a third non-hormone replacement therapy control group. Their results show poorer auditory function for all measures in the group treated with estrogen and progestin. These results suggest that progestin may negate any protective effect that estrogen may have on ARHL. A study by Stenberg, Wang, Sahlin, & Hulcrantz (1999) revealed widespread immunostaining of the estrogen receptors ERα and ERβ in cochlear structures in CBA mice. Estrogen is known to have widespread effects on physiology and this finding suggests that it may have a direct influence on cochlear structures. A study by Hulcrantz, Stenberg, Fransson, & Canlon (2000) using a mutant mouse, X,O "Turner mouse" which produces no estrogen, further supports the idea of estrogen influence on cochlear structure and function. They found a loss of OHC, primary afferent pathology, increased ABR thresholds, and decreased DPOAE amplitudes relative to controls. The idea that estrogen may provide a protective effect for cochlear structures is further bolstered by the results of Guimaraes et al. (2004). Using both ABR and DPOAE, they found differences in auditory sensory function pre and post menopause in female CBA mice. Females had lower ABR thresholds and larger amplitude DPOAEs than males at middle and old ages but much of this advantage was lost following menopause. The role of female hormones on inner ear structure and function is poorly understood but there is growing evidence suggesting that female hormones may have a protective effect against ARHL.

In summary, gender differences in auditory sensitivity are apparent throughout the lifespan. Gender differences in ARHL are reported in humans and animal models and research suggests that the female hormone estrogen may inhibit ARHL. More work is needed to understand the complex role of hormones in relation to ARHL.

Genetics and Age Related Hearing Loss (ARHL)

The association between inheritance and hearing loss has a long history but investigations of the epidemiology of hearing loss did not begin until the 19th century (Nance & Pandya, 2002). Hearing loss is one of the most common genetic disorders found in humans (Keats & Berlin, 2002) and genetic studies have identified a large and ever growing number of genes that contribute to both syndromic and non-syndromic hearing loss. These studies have revealed the heterogeneous nature of hearing loss and there is an increasing body of evidence concerning the additive and interactive effects of multiple genes upon auditory structures and function. The study of the genetic basis for hearing loss is potentially confounded by environmental causes (exposure to ototoxins, noise, and viral infection) and is difficult to study in humans due to heterogeneity of human genetics. Mice have been an invaluable tool in the attempt to control for environmental factors and genetic heterogeneity. Mice can be kept in a controlled environment, reproduce rapidly and in large numbers, and can be selectively bred which allows for the reduction of genetic heterogeneity.

Genetic studies of ARHL have illustrated the complex nature of ARHL inheritance. Throughout this discussion, ARHL will be used to signify the behavioral or physiological findings of a loss of auditory sensitivity (phenotype) and A*hl* will be used

to designate genes or loci related to ARHL (genotype). To date seven mutations that contribute to ARHL have been identified. Johnson, Erway, Cook, Willott, & Zheng (1997) were the first to identify a specific gene (A*hl*) that results in ARHL in mice. *Ahl* is a recessive, point mutation on the Cadherin 23 gene (*Cdh23*) located on Chromosome 10 in the C57BL/6J strain. The *Ahl* mutation is a single substitution at nucleotide *753 (A/G)* on the C*dh23* gene. This mutation occurs in approximately 80% of inbred strains with hearing loss (Drayton & Noben-Trauth, 2006) and is referred to as $Cdh23^{753A}$ (Noben-Trauth, Zheng, & Johnson, 2003). *Cdh23* is a gene that encodes cadherin, a protein necessary for inner ear development and maintenance of sensory cell structures such as stereociliary tip links (Siemens et al., 2004; Sollner et al., 2004) and transient lateral links between stereocilia (Michel et al., 2005; Lagziel et al., 2005). Mutations in *Cdh23* are associated with syndromic (Usher syndrome type 1D) and non-syndromic hearing loss (DFNB12) and have been shown to result in deafness and vestibular disorders in humans and mice (Bolz et al., 2001, Jones et al., 2004, 2005). Cochlear and vestibular cytoarchitectural abnormalities in strains that carry the *Ahl* mutation include: loss of IHC and OHC, loss of SG, degeneration of fibrocytes in the spiral ligament, disorganized stereociliary bundles, misplaced kinocilia, and disruption of stereociliary tip links (Di Palma, et al., 2001; Alagramam et al., 2001; Holme & Steel, 2002; Raphael, Kobayashi, & Dootz, 2001; Shiga et al., 2005).

Johnson and Zheng mapped a second mutation, called *Ahl2,* to chromosome 5 using backcross and linkage analysis in the NOD/LtJ strain. Johnson and Zheng concluded that the contribution of *Ahl2* to hearing loss is dependent on a predisposing *Ahl*

genotype and that the interaction between *Ahl* and *Ahl2* results in an earlier onset hearing loss than for *Ahl* alone. More studies are needed concerning *Ahl2* but the existing research suggests that *Ahl2* interacts with *Ahl* and accelerates the onset of hearing loss (Johnson & Zheng, 2002).

A third ARHL locus has been identified and designated *Ahl3*. Nemoto et al. (2004) identified and mapped *Ahl3* to chromosome 17. They showed that when the *Ahl3* gene is present along with *Ahl*, there is a protective effect and that hearing sensitivity is preserved until late in life. In contrast to *Ahl* and *Ahl2*, it appears that *Ahl3* does not result in age-related hearing loss but functions to inhibit age-related hearing loss.

Zheng, Ding, Yu, Salvi, & Johnson, (2007) identified and mapped a fourth ARHL locus, designated *Ahl4,* on Chromosome 10 of the A/J inbred mouse strain. The A/J strain harbors the $Cdh23^{ahl}$ (*Ahl*) mutation that is present in numerous strains and results in ARHL. The onset of hearing loss in the A/J strain is much earlier than other strains that carry *Ahl* alone. A/J mice show reduced cochlear sensitivity at very young ages (following weaning) and by 3 months of age have severe hearing loss at all frequencies. While information for ages as young as immediately following weaning is not available for the NOD NON strain, which carries *Ahl* and *Ahl2,* this strain also shows a severe to profound hearing loss by 3 months age. Zheng et al.'s genetic analysis of a backcross strain provides a compelling argument that *Ahl4* contributes to the accelerated onset of hearing loss in the A/J strain. Scanning electron microscopy images of macular otoconia in the A/J strain taken in our laboratory show abnormal otoconia and poor macular sensitivity by mid-age in this strain (Jones et al., 2006).

Dreyton and Noben-Trauth (2006) investigated the genetic basis of hearing loss in the non-inbred strain Black Swiss and identified two loci (*Ahl5 and Ahl6*) that contribute to hearing loss in this strain. *Ahl5* and *Ahl6* were localized to chromosome 10 and 18, respectively. Black Swiss mice demonstrate an early onset hearing loss which begins in the high frequencies and eventually reaches profound levels at all frequencies.

The most recent ARHL locus to be identified is *Ahl8* (*Ahl7* is reserved) in the DBA/2J strain (Johnson, Zheng, & Noben-Trauth, 2006). The specifics of *Ahl8* have not been published beyond the fact that it results in accelerated hearing loss and is located on the distal portion of Chromosome 11.

In summary, there are seven known loci related to ARHL and one gene has been identified. These seven loci have different auditory phenotypes. The *Ahl* mutation, in the absence of other ARHL mutations, results in an early to mid-life onset, progressive hearing loss that begins in the high frequencies. The *Ahl2* and *Ahl4* mutations accelerate ARHL when present in conjunction with *Ahl*. The *Ahl3* mutation inhibits ARHL when present along with *Ahl*. *Ahl5* and *Ahl6* have been identified in non-inbred strains and result in early onset hearing loss. The *Ahl8* locus is present on Chromosome 11 and accelerates ARHL in the DBA/2J strain.

Vestibular Structural Change with Aging

Morphological changes in the vestibular epithelium due to aging have been investigated in humans and in several animal species. These studies have identified structural changes in the sensory epithelium of the macular organs and the semi-circular canal (SCC) organs but findings are mixed depending on the species utilized. Rauch,

Velazquez-Villaseñor, Dimitri, & Merchant (2001) performed a study on a large sample (n = 67) of human temporal bones using interference contrast microscopy in order to establish normative data for type I and II hair cells as a function of age. The results showed a continuous decrease in both cell types from birth to 100 years of age. Type I hair cell densities for the crista decreased at a faster rate than type I hair cells densities of the macular organs. Type II hair cells counts decreased at a similar rate for the five sensory organs. The findings of Gopen, Lopez, Ishiyama, Baloh, & Ishiyama (2003), using an unbiased stereology technique to count type I and II hair cells of the utricle, conflict with the results of Rauch et al, (2001). Gopen et al. found no age effect on total hair cell counts in the utricle but the authors conceded that a broader age range than that used (42-96 years) may be needed to establish an age effect. Shiga et al. (2005) performed hair cell counts on the horizontal SCC in C57BL/6J mice (ages 3 to 60 weeks) and found that hair cell densities decreased by an average of 30% in the oldest animals. Similar studies of hair cell counts have been performed in gerbils, rats, and mice and findings differ based on the species and genetic strain used. Kevetter, Zimmerman, & Leonard (2005) found no age effect on hair cell counts in the crista ampullaris of Mongolian gerbils. Nakayama, Helfert, Konrad, & Caspary (1994) performed an ultrastructural study of the vestibular epithelium of Fischer 44 rats and found a significant loss of hair cells, as well as a reduction in the number and integrity of stereocilia and kinocilia in the crista ampullaris. Interestingly, these age-related pathological changes were not seen in the macular organs. Park, Hubel, and Woods (1987) measured hair cell densities of vestibular epithelia in C57BL/6NNia mice. They reported age related hair

cell pathology and decreased hair cell counts in the five vestibular organs. In addition to reduced hair cell counts, other age related pathological changes in vestibular epithelia are reported in the literature. These changes include cytoplasmic protrusions/ herniations (Gleeson, Felix, & Johnsson, 1990; Bloom & Hultcrantz, 1994), disarranged and fused stereocilia (Rosenhall & Ruben, 1975: Bloom & Hultcrantz, 1994), the presence of giant cilia (Nakayama et al., 1994; Rosenhall & Ruben, 1975; Bloom & Hultcrantz, 1994), the accumulation of lipofuscin (a melanin like substance which is a by product of metabolic processes and considered a wear-and-tear pigment) in sensory and supporting cells (Rosenhall & Ruben, 1975; Anniko, 1983; Park et al., 1987; Gleeson et al., 1990), membrane bound inclusions of sensory cells (Park et al., 1987; Gleeson et al., 1990; Anniko, 1983), neural degeneration (Gleeson et al., 1990, Rauch et al., 2001), reduced blood flow to the utricle (Lyon & Davis, 2001), reduced number of globular substances (which are secreted precursors to mature otoconia) (Suzuki, Ikeda, & Takasaka et al., 1997), age related degeneration of otoconia (Campos, Canizares, Sanchez-Quevedo, & Romero, 1990), and increased fragility and disintegration of the cuticular plate (Anniko, 1983; Bloom & Hultcrantz, 1994). Reports of widespread structural changes in vestibular organs support the notion that vestibular function should decline as a result of age.

Vestibular Functional Changes with Aging

Balance is a complex process that involves input from and integration of multiple sensory and motor systems. Sensory input is necessary for monitoring of body position in relation to gravity and surroundings. The visual, somatosensory, and vestibular systems provide this sensory input. Vestibular end organs make an important contribution to

balance and their structural degeneration and reduced function due to aging is likely an important factor in the increased incidence of falls in the elderly. Compensation by the central nervous system and other sensory systems may conceal peripheral vestibular dysfunction leading to many undiagnosed cases of vestibular disorders.

Studies that have utilized functional measures to assess vestibular aging have typically relied on the vestibuloocular reflex (VOR), optokinetic response (OKN), vestibular evoked myogenic potentials (VEMP), otolith-ocular responses and tests of posture to make inferences about vestibular function. These measures can all be classified as indirect measures of vestibular function because they rely on sensory input (postural measures rely on input from three separate sensory systems), integration of output to the central nervous system, and motor output. As a result, they do not readily allow for the separation of central and peripheral influences on the motor output and the status of vestibular end organs must be inferred from the observed motor response. In studies of the VOR and OKN in the elderly, Paige (1992,1994) and Baloh, Jacobson, & Socotch (1993) reported decreases in VOR gain (a ratio of eye velocity to head velocity), increased VOR phase lead, decreased ability to suppress the VOR with vision, and lower OKN slow-phase velocity saturation. Studies of the effect of age on VEMP describe age related decreases in amplitude (Basta, Todt, & Ernst, 2007; Brantberg, Granath, & Schart, 2007; Su, Huang, Young, & Cheng, 2004, Ochi & Ohashi, 2003), increased threshold (Ochi & Ohashi, 2003), and increased latency (Brantberg et al., 2007; Su et al., 2004). Furman and Redfern (2001) demonstrated age effects on two otolith related measures.

1) The otolith-ocular response, assessed with off vertical axis rotation (OVAR), demonstrated increased modulation and reduced bias with age, suggesting increased fluctuations in slow component eye velocity produced by OVAR. 2) The SCC-otolith interaction measure demonstrated that elderly subjects showed reduced ability to shorten VOR time constants with post rotary head tilt. Furman and Redfern (2001) suggested that these two findings are similar to those seen in subjects with vestibulo-cerebellum dysfunction who also display reduced central inhibition on these measures. They argue that the age related changes observed were due to degeneration of central structures responsible for processing of gravity receptor information rather than degeneration of peripheral vestibular function. Tests of postural control, utilizing computerized dynamic posturography (CDP), have demonstrated postural instability in the elderly as a result of increased muscle response latency (Goebel, 2001; Baloh, Spain, Socotch, Jacobson, & Bell, 1995; Borah, et al., 2007). Decreases in postural control may be due to deterioration of peripheral or central mechanisms.

In summary, the measures historically used to assess peripheral vestibular function (VOR, OKN, VEMP, otolith-ocular reflex, CDP) reflect sensory input, integration in the central nervous system and motor output to the eyes and postural muscles. As a result, these measures are indirect measures of inner ear vestibular function and functional changes due to peripheral or central mechanisms may not be readily distinguishable. It is well known that visual acuity, somatosensory, and central nervous system regions degenerate with increasing age. Baloh et al. (1993) and Paige (1992) have suggested that age-related degeneration of oculomotor and vestibuloocular central

nervous system pathways are responsible for the age related declines reported for indirect vestibular measures.

Research by Shiga et al., (2005) highlights the fact that central mechanisms may compensate for peripheral deficits. They found an age dependent progressive decrease in the number of sensory cells of the lateral semicircular canal in C57 mice yet no functional decline was seen in VOR gain. The decrease in the number of peripheral sensory cells without concomitant reduction in VOR gain strongly suggests central compensation for degeneration of peripheral structures. While there are no direct measures available to assess human inner ear vestibular function, non-invasive direct measures of macular organ function are possible in animal models.

Short latency vestibular evoked potentials (VsEP) are compound action potentials generated by the vestibular nerve and vestibular brainstem relays (Nazareth & Jones, 1998; Jones & Jones, 1999) in response to linear or angular acceleration. The VsEP in response to linear acceleration consists of a series of positive and negative peaks occurring within 10 ms of stimulus onset. Far field VsEPs are recorded at the scalp and have been shown to be critically dependent on the utricle and saccule (Jones & Pederson, 1989; Jones, Erway, Bergstrom, Schimenti, & Jones, 1999). The VsEP response is resistant to intense acoustic masking and extirpation of the cochlea but is abolished following bilateral labyrinth-thectomy, toxic insult to the macular sensory cells, and pharmacological blockage of labyrinth. Peaks PI and NI are generated by the peripheral portion of the vestibular branch of cranial nerve eight and later peaks correspond to more medial generation sites in the vestibular brainstem relays and cerebellum (Nazareth &

Jones, 1998; Jones & Jones, 1999). VsEP intensity functions are characterized by increasing amplitudes and decreasing latency as stimulus intensity increases. The dynamic range for VsEP in mice is relatively limited (generally less than 40 dB re: 1 g/ms) when compared to auditory dynamic range (generally in excess of 70 dB pe SPL). VsEP measures provide an objective direct measure of gravity receptor sensitivity, the number and synchrony of firing of vestibular primary afferents, and the relative amount of time required for: 1) sensory transduction of linear accelerations 2) transmission across the first synapse, 3) neural conduction time through the vestibular nerve and brainstem relays. VsEP measures of peripheral vestibular function in animal models suggest decreased sensitivity of the gravity receptor organs and potential changes in neural timing and conduction velocity of primary afferents with age (Jones et al., 2006).

A Comparison of Auditory and Vestibular Aging in Humans and Animal Models

There are many advantages to the use of mouse models. The biology of the mouse is the most studied of any mammal commonly used in auditory research (Henry & McGinn, 1992). Mice are relatively inexpensive to acquire and maintain. Use of animal models permits greater control of environmental and genetic factors that are not easily controlled in humans. The mouse has a long history as a model for human hearing and balance disorders (e.g. Yerkes, 1907; Lord & Gates, 1929; Lyon, 1953; Steel, 1995; Petit, Levilliers, & Hardelin, 2001; Ahituv & Avraham, 2002) and the genetics for many strains are well characterized. Standardization makes the same strain available to many researchers. The use of mice for auditory research has lead to numerous mouse models of syndromic and nonsyndromic hearing loss in humans and facilitated the study of genetic

and environmental causes of hearing loss, including ARHL. Changes in auditory and vestibular function can be measured over the whole lifespan due to the relatively short life of the mouse species (2 to 3 years). Tissue samples are immediately available to investigate the anatomical basis for functional change. Mice were chosen specifically for the proposed research because 1) they are mammals; 2) they have vestibular organs similar to humans; 3) they are the species specifically identified by the National Institute of Health for characterizing nervous system function; 4) data concerning morphology of the vestibular system in several genetic mutants are available; 5) several mouse strains have been identified as the genetic models for humans diseases and disorders, including inner ear disorders; 6) genetic studies can be conducted readily with mice; 7) genome data are readily available for the mouse; 8) only living organisms have vestibular systems. At the present time, there is insufficient knowledge about normal and abnormal vestibular ontogeny to create inanimate models.

The peripheral auditory system of man and mammalian animal models (guinea pig, rat, cat, monkey, and mice) although similar, are not identical. Small anatomical differences exist. These difference may include but are not limited to: the length and width of the basilar membrane, the number of IHC and OHC, the length of stereocilia of both types of hair cells, the innervation pattern and number of afferent and efferent nerve terminals to each hair cell type, the degree of branching of efferent fibers and number of synapses per afferent nerve terminal, the presence or absence of pre-synaptic bodies, the number of spiral ganglion cells and cochlear nerve fibers, the percentage of myelinated SG and variability in the presence of synapses on SG (Felix, 2002; Nadol, 1988). The

dynamic range for frequency selectivity of mice is larger than that measured in man (5 to 80 kHz and 20 Hz to 20 kHz for mice and man respectively). However, the anatomy and physiology of the inner ear of mice and other mammalian models are representative of the form and function of the human inner ear. The advantages offered by the use of murine models mouse model far outweighed the disadvantages of anatomical and function differences. We chose to study vestibular function in murine models of ARHL based on these advantages.

How might aging of auditory and vestibular function compare? We are aware of only two studies that compared age related functional change in the two sensory systems simultaneously. Enrietto, Jacobson, and Baloh (1999) measured hearing and VOR in a longitudinal study of human subjects. They showed that hearing declined about 1 dB per year, VOR gain decreased an average of 0.05 per year (for high velocity stimuli), and phase lead (for high velocity stimuli) increased an average of 2.3 deg/sec per year over the five-year study. They concluded that the age-related changes in hearing were not correlated with the age-related changes in the VOR and suggest that the vestibular and auditory systems in humans may not age at the same rate. However, the researchers acknowledged that the differences in auditory and vestibular functional aging might have been due to both peripheral and central mechanisms, not the periphery alone. Shiga et al. (2005) examined age related changes in auditory and vestibular morphology and function in C57BL/6J mice using VOR gain as a measure of vestibular function and ABR as a measure of cochlear function. Histology was performed to examine the anatomical correlates of cochlear and vestibular function. Histology showed sensory degeneration of

the cochlea with a corresponding decrease in auditory sensitivity. A decrease in lateral semicircular canal hair cell density, beginning between 12 and 24 weeks of age was reported. At 24 weeks of age, hair cells counts were reduced by 80%, compared to those measured at 3 weeks, but no corresponding decrease in VOR gain was observed. Based on these findings, the investigators suggested that the mechanisms for vestibular functional changes differ from those for the cochlea. However, they acknowledged the use of an indirect measure of vestibular function and the possibility that central plasticity may have compensated for the observed peripheral deficit.

The direct measurement of vestibular function is currently limited to VsEP in animals (Figure 3). Research using direct measures of inner ear sensory function: otoacoustic emissions (OAE), auditory brainstem response (ABR), and vestibular evoked potentials (VsEP) in mice has shown differences in auditory and vestibular function between various mutant strains, suggesting an important genetic influence on functional aging (Jimenez et al., 1999; Johnson et al., 2000; Jones et al., 2006). Jones et al. (2006) measured auditory and vestibular function in inbred mouse strains and the results showed some interesting trends. Some strains (e.g., BUB/BnJ, NOD/LtJ, A/J) showed severe hearing loss and severe vestibular impairment at the ages tested. Other strains (e.g., DBA/2J, A/WySnJ, NOD.NONH2kb, A/HeJ) had severe vestibular impairment with relatively little change in hearing at the ages tested. Three strains (MRL/MpJ, Ce/J, SJL/J) had significant vestibular loss with no concomitant hearing loss (at least to 32 kHz). These results demonstrate that genetic background plays a role in age-related changes in the two sensory systems and that functional change in one sensory system

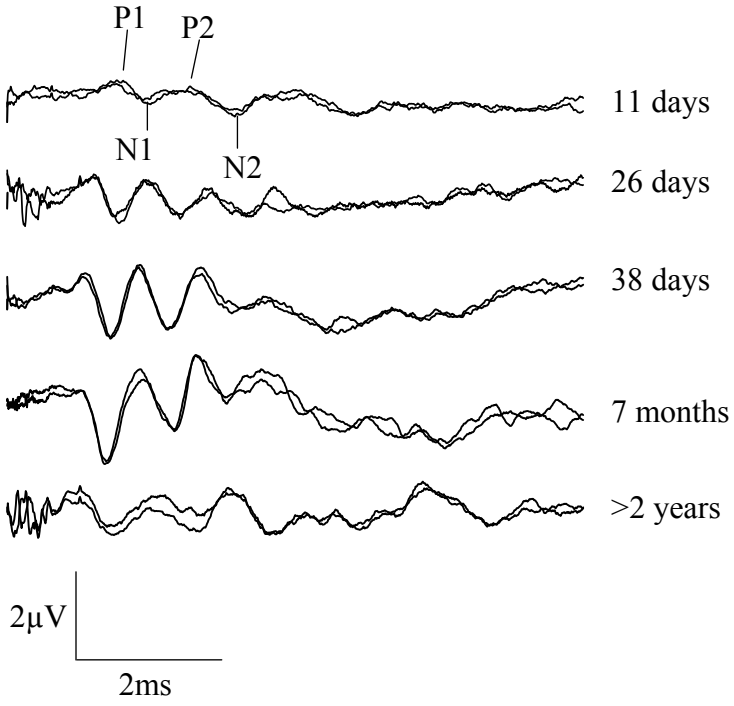


Figure 3. Linear VsEPs are shown for five different mice as a function of age. Responses are resolved first at ages 8 to 10 days after birth. PI, PII, NI, and NII peaks are labeled. Amplitudes increase and latencies become shorter during maturation. Age related change in waveform morphology are apparent at advanced age (>2 years).

does not obligate change in the other. Inferences concerning age related functional change in these strains are limited by the small numbers of animals used (4 or less) and the fact that measurements of auditory and vestibular function were not made in the same animal.

In summary, much is known about structural and functional change in the auditory system with aging but little is known about age-related changes in the vestibular system. Furthermore, even less is known about the relationship between aging of the two modalities.

A Short Statement about the Nature of the Problem

What little is known about age-related changes in the peripheral vestibular system is based primarily on structural changes and indirect measures of vestibular function. The effects of age, gender, and genetic background as related to vestibular functional aging are not known. Genetic background is known to predispose one to disease or disorder, including ARHL (Johnson et al., 1997). Comparison of auditory and vestibular functional changes due to aging, using indirect measures, has suggested that functional changes in the two sensory systems may occur independently. In reality, we are poorly informed about functional aging of the vestibular periphery.

Research Questions

1) Are there changes in macular function due to age alone?

   If so, is the time course the same as for auditory change?

2) Are there qualitative changes in macular otoconia that may explain functional change?

3) Do *Ahl* strains show age related changes in macular function?

   If so, are there qualitative changes in macular otoconia that may explain functional change?

4) If change is found in macular function in *Ahl* strains, how do these changes compare to those seen in auditory function?

5) Are age related changes in vestibular and auditory function different between males and females?

The proposed research tested the following hypotheses:

1) Functional changes in the macular organs, due to age alone, are concurrent with age related functional changes in the cochlea.
2) Mouse strains, which harbor A*hl* genetic mutations, will show age related change in macular function concurrent with ARHL.
3) Change in macular and cochlear function, due to age alone, is the same for males and females.

How were these hypotheses tested?

We used VsEP measures to assess macular function and ABR and DPOAE measures to assess auditory function. Scanning electron microscopy (SEM) was used to assess macular otoconia. Auditory and vestibular function were tested in CBA/CaJ (age alone model) and C57BL/6J, CE/J, and NOD.NON-H2nb1/LtJ (three *Ahl* strains) mice from 1.1 to 23.8 months of age. Temporal bones were dissected at the end of the functional experiments, fixed and processed for SEM imaging. SEM images were taken for representative samples of the macular organs from the young, middle aged and old

animals from each strain. This was done in an attempt to characterize any age related changes in the number and/or distribution of macular otoconia. ABR and VsEP response thresholds, latencies, and amplitudes were quantified and plotted as a function of age. ABR and VsEP thresholds were normalized in order to compare function between modalities. Linear regression was performed on raw ABR and VsEP thresholds, normalized ABR and VsEP thresholds, and on the difference between male and female thresholds for the two measures. Student' T-tests were performed on the slope and intercept of the linear regression line in order to compare auditory and macular sensitivity and age related change in sensitivity within and between strains and genders. DPOAE amplitude was plotted as a function of age and geometric mean frequency.

CHAPTER II: METHODS

Overview of the Experiment

The present research tested the following hypotheses: 1) functional changes in the macular organs, due to age alone, are concurrent with age related functional changes in the cochlea 2) mouse strains, which harbor A*hl* genetic mutations, will show age related change in macular function concurrent with ARHL 3) change in macular and cochlear function, due to age alone, is the same for males and females.

We used VsEP measures to assess macular function. VsEPs are critically dependent on utricular and saccular function and provide an indication of the sensitivity of macular end organs, as well as the timing and synchrony of firing of vestibular afferents. ABR and DPOAE measures were used to assess auditory function. ABR provides an objective measure of auditory sensitivity and the timing and synchrony of firing of auditory afferents. DPOAEs provided an additional measure of cochlear function (more specifically OHC function). The three measures were taken in a single session from the same mouse. Following functional measures, temporal bones were harvested and the macular organs were prepared for imaging with scanning electron microscopy.

ABR and VsEP thresholds were normalized in order to make comparisons between modalities and descriptive statistics were generated. Normalized ABR and VsEP threshold slopes and intercepts were compared using linear regression and student's T-test. Comparisons were limited to ABR 8 kHz due to the fact that *Ahl* mice exhibit hearing loss in the mid and high frequencies from a very early age resulting in a high number of "no response" for mid and high frequency stimuli. Peak latencies (i.e., P1, P2,

and P3) and peak to peak amplitudes (P1-N1, P2-N2, P3-N3), were quantified for responses at the maximum stimulus intensity and were plotted as function of age. DPOAE amplitude and noise floor were plotted for each strain as a function of age and 2f1-f2 geometric mean frequency. Extensive statistical analysis was not performed on DPOAE data as the main purpose of DPOAE measures in the current study was to confirm the cochlea as the site of lesion.

In summary, the CBA/CaJ strain (no known ear related genetic mutation) served as the model for age alone and the C57, CE/J, and NOD NON strains served as ARHL models. Auditory and vestibular thresholds, latencies, and amplitudes were quantified and plotted as a function of age. DPOAE responses were plotted as a function of age and geometric mean frequency. Within strain comparisons were made between auditory and vestibular function. Between strain comparisons were made for vestibular function. A between-strain auditory comparison was not made as each strain carries a unique genetic background so any auditory functional differences observed between strains was not surprising and in fact were expected (strain differences are well documented in the literature). Temporal bones were dissected at the end of the functional experiments, fixed and processed for imaging with SEM. A qualitative comparison was made between the macular organs and otoconia of "young" and "old" mice to determine if age related changes in macular otoconia would explain any observed loss of macular sensitivity.

Animals

This study utilized a murine model. Four inbred mouse strains were used: C57BL/6J, CE/J, CBA/CaJ and NOD.NON-H2nb1/LtJ. The C57BL/6J, CE/J, CBA/CaJ,

and NOD/NON inbred mouse strains were chosen because some functional information (although limited information in many ways) is available for at least one of the sensory modalities for each of these strains (see Table 1). The primary focus of this project was to characterize age related change in auditory and vestibular function over the majority of the lifespan of the selected murine models. For this reason we chose linear regression as the statistical tool. Animals were not separated into arbitrary age groups for analysis of ABR and VsEP data. DPOAE data was divided into bimonthly age groups for averaging and plotting of DP grams. The age groups were divided as follows: <2, 2-3, 4-5, 6-7, 8-9, 10-11, 12-13, 14-15, 16-17, 18-19, 20-21, 22-23. Approximately 8-10 mice were tested for each age group. ABR, VsEP, and DPOAE were measured once in each animal, after which the animal was killed in order to avoid affects of repeated anesthesia and to obtain temporal bones at the various ages for SEM.

The mouse strains were chosen based on their known genetic background and existing data for vestibular and auditory function. The CBA/CaJ strain was chosen because it is has no known ear related genetic mutations and is a useful model for examining the effect of age alone on inner ear function. The C57BL/6J and the CE/J strains were chosen because they are known to carry the *Ahl* mutation, which is expressed in the inner ear with age, and demonstrate ARHL. The NOD.NON-H2nb1/LtJ strain was chosen because it is known to carry the *Ahl* and *Ahl2* mutations, both of which affect the inner ear with age. Table 2 lists the number of data points for each measure for each strain.

Table 1. *Strains utilized and pre- existing knowledge of auditory and vestibular function and genetic mutations. Direct comparisons between auditory and vestibular functional aging in these strains are limited to small n and few ages.*

Strain	Vestibular function	Auditory function	Known mutations
C57BL/6J	Normal gravity receptor function through 190 days of age. Elevated VsEP thresholds at 389 days of age (Jones et al., 2006)	Age related, progressive hearing loss (Henry, 1980). Apparent gender differences in auditory aging (Henry, 2002).	*Ahl* on chromosome 10 (Johnson et al. 1997; 2000).
CE/J	May have significant gravity receptor dysfunction by 90 days old (Jones et al., 2006)	Normal hearing at less than 13 weeks of age (Zheng et al., 1999; www.jax.org).	*Ahl* on chromosome 10 (Johnson et al., 1997; 2000).
CBA/CaJ	Normal macular function to 166 days (Jones et al., 2005).	Retain normal hearing and OAE to advanced age (Jimenez et al., 1999; Frisina, 2001). Potential gender differences (Guimaraes et al., 2004; Henry, 2004)	None known
NOD.NON-H2nb1/LtJ	Potential gravity receptor dysfunction by 66 days of age.	Early onset profound hearing loss (Jones et al., 2006).	*Ahl* on chromosome 10 (Johnson et al., 1997; 2000). *Ahl2 on* chromosome 5 (Johnson & Zheng, 2002)

Table 2. *Number of data points per measure for each strain. Instances in which the measure was performed but no response was present are included (no responses).*

Strain	8 kHz	16 kHz	32 kHz	41.2 kHz	VsEP	DPOAE
CBA/CaJ	129	130	131	130	118	121
C57BL/6J	147	147	148	145	133	144
CE/J	90	92	92	92	95	96
NOD.NON-H2nb1/LtJ	22	22	22	21	19	22

Animal Preparation

Studies were conducted at East Carolina University. Use of animals in this work was approved by the Institutional Animal Care and Use Committee (AUP #P028) and national and international regulations regarding the use of animals were followed. Ambient noise levels in the animal housing area were monitored through out the study to assure that noise levels were below those which might result in noise induced hearing loss (See Appendix M). Sibling breeding pairs were obtained from The Jackson laboratory (Bar Harbor, ME). Animals were housed and maintained using standard husbandry until appropriate age for testing. During testing mice were anesthetized with ketamine (120 to 126 mg/kg) and xylazine (10 to 14 mg/kg) injected intraperitoneally. Core body temperature was maintained at 37.0 ± 0.2°C using a homeothermic heating blanket and rectal thermocouple (FHC Inc., Bowdoin, ME). Mice were excluded from the study if they showed abnormal outer or middle ear appearance, as determined by examination with an operating microscope under anesthesia. Any signs of ruptured tympanic membrane, excessive cerumen, redness or inflamed tympanic membrane lead to exclusion. In total, three mice were excluded for a ruptured tympanic membrane. During evoked potential testing, subcutaneous needle electrodes were placed just posterior to the lambdoidal suture (noninverting), behind the left pinna (inverting) and at the neck (ground). A noninvasive head clip was used to secure the head to a mechanical shaker for delivery of vestibular stimuli. Auditory stimuli were delivered via plastic tubing secured at the entrance of the external acoustic meatus. The three measures (VsEP, ABR, and DPOAE) were performed during the same session after which the mice were euthanized.

Measurements began with DPOAEs followed by ABR and VsEPs. This order was chosen in an attempt to limit exposure to high stimulus levels that could result in a temporary threshold shift. Stimulus levels for DPOAE were limited to 60 dB SPL compared to ABR maximum stimulus levels which could be as high as 100 dB SPL. The intense forward masker that was presented during VsEPs has an average intensity level ranging from 94 to 100 dB SPL. Test frequencies for ABR and DPOAE were tested in a random order. The time required for recording VsEP and DPOAE was consistent from mouse to mouse and was typically 30 minutes per measure. The time required to record the ABR thresholds varied depending on the sensitivity of the auditory system. When good auditory sensitivity was present, more stimulus presentations were required to determine threshold and as a result testing required more time. Recording time for the four frequencies did not generally exceed 45 minutes in mice with good auditory sensitivity and in mice with poor auditory sensitivity testing was as short as 30 minutes. In general, the three measures were completed in one mouse in less than two hours.

Recording Protocol for Evoked Potentials

Single channel signal averaging was used to record VsEP and ABR response waveforms. Offline analysis was used to determine response thresholds, response peak latencies, and peak-to-peak amplitudes. Subcutaneous electrodes were placed as described above. The electroencephalographic activity was amplified (200,000X, Grass P511), band pass filtered (300 to 3000 Hz, -6 dB points, Grass P511) and digitized (10 μs per point for 1024 points, TDT AD1) beginning at stimulus onset (TDT TG6 triggers system). 256 samples were averaged to produce the final VsEP or ABR waveform.

Stimuli for VsEPs were linear jerk pulses that physically moved the mouse's head. ABR stimuli were short duration tone bursts. Stimuli for VsEP and ABR are described in detail below.

Vestibular Evoked Potential (VsEP) Stimulus

A linear jerk pulse generated by Tucker Davis Technologies (TDT, Alachua, FL) modules (TG6, DA3-2, PA4) and controlled by custom software (Jones & Jones, 1996) was used to elicit VsEPs. The jerk pulse was generated using a linear voltage ramp of 2 ms duration, routed through a power amplifier (Peavey, Meridian, MS) which drove a mechanical shaker (Labworks Inc., Model E2-203, Costa Mesa, CA). The applied voltage produced an acceleration ramp, which was measured by an accelerometer (Endevco Inc., San Juan Capistrano, CA), 10mV/g where g = 9.81 m/sec^2. Output of the accelerometer was measured using a custom built electronic differentiator; the output of which was monitored with an oscilloscope. The linear jerk pulses were coupled to the head through a custom platform mounted atop the shaker. Systematically increasing or decreasing the amplitude of the stimulus waveform controlled the intensity of the stimulus. Amplitude of the jerk stimuli was recorded as the mean peak jerk level (measured using an oscilloscope) and was expressed in dB relative to 1.0 g/ms. Magnitude of the stimulus was calibrated to produce an onset jerk magnitude of 1.0 g/ms with an attenuator setting of 0 dB. Stimulus amplitude ranged from –18 to +6 dB re: 1.0 g/ms and was adjusted in 3 dB steps. Maximum stimulus amplitude (+ 6 dB re: 1.0 g/ms) resulted in a jerk amplitude of 2 g/ms and platform displacement of approximately 26 μm at peak acceleration. Jerk stimuli had two directions of motion: normal and inverted. Mice were placed in a supine

position with nose up. The axis of motion for normal polarity began with upward movement (naso-occipital +X). Inverted stimulus polarity began with downward movement (naso-occipital -X). Responses were collected for both normal and inverted polarities with the resulting waveforms averaged online to produce the final waveform for analysis.

Auditory Brainstem Response (ABR) Stimulus

Tone burst stimuli were used for ABR testing. Continuous pure tone stimuli were generated by a digital signal analyzer (Stanford Research Systems, SR785, Sunnyvale, CA) and shaped and controlled using custom software and Tucker Davis Technologies (TDT, Alachua, FL) modules (TG6, SW2, PA4). Tone bursts at 8, 16, 32, and 41.2 kHz had a 1.0 ms rise-fall times with 1.0 ms plateau (3 ms total duration). Stimuli were presented to the left ear via commercial OAE tips (Etymotic, ER10D-TO5 5mm, Elk Grove Village, IL) and PE-25 tubing. Auditory stimuli were presented at a rate of 17 stimuli/s. Intensity series were collected with a descending series of stimulus intensities (6 or 12 dB steps) beginning at +6 dB. The average maximum stimulus levels were 97, 90, 78, and 92 dB pe SPL for 8, 16, 32, and 41.2 kHz respectively. Stimulus levels were decreased until no response was visible and then dropped an additional 6 dB, in order to ensure that a sub-threshold level has been reached. The left ear only was tested.

Distortion Product Otoacoustic Emission (DPOAE) Stimulus

Methods for DPOAE stimuli and recording were similar to Jiminez et al. (1999) and Guimareaes et al. (2004). Stimuli for DPOAEs were generated and shaped using custom software and modules from TDT (TG6, PA4, and SW2). Pure tone frequencies

(f1, f2, f2/f1 ratio = 1.25), at equal levels (L1 = L2 = 60 dB SPL), 150 ms duration, were generated with independent sources (HP Agilent 33220A signal generators, Santa Clara, CA) and routed through separate drivers to mix acoustically in the ear canal. Stimuli were calibrated in a 0.35 ml coupler. Stimulus frequencies for the primaries were such that geometric mean (GM = $(f1 \times f2)^{0.5}$) frequencies ranged from 6.0 to 48.5 kHz (at least 8 frequencies per octave). All frequencies (f1, f2, 2f1-f2) are listed in Table 3.

Recording Protocol for DPOAE

During each study, ear canal sound pressure was recorded with a low noise probe microphone (Etymotic ER 10B+). The microphone output was amplified 10X (ER10B+ preamplifier) and input to a dynamic signal analyzer (Stanford Research Systems SRS785) for sampling (at 200 kHz) and fast Fourier transform (FFT). The amplitude of f1, f2, and the 2f1-f2-distortion product was measured from the FFT waveform. The noise floor was calculated as the average amplitude in the five frequency bins above and below the 2f1-f2 component. The recording system was tested periodically in the 0.35 ml coupler to confirm the absence of artifactual distortion.

ABR and DPOAE Microphone and Stimuli Calibration

Stimuli for ABR testing were calibrated using a Bruel & Kjaar (B & K) ¼" microphone (4954, Nærum, Denmark), Nexus amplifier (2690), custom software, and 0.35 ml custom coupler. During calibration the probe tube assembly (housing the ER10B+ microphone and exit ports for sound) was inserted into one end of the custom coupler (0.35 ml volume) and the B & K microphone was inserted into the other end. The output of the ER10B+ microphone was amplified (10X) and routed to one channel of the

Table 3. *DPOAE primaries and 2f1-f2 frequencies. Table 3 lists the two primary stimulus frequencies introduced into the ear canal and the 2f1-f2 response frequency.*

f1	f2	2f1-f2	geometric mean frequency
5.2	6.4	4	5.77
6	7.6	4.4	6.75
6.8	8.4	5.2	7.56
8	10	6	8.94
9	11.2	6.8	10.03
9.92	12.24	7.6	11.01
10.6	13	8.2	11.74
12	15	9	13.42
13	16.2	9.8	14.51
14	17.6	10.4	15.7
16	20	12	17.89
17	21.2	12.8	18.98
19	23.6	14.2	21.27
21	26.2	15.8	23.46
22	27.6	16.4	24.64
24	30	18	26.83
26	32.4	19.6	29.02
28	34.8	21.2	31.22
30	37.6	22.4	33.59
32	40	24	35.78
34.8	43.6	26	38.95
37	46	28	41.26
39	48.6	29.4	43.54
41.2	51.4	31	46.02
42.6	53.2	32	47.61

SRS785 signal processor. The output from the B & K microphone was routed to a second channel on the SRS785 signal analyzer. The Visual Basic custom software controlled stimulus generation and signal analysis for DPOAE stimuli. Two Tucker Davis SW2 modules were set for a rise fall time of 5 ms and 140 ms plateau, for a total duration of 150 ms. Each stimulus frequency was presented and the PA4 attenuation was adjusted until a level of 60 dB SPL was achieved for the B & K channel. The PA4 attenuation level and the ER10B+ output level from the other channel of the SRS785 signal analyzer were saved as a probe tube calibration file (PTC) and the ER10B+ microphone sensitivity was calculated for each frequency. This PTC file served two purposes. First, the PTC file was loaded into the custom Visual Basic software and was used to calculate the target dB SPL for the ER10B+ microphone output. This assured that a level of 60 dB SPL was achieved for both primary tones presented during DPOAE testing. Secondly, the calibration values were used to determine stimulus presentation level for the four stimulus frequencies (8, 16, 32, and 41.2 kHz) presented during ABR testing. During ABR testing the probe tip was place at the mouse ear canal and the ER10B+ microphone measured the stimulus sound pressure level. The output from the microphone was routed to an oscilloscope and a peak-to-peak voltage was manually measured and recorded. Knowing the ER10B+ microphone sensitivity for each frequency allowed us to convert the measured peak-to-peak voltage into a peak equivalent dB SPL (dB pe SPL) for each ABR tone burst stimulus. The actual SPL varied from mouse to mouse (due to ear canal properties) but the microphone sensitivity determined during the calibration allowed us to calculate the actual SPL for each ABR stimulus frequency for each study.

Data Analysis

ABR and VsEP waveforms were plotted as intensity series and peaks P1, P2, P3, N1, N2, and N3 were identified (Figures 4 and 5). ABR and VsEP thresholds, peak latency, and peak to peak amplitude were quantified. Amplitude and latency measures for the first three peaks (P1, P2 and P3) of the response waveform were quantified for the + 6 dB re: 1.0 g/ms stimulus level. Threshold was defined as the intensity midway between the minimum stimulus intensity that produced a discernible response and the maximum stimulus intensity that did not result in a visible response. Response peak latency was defined as the time, in ms, from onset of the stimulus to the appearance of each positive peak (P1, P2 and P3). Peak-to-peak amplitude, (P1-N1, P2-N2, and P3-N3) measured in micro volts (μV), represented the difference between each positive peak and its respective negative peak.

In order to compare cochlear and macular sensitivity it was necessary to normalize threshold values. We chose to normalize threshold values in terms of a percent change in auditory and macular dynamic range. Dynamic range was defined as the range from the best threshold measured for the CBA/CaJ strain to the most intense stimulus level possible without inducing a temporary or permanent threshold shift. The CBA strain harbors no genetic mutations, maintains hearing until late in life, and will serve as a baseline for "normal" hearing. Normalizing thresholds relative to the CBA strain also allowed a direct comparison between the four strains. The upper level of the dynamic range was based on a pilot study conducted to determine the level at which ABR stimuli may cause temporary or permanent threshold shift. The results of this pilot study

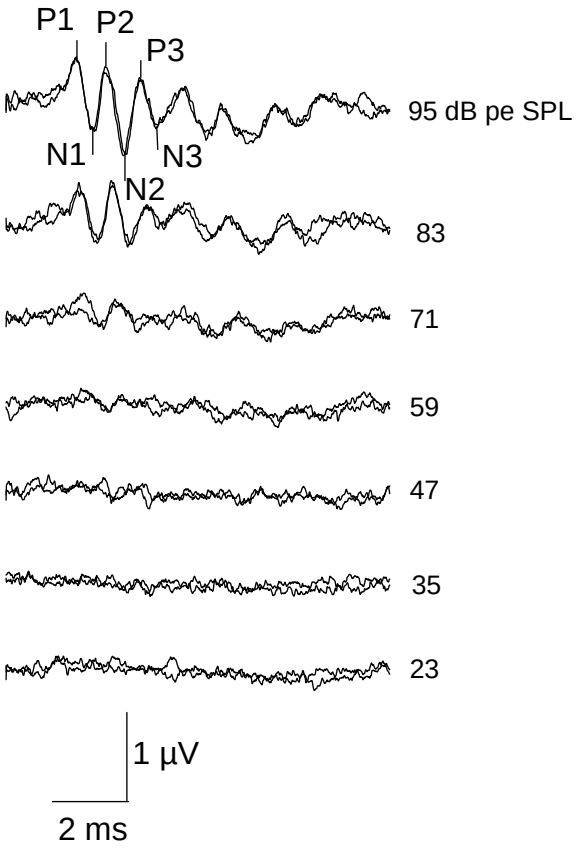


Figure 4. Representative ABR waveforms for 16 kHz. Peaks P1, P2, P3, N1, N2, and N3 are labeled and stimulus intensity is labeled on the right. Threshold for this intensity series was 41 dB pe SPL.

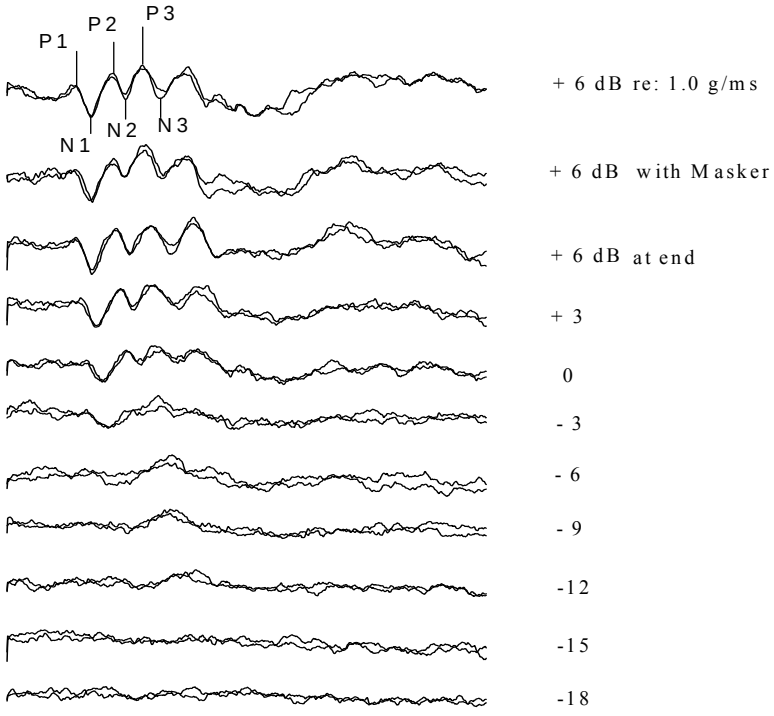


Figure 5. Representative VsEP intensity series. Peaks P1, P2, P3, N1, N2, and N3 are labeled and stimulus amplitude for each waveform is indicated on the right. VsEP threshold for this intensity series was – 13.5 dB re: 1.0 g/ms.

suggested that levels greater than 100 dB SPL resulted in temporary threshold shifts; so 100 dB SPL was used as the upper limit for the dynamic range. The lower limit for dynamic range was set by the best thresholds for the CBA/CaJ strain, which were 12.7, 13.1, 4.9, and 25.39 dB pe SPL for the test frequencies of 8, 16, 32, and 41.2 kHz, respectively. The lowest VsEP threshold measured was – 13.5 dB re: 1.0 g/ms and in this author's experience sustained linear accelerations more intense than + 6 dB re: 1.0 g/ms can dislodge macular otoconia. So the upper limit for VsEP dynamic range was set as +6 dB re: 1.0 g/ms. The resulting dynamic range was 19.5 dB re: 1.0 g/ms. To normalize a given threshold (y) the following formula was used: $x = [(y – z) / k]*100$ where x = the normalized value, z = the lowest threshold measured and k is the dynamic range.
For example, with an absolute threshold of -7.5 dB:

$$x = [(-7.5 - (-13.5) / 19.5] * 100$$

$$x = [6/19.5] * 100$$

$$x = 30.77\%$$

Within strain comparisons were made between auditory and vestibular thresholds. Between strain comparisons were made for vestibular function. A between-strain auditory comparison was not made. Auditory differences between strains are well described in the literature so any auditory functional differences observed between strains was not surprising and in fact were expected. A student's T-test was performed to determine if there were significant differences in the linear regression slopes or intercepts between: 1) auditory thresholds between males and females within each strain 2) VsEP

thresholds between males and females within each strain 3) VsEP thresholds between strains 4) ABR and VsEP thresholds within each strain.

The mean DPOAE amplitude and noise floor were plotted for each age group as a function of age and geometric mean frequency. Statistical analysis was not performed on DPOAE data, as the main purpose of DPOAE measures was to characterize age related changes in OHC function and to confirm the cochlea as the site of lesion.

Figure 6. ABR Stimulus Generation and Presentation

Stanford Research Systems (SRS) 785

- Wave: Sine
- Test frequency: 8,16,32, or 41.2 kHz
- Amplitude: 10 Vp-p
- Offset: 0 Mv

Tucker Davis Technology Timing Generator (TG6)

- 0.01 ms/pnt
- 0 start
- 3000 μs duration

Tucker Davis Technology (TDT) SW2 Switch

- Shape: Cos2
- Duration: 0
- Trigger: Enable ↑
- Rise Fall: 1 ms

Tucker Davis Technology (TDT) PA4 Attenuator

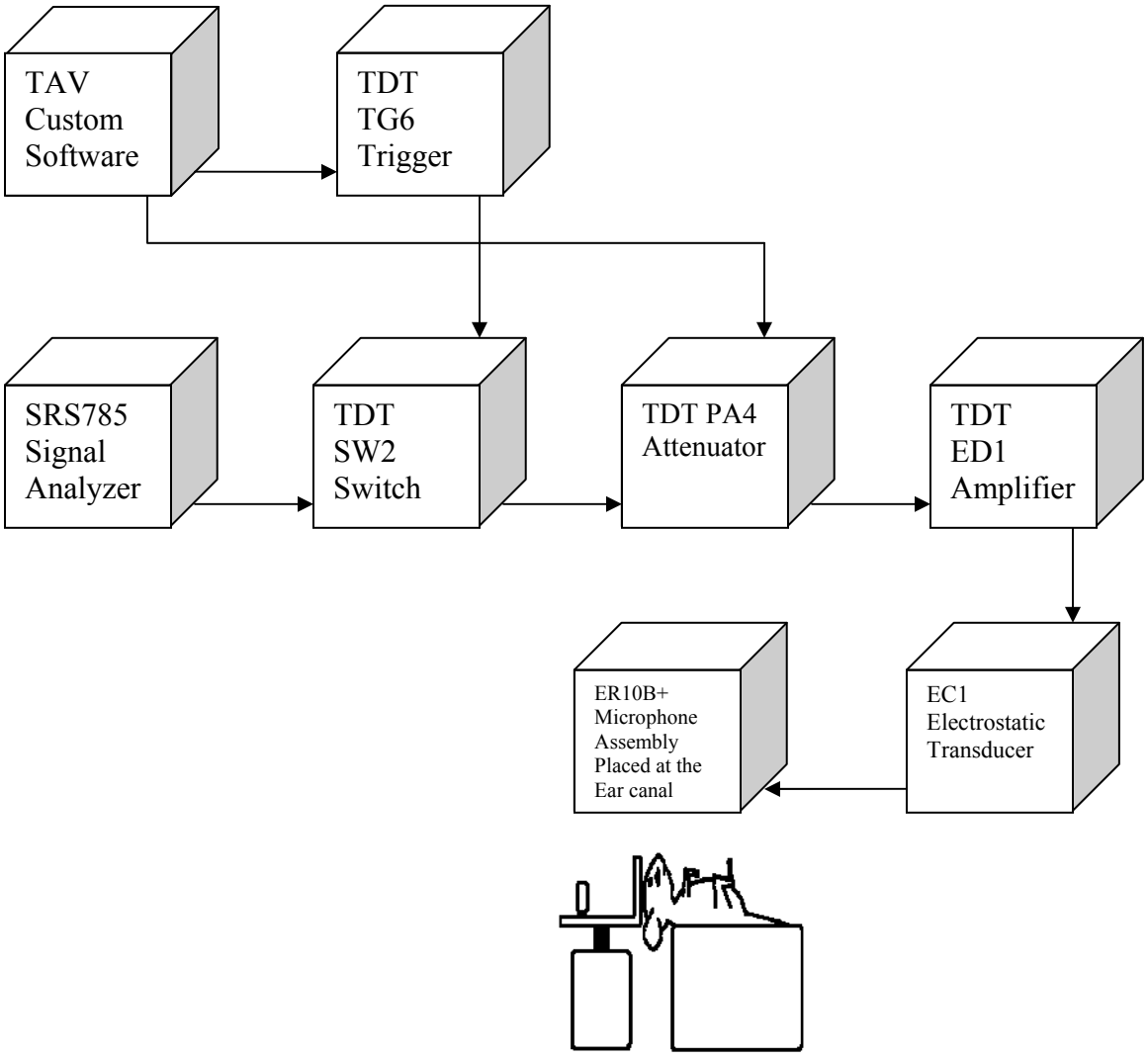
- TAV software controls PA4 and attenuation of tone burst
- Stimulus attenuation is adjusted from +6 to sub-threshold level

Tucker Davis Technology (TDT) ED1 Amplifier

- 0 dB gain

Tucker Davis Technology (TDT) EC1 electrostatic transducer

- Frequency Response: +/-9 dB from 4 kHz to 110 kHz
- Typical output (9.9V peak input): EC1 90 dB SPL, +/- 9.9 V 5 kHz signal
- Maximum Output: 110 dB SPL at 10 cm


TAV
Custom
Software
TDT
TG6
Trigger
SRS785
Signal
Analyzer
TDT
SW2
Switch
TDT PA4
Attenuator
TDT
ED1
Amplifier
ER10B+
Microphone
Assembly
Placed at the
Ear canal
EC1
Electrostatic
Transducer

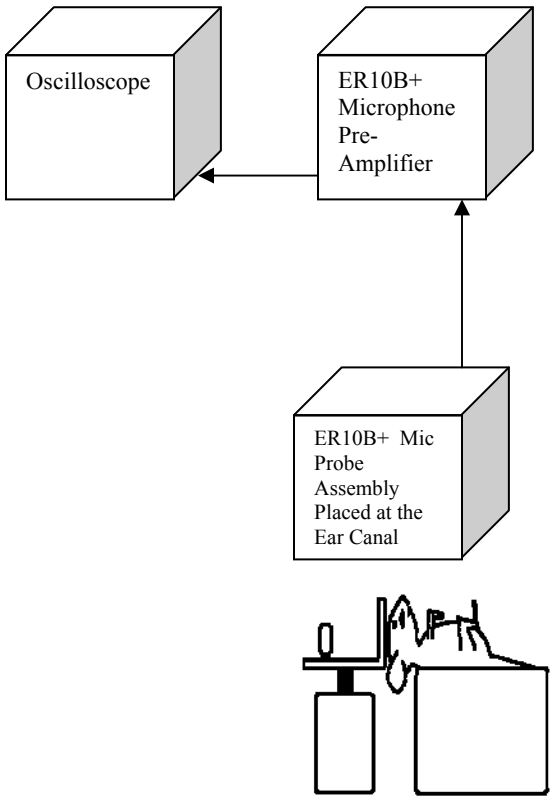


Figure 7. ABR Stimulus Monitoring

Etymotic ER10B+ pre-amplifier

- 10X

Hewlett Packard 54602B Oscilloscope

- 1 v/div
- 1.0 ms/div

Figure 8. ABR Recording

TAV Custom Software

- 17 stimuli per sec
- 128 sweeps
- 10 conversion time
- 1024 pnts after trig PTD
- 5 RC AD volt range
- 4096 RC # AD bits
- 65536 TDT ADbits
- 20 Max Amp rej level (μv)
- 68 Sweeps per display
- 300 Hz Ch1 lf cutoff
- 0 dB applied voltage
- 10 μs sample rate
- 1024 pnts/swp/ch
- 1 channel
- 1024 RC buffer size
- 0 RC trace delay
- 1220.7 RC V/AD bit; μv/bit
- 20 TDT AD voltage range
- 305.17 TDT μv/AD bit
- -20 Min Amp rej level (μv)
- 200,000 Ch1 gain
- 3000 Hz Ch1 hf cutoff
- 150 Max # rejects

Tucker Davis Technology Timing Generator (TG6) Channel 4

- 0.01 ms/pnt
- 0 start
- 3000 μs duration

Grass P511 Biological Amplifier

- calibrator 1
- cal D.C.
- ½ Amp lo Freq 300 Hz
- Amp 200,000
- ½ Amp hi Freq 3 kHz
- 60 Hz filter in

Tucker Davis Technology (TDT) AD1 Analogue to digital converter

- 64 sweeps
- 10 μs/pnt

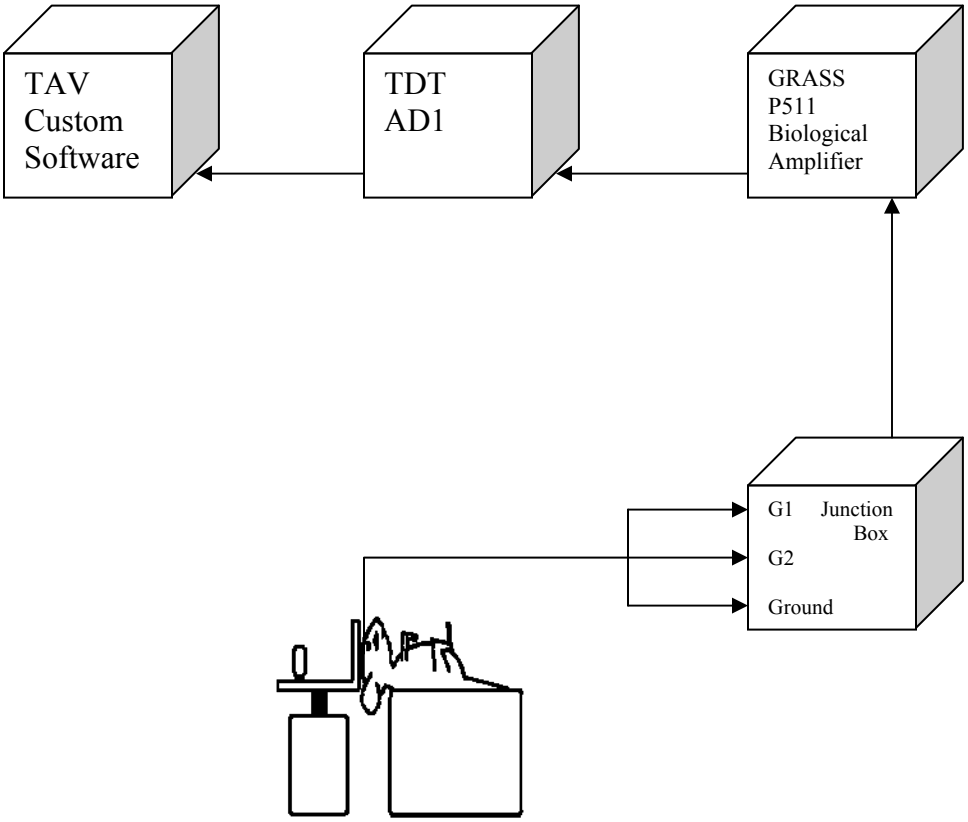

TAV
Custom
Software
TDT
AD1
GRASS
P511
Biological
Amplifier
G1
Junction
Box
G2
Ground

Figure 9. DPOAE Stimulus Generation and Presentation

Visual Basic Custom Software

- 1 dB accept limit
- 5 V peak applied voltage
- 3.06 stimuli/s
- 60 dB SPL target level
- 20 µPa acoustic reference level

HP Agilent 33220A arbitrary waveform generators

- controlled by the Visual Basic custom software
- waveform: sine
- 5 Vp-p

Tucker Davis Technology Timing Generator (TG6) Channel 5

- 0.01 ms/pnt
- 0 start
- 15000 µs duration

Tucker Davis Technology (TDT) SW2 Switch

- Shape: Cos2
- Duration: 0
- Trigger: Enable ↑
- Rise Fall: 5 ms

Tucker Davis Technology (TDT) PA4 Attenuator

- Visual basic software controls PA4 and attenuation of tones
- Stimulus attenuation is adjusted by Visual Basic until a level of 60 dB SPL is achieved for f1 and f2

Tucker Davis Technology (TDT) ED1 Amplifier

- 0 dB gain

Tucker Davis Technology (TDT) EC1 electrostatic transducers

- Frequency Response: +/-9 dB from 4 kHz to 110 kHz
- Typical output (9.9V peak input): EC1 90 dB SPL, +/- 9.9 V 5 kHz signal
- Maximum Output: 110 dB SPL at 10 cm

Etymotic ER10B+ pre-amplifier

- 10X

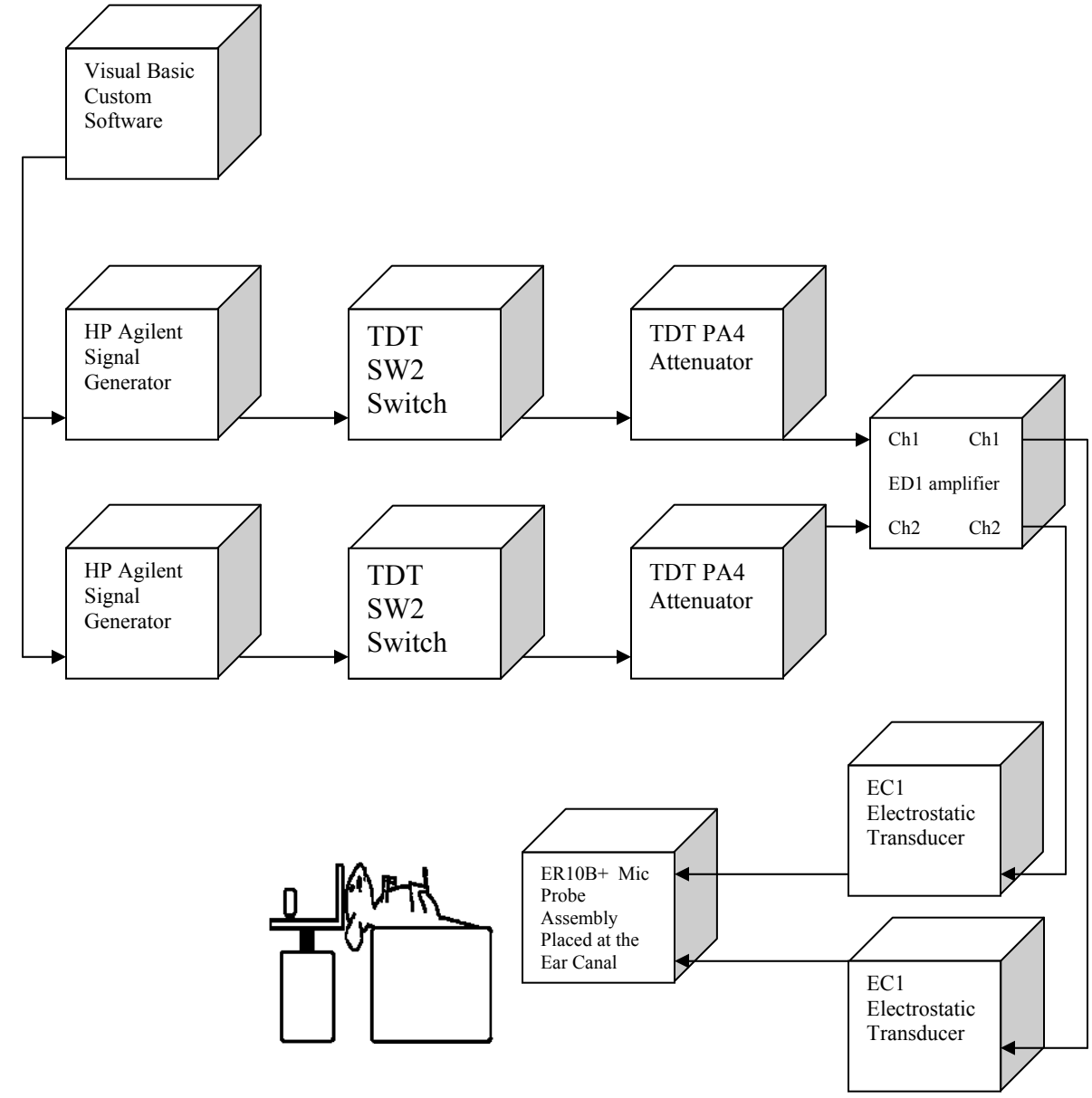

Visual Basic
Custom
Software
HP Agilent
Signal
Generator
TDT
SW2
Switch
TDT PA4
Attenuator
Ch1
Ch1
ED1 amplifier
Ch2
Ch2
HP Agilent
Signal
Generator
TDT
SW2
Switch
TDT PA4
Attenuator
EC1
Electrostatic
Transducer
ER10B+ Mic
Probe
Assembly
Placed at the
Ear Canal
EC1
Electrostatic
Transducer

Figure 10. DPOAE Recording

ER10B+ preamplifier

- 10X

Stanford Research Systems (SRS) 785 signal analyzer

- 6.4 kHz Span
- 125 acquisition time
- 24 dB V peak
- 102.4 kHz base frequency
- B display time 1
- 8 line width
- 800 FFT line
- -8 dB V peak
- A display FFT
- Input range -20 dB V peak

Visual Basic custom software

- 1 dB accept limit
- 3.06 stimuli/s
- 20 µPa acoustic reference level
- 5 v peak applied voltage
- 60 dB SPL target level

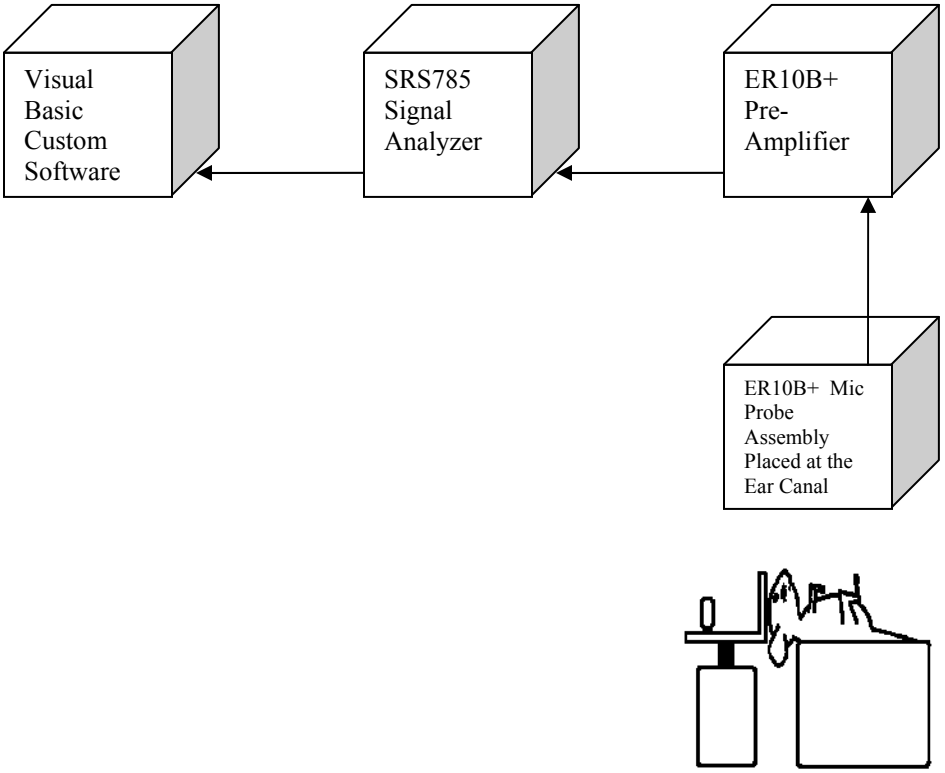

Visual Basic Custom Software
SRS785 Signal Analyzer
ER10B+ Pre-Amplifier
ER10B+ Mic Probe Assembly Placed at the Ear Canal

Figure 11. VsEP Stimulus Generation

TAV Custom Software

- 17 stimuli per sec
- 128 sweeps
- 10 RC trigger type
- 1024 RC buffer size
- 0 RC trace delay
- 1220.7 RC V/AD bit; µv/bit
- 20 TDT AD voltage range
- 305.17 TDT µv/AD bit
- -20 Min Amp rej level (µv)
- 200,000 Ch1 gain
- 3000 Hz Ch1 hf cutoff
- 150 Max # rejects
- 1024 pnts/swp/ch
- 1 channel
- 10 conversion time
- 1024 pnts after trig PTD
- 5 RC AD volt range
- 4096 RC # AD bits
- 65536 TDT ADbits
- 20 Max Amp rej level (µv)
- 68 Sweeps per display
- 300 Hz Ch1 lf cutoff
- 0 dB applied voltage
- 10 µs sample rate

Tucker Davis Technology (TDT) PA4 Attenuator

- TAV software controls PA4 and attenuation of jerk stimulus

Tucker Davis Technology Timing Generator (TG6) Channel 3

- 0.01 ms/pnt
- 0 start
- 3000 µs duration

Tucker Davis Technology (TDT) DA3-2 digital to analogue converter

- Acceleration time 2 ms
- Total time 100 ms
- Conversion time 20 µs/pnt

Grass P511 Biological Amplifier

- calibrator 1
- ½ Amp lo Freq 300 Hz
- Amp 200
- 60 Hz filter in
- cal D.C.
- X 1000
- ½ Amp hi Freq 3 kHz

Tucker Davis Technology (TDT) AD1 Analogue to digital converter

- 64 sweeps
- 10 µs/pnt

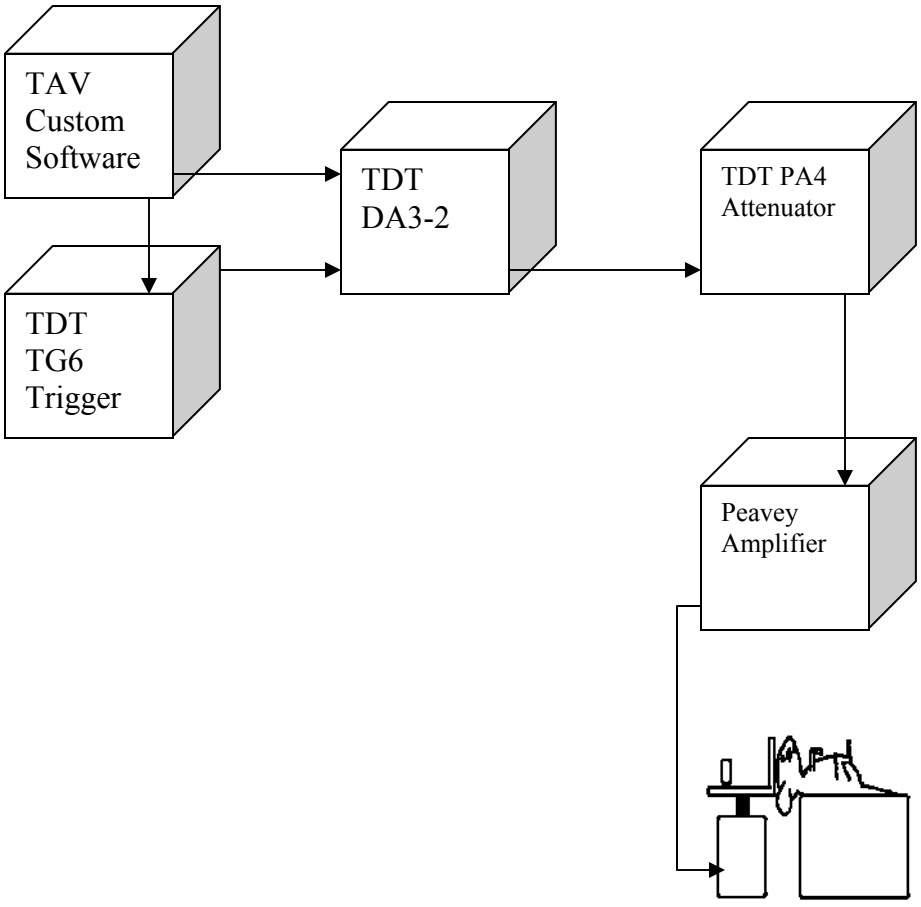

TAV
Custom
Software
TDT
DA3-2
TDT PA4
Attenuator
TDT
TG6
Trigger
Peavey
Amplifier

Figure 12. VsEP Masker Generation

Stanford Research Systems (SRS) 785

- Noise: white noise
- Amplitude: 1 V
- Offset: 0 Mv

Tucker Davis Technology Timing Generator (TG6) Channel 3

- 0.01 ms/pnt
- 0 start
- 15000 µs duration

Tucker Davis Technology (TDT) SW2 Switch

- Shape: Cos2
- Duration: 0
- Trigger: Enable↓
- Rise Fall: 0.5 ms

Peavey Amplifier

Frequency Devices (Ottawa, IL) high pass filtering

- 20 kHz knee point

Radio Shack Super-tweeter

Midrange speakers

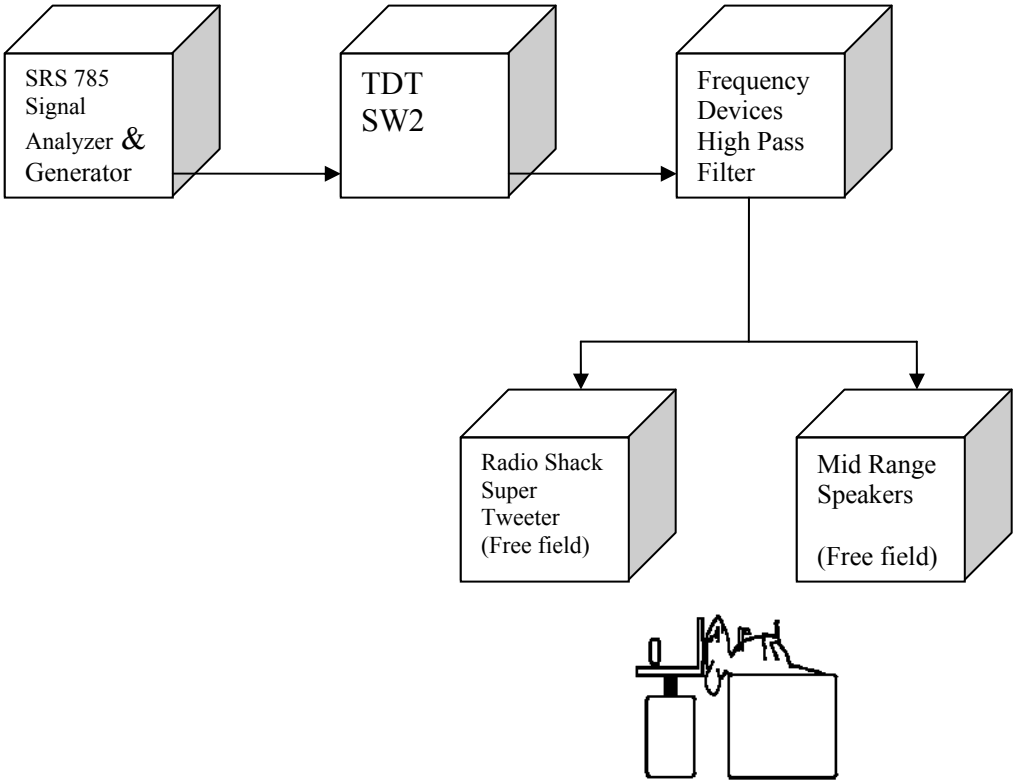

SRS 785
Signal
Analyzer &
Generator
TDT
SW2
Frequency
Devices
High Pass
Filter
Radio Shack
Super
Tweeter
(Free field)
Mid Range
Speakers
(Free field)

Figure 13. VsEP Jerk Stimulus Monitoring

Accelerometer amplifier

- 10 mV/g

Custom differentiator

- Calibrated 0.4 V = 1.0 g/ms

Hewlett Packard 54602B Oscilloscope

- 0.2 v/div
- 1.0 ms/div

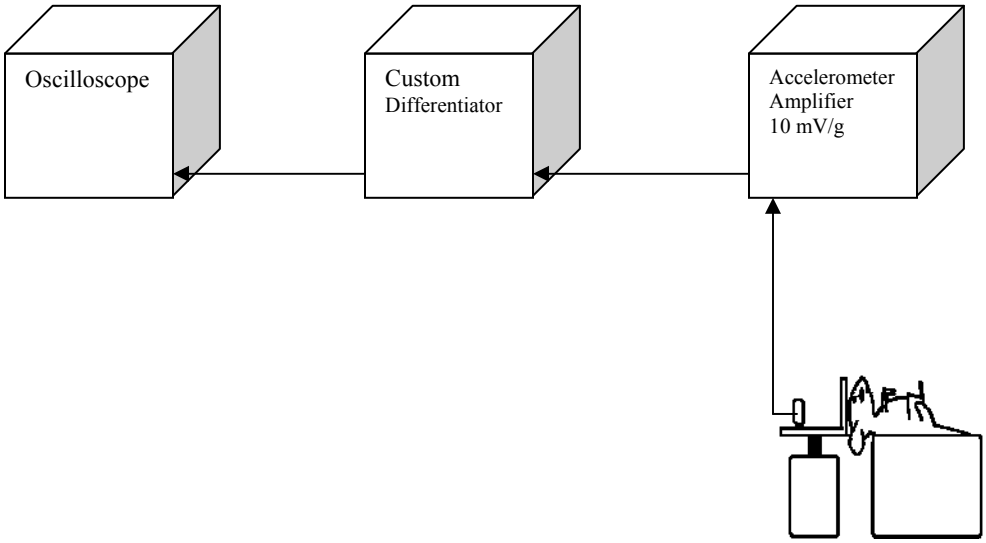


Figure 14. VsEP Recording

TAV Custom Software

- 17 stimuli per sec
- 1 channel
- 1024 RC buffer size
- 0 RC trace delay
- 1220.7 RC V/AD bit; µv/bit
- 20 TDT AD voltage range
- 305.17 TDT µv/AD bit
- -20 Min Amp rej level (µv)
- 200,000 Ch1 gain
- 3000 Hz Ch1 hf cutoff
- 150 Max # rejects
- 128 sweeps
- 1024 pnts/swp/ch
- 10 RC trigger type
- 1024 pnts after trig PTD
- 5 RC AD volt range
- 4096 RC # AD bits
- 65536 TDT ADbits
- 20 Max Amp rej (µv)
- 68 Sweeps per display
- 300 Hz Ch1 lf cutoff
- 0 dB applied voltage
- 10 µs sample rate
- 10 conversion time

Tucker Davis Technology (TDT) PA4 Attenuator

- TAV software controls PA4 and attenuation of jerk stimulus

Tucker Davis Technology Timing Generator (TG6) Channel 3

- 0.01 ms/pnt
- 3000 µs duration
- 0 start

Tucker Davis Technology (TDT) DA3-2 digital to analogue converter

- Acceleration time 2 ms
- Conversion time 20 µs/pnt
- Total time 100 ms

Grass P511 Biological Amplifier

- calibrator 1
- ½ Amp lo Freq 300 Hz
- Amp 200
- 60 Hz filter in
- cal D.C.
- X 1000
- ½ Amp hi Freq 3 kHz

Tucker Davis Technology (TDT) AD1 Analogue to digital converter

- 64 sweeps
- 10 µs/pnt

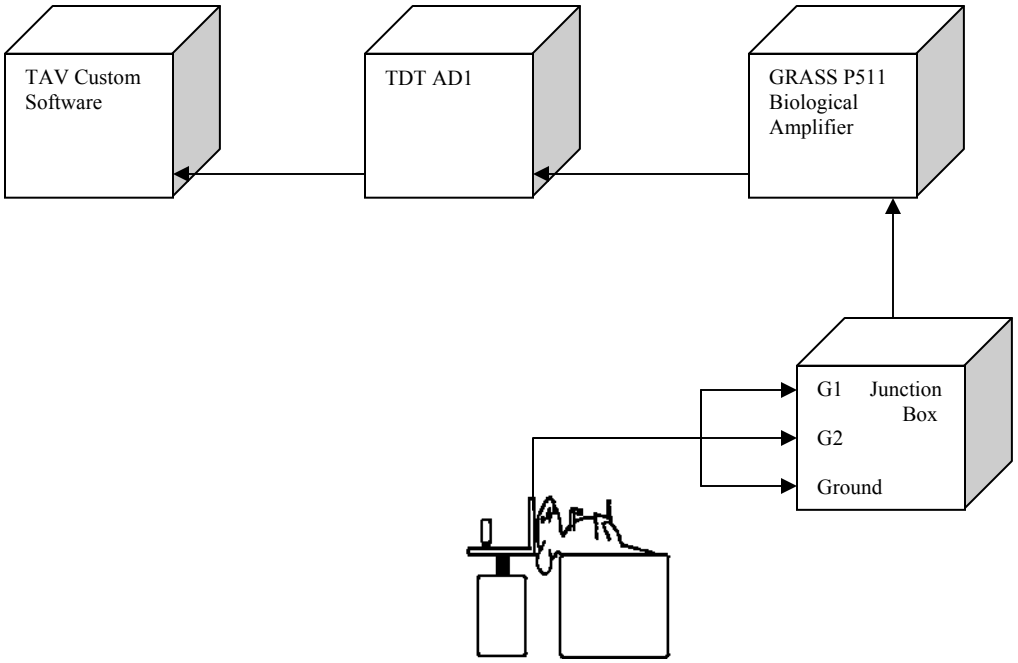

TAV Custom Software
TDT AD1
GRASS P511 Biological Amplifier
G1
Junction Box
G2
Ground

Figure 15. Stimulus and Microphone Calibration for ABR and DPOAE Stimuli

Visual Basic Custom Software

- 1 dB accept limit
- 3.06 stimuli/s
- 20 µPa acoustic reference level
- 5 v peak applied voltage
- 60 dB SPL target level

HP Agilent 33220A arbitrary waveform generators

- controlled by the Visual basic custom software
- waveform: sine
- 5 Vp-p

Tucker Davis Technology Timing Generator (TG6) Channel 5

- 0.01 ms/pnt
- 15000 µs duration
- 0 start

Tucker Davis Technology (TDT) SW2 Switch

- Shape: Cos2
- Trigger: Enable ↑
- Duration: 0
- Rise Fall: 5 ms

Tucker Davis Technology (TDT) PA4 Attenuator

- Visual basic software controls PA4 and attenuation of tones
- Stimulus attenuation is adjusted by Visual Basic until a level of 60 dB SPL is achieved for f1 and f2

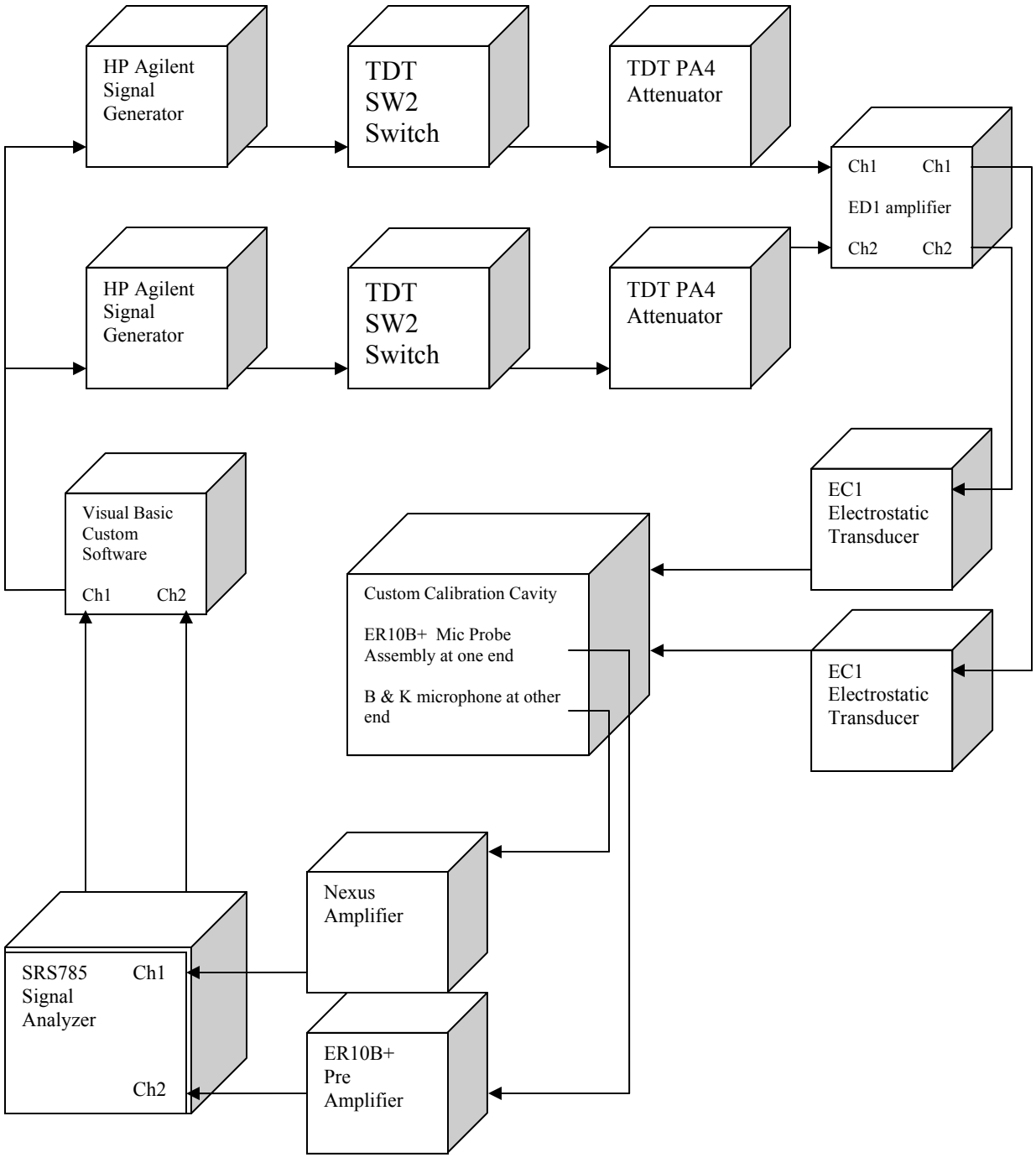
ER10B+ microphone

- 50 mv/Pa sensitivity
- No band pass filter
- 10 X gain

Brüel & Kjær ¼ microphone

Nexus amplifier

- 316 MV/Pa
- Gain 1
- 20 Hz– 100 kHz band pass filter


HP Agilent Signal Generator
TDT SW2 Switch
TDT PA4 Attenuator
Ch1 Ch1
ED1 amplifier
Ch2 Ch2
HP Agilent Signal Generator
TDT SW2 Switch
TDT PA4 Attenuator
Visual Basic Custom Software
Ch1 Ch2
EC1 Electrostatic Transducer
Custom Calibration Cavity
ER10B+ Mic Probe Assembly at one end
B & K microphone at other end
EC1 Electrostatic Transducer
Nexus Amplifier
SRS785 Signal Analyzer
Ch1
Ch2
ER10B+ Pre Amplifier

CHAPTER III: GRAVITY RECEPTOR AGING IN THE CBA/CaJ STRAIN; A COMPARISON TO AUDITORY AGING

Abstract

The CBA/CaJ strain is a widely studied mouse strain with no known genetic mutations affecting the inner ear, it maintains hearing sensitivity to late in life, and is commonly used as a genetic background for creating new genetic strains. The purpose of the present research was to characterize the effect of age and gender on gravity receptor function in the CBA/CaJ mouse strain and compare functional change between auditory and vestibular modalities. Vestibular evoked potentials (VsEP), auditory brainstem response (ABR) and distortion product otoacoustic emissions (DPOAE) were measured in 131 CBA/CaJ mice ranging in age from 1.7 to 23.8 months. VsEP thresholds increased significantly ($p<0.001$) as a function of age and deteriorated by an average of 0.39 dB re: 1.0 g/ms per month. At the oldest ages mice showed an average loss of 48 % of VsEP dynamic range. No loss of otoconia was observed that could account for the loss of macular sensitivity. No gender differences were found for VsEP measures. ABR thresholds increased significantly ($p<0.001$) as a function of age and decreased by 1.35, 1.38, 1.15, and 0.81 dB pe SPL per month for 8, 16, 32, and 41.2 kHz, respectively. These increases correspond to an average decrease in ABR dynamic range of 25 to 35%. The present study found no difference in the rate of decline in auditory function between males and females. DPOAE responses were present at all ages including the oldest ages tested but showed some decrease in amplitude consistent with ABR results. Macular and auditory sensitivity decreased with age in the CBA/CaJ strain but function is maintained

for both modalities even to advanced ages. The rate of change in auditory sensitivity varied by frequency and declined at a slower rate than macular sensitivity for all ABR test frequencies. When the rate of change for macular sensitivity and ABR 8 kHz was compared there was a significantly different rate of change ($p < 0.0001$), with macular function declining at a faster rate than auditory function. The current findings agree with previous reports of structural degeneration of the cochlea and vestibular organs, such as decreased hair cell densities and loss of primary afferents. Age related decreases in macular sensitivity should be considered when using the CBA/CaJ strain for vestibular studies.

Introduction

The inner ear houses the organs for hearing as well as balance. Age related declines in auditory function are well known and age related hearing loss (ARHL) is one of the most common causes of hearing impairment, affecting 50% of the population by age 80 (Gorlin, Toriello, & Cohen, 1995; Morton, 1991). The effects of age on vestibular function are much less understood. Studies by the National Institute on Deafness and other Communication Disorders estimated that 3.4 % of the U.S. adult population (6.2 million people) suffers from chronic dizziness and/or imbalance (Hoffman & Sklare, 2003). The use of mice for auditory research has lead to numerous mouse models of syndromic and non-syndromic hearing loss in humans and has facilitated the study of environmental and genetic causes of hearing loss, including ARHL.

The effect of age on inner ear morphology has been widely studied and structural degeneration in the cochlea and vestibular organs have been reported in animal models and humans. Common age-related cytoarchitectural changes in the cochlea of rats, mice, guinea pigs, monkeys and humans include: loss of outer and inner hair cells (Spongr, et al., 1997) loss of supporting cells (Nadol, 1979), reduced vascular supply (Saitoh, et al., 1995; Spicer & Schulte, 2005; Brown, et al., 1995), degeneration and loss of sensory cells (Willott, et al., 1991; Mizuta, et al., 1993: Spongr et al., 1997; Francis, et al., 2003), reductions in the number and integrity of afferent and efferent neurons (Felder & Schrott-Fischer, 1995), atrophy of the stria vascularis and spiral lamina (Suzuki, et al., 2006). Vestibular structural changes reported with aging include: a reduction in the number and integrity of sensory cells (Gleeson & Felix, 1987; Nakayama, et al., 1994; Rosenhall &

Rubin, 1975; Rauch, et al., 2001; Shiga, et al., 2005), reduced number of type 1 and type 2 hair cells (Gleeson & Felix 1987), reduced blood supply to vestibular sensory organs (Lyon & Wanamaker, 1993; Lyon & Davis, 2002), reduced number of globular substances (which are secreted precursors to mature otoconia) (Suzuki et al., 1997). Animal models have played an important role in identifying inner ear structural changes and factors that contribute to functional decline with age.

Auditory function has been well characterized in the CBA/CaJ strain, which maintains auditory sensitivity until late in life (Henry & McGinn, 1992; Zheng, Johnson, & Erway, 1999; Vasquez, Luebke, & Martin, 2001; Prosen, Dore, & May, 2003; Ohlemiller & Gagnon, 2004). Hearing sensitivity and outer hair cell function are maintained until 2 years of age as evidenced by DPOAE amplitudes (Guimaraes, et al., 2004), frequency tuning (as measured by behavioral testing and notched noise maskers) (May, Kimar, & Prosen, 2006) and ABR thresholds (Li & Borg, 1991; Guimaraes et al., 2004).

An interaction between age and gender for auditory function has been reported in humans and mice and include findings such as: young women generally have better hearing than young men (Corso, 1963; Rosen, Plester, & El-Mofty, 1964; Northern, Downs, Rudmose, Glorig & Fletcher, 1972; Surjan, 1973) and ARHL may progress faster in men than women (Corso, 1963; Pederson, Rosenhall, & Moller, 1989; Wallhagen, Strawbridge, Cohen, & Kaplan, 1997; McFadden & Pasanen, 1998; Gates & Cooper, 1999). Henry (2004) measured ABR and cochlear nerve envelope response in the CBA/CaJ and C57BL/6J strains and reported gender differences in hearing sensitivity. In

the CBA/CaJ strain he reported that at advanced age (~350 days) males show poorer high frequency hearing than females (on average 5-10 dB SPL poorer). In the C57 strain, Henry reported that males had better auditory sensitivity at all ages. Guimaraes et al., (2004) reported an auditory gender difference in CBA mice, only for the old age group, using a between group design and ANOVA. It has been suggested that the female hormone estrogen may have a direct influence cochlear function and this idea is supported by structural data from Stenberg, Wang, Sahlin, & Hulcrantz, (1999) that revealed widespread immunostaining of the estrogen receptors ERα and ERβ in cochlear structures in CBA mice. Estrogen is known to have widespread effects on physiology and this finding suggests that it may have a direct influence on cochlear structures. A study by Hulcrantz, Stenberg, Fransson, & Canlon (2000) using a mutant mouse, X,O "Turner mouse" which produces no estrogen, further supports the idea of estrogen influence on cochlear structure and function. They found a loss of OHC, primary afferent pathology, increased ABR thresholds, and decreased DPOAE amplitudes relative to controls. The role of female hormones on inner ear structure and function is poorly understood but there is growing evidence suggesting that female hormones may have a protective effect against ARHL.

Are there gender differences in vestibular function? A search of the literature reveals no reports of gender differences in vestibular structure or function.

Are there age related changes in vestibular function? Studies that have investigated the effect of age on vestibular function in humans have typically utilized indirect measures such as the vestibulo-ocular-reflex (VOR), optokinetic response (OKN), the

otolith-ocular reflex, visual-vestibular responses and tests of posture to make inferences about vestibular sensory function (Paige, 1992, 1994; Baloh, et al., 1993; Goebel, 2001; Enrietto, et al., 1999; Shiga et al., 2005; Furman & Redfern, 2001). In general, these studies have reported decreases in VOR gain, increased phase lead, decreased ability to suppress the VOR with vision, less foreshortening of the VOR time constant by post-rotary head tilt, and lower OKN slow-phase velocity saturation. Investigations by Jones et al. (2005, 2006) using direct measures of auditory and vestibular function in a variety of inbred mouse strains suggested that genetic background may play a role in age-related changes in the two sensory systems and that functional change in one sensory system does not obligate change in the other. Shiga et al., (2005) measured ABR and VOR and examined cochlear and semicircular canal sensory structures in C57BL/6J mice which carry *Ahl*, a cadherin23 mutation that affects cadherin expression in inner ear sensory structures and results in ARHL. They found degeneration of sensory structures in both the cochlea and semicircular canals but only found significant functional change in ABR. Their results could be explained by the fact that the VOR response is an indirect measure requiring peripheral input, central integration, and motor output. Using the VOR measure it is not possible to separate central and peripheral pathology or to calculate the effect of central compensation for peripheral deficits.

In the current study we posed the question: *"What is the effect of age alone on the inner ear sensory modalities?"* and used an inbred strain with no known mutations (CBA/CaJ) and a direct measure of vestibular function, the VsEP. We hypothesized: 1) changes in the macular function, due to age alone, are concurrent with age related

functional changes in the cochlea 2) changes in macular function, due to age alone, is the same for males and females.

Auditory and vestibular function (more specifically macular function) was characterized in 131 CBA/CaJ mice ranging in age from 1.7 to 23.8 months of age. ABR and DPOAE were used to assess auditory function and vestibular evoked potentials (VsEP) were used to assess macular function. Response parameters were compared between genders and across age. Temporal bones were harvested following functional studies and scanning electron microscopy (SEM) was used to qualitatively characterize macular otoconia.

Methods

Animals and Animal Preparation

Studies were conducted at East Carolina University. Use of animals in this work was approved by the Institutional Animal Care and Use Committee and in accordance with national and international regulations regarding the use of animals in research. Ambient noise levels in the animal housing area were monitored through out the study to assure that levels were below those which might result in noise induced hearing loss (See Appendix M). Sibling breeding pairs were obtained from The Jackson laboratory (Bar Harbor, ME). Animals were housed and maintained in standard husbandry until appropriate age for testing.

During testing mice were anesthetized with ketamine (120 to 126 mg/kg) and xylazine (10 to 14 mg/kg) injected intraperitoneally. Body core temperature was maintained at 37.0 ± 0.2°C using a homeothermic heating blanket and rectal

thermocouple (FHC, Inc.). Mice were excluded from the study if there was any indication of potential outer or middle ear dysfunction (i.e. redness or inflammation of the external ear canal or tympanic membrane (TM), TM perforation, visible fluid in the middle ear space, or excessive cerumen) as determined by examination with an operating microscope under anesthesia. Three mice were excluded due to a perforated TM. During ABR and VsEP testing, subcutaneous needle electrodes were placed just posterior to the lambdoidal suture (noninverting), behind the left pinna (inverting) and at the neck (ground). A noninvasive head clip was used to secure the head to a mechanical shaker for delivery of vestibular stimuli. Auditory stimuli for ABR and DPOAE were delivered via a probe assembly placed at the entrance of the external acoustic meatus. DPOAE, ABR and VsEP were performed in the same mouse, during the same test session, after which the mouse was euthanized. Temporal bones were harvested from mice representative of young, middle aged, and advanced age groups for imaging via scanning electron microscope. Stimuli for VsEP, ABR, and DPOAE are described in detail below.

Vestibular Evoked Potentials (VsEP)

VsEP stimulus and recording methods were similar to Jones et al., (2005). A linear jerk pulse was generated using a linear voltage ramp (2 ms duration) routed through a power amplifier, which drove a mechanical shaker (Labworks, Inc. Model E2-203). The applied voltage produced an acceleration ramp, which was measured by an accelerometer (Endevco, Inc., 10mV/g where g = 9.81 m/s^2). Output of the accelerometer was measured using an electronic differentiator; the output of which was monitored with an oscilloscope (Hewlett Packard 54602B). The linear jerk pulses were coupled to the

head through a custom platform mounted atop the shaker. Systematically increasing or decreasing the amplitude of the stimulus waveform controlled the amplitude of the stimulus applied to the head. Amplitude of the jerk stimuli was recorded as the mean peak jerk level (measured using an oscilloscope) and was expressed in dB relative to 1.0g/ms. Stimulus amplitude ranged from –18 to +6 dB (re: 1.0g/ms) and was adjusted in 3 dB steps. Jerk stimuli had two directions of motion: normal and inverted. Mice were placed in a supine position with nose up and stimuli were presented in the naso-occipital axis. Normal polarity began with upward movement (naso-occipital +X). Inverted stimulus polarity began with downward movement (naso-occipital -X). Responses were collected for both normal and inverted polarities and the resulting waveforms were averaged online to produce the final waveform for analysis.

Auditory Brainstem Response (ABR)

Tone burst stimuli were used to elicit ABR responses. Continuous pure tone stimuli (10 Vp-p voltage) were generated by a digital signal analyzer (Stanford Research Systems SR785) and shaped/controlled using custom software and Tucker Davis Technologies (TDT, Alachua, FL) modules (TG6, SW2, PA4). Tone bursts at 8, 16, 32, and 41.2 kHz had a 1.0 ms rise-fall times with 1.0 ms plateau (3 ms total duration). During ABR testing a probe assembly was inserted into the mouse ear canal and a calibrated ER10B+ microphone was used to measure sound level. The output from the ER10B+ was routed to a preamplifier and an oscilloscope and the acoustic peak-to-peak voltage was recorded for the maximum stimulus level (+6 dB). Peak equivalent SPL (pe SPL) was later calculated based on the microphone sensitivity as determined by

calibration in a custom 0.35 ml coupler. Based on these measures, the average maximum stimulus levels (at +6 dB) were 97, 90, 78, and 92 dB pe SPL for 8, 16, 32, and 41.2 kHz respectively. Stimuli were presented to the left ear via commercial OAE tips (Etymotic ER10D-TO5 5mm) and PE-25 tubing. Auditory stimuli were presented at a rate of 17 stimuli/s. Intensity series were collected with a descending series of stimulus intensities (6 or 12 dB steps). Stimulus levels were decreased until no response was visible and then dropped an additional 6 dB, in order to ensure that a sub-threshold level had been reached.

Distortion Product Otoacoustic Emissions (DPOAE)

DPOAEs provide an objective measure of cochlear function and are critically dependent on outer hair cell function (Brownell, 1990). Methods for DPOAE stimuli and recording were similar to Jimenez, et al., (1999) and Guimaraes et al., (2004). Stimuli for DPOAEs were generated and shaped using custom software and modules from TDT (TG6, PA4, and SW2). Pure tone frequencies (f1, f2, f2/f1 ratio = 1.25), at equal levels (L1 = L2 = 60 dB SPL), 150 ms duration, were generated with independent sources (HP Agilent 33220A signal generators) and routed through separate drivers to mix acoustically in the ear canal (via commercial OAE tips (Etymotic ER10D-TO5 5mm) and PE-25 tubing) placed securely at the external acoustic meatus. Stimulus frequencies for the primaries were such that the geometric mean ($GM = (f1 \times f2)^{0.5}$) frequencies ranged from 6.0 to 48.5 kHz (at least 8 frequencies per octave). During each study, ear canal sound pressure was recorded with a low noise probe microphone (Etymotic ER 10B+). The microphone output was amplified 10X (ER10B+ preamplifier) and input to a

dynamic signal analyzer (Stanford Research Systems SRS785) for sampling (at 200 kHz) and fast Fourier transform (FFT). The amplitude of f1, f2, and the 2f1-f2-distortion product was measured from the FFT waveform. The noise floor was measured as the average amplitude in the five frequency bins above and below the 2f1-f2 component. The recording system was tested periodically in the 0.35 ml coupler to confirm the absence of artifactual distortion.

ABR and DPOAE Microphone and Stimulus Calibration

Stimuli for ABR testing were calibrated using a Bruel & Kjaar (B & K) ¼" microphone (4954, Nærum, Denmark), Nexus amplifier (2690), custom software, and 0.35 ml custom coupler. During calibration the probe tube assembly (housing the ER10B+ microphone and exit ports for sound) was inserted into one end of the custom coupler (0.35 ml volume) and the B & K microphone was inserted into the other end. The output of the ER10B+ microphone was amplified (10X) and routed to one channel of the SRS785 signal processor. The output from the B & K microphone was routed to a second channel on the SRS785 signal analyzer. The custom software controlled stimulus generation and signal analysis for DPOAE stimuli. Two Tucker Davis SW2 modules were set for a rise fall time of 5 ms and 140 ms plateau, for a total duration of 150 ms. Each stimulus frequency was presented and the PA4 attenuation was adjusted until a level of 60 dB SPL was achieved for the B & K channel. The PA4 attenuation level and the ER10B+ output level from the other channel of the SRS785 signal analyzer were saved as a probe tube calibration file (PTC) and the ER10B+ microphone sensitivity was calculated for each frequency. This PTC file served two purposes. First, the PTC file was

loaded into the custom software and was used to calculate the target dB SPL for the ER10B+ microphone output. This assured that a level of 60 dB SPL was achieved for both primary tones presented during DPOAE testing. Secondly, during ABR testing the calibration values were used to determine the stimulus presentation level for the four stimulus frequencies (8, 16, 32, and 41.2 kHz) as measured in the ear canal. During ABR testing the probe tip was place at the mouse ear canal and the ER10B+ microphone measured the stimulus sound pressure level. The output from the microphone was routed to an oscilloscope and a peak-to-peak voltage was manually measured and recorded. Knowing the ER10B+ microphone sensitivity for each frequency allowed us to convert the measured peak-to-peak voltage into a peak equivalent dB SPL (dB pe SPL) for each ABR tone burst stimulus. The actual SPL varied from mouse to mouse (due to ear canal properties) but the microphone sensitivity determined during the calibration allowed us to calculate the actual SPL for each ABR stimulus frequency for each study.

Overview and Averaging

Measurements began with DPOAEs followed by ABR and VsEP. This order was chosen in an attempt to limit exposure to high sound pressure levels that could result in a temporary auditory threshold shift. Stimulus levels for DPOAE are limited to around 60 dB SPL compared to ABR maximum stimulus levels, which were as high as 100 dB pe SPL. An intense forward masker is presented during VsEPs that has an average intensity level as high as 97 dB SPL. VsEP were performed last in order to negate the possibility of a temporary auditory threshold shift from exposure to the intense masker during VsEP

testing. Test frequencies for ABR and DPOAE were tested in random order. In general, the three measures were completed in one mouse in less than two hours.

Single channel signal averaging was used to record VsEP and ABR response waveforms. Offline analysis was used to determine response thresholds, response peak latencies, and peak-to-peak amplitudes. Subcutaneous electrodes were placed as described previously. The electroencephalographic activity was amplified (200,000X, Grass P511, West Warwick, RI), band pass filtered (300 to 3000 Hz, -6 dB points, Grass P511) and digitized (10 μs per point for 1024 points, TDT AD1) beginning at stimulus onset (TDT TG6 triggers system). 256 samples were averaged to produce the final VsEP or ABR waveform. Stimuli for VsEPs were linear jerk pulses that physically moved the mouse's head. ABR stimuli were short duration tone bursts. ABR and VsEP threshold, peak latency, and peak to peak amplitude were quantified. Amplitude and latency measures for the first three peaks (P1, P2 and P3) of the response waveform were quantified. Threshold was defined as the intensity midway between the minimum stimulus intensity that produced a discernible response and the maximum stimulus intensity that did not result in a visible response. Response peak latency was defined as the time, in milliseconds, from onset of stimulus to the appearance of each positive peak (P1, P2 and P3). Peak-to-peak amplitude, (P1-N1, P2-N2, and P3-N3) measured in microvolts (μv), represented the difference between each positive peak and its respective negative peak.

Data Analysis

In order to compare cochlear and macular sensitivity it was necessary to normalize ABR and VsEP threshold values. We chose to normalize threshold values in terms of a percent change in the dynamic range. Dynamic range was defined as the range from the best threshold measured for the strain to the most intense stimulus level possible without causing a permanent threshold shift with a brief exposure. A pilot study was conducted to determine the level at which ABR stimuli may cause a threshold shift. The results of the pilot study suggested that levels greater than 100 dB pe SPL could result in a threshold shift with a ten minute exposure. Therefore 100 dB pe SPL was used as the upper limit for the dynamic range. The lower limit for auditory dynamic range was set by the best threshold obtained for each frequency (12, 13.1, 4.9, and 25.4 dB pe SPL for the test frequencies 8, 16, 32, and 41.2 kHz respectively). The lowest VsEP threshold measured was – 13.5 dB (re; 1 g/ms) and the maximum non-noxious stimulus presentation level was + 6 dB re: 1.0 g/ms. The resulting dynamic range was 19.5 dB. To normalize a given threshold (y) the following formula was used:

$$x = \frac{y - z}{k} * 100$$

where x = the normalized value in percent, y = absolute threshold to be normalized, z = the lowest threshold measured and k is the dynamic range.

For example, with an absolute threshold of -7.5 dB:

$$x = [(-7.5 - (-13.5) / 19.5] * 100$$

$$x = [6/19.5] * 100$$

$$x = 30.77\%$$

Auditory and macular function were compared between male and female mice using linear regression and a student's T test (Zar, 1984) was performed to determine if there was a significant difference in the linear regression slopes or intercepts for males and females. The rate of age related change in normalized ABR and VsEP thresholds was compared within strain using linear regression and a student's T-test. The mean DPOAE amplitude and noise floor were plotted for each age group as a function of geometric mean frequency but no statistical analysis was performed.

Structural Analysis

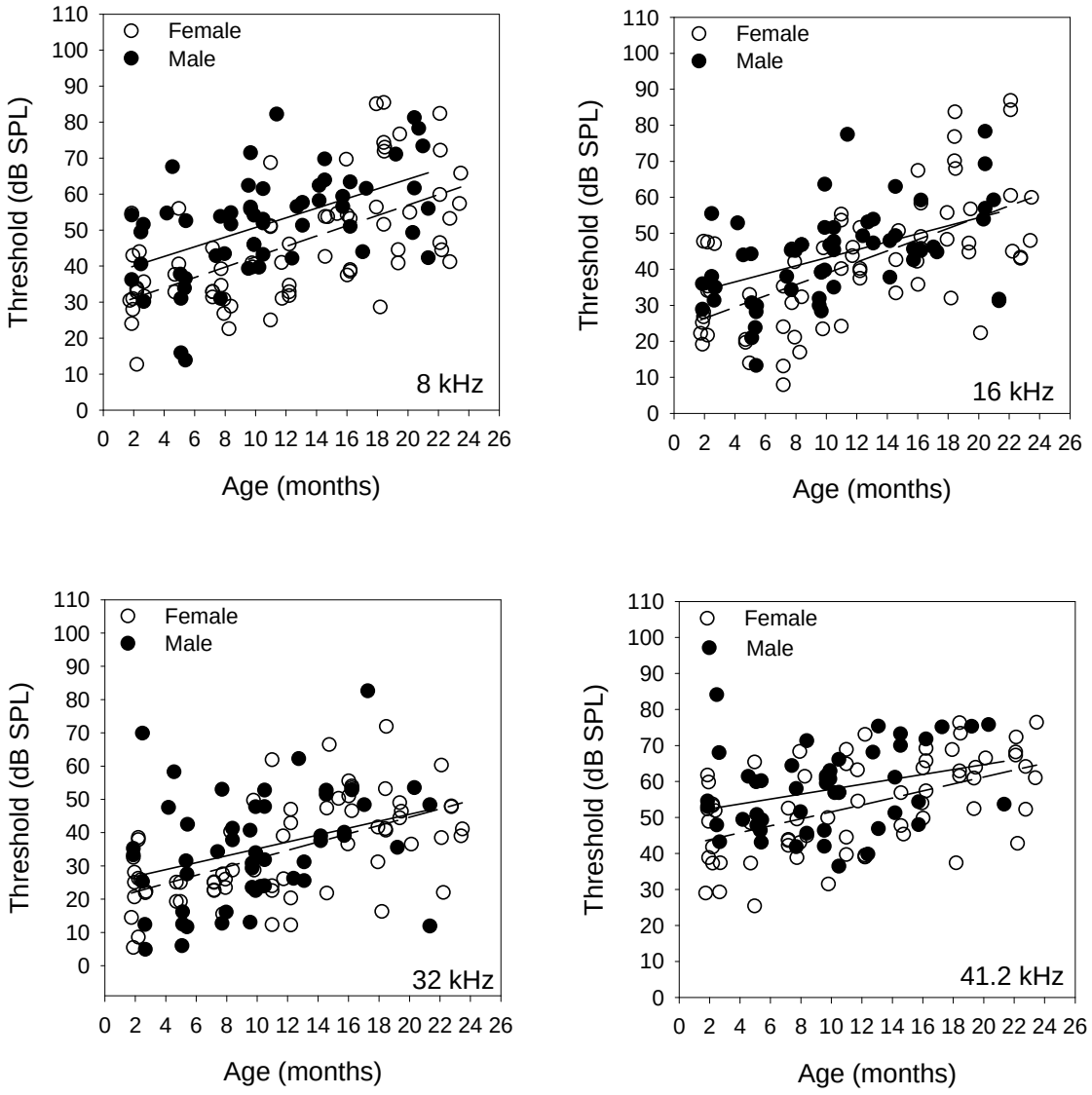
Temporal bones from young, middle aged, and old mice were dissected at the end of the functional experiments and fixed using 4% paraformaldehyde and 2% glutaraldehyde. Following 24 hrs in fixative the temporal bones were dehydrated using graded concentrations (70, 90, 95, and 100%) of ethyl alcohol. After a minimum of 24 hours in 100 % ethyl alcohol, the macular organs were micro-dissected from the temporal bone and mounted on aluminum stubs, critical point dried (Bal-Tec CPD 030, Liechtenstein), and sputter coated (Anatech LTD Hummer 6.6, San Diego, Ca.). Specimens were imaged with a scanning electron microscope (FEI Quanta 200, Hillsboro, Oregon).

Results

Auditory Results

No difference was found between female and male slopes or intercepts for ABR threshold at 8 kHz (Fig. 16) so data for the two genders were pooled for further analysis. The slope of the linear regression model for the difference between females and males

Figure 16. ABR thresholds female vs. males. ABR threshold is plotted as a function of age for the four ABR test frequencies. Linear regression analysis found no significant difference between the slopes or intercepts for males and females. Linear regression equations were y = 28.2 + 1.44x for females (the 95 % confidence interval for slope was 0.99 to 1.88) and y = 37.3 + 1.34x for males (the 95 % confidence interval for slope was 0.77 to 1.91).



is 1.03, meaning that the estimated average difference between genders is 1.03 dB per month. The 95 % confidence interval for the difference in the slope for female and males was –0.77 to 2.80. We would argue that an average difference in threshold of 1.03 dB per month between females and males does not represent a practical difference. ABR responses were generally present for all test frequencies for all age groups. ABR thresholds increased significantly as a function of age for the four ABR frequencies and increases averaged 1.35 (df (1,126), $F = 55.18$, $p<0.0001$, $R^2= 0.31$), 1.38 (df (1,122), $p< 0.0001$, $R^2= 0.31$), 1.15 (df (1, 117) $F = 31.16$, $p< 0.0001$, $R^2= 0.21$) , and 0.81 (df (1,114), $F = 22.9$, $p<0.0001$, $R^2= 0.16$) dB pe SPL per month for 8, 16, 32, and 41.2 kHz, respectively (Fig. 17, 18). This represents an average decrease of 25 to 35% in auditory dynamic range over the lifespan of CBA mice. P1 latency decreased an average of 0.009, 0.007, 0.005, and 0.004 ms per month for 8, 16, 32, and 41.2 kHz, respectively (Fig. 19). P1-N1 amplitudes decreased an average of 0.06, 0.02, 0.02, and 0.004 μV per month for 8, 16, 32, and 41.2 kHz respectively (Fig. 20).

DPOAE amplitudes decreased an average of 30% with age but even at the oldest ages tested remained within the 95% population range for the youngest group (Fig. 21). DPOAE results indicating decreased cochlear outer hair cell function and are in agreement with ABR findings of decreased auditory sensitivity. Table 4 lists the number of data points attained for each measure.

Macular Results

VsEP thresholds increased as a function of age but no significant difference was found for the slope or intercept for females and males (Fig. 22). Female and male datum was

Figure 17. ABR threshold and % responding. ABR thresholds increased significantly as a function of age for the four ABR frequencies and increases averaged 1.35 (df (1,126), F = 55.18, $p<0.0001$, $R^2= 0.31$), 1.38 (df (1,122), $p< 0.0001$, $R^2= 0.31$), 1.15 (df (1, 117) F = 31.16, $p< 0.0001$, $R^2= 0.21$) , and 0.81 (df (1,114), F = 22.9, $p<0.0001$, $R^2= 0.16$) dB pe SPL per month for 8, 16, 32, and 41.2 kHz, respectively.

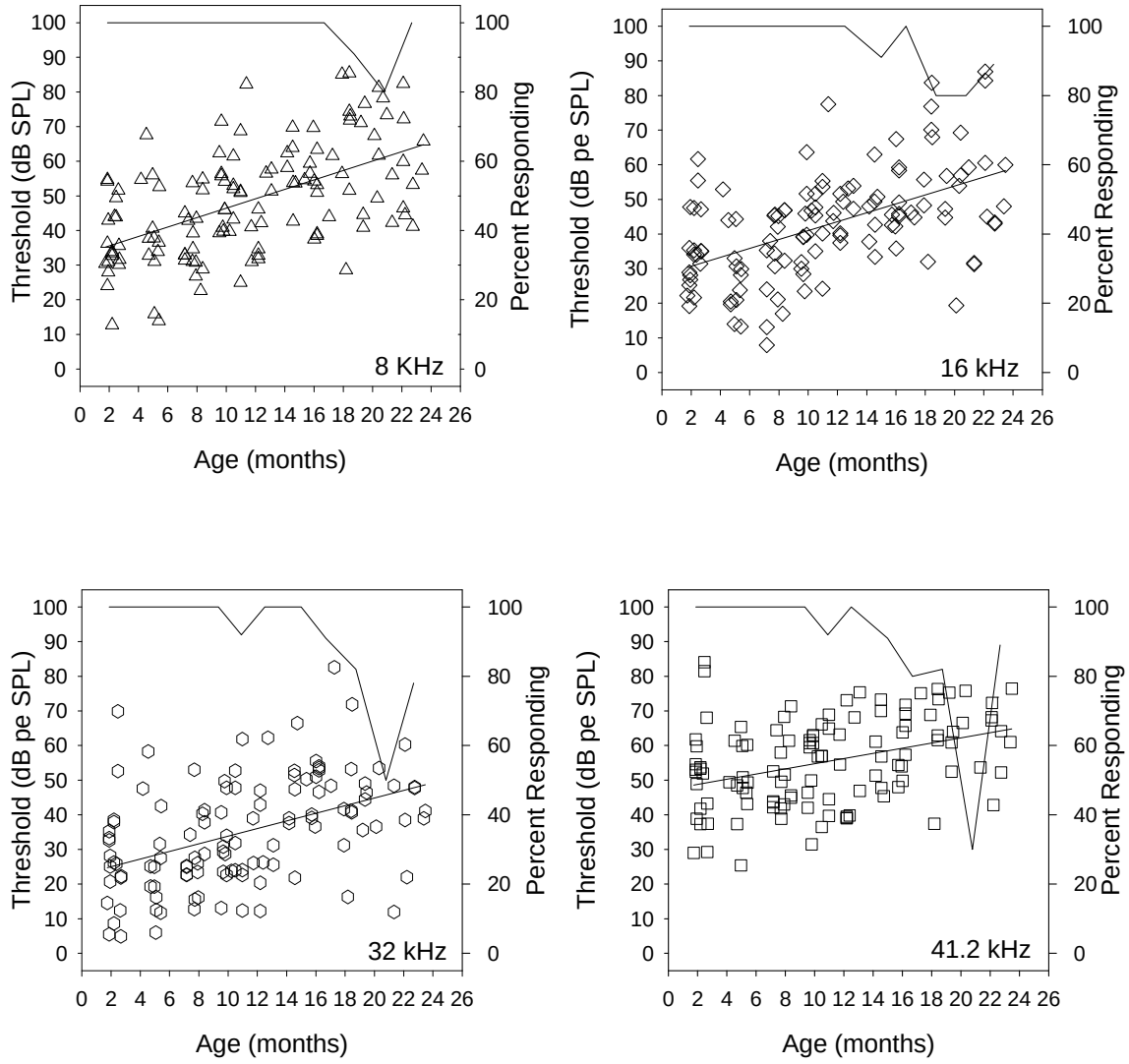


Figure 18. Audiogram. Average ABR thresholds plotted against ABR stimuli frequency for the data grouped into 12 age groups. Mean ages are listed at the right.

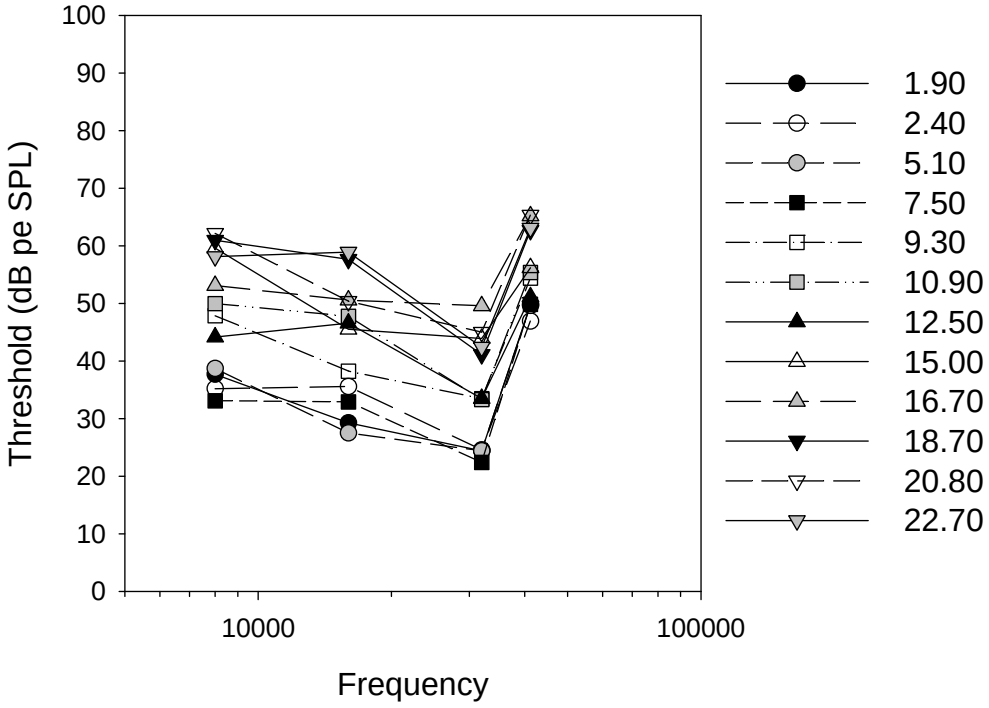


Figure 19. ABR latency. ABR P1(○), P2 (□), and P3 (Δ) latency as a function of age. P1 latency decreased an average of 0.009, 0.007, 0.005, and 0.004 ms per month for 8, 16, 32, and 41.2 kHz, respectively. 8 kHz P1 latency linear regression equations were y = 0.99 + 0.009x, y = 0.84 + 0.007x, y = 0.73 + 0.005x, and y = 0.7 + 0.004 for 8, 16, 32, and 41.2 kHz respectively.

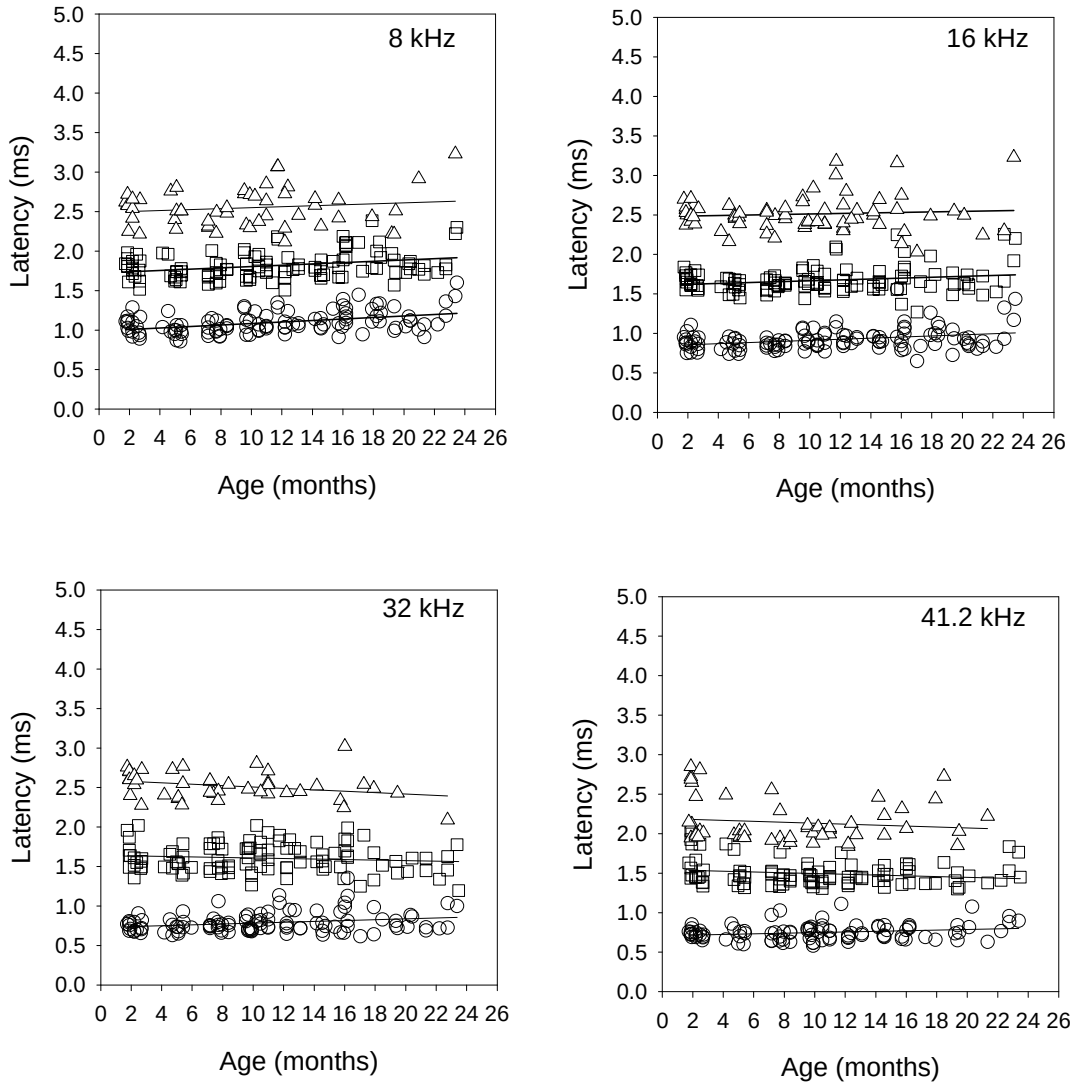


Figure 20. ABR amplitude. P1-N1 amplitude at maximum stimulus levels as a function of age. P1-N1 amplitudes decreased an average of 0.015, 0.023, 0.017, and 0.004 µV per month for 8, 16, 32, and 41.2 kHz respectively. Linear regression equations were y = 0.6 – 0.015x, y = 0.78 – 0.023x, y = 0.62 – 0.017x, and y = 0.35 – .004x for 8, 16, 32, and 41.2 kHz.

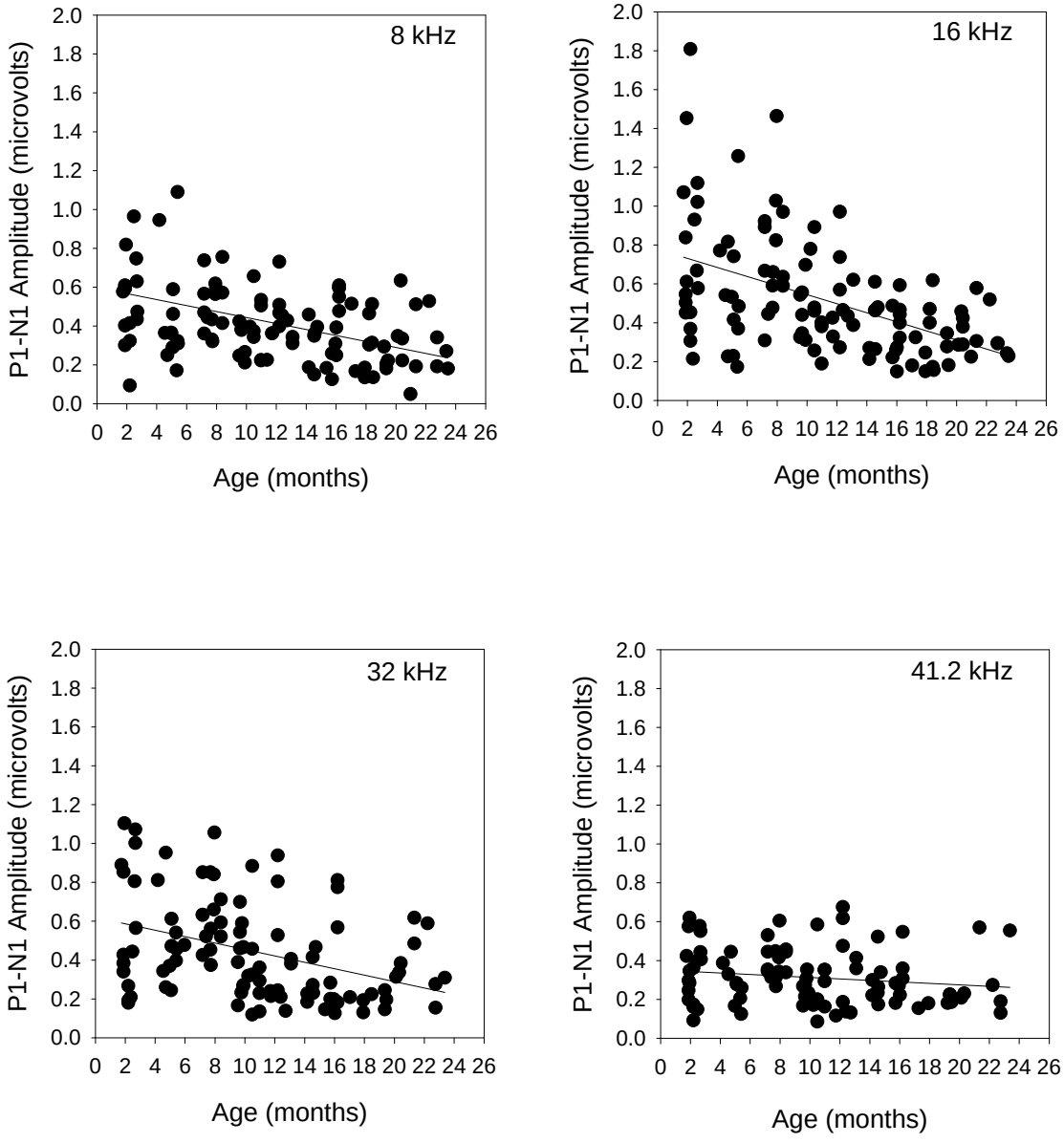


Figure 21. DPOAE amplitudes. Average DPOAE amplitude as a function of geometric mean frequency. The dashed line represents the 95% population range for the 2 to 3 month age group.

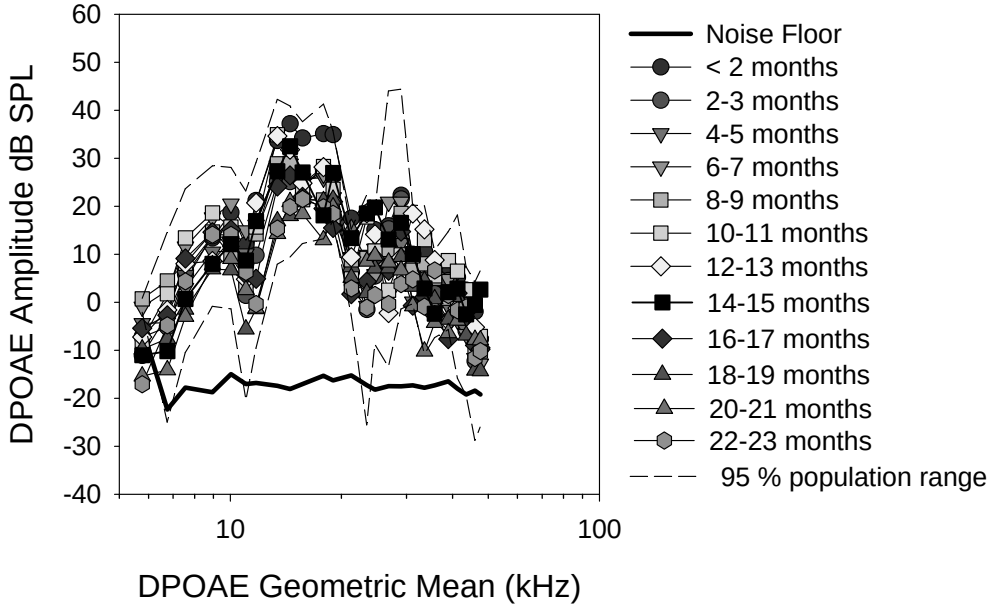
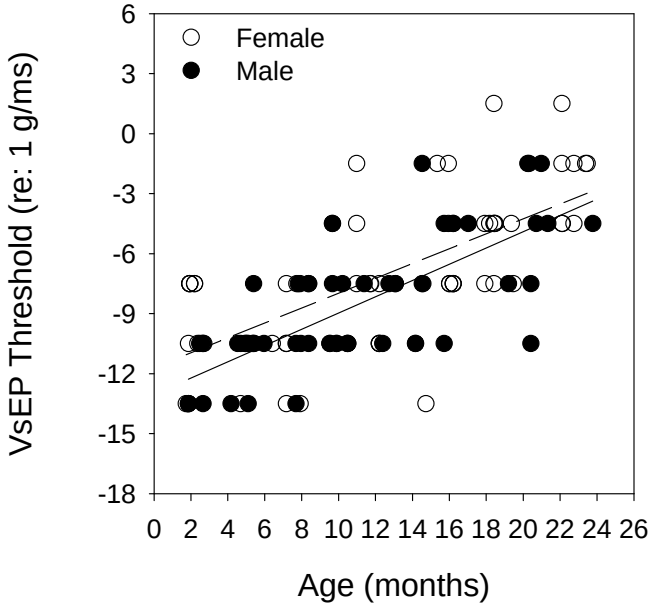


Table 4. *Number of data points for each measure. The table indicates the number of mice tested for each measure and includes cases where the measurement was performed but there was no measurable response (no response).*

	8 kHz	16 kHz	32 kHz	41.2 kHz	VsEP	DPOAE
Male	58	59	59	58	56	56
Female	71	71	72	72	62	65
Total	129	130	131	130	118	121

Figure 22. VsEP Gender. Female and male VsEP threshold as a function of age. Linear regression equations were y = -11.7 + 0.37x (the 95 % confidence interval for slope was 0.27 to 0.47) and y = -13 + 0.41x (the 95 % confidence interval for slope was 0.30 to 0.52) for females and males, respectively. No significant difference was found between females and males for the slope or intercept of the linear regression line.



pooled for further analysis. VsEP responses were generally present for all age groups (Fig. 23, 24). VsEP thresholds increased significantly as a function of age (df (1,115), F = 112.1, $p<0.0001$, $R^2= 0.49$) by an average of 0.39 dB (re: 1.0 g/ms) per month and showed an average loss of 48 % of dynamic range at the oldest ages. VsEP P1 latency showed small, yet significant increases (df (1,106), F = 25.47, $p<0.0001$, $R^2= 0.19$) (Fig. 25). P1-N1 amplitude decreased significantly (df (1,106), F = 25.45, $p<0.0001$, $R^2= 0.19$), by an average of 0.02 μv per month as a function of age (Fig. 26).

In regard to otoconia, we found no qualitative difference in macular otoconia between young and old mice that would explain the observed decline in macular function. Scanning electron microscopy images of macular otoconia showed dense populations of otoconia in the utricle and saccule at advanced age, with similar morphology to that seen at younger ages (Fig. 27).

VsEP and ABR thresholds both increased significantly as a function of age. A comparison of the rate of change (linear regression slopes of normalized thresholds) between the two measures, by student's t-test, revealed a significant difference ($p < 0.001$) (Fig. 28). Macular sensitivity declined faster than auditory sensitivity for all frequencies. In spite the statistical significance, it is apparent from the plot that the loss of sensitivity occurred a similar rate in the two modalities. This finding suggests that while there are subtle differences, overall the rate of age related change in auditory and macular sensitivity are similar in the CBA strain.

Figure 23. Representative VsEP waveforms. Representative VsEP waveforms for + 6 dB re: 1.0 g/ms for mice at 5 selected ages.

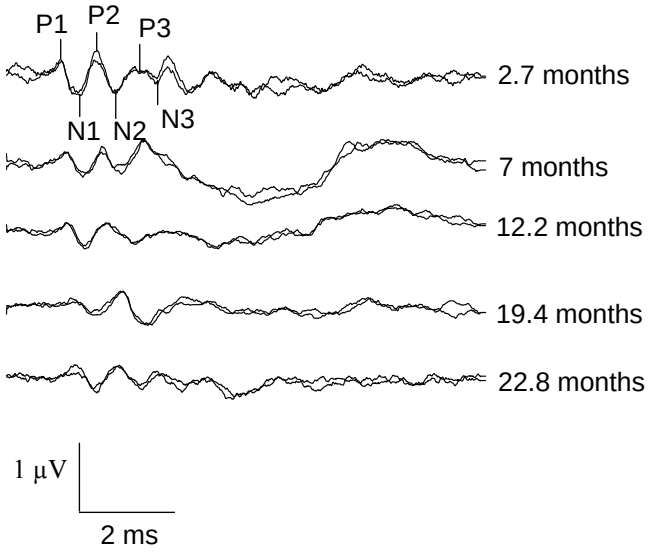


Figure 24. VsEP threshold. Pooled male and female VsEP threshold. VsEP thresholds increased significantly as a function of age (df (1,115), F = 112.1, $p<0.0001$, $R^2= 0.49$) by an average of 0.39 dB (re: 1.0 g/ms) per month and showed an average loss of 48 % of dynamic range at the oldest ages. The linear regression equation was y = -12.4 + 0.39X. Of 119 animals tested, two had no response (one at 18.4 months and a second at 20.4 months).

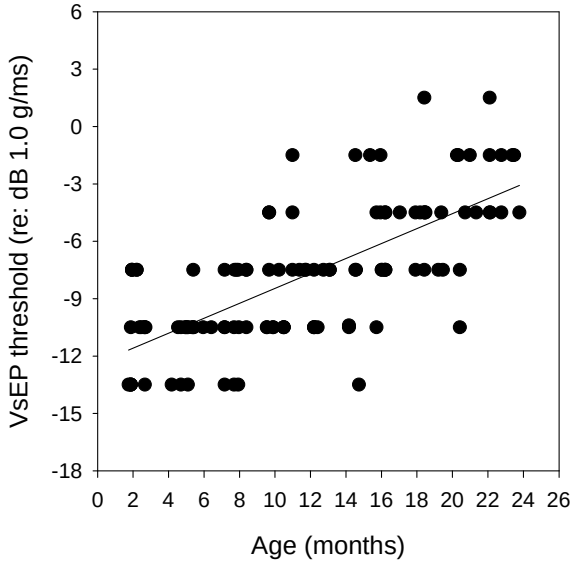


Figure 25. VsEP latency. VsEP P1 (○), P2 (□), and P3 (Δ) latency as a function of age. VsEP P1 latency showed small, yet significant increases (df (1,106), F = 25.47, $p<0.0001$, $R^2= 0.19$). P2 and P3 increased by an average of 0.01 and 0.01 ms per month. Linear regression equations were y = 1.3 + 0.01x, y = 2.13 + 0.01x, and y = 3.07 + 0.01x for P1, P2, and P3 respectively.

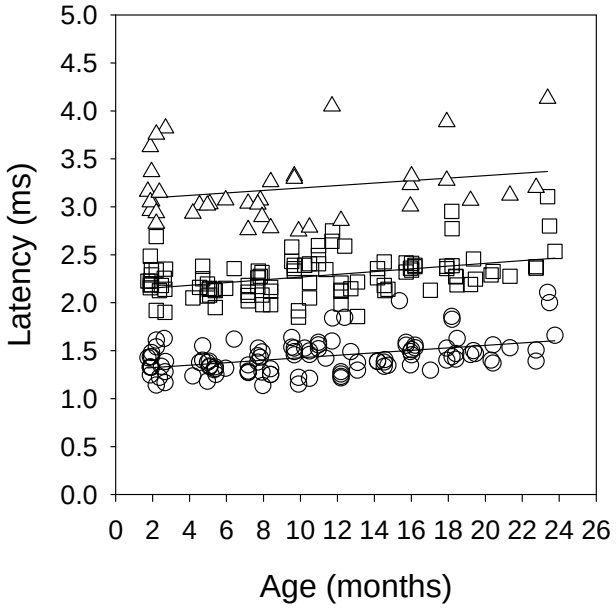


Figure 26. VsEP amplitude. VsEP P1-N1 amplitude decreased significantly as a function of age (df (1,106), F = 25.45, $p<0.0001$, $R^2= 0.19$), by an average of 0.02 μv per month as a function of age. The linear regression equation was $y = 0.77 – 0.02x$.

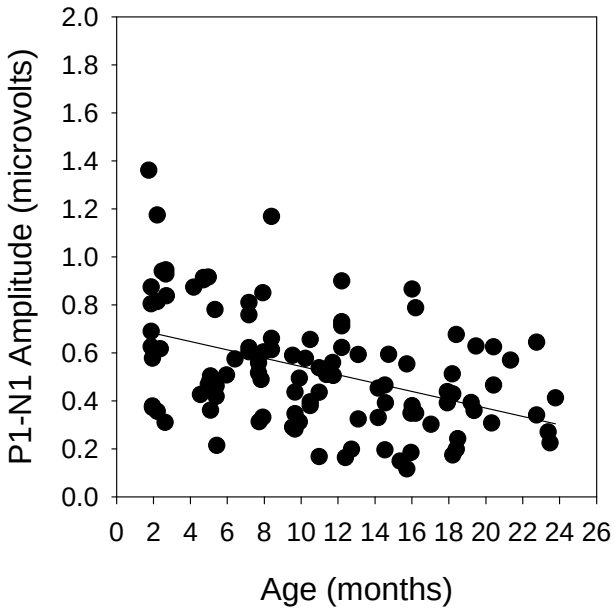
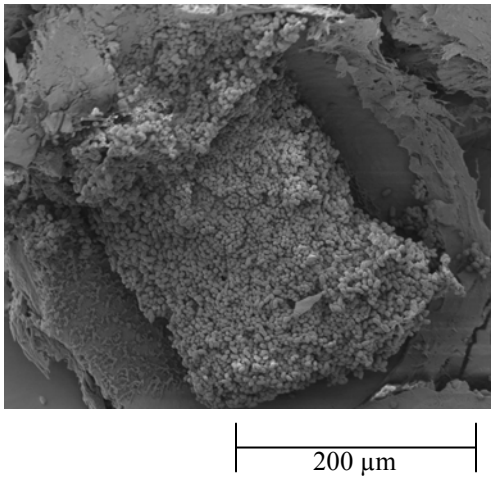
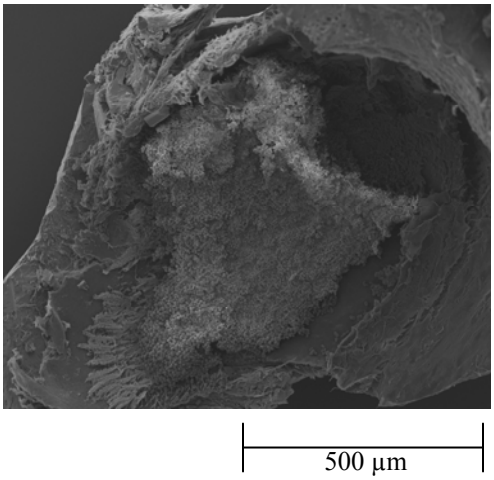


Figure 27. SEM Images**.** Scanning electron microscopy images of the utricle and saccule from 18 month old mice (top two images) and 12.2 month old mice (bottom two images).

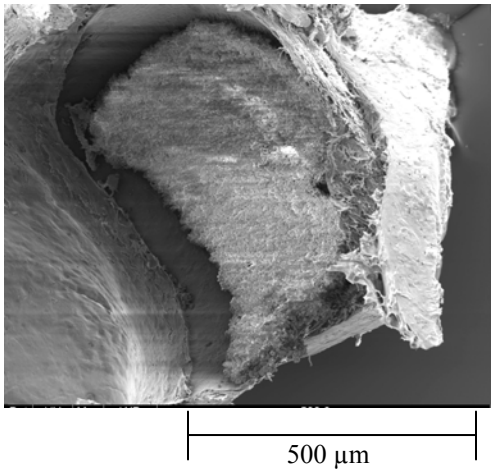
Saccule



Utricle



Saccule



Utricle

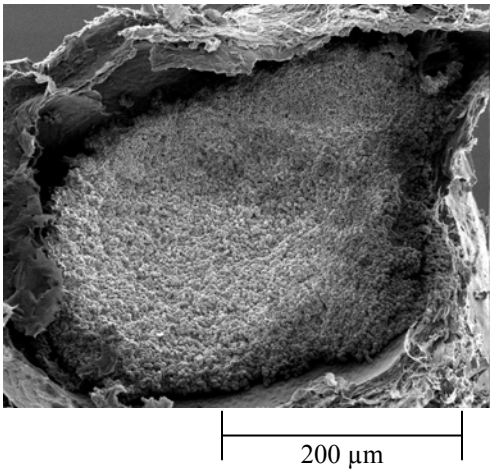
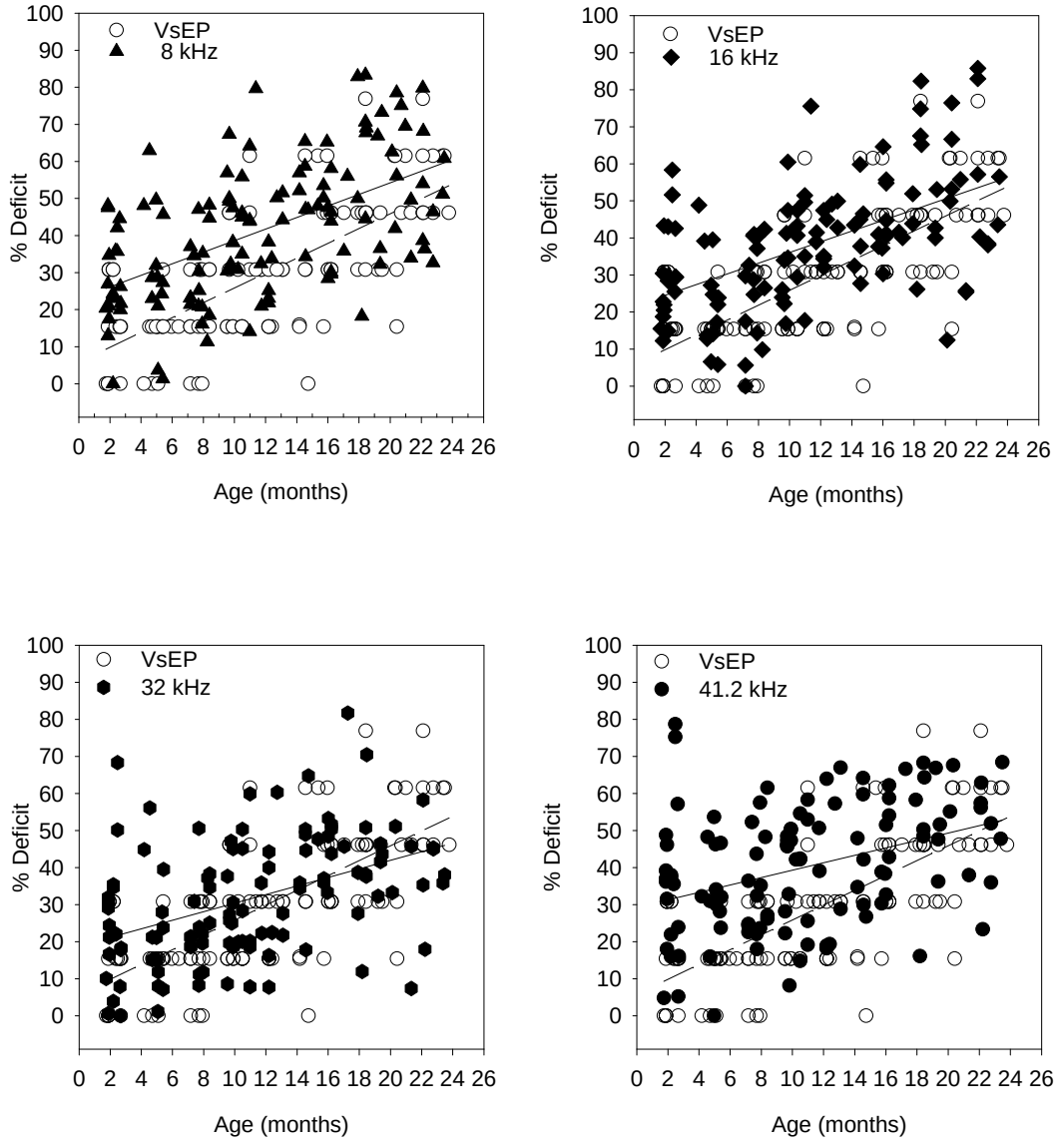


Figure 28. Auditory and macular comparison. Normalized VsEP and ABR thresholds plotted as a function of age. The average monthly decline in dynamic range was 2.17 % for VsEP (dashed line) and 1.77, 1.58, 1.23, and 1.08 % for 8, 16, 32, 41.2 kHz (solid lines), respectively. There was significant difference in the rate of change in VsEP and 8 kHz thresholds ($p<0.0001$). The 95% confidence interval for VsEP percent deficit slope was 1.76 to 2.59 and for 8 kHz slope was 1.32 to 2.22.



Discussion

The CBA/CaJ strain is widely used to investigate the environmental and genetic causes of hearing and balance disorders. It serves as the background strain for development of many mutant inbred strains that facilitate the study of the genetic origins of syndromic and non-syndromic auditory and vestibular disorders. It is important that the hearing and balance function be well characterized over the entire lifespan of this important inbred strain. The present study's auditory findings are in agreement with previous studies showing a gradual decline in auditory sensitivity and outer hair cell function. Vestibular results also show a slow progressive loss of macular sensitivity that has a significantly different rate of change than the observed auditory decline. Auditory function declined faster than macular function for three of the four ABR test frequencies. The current auditory findings are in agreement with previous studies that show increasing ABR thresholds beginning in mid-life, and reduced DPOAE amplitudes as a function of age; however we did not find any significant gender differences in auditory function as reported by others.

This study provides unique knowledge concerning the effect of age on gravity receptor function and how it relates to cochlear functional change with age. The results indicate a gradual decline in both modalities that follow different time courses. ABR results (increased threshold, small increases in latency, and small decreases in amplitude) along with DPOAE findings (reduced response amplitudes as a function of age) suggest the cochlea, and more specifically cochlear outer hair cells, as the source of declining auditory function. These findings are in agreement with reports of progressive age-related

decreases in inner and outer hair densities (Spongr et al., 1997) and loss of spiral ganglion fibers (Ohlemiller & Gagnon, 2004).

VsEP results (decreased sensitivity, small increases in latency, and decreases in amplitude) reveal a gradual decline in macular sensitivity that parallels the decrease in auditory sensitivity. Age related changes in vestibular sensory organs (pathology and loss of type I and II hair cells, pathology and loss of stereocilia/kinocilia, reduced blood flow, changes in the globular substance and otoconia, and increased presence of lipofuscin) are reported in humans and animal models (rats, guinea pigs, monkeys and C57 mice). Gravity receptor structural studies in the CBA/CaJ strain however, are not reported in the literature. Future morphological studies concerning age related changes in the macular organs of CBA/CaJ mice may identify the source of age related decline in gravity receptor sensitivity.

Could age related change in the number, distribution, or density of macular otoconia be responsible for the observed loss of macular sensitivity? There are reports in the literature of age-related morphological changes in macular otoconia (Jang, Hwang, Shin, Bae, & Kim, 2006; Campos, Canizares, Sanchez-Quevedo, & Romero, 1990) and the globular substance (Suzuki et al., 1997), which is involved in otoconia formation. It is conceivable that pathological changes in otoconia turnover/production could lead to otoconia with reduced density, resulting in increased VsEP thresholds. Scanning electron microscopy may not reveal this hypothetical change in otoconia density. The scanning electron microscopy images of macular otoconia in this study show no qualitative difference between the macular otoconia of young and old mice that would explain the

loss of macular sensitivity. The finding of reduced sensitivity of both the cochlea and macular organs suggests that there are global changes in the peripheral membranous labyrinth of CBA mice that affect both modalities. What broad acting structural changes might explain the observed functional decline of the cochlea and macular organs?

Age related structural changes in the cochlea and vestibular organs have been studied in depth in humans and a variety of animal models and are generally placed into several conceptual categories; 1) neural, involving primarily a loss of afferent neurons 2) metabolic, involving blood supply and non-sensory structures (stria vascularis, spiral lamina, vestibular dark cells, etc.) responsible for the endolymphatic potential 3) sensory, involving hair cells, stereocilia/kinocilia, or sensory supporting cells (Schuknecht & Gacek, 1993). Schuknecht's human temporal bone studies have shown that these pathologies may exist independently and concurrently. Studies focusing on the effect of age on cochlear and vestibular sensory structures have identified a myriad of pathologies including: loss of inner and outer hair cells in the cochlea, loss of type I and type II vestibular hair cells, missing/disarranged/fused stereocilia and kinocilia, hair cell cytoplasmic protrusions, giant hair cells, degeneration of structures responsible for the endolymphatic potential such as the stria vascularis and spiral ligament, increased fragility of the cuticular plate, decreases in otoconia turnover, loss of primary afferent fibers, and the accumulation of a "wear and tear" pigment known as lipofuscin.

One global change that could potentially affect cochlear and vestibular function involves the unique extracellular fluid known as endolymph that fills the membranous labyrinth. Endolymph has a high concentration of potassium, which results in a relatively

strong positive charge relative to the sensory hair cells of the cochlea and vestibular organs. This electrical gradient is known as the endolymphatic potential (EP) and both sensory systems are dependent upon this electrical charge for sensory transduction. It is possible that pathological changes in the EP or the structures that maintain the EP (stria vascularis and spiral ligament in the cochlea and dark cells in the vestibular organs) could effect both cochlear and vestibular function. Studies that have focused on the endolymphatic potential (EP) and the cochlear structures responsible for the ion pumping necessary to maintain the EP (Gratton & Schulte, 1995; Gratton, Schmiedt, & Schulte, 1996; Gratton & Schulte, 2003; Hequembourg & Liberman, 2001; Lang, Schulte, & Schmiedt, 2003; Ohlemiller, Lett & Gagnon, 2006) have identified degenerative changes in the stria vascularis and spiral ligament which are believed to maintain the EP in the cochlea. A search of the literature fails to find investigations on the effect of age on vestibular dark cells, which are responsible for ion pumping in the vestibular labyrinth. These studies are in agreement that there are age-related degenerative changes in the stria vascularis and spiral ligament; however the results concerning the EP itself are mixed. The EP is inherently difficult to measure and varies by measurement site, species, and genetic strain and the findings concerning the EP itself vary depending on the animal model and genetic strain utilized; e.g. gerbils and BALB/cJ mice show a reduced EP as function of age (Gratton et al., 1996) while the C57 and CBA/CaJ mouse strains show no change or limited change in the EP. (Lang et al., 2002; Ohlemiller et al. 2006; Wu & Marcus, 2003, Sha et al., 2008). The finding that the EP does not decrease with age in the CBA strain makes it unlikely that this is a source of decreased auditory and macular

sensitivity seen with aging. The ion pumping that occurs in the cochlear and vestibular structures is a “global” metabolic process that takes place in the membranous labyrinth. Other metabolic processes occur at the cellular level and degenerative changes in these cellular processes are another possible explanation for age related decreases in function.

Pathology of the cochlear hair cells (primarily OHC) and their accompanying stereocilia are widely accepted as a primary cause of age related hearing loss and loss of spiral ganglion fibers is generally considered to be secondary to the loss of hair cells. However, the specific mechanisms behind hair cell degeneration and neuronal loss are not known. One possible explanation is the membrane hypothesis of aging, also known as the mitochondrial clock theory. This theory proposes that reduced vascular supply, an increase in reactive oxygen metabolites (ROM) and accumulation of mitochondrial deletions result in apoptosis of sensory structures in the inner ear. Cochlear and vestibular hair cells do not regenerate in humans therefore the maintenance and repair of these sensory structures is critical for maintaining cochlear and vestibular function with advancing age. Vascular changes in the cochlea and vestibular organs, including decreased vascular plasticity and permeability (Prazma, Carrasco, Butler, Waters, Anderson, & Pillsbury, 1990; Seidman, et al., 1996; Lyon & Wanamaker, 1993; Gratton et al, 1996), decreased capillary diameter and blood flow (Lyon & Davis, 2002) result in reduced oxygen and nutrient delivery and elimination of cellular metabolic by products (Rosenhall & Rubin, 1986; McFadden, Ding, & Salvi, 2001) and an increase in ROM. ROM are toxic molecules that can damage tissue and have been shown to damage mitochondrial DNA (mtDNA) and result in specific mtDNA deletions (mtDNA del 4977

in humans and mtDNA del 4834 in rats). These mtDNA mutations are directly proportional to aging (Liu, Kong, & Liu, 2003; Markaryan, Nelson, & Hinojosa, 2008) and as these mutations accumulate with advancing age, the mitochondrial metabolic processes become progressively less efficient. One common finding in histological studies of the effect of age on cochlear and vestibular structures is the presence of lipofuscin (Anniko, 1983; Bohne, Gruner, & Harding, 1990; Igarashi & Ishii, 1990; Li & Hulcrantz, 1994; Gleeson, et al., 1990; Rosenhall & Rubin, 1975; Park, Hubel, & Woods, 1987), a wear-and-tear pigment that is a by product of cellular metabolic processes. The lipofuscin pigment results from peroxidation and may have negative affects on cellular function (Jung, Bader, & Grune, 2007). Its presence indicates decreased efficiency of cellular metabolic processes which may be indicative of membrane damage or mitochondrial/lysomal damage. Mitochondria are a critical component of cellular energy production and an age-related increased in mitochondrial deletions, and resulting decrease in cellular metabolic efficiency, is one possible explanation for age-related apoptosis of cochlear and vestibular sensory structures.

In summary, the current study found that CBA mice maintain macular function until late in life but demonstrate a gradual progressive decline in macular sensitivity that is slightly accelerated relative to the time course observed for declines in auditory sensitivity. We found no significant gender differences in auditory or macular function and qualitative changes in macular otoconia were ruled out as possible explanations for decreased macular sensitivity. Decreased vascular supply, decreased metabolic efficiency, increased ROM production, increased numbers of mtDNA deletions, and

eventual apoptosis of cochlear and vestibular hair cells and afferent neurons is one possible explanation for the observed reduction in auditory and vestibular sensitivity. The observed decline in macular sensitivity should be considered when using the CBA strain for vestibular studies.

CHAPTER IV: AGE EFFECTS ON GRAVITY RECEPTOR FUNCTION IN TWO MOUSE STRAINS WITH *Ahl* MUTATIONS

Abstract

The C57BL/6J (C57) and CE/J strains both carry a Cadherin23 (Cdh23) mutation (*Ahl*), which affects inner ear auditory structures (Bolz, von Brederlow, & Ramirez, 2001; Di Palma et al., 2001) and results in age related hearing loss (ARHL) (Johnson, et al., 1997). The effect of *Ahl* on vestibular structures and function is not well understood. The purpose of the present study was to characterize the effect of age and gender on macular function in the C57 and CE/J strains and to compare functional change between auditory and vestibular modalities and genders. Vestibular Evoked Potentials (VsEP), Auditory Brainstem Response (ABR), and Distortion Product Otoacoustic Emissions (DPOAE) were measured in 148 C57BL/6J mice, ages 1.01 to 23.8 months, and 96 CE/J mice, ages 1.0 to 20.6 months. ABR and DPOAE were used to assess cochlear function and VsEP testing was used to assess macular function. Thresholds, peak latencies, and peak-to-peak amplitudes were quantified for VsEP and ABR responses. DPOAE amplitude was plotted as a function of geometric mean frequency and age. The macular organs from young, mid-life, and old animals were harvested, prepared, and imaged using scanning electron microscopy and a qualitative comparison of macular otoconia was made.

VsEP and ABR thresholds increased significantly ($p<0.0001$) as a function of age in both strains. No significant gender difference was found for ABR or VsEP threshold linear regression slopes or intercepts for either strain. Average decreases of 0.12 dB re:

1.0 g/ms and 0.47 dB re: 1.0 g/ms per month were seen in VsEP thresholds for the C57 and CE/J strains, respectively. The rate of change in macular sensitivity was significantly different ($p<0.0001$) between the two strains. Average total loss of dynamic range for VsEP at the oldest ages was 21 % for C57 (22-23 months of age) and 56 % for CE/J (20-21 months of age). No loss of otoconia was observed that could account for the loss of macular sensitivity. ABR thresholds decreased an average of 2.75, 2.76, 4.14, and 1.37 dB pe SPL per month for the C57 strain and 2.07, 2.69, 0.97, 0.41 dB pe SPL per month for the CE/J strain for 8, 16, 32, and 41.2 kHz, respectively. The average decrease in auditory dynamic range for the oldest age groups was 100% for both strains. DPOAE amplitudes were initially more robust in the C57 strain than the CE/J strain and declined as a function of age in both strains in agreement with ABR results.

A comparison of VsEP and 8 kHz ABR linear regression slopes for normalized thresholds shows that the rate of decline of auditory and macular sensitivity is significantly different ($p< 0.001$) in both strains. This trend was more pronounced in the C57 strain.

In summary, our results show; 1) no gender differences for auditory or macular function 2) OHC dysfunction (and hearing loss) begins earlier in the CE/J strain than the C57 strain 3) macular function decreases at a faster rate in the CE/J strain than in the C57 strain 4) auditory function declines at a faster rate than macular function in both strains (this finding is more pronounced in the C57 strain). 5) aging of one inner ear sensory system does not predict aging of the other inner ear sensory system.

The minimal loss of macular sensitivity in the C57 strain suggests that this strain may harbor an unknown mutation that serves to inhibit degeneration of macular structures and preserves macular sensitivity at advanced age.

Introduction

The inner ear houses the organs for hearing and balance. The organ of Corti in the cochlea transduces sound while the maculae in the vestibule and the cristae in the semicircular canals transduce linear and angular motion of the head, respectively. Deficits in these sensory systems or their neural pathways can lead to hearing impairment and/or balance disorders. Age related hearing loss (ARHL) is the most common type of hearing impairment in humans affecting 50% of the population by age 80 (Gorlin, et al., 1995; Morton, 1991). While many studies have examined the prevalence and impact of presbycusis on society (e.g., decreased potential earnings, increased social isolation, and cost of treatment) (Mulrow et al. 1990; Lutman, 1990; Hinchcliffe, 1990; Stach, et al., 1990; Gates, & Cooper, 1991; Mohr, et al., 2000; Strawbridge, et al., 2000) relatively little has been done to investigate the effects of vestibular dysfunction on society or public health. Studies by the National Institute on Deafness and other Communication Disorders estimated that 3.4 % of the U.S. adult population (6.2 million people) suffers from chronic dizziness and/or imbalance (Hoffman & Sklare, 2003) and an estimated 7 million people a year seek care for disequilibrium and/or vertigo. Vestibular end organs make an important contribution to balance and their degeneration due to aging may be an important factor in the increased incidence of falls in the elderly. Functional changes in the auditory and vestibular systems due to aging are influenced by factors such as noise exposure, exposure to ototoxins, ear disease (e.g., see review by Gratton & Vazquez, 2003), the central auditory system (Willott, et al., 2001), lifestyle (diet, exercise, etc) (Willott et al., 2001),

genetic make up (Johnson, et al., 1997; Li, 1992; Fransen, et al., 2003), and gender (Guimaraes, et al., 2004; Henry, 2004).

Gender differences in auditory function, including ARHL, are reported in animal models as well as humans and include reports that young women generally have better hearing than young men (Corso, 1963; Rosen, et al., 1964; Northern, et al., 1972; Surjan, 1973), ARHL progresses faster in men than women (Corso, 1963; Pederson, et al., 1989; Wallhagen, et al., 1997; McFadden & Pasanen, 1998) and that female mice lose hearing earlier than males in mouse models of early onset ARHL (Henry, 2004). Guimaraes, et al. (2004), measured both ABR and DPOAEs in CBA mice, and found that female mice have lower thresholds and maintain DPOAE amplitudes at advanced age compared to males. This suggests that female hormones may have a protective effect against ARHL. Mock, Jones, & Jones (unpublished) however, found no gender differences in 131 CBA/CaJ mice ranging in age from 1.7 to 23.8 months. Mock, Jones, & Jones gender analysis was performed on ABR threshold only using linear regression and student's T-test in contrast to Guimaraes et al., who analyzed ABR and DPOAE, separated subjects into arbitrary age groups, and utilized ANOVA.

Genetic studies have identified a large and ever growing number of mutations that contribute to both syndromic and non-syndromic hearing loss and have revealed the heterogeneous nature of hearing loss. To date eight mutations that contribute to ARHL have been identified. Johnson et al. (1997) were the first to identify a specific mutation (*Ahl*) that results in ARHL in mice. The *Ahl* mutation is a recessive, single nucleotide mutation at *753 (A/G)* on the *Cdh23* gene on Chromosome 10 in the C57BL/6J strain.

This mutation occurs in approximately 80% of inbred strains with hearing loss (Drayton and Noben-Trauth, 2006) and is referred to as *Cdh23*^{753A} (Noben-Trauth, Zheng, & Johnson, 2003). *Cdh23* is a gene that encodes cadherin, a protein necessary for inner ear development and maintenance of sensory cell structures such as stereociliary tip links (Siemens et al., 2004; Sollner et al., 2004) and transient lateral links (Michel et al., 2005; Lagziel et al., 2005). Mutations in *Cdh23* are associated with syndromic (Usher syndrome type 1D) and non-syndromic hearing loss (DFNB12) and have been shown to result in deafness and vestibular disorders in humans and mice (Bolz et al., 2001, Jones et al., 2004, 2005). Cochlear and vestibular cytoarchitectural abnormalities reported in strains that carry the *Ahl* mutation include: loss of inner and outer cochlear hair cells, loss of spiral ganglion cells, degeneration of fibrocytes in the spiral ligament, disorganized stereociliary bundles, misplaced kinocilia, and disruption of stereociliary tip links (Di Palma et al,. 2001; Alagramam et al., 2001; Holme & Steel, 2002; Raphael, Kobayashi, & Dootz, 2001; Shiga et al., 2005).

A second mutation, named *Ahl2,* was subsequently mapped to Chromosome 5 using backcross and linkage analysis in the NOD/LtJ strain. Johnson and Zheng (2002) concluded that the contribution of *Ahl2* to hearing loss is dependent on a predisposing *Ahl* genotype and that the interaction of *Ah1* and *Ahl2* results in an earlier onset of hearing loss than for *Ahl* alone.

A third ARHL locus (*Ahl3)* was identified by Nemoto et al. (2004) and fine mapped to Chromosome 17 by Morita et al. (2007). *Ahl3*, when present along with *Ahl*, has a protective effect against early onset hearing loss and hearing sensitivity is preserved

until late in life. In contrast to *Ahl* and *Ahl2*, it appears that the *Ahl3* mutation does not result in age-related hearing loss but functions to inhibit age-related hearing loss.

Zheng et al. (2007) identified and mapped a fourth ARHL locus, designated *Ahl4,* on Chromosome 10, of the A/J inbred mouse strain. The A/J strain harbors *Ahl,* as well as *Ahl4,* and exhibits early onset hearing loss relative to strains with *Ahl* alone. Using genetic analysis of a backcross strain Zheng et al. provide a compelling argument that *Ahl4* contributes to the accelerated onset of hearing loss in the A/J strain. Studies of this strain in our laboratory have revealed poor macular sensitivity and abnormal macular otoconia in this strain (Jones, Jones, Johnson, Yu, Erway, & Zheng, 2006).

Dreyton and Noben-Trauth (2006) investigated the genetic basis of hearing loss in the non-inbred Black Swiss strain and identified two loci (*Ahl5 and Ahl6*) that contribute to hearing loss in this strain. *Ahl5* and *Ahl6* were localized to chromosome 10 and 18, respectively. Black Swiss mice demonstrate an early onset hearing loss which begins in the high frequencies and eventually reaches profound levels at all frequencies.

The latest ARHL mutation to be identified is *Ahl8* (*Ahl7* is reserved) by Johnson et al. in the DBA/2J strain (Johnson, Zheng, & Noben-Trauth, 2006). *Ahl8* results in accelerated hearing loss and occurs on the distal portion of chromosome 11.

While the effect of *Ahl* mutations on auditory function is well studied, their effect on vestibular function is much less understood. A study by Shiga et al. (2005) characterized age related changes in auditory and vestibular structures and function in the C57 strain which carries *Ahl*, using vestibulo ocular reflex (VOR) gain as a measure of vestibular function and ABR as a measure of cochlear function. Histology showed a 30%

decrease in hair cell counts in the horizontal semi-circular canal crista, a significant loss of inner and outer hair cells in the basal cochlea, and 50% reduction in spiral ganglion densities. Their functional results show a significant decrease in auditory sensitivity but no corresponding decrease in VOR gain was observed. Based on these findings, the investigators suggested that the mechanisms for vestibular functional change might differ from those for the cochlea. They conceded however, that VOR is an indirect measure of vestibular function and that central mechanisms may compensate for peripheral degeneration. Investigations by Jones et al. (2005, 2006) using direct measures of auditory (ABR) and vestibular function (VsEP) in inbred mouse strains showed some interesting trends. Some strains (e.g., BUB/BnJ, NOD/LtJ, A/J) showed severe hearing loss and severe vestibular impairment at the ages tested. Other strains (e.g., DBA/2J, A/WySnJ, NOD.NONH2kb, A/HeJ) had severe vestibular impairment with relatively little change in hearing at the ages tested. Three strains (MRL/MpJ, CE/J, SJL/J) had significant vestibular loss with no concomitant hearing loss (at least to 32 kHz). These studies were limited to small sample sizes and few ages. However, the results highlight the importance of genetic background on age-related changes in the two sensory systems and suggest that functional change in one sensory system does not obligate change in the other.

What is the effect of the *Ahl* mutation on macular function and does macular function decline at the same rate as auditory function? Furthermore, are there gender differences in auditory and macular aging in these two *Ahl* strains? To answer these questions we measured cochlear and macular function in female and male C57 and CE/J

mice, both of which carry the *Ahl* mutation, and compared within strain functional changes, between the two genders and the two modalities. VsEP was used to assess macular function and ABR and DPOAE were used to characterize cochlear function. Temporal bones were harvested, dissected and prepared for SEM following functional measures. SEM images of the utricle and saccule from young and old mice were qualitatively compared to determine if any age related functional changes might be explained by changes in distribution or number of macular otoconia present.

We hypothesized the following:

1) Macular sensitivity will decline as a function of age.
2) Declines in auditory and macular function will occur at the same rate within strain and between strains.
3) The time course for ARHL will be different between females than males.

Methods

Animals and Animal Preparation

Studies were conducted at East Carolina University. Use of animals in this work was approved by the Institutional Animal Care and Use Committee and in accordance with national and international regulations regarding the use of animals in research. Ambient noise levels in the animal housing area were monitored through out the study to assure that noise levels were below those which might result in noise induced hearing loss (See Appendix K). Sibling breeding pairs were obtained from the Jackson laboratory (http://www.jax.org/). Animals were housed and maintained in standard husbandry until

appropriate age for testing was reached. Table 5 indicates the number of animals tested for the different measures and includes cases when there was no measurable response.

During testing mice were anesthetized with ketamine (120 to 126 mg/kg) and xylazine (10 to 14 mg/kg) injected intraperitoneally. Body core temperature was maintained at 37.0 ± 0.2°C using a homeothermic heating blanket and rectal thermocouple (FHC Inc, Bowdoin, ME). Mice were excluded from the study if there was any indication of potential outer or middle ear dysfunction (i.e. redness or inflammation of the external ear canal or tympanic membrane (TM), TM perforation, visible fluid in the middle ear space, excessive cerumen) as determined by examination of the external acoustic meatus and tympanic membrane with an operating microscope under anesthesia. During ABR and VsEP testing, subcutaneous needle electrodes were placed just posterior to the lambdoidal suture (noninverting), behind the left pinna (inverting) and at the neck (ground). A noninvasive head clip was used to secure the head to a mechanical shaker for delivery of vestibular stimuli. Auditory stimuli were delivered via a probe assembly placed at the entrance of the left external acoustic meatus. DPOAE, ABR and VsEP were performed during the same session after which the mouse was euthanized. Temporal bones were harvested from mice representative of young, mid-life, and advanced age groups for imaging with a scanning electron microscope. Stimuli for VsEP, ABR, and DPOAE are described in detail below.

Vestibular Evoked Potentials (VsEP)

VsEPs were used to measure macular sensitivity to linear accelerations and characterize macular function. VsEPs are an objective measure of macular sensitivity, are

Table 5. *Number of data points for each measure. The table indicates the number of mice tested for each measure and includes cases where the measurement was performed but there was no measurable response (no response).*

Strain	8 kHz	16 kHz	32 kHz	41.2 kHz	VsEP	DPOAE
C57BL/6J	75 females 72 males	75 females 72 males	73 female 75 male	72 female 73 males	66 females 67 males	144 total
	147 total	147 total	148 total	145 total	133 total	
CE/J	44 female 46 male	44 female 48 male	44 female 48 male	44 female 48 male	44 female 51 male	96 total
	90 total	92 total	92 total	92 total	95 total	

critically dependent on the macular organs (utricle and saccule), the vestibular portion of cranial nerve VIII, and vestibular brainstem relays. VsEP peak latency and peak to peak amplitude are determined by the number, timing and the synchrony of firing of vestibular afferents and the integrity of vestibular brainstem relays. VsEP stimuli and recording methods were similar to Jones et al. (2005). A linear jerk pulse generated by Tucker Davis Technologies (TDT, Alachua, FL) modules (TG6, DA3-2, PA4) and controlled by custom software (Jones and Jones, 1996, 1999; Jones et al., 1997, 1998a,b; 2002) was used to elicit VsEPs. The jerk pulse was generated using a linear voltage ramp of 2 ms duration, routed through a power amplifier, which drove a mechanical shaker (Labworks, Inc. model E2-203). The applied voltage produced an acceleration ramp, which was measured by an accelerometer (Endevco, Inc., 10mV/g where g = 9.81 m/s^2). Output of the accelerometer was measured using an electronic differentiator; the output of which was monitored with an oscilloscope. The linear jerk pulses were coupled to the head through a custom platform mounted atop the shaker. Systematically increasing or decreasing the amplitude of the stimulus waveform controlled the intensity of the stimulus. Amplitude of the jerk stimuli was recorded as the mean peak jerk level (measured using an oscilloscope) and was expressed in dB relative to 1.0g/ms. Magnitude of the stimulus was calibrated to produce an onset jerk magnitude of 1.0 g/ms with an attenuator setting of 0 dB. Stimulus amplitude ranged from –18 to +6 dB re: 1.0g/ms and was adjusted in 3 dB steps. Maximum stimulus amplitude (+ 6 dB re: 1.0 g/ms) resulted in jerk amplitude of 2 g/ms and a platform displacement of approximately 26 μm at peak acceleration. Jerk stimuli had two directions of motion: normal and inverted. Mice were

placed in a supine position with nose up. The axis of motion for normal polarity began with upward movement (naso-occipital +X). Inverted stimulus polarity began with downward movement (naso-occipital -X). Responses were collected for both normal and inverted polarities and the resulting waveforms were averaged online to produce the final waveform for analysis.

Auditory Brainstem Response (ABR)

ABR is an objective measure of cochlear sensitivity and the timing and synchrony of firing of auditory afferent neurons. Tone burst stimuli were used to illicit ABR responses. Continuous pure tone stimuli were generated by a digital signal analyzer (Stanford Research Systems SR785, Sunnyvale, CA.) and shaped/controlled using custom software and Tucker Davis Technologies (TDT, Alachua, FL) modules (TG6, SW2, PA4). Tone bursts at 8, 16, 32, and 41.2 kHz had 1.0 ms rise-fall times with 1.0 ms plateau (3 ms total duration). During ABR testing the probe tip was inserted into the mouse ear canal and an ER10B+ microphone measured the stimulus sound pressure level. The output from the microphone was routed to an oscilloscope and a peak-to-peak voltage was recorded. Peak equivalent SPL (pe SPL) was later calculated based on the microphone sensitivity as determined by a calibration in a custom coupler. The average maximum stimulus levels were 97, 90, 78, and 92 dB pe SPL for 8, 16, 32, and 41.2 kHz respectively. Stimuli were presented to the left ear via commercial OAE tips (Etymotic ER10D-TO5 5mm, Elk Grove, IL.) and tapered at the ear canal with PE-25 tubing. Auditory stimuli were presented at a rate of 17 stimuli/sec. Intensity series were collected with a descending series of stimulus intensities (6 or 12 dB steps) beginning at +6 dB.

Stimulus levels were decreased until no response was visible and then dropped an additional 6 dB, in order to ensure that a sub-threshold level had been reached.

Distortion Product Otoacoustic Emissions (DPOAE)

DPOAEs provide an objective measure of cochlear function and are critically dependent on outer hair cell function (Brownell, 1990). Methods for DPOAE stimuli and recording were similar to Jimenez et al. (1999) and Guimaraes et al. (2004). Stimuli for DPOAEs were generated and shaped using custom software and modules from TDT (TG6, PA4, and SW2). Pure tone frequencies (f1, f2, f2/f1 ratio = 1.25), at equal levels (L1 = L2 = 60 dB SPL), 150 ms duration, were generated with independent sources (Hewlett Packard Agilent signal generators model 33220 A, Santa Clara, Ca.) and routed through separate drivers to mix acoustically in the ear canal via commercial OAE tips (Etymotic ER10D-TO5 5mm), tapered at the ear canal with PE-25 tubing, and placed securely in the external acoustic meatus. Stimulus frequencies for the primaries were such that the geometric mean ($GM = (f1 \times f2)^{0.5}$) frequencies ranged from 6.0 to 48.5 kHz (at least 8 frequencies per octave). During each study ear canal sound pressure was recorded with a low noise probe microphone (Etymotic ER 10B+). The microphone output was amplified 10X (ER10B+ preamplifier) and input to a dynamic signal analyzer (Stanford Research Systems SRS785) for sampling at 200 kHz and fast Fourier transform (FFT). The amplitude of f1, f2, and the 2f1-f2-distortion product was measured from the FFT waveform. The noise floor was calculated as the average amplitude in the five frequency bins above and below the 2f1-f2 component. The recording system was tested periodically in the 0.35 ml coupler to confirm the absence of artifactual distortion.

ABR and DPOAE Microphone and Stimulus Calibration

ABR and DPOAE stimuli were calibrated monthly using a 0.35 ml custom coupler, ¼" B&K microphone with Nexus amplifier, and custom software. During calibration the probe tube assembly (housing the ER10B+ microphone and exit ports for sound) was inserted into one end of the custom coupler (0.35 ml volume) and the ¼ B & K microphone was inserted into the other end. The output of the ER10B+ microphone was amplified (10X) and routed to one channel of the SRS785 signal processor and the output from the ¼ B & K microphone was amplified and routed to a second channel on the SRS785 signal analyzer. Each stimulus frequency was presented and the attenuation was adjusted until a level of 60 dB SPL was achieved for the B & K channel. The attenuation level and the ER10B+ output level from the other channel of the SRS785 signal analyzer were saved as a probe tube calibration file (PTC) and the ER10B+ microphone sensitivity was calculated for each frequency. This PTC file was used to set the target dB SPL for the ER10B+ microphone output during each DPOAE study. This assured that a level of 60 dB SPL was achieved for both primary tones presented during DPOAE measures. The PTC file was also used to calculate the ER10B+ sensitivity, which is needed to calculate dB pe SPL for ABR stimulus intensity. During ABR studies, the ER10B+ microphone output was routed to an oscilloscope (Hewlett Packard 54602B) and peak-to-peak amplitude was recorded. Stimulus intensity (dB pe SPL) was later calculated based on the stimulus peak-to-peak amplitude and microphone sensitivity at that frequency.

Overview and Averaging

Measurements began with DPOAE followed by ABR and VsEPs. This order was chosen in an attempt to limit exposure to high sound pressure levels that could result in an auditory threshold shift. Stimulus levels for DPOAE are limited to around 60 dB SPL compared to ABR maximum stimulus levels, which can be as high as 100 dB pe SPL. An intense forward masker is presented during VsEPs that has an average intensity level as high as 97 dB SPL. Vestibular evoked potentials were performed last in order to negate the possibility of noise-induced damage to the auditory system from exposure to the intense broad-band masker during VsEP testing. The broad-band masker is not required for VsEP recording but demonstrates that there is no auditory contribution or contamination in the VsEP waveforms. Test frequencies for ABR and DPOAE were tested in random order. In general, the three measures were completed in one mouse in less than two hours.

Single channel signal averaging was used to record VsEP and ABR response waveforms. Offline analysis was used to determine response thresholds, response peak latencies, and peak-to-peak amplitudes. Subcutaneous electrodes were placed as described previously. The electroencephalographic activity was amplified (200,000X, Grass P511), band pass filtered (300 to 3000 Hz, -6 dB points, Grass P511, West Warwick, RI) and digitized (10 μs per point for 1024 points, TDT AD1) beginning at stimulus onset (TDT TG6 triggers system). 256 samples were averaged to produce the final VsEP or ABR waveform. Stimuli for VsEPs were linear jerk pulses that physically moved the mouse's head. ABR stimuli were short duration tone bursts. ABR and VsEP

threshold, peak latency, and peak to peak amplitude were quantified. Amplitude and latency measures for the first three peaks (PI, PII and PIII) of the response waveform were quantified. Threshold was defined as the intensity midway between the minimum stimulus intensity that produced a discernible response and the maximum stimulus intensity that did not result in a visible response. Response peak latency was defined as the time, in milliseconds, from onset of the stimulus to the appearance of each positive peak (PI, PII and PIII). Peak-to-peak amplitude, (PI-NI, PII-NII, and PIII-NIII) measured in microvolts (μv), represented the difference between each positive peak and its respective negative peak.

Data Analysis

In order to compare cochlear and macular sensitivity, ABR and VsEP thresholds were normalized to establish a common unit of measure. We chose to normalize threshold values in terms of a percent change in auditory and macular dynamic range. Dynamic range was defined as the range from the best threshold measured previously in the CBA/CaJ strain to the most intense stimulus level possible without inducing a temporary threshold shift or permanent damage (e.g. noise induced hearing loss or dislodging macular otoconia). The CBA strain harbors no genetic mutations, maintains hearing until late in life, and served as a baseline for "normal" hearing. ABR and VsEP absolute threshold values were normalized relative to the dynamic range established for the CBA strain. Normalizing thresholds relative to the CBA strain allows a direct comparison between the two *Ahl* strains (C57 & CE/J) and between the two *Ahl* strains the control strain (CBA). The upper level of the dynamic range was determined

based on a pilot study that was conducted to determine the level at which ABR stimuli may cause temporary or permanent threshold shift. The results of this pilot study suggested that levels greater than 100 dB pe SPL might result in threshold shifts; so 100 dB pe SPL was used as the upper range for auditory dynamic range. The lower limit for dynamic range was set by the best thresholds measured in the CBA/CaJ strain and were 12.7, 13.1, 4.9, and 25.39 dB pe SPL for the test frequencies of 8, 16, 32, and 41.2 kHz, respectively. The lowest VsEP threshold measured was – 13.5 dB re: 1 g/ms and the most intense non-noxious sustained stimulus presentation level is + 6 dB re: 1 g/ms. The resulting dynamic range was 19.5 dB re: 1 g/ms. To normalize a given threshold (y) the following formula was used:

$$x = \frac{y - z}{k} * 100$$

where x = the normalized value, z = the lowest threshold measured and k is the dynamic range.

For example, with an absolute threshold of -7.5 dB:

$$x = [(-7.5 - (-13.5) / 19.5] * 100$$

$$x = [6/19.5] * 100$$

$$x = 30.77\%$$

Age related changes in auditory sensitivity were compared between genders within strain. Age related changes in macular sensitivity were compared between genders within strain and between strains. The rate of change between auditory and macular sensitivity was compared within strain using linear regression and student's T-test. Linear

regression analysis was performed to determine if there were significant differences in the linear regression slopes or intercepts of; female and male auditory thresholds, female and male macular thresholds, between auditory and macular thresholds within strain, and macular thresholds between strains. The mean DPOAE amplitude and noise floor were plotted for each age group as a function of age and geometric mean frequency. Extensive statistical analysis will not be performed on DPOAE data, as the main purpose of DPOAE measures was to characterize age related change in OHC function and to confirm the cochlea as the site of lesion.

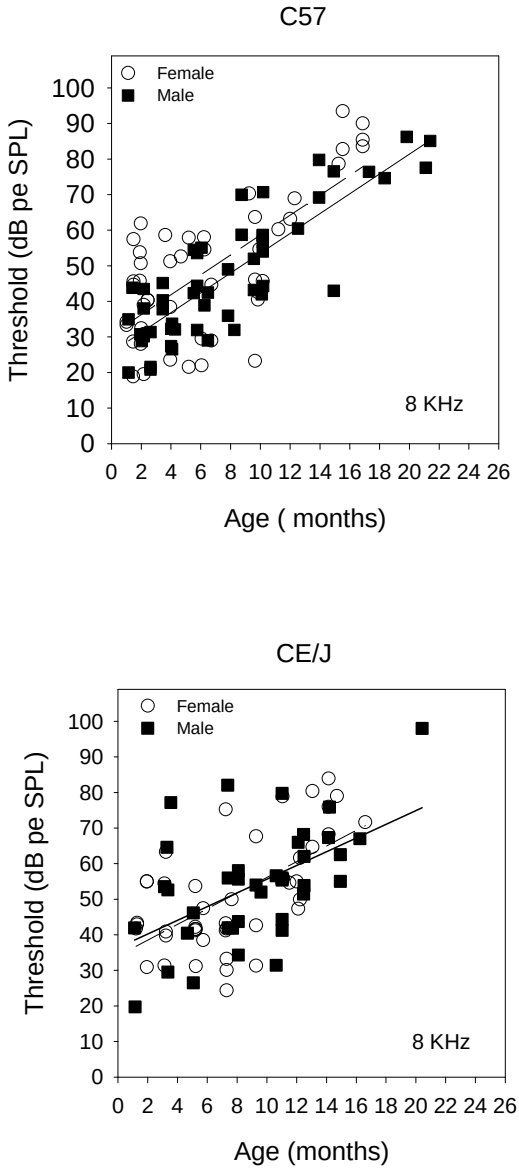
Structural Analysis

Temporal bones from young, middle, and old mice were dissected at the end of the functional experiments and fixed using 4% paraformaldehyde and 2% glutaraldehyde. Following 24 hours in fixative the temporal bones were then dehydrating using graded concentrations (70, 90, 95, and 100%) of ethyl alcohol. After a minimum of 24 hours in 100 % ethyl alcohol, the macular organs were micro-dissected and mounted on aluminum stubs, critical point dried (Bal-Tec CPD 030, Liechtenstein), and sputter coated (Anatech LTD Hummer 6.6, San Diego, Ca.), before imaging with a scanning electron microscope (FEI Quanta 200, Hillsboro, Oregon).

Results

ABR thresholds increased significantly as a function of age for both strains. No significant gender difference was found for either strain for ABR threshold slopes or intercepts for 8 kHz (Fig. 29), so ABR data for the two genders were pooled for further analysis. Regression analysis for ABR and VsEP threshold comparisons between genders

Figure 29. Female and Male 8 kHz Threshold Comparison. Linear regression equations were: C57: females y = 30.6 + 2.86x, males y = 25.7 + 2.78x (confidence intervals were 2.04 to 3.67 and 2.29 to 3.28 for females and males respectively). CE/J: females y = 34.1 + 2.20x males y = 36.4 + 1.93x (confidence intervals were 1.33 to 3.08 and 0.92 to 2.94 for females and males respectively). No significant differences were found for slope or intercept between females and males of either strain.



was limited to data for ages less than 12 months due to the large number of missing data points (no responses) after 12 months of age. ABR and VsEP gender regression analysis focused on the difference between slopes of female and male VsEP and 8 kHz thresholds. In C57 mice, the slope of the linear regression model for the difference between females and males was 1.15, meaning that the estimated average difference between genders was 1.15 dB per month. The 95 % confidence interval for difference in the slope for female and males was also calculated and values were from - 0.44 to 2.7. In CE/J mice, the slope of the linear regression model for the difference between females and males was 0.09, meaning that the estimated average difference between genders was 0.09 dB per month. The 95 % confidence interval for the difference in the slope for CE/J female and males was also calculated and values were from –2.24 to 2.05. We would argue that an average difference between females and males thresholds of 1.03 dB and 0.09 dB per month for C57 and CE/J strains respectively, does not represent a practical difference. The ABR and VsEP data for both genders was pooled for further analysis.

ABR thresholds increased significantly as a function of age for both strains for all frequencies (with the exception of 41 kHz for CE/J) (Fig. 30, 31). Auditory sensitivity at the young ages was similar for the two strains for 8 and 16 kHz; however the CE/J showed elevated thresholds at 32 and 41.2 kHz at young ages relative to the C57 strain (Fig. 31). C57 thresholds increased an average of 2.75, 2.76, 4.14, and 1.37 dB pe SPL per month and CE/J thresholds increased an average of 2.07, 2.69, 0.97, and 0.41 dB pe SPL per month for the test frequencies of 8, 16, 32, and 41.2 kHz, respectively. These average increases in threshold must be interpreted cautiously due to the large number of

Figure 30. ABR Audiogram for Both Strains. Data points represent the mean ABR threshold for each age group (listed on the right).

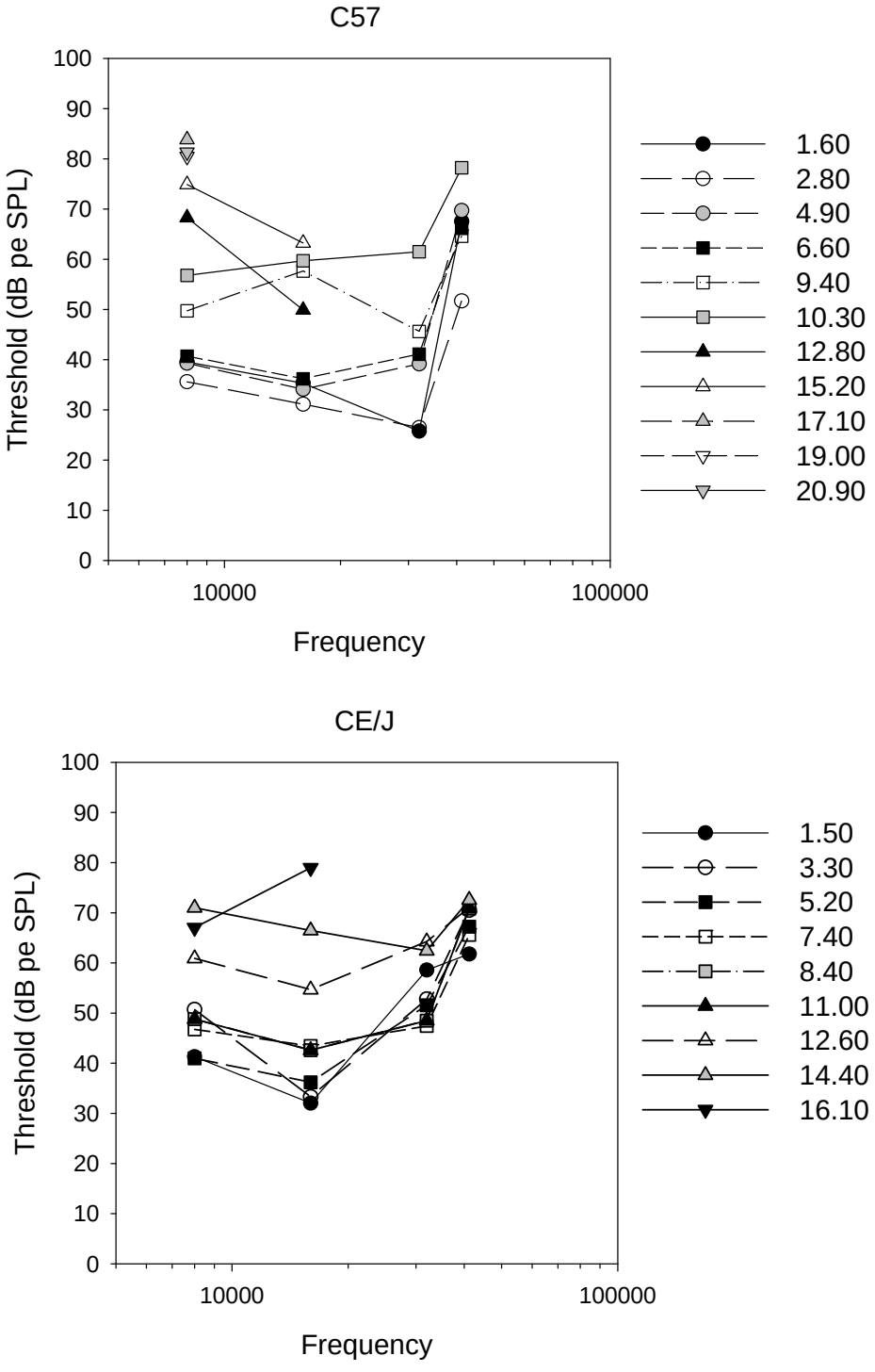
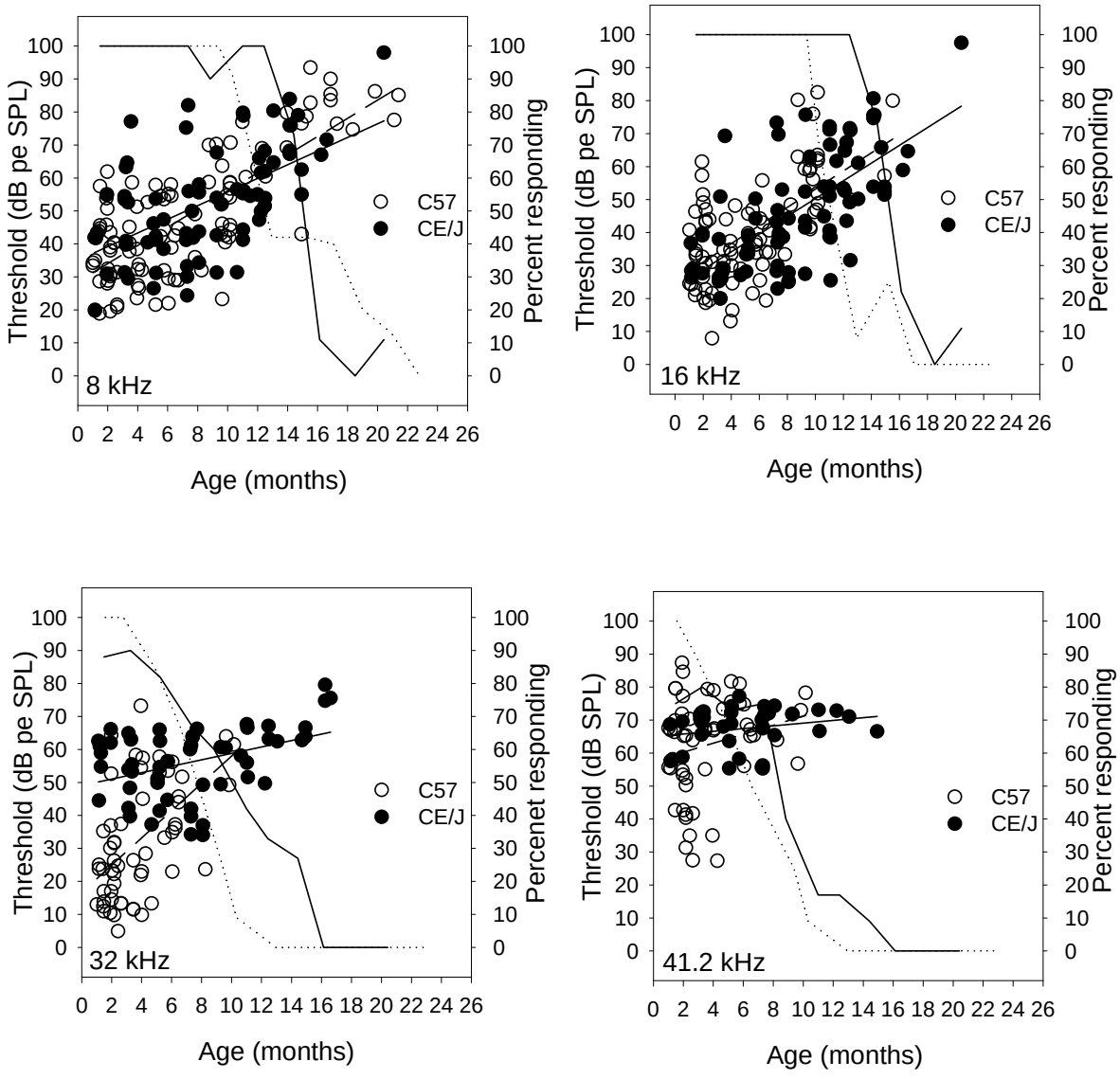


Figure 31. Comparison of ABR Thresholds. ABR threshold for the four test frequencies plotted as a function of age. Auditory sensitivity and rate of change for the two strains were similar for 8 and 16 kHz. The CE/J strain exhibits poor sensitivity for 32 and 41.2 kHz even at the youngest ages. The right axis and line plots (dotted line for C57 and solid line for CE/J) reflect the percent of animals tested with a response (percent responding).



missing data points that occur as hearing loss progresses with age and reaches a level where no ABR response can be measured. P1 peak latencies increased an average of 0.03, 0.03, 0.03, and 0.04 ms per month in the C57 strain (Fig. 33) and decreased an average of 0.002, 0.007, 0.0007, and 0.002 ms per month in the CE/J strain for 8, 16, 32, and 41.2 kHz respectively (Fig. 33).

P1-N1 amplitude declined in both strains; an average of 0.06, 0.07, 0.11, and 0.04 μv per month for C57 and 0.06, 0.07, 0.02, and 0.009 μv per month for CE/J for 8, 16,32, and 41.2 kHz respectively (Fig. 34).

The VsEP response was generally present for all age groups (Fig. 35) in both strains and no significant gender differences were found for VsEP linear regression intercepts or slopes (Fig. 36). Macular sensitivity was similar for both strains at the earliest ages tested but the rate of decline in VsEP thresholds was significantly faster in the CE/J strain ($p<0.001$)(Fig. 37). The C57 strain showed an average decrease of 0.12 dB re: 1 g/ms per month compared to 0.47 dB re: 1 g/ms per month for the CE/J strain. A student's T-test comparing the slope of the linear regression line for VsEP threshold for the two strains, shows a significant difference ($p< 0.001$) in the rate of change of macular sensitivity between the two strains. VsEP P1 latency was similar for the two strains and increased as a function of age by an average of 0.01 and 0.008 ms per month for C57 and CE/J respectively (Fig. 38). VsEP P1-N1 amplitude decreased as a function of age by an average of 0.01 μv per month for both strains (Fig. 39).

Figure 32. ABR P1 Latency Comparison. ABR P1 latency plotted as a function of age for the two strains for the four ABR test frequencies. Least squares linear regression are indicated by the dashed (C57) and solid (CE/J) lines. Stable P1 latencies suggest minimal age related change in synaptic mechanisms and activation of primary afferents.

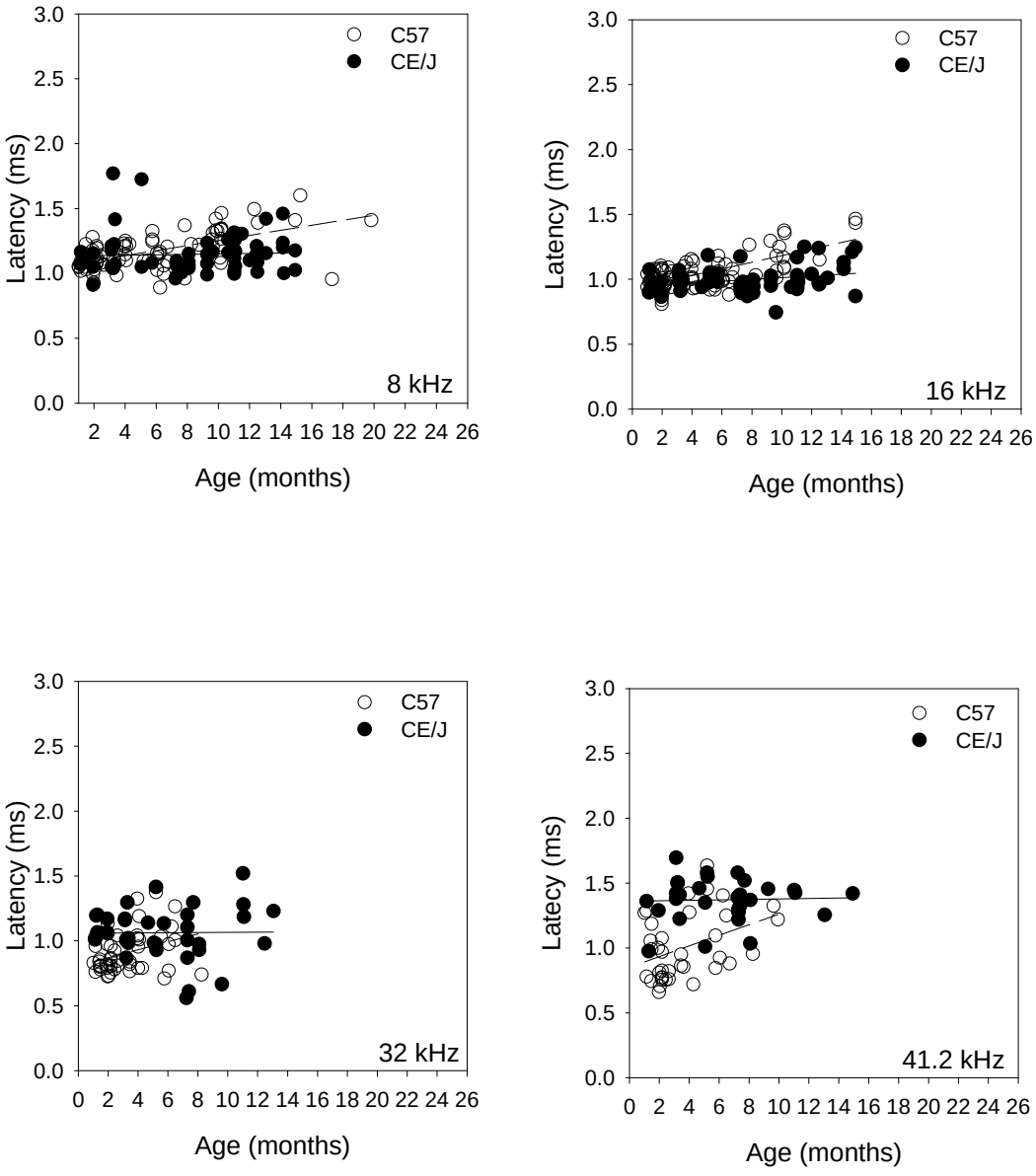


Figure 33. ABR P1-N1 Amplitude Comparison. ABR P1-N1 amplitude plotted against age for the C57 and CE/J strains for the four ABR test frequencies. There was a significant decrease ($p < 0.0001$) in P1-N1 amplitudes as a function of age for both strains. Significant decreases in P1-N1 amplitude suggests a dramatic reduction in the number of hair cells and primary afferents contributing to the response.

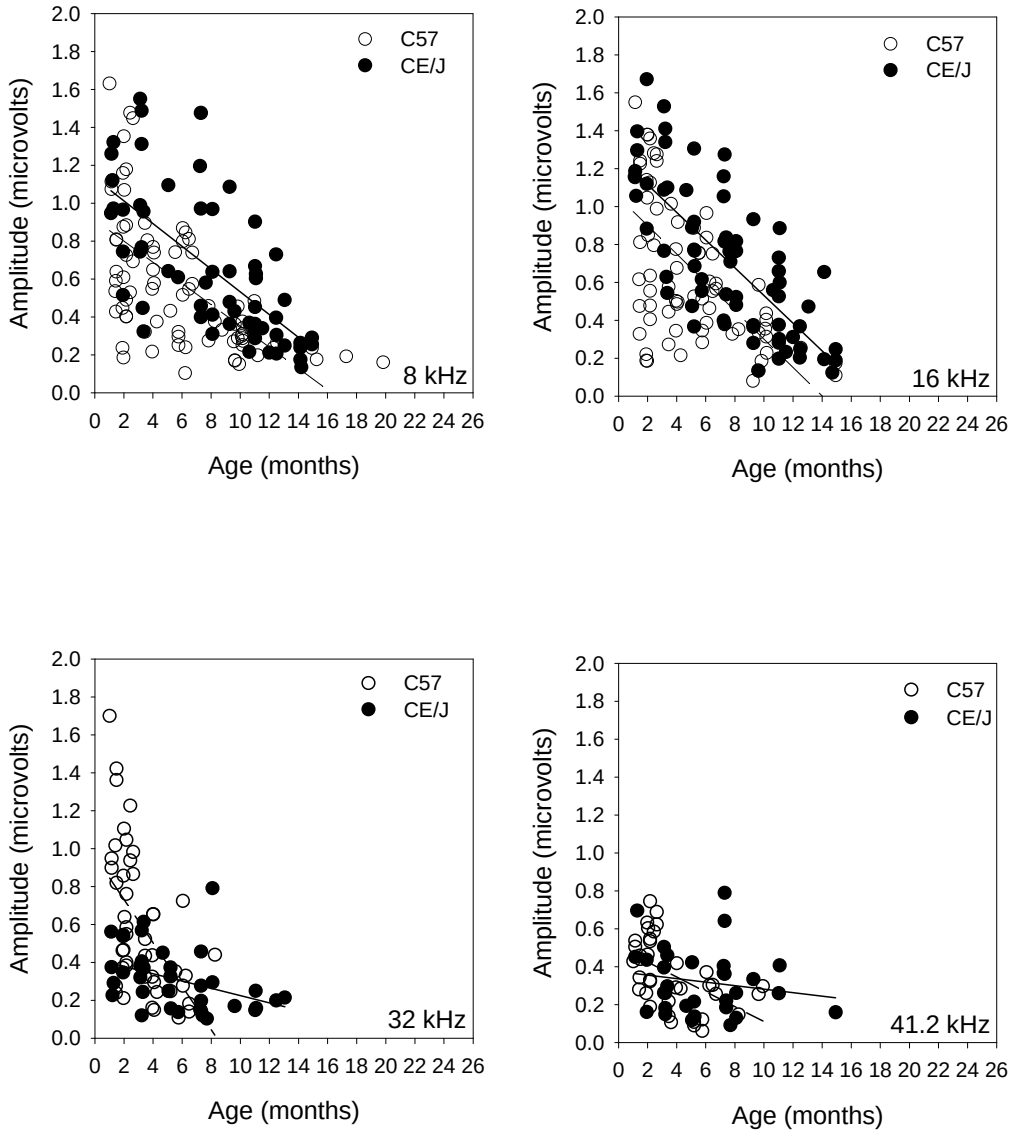


Figure 34. Representative VsEP Waveforms. Representative VsEP waveforms for the two strains at selected ages.

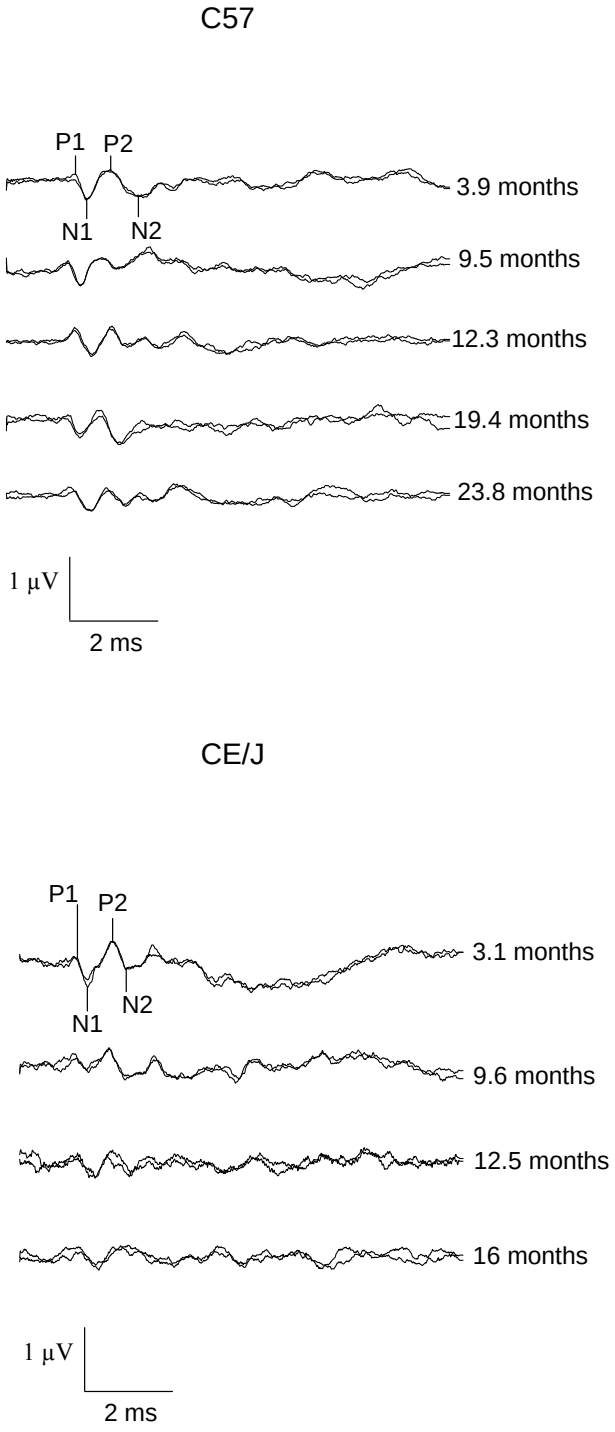


Figure 35. Comparison of Female and Male VsEP Threshold. A comparison of female and male VsEP thresholds for the two strains. Linear regression equations were: C57: females y = -11.02 + 0.09x and males y = -11.84 + 0.15x (the 95 % confidence intervals were 0.17 to 0.18 for females and 0.06 to 0.23 for males). CE/J: females y = -10.4 + 0.53x and males y = -10 + 0.43x (the 95 % confidence intervals were 0.37 to 0.70 for females and 0.28 to 0.57 for males). No significant gender difference was found for slope or intercept for either strain.

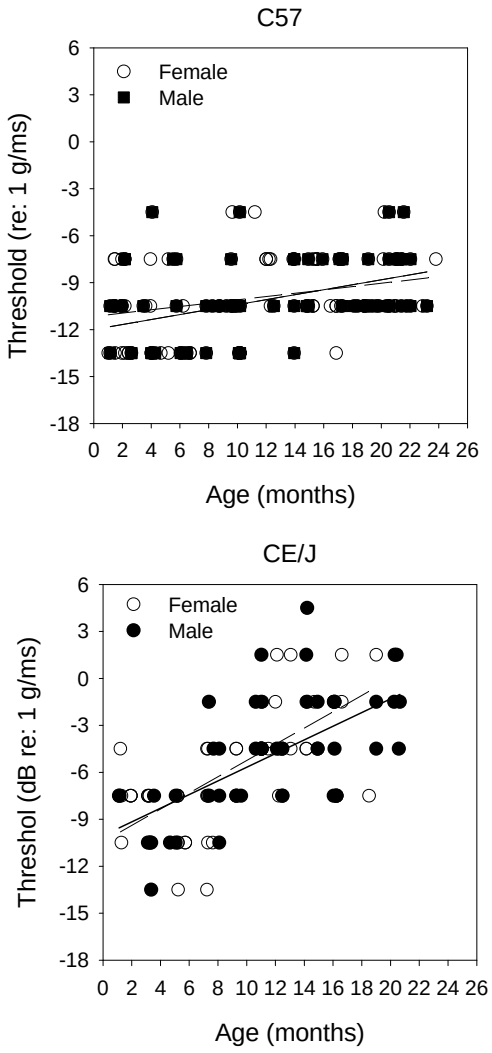


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Figure 36. Comparison of VsEP threshold. A comparison of age related change in VsEP thresholds between the two strains. Linear regression equations were: C57: y = -11.4 + 0.12x and CE/J: y = -10.19 + 0.46x (the 95% confidence intervals for slope were 0.06 to 0.18 and 0.36 to 0.57 for C57 and CE/J, respectively). Intercepts were not significantly different. The slopes of the linear regression lines were significantly different ($p<0.0001$). All 149 C57 mice tested had a VsEP response. Out of 102 CE/J mice tested two had no response (one at 7.4 months and one at 14.2 months).

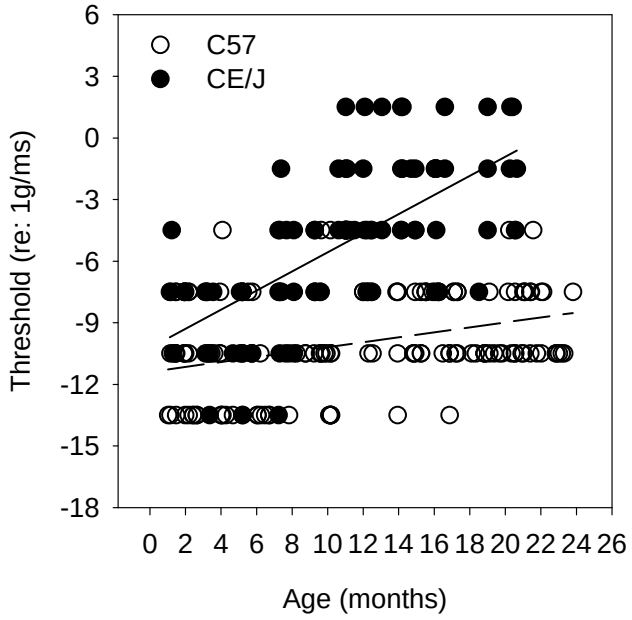


Figure 37. Comparison of VsEP P1 Latency. A comparison of VsEP P1 latency between the two strains. Linear regression equations were: C57: y = 1.29 + 0.01x. CE/J: y = 1.26 + 0.01x (the 95% confidence intervals were 0.006 to 0.014 and 0.004 to 0.013 for C57 and CE/J respectively). No significant difference was found for intercept or slope between the two strains. Small changes in P1 latencies suggest minimal age related change in synaptic processes and activation of primary afferents.

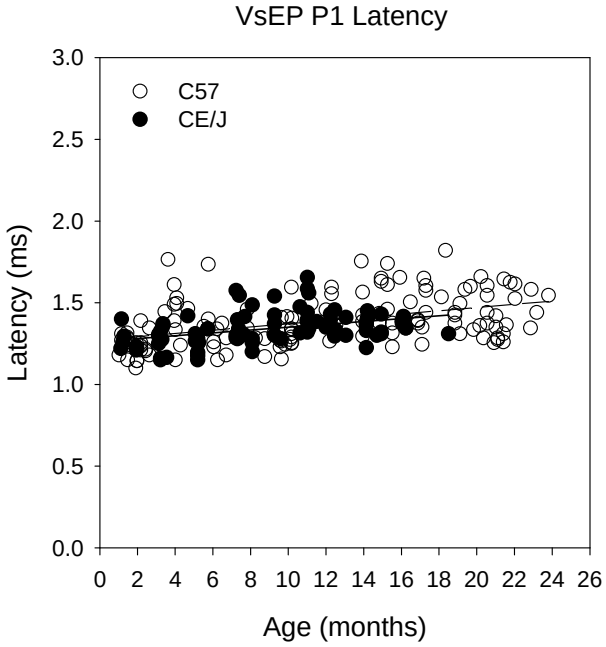
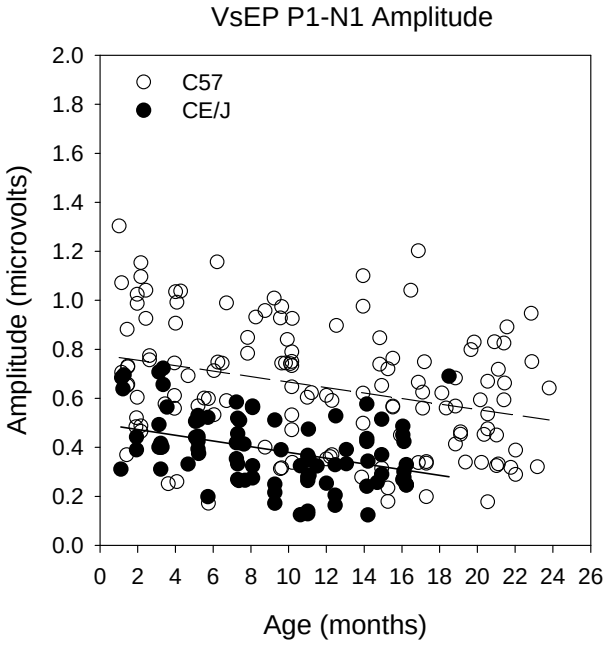


Figure 38. Comparison of VsEP P1-N1 Amplitude. A comparison of VsEP P1-N1 amplitude between the two strains. Amplitude declined significantly ($p<0.0001$) as a function of age in both strains. Linear regression equations were: C57: $y = 0.78 – 0.01x$ and CE/J: $y = 0.50 – 0.01x$. Significant decreases in P1-N1 amplitude suggests a dramatic reduction in the number of hair cells and primary afferents contributing to the response.



SEM images of the utricle and saccule from young and old animals show no apparent qualitative difference in macular otoconia of young and old mice that would explain a decrease in macular sensitivity in either strain (Fig. 40). SEM images of the macular organs showed full coverage of the utricular and saccular epithelia with dense populations of otoconia at young and advanced ages. This finding suggests that the observed age related decrease in macular sensitivity is not due to a loss of otoconia.

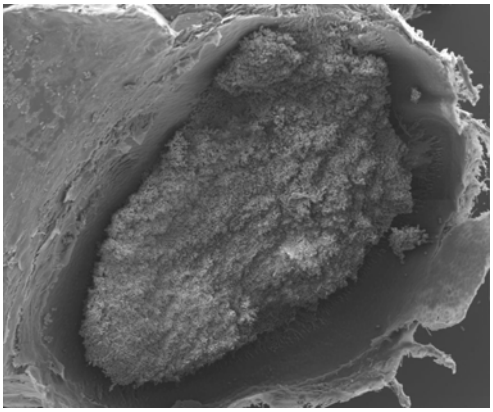
A student's T-test was used to compare VsEP and ABR threshold slopes within-strain and this test revealed a significant difference ($p < 0.0001$) in the rate of change for the two modalities for both strains (Fig. 41). The comparison is based on 8 kHz only because both strains exhibit or develop high frequency hearing loss very young. A significant difference was found for both strains but the relationship between auditory and macular change is very different between the two strains. In the CE/J strain, the linear regression slopes for VsEP and ABR normalized thresholds were 2.15 and 2.37, respectively (Fig. 41). In the C57 strain, the linear regression slope for normalized VsEP and ABR thresholds were 0.62 and 3.15, respectively (Fig. 41). The difference in the rate of change between auditory and macular sensitivity is greater in the in C57 strain than the CE/J strain.

DPOAE amplitudes decrease as a function of age in both strains (Fig. 42). The C57 strain exhibits larger amplitude DPOEA than the CE/J strain at all ages, except < 2 months. DPOAE amplitudes in the CE/J strain generally fall below "normal" levels (as established by the 95% population range for the CBA strain) for all

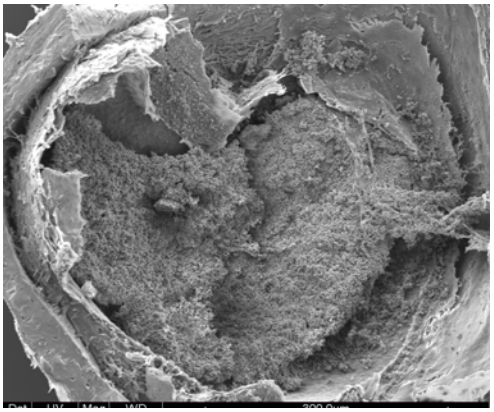
Figure 39. Scanning Electron Microscopy Images of the Macular Organs. Representative SEM images of the utricle and saccule for old and young mice of both strains. The first set of images are from 20.9 month old C57 mice. The second set of images are from 10 month old C57 mice. The third set are from 39 day old CE/J mice and the fourth set are from 14.9 and 16 month old CE/J mice.

C57

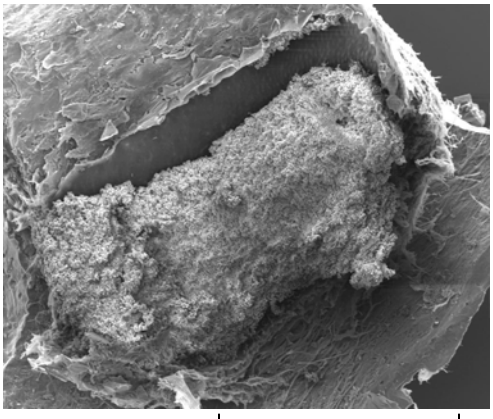
Saccule



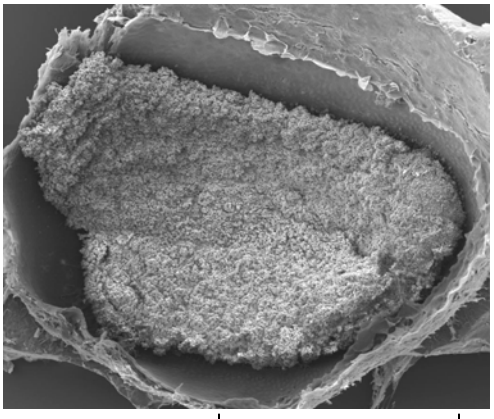
Utricle



Saccule

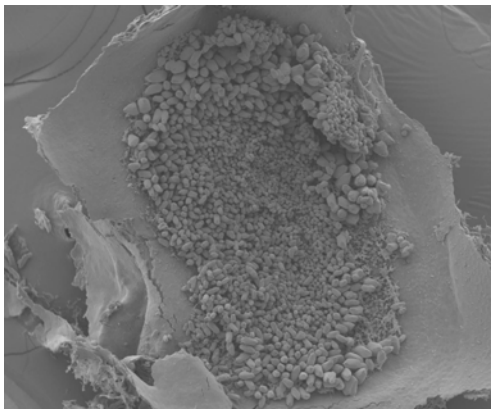


Utricle



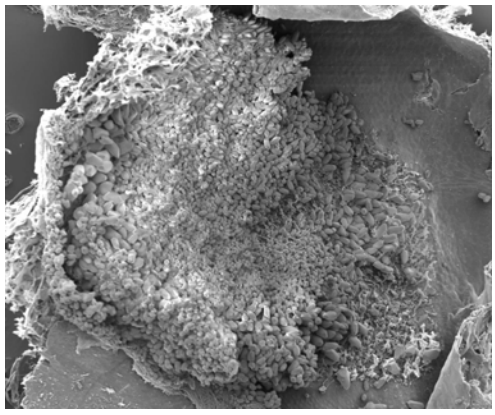
CE/J

Saccule



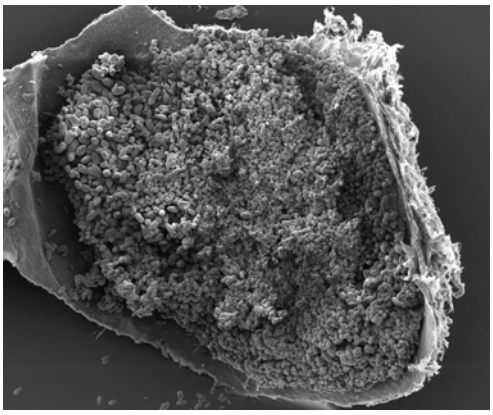
300 µm

Utricle



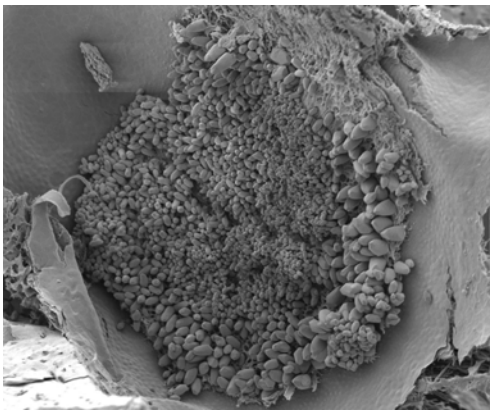
200µm

Saccule



300 µm

Utricle



300 µm

Figure 40. Comparison of Age Related Change in Auditory and Macular Sensitivity. Normalized VsEP and ABR thresholds plotted against age for both strains. Linear regression equations were: C57: 8 kHz: y = 18.1 + 3.15x. VsEP: y = 10.76 + 0.62x. CE/J: 8 kHz: y = 25.6 + 2.37x. VsEP: y = 18.51 + 2.15x. There was a significant difference between 8 kHz and VsEP slopes for both strains but the difference in rate of change between auditory and macular sensitivity is more pronounced in the C57 strain.

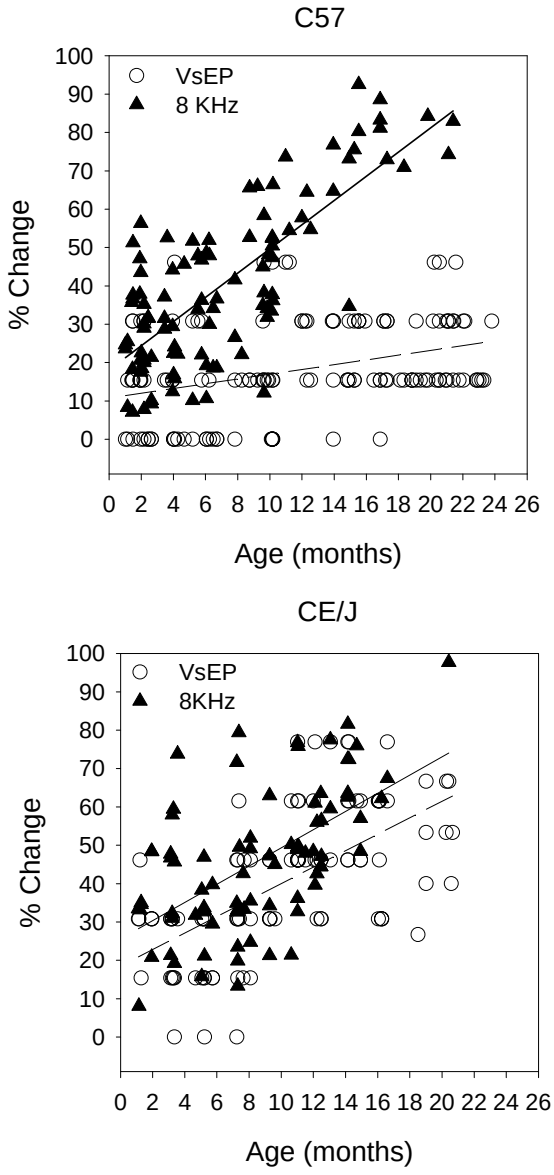
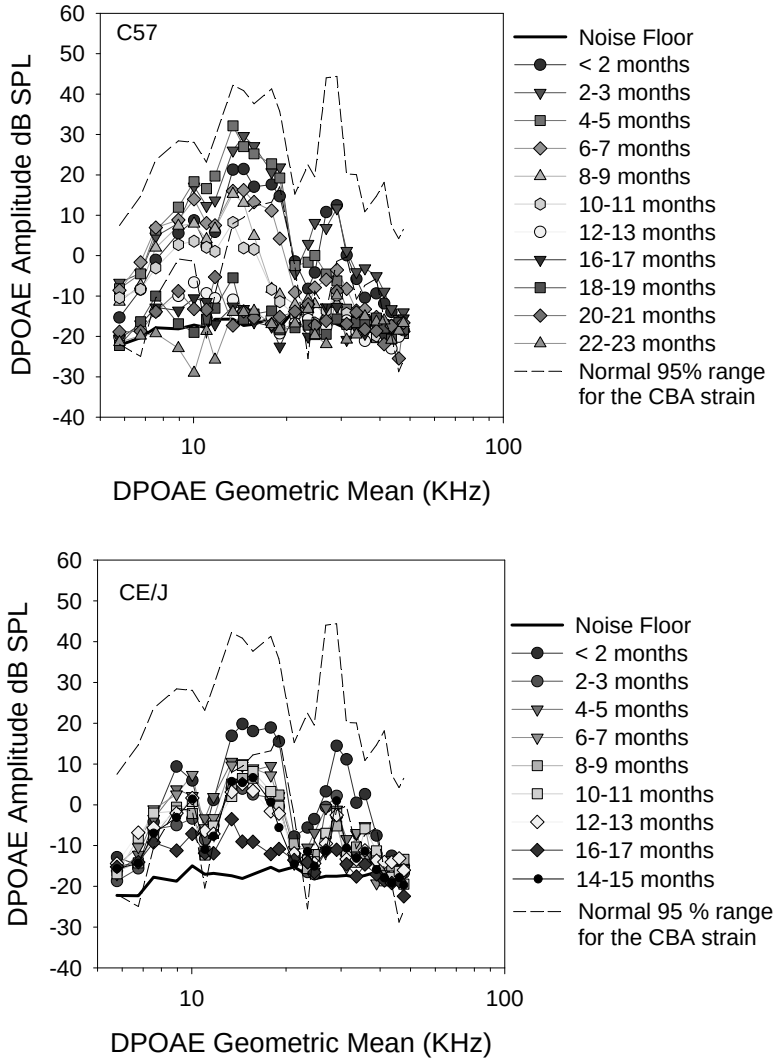


Figure 41. DPOAE Plots. DPOAE amplitude as a function of geometric mean frequency for the C57 and CE/J strains. Ages represents the mean age for the mice in each bimonthly age group. The black dashed line represents the 95% population range for DPOAE amplitudes for the 2 to 3 month age group for the CBA strain. Outer hair cell function declines with age in both strains but is reduced in the CE/J strain from an early age.



frequencies for all age groups except the youngest group. This is an indication that outer hair cell function is reduced from an early age in the CE/J strain.

Discussion

The current ABR and DPOAE findings are in good agreement with the auditory structural degeneration and age related functional decline previously reported in both strains. Auditory structural and functional changes due to age have been well characterized in the C57 strain, which is widely accepted as an animal model for early onset ARHL, and gender differences in auditory function have been reported in this strain (Henry, 2004). Henry did not perform statistical analysis and characterized gender differences based on line plots of ABR and cochlear nerve envelope thresholds for mice at selected ages. Henry's line plots suggest that male C57 mice tend to have more sensitive hearing than females prior to hearing loss onset. In the current study, ABR thresholds versus age scatter plots for the C57 strain also show that males tend to have more sensitive hearing than females, even though no statistical difference was found. ABR gender plots for the CE/J strain show similar auditory sensitivity and rate of change between female and male mice.

There is growing structural and functional data that supports the idea of auditory gender differences in mouse models of ARHL and it has been suggested that the female hormone estrogen may have a direct influence on cochlear function. This idea is supported by structural data from Stenberg et al. (1999) that revealed widespread immunostaining of the estrogen receptors ERα and ERβ in cochlear structures in CBA mice. Estrogen is known to have widespread effects on physiology and this finding

suggests that it may have a direct influence on cochlear structures. Further evidence is provided by Hulcrantz et al. (2000) who characterized cochlear structure and function in the X,O "Turner mouse" which produces no estrogen. They found a loss of outer hair cells (OHC), primary afferent pathology, increased ABR thresholds, and decreased DPOAE amplitudes relative to controls. Guimaraes et al. (2004) provide further evidence for the protective effects of estrogen on auditory function. Using both ABR and DPOAE, they found differences in auditory function pre and post menopause in female CBA mice. Females had lower thresholds and larger amplitude DPOAEs than males at middle and old ages but this advantage was lost following menopause. Study of the CBA strain in our laboratory, found that females tended to have lower ABR thresholds than males but no significant statistical difference was found between the genders. The role of female hormones on inner ear structure and function is poorly understood but there is growing evidence suggesting that female hormones may have a protective effect against ARHL. While previously described C57 strain auditory gender differences were not confirmed with statistical analysis there are small gender differences apparent in ABR threshold scatter plots.

The effect of gender on vestibular structures and function is poorly understood. A search of the literature reveals no reports of gender differences in vestibular structures or function in relation to aging. We found no significant differences for macular sensitivity or the rate of change in macular sensitivity between the two genders for either strain.

The auditory structural and functional changes seen in mice that carry *Ahl* may be explained by the effect of *Ahl* on cadherin23 expression in cochlear sensory structures

such as stereocilia, and transient and lateral tip links (Bolz et al., 2001; Bork et al., 2001; Di Palma et al., 2001). Cadherin23 is also expressed in vestibular stereocilia/kinocilia so it is reasonable to expect vestibular structural and functional degeneration with age as well. Vestibular structural pathologies have been identified in mice carrying Cdh23 mutations, including C57 (Shiga et al., 2004), modified deafwaddler (Noben-Trauth et al., 2003), waltzer (Di Palma et al., 2001), and Ames waltzer (Raphael et al., 2001) yet no significant functional change was found in C57 vestibular function using the VOR measure (Shiga et al., 2004). VOR relies on peripheral input, central integration and motor output and central compensation for peripheral deficits may explain the poor correlation between peripheral structural degeneration and functional results. Shiga et al. (2004) did not assess macular function. The current study utilized VsEP, which does not rely on central integration of peripheral input or motor output and therefore assesses peripheral function directly. The present study found an age related decline in macular sensitivity in both strains (21% and 56% loss of dynamic range at the oldest ages for C57 and CE/J, respectively), with a more rapid decline in the CE/J strain (0.12 dB vs. 0.47 dB per month decrease for C57 and CE/J respectively). No qualitative difference was observed between young and old animals, of either strain, that would explain the observed macular functional decline. Reports in the literature concerning age related change in macular otoconia suggest structural (Ross, Peacor, Johnsson, & Allard, 1976; Jang et al., 2006) & ultra-structural changes (Campos, Canizares, Sanchez-Quevedo, & Romero, 1990), and an increased presence of giant otoconia, occurring more frequently on the edges of the macular surface (Suzuki, Katsuhisa, & Takasaka, 1997). In the current

study our assessment of macular otoconia was limited to a gross characterization of macular otoconia. Hypothetically, age related changes in otoconia production/turnover could result in ultra-structural differences (such as reduced Ca+ concentrations reported by Campos et al. (1990) in rats) in otoconia that could result in reduced otoconia density and decreased sensitivity to linear acceleration. It is unknown if this type of ultra-structural change would be apparent in SEM images. Suzuki et al., (1997) compared the globular substance (secreted precursors to mature otoconia) between young and old C57 mice (they did not assess otoconia numbers or structure directly) and their finding suggest reduced otoconia turnover in C57 mice. This is in sharp contrast to our findings of widespread coverage and dense concentrations of otoconia in the utricle and saccule of old C57 and CE/J mice. The observed decline in vestibular sensitivity therefore, may be due to degeneration of other macular structures such as hair cells (Anniko, 1983; Park et al., 1987; Nakayama et al., 1994), stereocilia/kinocilia (Rosenhall & Rubin, 1975; Nakayama et al., 1994), and/or primary afferents (Gleeson, et al., 1990) which are reported in the literature.

Work with the CBA strain has demonstrated age related decreases in auditory and macular function due to age alone (see the Chapter 4: *Gravity Receptor Aging in the CBA/CaJ Strain; A Comparison to Auditory Aging*). Age related auditory dysfunction is accelerated in *Ahl* strains, likely due to the *Ahl* mutation, but vestibular findings vary in the C57 and CE/J strains. Siemens et al. (2003) and Sollner et al. (2004) have shown that cadherin 23 is an essential component of cochlear and vestibular hair cell tip links and

lateral links and is necessary for the development and maintenance of mechanotransduction by these sensory structures.

If *Ahl* effects *Cdh23* expression in both cochlear and vestibular hair cells/stereocilia, why do auditory and macular function decline at significantly different rates in the C57 and CE/J strains and why does macular sensitivity decline at a significantly faster rate in the CE/J strain? A reasonable genetic explanation for these findings is the presence of an unknown genetic modifier in the C57 strain that modifies *Cdh23* expression in the vestibular macula and inhibits degeneration of macular structures/function. Genetic modifiers are "heritable factors capable of modifying the phenotype of a mutant gene without having an obvious effect on the normal condition" (Johnson, Zheng, & Noben-Trauth, (2006). Genetic modifiers are prevalent in all genetically mixed populations and their aggregate influence on a mutant phenotype is known as the *genetic background effect* or the *strain background effect* when referring to inbred strains. It is conceivable that the C57 strain carries a genetic modifier that is not present in the CE/J strain, which results in differential expression of the $Cdh23^{753A}$ mutation in cochlear and macular structures and results in preserved macular structures and function in the C57 strain. Further study is needed to: 1) pinpoint the genetic and anatomical sources of macular decline 2) identify the mechanisms for macular preservation in the C57 strain and 3) to determine the source of such disparate declines in macular function between strains carrying the *Ahl* mutation.

CHAPTER V: DISCUSSION

Mouse Models: Why?

Animal models play an important role in auditory and vestibular research and their use has resulted in a wealth of knowledge concerning the genetic and environmental causes of hearing loss and balance disorders. The mouse model has been of particular importance in the search for genetic contributions to hearing loss and balance disorders and a large and ever growing number of mutations that result in syndromic and non-syndromic hearing loss and balance disorders have been identified. The study of age effects on auditory and vestibular function in humans is hampered by individual differences in terms of lifestyle, the inherent heterogeneity of human genetics, and environmental factors such as infection, disease, physical insult, noise, and exposure to ototoxins. Anatomical and functional changes in the auditory system associated with aging are well studied and described in the literature. The effect of age on the vestibular system is much less understood and research in humans is impeded by the lack of a direct measure of vestibular function. The mouse model allows researchers to address many of these challenges to experimental control because mice can be selectively bred and housed in a controlled environment, function can be readily measured over the entire lifespan, and a direct measure of vestibular function is available. In order to develop appropriate mouse models for auditory and vestibular aging, it is necessary to accurately describe function over the whole lifespan of the potential mouse model.

Summary of "Age Alone" Study

The goals of this project regarding age effects alone (utilizing the CBA/CaJ strain) were:

1) to describe the effect of age alone on auditory and vestibular function

2) to describe the effect of age and gender on auditory and vestibular function

3) to compare the time course of changes between auditory and vestibular function.

The results of auditory measures in the current study are in good agreement with previously published structural studies that have identified decreases in the number of cochlear hair cells and reduced spiral ganglion densities with aging. Our results replicated previous studies in terms of the onset and rate of change of auditory function. The finding that CBA exhibit a slow progressive decline in auditory sensitivity for all frequencies, which begins around midlife, supports the widely held belief that the CBA strain is a good control strain for auditory measures, and genetic background for creation of new strains.

Gender

The current study differs from previous studies regarding gender differences in auditory sensitivity and change over time. Henry (2004) reported that male CBA mice showed poorer high frequency sensitivity than females at 1 year of age based on the ABR audiogram although no statistical analysis was performed. Henry (2002) reported that female C57 mice had poorer auditory sensitivity than males. Guimaraes et al. (2004) reported a gender difference in CBA mice, only for the old age group, using arbitrary age groups and ANOVA. Our results show no significant difference for the

slope or intercept of the linear regression line between male and female CBA mice. However, the ABR scatter plots for CBA do reveal small gender differences that replicate the trends reported by Henry (2002, 2004) and Guimaraes (2004). In the current study, male CBA mice tended to have poorer auditory sensitivity than females. Henry (2002, 2004) did not perform statistical analysis and his conclusions are based on characterization of scatter plots. Guimaraes et al., (2002) separated subjects into arbitrary young, middle, and old, age groups and only found a statistically significant ABR gender difference in the oldest group using ANOVA. It is possible that we did not find a significant gender difference like Guimaraes et al., 2002 for two reasons. 1) Their oldest age group included mice from 24 to 29 months of age. We did not measure mice older than 24 months. 2) They separated mice into arbitrary age groups and compared groups using ANOVA. We chose to use linear regression analysis and student's T-test to compare slopes.

Auditory Aging

Overall, auditory sensitivity showed a gradual decline and DPOAE results indicate decreased OHC function as once source of decreased auditory sensitivity. It is well established that the cochlea of CBA mice show age related structural change and gradual age related functional changes. What was not known before the current study was the effect of age and gender on macular function and the relationship between auditory and vestibular functional aging.

Macular Aging

The information gained by measuring macular function over the majority of the lifespan of the CBA mouse is completely new. The limited functional data available previously for the CBA strain suggested that macular function was maintained until at least 5 months of age. Our results show that there is gradual decline in macular sensitivity (an average of 0.39 dB per month) and that by 23 months of age there is an average loss of 48% of the dynamic range for VsEP. This slow progressive loss of macular function must be taken into consideration when utilizing the CBA strain as a control for vestibular studies or as a background strain for creation of new mouse strains.

SEM Findings

Scanning electron microscopy imaging of the macular organs rules out gross qualitative change in macular otoconia as possible explanations for the observed functional decline. Previous reports of age related change in otoconia include: reduced numbers of globular substance (substance secreted from the statoconial layer that develop into mature otoconia) in old C57 mice, which suggests reduced otoconia turnover (Suzuki et al., 1997) 2) ultra structural changes in otoconia morphology such as: increased fracturing, pitting, fragmentation, collapse, and weakened or broken linking filaments links (Jang et al., 2006; Campos et al., 1990) and 3) increased presence of giant otoconia, occurring more frequently on the edges of the macular surface. These studies suggest age related change at the cellular level in the macular organs and it is possible that these changes could result in otoconia with reduced

density. Reduced otoconial density could conceivably result in higher thresholds to linear acceleration. It is unknown if this hypothetical reduction in otoconia density would be revealed in SEM images. A reduction in otoconia density therefore cannot be ruled out as a possible explanation for age related reduction in macular sensitivity.

Global Membranous Labyrinth Change

The finding that auditory and macular sensitivity both decline in the CBA strain leads us to the question: W*hat global change in the membranous labyrinth might affect the auditory and vestibular organs equally*? Morphological studies of the inner ear have identified widespread age related change in the cochlea and vestibular organs. Reports of cochlear degeneration with age include: loss of inner and outer hair cells, missing/disarranged/fused stereocilia, increased fragility of the cuticular plate, reduced blood flow, degeneration of structures responsible for the maintenance of the endolymphatic potential such as the stria vascularis and spiral ligament, reduction in the density of primary afferent fibers, hair cell cytoplasmic protrusions, giant hair cells, the accumulation of a "wear and tear" pigment known as lipofuscin, and increased numbers of mitochondrial mutations. In the vestibular organs reports of age related structural change include: loss of type I and type II vestibular hair cells, missing/disarranged/fused stereocilia and kinocilia, hair cell cytoplasmic protrusions, giant hair cells, increased fragility of the cuticular plate, reduced primary afferent densities, reduced blood flow, morphological changes in macular otoconia, and reduced numbers of globular substances and otoconia turnover.

Schuknecht and Gacek's Classification

Age related degeneration of membranous labyrinth structures have typically been placed into several conceptual categories; 1) neural, involving primarily a loss of afferent innervation 2) metabolic, involving blood supply and non-sensory structures (stria vascularis, spiral ligament, vestibular dark cells, etc.) responsible for producing the endolymphatic potential 3) sensory, involving hair cells, stereocilia/kinocilia, or sensory supporting cells (Schuknecht & Gacek, 1993). Schuknecht's human temporal bone studies have shown that these pathologies may exist independently and concurrently. Pathology of the cochlear hair cells (primarily OHC) and their accompanying stereocilia are widely accepted as a primary cause of age related hearing loss and loss of spiral ganglion fibers is generally considered to be secondary to the loss of hair cells. The specific mechanisms behind hair cell degeneration and neuronal loss are not known. Several researchers have suggested that pathological changes in the stria vascularis and spiral ligament, which are critical structures to the maintenance of the endolymphatic homeostasis, are precursors to hair cell degeneration and neuronal loss (Ohlemiller & Gagnon, 2004; Hequembourg & Liberman, 2001; Gratton & Schulte, 1995; Spicer & Schulte, 2005). While there is strong evidence for age related degeneration of these structures, the results from studies of the EP itself are mixed depending on the location of measurement, species studied, and genetic strain utilized. The EP is inherently difficult to measure and varies by measurement site, species, and genetic strain and the findings concerning the EP itself vary depending on the animal model and genetic strain utilized; e.g. gerbils and BALB/cJ mice show a reduced EP as

function of age (Gratton et al., 1996) while the C57 and CBA/CaJ mouse strains do not (Lang et al., 2002; Ohlemiller et al. 2006; Wu & Marcus, 2003, Sha et al., 2008). The finding that the EP does not decrease with age in the CBA or C57 strains makes it unlikely that this is the source of decreased auditory and macular sensitivity seen with aging in these strains.

Mitochondrial Clock Theory

The maintenance of endolymphatic homeostasis is one type of metabolic process that occurs in the cochlea and vestibular organs. Another type of metabolic process occurs at the cellular level in all sensory and supporting cells of the cochlea and vestibular organs. Cellular metabolic activity involves mitochondria and aerobic respiration to create energy for cellular activity. Mitochondria produce cellular energy for virtually every cell in the body through mitochondrial oxidative phosphorylation and mitochondria are unique in that they contain their own DNA (mtDNA) (Anderson, et al., 1955; Chance & Williams, 1981). The membrane theory of aging, or mitochondrial clock theory, maintains that as animals age mtDNA mutations/deletions increase due to damage from reactive oxygen metabolites (ROM), which are toxic enzymes produced as by products of oxidative phosphorylation. An increase in mtDNA mutations/deletions and decreased metabolic efficiency eventually lead to a cellular "energy crisis" and cell death (apoptosis) (Harmon, 2002). Increased ROM production and mtDNA mutations/deletions are associated with reduced blood flow (Ohlemiller & Dugan, 1999), aging (Bai et al., 1997), and specific genetic mutations (Niu, Trifunovic, Larsson, & Canlon, 2007; Mcfadden, Ding, & Salvi, 2001; Yamasoba et al., 2007) and

commonly occur in post mitotic tissues that have high energy requirements (e.g. brain, heart, eyes, inner ear, muscle, and kidneys). An increased rate of mtDNA mutations/deletions in the cochlea with aging is supported by direct measures of cochlear tissues using polymerase chain reaction (PCR) (Wu, Du, Wang, Niu, & Jin, 2002, Markaryan, Nelson, & Hinojosa, 2008, Han, Han, & Jiang, 2000, Bai et al., 1997) and DNA microarrays (Yamasoba et al., 2007). Indirect evidence for decreased cochlear metabolic efficiency and increased ROM production as a result of aging includes: increased presence of lipofuscin (a wear-and-tear pigment that is a by product of cellular metabolic processes) in inner ear structures, reduced immunohistochemical staining for key molecules and proteins that detoxify ROM (SOD (Cu/Zn), SOD (Mn), Catalase, Glutathione perioxidase) (Staecker et al., 2001; Jiang et al., 2007; Lautermann, Crann, McLaren, & Schacht, 1997), poor auditory sensitivity in mutant strains with a genetic predisposition for increased mtDNA mutations/deletions (McFadden et al, 2001; Yamasoba et al., 2007; Niu et al., 2007), and amelioration of oxidative stress through use of antioxidants or dietary restriction (Seidman, Bai, khan, & Quirk, 2001; Seidman, 2000; Le & Keithley, 2007, Someya, Yamasoba, Weindruch, Prolla, & Tanokura, 2007).

A search of the literature found no direct evidence for decreased metabolic efficiency, increased ROM production, or mitochondrial mutations/deletions in the vestibular organs as an effect of age. Many studies however have reported the presence of lipofuscin in sensory (more pronounced in type I hair cells than type II hair cells) and supporting structures (Rosenhall & Rubin, 1975; Park et al., 1987; Gleeson et al., 1990;

Anniko, 1983) providing indirect evidence for reduced metabolic efficiency in vestibular sensory structures as an effect of age. A study by Lyon and Jensen (2001) in rats indicates that the blood flow to the cochlea is approximately double that of any of the individual vestibular organs and blood flow to each of the vestibular organs is roughly equal. The large vascular supply to the cochlea, relative to the macular organs, suggests that the metabolic demands of the cochlea are higher than those of the vestibular organs and would indicate that the cochlea may be at higher risk for mtDNA mutations/deletions and resulting apoptosis. This idea is not borne out by our functional findings in the CBA strain however. In spite of significant statistical difference between ABR and VsEP normalized threshold slopes, CBA results show functional decline that is similar for the cochlea and macular organs. In general, macular function declined at a slightly faster rate than auditory function in this strain but overall the rate of change in the two modalities is similar. A study of mtDNA mutations/deletions that included the vestibular organs as well as the cochlea in the CBA strain would further our understanding of inner ear degeneration at the cellular level and help elucidate the origin of the metabolic, sensory, and neuronal degeneration seen in the inner ear with aging.

Summary of Ahl Models

The primary goal of the current study was to characterize macular function in three inbred strains known to harbor *Ahl* mutations and determine the effect of *Ahl* and *Ahl2* on vestibular function. We were successful in establishing breeding colonies and measuring function in two strains that harbor *Ahl* (C57 and CE/J) but had limited

success in breeding sufficient numbers of the strain that carries *Ahl* and *Ahl2* (NOD. NON).

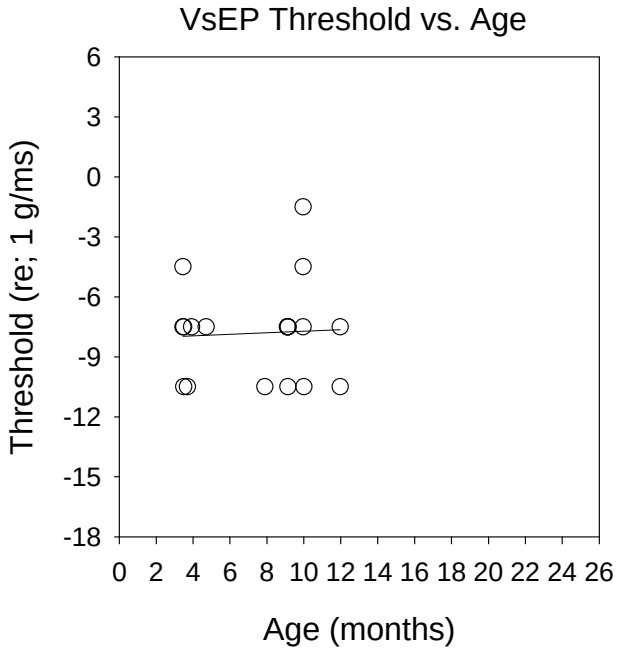
NOD. NON

The minimal functional data (22 mice to 11 months of age) collected for the NOD. NON strain shows that they have profound hearing loss from an early age (there were only 5 measurable ABR threshold in 22 animals tested) and maintain macular sensitivity out to 11 months of age (Fig. 42). These auditory results are in good agreement with previous auditory reports of profound hearing loss very early in life for this strain (Johnson & Zheng, 2002). The maintenance of macular function to mid-life is new information concerning vestibular function in a strain carrying *Ahl* and *Ahl2*. This finding indicates that macular function does not follow the rapid degeneration that occurs in auditory function when both *Ahl* and *Ahl2* are present. A larger sample size is needed to determine if this preliminary finding is correct and to characterize macular function beyond 11 months of age.

C57 and CE/J

We chose to use the C57 and CE/J strains to assess the effect of *Ahl* on vestibular function. The *Ahl* mutation effects *Cdh23* expression (a gene which encodes a family of proteins necessary for inner ear development/maintenance of sensory cell structures (Di Palma, Pellegrino, & Noben-Trauth, 2001). Immunostaining studies in mice, rats, frogs, and fish have identified *Cdh23* as a key component in cochlear and vestibular stereocilia and kinocilia ontogeny but the role of *Cdh23* later in the lifespan remains controversial. Seimens et al. (2004), Sollner et al. (2004), and Rzadzinska, Derr, Kachar, &

Figure 42. VsEP vs. Age for the NOD. NON strain. 100% of animals measured had a VsEP response. The limited data collected suggest that macular sensitivity is maintained until mid-life in the presence of the *Ahl* and *Ahl2* mutations in the NOD. NON strain.



Noben-Trauth et al., (2005) present evidence that Cdh23 is a component of tip links and is present in adult mouse cochlear stereocilia; while Boeda et al. (2002) and Lagziel et al. (2005) present evidence that Cdh23 is absent from stereocilia but remains present in Reissner's membrane (RM), in hair cell centrosomes, and in areas of kinocilia-stereocilia attachment. While age related morphological changes in the C57 cochlea have been well described (loss of inner and outer hair cells, missing/disarranged/fused stereocilia, increased fragility of the cuticular plate, reduced blood flow, degeneration of the stria cytoplasmic protrusions, giant hair cells, the accumulation of a "wear and tear" pigment known as lipofuscin, and increased numbers of mitochondrial mutations), the precise role of Cdh23 in age related cochlear dysfunction is unknown. The fact that C57 mice have "normal" hearing and macular sensitivity at young ages and maintain macular sensitivity while losing hearing suggests that *Cdh23* participation during cochlear and macular development is normal. This suggests that *Cdh23* expression later becomes abnormal for maintenance of cochlear stereocilia structures while remaining normal in macular structures. The CE/J strain loses auditory and macular sensitivity at a similar rate suggesting that *Cdh23* expression may become abnormal in both the cochlea and macular organs as a function of age. Interestingly, the rate of change in macular sensitivity for the C57 strain is significantly slower than the of the CBA strain; 0.12 dB loss per month and 21% total loss of dynamic range in C57 vs. 0.39 for CBA and 48 % for CBA (Fig. 43). The VsEP response appears more robust in C57 mice at advanced age compared to the CBA response (Fig. 44). In the CE/J strain, auditory and macular function declined at a similar rate suggesting that cochlear and vestibular stereocilia/kinocilia degenerate

Figure 43. A comparison of VsEP threshold for the C57 and CBA strains. The linear regression equations were y = -11.4 + 0.12x for C57 and y = -12.4 + 0.39x for CBA. The slope of the linear regression line for the two strains is significantly different ($p<0.001$).

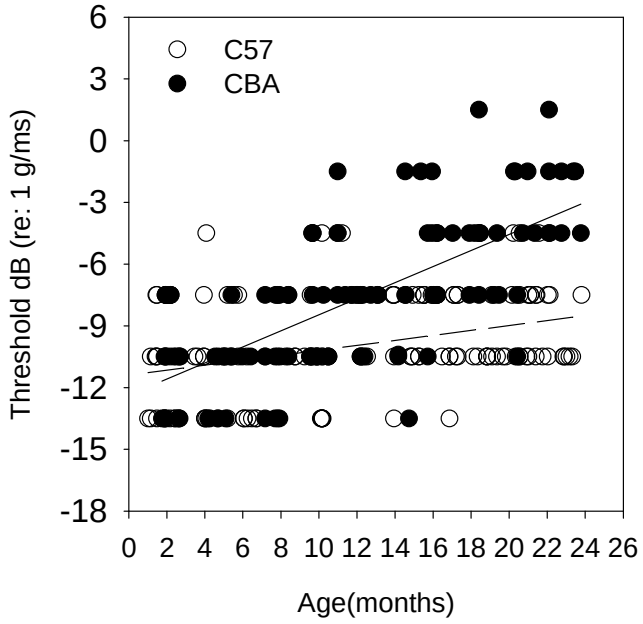
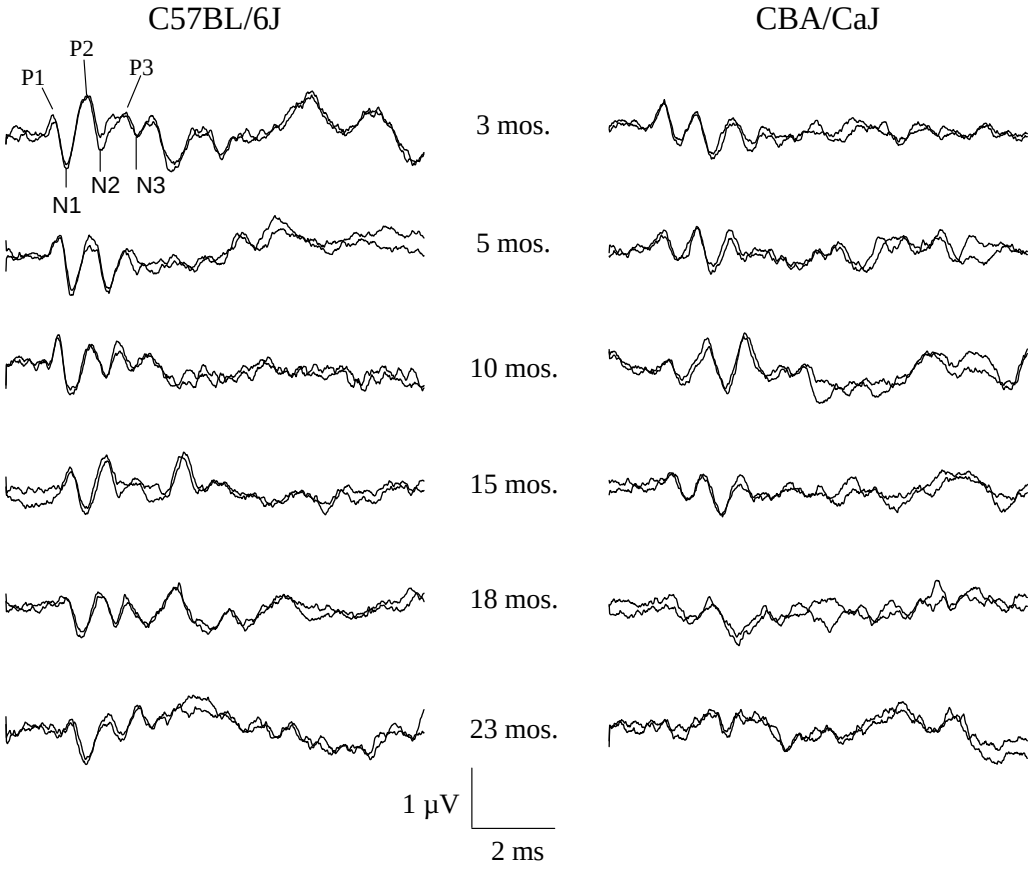


Figure 44. Representative waveforms for 6 ages for the C57 and CBA strains. Peaks P1, P2, P3, N1, N2, and N3 are labeled.



simultaneously in this strain. No significant difference was found for the rate of change in macular sensitivity between the CE/J and CBA strains, 0.58 dB loss per month reaching 48% total loss of dynamic range for CE/J versus 0.39 dB loss per month reaching 48 % total loss of dynamic range for CBA (Fig. 45). The slope of the linear regression lines for VsEP threshold between C57 and CE/J were significantly different (Fig. 46), suggesting distinct rates of change in macular sensitivity for these two *Ahl* strains. Figures 47 and 48 compare the rate of age related change in VsEP and 8 kHz ABR thresholds between the three strains.

C57 Genetic Modifier?

The finding that macular aging occurs at different rates in these two *Ahl* strains suggests that the effect of the *Ahl* mutation on *Cdh23* expression in vestibular hair cells/stereocilia is distinct in these two strains. A reasonable genetic explanation for this finding is the presence of an unknown genetic modifier, in the C57 strain, that modifies *Cdh23* expression in the vestibular macula and inhibits degeneration of macular structures/function. Genetic modifiers are “heritable factors capable of modifying the phenotype of a mutant gene without having an obvious effect on the normal condition” (Johnson, Zheng, & Noben-Trauth, 2006). Genetic modifiers are prevalent in all genetically mixed populations and their aggregate influence on a mutant phenotype is known as the *genetic background effect* or the *strain background effect* when referring to inbred strains. It is conceivable that the C57 strain carries a genetic modifier that is not present in the CE/J strain, which results in differential expression of the *Cdh23*^{753A}

Figure 45. A comparison of VsEP threshold for the CE/J and CBA strains. The linear regression equations were y = -10.2 + 0.47x for CE/J and y = -12.4 + 0.39x for CBA. The slope of the linear regression line for the two strains is not significantly different.

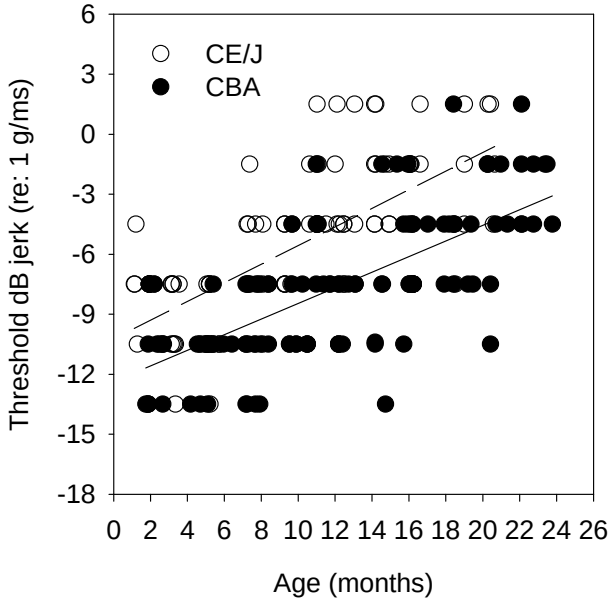


Figure 46. A comparison of VsEP threshold for the CE/J and C57 strains. The linear regression equations were y = -10.2 + 0.47x for CE/J and y = -11.4 + 0.12x for C57. The slope of the linear regression line for the two strains is significantly different ($p < 0.0001$).

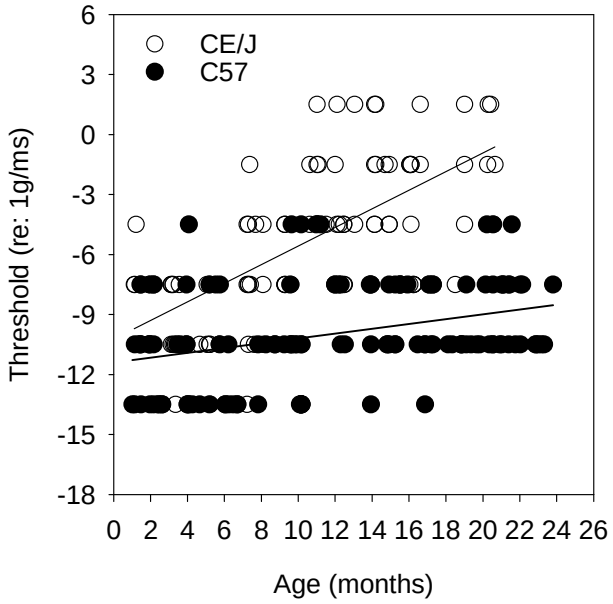


Figure 47. VsEP comparison between the CBA, C57, and CE/J strains. VsEP threshold plotted as a function of age for the CBA, C57, and CE/J strains. Linear regression equations were y = -11.4 + 0.12x for C57 (dotted line), y = -10.2 + 0.47x for CE/J (solid line), and y = -12.4 + 0.39x for CBA (long dashed line) strains respectively.

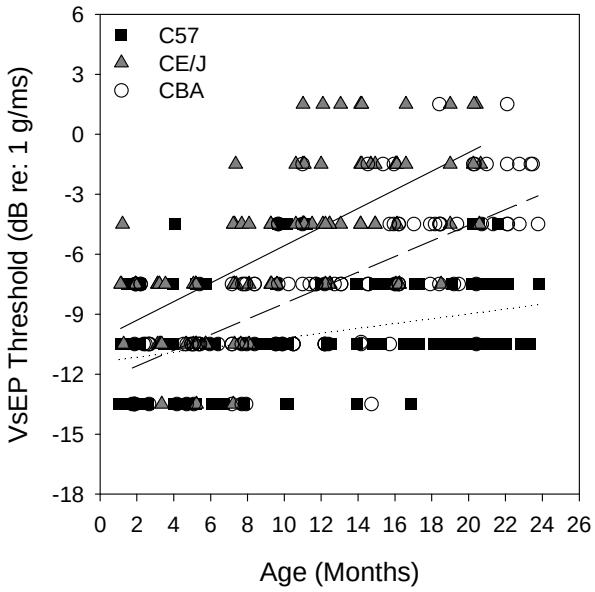
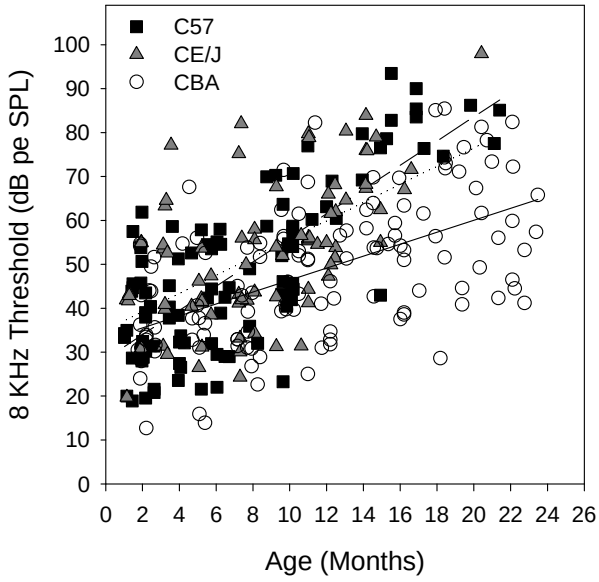


Figure 48. 8 kHz ABR threshold comparison between CBA, C57, and CE/J strains. 8 kHz threshold plotted as a function of age for the C57, CE/J, and CBA strains. The linear regression equations were y = 28.5 + 2.8x, y = 35.1 + 2.1x, and y = 32.8 + 1.36x for the C57 (dotted line), CE/J (long dashed line), and CBA (solid line) strains respectively. There is a large number of missing data points for advanced ages in the C57 and CE/J strains which results in a slope that underestimates the rate of decline in auditory sensitivity.



mutation in cochlear and macular structures and results in preserved macular structures and function in the C57 strain. Further study is needed to determine if such a genetic modifier exists in the C57 strain and explains such a disparate decline in macular function between the two strains.

Limitation of the Study and Implications for Future Research

Limitation of the Study

The vestibular measure used in the current study assesses the integrity of the macular organs and provides no direct information concerning the functional status of the SCC. Due to the complex morphological polarization vectors of macular hair cells it is likely that during both macular organs are stimulated by linear accelerations and both macular organs contribute to the response. The VsEP measures in the current study utilized a linear acceleration stimulus. The macular organs are vestibular structures that are primarily stimulated by gravitation force and linear accelerations and are the primary contributors to linear acceleration VsEPs (Nazareth & Jones, 1998; Jones & Jones, 1999). As a result, linear acceleration VsEPs originate primarily in the macular organs and the contribution of the SCC is likely minimal. Morphological studies that have assessed age related change in each of the five vestibular organs have shown mixed results, depending on the species and strain utilized. There are studies that have shown disparate changes in the SCC and the macular organs (Rauch, et al. 2001; Merchant, et al., 2000). The present study does not contribute any useful information for determining if aging effects are equivalent for the SCC and macular organs.

It was discussed previously that age related structural change in the macular organs are reported in the literature. These reports include 1) reduced numbers of globular substance (substance secreted from the statoconial layer that develop into mature otoconia) in old C57 mice, which suggests reduced otoconia turnover (Suzuki et al., 1997) 2) ultra structural changes in otoconia morphology such as: increased fracturing, pitting, fragmentation, collapse, and weakened or broken linking filaments links (Jang et al., 2006; Campos et al., 1990) and 3) increased presence of giant otoconia, occurring more frequently on the edges of the macular surface. These studies describe gross and ultra structural age related changes in otoconia and suggest degeneration at the cellular level in otoconia production. Such gross or ultra structural changes could conceivably result in otoconia with reduced density and result in higher thresholds to linear acceleration. A search of the literature fails to reveal studies regarding age related change in otoconia density. It is unknown if this hypothetical reduction in otoconia density would be revealed in SEM images. A reduction in otoconia density therefore cannot be ruled out as a possible explanation for age related reduction in macular sensitivity. SEM imaging in the current study was limited to views of the whole otoconial surface rather than individual otoconia or small groups of otoconia. As a result, images do not have the resolution necessary to identify structural or ultra structural changes at the level of individual otoconia. Though limited in resolution, these images do reveal large numbers of widely distributed, dense inclusions of otoconia in the utricle and saccule of old mice of all three strains with no apparent difference between young and old macular otoconia.

Research and Clinical Implications

Research Implications

In the discussion of age related changes in macular sensitivity of the CBA strain (our “age alone” model), we suggested that the changes seen in auditory and macular function may be due to reduced efficiency of metabolic processes, increased ROM production, and cumulative damage to mtDNA. Future study into the role of oxidative stress on macular structures in the CBA strain might help identify the role of oxidative stress in age related declines in macular sensitivity.

In the current study, the C57 strain auditory results replicate previous studies showing early onset hearing loss and our VsEP results indicate a slow decline in macular sensitivity reaching an average loss of 21 % in the oldest age group. We hypothesized that C57 auditory and macular function would decline at similar rates, as it does in the CBA strain and the second *Ahl* strain (CE/J) measured. This hypothesis was not borne out by our findings and auditory and macular function was shown to decline at significantly different rates in the C57 strain. Park, Hubel, and Wood (1987) reported on detailed morphological study of the vestibular organs of young and old C57BL/6NNia mice and describe an average 14 % and 19% age related loss of hair cell densities for the utricle and saccule respectively. The elderly mice in their study were between 29 and 31 months. They did not assess cochlear structures but other studies have reported losses of 80% of OHC and 100% in the base and 20% of IHC in the apex by 26 months of age (Spongr et al., 1997). It would be interesting to perform similar studies in mice closer to the oldest ages (23 months) used in our study and compare hair

cell densities to our functional data. Disparate rates of age related change in auditory and macular function, in the presence of a *Cdh23* mutation, suggests an unknown genetic modifier of the *Ahl* mutation in the macular organs of the C57 strain. Future genetic studies may identify this hypothetical genetic modifier of *Ahl* expression in C57 macular organs.

DPOAE measures were performed in the current study to characterize age related change in OHC function, to confirm the cochlea as the site of lesion, and to provide an indication of where in the cochlea the pathology is occurring (e.g. IHC vs. OHC). As a result, we measured and describe DPOAE amplitudes only. Future studies that utilize other DPOAE parameters, (threshold, input/output functions, phase, contralateral suppression, etc.) and focus on the time period surrounding the epoch when functional decline begins, may identify alterations in DPOAE that proceed (and therefore might predict) hearing loss.

Clinical Implications

The development of appropriate animal models is a critical step in determining factors that contribute to age related declines in auditory and vestibular function. Furthermore, animal models are a valuable tool for determining the genetic, structural, and physiological pathogenesis of age related declines in inner ear function. The indirect nature of existing vestibular measures in humans and central compensation for peripheral deficits makes it difficult to definitively separate peripheral and central pathologies. The findings of the current study, using a direct vestibular measure, for the CBA strain (our model for age alone) indicate similar rates of change in auditory and

macular sensitivity. If this finding is generalized to humans, it would suggest that patients diagnosed with ARHL are likely to suffer decreased peripheral vestibular function concomitantly. Unfortunately, audiological tests that differentiate between genetic and acquired hearing loss are not currently available. Even if such a diagnosis were possible, such generalizations must be made cautiously. The findings from the C57 strain (profound hearing loss and maintenance of macular sensitivity) highlights the fact that a genetic predisposition for change in one inner ear modality does not obligate change in the other inner ear modality. Clinicians must be cognizant of the role that genetic background plays in ARHL and vestibular dysfunction. It is conceivable that in the future, genetic profiles may be an integral part of medical records and clinicians may direct diagnostic testing and interventions based on an individual's known genetic background.

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APPENDIX A: CBA/CaJ RAW EXPERIMENTAL DATA

Study	Gender	Study Date	Age (months)	VsEP	ABR 8k	ABR 16k	ABR 32k	ABR 41.2
Age27	F	7/21/2005	1.94	-7.5	30.96	47.78	28.1	59.8
Age28	F	7/21/2005	1.94	-7.5	27.96	28.13	20.7	38.84
Age29	F	7/21/2005	1.94	-7.5	42.96	26.78	25.08	48.9
Age70	F	10/28/2005	1.87	-13.5	54.67	25.19	32.58	61.75
Age71	F	10/28/2005	1.87	-10.5	24	19.17	5.5	52.4
Age72	F	10/28/2005	1.74	-13.5	30.46	22.2	14.5	28.98
Age73	M	10/28/2005	1.87	-13.5	36.24	35.95	33.24	54.59
Age74	M	10/28/2005	1.87	-13.5	54.2	28.9	35.24	53
Age55	F	9/16/2005	2.20	-7.5	12.7	21.65	8.6	37.3
Age86	M	11/15/2005	2.47	-10.5	40.61	31.98	46.28	52.39
Age87	M	11/15/2005	2.47	NA	40.61	37.98	25.28	47.89
Age89	F	11/22/2005	2.70	-10.5	31.61	34.98	22.28	37.39
a225	F	10/13/2006	2.20	-7.5	33.46	47.53	37.88	41.77
a226	F	10/13/2006	2.20	NA	32.79	34.07	26.24	53.7
a227	F	10/13/2006	2.20	NA	33.89	35.3	38.53	53.27
a228	F	10/18/2006	2.37	-10.5	44	34.03	25.88	51.9
a292	M	1/11/2007	2.63	-10.5	51.6	31.4	12.4	68
a294	M	1/12/2007	2.66	-13.5	30.18	35.1	4.9	43.23
a295	F	1/12/2007	2.66	-10.5	35.63	47.1	21.9	29.27
Age5	M	1/11/2005	4.54	-10.5	67.61	43.98	58.28	61.39
Age8	M	2/3/2005	5.95	-10.5	NA	NA	NA	NA
Age20	F	5/3/2005	4.96	-10.5	40.61	13.98	19.28	25.39
Age38	F	8/25/2005	4.70	-10.5	37.7	20.5	19.3	48.5
Age39	F	8/25/2005	4.70	-13.5	32.8	19.7	25.1	37.3
Age46	M	9/8/2005	5.06	-10.5	37.78	44.3	6	59.9
Age47	M	9/9/2005	5.10	-13.5	31	20.95	12.5	50.8
Age48	M	9/9/2005	5.10	-10.5	15.9	30.67	16.2	47.8
Age49	M	9/13/2005	5.33	-10.5	33.87	23.78	31.54	46.4
Age50	M	9/15/2005	5.39	-10.5	36.57	28.17	27.52	60.18
Age51	M	9/15/2005	5.39	-7.5	13.9	13.27	11.7	43.1
Age85	M	11/11/2005	4.18	-13.5	46.41	34.98	22.26	49.42
a285	F	1/3/2007	4.96	-10.5	56	33	25	65.4
a300	M	1/19/2007	5.42	-10.5	52.6	29.86	42.48	49.3
Age12	F	2/17/2005	6.41	-10.5	NA	NA	NA	NA
Age19	M	4/21/2005	7.82	-7.5	NA	NA	NA	NA
Age26	M	7/15/2005	7.40	NA	42.9	37.98	34.28	64.39
Age32	F	7/26/2005	7.73	NA	39.23	30.67	15.52	38.84
Age33	F	7/26/2005	7.73	-7.5	34.66	45.59	27.6	49.56
a243	F	11/2/2006	7.17	-10.5	20.9	24	13.1	55.58
a244	F	11/2/2006	7.17	-10.5	20.9	23.28	10.6	42.2
a245	F	11/2/2006	7.17	-10.5	19.39	13.1	10.75	52.55
a246	F	11/2/2006	7.17	-13.5	33.02	31.9	25.2	31.86
a286	M	1/3/2007	7.69	-10.5	31	34.36	12.75	41.86
a287	M	1/3/2007	7.69	-13.5	53.78	45.3	53	58
a290	F	1/10/2007	7.92	-13.5	30.8	21.1	23.5	43
a291	F	1/10/2007	7.92	-7.5	26.8	42.1	26	68.3
a293	M	1/11/2007	7.96	-10.5	43.46	45.2	16.1	51.6

Study	Gender	Study Date	Age (months)	VsEP	ABR 8k	ABR 16k	ABR 32k	ABR 41.2
Age18	F	4/14/2005	8.25	NA	22.61	16.98	40.28	61.39
age61	M	9/23/2005	9.67	-4.5	55.75	39.26	29.36	60.7
age62	M	9/23/2005	9.67	-4.5	56.38	28.4	23.52	61.55
age63	M	9/23/2005	9.67	-7.5	71.49	39.13	30.8	59.5
a126	M	3/9/2006	9.53	-10.5	62.4	29.95	40.7	46.4
a127	M	3/9/2006	9.53	-10.5	39.3	31.88	13.06	42
a168	M	5/4/2006	9.90	-10.5	54.16	63.6	33.94	62.76
a169	M	5/4/2006	9.90	-10.5	45.99	39.77	22.6	63
a170	M	5/4/2006	9.90	NA	45.99	51.6	47.78	60.7
a302	F	3/7/2007	9.76	NA	40.86	23.43	49.7	49.9
a304	F	3/8/2007	9.80	NA	39.75	45.9	28.7	31.47
a307	F	3/22/2007	8.38	-7.5	28.86	32.3	28.7	44.9
a308	M	3/22/2007	8.38	-7.5	51.7	46	41.3	45.6
a309	M	3/22/2007	8.38	-10.5	54.8	46.86	37.83	71.3
Age75	F	11/2/2005	10.98	NA	51.2	55.3	24	68.9
Age76	F	11/2/2005	10.98	-7.5	25	30.2	12.34	44.5
Age77	F	11/2/2005	10.98	-1.5	68.73	53.6	61.88	64.9
Age78	F	11/2/2005	10.98	-4.5	50.95	40.13	22.6	39.68
a128	M	3/9/2006	10.22	-7.5	39.67	46.75	23.8	56.86
a129	F	3/9/2006	11.70	-7.5	41	43.75	39	63.2
a133	F	3/10/2006	11.74	-7.5	30.99	46.11	26.1	54.53
a152	M	4/7/2006	10.49	NA	61.48	51.6	47.8	66.12
a303	M	3/8/2007	10.49	-10.5	52	35	23.99	57
a310	M	3/22/2007	10.49	-10.5	52.99	45.37	52.77	56.89
a311	M	3/22/2007	10.49	-10.5	43.25	47.7	31.85	36.45
a332	M	4/25/2007	11.38	-7.5	82.23	77.48	NR	NR
a132	M	3/10/2006	12.39	-1.5	42.19	49.32	26.27	39.81
a156	M	4/20/2006	13.08	-4.5	57.68	53.9	31.2	63.36
a157	M	4/20/2006	13.08	-7.5	51.33	47.28	25.58	46.9
a171	F	5/8/2006	12.20	-7.5	34.68	39.6	12.24	39
a172	F	5/8/2006	12.20	-10.5	32.84	40.2	20.36	39.47
a173	F	5/8/2006	12.20	-7.5	46.14	51.6	47	73.08
a336	F	4/25/2007	12.20	-10.5	31.79	37.46	42.93	39.09
a434	M	8/23/2006	12.72	-7.5	56.55	53.15	62.25	68.12
Age36	F	8/19/2005	15.35	-1.5	65.46	NR	50.28	NR
age64	M	10/11/2005	14.17	-10.5	62.4	47.95	37.6	61.13
age65	M	10/11/2005	14.17	-7.5	58.17	37.79	39	51.3
age66	M	10/12/2005	15.72	-4.5	59.4	42.6	39.09	54.36
age67	M	10/12/2005	15.72	-4.5	56.5	45.6	40.1	48
a187	F	7/19/2006	14.56	-7.5	53.8	42.67	47.38	56.86
a188	F	7/19/2006	14.56	NA	42.67	33.4	21.84	47.72
a275	F	12/27/2006	15.95	-4.5	69.67	42.2	36.59	54
a276	F	12/27/2006	15.95	-4.5	NA	NA	NA	NA
a430	M	6/21/2006	14.53	-7.5	63.92	49.35	51.48	73.26
a431	M	6/21/2006	14.53	-1.5	69.78	62.98	52.7	69.98
a432	F	6/21/2006	14.73	-13.5	53.69	50.73	66.48	45.34
Age59	F	9/21/2005	17.92	-4.5	56.38	48.28	41.68	NR

Study	Gender	Study Date	Age (months)	VsEP	ABR 8k	ABR 16k	ABR 32k	ABR 41.2
age60	F	9/21/2005	17.92	-7.5	85.07	55.72	31.2	68.86
Age68	F	10/21/2005	16.01	-4.5	54.24	67.42	52.53	63.8
Age69	F	10/21/2005	16.01	-1.5	37.5	35.8	51	49.8
a197	M	8/21/2006	17.26	NA	61.58	44.8	63.84	75.11
a229	M	10/23/2006	17.03	NA	43.97	46.19	48.39	NA
a231	M	10/23/2006	17.03	-4.5	NA	NA	NA	NA
a445	F	10/30/2007	16.21	-7.5	38.56	49.1	46.58	69.2
a446	F	10/30/2007	16.21	-7.5	53.2	45.8	53.45	57.34
a447	F	10/30/2007	16.21	-7.5	39	58.36	54	65.7
a448	M	10/30/2007	16.21	-4.5	51	59.2	52.85	71.77
a449	M	10/30/2007	16.21	NA	63.39	45.2	NR	NR
Age21	F	5/3/2005	18.18	-4.5	28.61	31.98	16.28	37.39
Age54	M	9/16/2005	19.20	-4.5	71.1	NR	35.6	75.3
Age56	F	9/21/2005	19.36	-4.5	40.85	47.23	44.44	60.9
Age88	F	11/22/2005	18.48	-4.5	64.61	49.98	43.28	73.39
a184	F	7/14/2006	19.46	-4.5	76.66	56.76	46.42	63.92
a232	F	10/24/2006	19.36	NA	44.55	44.75	49	52.44
a450	F	10/31/2007	18.41	NA	NR	NR	NR	NR
a451	F	10/31/2007	18.41	-4.5	85.44	76.83	NR	76.3
a452	F	10/31/2007	18.41	-4.5	74.36	70.1	41.19	61.53
a453	F	10/31/2007	18.41	1.5	51.58	NA	53.19	62.9
a454	F	11/1/2007	18.44	-4.5	71.86	83.7	40.7	NR
Age34	M	7/27/2005	20.98	-1.5	73.39	59.25	NR	NR
Age35	M	7/27/2005	20.25	-1.5	NR	NR	NR	NR
a198	M	8/21/2006	20.71	-4.5	78.24	NR	NR	NR
a233	F	10/25/2006	20.12	NA	54.94	22.34	36.54	66.52
a277	M	12/27/2006	21.34	-4.5	42.3	31.65	23.9	53.68
a278	M	12/27/2006	21.34	NA	56	31.26	48.39	NR
a463	M	12/7/2007	20.32	-1.5	49.3	53.9	53.5	75.8
a464	M	12/10/2007	20.42	-4.5	61.68	56.9	62.67	NR
a465	M	12/10/2007	20.42	-4.5	81.25	69.27	NR	NR
a466	M	12/10/2007	20.42	NA	NR	78.3	NR	NR
a123	F	3/6/2006	23.38	-1.5	57.36	48	39	61
a125	F	3/9/2006	23.47	-1.5	65.79	59.95	41.08	76.42
a234	F	10/25/2006	22.75	-4.5	41.2	43.34	47.75	52.24
a235	F	10/25/2006	22.75	-1.5	53.24	43	48	64.13
a258	M	11/22/2006	23.77	-4.5	NA	NA	NA	NA
a284	F	1/3/2007	22.22	NA	44.5	45	22	42.8
a455	F	11/6/2007	22.09	-4.5	46.48	60.54	38.5	67.28
a456	F	11/6/2007	22.09	-1.5	59.86	84.3	60.29	68.2
a457	F	11/6/2007	22.09	1.5	82.4	86.9	NR	NR
a458	F	11/7/2007	22.13	-4.5	72.2	NR	NR	72.3

APPENDIX B: C57BL/6J ABR and VsEP RAW DATA

Study	Gender	Study Date	Age (months)	VsEP	ABR 8k	ABR 16k	ABR 32k	ABR 41.2
a176	F	5/26/2006	1.91	-10.5	45.78	61.5	10.4	71.9
a177	F	5/26/2006	1.91	NA	53.8	57.28	30.07	87.35
a221	F	10/12/2006	1.45	-7.5	18.89	22.7	12.39	42.68
a222	F	10/12/2006	1.45	-7.5	28.66	46.36	35.18	69.83
a238	M	10/27/2006	1.94	-10.5	30.76	32.6	36.88	54.68
a268	F	12/16/2006	1.02	NA	33.35	40.77	NA	55.65
a269	F	12/16/2006	1.02	-13.5	34.24	24.5	12.96	67.58
a270	M	12/18/2006	1.15	-13.5	20	26.3	23.78	55.4
a271	M	12/18/2006	1.15	-10.5	34.96	24.24	25	66.99
a279	M	12/28/2006	1.41	-10.5	43.86	21	23.78	66.36
a280	F	12/28/2006	1.48	-7.5	44.66	33.59	16.97	79.58
a281	F	12/28/2006	1.48	-10.5	57.46	32.94	13.88	67.29
a282	F	12/28/2006	1.48	-7.5	45.57	34.6	10.95	79.58
a296	F	1/19/2007	1.97	NA	NA	NA	NA	NA
a297	F	1/19/2007	1.97	-7.5	28	26.5	17	53.38
a298	F	1/19/2007	1.97	-13.5	61.89	51.49	64.37	77.27
a299	F	1/19/2007	1.97	-10.5	50.68	28.68	52.6	84.6
Age3	F	1/6/2005	3.62	-10.5	58.61	43.98	58.28	79.39
Age40	F	8/26/2005	3.95	-10.5	38.4	34.8	73.2	NR
Age41	F	8/26/2005	3.95	-10.5	23.55	13.1	21.89	34.99
Age42	F	8/26/2005	3.95	-7.5	51.25	37.38	54.46	NR
a199	F	8/24/2006	2.17	-13.5	19.52	22.75	26.24	31.29
a200	F	8/24/2006	2.17	-10.5	39	30.67	22.23	40.34
a201	F	8/24/2006	2.17	NA	30.8	18.67	9.76	50.25
a205	M	9/18/2006	2.17	-7.5	30.2	47	31.6	52.39
a206	M	9/18/2006	2.17	NA	38	43.58	19.3	41.27
a207	M	9/18/2006	2.17	-7.5	43.47	43.1	31.86	65.3
a211	F	9/20/2006	2.01	NA	32.43	49.1	14.48	42.68
a212	M	9/20/2006	2.04	NA	28.84	20.1	23.26	65.18
a217	M	10/9/2006	3.45	-10.5	45.13	34.75	26.36	55.07
a218	M	10/9/2006	3.45	NA	40.27	31.26	11.62	66.68
a219	M	10/9/2006	3.45	NA	37.77	29	11.46	67.44
a239	M	10/30/2006	2.63	-13.5	31.39	31.07	37.37	63.9
a240	M	10/30/2006	2.63	-13.5	21.57	21.59	13.35	41.68
a242	M	10/30/2006	2.63	NA	20.8	7.9	13.26	27.47
a305	F	3/21/2007	2.43	-13.5	40.25	19.44	4.9	34.9
a306	F	3/21/2007	2.43	-13.5	40.4	44	24.7	70.3
Age22	F	5/18/2005	4.67	-13.5	52.61	28.98	13.28	73.39
Age43	M	8/30/2005	4.08	-4.5	33.74	16.4	57.43	NR
Age44	M	8/30/2005	4.08	-13.5	26.53	24.57	45	67.4
a111	M	2/27/2006	5.75	NA	31.93	37.19	56.28	NR
a112	M	2/27/2006	5.75	-10.5	44.38	36.28	53.5	81
a113	M	2/27/2006	5.75	-7.5	53.55	33.9	NR	75
a160	M	4/26/2006	4.27	NA	32.11	48.15	28.4	27.3
a161	M	4/26/2006	4.27	-13.5	NA	NA	NA	NA
a202	M	9/14/2006	5.52	-7.5	54.5	47	NR	NR
a204	M	9/14/2006	5.52	NA	42.3	21.5	33.2	NR

Study	Gender	Study Date	Age (months)	VsEP	ABR 8k	ABR 16k	ABR 32k	ABR 41.2
a209	M	9/19/2006	4.01	-13.5	32.3	33.5	23	79
a210	M	9/19/2006	4.01	-13.5	27.4	29.99	9.8	66.88
a272	F	12/19/2006	5.19	-7.5	21.57	39.73	53	75.5
a273	F	12/19/2006	5.19	-13.5	57.86	45.7	57.76	81.68
a213	F	10/5/2006	6.21	-10.5	57.99	55.78	NR	NR
a223	M	10/12/2006	6.48	NA	29	19.38	45.7	65.13
a247	M	11/6/2006	6.25	-13.5	38.89	30.3	37.23	68.53
a248	F	11/6/2006	6.25	NA	54.55	42.97	36.17	NR
a254	F	11/13/2006	6.05	NA	29.5	37.9	22.95	56
a255	F	11/13/2006	6.05	-13.5	21.99	25.5	56.19	74.77
a256	M	11/20/2006	6.48	-13.5	42.5	34.07	44	67.14
a257	M	11/20/2006	6.05	-13.5	55.1	44.25	34.87	NR
a263	F	11/27/2006	6.71	-13.5	44.67	33.96	51.66	NR
a264	F	11/27/2006	6.71	-13.5	28.99	40.64	NR	65.4
a334	M	4/25/2007	7.82	-13.5	35.93	33.38	NR	NR
a335	M	4/25/2007	7.82	-10.5	49	no data	NR	NR
a162	M	4/27/2006	8.75	-10.5	69.94	80.18	NR	NR
a163	M	4/27/2006	8.75	-10.5	58.7	62.95	NR	NR
a164	F	4/28/2006	9.83	-10.5	42.52	56.14	49.24	72.97
a165	F	4/28/2006	9.83	NA	40.47	75.93	NR	NR
a166	M	5/3/2006	8.25	-10.5	31.99	48.4	23.62	63.97
a174	M	5/9/2006	9.57	-10.5	43.18	62	NR	NR
a175	M	5/9/2006	9.57	-7.5	51.97	58.75	NR	NR
a214	F	10/6/2006	9.63	-4.5	23.28	41	63.96	56.78
a215	F	10/6/2006	9.63	-10.5	63.66	41.66	NR	NR
a216	F	10/6/2006	9.63	-10.5	46.1	59.43	NR	NR
a220	F	10/11/2006	9.24	-10.5	70.29	59.28	NR	NR
a312	F	3/28/2007	9.93	-10.5	54.67	46.2	NR	NR
Age52	F	9/16/2005	11.21	-4.5	60.23	NR	NR	NR
a249	F	11/8/2006	10.16	-13.5	45.68	82.52	NR	NR
a250	M	11/8/2006	10.16	-13.5	58.44	62.4	NR	NR
a251	M	11/8/2006	10.16	-13.5	58.68	61.79	NR	NR
a252	M	11/9/2006	10.19	-10.5	44.3	NR	NR	NR
a253	M	11/9/2006	10.19	-13.5	70.68	48.86	NR	NR
a314	M	4/2/2007	10.09	-13.5	41.95	46.86	NR	NR
a317	M	4/4/2007	10.16	-13.5	54.06	63.5	NR	NR
a318	M	4/4/2007	10.16	-10.5	NR	NR	NR	NR
a319	M	4/4/2007	10.16	-4.5	56.76	51.9	61.49	78.23
a409	F	7/30/2007	10.98	-4.5	76.98	NR	NR	NR
Age30	F	7/22/2005	12.30	-7.5	68.95	NR	NR	NR
Age31	F	7/22/2005	12.30	-10.5	NR	NR	NR	NR
Age98	M	12/8/2005	13.94	-13.5	NR	NR	NR	NR
Age99	M	12/8/2005	13.94	-10.5	69.19	NR	NR	NR
Age100	M	12/8/2005	13.94	-7.5	79.75	NR	NR	NR
a101	M	12/8/2005	13.94	-7.5	NR	NR	NR	NR
a105	F	2/16/2006	13.87	-7.5	NR	NR	NR	NR
a192	M	7/21/2006	12.53	-10.5	NR	NR	NR	NR

Study	Gender	Study Date	Age (months)	VsEP	ABR 8k	ABR 16k	ABR 32k	ABR 41.2
a193	M	7/21/2006	12.53	NA	60.46	49.9	NR	NR
a316	F	4/4/2007	12.20	-7.5	NR	NR	NR	NR
a346	F	5/7/2007	12.00	-7.5	63.14	NR	NR	NR
a347	F	5/7/2007	12.00	-7.5	NR	NR	NR	NR
a116	F	3/2/2006	15.25	-10.5	NR	NR	NR	NR
a117	F	3/2/2006	15.25	-10.5	78.62	NR	NR	NR
a118	F	3/2/2006	15.25	-7.5	NR	NR	NR	NR
a119	F	3/3/2006	14.37	NA	NR	NR	NR	NR
a138	M	3/20/2006	14.93	-7.5	76.52	52.5	NR	NR
a141	M	3/20/2006	14.93	-10.5	42.96	57.22	NR	NR
a144	M	3/22/2006	15.91	NA	NR	NR	NR	NR
a145	M	3/22/2006	15.91	-7.5	NA	NA	NA	NA
a146	M	3/23/2006	14.83	-10.5	NR	NR	NR	NR
a148	M	3/23/2006	14.83	-10.5	NR	NR	NR	NR
a189	F	7/20/2006	15.52	-7.5	NR	NR	NR	NR
a190	F	7/20/2006	15.52	-7.5	93.46	NR	NR	NR
a191	F	7/20/2006	15.52	-7.5	82.79	79.97	NR	NR
a106	F	2/23/2006	17.19	-10.5	NR	NR	NR	NR
a107	F	2/23/2006	16.47	-10.5	NR	NR	NR	NR
a134	M	3/20/2006	17.29	-7.5	NA	NA	NA	NA
a135	M	3/20/2006	17.29	-10.5	NR	NR	NR	NR
a136	M	3/20/2006	17.29	-7.5	76.39	NR	NR	NR
a137	M	3/20/2006	17.29	NA	NR	NR	NR	NR
a195	M	8/17/2006	17.10	-7.5	NR	NR	NR	NR
a196	F	8/17/2006	17.10	-7.5	NR	NR	NR	NR
a265	F	11/30/2006	16.87	-10.5	83.55	NR	NR	NR
a266	F	11/30/2006	16.87	-13.5	90	NR	NR	NR
a267	F	11/30/2006	16.87	-10.5	85.39	NR	NR	NR
Age23	F	5/19/2005	19.36	-10.5	NR	NR	NR	NR
Age90	F	11/29/2005	18.84	-10.5	NR	NR	NR	NR
Age91	F	11/29/2005	18.84	-10.5	NR	NR	NR	NR
Age92	F	11/29/2005	18.84	-10.5	NR	NR	NR	NR
Age96	M	12/7/2005	19.10	-10.5	NR	NR	NR	NR
Age97	M	12/7/2005	19.10	-7.5	NR	NR	NR	NR
a109	M	2/24/2006	18.12	-10.5	NR	NR	NR	NR
a115	F	3/2/2006	19.66	-10.5	NR	NR	NR	NR
a120	M	3/3/2006	18.35	-10.5	74.64	NR	NR	NR
a155	M	4/17/2006	19.82	-10.5	86.23	NR	NR	NR
Age93	M	12/7/2005	20.55	-10.5	NR	NR	NR	NR
Age94	M	12/7/2005	20.55	-4.5	NR	NR	NR	NR
Age95	M	12/7/2005	20.55	-10.5	NA	NA	NA	NA
a104	M	2/16/2006	21.44	-7.5	NR	NR	NR	NR
a114	F	2/27/2006	20.22	-4.5	NR	NR	NR	NR
a131	M	3/9/2006	20.55	-7.5	NR	NR	NR	NR
a142	F	3/22/2006	21.76	-10.5	NR	NR	NR	NR
a149	F	3/23/2006	21.01	-7.5	NR	NR	NA	NA
a150	F	3/23/2006	21.01	-10.5	NR	NR	NR	NR

Study	Gender	Study Date	Age (months)	VsEP	ABR 8k	ABR 16k	ABR 32k	ABR 41.2
a158	M	4/25/2006	21.40	-10.5	NR	NR	NR	NR
a159	M	4/25/2006	21.40	-7.5	85.1	NR	NR	NR
a167	F	5/3/2006	20.35	-10.5	NR	NR	NR	NR
a178	M	5/26/2006	21.11	-7.5	NR	NR	NR	NR
a179	M	5/26/2006	21.11	-7.5	77.53	NR	NR	NR
a208	M	9/18/2006	21.57	-4.5	NR	NR	NR	NR
a236	F	10/27/2006	20.15	-7.5	NR	NR	NR	NR
a315	F	4/2/2007	20.91	-10.5	NR	NR	NR	NR
a103	F	2/15/2006	22.88	-10.5	NR	NR	NR	NR
a108	F	2/23/2006	23.80	-7.5	NR	NR	NR	NR
a110	M	2/24/2006	23.18	-10.5	NR	NR	NR	NR
a121	M	3/6/2006	22.03	-10.5	NR	NR	NR	NR
a122	M	3/6/2006	22.03	-7.5	NR	NR	NR	NR
a348	F	5/31/2007	22.85	-10.5	NR	NR	NR	NR
C57_2	M	7/23/2006	23.01	-10.5	NA	NA	NA	NA
C57_3	M	7/23/2006	22.13	-7.5	NA	NA	NA	NA
Old2	M	5/24/2006	23.31	-10.5	NA	NA	NA	NA

APPENDIX C: CE/J ABR and VsEP RAW DATA

Study	Gender	Study Date	Age (months)	VsEP	ABR 8k	ABR 16k	ABR 32k	ABR 41.2
a382	M	6/29/07	1.15	-7.5	19.75	28.45	44.5	57.54
a395	M	7/20/07	1.12	-7.5	41.9	36.78	62.58	68.65
a396	F	7/23/07	1.22	-4.5	41.7	26.67	60.99	NR
a397	F	7/23/07	1.94	-7.5	55	39.1	66.1	58.76
a398	F	7/23/07	1.94	-7.5	54.9	27.6	62	69.56
a399	F	7/23/07	1.94	-7.5	30.9	39.7	NR	NR
a402	F	7/25/07	1.28	-7.5	43.3	29.1	58.95	57.98
a404	F	7/25/07	1.28	-10.5	42.9	28.7	54.75	58.1
a321	M	4/12/07	3.12	-10.5	53.5	26.4	54	71.56
a322	F	4/12/07	3.12	-7.5	54.4	37.95	64.9	71.56
a323	F	4/12/07	3.12	-7.5	31.32	25.24	42.19	70.38
a324	F	4/13/07	3.22	-7.5	63.3	50.89	53.89	65.59
a325	F	4/13/07	3.22	-7.5	39.75	20	48.34	72.2
a326	F	4/13/07	3.22	-10.5	40.86	20	39.67	69.34
a327	M	4/17/07	3.29	-10.5	64.59	26	63	NR
a330	M	4/19/07	3.35	-13.5	29.48	29.13	55.45	72.53
a331	M	4/19/07	3.35	-10.5	52.59	27.86	53.26	70.76
a333	M	4/25/07	3.55	-7.5	77.14	69.3	NR	NR
a180	M	6/22/06	5.06	-10.5	46.15	28.11	51.1	55.34
a181	M	6/22/06	5.06	-7.5	26.5	33.34	49.88	63.62
a182	F	7/12/06	5.72	-10.5	47.4	50.2	44.7	58.24
a183	F	7/12/06	5.72	-10.5	38.48	44.25	56.39	77.23
a339	F	4/30/07	5.19	-10.5	41.23	33.55	66	NR
a340	F	4/30/07	5.19	-7.5	53.67	35.15	41.39	68.89
a341	F	4/30/07	5.19	-7.5	41.56	34.56	54.59	72.29
a342	F	4/30/07	5.19	-7.5	42.18	34.15	NR	NR
a343	M	5/1/07	4.67	-10.5	40.46	27.1	37.22	67.94
a344	F	5/1/07	5.23	-10.5	41.56	39.16	62.56	74.2
a345	F	5/1/07	5.23	-13.5	31.15	38.48	NR	NR
a349	F	6/4/07	7.63	-10.5	49.93	52.98	NR	NR
a350	F	6/4/07	7.30	-7.5	30.07	29.89	34.23	55.3
a351	F	6/4/07	7.30	-4.5	41.7	37	61.2	67.5
a352	F	6/4/07	7.30	-7.5	24.33	46.7	39.7	56.23
a353	F	6/4/07	7.30	-10.5	33.2	22.96	41.97	67.78
a354	F	6/5/07	7.23	-13.5	41.18	43.15	60.03	55.65
a355	F	6/5/07	7.23	-7.5	43.18	28.33	NR	70.08
a356	F	6/5/07	7.23	-4.5	75.24	73.3	NR	NR
a357	M	6/6/07	7.36	-1.5	82.03	69.7	NR	NR
a358	M	6/6/07	7.69	-4.5	41.84	38.58	66.2	72
a359	M	6/7/07	7.40	NA	55.96	38.77	63.67	71.39
a360	M	6/7/07	7.40	-7.5	41.95	39.74	63.98	74.2
a288	M	1/4/06	8.09	-7.5	58	25.3	36.96	NR
a289	M	1/4/06	8.09	-10.5	34.29	25	34	65.4
a328	M	4/18/07	9.60	-7.5	52	62.94	60.5	NR
a361	M	6/13/07	9.27	-7.5	53.95	27.46	NR	NR
a362	F	6/13/07	9.27	-7.5	31.28	43.48	49.41	71.89
a363	F	6/13/07	9.27	-4.5	42.61	41.49	60.69	71.72

Study	Gender	Study Date	Age (months)	VsEP	ABR 8k	ABR 16k	ABR 32k	ABR 41.2
a364	F	6/13/07	9.27	-4.5	67.66	52.38	NR	NR
a365	M	6/14/07	9.30	-7.5	NR	75.72	NR	NR
a405	M	7/27/07	8.09	NA	43.7	27.9	49.29	74.3
a406	M	7/27/07	8.09	-4.5	55.6	44.27	NR	NR
a373	M	6/25/07	11.01	-4.5	55.72	51.06	NR	NR
a374	M	6/25/07	11.01	1.5	55.98	40.64	NR	NR
a371	M	6/25/07	11.01	-4.5	41.24	38.74	NR	NR
a372	M	6/25/07	11.01	-4.5	55.37	71.15	67.64	NR
a375	M	6/25/07	11.01	-1.5	79.72	72.16	NR	NR
a376	M	6/25/07	11.01	-4.5	44.3	53.89	56	73.08
a377	F	6/26/07	11.05	-4.5	78.85	74.35	66.58	NR
a378	F	6/27/07	11.08	-4.5	55.74	38.48	NR	NR
a379	F	6/27/07	11.08	-1.5	56	25.46	51.58	66.66
a380	M	6/27/07	10.62	-4.5	31.4	44.93	NR	NR
a381	M	6/27/07	10.62	-1.5	56.55	53.89	58.09	NR
a429	F	8/8/07	11.51	-4.5	54.57	61.67	NR	NR
a368	F	6/22/07	13.05	1.5	80.36	61.09	NR	NR
a369	F	6/22/07	13.05	-4.5	64.66	50.15	62.46	71.03
a366	M	6/18/07	12.49	NA	53.84	71	NR	NR
a367	M	6/18/07	12.49	-7.5	62	31.58	63.18	NR
a400	F	7/25/07	12.00	-1.5	54.97	53.36	NR	NR
a425	M	8/22/07	12.46	-4.5	68.16	71.58	67.1	NR
a426	M	8/22/07	12.46	-7.5	51.5	49.24	NR	NR
a428	M	8/22/07	12.46	-4.5	51.5	49.24	NR	NR
a467	F	12/13/07	12.10	1.5	47.3	52.6	NR	NR
a468	M	12/13/07	12.10	-4.5	66	64.8	NR	NR
a469	F	12/17/07	12.23	-7.5	61.6	67.3	49.7	NR
a470	F	12/17/07	12.23	-4.5	49.9	43.5	NR	72.8
a320	F	4/11/07	14.70	-1.5	79	65.8	62.8	NR
a329	M	4/18/07	14.93	-1.5	62.49	51.5	66.59	NR
a384	M	7/9/07	14.14	1.5	67.32	53.87	NR	NR
a385	F	7/9/07	14.14	-4.5	83.92	80.68	NR	NR
a386	F	7/9/07	14.14	-1.5	68.2	74.67	NR	NR
a387	F	7/9/07	14.14	-4.5	75.95	75.46	NR	NR
a389	M	7/11/07	14.20	-1.5	NR	NR	NR	NR
a390	M	7/11/07	14.20	NA	NR	75.65	NR	NR
a391	M	7/11/07	14.20	4.5	75.88	NR	NR	NR
a392	M	7/12/07	14.93	-4.5	54.98	53.85	63.86	66.56
a393	M	7/12/07	14.93	-4.5	NR	NR	NR	NR
a435	F	10/10/07	16.04	-1.5	NR	NR	NR	NR
a436	F	10/10/07	16.04	-1.5	NR	NR	NR	NR
a437	M	10/10/07	16.04	-7.5	NR	NR	NR	NR
a438	M	10/12/07	16.11	-1.5	NR	NR	NR	NR
a439	M	10/12/07	16.11	-4.5	NR	NR	NR	NR
a440	M	10/12/07	16.11	-1.5	NR	NR	NR	NR
a441	M	10/16/07	16.24	-7.5	NR	NR	NR	NR
a442	M	10/16/07	16.24	-7.5	67	76.73	NR	NR

Study	Gender	Study Date	Age (months)	VsEP	ABR 8k	ABR 16k	ABR 32k	ABR 41.2
a443	M	10/16/07	16.24	-7.5	NR	81.2	NR	NR
km2	F	1/18/08	16.37	NA	NR	NR	NR	NR
km3	F	1/25/08	16.60	1.5	NR	NR	NR	NR
km4	F	1/25/08	16.60	-1.5	71.6	77.5	NR	NR
a444	F	10/29/07	18.51	-7.5	NR	NR	NR	NR
km5	M	3/20/08	18.67	NA	NA	NA	NA	NA
km6	M	3/20/08	18.67	NA	NA	NA	NA	
km7	F	3/30/08	19.00	1.5	NA	NA	NA	NA
km8	M	3/30/08	19.00	-1.5	NA	NA	NA	NA
km9	M	3/30/08	19.00	-4.5	NA	NA	NA	NA
km12	M	4/24/08	20.38	NA	NR	NR	NR	NR
km13		4/24/08	20.38	NA	NA	NA	NA	NA
km14	M	5/2/08	20.65	-1.5	NA	NA	NA	NA
km15	M	5/3/08	20.42	1.5	98	83	NR	NR
km16	M	5/3/08	20.42	NA	NA	NA	NA	NA
km18	M	5/7/08	20.25	-1.5	NR	NA	NA	NA
km19	M	5/8/08	20.28	1.5	NR	NR	NR	NR
km20	M	5/8/08	20.58	NA	NA	NA	NA	NA
km21	M	5/8/08	20.58	-4.5	NA	NA	NA	NA

APPENDIX D: NOD.NON-H2nb1/LtJ ABR and VsEP RAW DATA

Study	Gender	DOB	Study Date	Age (months)	VsEP	ABR 8k	ABR 16k	ABR 32k	ABR 41.2
a411	F	04/18/07	08/01/07	3.45	-4.5	NR	NR	NR	NR
a412	F	04/18/07	08/01/07	3.45	-7.5	NR	NR	NR	NR
a413	F	04/18/07	08/02/07	3.48	-7.5	NR	NR	69.45	NR
a414	F	04/18/07	08/02/07	3.48	-7.5	63.6	NR	NR	NR
a422	M	04/18/07	08/08/07	3.68	-10.5	NR	NR	NR	NR
a424	M	04/18/07	08/15/07	3.91	-7.5	NR	NR	NR	NR
a274	M	07/31/06	12/21/06	4.70	-7.5	NR	NR	NR	NR
a313	F	07/31/06	03/28/07	7.89	-10.5	72	NR	NR	NR
a415	M	11/02/06	08/06/07	9.11	-7.5	NR	NR	NR	NR
a416	M	11/02/06	08/06/07	9.11	NA	NR	NR	NR	NR
a417	F	11/02/06	08/07/07	9.14	-10.5	NR	NR	NR	NR
a418	F	11/02/06	08/07/07	9.14	NA	NR	NR	NR	NR
a419	F	11/02/06	08/07/07	9.14	-7.5	NR	NR	64.4	NR
a420	M	11/02/06	08/07/07	9.14	-7.5	NR	NR	64.2	NR
a421	M	11/02/06	08/07/07	9.14	-7.5	NR	NR	NR	NR
a459	M	01/10/07	11/09/07	9.96	-1.5	NR	NR	NR	NR
a460	M	01/10/07	11/09/07	9.96	-4.5	NR	NR	NR	NR
a461	M	01/10/07	11/09/07	9.96	-7.5	NR	NR	NR	NR
a462	F	01/10/07	11/10/07	9.99	-10.5	NR	NR	NR	NR
a407	F	07/31/06	07/30/07	11.97	-10.5	NR	NR	NR	NR
a408	M	08/14/06	07/30/07	11.51	NA	NR	NR	NR	NR
a410	M	07/31/06	07/30/07	11.97	-4.5	NR	NR	NR	NR

APPENDIX E: CBA/CaJ DPOAE AVERAGE AMPLITUDES BY AGE GROUP

Geometric Mean Frequency	<2 Months	2-3 Months	4-5 Months	6-7 Months	8-9 Months	10-11 Months	12-13 Months	14-15 Months	16-17 Months	18-19 Months	20-21 Months	22-23 Months	Mean noise floor
5.77	-11.09	-7.25	-4.43	-0.96	0.71	-7.93	-7.25	-11.05	-5.43	-9.77	-16.57	-12.54	-22.23
6.75	-2.64	-5.18	-4.25	2.40	4.42	1.72	-1.61	-10.23	-2.75	-7.57	-18.92	-4.83	-22.33
7.56	5.83	6.55	2.90	11.56	8.10	13.37	8.01	0.64	9.10	-1.52	-4.63	4.33	-17.76
8.94	13.35	13.77	10.45	17.61	14.64	18.59	8.59	7.97	14.58	6.92	2.46	14.06	-18.74
10.04	18.64	13.30	10.66	20.47	9.57	15.67	13.00	12.08	15.13	6.70	2.86	14.03	-14.98
11.02	11.86	1.38	10.11	14.89	4.47	3.69	4.78	8.68	5.72	-5.60	0.49	6.30	-17.05
11.74	21.14	9.74	14.68	16.05	14.20	15.64	20.72	16.91	4.84	-1.37	3.74	-0.37	-16.80
13.42	33.65	25.08	25.93	28.41	28.83	34.89	34.61	27.29	24.11	16.89	10.57	15.36	-17.43
14.51	37.13	25.11	27.42	28.80	28.95	32.28	31.77	32.50	26.43	18.03	18.98	19.82	-18.09
15.70	34.16	24.92	25.70	24.88	21.34	23.69	24.76	27.01	21.93	21.50	13.64	21.49	-17.06
17.89	35.10	27.24	25.90	26.68	21.30	28.26	28.13	18.09	19.50	21.11	5.35	19.78	-15.36
18.98	34.88	26.42	21.83	25.76	17.76	23.54	20.92	26.92	15.46	21.43	17.28	18.43	-16.29
21.26	17.45	6.27	9.31	9.27	8.81	4.86	9.37	13.33	1.64	5.10	19.81	2.84	-15.25
23.46	18.53	-1.54	9.80	-0.84	9.12	17.73	18.56	18.49	4.28	1.94	11.27	-1.14	-17.29
24.64	15.09	5.40	11.08	5.77	9.13	10.67	14.13	19.73	9.20	7.05	9.06	1.49	-18.21
26.83	15.91	15.26	7.82	20.86	10.98	2.51	-2.25	13.07	6.56	7.08	12.85	-0.37	-17.51
29.02	22.24	21.49	12.25	20.88	18.49	9.87	9.29	16.55	14.69	9.47	11.31	3.76	-17.55
31.22	9.58	9.95	0.04	5.82	6.74	9.64	18.47	10.00	-0.73	3.24	2.76	4.74	-17.32
33.59	10.59	4.61	2.67	4.28	7.99	13.01	15.17	2.88	1.06	4.46	-8.16	-0.92	-17.81
35.78	4.55	1.74	-1.61	6.27	4.99	5.48	8.79	-2.41	-2.70	0.66	-8.39	6.61	-17.35
38.95	7.47	4.49	-0.84	4.78	0.83	8.64	-3.91	2.12	-7.69	-3.58	-3.71	1.52	-16.50
41.26	5.13	1.18	-3.73	6.47	0.56	6.47	-1.21	2.83	1.88	1.13	-9.10	-1.70	-18.03
43.54	2.48	-6.01	-5.78	-6.69	-1.43	2.56	-0.92	-2.52	-2.05	-6.91	-2.43	-1.06	-19.22
46.02	-2.00	-12.28	-6.92	-11.69	-7.69	-10.03	-5.24	-0.59	-8.99	-14.18	-9.31	-11.63	-18.44
47.61	-7.74	-9.77	-8.28	-12.84	-10.19	-7.15	-9.50	2.60	-9.82	-14.34	-9.37	-10.18	-19.24

APPENDIX F: C57BL/6J DPOAE AVERAGE AMPLITUDES BY AGE GROUP

Geometric Mean Frequency	<2 Months	2-3 Months	4-5 Months	6-7 Months	8-9 Months	10-11 Months	12-13 Months	14-15 Months	16-17 Months	18-19 Months	20-21 Months	22-23 Months	Mean noise floor
5.77	-11.09	-7.25	-4.43	-0.96	0.71	-7.93	-7.25	-11.05	-5.43	-9.77	-16.57	-12.54	-22.23
6.75	-2.64	-5.18	-4.25	2.40	4.42	1.72	-1.61	-10.23	-2.75	-7.57	-18.92	-4.83	-22.33
7.56	5.83	6.55	2.90	11.56	8.10	13.37	8.01	0.64	9.10	-1.52	-4.63	4.33	-17.76
8.94	13.35	13.77	10.45	17.61	14.64	18.59	8.59	7.97	14.58	6.92	2.46	14.06	-18.74
10.04	18.64	13.30	10.66	20.47	9.57	15.67	13.00	12.08	15.13	6.70	2.86	14.03	-14.98
11.02	11.86	1.38	10.11	14.89	4.47	3.69	4.78	8.68	5.72	-5.60	0.49	6.30	-17.05
11.74	21.14	9.74	14.68	16.05	14.20	15.64	20.72	16.91	4.84	-1.37	3.74	-0.37	-16.80
13.42	33.65	25.08	25.93	28.41	28.83	34.89	34.61	27.29	24.11	16.89	10.57	15.36	-17.43
14.51	37.13	25.11	27.42	28.80	28.95	32.28	31.77	32.50	26.43	18.03	18.98	19.82	-18.09
15.70	34.16	24.92	25.70	24.88	21.34	23.69	24.76	27.01	21.93	21.50	13.64	21.49	-17.06
17.89	35.10	27.24	25.90	26.68	21.30	28.26	28.13	18.09	19.50	21.11	5.35	19.78	-15.36
18.98	34.88	26.42	21.83	25.76	17.76	23.54	20.92	26.92	15.46	21.43	17.28	18.43	-16.29
21.26	17.45	6.27	9.31	9.27	8.81	4.86	9.37	13.33	1.64	5.10	19.81	2.84	-15.25
23.46	18.53	-1.54	9.80	-0.84	9.12	17.73	18.56	18.49	4.28	1.94	11.27	-1.14	-17.29
24.64	15.09	5.40	11.08	5.77	9.13	10.67	14.13	19.73	9.20	7.05	9.06	1.49	-18.21
26.83	15.91	15.26	7.82	20.86	10.98	2.51	-2.25	13.07	6.56	7.08	12.85	-0.37	-17.51
29.02	22.24	21.49	12.25	20.88	18.49	9.87	9.29	16.55	14.69	9.47	11.31	3.76	-17.55
31.22	9.58	9.95	0.04	5.82	6.74	9.64	18.47	10.00	-0.73	3.24	2.76	4.74	-17.32
33.59	10.59	4.61	2.67	4.28	7.99	13.01	15.17	2.88	1.06	4.46	-8.16	-0.92	-17.81
35.78	4.55	1.74	-1.61	6.27	4.99	5.48	8.79	-2.41	-2.70	0.66	-8.39	6.61	-17.35
38.95	7.47	4.49	-0.84	4.78	0.83	8.64	-3.91	2.12	-7.69	-3.58	-3.71	1.52	-16.50
41.26	5.13	1.18	-3.73	6.47	0.56	6.47	-1.21	2.83	1.88	1.13	-9.10	-1.70	-18.03
43.54	2.48	-6.01	-5.78	-6.69	-1.43	2.56	-0.92	-2.52	-2.05	-6.91	-2.43	-1.06	-19.22
46.02	-2.00	-12.28	-6.92	-11.69	-7.69	-10.03	-5.24	-0.59	-8.99	-14.18	-9.31	-11.63	-18.44
47.61	-7.74	-9.77	-8.28	-12.84	-10.19	-7.15	-9.50	2.60	-9.82	-14.34	-9.37	-10.18	-19.24

APPENDIX G: CE/J DPOAE AVERAGE AMPLITUDES BY AGE GROUP

Geometric mean frequency	<2 Months	2-3 Months	4-5 Months	6-7 Months	8-9 Months	10-11 Months	12-13 Months	14-15 Months	16-17 Months	Mean noise floor
5.77	-12.89	-18.72	-17.01	-14.87	-16.22	-16.92	-14.42	-15.70	-15.22	-22.23
6.75	-14.80	-15.63	-12.24	-10.37	-14.26	-8.18	-6.85	-14.33	-14.07	-22.33
7.56	-4.22	-6.08	-3.45	-1.10	-7.85	-2.03	-5.65	-6.99	-9.26	-17.76
8.94	9.35	-5.03	3.83	2.55	-0.59	-1.56	-2.08	-3.16	-11.25	-18.74
10.04	5.90	-3.41	2.16	7.34	-2.12	0.82	1.64	1.40	-7.15	-14.98
11.02	-6.68	-8.53	-3.28	-5.70	-12.37	-11.42	-6.47	-10.95	-11.87	-17.05
11.74	1.16	-5.11	1.92	-3.25	-7.05	-5.23	-7.11	-7.75	-11.88	-16.80
13.42	16.87	4.00	10.55	9.65	5.06	2.03	3.11	5.67	-3.56	-17.43
14.51	19.79	3.91	10.21	9.61	6.43	9.64	5.08	5.40	-9.06	-18.09
15.70	18.06	2.52	8.40	8.94	6.47	8.15	3.37	6.61	-9.08	-17.06
17.89	18.91	1.71	9.55	7.21	2.52	3.25	-1.61	0.62	-12.08	-15.36
18.98	15.49	-0.47	2.12	0.93	2.39	-0.37	-2.19	-5.63	-10.85	-16.29
21.26	-7.82	-12.54	-11.10	-14.41	-9.59	-12.82	-11.96	-14.57	-13.54	-15.25
23.46	-5.62	-16.51	-10.49	-15.38	-15.62	-13.48	-14.09	-11.52	-14.08	-17.29
24.64	-3.55	-17.04	-6.88	-16.37	-12.22	-13.98	-14.37	-15.07	-16.55	-18.21
26.83	3.26	-0.55	-0.82	-11.26	-7.16	-6.97	-9.50	-11.29	-11.25	-17.51
29.02	14.40	2.14	-1.20	-3.82	-5.18	-2.18	-2.53	0.94	-11.06	-17.55
31.22	11.12	-12.33	-8.40	-15.30	-12.94	-12.17	-13.36	-10.64	-14.59	-17.32
33.59	0.47	-11.46	-7.26	-6.91	-10.36	-14.09	-13.47	-13.14	-17.59	-17.81
35.78	2.58	-14.24	-12.66	-5.21	-12.09	-5.88	-11.19	-11.39	-14.66	-17.35
38.95	-7.59	-12.12	-13.60	-19.24	-13.13	-11.32	-13.71	-15.93	-17.07	-16.50
41.26	-17.01	-14.49	-14.76	-15.98	-14.84	-18.66	-14.05	-17.96	-18.58	-18.03
43.54	-12.51	-14.22	-13.58	-17.90	-15.01	-17.79	-14.09	-19.18	-19.21	-19.22
46.02	-13.58	-15.28	-17.06	-15.81	-13.46	-16.34	-13.16	-17.91	-18.75	-18.44
47.61	-15.13	-16.70	-16.93	-15.79	-13.39	-19.49	-16.06	-19.77	-22.46	-19.24

APPENDIX H. CHEMICAL RECIPES

4% Paraformaldehyde, 2% Glutaraldehyde in 0.1 M PB

Desired Final Volume

Ingredients	50 mL	100 mL	250 mL	500 mL	1000 mL
Sodium Phosphate (monobasic)	0.13 g	0.26 g	0.66 g	1.31 g	2.62 g
Sodium Phosphate (dinobasic Heptahydrate))	1.09 g	2.17 g	5.43 g	10.85 g	21.70 g
Distilled water	See directions for appropriate volumes	See directions for appropriate volumes	See directions for appropriate volumes	See directions for appropriate volumes	See directions for appropriate volumes
Paraformaldehyde	2.00 g	4.00 g	10.00 g	20.00 g	40.00 g
2N NaOH	0.50 mL	1.00 mL	2.50 ml	5.00 mL	10.00 mL
50% Glutaraldehyde	1.00 mL	2.00 mL	5.00 mL	10.00 mL	20.00 mL

Directions

1. Dissolve sodium phosphate(monobasic) and sodium phosphate (dibasic heptahydrate) in distilled water.
2. Bring to volume with distilled water (1/2 total expected volume, e.g. 500mL for 1L 4% paraformaldehyde, 1% glutaraldehyde in 0.1 M PB). Label this solution 0.2 M PB.
3. Add 40g paraformaldehyde to 450 ml distilled water.
4. Heat solution to 55-60ºC on a hot stirring plate.
5. Add drops of 1M NaOH until solution clears, then filter. (usually ~10mL per 1L)
6. Add 500ml of 0.2M PB, pH 7.4 for 1L total volume.
7. Add 20ml of 50% gluteraldehyde (or 40mL if using 25% gluteraldehyde)
8. Dilute with distilled water to 1L. Bring to room temperature before proceeding.
9. Adjust pH to 7.2-7.4 with either NaOH or HCL.

APPENDIX I. ANIMAL CARE AND USE APPROVAL LETTER

Animal Care and Use Committee
East Carolina University
212 Ed Warren Life Sciences Building
Greenville, NC 27834
252-744-2436 office • 252-744-2355 fax

November 22, 2004

Sherri Jones, Ph.D.
Department of CSDI
Belk Annex
East Carolina University

Dear Dr. Jones:

Your Animal Use Protocol entitled, "Functional Aging of the Inner Ear Sensory Systems," (AUP #P028) was reviewed by this institution's Animal Care and Use Committee on 11/22/04. The following action was taken by the Committee:

"Approved as submitted"

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure

APPENDIX J. LIST OF EQUIPMENT MANUFACTURERS AND ADDRESSES

Systems Division - Anatech USA
21363 Cabot Blvd.
Hayward, CA 94545
Toll Free: 800-390-4449
Tel: 510-732-6961
Fax: 510-732-6971
E-mail: tawatts@anatechusa.com

BAL-TEC AG
Neugruet 7
FL-9496 Balzers
Principality of Liechtenstein
Tel. +423 3881 212
Fax +423 3881 260

Brüel & Kjær Sound & Vibration Measurement A/S
Skodsborgvej 307
DK-2850 Nærum
Denmark
Phone: +45 4580 0500
Fax: +45 4580 1405

Endevco Corporation
30700 Rancho Viejo Rd.
Juan Capistrano, CA 92675 San
Phone: 1-888-ENDEVCO

Etymotic Research, Inc.
61 Martin Lane
Elk Grove Village, IL 60007 USA
Phone: 847-228-0006
Toll-free 1-888-Etymotic (1-888-389-6684)
Fax: 847-228-6836

FEI Corporation
North America NanoPort
5350 NE Dawson Creek Drive
Hillsboro, Oregon 97124 USA
Phone: +1 (503) 726-7500
Sales: +1 (503) 726-7500
Sales Fax: +1 (503) 726-2615

FHC Inc.
1201 Main Street
Bowdoin, ME 04287
Tel: 207-666-8190, Canada & US 1-800-326-2905,
Fax: 207-666-8292
email: fhcinc@fh-co.com

Grass TechnologiesA
Astro-Med Industrial Park
600 East Greenwich Avenue
West Warwick, RI 02893 U.S.A.
phone: 401-828-4000 . fax: 401-822-2430
toll-free: 1-877-472-7779 (U.S.A. & Canada only)
e-mail: grass@astromed.com
web site: www.GrassTechnologies.com

Hewlett Packard Corporation
20555 SH 24P
Houston, Tx
Phone: 281-370-0670
Fax: 281-514-1740

Labworks, Inc.
2950 Airway, Ave., #A-16
Costa Mesa, CA 92626
U.S.A.Phone: (714) 549-1981
Fax: (714) 549-8041

Peavey Electronics Corporation
5022 Hartley Peavey Drive
Meridian, MS 39305
Customer Phone: 601-483-5365
Fax: 601-486-1278

Stanford Research Systems
1290-D Reamwood Avenue
Sunnyvale, CA 94089
Phone (408) 744-9040
Fax (408) 744-9049
Email: info@thinkSRS.com

Tucker Davis Technologies
11930 Research Circle
Alachua, FL 32615
USA
Phone: 386.462.9622
Fax: 386.462.5365
E-mail: info@tdt.com

Vibra-metrics
195 Clarksville Road
Princeton Jct, NJ 08550
USA
Phn: +1 609.716.4130
Fax: +1 609.716.0706

APPENDIX K. AVERAGE NOISE LEVELS IN ANIMAL HOUSING.

Average noise amplitude (dB SPL) plotted against the time of day in the animal housing area. There were 48 noise samples, comprised of 16 hour periods, taken on random days over a five month period. The data logging dosimeter had a minimum level of 65 dB SPL, any sound level measured that was lower than 65 dB was given a 65 db SPL value. Each data point represents the mean SPL for ten one minute samples. The logarithmic mean SPL for all samples was 66.8 dB SPL.