ABSTRACT

Title of Document: CHARACTERIZING THE AUDITORY PHENOTYPE OF NIEMANN-PICK, TYPE C DISEASE: A COMPARATIVE EXAMINATION OF HUMANS AND MICE

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Niemann-Pick disease, type C (NPC) is a rare (1:120,000-150,000) autosomal recessive lysosomal lipidosis resulting in a progressive and fatal neurological deterioration. There is much about the pathogenesis and natural history of this complex, heterogeneous disorder that remains unknown. Limited literature suggests auditory dysfunction is part of the phenotype, but an aspect of the disease process that is poorly understood and, indeed, has likely been underreported. Experiment one includes auditory data from 55 patients with NPC seen at the National Institutes of Health between 8/14/2006 and 12/27/2010. These data confirm a prevalent high frequency hearing loss that progressively worsens in at least some individuals. Retrocochlear involvement is

common, with abnormalities that suggest a profile of auditory neuropathy spectrum disorder in some patients. Analysis of late-onset cases suggests hearing loss is a premonitory symptom in this disease subcategory.

The investigation was expanded to include the mouse model for NPC (BALB/cNctr-*Npc1*^{m1N}/J), in which symptomatology is clinically, biochemically, and morphologically comparable with affected humans. There have been no previous reports of auditory function in NPC mice, although brainstem histopathology has been localized to the auditory pathway. Experiment two includes auditory brainstem response (ABR) and otoacoustic emission (OAE) data revealing a high frequency hearing loss in mutant NPC mice as early as postnatal day (p) 20, which becomes progressively poorer across the experimental lifespan. With support for both a cochlear and retrocochlear site of lesion, OAE level and ABR latency data provide surprising evidence for a disruption in maturational development of the auditory system in diseased animals, which may add a unique perspective on the role of NPC pathogenesis. This comparative, translational study has, for the first time, addressed comprehensively the existence of, and implications for, auditory dysfunction in NPC. Similar auditory phenotypes between affected humans and mutant mice should aid future efforts in refining site of lesion. In combination, these data support the auditory system as a useful marker for disease status and provide valuable prognostic and quality of life information for patients and their families.

CHARACTERIZING THE AUDITORY PHENOTYPE OF NIEMANN-PICK, TYPE C DISEASE: A COMPARATIVE EXAMINATION OF HUMANS AND MICE

By

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Dedication

This work is dedicated to our participants with NPC and their families, who have so selflessly given of their time, energy, and spirit, and who each day live the message they espouse: *persevere*.

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To my parents, Karen King Thompson and Barry King: thank you for showing me the value of hard work, humility, and kindness; "*On ne voit bien qu'avec le cœur. L'essentiel est invisible pour les yeux*." To my best friend, partner, and husband, John Daniel Stump: thank you, more than any other, for your support and love.

Table of Contents

List of Tables

List of Figures

CHAPTER 1: INTRODUCITON

Niemann-Pick, type C disease (NPC) is a rare (1:120,000-150,000), genetic lysosomal lipidosis with a hallmark neurological deterioration that is fatal in all cases. It is part of a family of metabolic storage disorders resulting in a pathological accumulation of lipids in numerous organs and systems throughout the body. The primary metabolic defect in NPC is abnormal cholesterol trafficking from lysosomes (Liscum & Faust, 1987; Liscum, Ruggiero, & Faust, 1989), and the characteristic phenotype includes hepatic dysfunction and neurological decline. Currently, there is no effective treatment or cure.

Despite major developments over the last century in elucidating the metabolic and genetic bases of NPC, there is much about this disease that remains a mystery. This is, in part, due to the inherent difficulty associated with studying such a complex and heterogeneous disorder. Among the challenges for researchers and clinicians investigating NPC are the highly variable ages of onset and diagnosis, and the broad, multifaceted clinical spectrum associated with the disease.

It is now known that NPC is the result of biallelic mutations in either *NPC1* or *NPC2* (Carstea, Morris, Coleman, Loftus, Zhang, Cummings, et al., 1997; Naureckiene et al., 2000) and, while 95% of all affected individuals have mutations in *NPC1*, there appears to be no significant difference in how the disease manifests between mutations in either of these genes (e.g., Ikonen, & Hölttä-Vuori, 2004). The ensuing biochemical defect is an over-accumulation of exogenous and unesterified cholesterol within cells and tissues throughout the body, including areas with high concentrations of lipids within the brain, such as myelin and neural plasma membranes (Vincent, Bu, & Erickson, 2003).

The early symptoms of NPC commonly involve the hepatic system; however, diagnosis is typically delayed until the onset of neurological manifestations, which may include, among other complications, cerebellar ataxia, dysarthria, dysphagia, dystonia/hypotonia, cataplexy, and seizures. In some cases, neurological involvement may progress to states of psychosis and dementia.

Preliminary evidence from limited literature on the subject suggests the auditory system is affected during the course of this disease (e.g., Pikus, 1989), although the full natural history of the auditory phenotype is unknown. Indeed, auditory manifestations have likely been underreported given the difficulty in obtaining behavioral audiological evaluations in a neurologically compromised population and the inability of many affected individuals to self-report hearing difficulties.

The handful of case studies and articles in the literature describing hearing in NPC includes sporadic descriptions of high-frequency peripheral hearing loss, acoustic reflex abnormalities, and auditory brainstem response (ABR) dysfunction, although none are depicted with any great detail. As a result, there remains a clear need to examine comprehensively the auditory phenotype in NPC characterizing both how the dysfunction manifests and if, like the global neurological phenotype, it is progressive.

In recent years, the discovery of several animal models for NPC has significantly advanced understanding of the disease and the ability to examine its effect on various biological systems. Research with animal models offers many advantages over those challenges posed by studying a genetically heterogeneous species, such as humans, where quantifying the contribution of a specific genetic mutation is difficult. The mouse model for NPC, in which disease manifestations are clinically, biochemically, and

morphologically comparable with affected humans (Kolodny, 2000), has emerged as an invaluable species for studying this disorder. However, unlike humans with NPC, there are no reports documenting auditory function in the NPC mouse. Luan et al. (2008) identified histological brainstem pathology localized to the auditory pathway in NPC mice, which is the best evidence to date suggesting the auditory system may be compromised in this model. Further exploration is required to confirm an auditory phenotype in the mouse model for NPC and establish the extent to which it is comparable to findings in humans.

The principal goal of the present study was to evaluate comprehensively the auditory phenotype in NPC to understand better the natural history of the disease and its effect on the auditory system. With this aim in mind, auditory function in both affected humans and mice was examined. Studying auditory function in humans with NPC provides unique insights into the behavioral manifestations and quality of life issues for these individuals, and working with the mouse model allows a focused examination of the effects of a specific genetic mutation on the auditory system not possible to obtain in other heterogeneous populations. The synergistic combination of the two projects will create the greatest contribution of knowledge for patients, clinicians, and researchers than either alone.

CHAPTER 2: AUDITORY FUNCTION IN HUMANS WITH NIEMANN-PICK DISEASE, TYPE C (NPC)

Review of the Literature

Overview

In 1914 a German pediatrician named Albert Niemann published a case study describing a Jewish infant with neurodegeneration and hepatosplenomegaly (Niemann, 1914). He noted that the child's symptoms were similar to those observed in Gaucher disease and believed he was seeing a new variant of the known lipid storage disorder. More than a decade later, Ludwig Pick isolated the new disease by characterizing its unique histopathology, determining it was distinct from Gaucher disease (Pick, 1933), and labeled it Niemann-Pick disease (NPD). Initially, Pick believed this novel neurological malady was fatal in all patients by the time they reached the age of two years; however, in 1958 Crocker and Farber published a seminal account of 18 patients with NPD ranging in age from four months to 19 years. Crocker (1961) later divided these 'atypical cases' into four distinct subgroups based on their clinical and biochemical phenotypes, and genealogy: type A, B, C, and D.

Today, NPD is known as a group of autosomal recessive, metabolic lipid storage disorders that cause an accumulation of harmful amounts of lipids in various organs and tissues throughout the body. Lipids, or fat molecules, are vital components to all biological cells, most critically involved in cellular structure and function. The arrangement and distribution of lipids can vary significantly, especially in membranes within the central nervous system (CNS), but also in organs and peripheral tissues (Percy, Shapiro & Kaback, 1979). In NPD, the over accumulation of lipids in cells, which occurs

in all subtypes of the disease, is not the result of abnormal molecular structure or production. Instead, NPD is caused by impairments in the mechanisms responsible for lipid breakdown, or transportation away from a cell.

It is now understood that the cluster of diseases that comprise NPD represents two primary categories of dysfunction. The first are those with a deficiency in acid sphingomyelinase (ASM). This enzyme is charged with breaking down the lipid sphingomyelin, and is deficient in NPD types A and B. The second category includes those cases with defects in intracellular transportation of cholesterol, observed in types C and D (Kolodny, 2000). Both cholesterol and sphingomyelin are types of lipids housed within cell membranes of most, if not all animal tissues. Although there are only two principal categories of metabolic dysfunction, types of NPD can differ significantly in their pathophysiology and clinical manifestations.

All forms of NPD are associated with hepatosplenomegaly (enlargement of the liver and spleen). NPD, type A (NPA) and type B (NPB), both caused by an ASM deficiency, differ significantly in disease presentation and progression. NPA is an acute, neurovisceral form (Elleder & Cihula, 1983), with onset during infancy, severe neurological manifestations, and rapid progression toward death. NPA occurs more frequently than NPB, with a disproportionately high prevalence in the Ashkenazi Jewish population (Weinstein, 2007). NPB has a juvenile onset, does not involve the nervous system, and survival into adulthood is common (Pavlu-Pereira et al., 2005). Respiratory problems secondary to pulmonary infiltration occur frequently in NPA and NPB.

The NPD variant, labeled type D by Crocker (1961) has only been reported in French Canadian descendants of an Acadian couple married in the 1600s in what is today

known as Nova Scotia (Winsor & Welch, 1978). Other than this distinct ancestry, the type D variant of NPD is phenotypically indistinguishable from NPD type C (NPC) and shares a common molecular aberration. Consequently, type D is no longer recognized as a distinct NPD classification (Vanier & Millat, 2003).

NPC, which is the primary focus of this paper, is a complex, heterogeneous disorder characterized by hepatosplenomegaly and a neurological deterioration that is ultimately fatal. The incidence of NPC is estimated to be one in every 120,000 to 150,000 individuals; however, because it is so rare and because it is frequently misdiagnosed, this may be an underestimation. NPC is panethnic (Vanier & Millet, 2003) and occurs more frequently than NPA and NPB combined (Ikonen, & Hölttä-Vuori, 2004).

A major breakthrough in the science of NPD occurred with identification of the defective ASM enzyme in NPA and NPB (Brady, Kanfer, Mock, & Fredrickson, 1966). This significant advancement, however, paradoxically delayed the discovery of the metabolic bases of NPC for years as scientists labored to connect NPC with a sphingomyelinase deficiency. It was not until 1984 that Pentchev and colleagues initially linked NPC with cholesterol metabolism. Following this connection, Liscum published two seminal articles (Liscum & Faust, 1987; Liscum et al., 1989) establishing, for the first time, that the specific metabolic defect in NPC involves cholesterol trafficking from lysosomes (for review see Pentchev, 2004).

Perhaps the most significant recent development in NPC research came with the discovery of the underlying genetic defects. The *NPC1* mutation (18q11) was identified through positional cloning by Carstea and colleagues in 1997. This discovery created a

flurry of research into the causative molecular pathology of NPC. As of the year 2000, 36 *NPC1* mutations had been described in the literature (Greer, et al., 1998,1999; Millat, et al., 1999; Yamamoto, et al., 1999). By 2003, the number more than tripled (Vanier & Millat, 2003), and today *NPC1* is associated with over 240 disease-causing allelic mutations (Runz, 2009). During this period, strong evidence emerged to suggest the existence of a second gene linked to NPC. Vanier, Duthel, Rodriguez-Lafrasse, Pentchev, and Carstea (1996) reported a linkage analysis on five families with NPC that suggested a second causative locus. Naureckiene et al. (2000) ultimately identified a second mutation in *HEI/NPC2*, most commonly referred to as the *NPC2* mutation (14q24.3). More than 95% of patients with NPC have mutations in *NPC1,* however there appears to be no significant difference in how the disease manifests in those with mutations in either *NPC1* or *NPC2.* While some investigators suggest *NPC2* causes more severe symptoms and pronounced pulmonary involvement compared to *NPC1* (Millat, et al., 2001), most reports state that the two complementation groups are clinically and biochemically indistinguishable (e.g., Ikonen, & Hölttä-Vuori, 2004).

Molecular aberrations in *NPC1* and *NPC2* have complex and multifaceted outcomes on cellular function. The primary biochemical defect is abnormal processing of low-density lipoprotein (LDL)-derived cholesterol, which is partly responsible for regulating cholesterol synthesis within tissues of the body. As a result, affected cells are unable to transport exogenous cholesterol out, and accumulate a harmful amount of unesterified cholesterol (Ikonen, & Hölttä-Vuori, 2004). Cholesterol accumulation is the dominant cellular phenotype, although multiple other lipids can accrue within affected tissues, including sphingolipids and gangliosides (Sturley, Patterson, Balch, & Liscum,

2004), both critical molecules in signal transmission through cells and neural tissues. There are high concentrations of both cholesterol and sphingolipids within the brain, specifically in myelin and neural plasma membranes comprised of fat molecules and proteins known as lipid rafts (Vincent et al., 2003).

While many individuals with NPC are initially diagnosed based on their clinical presentation, the definitive diagnosis is made through cytological and biochemical assessment. Most commonly, skin fibroblast samples are stained with the antibiotic filipin, which binds to unesterified cholesterol and causes a distinct fluorescent output. A second diagnostic approach is biochemically monitoring fibroblasts to assess the rate of LDL-cholesterol esterification (e.g., Morris & Carstea, 1998). Of these two diagnostic techniques, the filipin test has higher rates of sensitivity and specificity (Vanier & Millat, 2003).

The large age range for both the onset and diagnosis of NPC, coupled with a broad clinical spectrum, creates significant challenges for researchers investigating the natural history of this disease. The classical onset of neurological symptoms begins in the late-infantile and juvenile years, with death occurring in the second decade (e.g., Garver et al., 2007). However, the age of presentation, and the subsequent age of diagnosis, may range from the perinatal period to adulthood (e.g., Vanier & Millat, 2003). Given this variability, some researchers have suggested a further breakdown of the NPC phenotype to subgroups of neonatal, childhood, or adult presentation (Imrie et al., 2007).

The initial clinical symptoms of NPC may be neurological or psychiatric (Vanier & Millat, 2003), but most commonly begin in the hepatic system and manifest as

neonatal jaundice and early-childhood hepatosplenomegaly. The hallmark dysfunction is neurological, however, and can include cerebellar ataxia and motor impairment, speech and swallowing disorders (e.g., dysarthria, dysphagia,), dystonia, hypotonia, cataplexy, and seizures. Neurological symptoms may progress to states of psychosis and dementia. Demyelinating polyneuropathy has been reported in several cases of NPC, and is purported to have a higher incidence within the disease than is currently recognized (Zafeiriou et al., 2003). The finding of supranuclear vertical gaze palsy is practically pathognomonic for NPC (e.g., Fink et al., 1989; Uc, Wenger, & Jankovic, 2000; Zafeiriou et al., 2003), and may be an early indication of neurological involvement when it is detected. Additional oculomotor dysfunction secondary to specific brainstem neuropathology has been reported (Solomon, Winkelman, Zee, Gray, & Büttner-Ennever, 2005).

Auditory function in NPC

In light of the systemic nature of NPC, it is doubtful the auditory system remains unaffected during the course of this disease. Indeed, there are limited accounts of auditory manifestations in NPC; however, reports of auditory function in any of the NPD variants are sparse. The overwhelming majority of NPC case studies fail to evaluate auditory function unless the patient specifically presented with complaints of hearing loss. Even in such cases, comprehensive audiological evaluations were typically deferred, or not reported. It is clear that this aspect of the phenotype has not been thoroughly examined. Moreover, a pediatric, neurologically compromised population poses significant challenges to obtaining behavioral auditory assessments, and it is likely many individuals with NPC are unable to self-report hearing difficulties. This all

suggests that the limited auditory data published to date may underrepresent the actual auditory phenotype in NPC.

A review of previously reported auditory manifestations in NPC reveals a modest collection of case reports and natural history studies. Often these are limited or vague reports of auditory dysfunction, such as the description of an 18-month-old African child with NPD, type unknown, who displayed, "marked delay in motor, social, speech, and hearing progress …" (Singer, Lowe, & Cmelik, 1972). Schneider et al. (2001) reported a 33 year old woman with adult onset NPC who had normal, "acoustically… evoked potentials …", and Palmeri and colleagues (2005) described a 16 year old male with NPC whose ABR showed increased I-V and III-V interpeak latencies bilaterally, although no additional information was provided.

Sévin et al. (2007) published a comprehensive review of adult-onset NPC, in which they reported findings from 13 unrelated patients. On average, the age of onset of neuropsychiatric symptoms was 25 years and the age at death was 38. Detailed magnetic resonance imaging (MRI) showed atrophy in, among other locations, the cortex, cerebellum, corpus callosum, and brainstem. Three (23%) of their patients were identified as having "perceptual deafness," a finding the authors suggest indicates deep brain involvement. In two of these cases, "deafness" was a premonitory symptom in childhood preceding the onset of neurological signs. The hearing metric was not identified and standard descriptions of hearing (e.g., type, degree) were not provided. One patient presented with, "an unexplained perceptive hypoacousia." Another had a family history of "deafness" that followed a recessive inheritance pattern. Hearing loss in the third patient was associated initially with mild head trauma, prior to disease onset.

These three case reports of adult-onset NPC that include auditory manifestations (Sévin et al., 2007) are a unique addition to the literature, especially considering that in two of three cases hearing loss was an early-onset symptom. However, specific conclusions about site of lesion and auditory pathophysiology cannot be made due to a lack of detail in how hearing was measured and use of the generic label "deafness" applied to all three cases.

Garver et al. (2007) presented natural history data collected by questionnaire that described clinical and overall health in a cohort of patients with NPC. The 83-question survey was mailed to 136 families affected by NPC living in the United States, of whom 87 (64%) responded. Parents/caregivers and physicians of individuals with the disease provided the responses. The average age of diagnosis for NPC was 10.4 years and the average age of death was 16.2 years. Although not stated directly, this implies most of the participants in this study presented with the classical onset of NPC. In a section on general medical and developmental problems experienced by affected individuals, three questions asked specifically whether the child with NPC has hearing problems, ringing in the ears, and/or dizziness/vertigo complaints. "Hearing problems" were reported in 13 (15%) of the respondents, "ringing in the ears" in one (1%), and "dizziness or vertigo" in five (6%). Seventy-five (87%) respondents reported at least slight-to-moderate difficulty with "Language," with more than half describing severe or difficult problems; however no attempt was made to correlate these communication issues with hearing impairment.

The data presented by Garver et al. (2007) are another indication that auditory manifestations are a part of the global NPC phenotype, and that hearing loss may be an overlooked finding. In general, anamnestic reports of hearing lack the objectivity

necessary to make conclusive statements regarding diagnosis and etiology. Many patients without neurological involvement who have mild hearing loss are unaware of it, and consequently, underreport the symptom (e.g., Reilly, Troiani, Grossman, & Wingfield, 2007). It seems probable these individuals would have been limited in their ability to report subjective auditory dysfunction, and that the data presented may underestimate the frequency and, perhaps, the severity of the problem.

Several earlier reports of auditory dysfunction in patients with NPC provide somewhat greater detail than those discussed previously. Aisen, Rapoport, and Solomon (1985) published a report of ABR findings in two brothers under the age of four years with NPC. Neither sibling presented with any auditory complaints or clinical evidence of brainstem dysfunction. ABRs were obtained with moderate stimulus intensity ("60 dBSL") at a click rate of 11 per second. Both brothers showed prolonged I-III interwave latencies, a normal absolute latency for wave V, and all other interpeak latencies within normal limits. In spite of disparate neurological manifestations and overall disease severity between the two brothers, the ABR results for both boys were remarkably similar and suggested a delay in the neural conduction time between the auditory nerve and the cochlear nucleus. The authors suggest it is possible that the delay extends as far along in the system as the superior olivary complex.

Fink et al. (1989) analyzed neurological symptomatology in 22 patients with NPC, including the auditory phenotype. Their population consisted of 10 males and 12 females ranging in age from 18 months to 27 years. Twenty-one of these patients were given audiological evaluations that included behavioral assessment for speech and pure-tone stimuli, acoustic immittance measures, and ABR measures. There were no

additional methodological details provided on how auditory data were collected. They reported a pervasive high-frequency hearing loss in 14 individuals that was presumably sensorineural in nature. Although tympanometry was unremarkable, abnormal (i.e., absent or elevated) acoustic reflexes and/or positive acoustic reflex decay were observed in 17 of 21 patients. ABR abnormalities included eight individuals with prolonged I-V interpeak latencies, and three individuals with prolonged I-III interpeak latencies. The authors note that 10 people presented with an abnormal ABR, but it is unclear whether these 10 individuals included those with prolonged interpeak latencies already presented, or if they represent other patients with abnormalities. Additional specific ABR findings were not described. Although Fink and colleagues offer one of the few audiologic profiles in patients with NPC, their report is limited in its detail and scope. As a result, it is difficult to draw concrete conclusions beyond a very general description of the phenotype.

A report by Pikus (1989) is the only published account focusing solely on comprehensive audiological findings in patients with NPC. This brief document describes a cohort of 28 patients with NPC ranging in age from two to 37 years. Audiological assessment included behavioral evaluation of hearing using speech and pure-tone stimuli, tympanometry and acoustic reflex testing, and measurement of the ABR. No specific methodological details or stimulus/recording parameters were provided.

Twenty-one (75%) of 28 patients exhibited hearing loss of varying degrees, which was almost always confined to the high frequency test region. While the type of hearing loss was not reported, conductive pathology is unlikely in light of normal tympanometry

in all patients. The author notes the hearing loss "appears to progress over time and with increasing severity of somatic symptoms," however there was no explanation of the basis for this statement. Specifically, it is unclear whether the conclusion is made from longitudinal data, which were not reported, cross-sectional analysis, or anecdotal observation.

ABR findings were abnormal in 15 patients (53%) and, although the abnormalities observed across the population were labeled as "diverse," specific descriptions are lacking. Prolonged I-V interpeak latencies, overall dysmorphic waveforms, and normal V/I amplitude ratios are the only details provided. A prevalent finding in this sample was abnormal acoustic reflexes, observed in 23 (85%) patients, and it is described as, "an early disturbance." The author concludes that, given the age of this cohort, abnormal acoustic reflex findings may serve as an early indication of NPC onset. However, the age range of this group (2 to 37 years) does not suggest they represent an especially young cohort of patients with NPC considering the classical late infantile to juvenile onset of the disease. In order to conclude that aberrant acoustic reflex findings are an initial symptom, there should be data to support that the cohort in question is actually in the early stages of the disease. This is not likely to be the case in the population reported by Pikus, however there are no data provided to dispute conclusively or confirm this claim.

Higgins et al. (1992) reported similar audiological findings to Fink et al. (1989) and Pikus (1989). They observed increased ABR latencies and abnormal acoustic reflexes in their sample of NPC patients. While the size of each cohort reported by Higgins (1992), Fink et al. (1989), and Pikus (1991) varies slightly, these three reports

originated from the same institution (National Institutes of Health), the findings and manner in which they are reported are strikingly similar, and at least one individual (Pikus) who is an audiologist is an author on all three studies. Thus, it is not clear that these reports represent three separate samples of individuals with NPC. An initial review of the audiology literature on NPC may lead some to conclude that ABR abnormalities and aberrant acoustic reflex findings are a pervasive finding in the disease given their consistent presence across three distinct publications. If, in fact, there is overlap between the reported samples, it would suggest that replication of these findings in additional independent cohorts is necessary.

It is worth noting a small collection of articles describing histological analyses in the temporal bones of patients with NPD, most likely type A. Druss (1932) reported proliferation of Niemann-Pick cells in the auditory nerve and degeneration in the ganglia of the seventh and eighth cranial nerves. Oppikofer (1935) reported no anatomical abnormalities in the inner ear of a child with severe hearing loss and concluded the origins of the loss were based on some central dysfunction. Bachor et al. (1997) described the temporal bones of three patients, aged 6 months, 7 months, and 2.5 years at the time of death, all with NPA. Light microscopy revealed abnormal lipid storage in the ganglion cells and changes in the organ of Corti, including alterations in the stria vascularis and missing or deformed hair cells. Behavioral and electrophysiological evaluations of hearing were not performed on any of the patients. While these studies describe the histopathological findings in the temporal bones of patients with NPA, they may provide valuable insight into the unknown effects of NPC on the auditory system. If

nothing else, they offer further evidence of pathological invasion of the inner ear and auditory system in a lipid storage disease.

Although the research describing hearing in humans with NPC is limited, auditory dysfunction does appear to be part of the disease process. A clear picture of the auditory manifestations is still missing, however, and there have been no definitive reports on whether the auditory phenotype is progressive, despite the known neurodegenerative characteristics of the disorder. If a clear and consistent phenotype emerges, it is possible the auditory system may become a useful benchmark for future pharmacological and therapeutic interventions for NPC. Patterson, Vecchio, Prady, Abel, and Wraith (2007) published findings from a therapeutic study in which hearing sensitivity was one outcome measure. The purpose was to evaluate the efficacy and potential side effects of the experimental treatment miglustat (Zavesca), a small iminosugar that can cross the blood-brain barrier, on patients with NPC. They monitored several clinical markers, including hearing sensitivity. Auditory acuity was based on the ability of the participant to hear either a ticking watch or a Manchester rattle, and a 256 Hz tuning fork. Each device was "held at 30 cm or less from the external auditory canal." If the patient was able to hear the sound source at 30 cm they were classified as normal. Any distance less than 30 cm was abnormal, unless they were unable to hear the sound source at all, in which case they were labeled "deaf."

By these definitions, auditory acuity at baseline prior to treatment was normal in 31 out of 40 (78%) ears in the treatment group receiving miglustat. A control group of patients with NPC not receiving treatment was tested and hearing was reportedly normal in all nine patients. In as much as it was measured, hearing remained stable in patients

treated with miglustat, whereas two patients in the control group eventually presented with "deafness."

The measurement of hearing sensitivity in this study is invalid, rendering it useless for either clinical or research purposes. However, it raises the question of how the results would have been interpreted if the group receiving miglustat exhibited significant changes in hearing. With no basic understanding of the natural history of the auditory phenotype, it is impossible to discern what is an expected change due to disease progression and what may be a direct adverse event from the drug. If the conclusion were the latter, the development and proliferation of a potentially safe treatment may be delayed.

While the audiological limitations of this study are obvious, the authors note that hearing impairment in NPC is easily overlooked and an important aspect in quality of life. These data, at the very least, provide additional evidence of auditory dysfunction in NPC and appear to be the first attempt in which hearing sensitivity was used as a benchmark for monitoring intervention in this disease. A clear understanding of the natural history of the auditory phenotype in NPC will only improve the efficacy of future, similar endeavors.

Summary

The purpose of experiment one is to evaluate further the auditory phenotype in humans with NPC. There are a number of remaining obstacles to understanding this rare, devastating disease, including established clinical markers for its onset and progression, and there is no effective treatment or cure; interventions at this time are focused on palliative care. The literature provides enough preliminary evidence to suggest auditory

dysfunction is a part of the NPC phenotype, and the case has been made that it is likely an underreported manifestation. The natural history of the auditory phenotype, however, remains unknown, which hinders prognostic counseling for patients and families, and the etiology of the hearing loss is unclear, although preliminary data suggest it may be complex and widespread throughout the auditory system.

Research Questions and Hypotheses

The overarching question is whether or not the pathophysiological processes of NPC detrimentally impact the auditory system in humans and, if so, in what ways?

Research Questions

1. Do patients with NPC exhibit peripheral hearing loss and/or evidence of central auditory nervous system (CANS) dysfunction?
    a. If hearing loss exists, is it localized to the conductive or sensorineural pathway?
    b. If CANS dysfunction exists, can it be localized to the auditory brainstem?
    c. Is auditory system dysfunction observed consistently in a sample of patients with NPC?
        1.) Is auditory dysfunction present in the majority of this sample?
        2.) Is auditory dysfunction stable, progressive, or fluctuating among this sample?
        3.) If variability exists in the presence/absence or natural history of auditory dysfunction, are there individual factors that contribute to this variability, specifically: age of disease onset (defined as age at first reported symptom), age at the time of the test,

duration of disease, gender, or the use of the experimental therapeutic medication miglustat (Zavesca) for disease treatment?

Hypotheses

It is hypothesized that patients with NPC will exhibit both peripheral hearing loss and evidence of CANS dysfunction, and that auditory manifestations will be consistent across the majority of a sample of patients with the disease. NPC is associated with CNS and brainstem pathology, as well as neuropathy in multiple peripheral nerves and dysfunction in CNS myelin (Vincent et al., 2003). This suggests a high potential for abnormal retrocochlear findings in patients with NPC. A mixed peripheral/CANS site of lesion may manifest as elevated air-conduction and bone-conduction behavioral thresholds, elevated acoustic reflex thresholds and/or abnormal acoustic reflex adaptation, absent otoacoustic emissions or a loss of transient-evoked otoacoustic emission (TEOAE) suppression over time, or increased neural conduction time on the ABR relative to published normative data (Issa & Ross, 1995; Joseph, et al, 1987). No significant conductive pathologies are anticipated in this cohort.

It is hypothesized that auditory dysfunction will be progressive in patients with NPC, in light of the well-established progressive nature of the disease, although it is unclear if this will manifest over the experimental time frame ranging from zero months to four years of follow-up. NPC is a highly pleiotropic disease, which suggests influences from undiscovered genetic and environmental modifiers. These potential confounding variables, in addition to the challenge of obtaining behavioral audiologic

data in neurologically compromised participants, may make the progressive nature of the auditory phenotype difficult to determine in a human population.

It is hypothesized that no significant effects of independent variables on hearing will be observed in this group, specifically: age of disease onset (defined as the age at first reported symptom), age at the time of test, duration of the disease, gender, or the use of miglustat for disease treatment. Both age and gender have been shown to effect hearing (e.g., Pearson, et al., 1995) and should be considered when interpreting auditory data. However, the age of NPC disease onset can range from the perinatal period to adulthood (Vanier & Millat, 2003), suggesting this variable and variables related to it (e.g. duration of disease) are unlikely to correlate strongly with auditory function. Similarly, gender effects are not an established part of the NPC phenotype, and, consequently, this variable is not hypothesized to significantly affect experimental auditory outcomes. Miglustat is an enzyme inhibitor currently approved by the Food and Drug Administration to treat Gaucher disease, another lipid storage disorder. Some patients with NPC elect to use miglustat, although off-label treatment for this disease is experimental. Its use in this cohort cannot be controlled and will vary between patients. Because of this variability, and because no robust empirical effects of miglustat for treating NPC have been reported, it is hypothesized that it will have no significant effect on hearing.

Experiment One

Methods

Participants. Participants were those patients with NPC disease admitted to the NIH Clinical Center for evaluation in the ongoing protocol entitled, "Evaluation of Biochemical Markers and Clinical Investigation of Niemann-Pick Disease, type C" (NCT00344331). This protocol was approved by the Institutional Review Board of the National Institute of Child Health and Human Development (NICHD) and began in June 2006. Recruitment for the NIH protocol is through both parent and professional organizations, and handled by the principal investigator, Dr. Forbes Porter, and under the auspices of the NICHD. The Institutional Review Board of the University of Maryland College Park approved this work (07-0675, PAS# 1868.3).

Once a patient was enrolled in the NIH protocol, they were eligible for participation in this experiment. Based on the NIH inclusionary and exclusionary criteria, all patients with an established diagnosis of NPC (biochemical or molecular) were considered for this study. Patients were excluded if they could not travel to the NIH because of their medical condition or were too ill to be cared for at home. Patients with rapidly progressive neonatal cholestasis were also excluded. Patients with stage 4 disease status (non-ambulant with vegetative disturbances) were not enrolled. Patients were excluded if they were pregnant (a negative urine pregnancy test was required for any menstruating female before participation in this study and at each NIH Clinical Center admission). Otherwise, patients of any age, sex, or ethnic background were eligible for this study. There are no auditory-based inclusionary or exclusionary criteria.

Equipment. Age- and ability-appropriate behavioral audiological data, as well as electrophysiological data obtained in unsedated patients (tympanometry, OAEs, and ABR), were collected in double-walled sound suites in accordance with the American National Standards Institute criteria (ANSI, 2010). For patients who were unable to cooperate for unsedated electrophysiological analyses, sedated testing took place in either an operating room or a special procedure room, during which sedation was administered and monitored by licensed anesthesia personnel, and noise levels were kept to a minimum.

Behavioral audiological data were collected using a Grason-Stadler (GSI-61) diagnostic audiometer. Middle ear function was measured using an acoustic immittance unit (GSI Tymp Star, GSI-33). OAEs were measured using an Otodynamics Echoport Otoacoustic Emission System supported by ILO V6 Clinical OAE software on a Dell laptop computer. ABR data were collected using Audera software and supported on a Dell laptop computer.

Procedure. Details of the entire NIH protocol entitled, "Evaluation of Biochemical Markers and Clinical Investigation of Niemann-Pick Disease, type C" (NCT00344331) are not included in this document but are available for review. The co-chair for this dissertation, Dr. Carmen Brewer, and the student investigator, Kelly King, are associate investigators on the NIH protocol.

Admissions (including hospital stay) at the NIH lasted approximately 4-5 days and occurred every six months or once per year, as determined by Dr. Forbes Porter. Numerous clinical, biochemical, and radiological evaluations took place during the patient's visit to the NIH.

Multiple considerations were made when selecting an appropriate test battery to determine the natural history of this disease. Evidence exists for widespread dysfunction in the auditory system, potentially involving numerous peripheral and central sites. Thus, an extensive test battery was required. It was also imperative to consider the ability and neurological status of the population in question. Depending on the age of onset and neurological involvement in an individual at the time of testing, the acquisition of behavioral audiological data can be limited, if not impossible. Furthermore, in patients with significant neurological involvement, obtaining certain types of electrophysiological data may also be compromised.

With these issues in mind, the following describes the test battery employed to define the auditory phenotype in NPC. It should be noted that the acquisition of each measure was not possible in all patients. Justification for each test, as well as procedural methodology, is included.

1. Evaluation of middle ear function and the acoustic reflex arc (acoustic immittance): tympanometry, ipsilateral and contralateral acoustic reflexes, and acoustic reflex adaptation (decay).

These are quick, non-invasive measures that provide critical information on the health and integrity of the middle ear system and the acoustic reflex arc, which involves cranial nerves VII and VIII, as well as the cochlear nuclei, superior olivary complex, and facial motor nuclei. These tests provide important contributions to determining site of lesion along the auditory pathway.

Procedure: Otoscopy was performed prior to testing. A single-frequency (226 Hz) tympanogram was obtained to measure tympanometric peak pressure (TPP), ear canal volume, and peak-compensated static admittance. Ipsilateral and contralateral acoustic reflex thresholds were determined at stimulus frequencies of 500, 1000, and 2000 Hz. Acoustic reflex adaptation was evaluated by presenting a pure-tone (500, 1000 Hz) at a level 10 dB above the acoustic reflex threshold for 10 seconds in the contralateral stimulus condition. The intensity of the test stimulus did not exceed 110 dB HL in either of these measures.

The acoustic reflex threshold was defined as the lowest intensity level of a stimulus that elicited a $\geq$ 0.02 mmho decrease in admittance, and which replicated in order to be considered a valid reflex. If no repeatable acoustic reflex was measured at these levels, the reflex was considered absent.

Acoustic reflex adaptation is a reduction in the magnitude of change in admittance during a 10 second period of stimulus presentation. This was considered positive (abnormal) if the change in admittance decreased by $\geq$ 50% during the 10 second stimulus presentation, indicating failure of the stapedius muscle to maintain its contraction over time.

2. Pure-tone thresholds and suprathreshold speech recognition performance

A comprehensive audiological evaluation includes the behavioral pure-tone audiogram. Accurate and thorough interpretation of objective electrophysiological results is contingent on these data, and the audiogram is a useful tool in counseling patients and family members.

Suprathreshold speech recognition performance is also a standard component of the audiological evaluation to establish the possible effects of reduced audibility and signal distortion associated with the hearing loss on speech understanding. Extremely poor speech recognition performance that is disproportionate to the degree of peripheral hearing loss may be an indication of a retrocochlear site of lesion.

Procedure: Whenever possible, ear-specific data were obtained. An attempt was made to measure pure-tone air-conduction thresholds at .25, .5, 1, 2, 3, 4, 6, and 8 kHz, and bone-conduction thresholds at .25, .5, 1, 2, 3, and 4 kHz. Threshold was considered the lowest intensity at which a person was able to detect correctly the stimulus 50% of the time upon ascending runs. Speech recognition/detection thresholds (SRT/SDT) to spondee words, and suprathreshold (e.g., 40 dB SL re: SRT, and 85 dB HL) word recognition using age-appropriate monosyllabic word tests (Northwestern University Test #6, NU-6; PBK-50; Word Intelligibility by Picture Identification, WIPI) were determined via monitored live voice, which allowed the examiner the ability to quickly engage fatiguing patients in an effort to extend the test session.

3. Otoacoustic emission (OAE) testing: distortion product (DP) OAEs, as well as transient evoked (TE) OAE suppression.

OAEs are low-level acoustic signals that originate in the cochlea; they can be recorded with sensitive microphones in the external auditory canal. OAEs can occur spontaneously, in the absence of an eliciting stimulus, or they can be evoked with a variety of different stimuli. DPOAEs are the result of two primary

tones of different frequencies (f1 and f2) presented simultaneously to the cochlea, and provide mid- and high-frequency (~1-8 kHz) information on the functional integrity of the cochlear outer hair cells. TEOAEs are often evoked using a click stimulus that elicits a broadband response up to approximately 4 kHz from the basilar membrane. This broadband response is then analyzed in half octave frequency bands. Suppression of the TEOAE response (level) can be observed when a contralateral masker is presented to the non-test ear. This is a non-invasive window into the function and integrity of central locations along the auditory pathway that regulate OAE production, specifically, the olivocochlear efferent system.

Procedure: Due to a risk of patient fatigue or lack of cooperation, emphasis was placed on obtaining DPOAEs first because they provide frequency-specific information across a broad range, from 842 to 7996 Hz. Following i*n situ* calibration in the ear canal, DPOAEs were measured by varying f2 in one-quarter octave decrements from 7996 to 842 Hz. In order to achieve a robust 2f1-f2 emission, an f2/f1 ratio of 1.2 (e.g., Abdala, 1996) was used, with L1 and L2 set at 65 dB SPL and 55 dB SPL, respectively (e.g., Stover, Gorga, Neely, Montoya, 1996). A minimum of three full frequency sweeps was accomplished prior to test termination, when possible. An emission was considered present if the DPOAE level was $\geq$ 6 dB above the noise floor.

In cooperative patients who were able to complete additional testing, TEOAE suppression was attempted. TEOAEs were first recorded with a nonlinear broadband click stimulus at a level of 84 (± 3) dB SPL presented 50 times per

second (e.g., Stover & Norton, 1993). In the nonlinear mode of the ILO software, the first few milliseconds of the response are removed from analysis to ensure no stimulus energy is contained within the response, consequently mitigating risk of stimulus artifact contamination. A stimulus check-fit was performed *in situ* in the ear canal prior to TEOAE recording to confirm an adequate stimulus was present. A total of 260 artifact-free averages were obtained to a broad-band stimulus prior to test termination. A nonlinear TEOAE was considered present if the signal-to-noise ratio (SNR) was ≥ 6 dB. If no nonlinear TEOAE was present, further testing for TEOAE suppression was halted.

If TEOAEs were present for at least one half-octave frequency band, and assuming the patient remained cooperative, measurement of TEOAE suppression was attempted. This was performed by placing a probe in the contralateral ear with a broadband white noise masker set to 65 dB SPL, while a linear click stimulus was presented to the test ear at 60 dB SPL. In the linear mode, none of the response window is removed following averaging, and thus contain some stimulus energy in the TEOAE; however, the linear mode is appropriate for TEOAEs at lower stimulus levels and is ideal for measuring suppression of the response, when small changes in TEOAE amplitude are expected. The delivery of stimuli and masker noise alternated between noise-on and noise-off conditions. Specifically, the stimulus alone was presented for ten seconds (noise-off), after which the noise was presented concurrently with the stimulus for ten seconds (noise-on). This interleaved presentation of noise-on, noise-off recording continued until 260 artifact-free averages are obtained for each condition. The

amount of overall TEOAE suppression across frequency bands to contralateral stimulation was calculated by subtracting TEOAE amplitude during the noise-on condition from the amplitude recorded during the noise-off condition. If a difference in amplitude between conditions of greater than or equal to 1 dB (e.g., Berlin, Hood, Hurley, & Wen, 1994) was observed, then suppression was considered present. Given the variability of suppression across individuals (De Ceulaer, et al., 2001), absence of TEOAE suppression was considered pathological only in cases when suppression was previously present.

4. Auditory Brainstem Response (ABR).

The ABR is an evoked electrical potential of neural origin, generated from the distal portion of the auditory nerve through the level of the lower brainstem in response to acoustic stimulation. Like other auditory evoked potentials (AEPs), it is recorded by computer-controlled signal summation and averaging techniques used to distinguish synchronous neural activity from random background electrical activity. The ABR can be recorded from a number of species and is routinely used in the diagnosis and management of auditory dysfunction in humans. Responses can be generated and amplified with broadband stimuli, such as clicks, or more frequency-specific tone bursts. Signals are bandpass filtered (e.g., 300 to 3000 Hz) and multiple recordings, or sweeps, are conducted to generate an averaged waveform response (e.g., Burkard, Shi, & Hecox, 1990).

Brainstem dysfunction is often associated with NPC and there is potential for involvement of the auditory nerve and demyelination. This is a useful test in determining the integrity of the auditory pathway and may add insight to what is

already known about the pathophysiological manifestations of NPC. In the event that behavioral testing is prohibited (because of patient factors), the ABR, in conjunction with other measures, may be used to infer hearing sensitivity.

Procedure: Neuro-otological ABRs were attempted on every patient in the protocol. For those patients who were able to sit quietly during testing, ABRs were obtained while they were awake, resting quietly, and in a seated or recumbent position. All other patients had ABRs performed while they were sedated and in a supine or lateral position. Disposable surface electrodes were placed in an Fz to earlobe (or mastoid) configuration, with a grounding electrode placed at Fpz. Broadband click stimuli were presented via insert earphones (Ear Tone ER-3A). Neuro-otologic ABRs were obtained using a high-level (85 or 95 dB nHL) stimulus intensity. Single polarity traces using negative versus positive polarity clicks (rarefaction and condensation, respectively) were acquired using a click rate of 8.3 per second. Test paradigms including a fast click rate of 63.3 per second (85/95 dB nHL, rarefaction) and a lower intensity stimulus of 65 dB nHL (8.3/second, rarefaction) were recorded to further establish neural integrity (Hall, 1992; Hood, 1998). A minimum of 1000 stimulus presentations were averaged for each ABR test run, and every condition was replicated to determine repeatability of the waveform.

A benefit of using single polarity clicks versus alternating polarity stimuli is that they allow the examiner to distinguish cochlear from neural responses. Specifically, there is potential observation of the cochlear microphonic (CM) during ABR testing. The CM is an alternating current change in voltage that

occurs when the basilar membrane is displaced, believed to originate from depolarization of the cochlear hair cells. This response will mimic the polarity of the incoming stimulus, whereas a neurogenic response will maintain its polarity regardless of changes to stimulus polarity. The ABR is not the ideal recording paradigm with which to observe the CM; therefore, absence of a CM during the ABR is not considered pathologic. However, it is useful to know when a CM is present so that cochlear responses are not inadvertently confused with those that are believed to be neurogenic in nature.

Two control runs were performed on each ear during ABR data collection. The first was a 0 dB nHL (8.3/second, rarefaction) stimulus, and the second was an 85/95 dB nHL (8.3/second, rarefaction) stimulus with the tubing of the insert earphone clamped. These control runs established the quality of the experimental traces by ensuring they were free from electrical and or physiological artifacts, and aided in the quantification of results.

Neuro-otologic ABRs were evaluated based on the presence of waves I, III, and V. Absolute and interpeak latencies of high level, low rate (85/95 dB nHL, 8.3/sec) stimulation were recorded, and overall waveform morphology was assessed. Responses obtained with faster click rates and lower intensity stimulation were evaluated to determine if there was an appropriate degradation of the response and prolongation of wave V, which helped ensure the response was neural in nature.

The presence/absence of cochlear microphonics was evaluated by comparing averages obtained using rarefaction and condensation clicks at a high intensity

(85/95 dB nHL) and low stimulus rate (8.3/second). Cochlear responses mimic the polarity of the incoming stimulus and will therefore reverse with changes in stimulus polarity. These are distinguished from neural responses that do not invert with changes in stimulus polarity.

The total audiologic evaluation was completed in approximately two hours. The order in which tests were administered loosely corresponds to the order in which they were presented within this document; however, this was ultimately dependent on patient cooperation and fatigue. The initial test ear was randomized within and across patients. The methods and stimuli used for behavioral data collection varied depending on the age and ability of the participant, which is standard for audiologic evaluations. Indeed, the ability of this population was highly variable given the early onset of the disease and the progressive neurological involvement, including psychosis and/or dementia. The test technique used during the baseline evaluation was maintained as the test technique for all follow-up visits, when possible.

Acoustic admittance measures, OAEs, and ABR are inherently objective measures of auditory function that can be administered consistently. Behavioral audiometry proved more difficult to administer in a consistent manner and to obtain complete results during each test session because either a patient was too young or too severely affected to complete the exam. In those patients for whom no behavioral hearing data could be obtained, Auditory Steady State Response (ASSR) measures were used to estimate peripheral hearing sensitivity for the purpose of determining appropriate

clinical intervention. The ASSR was obtained while the patient was sedated for additional procedures (e.g., imaging studies, Lumbar puncture).

Assessment of auditory midbrain and cortical function was not included in this protocol. Formal assessment of central auditory function would not be possible to obtain in the vast majority of patients with NPC while they are awake and would be unreliable or unattainable in a sedated patient. If these data could be obtained in a small number of patients with NPC, it would likely not be possible to extrapolate findings meaningfully to the larger population of patients.

Statistical Analyses. Data were maintained and analyzed using Microsoft Excel, the Statistical Package for Social Science Software (SPSS, v15), and the Proc MIXED SAS 9.1 software packages. Experiment one was a single-group design for analysis on an individual level and across the group of NPC patients. For those individuals with longitudinal data, their baseline audiological exam served as the reference to which all subsequent evaluations were compared.

To identify potential independent variables that may affect hearing, a mixed model linear regression analysis was used to evaluate effects of gender, use of the experimental therapeutic drug miglustat, and age. Three variables related to age were examined: age at disease onset (defined as age at first symptom), age at baseline testing, and time from disease onset. Because all three age variables are related, they were not included in the model together at any one time, but rather evaluated independently and with other non-age-related variables. The dependent variables to quantify hearing were pure-tone averages: .5/1/2 kHz, .5/1/2/4 kHz, .25/5 kHz, and 4/8 kHz. For this analysis, left and right ears were combined. For all statistical analyses $\alpha = .05$.

For patients less than 6 years of age, tympanometry was considered normal if the static admittance value was between 0.2 mmhos and 0.8 mmhos, if the tympanometric pressure peak was between -140 and +15 daPa, and the equivalent ear canal volume (V_{ec}) was between 0.4 to 1 cm^3. For patients 6 years of age and older, tympanometry was considered normal if the static admittance value was between 0.3 mmhos and 1.4 mmhos, if the tympanometric pressure peak was between -85 and 0 daPa, and the equivalent ear canal volume (V_{ec}) was between 0.6 to 1.5 cm^3 (Margolis & Heller, 1987). Ipsilateral and contralateral acoustic reflexes were considered normal if they were elicited at levels equal to or less than the 90^{th} percentile published by Gelfand, Schwander, and Silman (1990). Acoustic reflex decay was considered negative (normal) if the change in admittance did not decrease by $\geq$ 50% during stimulus presentation over a 10 second period of time. These measures were analyzed as categorical data and defined based on normal and abnormal criteria.

Pure-tone audiometric data were evaluated and the type, degree, and configuration of hearing loss were determined from criteria described by King et al. (2007) in Table I. Individual and mean data across the cohort were analyzed. Longitudinal change in hearing was evaluated individually, and cross-sectional data were used to evaluate hearing and age-related change.

Suprathreshold word recognition scores were collected initially to compare to the Speech Intelligibility Index (SII, ANSI S3.5-1997), based on the Articulation Index (AI, French & Steinberg, 1947). However, the small number of individuals for whom these data could be collected, the large range of patient performance, and the variation in the type of test administered (NU-6, WIPI, and PB-K word lists) limited the application of

the SII to this data set. In those patients able to provide word recognition scores at two presentation levels (moderate and high dB SL re: SRT), a rollover index was calculated from the phonetically balanced (PB) NU-6 word lists to differentiate cochlear versus eighth nerve lesions ($RI = PB_{max} - PB_{min} / PB_{max}$, where PB_{max} is the highest word recognition score for an ear and PB_{min} is the lowest). A RI of > 0.25 (Bess, Josey, & Humes, 1979) was considered as a positive sign for a retrocochlear lesion.

The presence or absence of DPOAEs and TEOAE suppression were documented in each participant and were used to determine site of lesion within an individual. Mean (SD) DPOAE level data for the group across test frequencies was calculated. Presence and absence of DPOAEs (6 dB SNR) were determined in those individuals without evidence of middle ear disease. TEOAE suppression was categorically analyzed as either present or absent, and the absence of TEOAE suppression was only considered pathological when it was present previously in an individual.

Interpretation of ABR absolute and interpeak latencies was based on normative data published by Joseph, et al. (1987) for those greater than 3 years of age and Issa and Ross (1995) for those less than or equal to 3 years. Descriptive statistical group data, including categorical interpretation (normal versus abnormal) for absolute and interpeak latencies are reported.

Table I. Operational definitions for normal hearing and type, degree, and configuration of hearing loss.

Classification		Criteria
*Type	Normal	Average AC thresholds ≤ 20 dB HL No A-B gaps > 10 dB
	Conductive	Average BC thresholds ≤ 20 dB HL Average A-B gap ≥ 15 dB
	Sensorineural	Average BC thresholds > 20 dB HL Average A-B gaps ≤ 10 dB
	Mixed	Average BC thresholds > 20 dB HL Average A-B gap ≥ 15 dB
	Unknown	Does not meet above criteria
†Degree	Normal	≤ 20 dB HL
	Mild	> 20 and ≤ 40 dB HL
	Moderate	> 40 and ≤ 70 dB HL
	Severe	> 70 and ≤ 95 dB HL
	Profound	> 95 dB HL
Configuration	Normal	All PT thresholds from 250-8000 Hz ≤ 20 dB HL
	Flat	< 15 dB difference between all thresholds from 250-8000 Hz
	LF-Ascending	≥ 15 dB difference between LF and better HF thresholds
	Mid-Frequency U-Shaped	≥ 15 dB difference between worst mid-frequency (1000-2000 Hz) thresholds and those of higher- and lower-frequencies
	HF-Gentle	15-29 dB difference between mean thresholds of 500 and 1000 Hz and mean thresholds of 4000 and 8000 Hz
	HF-Sharp	≥ 30 dB difference between mean thresholds of 500 and 1000 Hz and mean thresholds of 4000 and 8000 Hz
	Atypical	Does not meet above criteria

Note. AC, air conduction; BC, bone conduction; A-B, air-bone; PT, pure-tone; LF, low-frequency (250-500 Hz); HF, high-frequency (4000-8000 Hz). * Type of hearing loss was determined based on three-frequency (500, 1000, 2000 Hz) PT averages for AC and BC. † Degree of hearing loss was based on a four-frequency (500, 1000, 2000, 4000 Hz) PT AC average. From "Auditory Phenotype and Karyotype of 200 Women with Turner Syndrome," by K. King, et al., 2007, *Ear and Hearing, 28*(6), p.833.

Results

Fifty-five patients (26 males, 29 females) with NPC, confirmed via biochemical or molecular markers, were enrolled in this study between 8/14/2006 and 12/27/2010. Five sibling pairs, including one set of monozygotic twins, are included in the cohort. The mean age at enrollment was 11.6 years (SD =10.2 years), although there is large variability in age (minimum = 4 months, maximum = 51.3 years), as shown in Figure 1. Just over half (53%) of the cohort was less than 10 years of age, and the majority (80%) fall within a pediatric range of less than18 years. The average age of disease onset was 3.7 years (Figure 2) and, while the initial symptom most often involved the hepatic system, 16 individuals presented with early neurological findings (Table II).

Thirty-five (64%) patients were able to participate to some degree for behavioral testing, which resulted in data that varied from a single pure-tone threshold to a complete audiological evaluation. Alternatively, 20 patients were unable to provide any behavioral data, which was a reflection not only of a patient's age, but also disease status. Among these 20 cases, the age range varied from six months to 32 years.

Within the cohort of 55, five patients presented with a late-onset variant of the disease; these five patients ranged in age from 25 to 46 years in age at the time of their diagnosis. In addition to this atypical disease onset, these patients each presented with unique auditory histories and findings. Therefore, their data have been removed from the cohort to analyze separately.

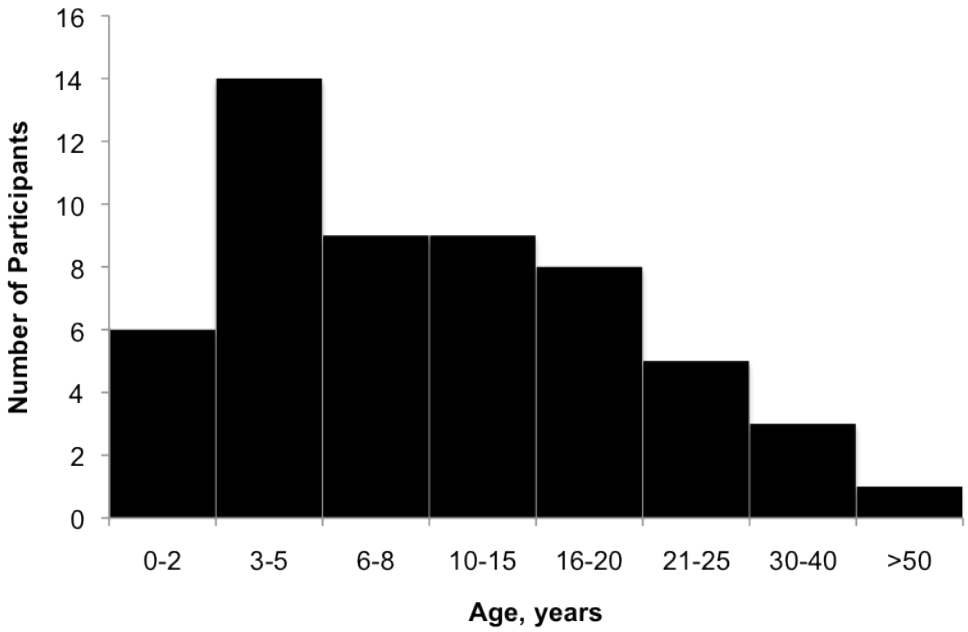


Figure 1. Age at enrollment of study for 55 patients with NPC.

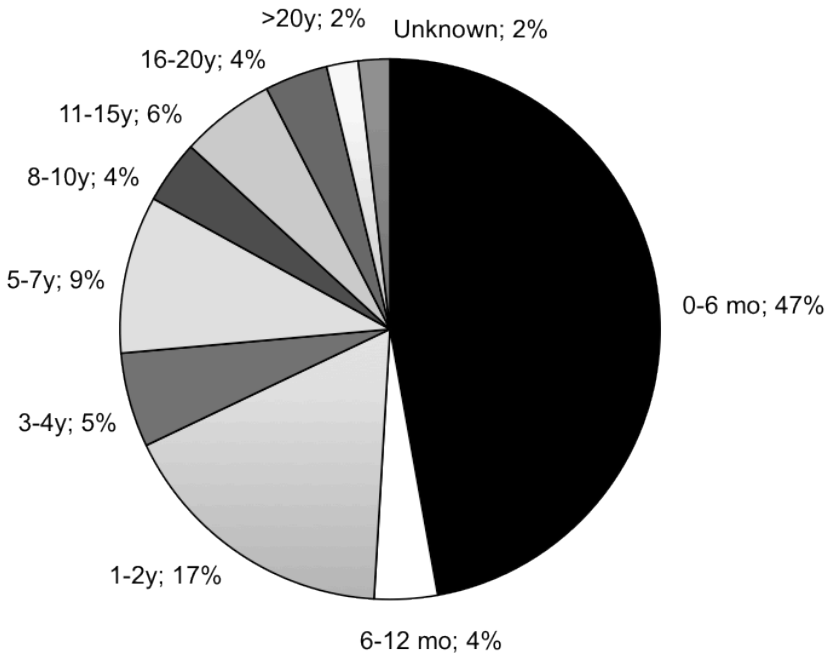


Figure 2. Age at initial symptom for 55 patients with NPC. (mo, months; y, years)

Table II. Presenting symptom, determined via oral histories and medical records provided to Dr. Forbes Porter.

Presenting Symptom (n)
Hepatic dysfunction / Jaundice (19)
Splenomegaly (17)
Learning delay (5)
Fetal ascites (3)
Clumsiness / Poor coordination (3)
Developmental delay (2)
Cognitive decline/ Slurred speech (1)
Depression (1)
Fine motor ataxia (1)
Hearing loss (1)
Vertical gaze palsy (1)
Psychosis (1)

Baseline audiometry findings. Mean (SD) pure-tone air-conduction thresholds at baseline for 31 patients (late-onset cases removed) ranging in age from three to 21 years ($\bar{x}$ =10.9, SD=5.6) are plotted in Figure 3, and reveal an average high-frequency hearing loss phenotype across the cohort. There are no obvious asymmetries between right and left ears. Mean data are closer to the cutoff for normal/abnormal than to the reference value for normal hearing (0 dB HL).

Because this is predominantly a pediatric population, the normative cut-off for hearing loss in an adult (Table 1) was modified to a normative cut-off of 15 dB HL that is more appropriately applied to children (Clark, 1981). When sufficient pure-tone data were collected to determine degree of hearing loss (.5/1/2/4 kHz PTA), 13 of 52 (25%) ears had a mild loss; the remaining ears (75%) were categorized as within normal limits. When percent of ears with hearing loss (>15 dB HL) by frequency is evaluated (Figure 4), approximately half of the sample exhibited hearing loss involving the high frequencies. When a high frequency average (.4/8 kHz) is considered (Table III), the degree of hearing loss ranged from within normal limits to a moderate loss.

Bone conduction data were obtained in fewer cases than air-conduction (Table III) because of patient fatigue and lack of cooperation. When bone conduction data (.5/1/2 kHz PTA) are not available, type of hearing loss cannot be determined. Thirty-one of 62 ears with pure-tone data had insufficient bone conduction data to determine type of hearing loss (.5/1/2 kHz PTA). Of the remaining 31 ears, 26 had no hearing loss (as operationally defined in Table I), two had conductive, and three had sensorineural hearing loss. In total, 23 patients were newly identified with hearing loss based on their

baseline evaluations: 15 by behavioral evaluations of hearing and 10 via sedated electrophysiological assessments of hearing (ASSR).

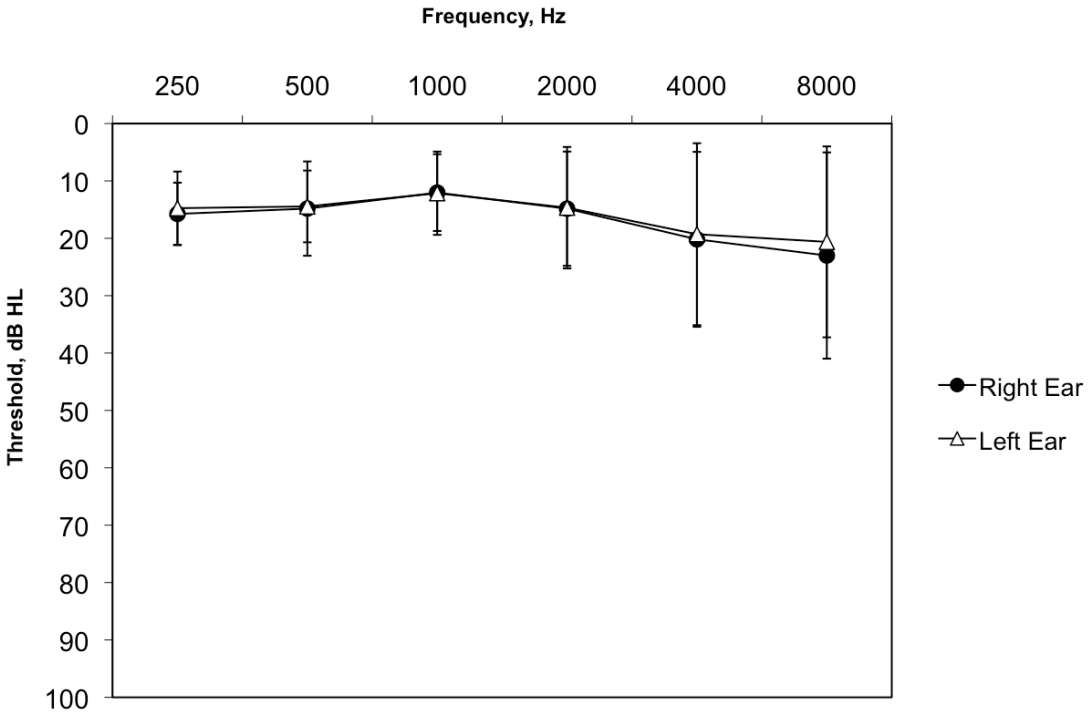


Figure 3. Mean (SD) pure-tone air-conduction thresholds for 31 patients ranging in age from 3 to 21 years ($\bar{x}$ =10.9; SD=5.6).

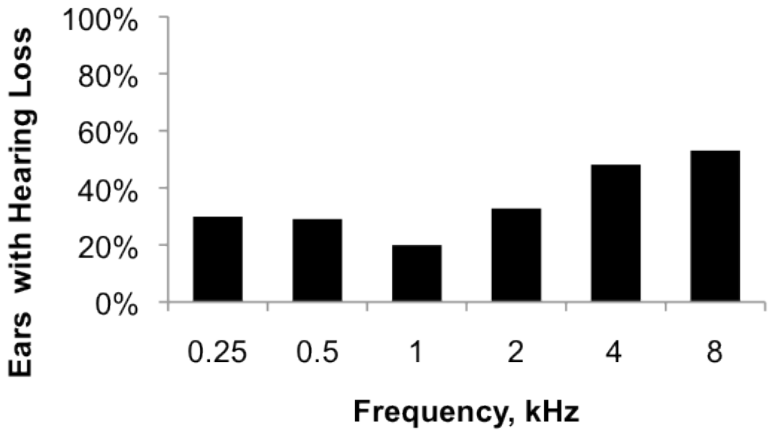


Figure 4. Percent of ears (based on 31 patients) with hearing loss (>15 dB HL) by frequency.

Table III. Pure-tone averages for air- and bone-conduction data from 31 patients with NPC.

	.5/1/2 AC (n=52)	.5/1/2 BC (n=31)	.5/1/2/4 AC (n=52)	.5/1/2/4 BC (n=30)	.25/5 AC (n=40)	.25/5 BC (n=28)	.4/8 AC (n=49)
Mean	13.1	10	14.9	12.2	14.1	9.5	20.7
SD	6.6	6.5	8.1	8.1	5.1	5.2	15.8
Range	1.7 - 28.3	-1.7 – 26.7	2.5 – 37.5	0 – 35	5 – 22.5	0 – 20	0 – 60

Note. Data are presented in dB HL, based on ears (n=). AC, air conduction; BC, bone conduction.

A mixed model linear regression analysis failed to reveal any relationships between hearing and the independent variables age (baseline, disease onset, time from disease onset), gender, or use of miglustat (Table IV). Mean (SD) thresholds by pure-tone averages are plotted for gender in Figure 5. At baseline, 12 patients tested positive for miglustat, 16 tested negative, and three samples were unavailable. Scattergrams by age showing the spread of distribution between miglustat groups are plotted for mid-frequency (.5/1/2/4 kHz) and high-frequency (4/8 kHz) pure-tone averages in Figure 6. The relationship between age at disease onset and these pure-tone averages at baseline is shown in Figure 7. Cross-sectional data showing the distribution of hearing (mid- and high-frequency pure-tone averages) by age at baseline are shown in Figure 8. There are no obvious associations between these variables associated with age and hearing.

Suprathreshold speech findings. Maximum suprathreshold speech recognition performance in quiet for 35 patients able to participate in testing is plotted in Figure 9. Overall, performance was consistent with peripheral hearing sensitivity. A rollover index (RI) was calculated for the 20 patients who could participate in monitored live voice NU-6 word testing at two levels (moderate and high dB SL re: SRT). The RI was considered significant if it was greater than 0.25 (Bess, et al.,1979) Five patients exhibited significant decline in their performance (rollover) when speech was presented at the higher intensity level; one patient has a late-onset variant of the disease, and the other four fall within the pediatric classical onset. In all cases, this finding supports retrocochlear dysfunction of the auditory system; however it should be noted that stimuli were administered via monitored live voice, and normative data are based on recorded material.

Table IV. Results of mixed model linear regression analysis of hearing for included predictor variables.

Dependent Variable	Predictors	B coefficient	S. E. (standard error)	T	Significance
.5/1/2 kHz					
	Miglustat	4.20	2.53	1.662	.111
	Gender	1.77	2.57	.688	.498
	Age at baseline	.253	.228	1.110	.278
	Age at onset	.395	.241	1.635	.118
	Time from onset	-.318	.331	-.961	.348
.5/1/2/4 kHz					
	Miglustat	4.84	3.11	1.557	.134
	Gender	2.59	3.19	.813	.424
	Age at baseline	.366	.281	1.305	.204
	Age at onset	.520	.310	1.676	.109
	Time from onset	-.241	.432	-.558	.583
4/8 kHz					
	Miglustat	6.67	6.56	1.017	.321
	Gender	3.82	6.30	.607	.550
	Age at baseline	.700	.585	1.197	.243
	Age at onset	.878	.632	1.389	.180
	Time from onset	-.193	.891	-.216	.831

Note. B coefficient estimates variation in hearing accounted for by the predictor, and the t statistic determines relative importance of the predictors in the model.

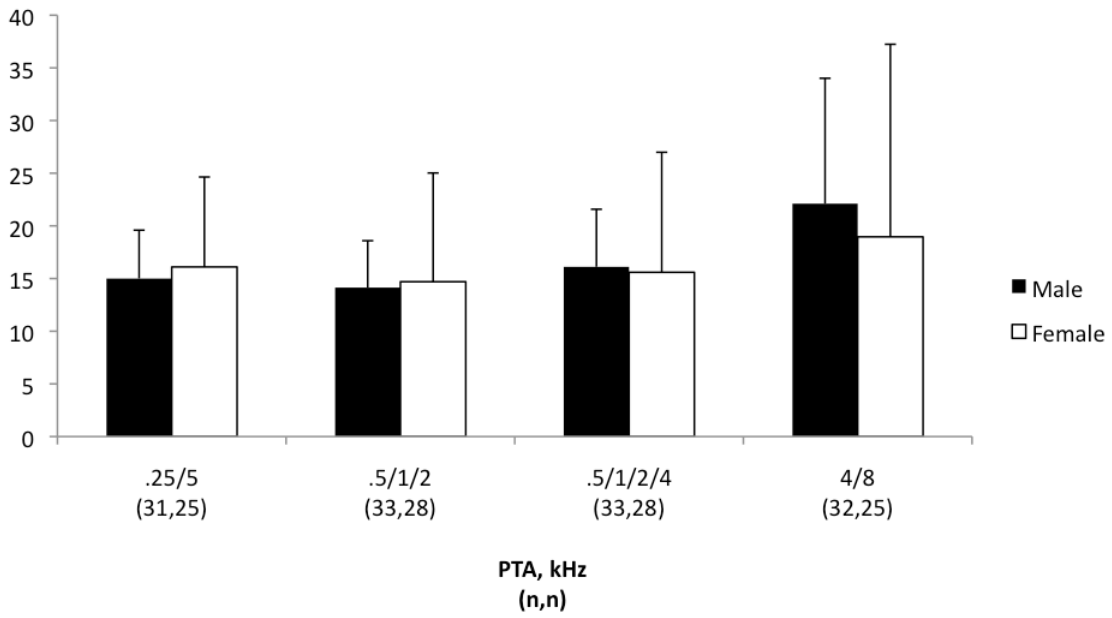


Figure 5. Mean (SD) PTA air-conduction thresholds for male and female participants. The n is provided for male and female ears (n,n), respectively.

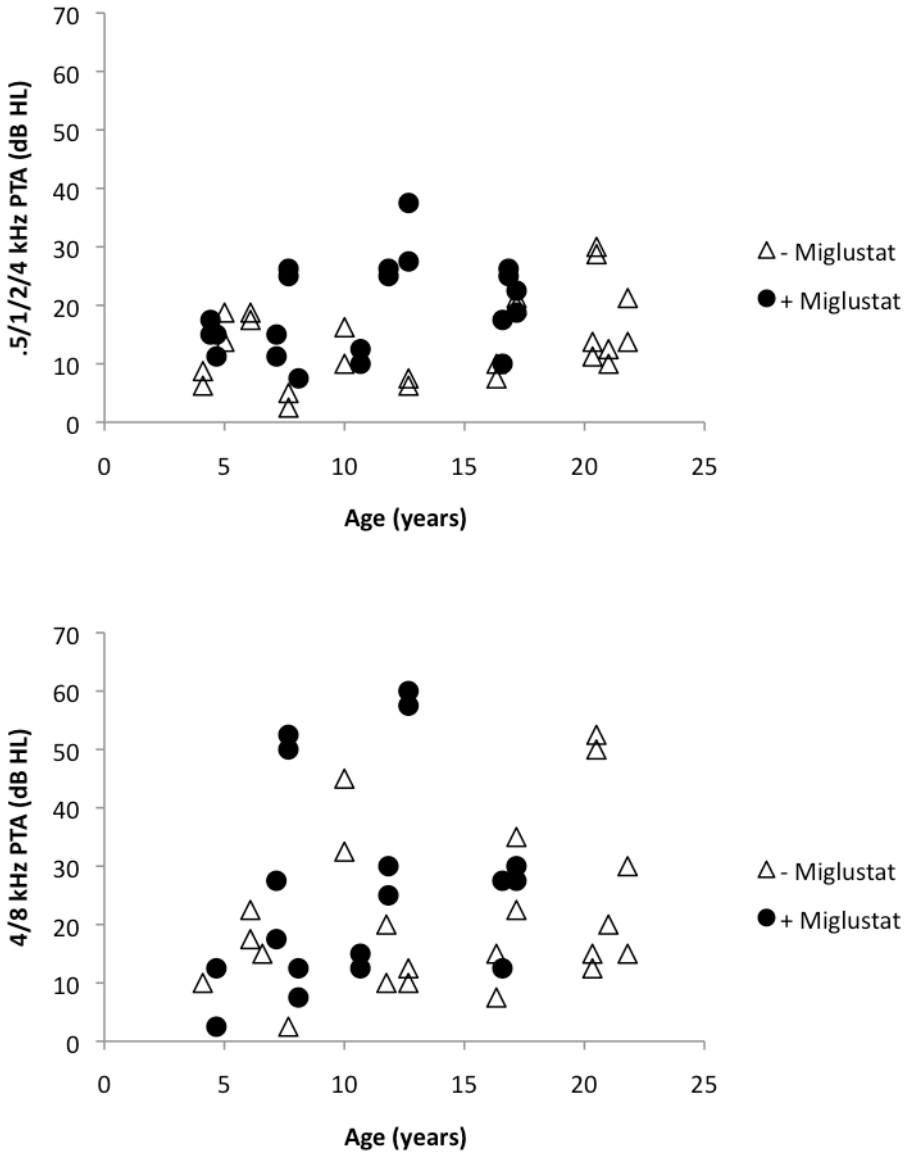


Figure 6. Hearing thresholds for a four-frequency (top panel) and high-frequency (bottom panel) PTA. Filled circles represent ears from 12 patients who tested positive for miglustat at baseline. Open triangles represent ears from 16 patients with no evidence of miglustat at baseline.

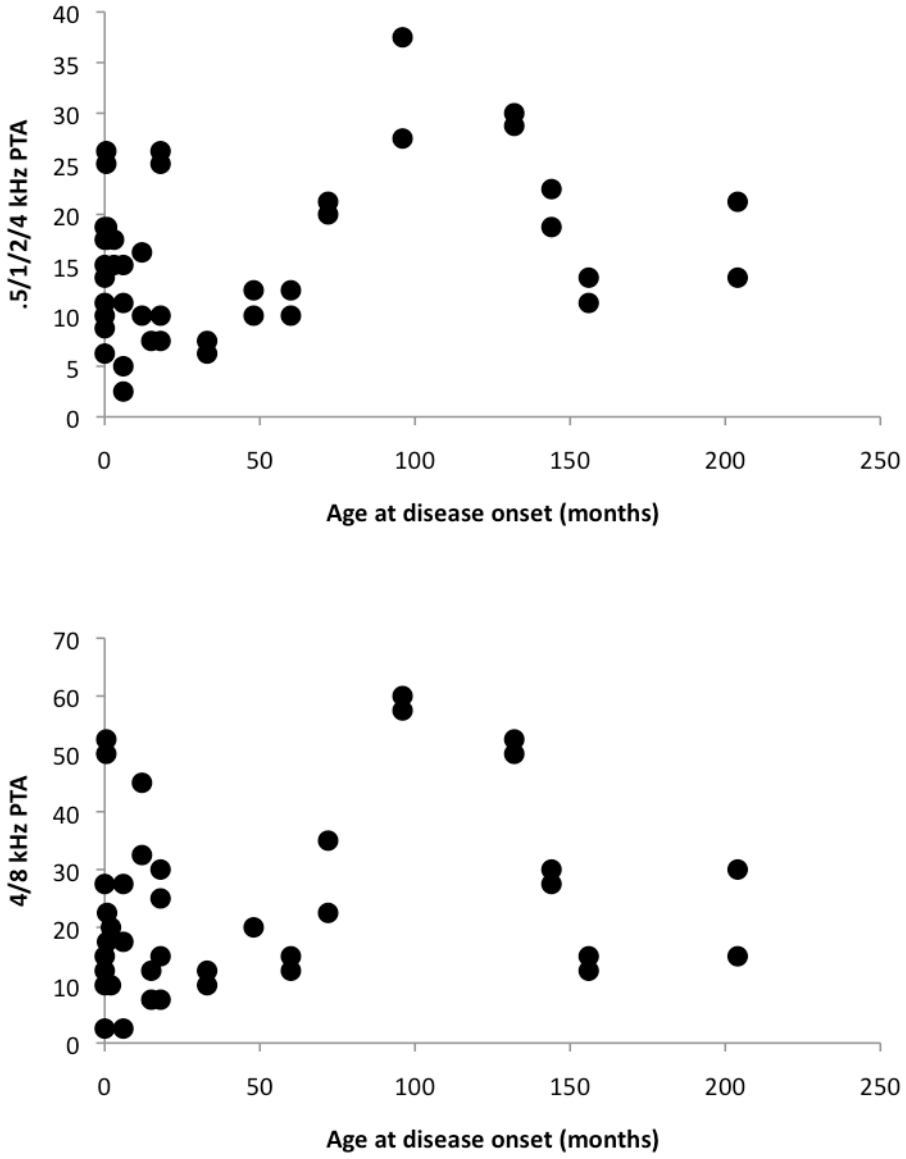


Figure 7. Hearing thresholds (ears) for a four-frequency (top panel) and high-frequency (bottom panel) PTA at baseline by the age of disease onset (based on patient report and review of medical records).

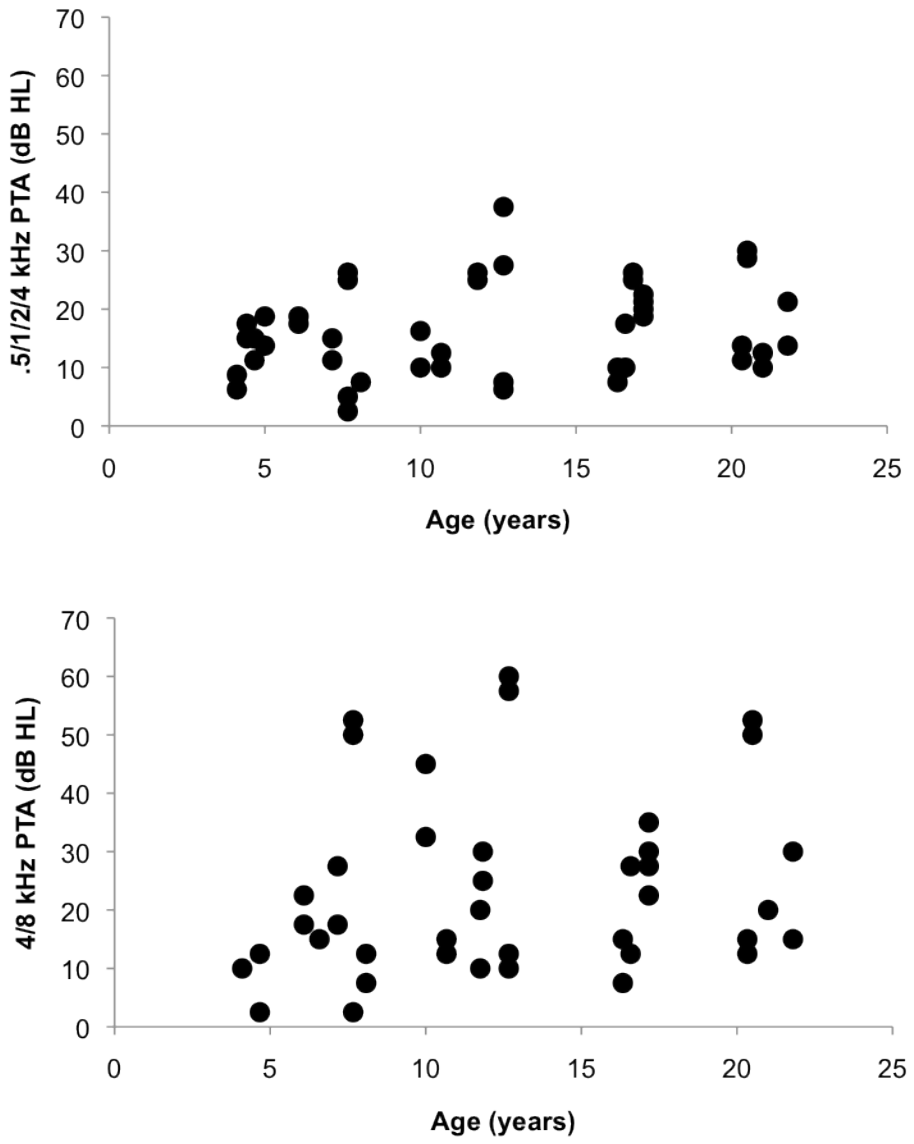


Figure 8. Hearing thresholds (ears) at baseline for both a four-frequency (top panel) and high-frequency (bottom panel) PTA by age.

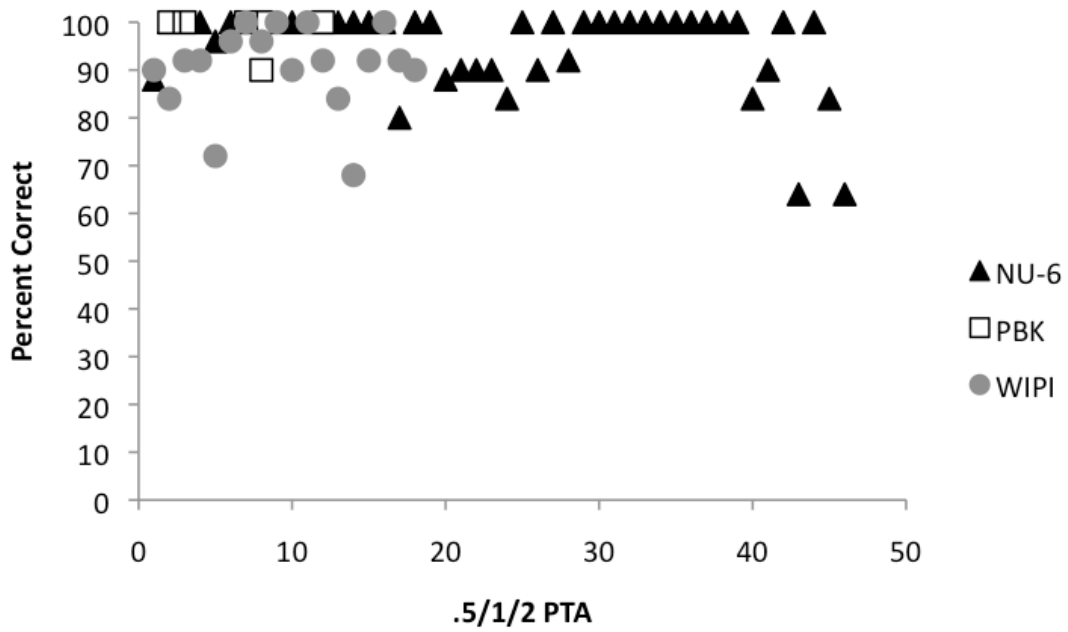


Figure 9. Maximum suprathreshold speech recognition performance in quiet by three-frequency PTA for 70 ears with NPC. NU-6, Northwestern University Test #6; PBK, Phonetically Balanced Kindergarten test; WIPI, Word Intelligibility by Picture Identification test.

Immittance findings. Immittance data were collected on 54 patients. Table V shows the percentage of normal and abnormal findings for tympanometry and the acoustic reflex. Overall, middle ear status fell within normal limits for tympanometry. The most common abnormality observed was TPP, which revealed both positive and negative pressure findings.

Acoustic reflex thresholds were abnormal (elevated or absent) in 92% of patients in whom these data could be collected. Sixty-three percent of these cases cannot be explained by either middle ear status or peripheral hearing thresholds. Although acoustic reflex adaptation could only be evaluated in a small number of individuals (n=10), adaptation was abnormal (positive) in four (40%) of these cases. Both the acoustic reflex threshold and adaptation findings suggest retrocochlear dysfunction of the auditory system for a significant percentage of the cohort in whom these data could be collected.

Otoacoustic emissions findings. DPOAEs were measured in all 55 patients (110 ears). In cases with significant middle ear dysfunction (i.e., outside the age-appropriate normative range for tympanometry), OAE data were removed from analysis. Late-onset cases are analyzed separately. Mean (SD) DPOAE level data from the remaining 82 ears are plotted in Figure 10. The large variability in DPOAE level is not surprising in such a heterogeneous population. Analysis of present versus absent DPOAEs by frequency across the cohort (Figure 11) revealed over half of ears had absent low frequency DPOAEs (842 Hz – 1000 Hz), because of an elevated noise floor (Figure 9). DPOAEs were present in 50% to 75% of ears at frequencies from 1189 Hz to 6727 Hz, with only 28% of emissions present at 7996 Hz, consistent with the high frequency hearing loss noted earlier.

Nonlinear TEOAEs were collected on 19 individuals in an attempt to test for TEOAE suppression. Of those 19, eight individuals had present nonlinear TEOAEs and were able to continue participation to measure for suppression. Seven of these individuals had measureable TEOAE suppression (≥ 1 dB) in their overall response; one patient had absent TEOAE suppression.

Table V. Immittance findings for 54 patients with NPC

Tympanometry Findings (n=108 ears)				
	WNL	Abnormal		CNT
		High	Low	
V_{ec} (cm^3)	79% (85)	18% (19)*	3% (4)	
Admittance (mmhos)	82% (88)	9% (10)	9% (10)	
		Negative	Positive	
TPP (daPa)	53% (57)	17% (18)	24% (26)	6% (7)*

Acoustic Reflex Findings (n=26 patients)			
	WNL	Abnormal	CNT
Acoustic Reflex Thresholds	8% (2)	92% (24)**	
Acoustic Reflex Adaptation	23% (6)	15% (4)	62% (16)

Note. WNL, within normal limits; CNT, could not test; $V_{ec,}$ equivalent ear canal volume; TPP, tympanometric peak pressure. *Includes four ears with intact, patent pressure equalization tubes **63% (15/24) cannot be explained by middle ear status or peripheral hearing thresholds.

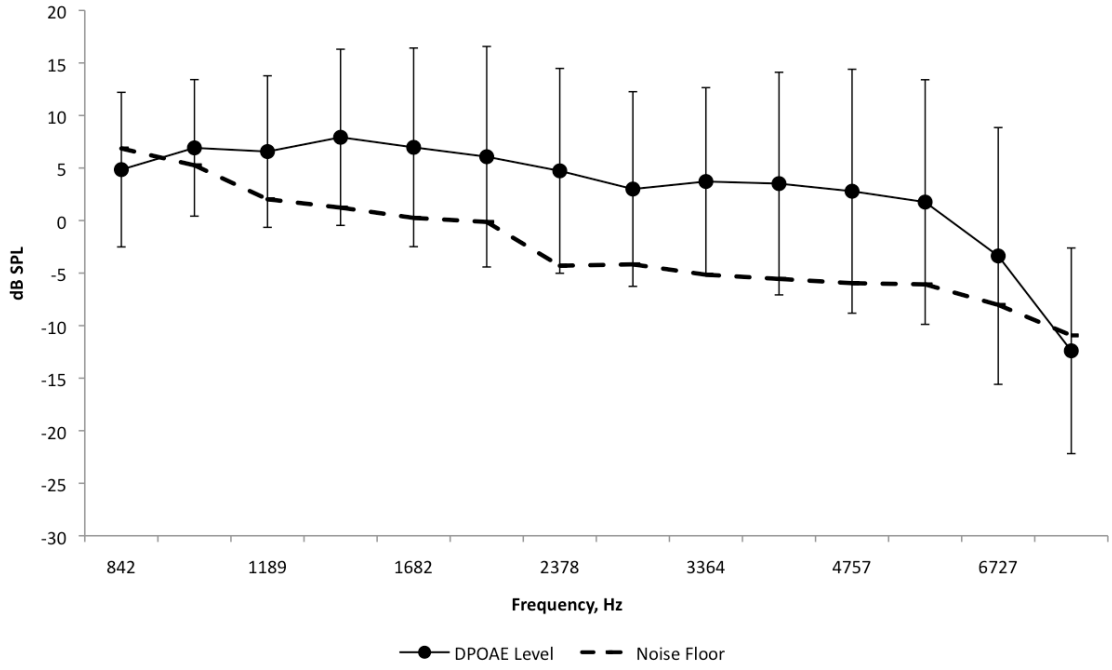


Figure 10. Mean (SD) DPOAE amplitude and noise floor from 82 ears with no significant middle ear disease (late-onset cases removed).

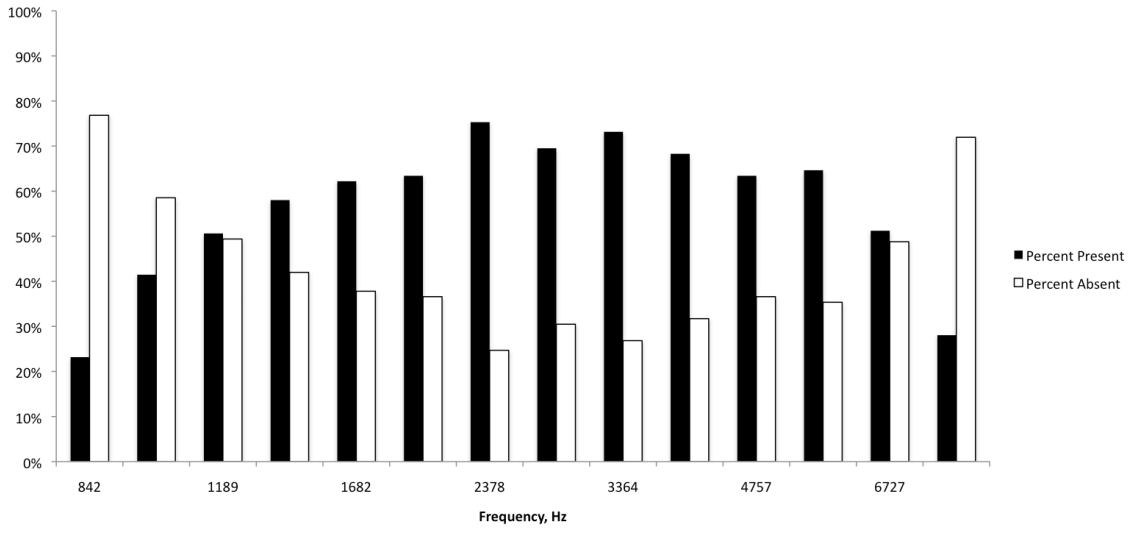


Figure 11. Percent of ears without significant middle ear disease (late-onset cases removed) with present (≥ 6dB SNR) and absent DPOAEs.

ABR findings. Baseline neuro-otologic ABRs were collected in 54 patients (107 ears). Table VI shows the number of ears (without late-onset cases, n=97 ears) with normal and abnormal findings for both absolute and interpeak latencies. Results reflect averaged (condensation and rarefaction) responses to ipsilateral stimulation of each ear. The most common ABR abnormalities observed were poor waveform morphology, characterized by absent waves I and III, and prolongation of the interpeak interval between waves I-III and I-V. Forty-one of 49 patients (84%) had an ABR abnormality in at least one ear. Of these, nine had normal hearing and abnormal ABR results; 11 had peripheral hearing loss, but ABR abnormalities were disproportionate to the degree of loss (e.g., mild high frequency hearing loss, present wave I, absent wave III); three patients had hearing loss that could account for the ABR abnormalities; and 18 patients had no behavioral peripheral hearing data with which to compare to their ABR abnormalities.

An additional salient finding on ABRs collected in this cohort were large and prolonged sinusoidal activity occurring in the first few milliseconds (e.g., 1-5 ms) of the recording, interpreted as cochlear microphonic (CM). Figure 12 shows ABRs collected using the same equipment and under the same paradigm from a 45-year-old female without NPC, and without neurological disease. Both the right and left ABRs are normal and the CM observed in the left ear is an appropriate duration (< 1 ms). There is no CM observed in the right ear. Because the ABR is not the ideal measurement to record the CM, its absence on the ABR is not considered a pathological finding.

Figure 13 shows the ABR traces from a six-year-old male with NPC. Large amplitude and prolonged CMs are observable in both the left and right ear responses.

Specifically, the CM in the left ear extends out to approximately 4 ms and the CM in the right ear extends out to just beyond 3 ms. Quantification of these responses is difficult; latency is the most efficient and accurate way to interpret data, and there are no normative criteria for CMs observed on the ABR. Based on the observation of hundreds of ABRs collected on the same equipment using the same test paradigm, a criterion of > 1 ms was established as an abnormal finding. Of 89 ears in which a CM was visible during the ABR recording, 34 (44%) showed evidence of prolonged responses. Many of theses responses were noted to be large in amplitude as well (e.g., Figure 13). These are interpreted as CM activity because the responses reversed polarity when the stimulus polarity was reversed. Neural responses do not reverse polarity with changes in stimulus polarity. Electrical artifact was ruled out via a control run when the same stimulus was delivered and the tubing of the insert earphone was clamped; in such cases activity interpreted as CM was not present.

Site of lesion. When each patient's collective findings are considered, an individual profile of a cochlear, retrocochlear, or mixed site of lesion is available for 40 patients. The remaining 15 patients lacked sufficient data to determine a site of lesion. Results of this analysis are shown in Table VII. Pure cochlear hearing loss (e.g., elevated pure-tone thresholds, absent DPOAEs, normal acoustic reflexes, normal ABR) was observed in 7% of patients. Retrocochlear dysfunction of the auditory system alone (e.g., pure-tone thresholds within normal limits, present DPOAEs, elevated/absent acoustic reflexes, abnormal ABR) occurred in 35% of patients. The most common single finding among these 40 individuals was a mixed (cochlear and retrocochlear) site of lesion.

Retrocochlear dysfunction with or without evidence of cochlear involvement occurred in 75% of patients. Eighteen percent had no abnormal findings.

Table VI. Absolute and interpeak latency data for ABRs collected on 49 patients (late-onset cases removed).

	Absolute Latencies			Interpeak Latencies		
	I	III	V	I-III	III-V	I-V
Normal latencies	59% (57)	57% (55)	76% (74)	45% (24)	85% (63)	62% (36)
Prolonged Latencies	1% (1)	18% (18)	18% (17)	55% (29)	15% (11)	38% (22)
Absent Waves	40% (39)	25% (24)	6% (6)	*	*	*

Note. Data are presented as % (ears). * Indicates interpeak latencies could not be calculated.

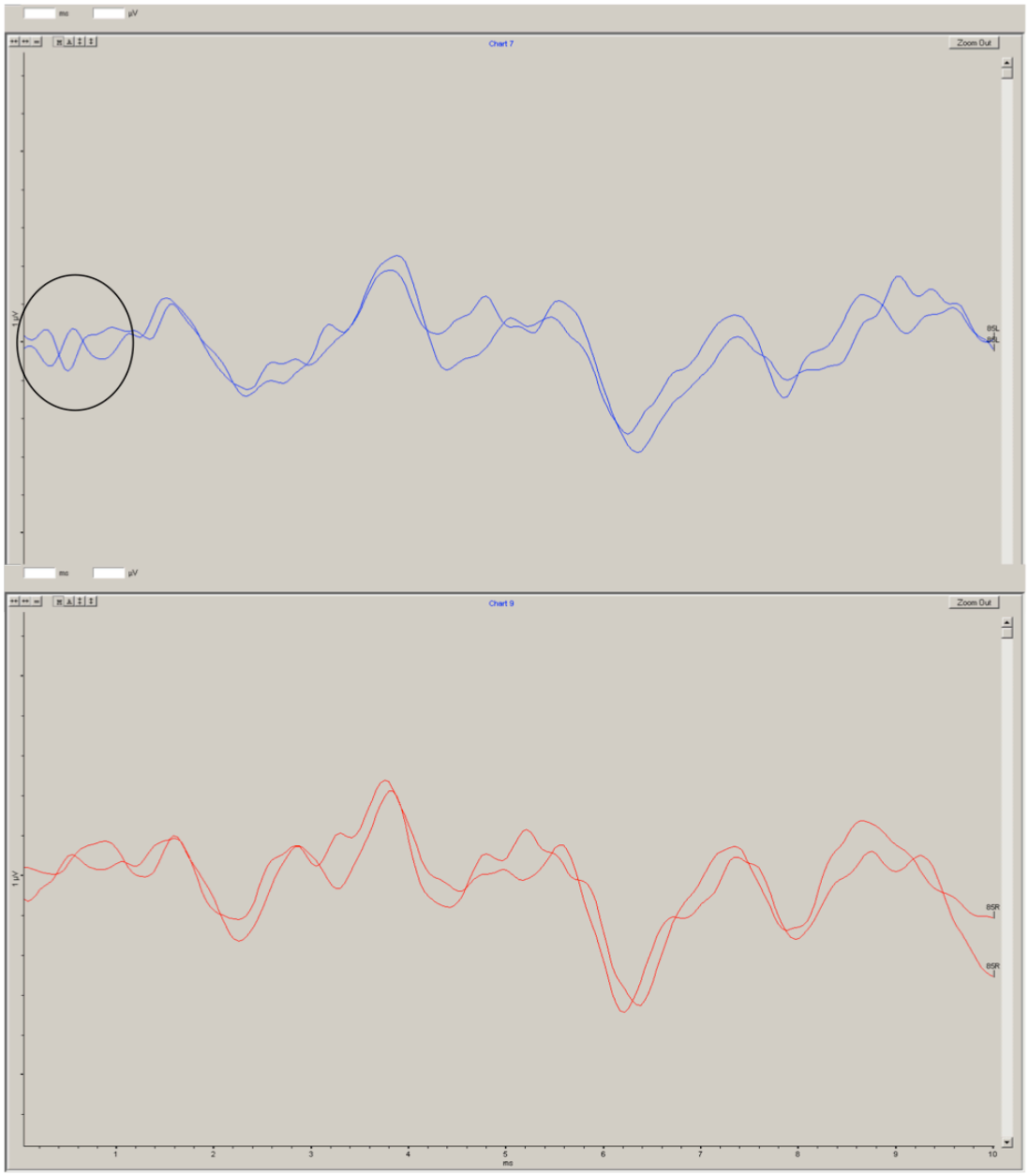


Figure 12. ABRs from a 45-year-old female without NPC or neurological disease, collected using the same equipment under the same test paradigm described in this study. ABRs are interpreted as normal, and the CM observed (circled area) following stimulation of the left ear (top panel) is considered appropriate in amplitude and duration. The CM is not observed following stimulation of the right ear (bottom panel).

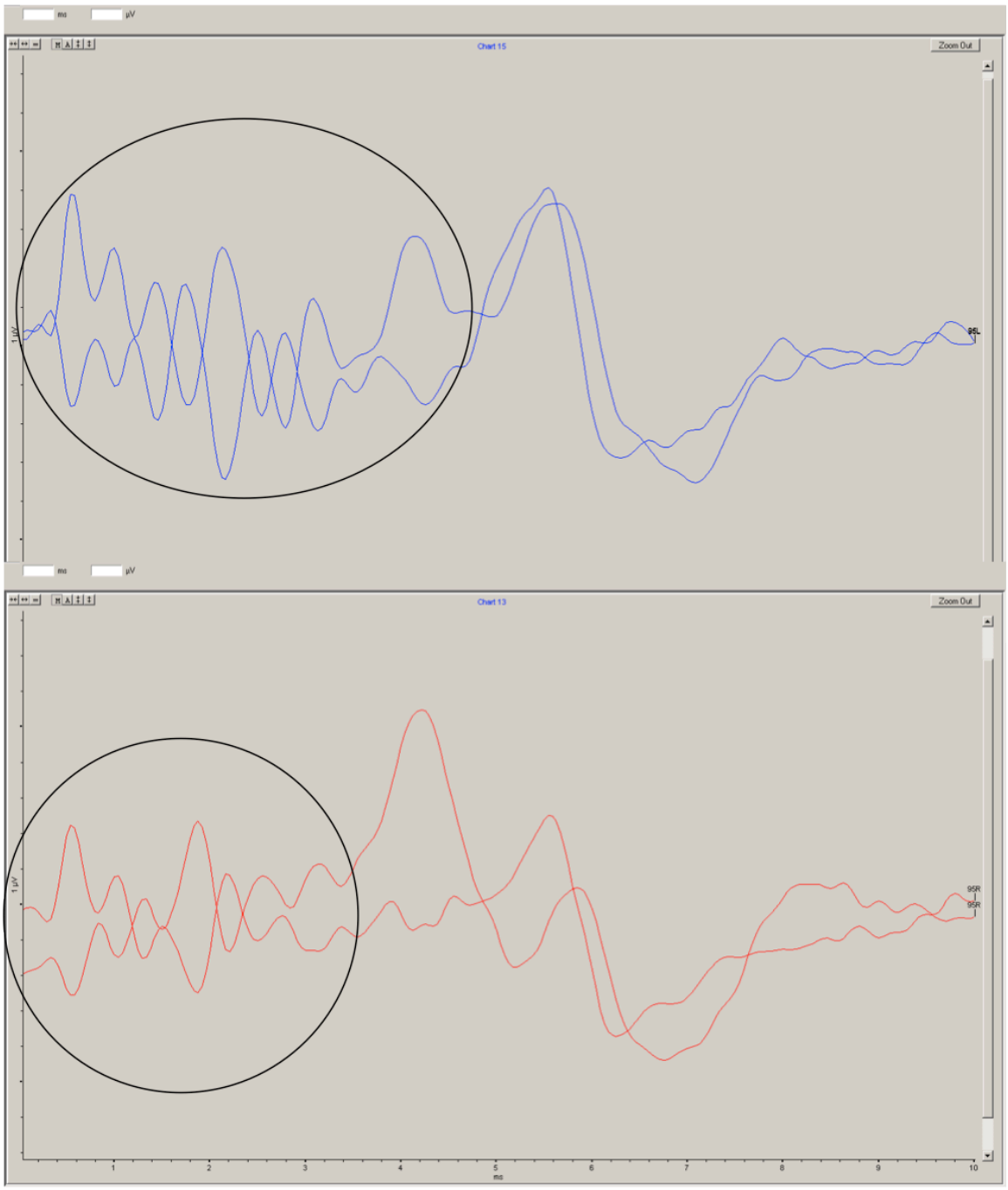
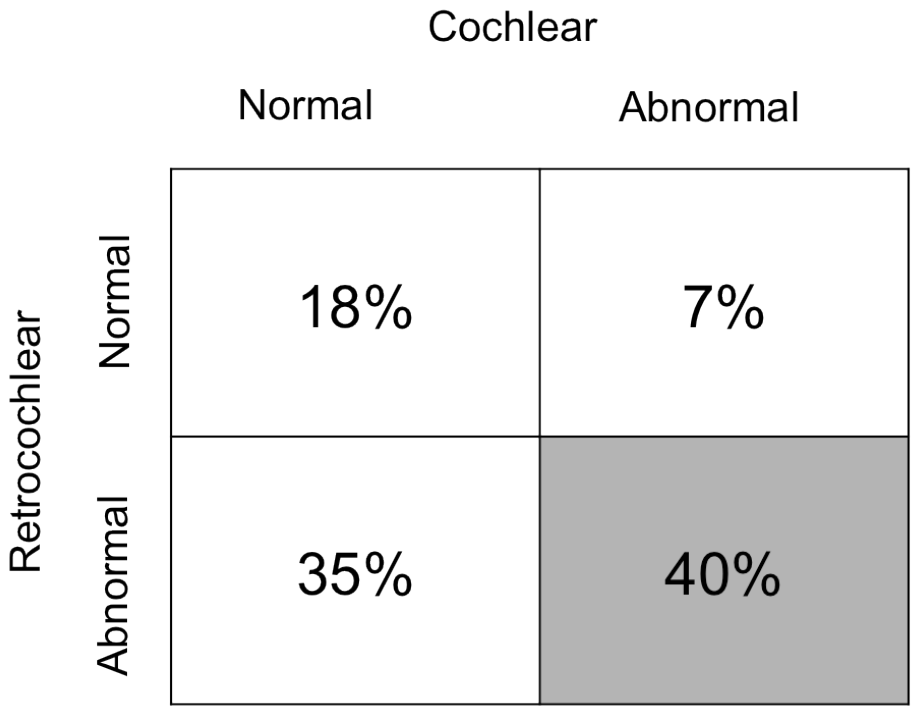


Figure 13. ABRs from a six-year-old male with NPC. Large amplitude and prolonged CMs are observed (circled area) in both the left (top panel) and right (bottom panel) ear responses.

Table VII. Percentage of cases (n=40) that are classified as either normal or abnormal for cochlear and retrocochlear dysfunction, and those cases (shaded area) that qualify as abnormal for both.



Longitudinal Data. Longitudinal data are available for 30 patients. The duration of follow-up is plotted in Figure 14 for 18 patients with longitudinal pure-tone data (top panel) and 26 patients with longitudinal ABR data (bottom panel) (late onset cases removed). The majority of patients in both subsets were followed for two or more years. Age and change in disease status resulted in patients able to participate reliably in some testing at baseline who were then unable to provide the same data at return visits, or vice versa. Because of this limitation, and the variability in the duration of follow-up, rigorous statistical analysis on any of the longitudinal data is not possible. Descriptive results are presented below.

Pure-tone findings: Longitudinal pure-tone data for a four-frequency and high-frequency pure-tone average, and for 8k Hz are plotted in Figure 15. Patients followed longitudinally ranged in age from four to 21 years at the time of their final follow-up audiogram. Hearing sensitivity in several ears is noted to improve (- change in hearing) from baseline, although most of these changes are not outside of test-retest variability (+/- 10 dB). No significant change in averaged mid-frequency (.5/1/2/4 kHz) hearing is noted; of 30 ears with data, only one had a clinically significant (>10 dB) decline in hearing of 12.5 dB. However, the high-frequency average reveals several ears that had significant decline in hearing sensitivity from baseline. Of 12 ears followed for 23 months or more, nine (75%) had a clinically significant (>10 dB) decline in hearing. The largest change in hearing for the high-frequency average (32.5 dB and 27.5 dB for the right and left ears, respectively) occurred in the individual followed for the longest period of time (135 months); she was 21 years at the time of her final audiogram. The trend of decline in hearing in the high-frequency average is stronger when only decline at 8k Hz is

viewed; eight of 12 ears (66%) showed clinically significant decline in hearing, with an average change of 29 dB. For individuals followed for less than a total of 23 months, only one ear at 2000 and 4000 Hz had a significant decline in hearing (15 and 20 dB, respectively). Of the 10 patients followed for at least a two-year period, who ranged in age from 6 to 21 years, eight have had clinically significant declines in hearing; the remaining two are highly functioning sisters.

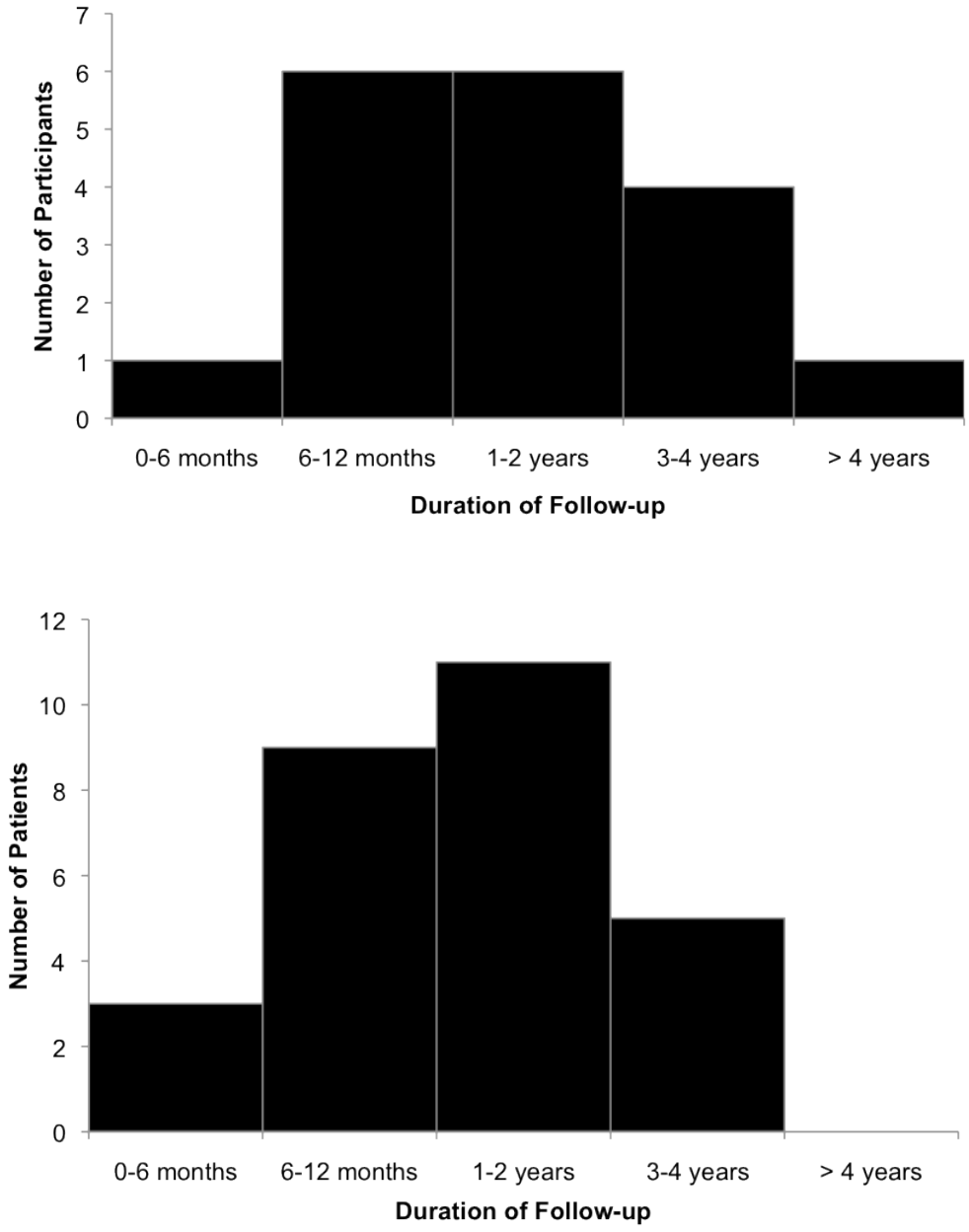


Figure 14. Duration of follow-up for 18 patients with longitudinal pure-tone data (top panel) and 26 patients with longitudinal ABR data (bottom panel).

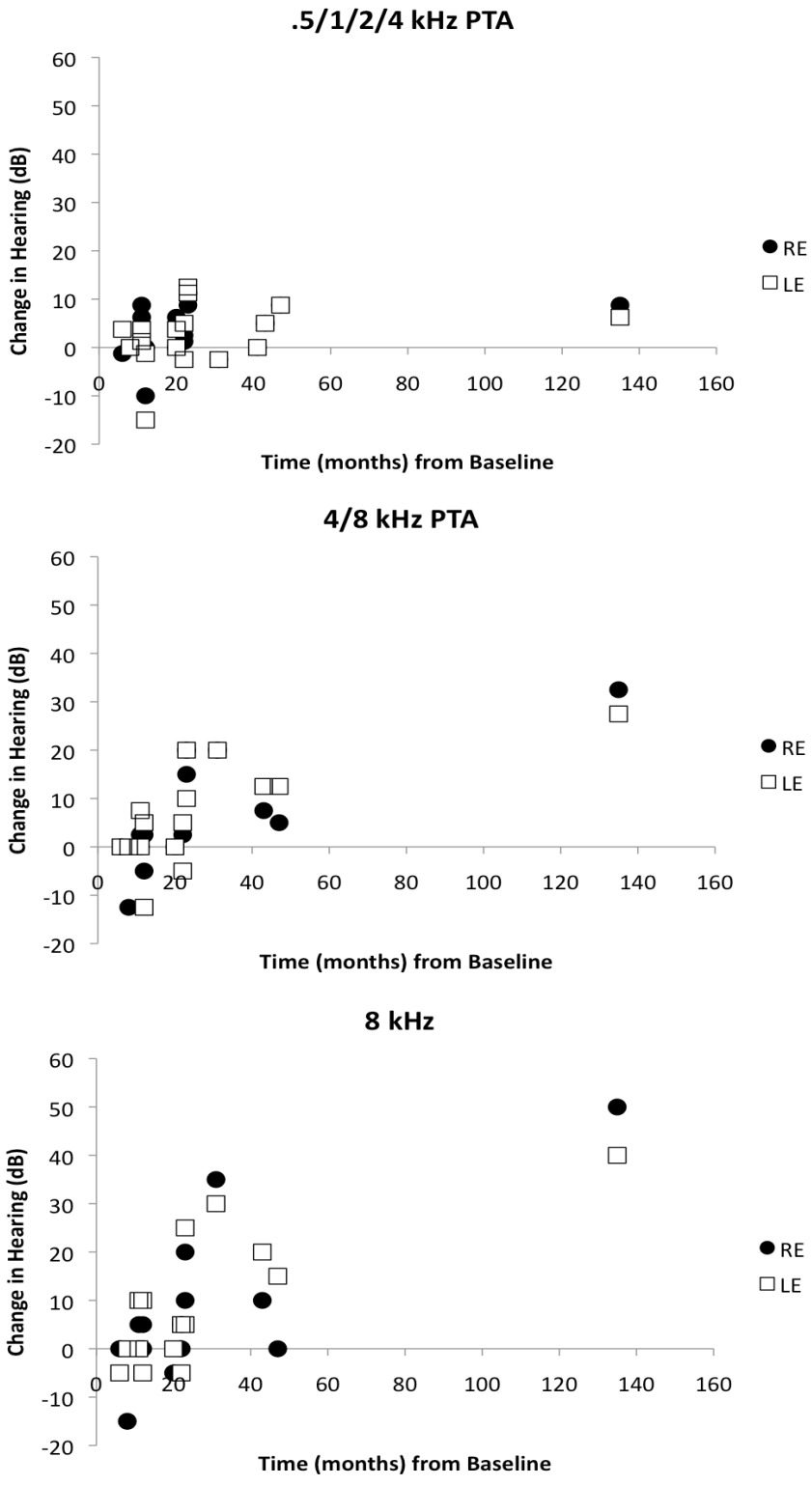


Figure 15. Change in hearing from baseline against duration of follow-up (months) for a four-frequency and high-frequency pure-tone average (PTA), and 8k Hz.

ABR findings: Twenty-six patients provided longitudinal ABR data, with an average duration of follow-up of 18 months. Clinically significant change (> 1 ms, e.g., Hood, 1998, Ornitz & Walter, 1975) from baseline was not observed in either ear of any individual for absolute latency (I, III, V) or interpeak latency (I-III, III-V, I-V). Change from baseline for these measures is plotted in Figures 16 and 17. A positive change indicates a prolongation in latency, and a negative change indicates a reduction in latency. There is no observable trend in the data, in either direction, to suggest an overall pattern of change in the cohort. However, there appears to be more stability in the response for the absolute latency of waves I and III, and a less stable response (both positive and negative change) for the absolute latency of wave V. A similar pattern was not observed on the interpeak latency data. ABR waves undergoing a categorical change from present to absent are not included in these figures.

Table VIII shows the number of ears from 12 individuals with longitudinal ABR data that underwent a categorical change from present to absent or normal to abnormal during the duration of follow-up. The most common finding was a loss (present to absent) of waves I and III.

OAE Suppression findings: Of the 8 individuals for whom OAE suppression could be tested; three provided longitudinal data. Two of the three individuals were followed for three years and had OAE suppression present in at least one ear on two occasions. The third patient was followed for one year, had measurable suppression at baseline bilaterally, and no measurable suppression in either ear on her follow-up visit. The two individuals who maintained their suppression are highly functioning sisters, and

neither of these girls showed evidence of a prolonged CM on their ABR. The third patient who lost suppression had a large CM in both ears on both visits.

This relationship between a prolonged CM and presence/absence of OAE suppression was explored because it is speculated that large and prolonged CMs may represent a lack of regulation by the olivocochlear efferent system on cochlear hair cell activity (Starr, Sininger, & Pratt, 2000). If OAE suppression functions as a non-invasive window into auditory efferent pathways, such as the olivocochlear efferent system, that regulate OAE production, the two measures may be related.

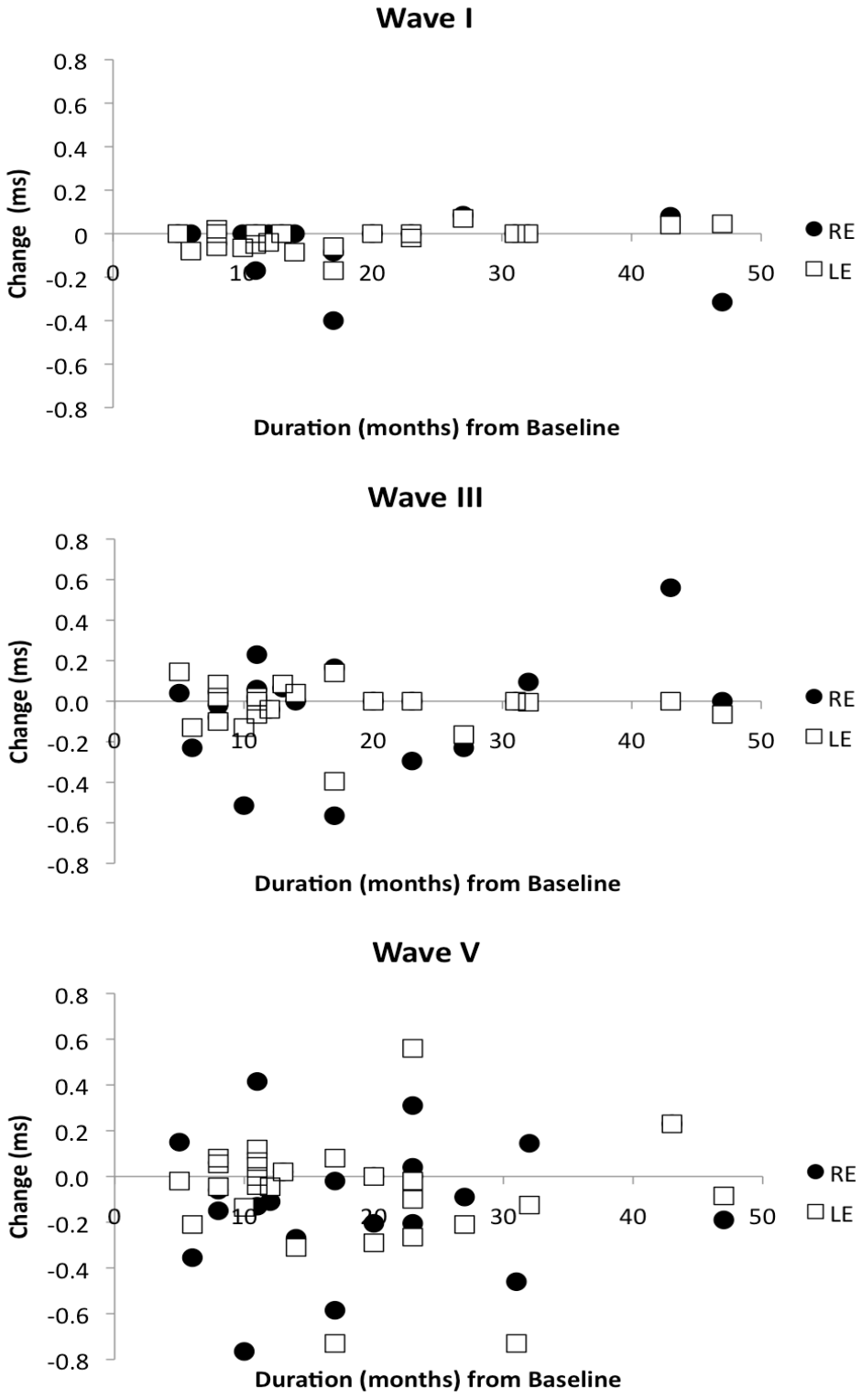


Figure 16. Change in ABR absolute latency from baseline by duration of follow-up (months) for waves I, III, and V.

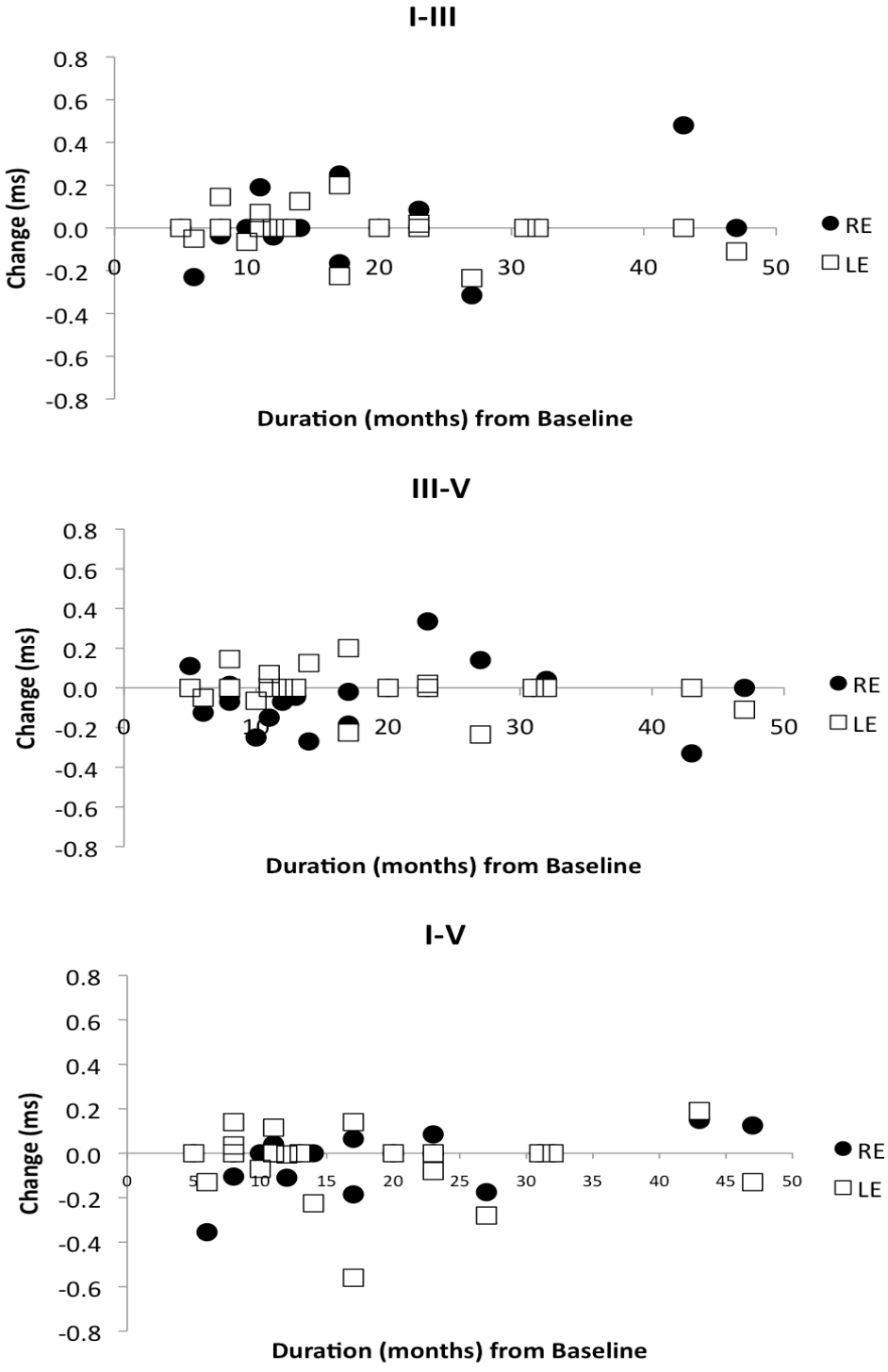


Figure 17. Change in ABR interpeak latency from baseline by duration of follow-up (months) for I-III, III-V, and I-V.

Table VIII. Categorical change in absolute and interpeak ABR latency findings from 26 patients (52 ears) with longitudinal data. The average duration of follow-up was 18 months.

Longitudinal ABR Change		
Present to Absent (ears)		
I	III	V
7	3	0
Normal to Abnormal (ears)		
I	III	V
0	2	3
I-III	III-V	I-V
4	1	0

Longitudinal Case Examples. Four longitudinal case examples are provided from individuals who were able to provide complete audiological evaluations at multiple points in time (Figures 18 through 22). Case one (Figure 18) shows threshold data from a male patient with NPC at six and eight years of age. At the baseline visit, he presented with a slight low-frequency conductive hearing loss secondary to Eustachian tube dysfunction. On his return visit at eight years of age, middle ear function and the low-frequency component resolved, however there was a new-onset precipitous decline in high-frequency hearing. Case two (Figure 19) shows threshold data from a male patient with NPC at seven and nine years of age. A significant decline in hearing was documented from 2k to 8k Hz bilaterally, although there was no patient or parental concern regarding a change in hearing and no concomitant change in middle ear function. Case three (Figure 20) shows threshold data from a male patient with NPC at seven and 11 years of age. When the patient presented at baseline, there were no parental or patient concerns regarding hearing sensitivity. At that time, a moderate high-frequency hearing loss was newly identified. Following identification of hearing loss at baseline, this patient was subsequently fit with bilateral hearing aids. At follow-up, a significant decline in hearing was documented at most test frequencies between 2k and 8k Hz, although there was no concern regarding a change in hearing, and no significant change in middle ear status was documented.

Case four (Figure 21) shows threshold data from a female patient with NPC at nine and 21 years of age (historical records available). A progressive decline in high frequency hearing was documented across multiple interim evaluations and a similar decline was observed bilaterally. The historical records available for this patient provide

the longest duration of follow-up in this cohort. The corresponding ABR data for this patient are shown in Figure 22. ABRs obtained in 1995 compared to those obtained in 2006 reveal a loss of waves I and III in the left ear and a total loss of the response in the right ear.

Case 1.

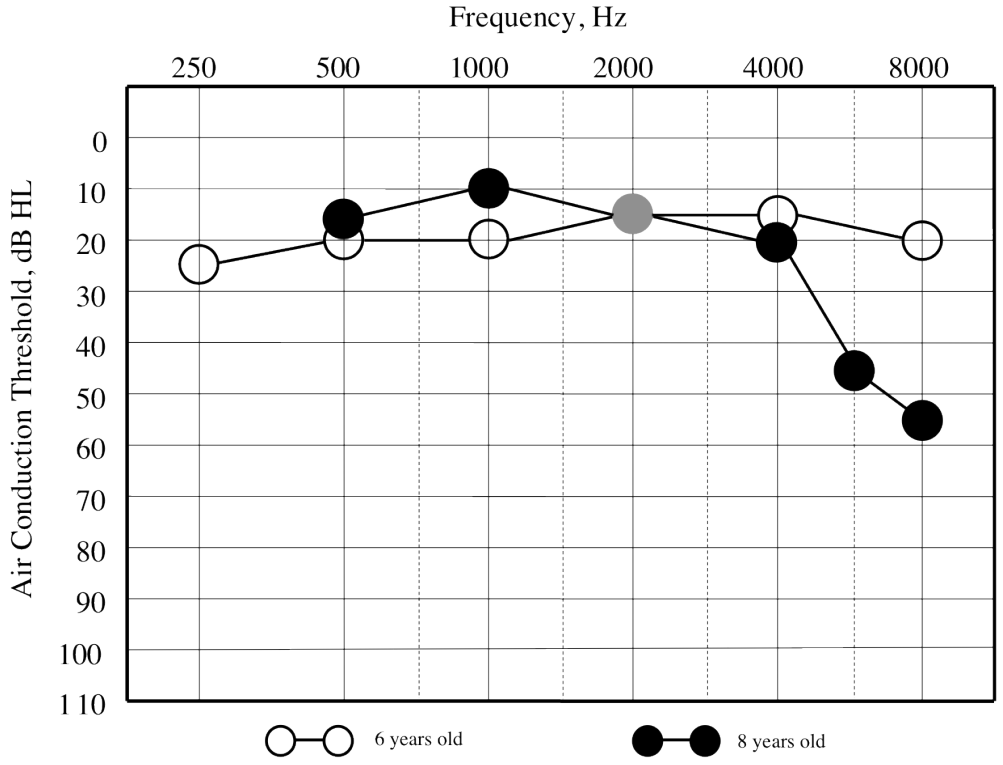


Figure 18. Right ear pure-tone air-conduction thresholds for a male patient with NPC at six and eight years of age. Similar results were observed in the left ear (data not shown).

Case 2.

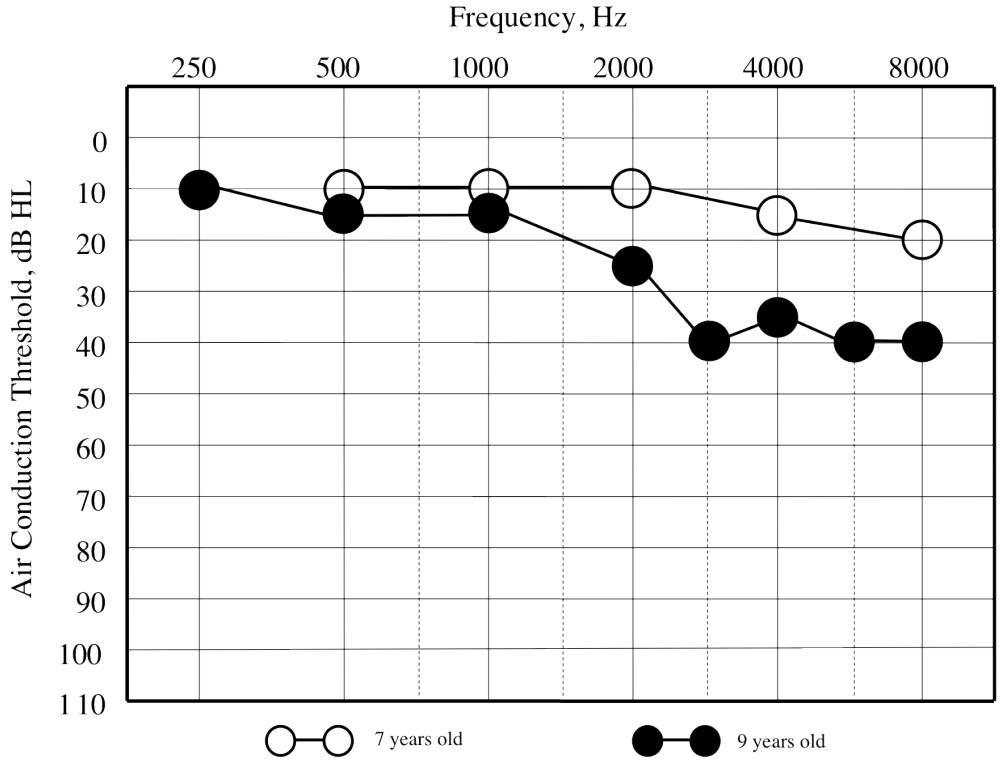


Figure 19. Right ear pure-tone air-conduction thresholds for a male patient with NPC at seven and nine years of age. Similar results were observed in the left ear (data not shown).

Case 3

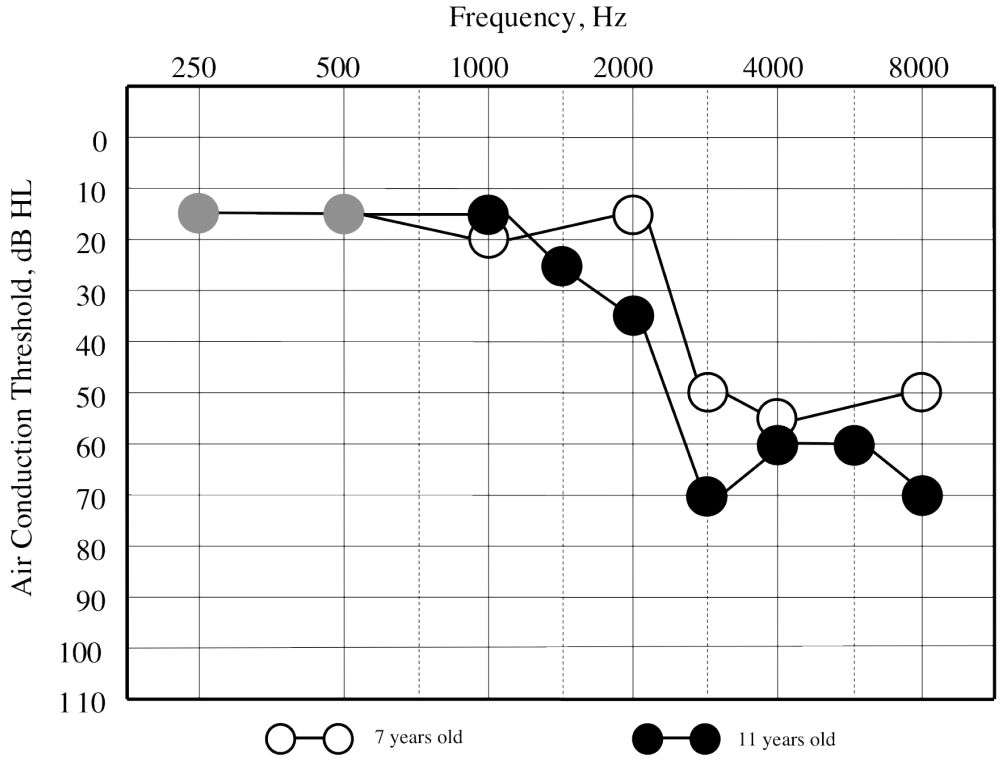


Figure 20. Left ear pure-tone air-conduction thresholds for a male patient with NPC at seven and 11 years of age. Similar results were observed in the left ear (data not shown).

Case 4.

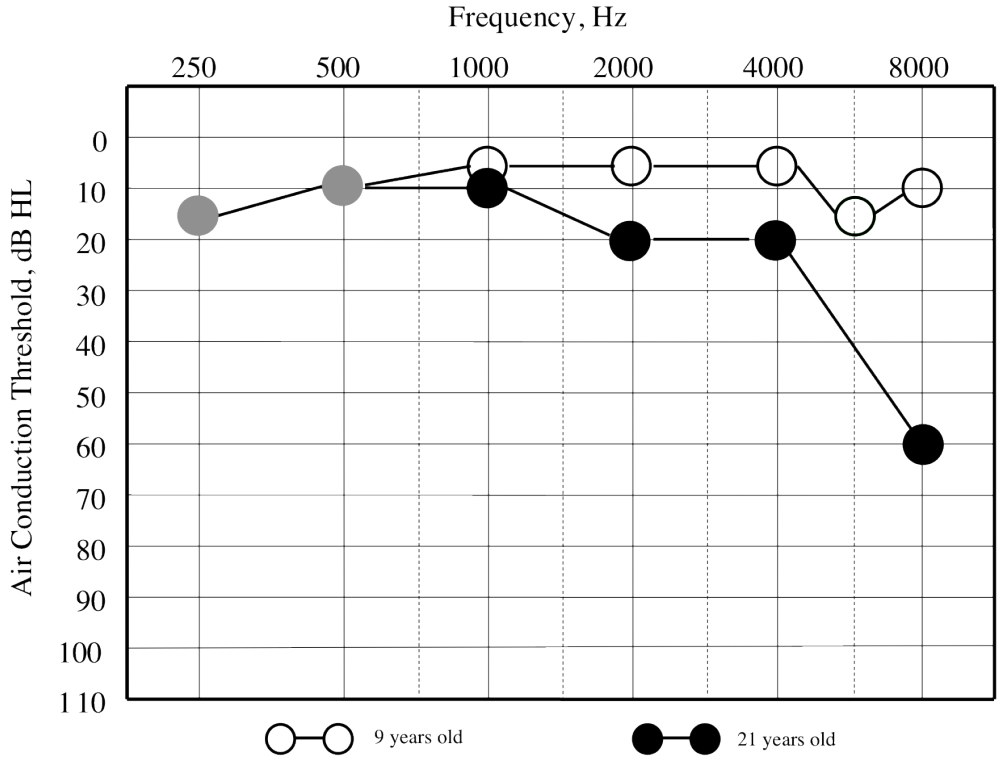


Figure 21. Right ear pure-tone air-conduction thresholds for a female patient with NPC at nine and 21 years of age. Similar decline was observed in the left ear (data not shown).

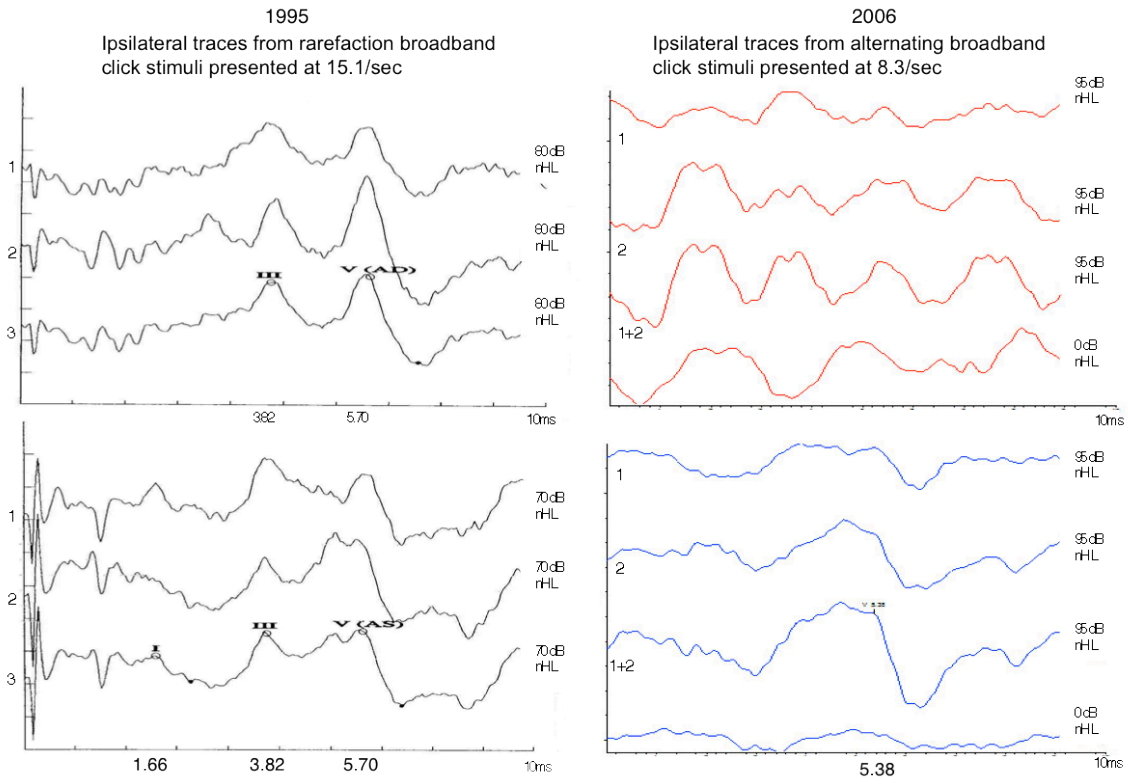


Figure 22. ABR results are from the patient described in longitudinal case 4, and correspond to pure-tone thresholds shown in Figure 21. Right ear responses are shown in the top panels, left ear responses are shown in the bottom panels.

Late-onset cases. Five patients presented with late-onset stages of NPC. Each presented with unique medical and auditory histories, and as such, each will be presented below as a case example. An audiogram key is provided in Appendix B. Table IX summarizes findings from the five late-onset cases of NPC in this cohort. All five patients have hearing loss, although two of the five were unaware of their loss at the time of baseline testing. In at least three of the cases hearing loss was an early symptom of the disease.

Case 1.

This is a 25-year-old female with NPC. She was diagnosed with hepatosplenomegaly at five years of age and bilateral hearing loss during middle school. Additional neurological decline during early adulthood ultimately led to the diagnosis of NPC several months prior to her NIH baseline evaluation (Figure 23). She has normal tympanometry and absent ipsilateral and contralateral acoustic reflexes bilaterally. Otoacoustic emissions are absent bilaterally. The ABR is absent in the left ear and only wave V is present in the right ear, which is consistent with the degree of peripheral hearing loss.

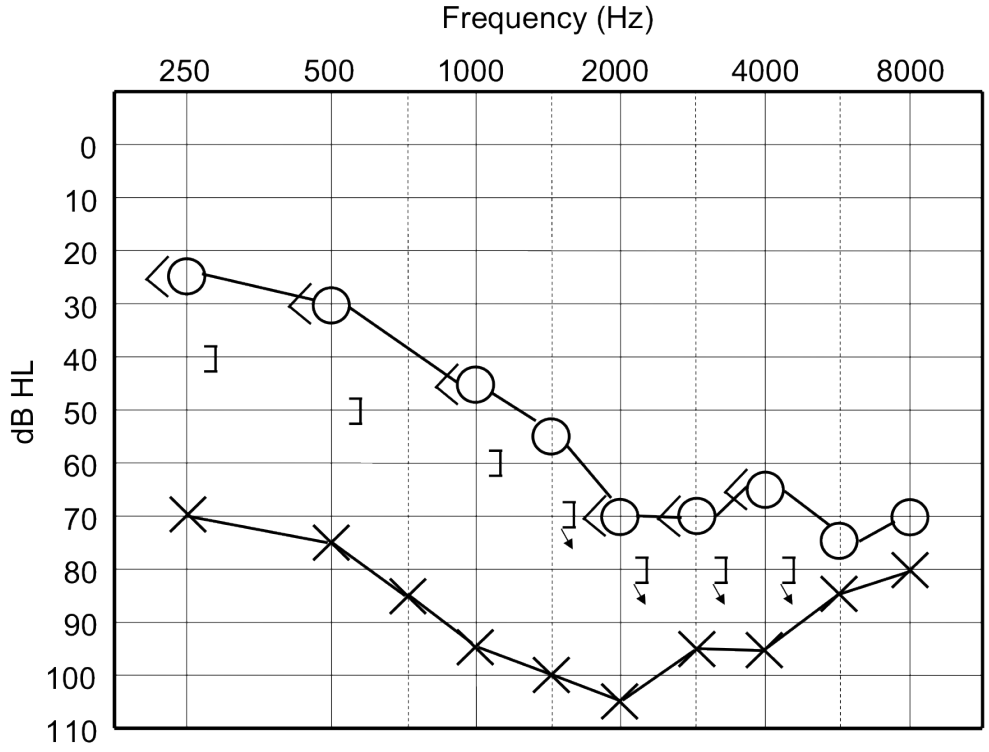


Figure 23. Baseline NIH audiogram for a 25 year old female with NPC. Low-frequency bone-conduction scores in the left ear are believed to be vibrotactile responses.

Case 2.

This is a 32-year-old male with NPC. The patient was diagnosed with a learning delay at age 8, and "idiopathic" high frequency hearing loss at age 9. He was diagnosed with NPC at 21 years of age after significant and rapid neurological decline. At his baseline NIH evaluation he had normal middle ear function and absent otoacoustic emissions bilaterally. He was unable to cooperate for acoustic reflex or behavioral hearing assessment. His ABR, obtained under sedation, was absent bilaterally. Historical audiograms indicate a significant deterioration in hearing up to age 18. Figure 24 is the most recent, complete audiogram for this patient, who is now 32 years old and unable to condition for behavioral evaluations.

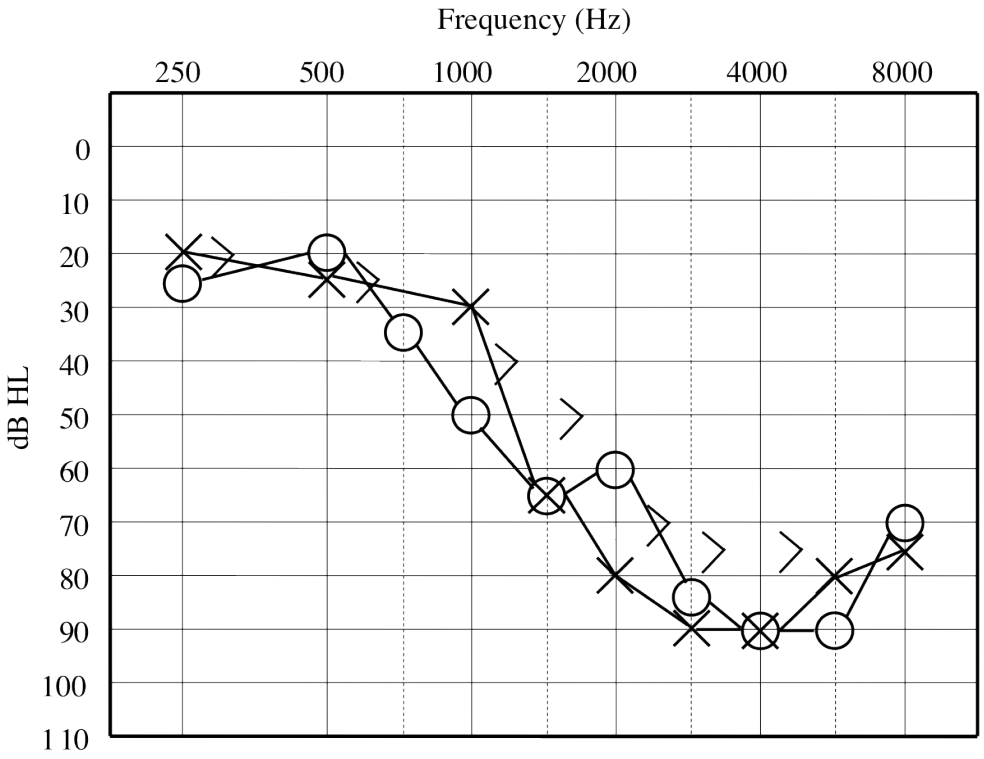


Figure 24. Audiogram for a male patient with late-onset NPC. Historical audiograms from the age of nine to 18 years, when the above audiogram was obtained, show a progressive deterioration in hearing bilaterally.

Case 3.

This is a 33-year-old female with NPC. She was initially diagnosed with schizophrenia at age 18; she later developed vertical gaze palsy, decreased motor skills, and difficulty ambulating. She was diagnosed with NPC at the age of 33 years. She presented to her baseline NIH evaluation, accompanied by her mother, with no concern regarding her hearing. Middle ear function was normal, but acoustic reflexes were elevated bilaterally, inconsistent with the degree of peripheral hearing loss. DPOAEs were consistent with behavioral pure-tone hearing thresholds (absent in the high frequencies). Waves I and III of the ABR were absent bilaterally and wave V was present at a normal absolute latency, which suggests a cochlear contribution to the poor ABR morphology.

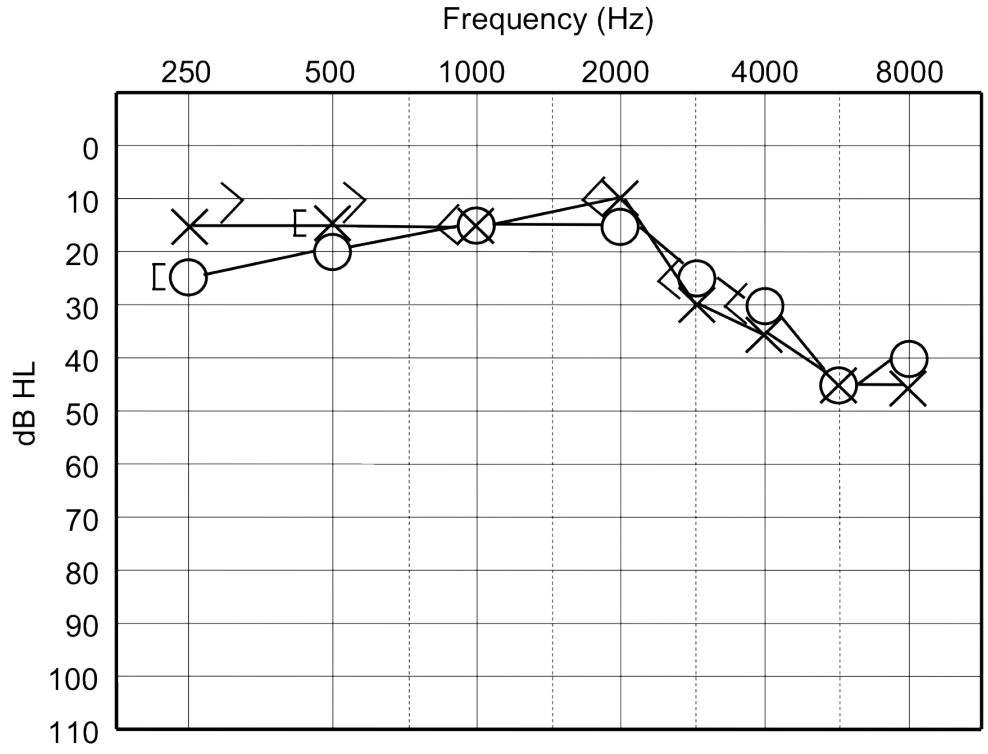


Figure 25. Baseline NIH audiogram from a 33-year-old female with NPC. Neither the patient nor her mother suspected hearing loss at the time of the evaluation.

Case 4.

This is a 35-year-old female with NPC. She was initially diagnosed with depression at age 18. Following the onset of progressive neurological involvement beginning around age 30, she was ultimately diagnosed with NPC at age 34. Neither she nor her mother had concern about her hearing prior to her baseline NIH evaluation (Figure 26). She presented with normal middle ear function and elevated/absent acoustic reflexes that were incongruous with pure-tone hearing thresholds, and positive (abnormal) acoustic reflex adaptation. DPOAEs were consistent with behavioral thresholds (high frequency hearing loss). Wave I of the ABR was absent bilaterally, but otherwise the ABR was within normal limits, which is consistent with a high frequency hearing loss of a cochlear origin.

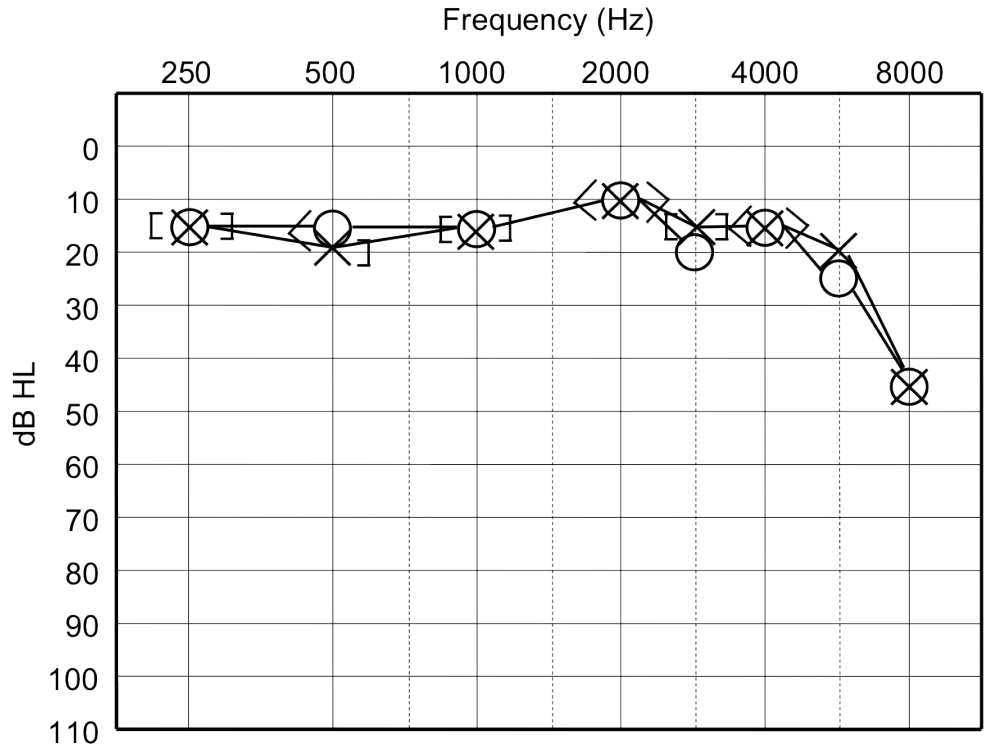


Figure 26. Baseline NIH audiogram from a 35-year-old female with NPC. Neither the patient nor her mother suspected hearing loss at the time of the evaluation.

Case 5.

This is a 51-year-old female with NPC. She was diagnosed with bilateral hearing loss at the age of 39. One year later she presented with slurred speech, ataxia, and difficulty swallowing; three years later she developed significant motor impairment. She was diagnosed with NPC at the age of 46. She is unaware of any change in hearing since the hearing loss was initially diagnosed. Audiological testing revealed normal middle ear function with absent ipsilateral and contralateral acoustic reflexes (.5/1/2 kHz), which was incongruous with low-frequency peripheral hearing thresholds. Suprathreshold word recognition testing revealed significant decline in performance (80% to 52%) in the right ear. DPOAEs were absent bilaterally. ABRs were absent bilaterally, which could not be explained entirely by peripheral hearing thresholds.

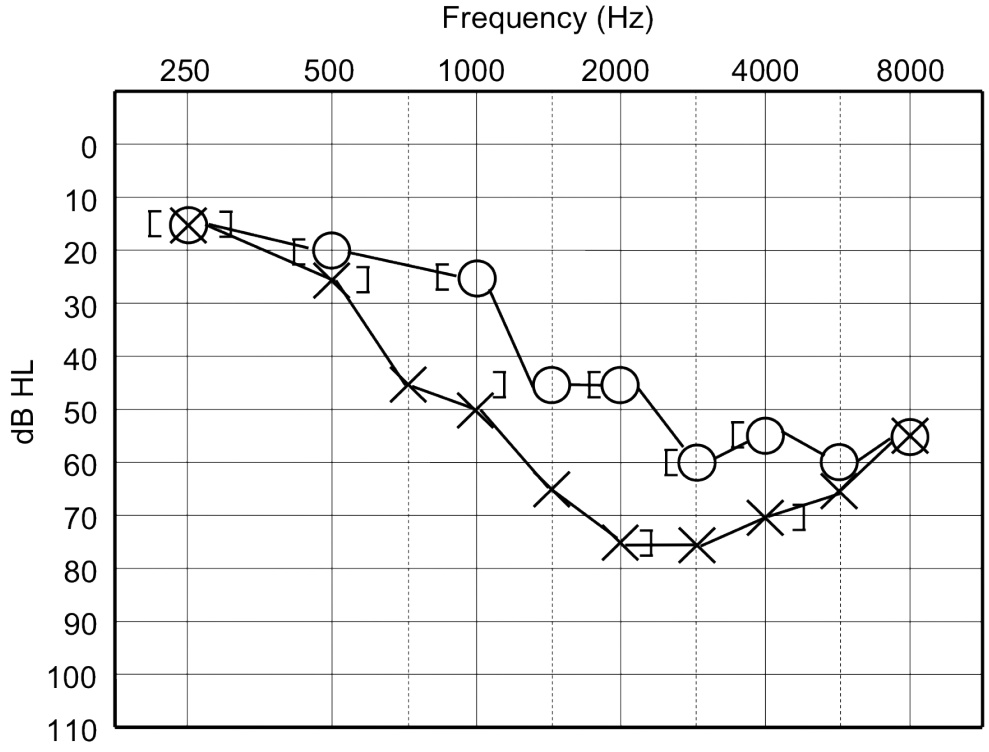


Figure 27. Baseline NIH audiogram for a 51-year-old female with NPC. The patient was aware of the hearing loss and fit with bilateral hearing aids.

Table IX. Summary of findings from five patients with late-onset NPC.

Gender	Age at Baseline (NIH)	Age at Dx of NPC	HL (Y/N)	
*Female	25	25	Y	Dx with hepatosplenomegaly at age 5; Dx with bilateral HL in middle school
*Male	32	21	Y	Dx with learning delay at age 8; Dx with bilateral HF HL at age 9
Female	33	33	Y	Dx with schizophrenia at age 18; later developed vertical gaze palsy, decreased motor skills, and difficulty ambulating; no concern about hearing
Female	35	34	Y	Dx with depression at age 18; onset of progressive neurological involvement beginning around age 30; no concern about hearing
*Female	51	46	Y	Dx with bilateral HL at age 39; 1 year later presented with slurred speech, ataxia, and difficulty swallowing; 3 years later, significant motor impairment

Note. Dx, diagnosed; HF, high frequency; HL, hearing loss. * identifies three of five cases for which hearing loss was an early symptom of the disease.

Summary and Discussion

The data presented here represent the largest cohort of patients with NPC in whom auditory function has been examined comprehensively. Results of the baseline auditory assessment support an auditory phenotype in humans with NPC. When mean data (Figure 3) for the cohort of 31 patients (late-onset cases removed) able to participate in behavioral testing are viewed, a high frequency hearing loss is evident. Insert earphones were used as the standard for data collection whenever possible, which mitigates the risk that findings represent an artifact from standing waves or collapsing external auditory canals. These findings corroborate previous reports from a small cohort of patients with NPC with high frequency hearing loss (Fink, et al., 1989; Pikus, 1991).

When a four-frequency pure-tone average is used to quantify hearing loss in this cohort, only 25% of ears are identified as having a hearing loss; however, approximately half of all ears had clinically significant hearing loss (>15 dB HL) at 4000 and 8000Hz. This observation suggests that standard mid-frequency pure-tone averages may be inadequate to capture accurately hearing loss in patients with NPC. Furthermore, early-onset hearing loss in this population may be missed easily, as common screening procedures for hearing in medical offices and academic settings typically do not test above 4000 Hz (e.g., McPherson, Law & Wong, 2010). Efforts during behavioral evaluations in this difficult-to-test population should emphasize collection of high frequency information first, when possible, with the knowledge that a complete behavioral exam may not be feasible.

The majority of hearing loss observed in this cohort with NPC appears to be sensorineural in nature. While ear-specific bone conduction thresholds were difficult to

obtain and are, consequently, limited, middle ear disease was not a prevalent finding in this group. The most common abnormality observed was on measures of TPP, which is a reflection of Eustachian tube dysfunction, and which is not uncommon in a pediatric population. Children are more susceptible to middle ear disease, in part, because of the horizontal positioning of their Eustachian tube, which regulates pressure in the middle ear cavity. An additional factor for consideration when interpreting middle ear function is that many participants travel to the NIH via commercial aircraft and are typically seen one to two days after arrival; residual effects from flight on middle ear status can occur, and may actually be greater in children (Mirza & Richardson, 2005). Previous reports from a single cohort (e.g., Fink, et al., 1989; Pikus, 1991) did not observe abnormal tympanometry findings, but normative criteria used for categorization were not specified. Middle ear disease is a concern for any pediatric population, and as a noninvasive, relatively quick assessment of middle ear status, tympanometry should play an important role in identifying possible conductive pathology in this population in whom collection of ear-specific bone-conduction pure-tone thresholds can be difficult.

In the current study, otoacoustic emission data support a cochlear site of lesion (sensory), specifically the outer hair cells, in ears with absent OAEs, when cases of middle ear dysfunction are removed. When low-frequency emissions ($\leq$ 1000 Hz) are discounted because of an elevated noise floor, DPOAE data suggest cochlear dysfunction in approximately one third of all participant ears tested; the most common range affected was the high frequency range, which corresponds with the high frequency pure-tone hearing loss observed in this group. While this is the first time OAE data have been reported in a cohort of patients with NPC, the findings are consistent with behavioral

thresholds in all cases. That is, ears with hearing loss had appropriately absent OAEs at frequencies corresponding to the hearing loss. This supports cochlear dysfunction as part of the auditory phenotype. OAEs can serve as an especially useful tool to either screen for peripheral hearing loss or help confirm behavioral thresholds in a young, neurologically compromised population, and should be included in every auditory assessment of patients with NPC.

In addition to evidence for a cochlear site of lesion in NPC, there is support for retrocochlear dysfunction of the auditory system in a large percentage of the cases. Twenty-five percent of patients who were able to provide word recognition responses at two presentation levels showed evidence of rollover in performance, suggesting retrocochlear involvement. Word recognition scores from ears with purely cochlear hearing loss tend to plateau or exhibit a slight decline in performance with an increased presentation level. Patients with retrocochlear dysfunction, however, tend to exhibit a dramatic decline in performance with increases in intensity beyond the level required to obtain their maximum performance (Jerger & Jerger, 1971).

Similarly, 63% of ears had abnormal acoustic reflex patterns that could not be explained by either middle ear status or peripheral hearing thresholds, suggesting retrocochlear involvement. Retrocochlear disorders are suspected in ears where the acoustic reflex threshold is either elevated or absent beyond what would be anticipated by cochlear hearing loss, and in the absence of middle ear disease. In addition, acoustic reflex adaptation, which was abnormal in 15% of cases, is also consistent with a retrocochlear site of lesion. These data are consistent with those from Fink, et al. (1989) and Pikus (1991) who also reported a high prevalence of acoustic reflex threshold and

adaptation abnormalities (81% and 85%, respectively). When middle ear status and peripheral hearing are accounted for, an abnormal acoustic reflex indicates a disturbance in the reflex arc comprising cranial nerves VII and VIII, the cochlear nuclei, and the superior olivary complex. Additional support for a neural site of lesion is found in the ABR results.

ABR abnormalities were observed in 84% of patients, of whom 49% had abnormalities that could not be explained by peripheral hearing status; an additional 44% had no peripheral hearing data with which to compare results. The most common findings were absent waves I and III and prolongation of interpeak latencies I-III and I-V While absence of early waves can be attributed to high frequency peripheral hearing loss, prolongation in interpeak latencies indicates a disturbance in transmission of the auditory nerve through the lower brainstem. Although specific ABR abnormalities in previous reports are limited, current data are not inconsistent with earlier findings that implicate a disturbance in the early components of the ABR waveform (Aisen, et al., 1985; Fink, et al., 1989; Palmeri, et al., 2005; Pikus, 1991) and suggest auditory nerve and auditory brainstem dysfunction.

A pattern of disproportionate neural findings that cannot be explained by degree of peripheral hearing loss implies that some patients with NPC fit the diagnostic criteria for auditory neuropathy spectrum disorder (ANSD). ANSD is a disorder of afferent transmission from the cochlea through the auditory nerve, believed to disrupt the temporal processing of a signal. The original description of the disorder involved elevated pure-tone thresholds, disproportionately poor speech discrimination abilities, absent acoustic reflexes, and absent ABRs that could not be explained by the degree of

peripheral hearing loss (Starr, Picton, Sininger, Hood, & Berlin, 1996). It is now widely accepted that ANSD encompasses a continuum of clinical findings and a wide range of etiologies (e.g., syndromic and nonsyndromic, neonatal hyperbilirubinemia, idiopathic) resulting in a depletion or dyssynchrony of afferent auditory nerve fibers, or both (Picton, 2011).

Today, the diagnostic criteria for ANSD include evidence of cochlear hair cell function (OAEs and/or cochlear microphonic) and abnormal processing of the auditory nerve, usually established through ABR abnormalities beginning with the early components (e.g., wave I) of the waveform (Picton, 2011). Additional useful tests include acoustic reflex thresholds and adaptation (Berlin, et al., 2005) and OAE suppression via a contralateral masker (Hood, Berlin, Bordelon, & Rose, 2003), both of which are reported to be abnormal in ANSD. Some children with ANSD exhibit large amplitude and prolonged latency CMs (Starr, et al., 2000; Starr, Sininger, Nguyen, Michalewski, Oba, Abdala, 2001).

Based on the above diagnostic criteria, 12 patients in the NPC cohort have audiometric findings consistent with ANSD. These 12 individuals have both intact cochlear function and abnormal ABR findings that begin in the early part of the waveform and that cannot be explained by peripheral hearing status. This number may be an underestimation of the prevalence of ANSD in NPC disease because 15 patients were unable to provide enough data to determine their inclusion or exclusion from this category.

A large number of participants (40%) exhibited a mixed, cochlear and retrocochlear site of lesion on the auditory test battery. An example of this finding is a

participant with elevated pure-tone thresholds, absent otoacoustic emissions (cochlear), elevated/absent acoustic reflex thresholds and/or abnormal ABR findings that could not be explained by the degree of peripheral hearing loss. This high proportion of patients with retrocochlear and cochlear involvement further lends support for NPC disease pathogenesis having a widespread and complex role within the auditory system.

Thirty-six percent (20/55) of the cohort who ranged in age from four months to 32 years were unable to provide any behavioral pure-tone data during examination, suggesting that, in addition to young age, disease status can be a significant barrier to obtaining a complete audiological evaluation in patients with NPC. In those patients for whom no behavioral data could be obtained, Auditory Steady State Response (ASSR) measures were used to estimate peripheral hearing sensitivity for the purpose of determining appropriate clinical intervention while the patient was sedated. These data were not included in the statistical analysis aimed at phenotyping NPC because of the variability associated with these predictions of behavioral thresholds (Yeung & Wong, 2007). ASSR thresholds have been shown to overestimate hearing loss in normal hearing ears and in ears with ANSD (Attias, Buller, Rubel, & Raveh, 2006). Nevertheless, ASSR, in conjunction with tympanometry and OAEs, was the only assessment option in about one-third of the cohort. Taken together, auditory evoked potentials and behavioral auditory testing identified hearing loss in 23 patients (42%) with NPC. When all patients with at least a mild sensorineural hearing loss are considered (32) this means that 72% of these participants were unaware of their hearing loss. This, in part, supports the hypothesis that hearing loss is an overlooked, underestimated component of the NPC phenotype and that more awareness by clinicians is needed.

The tremendous heterogeneity and relatively small size of the sample made identifying contributions of specific independent variables to observed hearing loss challenging. Only eight patients (15%) were able to participate in every component of the test battery. This limited number essentially precluded a meaningful mixed model regression analysis with multiple variables. Although gender, age, and miglustat were not identified in the current study as contributing to the variability in hearing sensitivity among the current NPC patients, definitive findings await a larger sample size (minimally five to ten participants per predictor variable).

There is evidence of progressive hearing loss accompanying NPC. This is supported by the subset of longitudinal data identifying clinically significant (>10 dB) change in high frequency hearing. Of 12 ears followed for 23 months or more, 75% experienced decline in hearing, and the ears that experienced the largest deterioration (>27 dB) were those followed for the longest period of time (~11 years). In addition, the four longitudinal case examples of patients who were able to provide multiple complete behavioral evaluations provide clear evidence of deterioration in hearing. Although the heterogeneity and small size of the sample preclude observation of robust evidence for progression, these individual case examples and the subset of data from patients followed for the longest amount of time cannot be ignored. Collectively, these data reveal for the first time that, like the global neurological phenotype, hearing in at least some patients with NPC is progressive. This finding supports the importance of regular audiologic monitoring in all patients with NPC regardless of a history of normal hearing.

Longitudinal ABR data fail to reveal any significant change (> 1 ms) in absolute or interpeak latencies regardless of the duration of follow-up. However, several ears

underwent categorical change from normal to abnormal when compared to normative data. The most common finding was a loss of waves I and III, and a prolongation in the I-III interval across an average duration of follow-up of 18 months. These data suggest detrimental longitudinal change in ABR morphology is possible in patients with NPC, and Case 4 of the longitudinal examples provides clear evidence of a decline in waveform morphology for the patient followed for the longest duration (12 years). It is possible that average duration of follow-up in the cohort (average 18 months) is not sufficiently long to view overt changes in ABR results across many patients. Nonetheless, the ABR is an excellent tool for monitoring auditory neural status and should be considered for patients with NPC, especially those unable to participate fully in behavioral evaluations.

Because of their unique disease progression and auditory findings, the data of five patients with late-onset NPC were removed from the overall cohort and analyzed separately. These patients have more hearing loss than the cohort average, and in three of the five cases hearing loss was an early symptom of the disease; the remaining two patients were unaware of their hearing loss. The only previous mention of hearing loss in late-onset cases of NPC was by Sévin et al. (2007), who identified three of 25 patients as having "perceptual deafness." Although the metric for hearing assessment was not identified, the authors reported that hearing loss was an early symptom of the disease in two of the three patients. These two cases, in conjunction with the current findings, support hearing loss as a premonitory symptom in late-onset cases of NPC. Consequently, the inclusion of NPC should be added to the differential diagnosis for patients of any age with subtle neurological symptoms (e.g., learning delay) and idiopathic hearing loss.

While these data provide clear evidence for an auditory phenotype in humans with NPC, the pitfalls associated with longitudinal data collection in an outbred, heterogeneous, neurologically compromised pediatric population are evident, and prevent the rigorous application of statistics to much of the data. Questions remain about the role of NPC mutations on auditory function, longitudinal progression of the neural phenotype, and the utility of the auditory system as a clinical marker for disease status and intervention efficacy. With these in mind, experiment two focuses on exploring auditory function in the mouse model for NPC.

CHAPTER 3: AUDITORY FUNCTION IN THE MOUSE MODEL FOR NIEMANN-PICK DISEASE, TYPE C (NPC)

Review of the Literature

Mouse Models for Human Biology

Overview. In the last 100 years, the mouse has emerged as the most prominent, well-studied animal model for understanding mammalian, specifically human, biology (for review see Morse, 2007). Its impressive status as an early leader among research models was evident in its selection to be the first organism targeted for complete genetic sequencing. Within the last 10 years, both the human and mouse genomes were completely sequenced, providing an invaluable blueprint for understanding normal and aberrant genetic structure and function. Today, mouse models exist for cancer, aging, immunology, metabolism, and inherited neurodegenerative diseases, among others. It is the animal model of choice in many areas of research.

Through the majority of the 20th century, genetic research with mice relied on the use of spontaneous, naturally occurring molecular mutations. However, the ability to transfer genes and deoxyribonucleic acid (DNA) material across organisms (transgenesis) led to the discovery of gene 'knockout' technology, further enhancing the link between mammalian genetics and biology. It is now also possible to replace an endogenous mouse gene with a new, exogenous molecular variant, using similar targeted insertion. With this gene knockout and knockin technology, it is possible to control the expression of a gene in a model organism - in this case, the mouse - to target its specific effect on biology.

Research with mice typically involves the use of inbred strains, whose lineage traces to a single ancestral pair, but who have been mated through their own siblings for at least 20 generations. The outcome is a genetically uniform animal model. Inbred strains of mice have led to remarkable discoveries in human heritability and biology, and are commonly used in research of the auditory system. Consequently, a great deal is known about normal and aberrant auditory structure and function in the mouse, including similarities in inheritance patterns of auditory pathologies between mice and humans (Brown & Steel, 1994). There are mouse models for outer and middle ear deformities, sensory hair cell defects, auditory CNS disorders, age-related hearing loss, and noise-induced hearing loss, to name a few.

The molecular and biological systems in this species are well understood and, in many ways, analogous to humans. The anatomical and physiological features of the mouse and human cochleae are comparable in function (Johnson & Zheng, 2002) and there is approximately 95 percent sequence homology between the species (McFadden, Ding, & Salvi, 2001). Moreover, they are characterized by a short lifespan (~18 to 24 months), rapid reproduction cycle (18 to 21 day gestational period), malleable breeding, and inexpensive care. For all of these reasons, the mouse model is ideal for understanding human biology and, in this case, auditory function or dysfunction.

Hearing in mice. The mouse is a highly vocal animal, which suggests its ability to communicate and auditory sensitivity play an important role in social and behavioral function. While some of these vocalizations occur at frequencies that are audible to humans, many are ultrasonic, upwards of 70 to 80 kHz (Nyby, 2001). Most commercially available transducers currently lack the ability to produce artifact-free

stimuli at these ultrasonic frequencies, which makes obtaining accurate auditory thresholds above 32 or 64 kHz difficult. However, it is reasonable to assume these animals have not developed the capability to produce sounds that they themselves are unable to detect. Considering the ultrasonic vocalizations that have been recorded from this species, it can be inferred that mice have high-frequency hearing that extends at least as high as 70 to 80 kHz. According to the Greenwood function (Greenwood, 1990), and under the assumption that the mouse cochlea is 7mm long (Ehret, 1983), the upper frequency limit of hearing for the mouse could be as high as 120 kHz.

The ability to determine both behavioral and electrophysiological auditory thresholds across an animal's frequency response range has increased over recent decades with improved technology. Heffner and Heffner (2007) published a review of audiograms for commonly used laboratory animals, including the mouse. They compared the range of hearing across species for frequencies audible at a level of at least 60 dB SPL. In addition, they documented the range of frequencies for which each animal is most sensitive, defined as those audible at a level of $\leq$ 10 dB SPL. Based on these operational definitions, the range of hearing for humans extends from 31 Hz to 17.6 kHz, with the best sensitivity between .25 and 8.1 kHz. In comparison, hearing in the domestic house mouse can range from 2.3 to 85.5 kHz, with a narrow range of sensitive hearing extending from 8 to 32 kHz, which is most sensitive at 16 kHz (Heffner & Masterton, 1980).

There are multiple techniques available to test auditory function in the mouse. Using behavioral techniques, it is possible to evaluate absolute thresholds, frequency and intensity difference limens, masking effects, and sound localization acuity in this species

(Heffner, Koay, & Heffner, 2001). However, the conditioning procedures for such assessments can be difficult and require prolonged training periods before data collection can begin. The acoustic startle reflex is another form of behavioral assessment, although even in alert and well-behaving animals the response is sensitive to environmental events in addition to effects from any underlying neurological or cognitive problems (Ison, 2001). In light of these issues, both the lifespan and the neurological integrity of the mouse must be considered before electing to conduct such behavioral procedures.

The most popular method to assess hearing in mice is the use of physiological and electrophysiological measures, specifically the ABR and otoacoustic emissions (OAE). These are two noninvasive measures that require no training or conditioning of the animal and, as such, generate quick results that lend themselves to producing longitudinal data over a lifetime. As with humans, OAEs and ABR alone in mice can only be used to infer information about auditory sensitivity, and provide no information about the animal's ability to discriminate sounds. Despite these limitations, the ABR, and more recently, distortion product OAEs (DPOAEs) have become the tools of choice for investigators studying auditory function in mice.

The presence of OAEs is believed to reflect the active biological processes within the cochlea, specifically, the electromotile properties of the outer hair cells (e.g., Probst, Lonsbury-Martin, Martin, & Coats, 1987). As observed in many species in which OAEs can be recorded, when outer hair cells are missing or severely damaged, such as in the case of significant hearing loss, emissions are absent. Further evidence of this relationship exists in mouse models as well. In the *Bronx waltzer* mouse, a mutant model in which cochlear outer hair cells are completely formed but only 20% of inner hair cells

are intact, clear and reliable 2f1-f2 emissions can be recorded (Horner, Lenoir, & Bock, 1985). However, *Wv/Wv* mutant mice that have defective cochlear outer hair cells but intact inner hair cells have absent DPOAEs (Schrott, Puel, & Rebillard, 1991).

Most studies of OAEs in mice have used the same instrumentation and stimulus recording techniques as those used with humans. This is, in part, due to a lack of research investigating effects of varying stimulus parameters on DPOAEs in mice, either in normal or impaired ears (Parham, Sun, & Kim, 2001). As in humans, the largest distortion product consistently reported is the 2f1-f2, or the cubic difference tone. Sun and colleagues (1997, 1998) offer one of the few investigations of DPOAEs with varying stimulus parameters in CBA/J and C57 mice, two commonly used strains in auditory research. They conclude optimal stimulus parameters for assessing cochlear impairment in these mice are: f2/f1 = 1.35, L2= 20 to 30 dB SPL, and L_1-L_2 = 25 dB. In general, across strains in normal mouse ears, it is accepted that the f2/f1 ratio resulting in the largest DPOAE level is near 1.2 when L_1-L_2 = 0 to 10 dB, but is > 1.25 when L_1-L_2 > 10 dB (e.g., Parham, 1997). Results can be less consistent when hearing loss is present (Parham et al., 2001), and therefore comparison of results across strains and with non-littermates should be interpreted with caution.

Currently, due to equipment limitations, the ability to measure DPOAEs in mice is restricted to primary frequencies below 20 kHz for most transducers. The detection threshold of the DPOAE is traditionally defined as the lowest primary level that produces an emission above the noise floor by a criterion amount (e.g., 3 or 6 dB). Because little is known about the correlation between hearing and DPOAEs in mice, DPOAEs are

interpreted commonly as either present or absent in order to infer the functional integrity of the cochlea.

There are some distinct differences between DPOAEs recorded in humans and in mice. Characteristics of the 2f1-f2 distortion product in the mouse are similar to other non-primate laboratory animals with respect to fine structure, input/output (I/O) functions, and effects of primary tone level and frequency separation (Parham, 1997). Typically, mice have higher level DPOAEs than humans, less pronounced fine structure, and fewer spontaneous and transient OAEs (Parham et al., 2001). Consequently, DPOAEs should be compared carefully between species, with these differences in mind.

The ABR in humans allows a gross estimation of the integrity of the auditory pathway up to and including the inferior colliculus, although far-field recording prohibits identification of specific nuclei or tracts as generation sites. Early work in the area of auditory evoked potentials (AEPs) included a seminal study by Henry (1979) in which surgically induced lesion techniques and click-evoked ABRs were used to determine murine generation sites for this AEP. He concluded that the first five positive peaks in the mouse ABR have the same generation sites as those that had previously been defined in the cat. Specifically, P1 of the ABR in the mouse results from the contribution of both the summating potential (cochlear origin) and the compound action potential (firing of the auditory nerve). The following four positive peaks, P2 through P5, correspond to the cochlear nucleus, the contralateral superior olivary complex, the lateral lemniscus, and the contralateral inferior colliculus, respectively.

Various ABR characteristics have been used to quantify the response in mice. These include threshold, overall waveform morphology, peak amplitude, and absolute

peak and interpeak latencies. Threshold determination has been the most popular and well-studied approach to phenotyping auditory function in mice. However, the ABR threshold will vary not only with the stimulus characteristics (e.g., click versus tone burst), but also with the age and the background strain of the mouse in question. For example, both the BALB/cJ (Willott et al., 1998) and the C57BL (Henry, 1984) strains exhibit early-onset age-related hearing loss beginning as soon as postnatal day (p) 65. Obviously, normative ABR data for these mice will differ significantly from CBA mouse data, for example, in which age-related cochlear hair cell loss is not observed until eight months of age (Ding, McFadden, & Salvi, 2001). For this reason, comparing ABR characteristics for the purposes of phenotyping mutant mice is best accomplished within littermates from the same colony and background strain. In this case, results of affected mutants can be directly compared with their wildtype littermates who do not carry the mutation in question, but who are part of the same genetic background. The wildtype littermates effectively serve as a control group for experimental questioning.

A correlation appears to exist between ABR threshold and DPOAE threshold, when the primary frequency f2 is considered; however this is not well understood and varies depending on the genetic background of the animals. Evidence suggests a weak correlation between the two measures in non-inbred CD1 mice with early-onset hearing loss, compared to a stronger association in C57BL/J6 mice (Parham et al., 2001). Much research is needed in this area to understand better the relationship between these measures of auditory function in the mouse. Investigations using both ABR and DPOAE will help to elucidate this relationship and further understanding of their independent and synergistic effects on auditory processing.

There is no direct method, currently, for evaluating the integrity of the middle ear system in the mouse. Immittance measures commonly employed in humans are not available yet for use with this species. Therefore, the risk of peripheral otitis media, while not very common, is a distinct possibility. Measurement of the OAE is dependent on both the forward and backward transmission properties of the outer and middle ear, and is therefore susceptible to effects of otitis media. The ABR is also at risk. Pahram et al. (2001) reported a 40 dB elevation in click-evoked ABR thresholds in CBA/J mice with otitis media compared to normal controls. This issue is confounded further by the inability to visually inspect the external auditory canal and tympanic membrane for signs of infection with hand-held otoscopes. With the exception of certain background strains that are genetically susceptible to otitis media, this is not a common occurrence in laboratory mice. The use of multiple experimental animals should negate the effect of any sporadic case(s) of otitis media on collapsed data from the cohort.

The mouse model of NPC. The discovery of several animal models of NPC in recent years has advanced the ability of researchers to investigate and resolve many remaining mysteries of this disease. Although microbial (Higaki, Almanzar-Paramio, & Sturley, 2004) and canine (Bundza, Lowden, & Charlton, 1979) models of NPC exist, most research has focused on the feline and mouse models, which both exhibit spontaneous genetic mutations homologous to humans with NPC. Disease manifestations are clinically, biochemically, and morphologically comparable between all three species (Kolodny, 2000), and the mouse model has emerged as the preeminent animal model for NPC. Two mouse models for mutations in *NPC1* exist: BALB/c-*npc1*^{nih} and C57BL/KsJ-*npc1*^{spm}. Much of the early work detailing cholesterol trafficking

defects in NPC involved the BALB/c strain, and this strain continues to be more widely used in various areas of research. As a result, a great deal is now known about the neuropathology of the disease in BALB/c background model.

The molecular, anatomical, and biochemical manifestations in NPC mice mimic those observed in humans with the disease. NPC mice show age-related Purkinje cell loss and demyelination in the corpus callosum (German et al., 2001; Weintraub et al., 1992). In addition, neuronal degeneration in the cerebellum and brainstem structures has been widely documented in this strain, and is consistent with the onset of tremors and ataxia well-noted in the phenotype. Homozygous mutant offspring have widespread cortical degeneration that is both spatially and temporally specific, such that affected nerve fibers (e.g., axons) appear to degenerate before cell bodies (Ong et al., 2001). There is also evidence for a global reduction in white matter tracts (Ory, 2000).

Minimal behavioral data have been obtained from the NPC mouse beyond describing overt phenotypic manifestations, with the majority of research focusing on the metabolic and neuropathological characteristics of the disease. Võikar, Rauvala, and Ikonen (2002) offer a rare description of cognitive deficits and development of motor impairments in the (BALB/c) *NPC1* mouse. Male and female homozygous NPC (-/-) mice showed retarded growth between p25 and p35. Their weight was similar to control mice up to p45, after which it rapidly decreased. Motor impairment was present between p28 and p42, prior to the onset of visible ataxia. Diseased mice displayed decreased exploratory activity and no habituation, in addition to learning and memory deficits, which suggest some degree of cognitive impairment. In several measures, male NPC (-/-) mice were more affected than females. The authors note that similar gender

differences have been reported elsewhere in other neurodegenerative mouse models with cerebellar involvement in which female mutant mice were less affected neurologically and showed less deterioration in their neuroanatomy than their male counterparts.

There have been no reports on auditory function in the NPC mouse model. However, Luan et al. (2008) documented brainstem pathology localized to the auditory pathway in the *NPC1* (BALB/c) mouse. They compared the brainstems of eight-week-old mutant *NPC1* (-/-) mice with a control group of littermates, comprised of carrier *NPC1* (+/-) and wild type (+/+) mice. The neural density of the ventral cochlear nucleus was significantly lower in mutant mice than controls. In addition, a proliferation of astrocytes was observed in the inferior colliculus and medial geniculate nucleus, which the authors suggest is an additional aspect of the neuropathology of NPC.

Currently, the BALB/cNctr-*Npc1*^{m1N}/J strain is sold by the Jackson Laboratory and has been procured by the National Institutes of Health for study. This strain of mice, hereafter referred to as the BALB/c or *NPC1* mouse, begins to show neurological symptoms including tremor and ataxic gate, together with poor food intake at approximately seven weeks of age. The life span is approximately 75 days (phenotypic information retrieved from the Jackson Laboratory, 1/18/09, http://jaxmice.jax.org/strain/003092.html).

Beyond the advantages previously mentioned, working with the *NPC1* mouse to help elucidate the auditory phenotype of the disease is attractive for a number of reasons. The challenges posed by studying a genetically heterogeneous species, such as humans, make quantifying the contribution of a specific genetic mutation challenging. Extensive longitudinal experiments in humans are fraught with logistical difficulties and it is

virtually impossible to find individuals with no exposure to the confounding influences of uncontrolled environmental factors. The short lifespan of the mouse makes it ideal for addressing questions of progression. Similar patterns in the neurological deterioration relative to lifespan have been documented in mice and humans with NPC, and there is no reason to believe the same would not be true of the auditory phenotype.

A mouse model for disease not only allows access to longitudinal audiological data in a reasonable period of time, but it does so in a controlled manner that is not possible when collecting data from humans. These mice are uniform in their molecular composition, they receive the same care and husbandry, and their environmental exposures to extraneous influences not only are controlled and limited, but also identical. If any of these factors affect the variability in the clinical phenotype observed in humans, which is most assuredly the case, then use of the mouse model will, by design, improve the likelihood of finding interpretable, meaningful results as compared to experimentation focused solely on humans with NPC.

Based on its molecular, biochemical, and clinical characteristics, the *NPC1* BALB/c mouse strain is an excellent model for describing both normal and abnormal function in humans with the disease. Indeed, many of the heuristic discoveries responsible for exposing the molecular and biochemical foundations of NPC have been aided or accomplished with the use of this model, and there is no doubt that future research, including new potential pharmacological and therapeutic interventions, will involve this species.

Summary

Experiment one identified an auditory phenotype in humans with NPC; high frequency hearing loss is common, and retrocochlear pathology involving the auditory brainstem tracts is pervasive. The hearing loss is progressive in at least some individuals, and those with late-onset disease may have auditory manifestations long before overt neurologic onset. It is unclear from the human data whether the neural component of the auditory dysfunction is progressive, although longitudinal data from one individual suggest it may be.

A mouse model with a spontaneous genetic mutation homologous to that observed in humans exists for NPC. Much of the phenotype in this model has been described previously and closely resembles clinical characteristics observed in humans with this disease, indicating it is a good animal model for studying NPC. However, to date, there has been no examination of auditory function in this mouse model. Detailed and comprehensive examination of the auditory manifestations of humans with NPC revealed a pattern of dysfunction, despite limitations in the data, and any correlation with findings in an animal model will only strengthen that investigation. It is possible that the auditory system may ultimately serve as a clinical marker for the progression of NPC and, potentially, as a benchmark for therapeutic intervention. This will remain speculative until impact of the disease on the auditory system is understood fully. It appears that comparative, translational research involving both humans and mice is the most effective means with which to answer this question.

Research Questions and Hypotheses

The overarching question is whether or not the pathophysiological processes of NPC detrimentally impact auditory system function in the *NPC1* mouse and, if so, how do audiologic findings in the mouse compare to those in humans with the disease?

Research Questions

1. Is there a significant difference in auditory function as measured by ABR thresholds and DPOAEs between the three experimental groups: homozygous *NPC1* mice (-/-), heterozygous carriers (+/-), and wildtype littermates (+/+)?
    a. If auditory function is poorer in *NPC1* mutant mice (-/-) than wildtype controls, can the site-of-lesion be localized using DPOAEs and ABR?
    b. Is there a significant effect of gender on hearing outcomes (DPOAEs and ABR) within the three experimental groups?
    c. Are the auditory findings within each group stable over the course of the experimental lifetime?

Hypotheses

It is hypothesized that *NPC1* mutant mice (-/-) will display an abnormal auditory phenotype compared to wildtype littermates. This hypothesis is based on known similarities between the phenotype in *NPC1* mutant mice (-/-) and humans with this disease, and because experiment one identified auditory dysfunction in affected humans. DPOAE and ABR data will evaluate the integrity of the auditory pathway through the auditory brainstem in this strain and should be sensitive to dysfunction. These measures have been successful in identifying auditory dysfunction in other mouse models for auditory research (e.g., Ohlemiller, Vogler, Daly, & Sands, 2001). Based on abnormal

histology localized to the auditory brainstem in this strain (Luan, 2008), it is hypothesized that dysfunction will at least be localized to the auditory brainstem and may manifest as significantly elevated ABR thresholds, prolonged absolute and interpeak latencies, and/or decreased waveform amplitude for mutant mice as compared to wildtype littermates. Additionally, *NPC1* mutant mice are anticipated to have significantly lower DPOAE amplitude compared to control animals based on DPOAE and pure-tone threshold findings in humans with NPC that indicate a concomitant cochlear site of lesion.

There is no strong evidence to suggest that *NPC1* heterozygous carriers (+/-) should have poorer hearing than wildtype control mice given the autosomal recessive inheritance pattern of the disease. However, some reports suggest subtle neurological dysfunction in heterozygous carriers of the *NPC1* mutation in both mice and humans (Josephs, Matsumoto, & Lindor, 2004; Yu, Ko, Yanagisawa, & Michikawa, 2005). Consequently, their auditory data will be analyzed separately. Significant differences in ABR (threshold and latency) and DPOAE data are not anticipated between heterozygous carriers (+/-) and wildtype littermates. Similarly, Võikar et al. (2002) observed differences in cognitive and developmental motor function in male and female *NPC1* mice (-/-). Differences in age-related hearing loss between male and female mice of the same strain have also been reported (e.g., Henry, 2004). This is not a common finding in this species, however, and gender differences in auditory function in any of the three experimental groups are not anticipated.

Given the neurodegenerative nature of the disease, it is hypothesized that the auditory phenotype in the *NPC1* mice (-/-) will deteriorate progressively across the

experimental lifespan. Progressive hearing loss would be consistent with other aspects of the phenotype reported in this model, including shortened lifespan, progressive ataxia, and progressive neurological dysfunction; it would also be consistent with the progressive hearing loss observed in humans. The lifespan of the *NPC1* mutant animal is approximately 75 days. The experimental lifespan is expected to be shorter in duration and is contingent on the animals' ability to maintain life independently without unnecessary pain or discomfort. As such, euthanasia criteria have been established and are described in the methodology section for this experiment.

Experiment Two

Methods

Participants. Heterozygous carriers (+/-) of the *NPC1* mutation (BALB/cNctr-*Npc1*^{m1N}/J strain) were mated to produce offspring that were homozygous (-/-) for the *NPC1* mutation, heterozygous carriers (+/-), and wildtype (+/+) littermates.

The Animal Care and Use Committee (ACUC) of the University of Maryland College Park (R-08-20), and the combined National Institute of Neurological Disorders and Stroke and NIDCD ACUC (1264-06) approved this work. Animals were housed at an NIH facility in Rockville, Maryland, and maintenance and care of the animals followed the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, Committee on Care and Use of Laboratory Animals, 1985). Twenty mice per group (genotype) were used in this experiment, for a total of 60 mice. The experimenter was blinded to the genotype of the animals during the early part of life prior to mutant mice becoming overtly symptomatic.

Equipment. DPOAEs were recorded with an ER-10C (Etymotic Research) speaker-probe assembly using the DP2000 DPOAE measurement system, version 3.0 (Starkey Laboratories). ABR stimuli were generated with the auditory-evoked Intelligent Hearing Systems (IHS) software and produced through a high-frequency, ear-specific transducer.

Procedure. Animals were anesthetized using a combined cocktail of ketamine and dormitor, administered via subcutaneous (SQ) injection. Total dosage of anesthesia was weight-dependent, and neither of these drugs is known to affect auditory function in this species. In order to protect the corneas of the mice while under sedation, an otic cream was applied to the animals' eyes. The postauricular areas of skin and head of the mouse were cleaned with disposable alcoholic wipes. The animal was placed on a heating pad in a sound-treated test booth. A thermometer ensured the temperature of the heating pad was maintained at 37 degrees Fahrenheit to preserve the body heat of the animal.

DPOAEs were recorded by placing the speaker-probe assembly in the external auditory canal of the animal. Two primary tones at a frequency ratio (f2/f1) of 1.2 were presented at L_1 = 65 dB SPL and L_2 = 55 dB SPL. Calibration of the primary tones took place *in situ.* The primary tones were varied in one-twelfth octave steps from 5297 to 10641 Hz, based on the frequency limitations of the speaker-probe assembly. DPOAE data were collected on both ears of each animal.

ABRs were evaluated by placing three subdermal needle electrodes: one at the forehead and one at each mastoid location. ABR thresholds were averaged using a negative polarity (rarefaction) click stimulus, as well as 8, 16, and 32 kHz tone bursts.

Averages were acquired at a rate of 21 per second, and a minimum of 400 artifact-free averages were obtained for each recording. Prior to beginning threshold searches, a high intensity (e.g., 110 dB SPL) suprathreshold response was collected and used to determine absolute and interpeak latencies, as well as waveform amplitude. The same number of averages (1024) was collected for the high intensity responses for every test session on each animal.

Threshold search began with administration of a 110 dB SPL signal for the click, 8, and 16 k Hz stimuli, and a 100 dB SPL signal for the 32k Hz tone burst. The stimulus intensity was decreased subsequently in 10 dB steps, followed by 5 dB steps at lower intensities near threshold, to determine the exact threshold of the response. Once threshold was reached, the response was repeated to determine replicability. Following collection of suprathreshold data and determination of thresholds for click and tone burst stimuli, the effects of sedation were reversed using an antecedent and the animal was returned to a recovery cage.

In light of the neurodegenerative phenotype, endpoint criteria for euthanasia were established to ensure that none of the animals suffered unnecessarily. They were as follows: 15% loss of total body weight or an observation that the animal was moribund, cachectic, or unable to obtain food or water. The animals were monitored daily by trained staff. No additional husbandry care (e.g., bladder secretion, intravenous delivery of food) was provided once endpoint criteria were met. Therefore, the temporal intervals of the experimental design were contingent on the lifespan and neurological condition of the homozygous mutant mice.

Based on these criteria, the experimental lifespan of the *NPC1* mutant (-/-) mice extended to p65. All mice were able to survive to this age; however, very few mutant animals were able to survive to p70 without meeting euthanasia criteria and requiring additional husbandry care. The timeframe for auditory testing was also limited by the development of the auditory system and weaning from the mother. The auditory system in the mouse is mature at approximately p17, and weaning usually occurs around p20. Therefore auditory testing began at p20 and was repeated every five days until p65, resulting in 10 longitudinal data points.

Five-day time intervals were selected based on the health and well being of the animals. Full recovery of the animals from anesthesia precludes testing at very short time intervals. Because auditory findings in NPC mice have not been published, guidance on a longitudinal test schedule was derived from longitudinal studies of other neurological measures in this mouse model. Võikar et al. (2002) observed longitudinal changes in cognitive function and motor impairment in NPC mice that were tested every seven days starting at P28 and ending at day P56. Significant changes in performance could be noted between individual test sessions, indicating that a five-day test interval of auditory function was reasonable and likely to produce meaningful data without causing unnecessary stress for the animals.

Statistical Analysis. Data were maintained and analyzed using Microsft Excel, the Statistical Package for Social Sciences Software (SPSS, v15) and the Proc MIXED SAS 9.1 software packages. For all statistical analyses, $\alpha = .05$. Descriptive statistical data were prepared for each experimental group.

To evaluate neural and cochlear function, change over time, and effect of disease within gender, DPOAE (averaged between the right and left ears) and ABR data were analyzed using a repeated measures model with auto-regressive correlation matrix structure (e.g., Crowder & Hand, 1990). This is similar to a typical repeated measures ANOVA but models time as an interval-scaled variable rather than a nominal-scaled variable. A typical repeated measures ANOVA requires the assumption of sphericity, typically modeled as compound symmetry. Compound symmetry assumes that the correlation between residuals (i.e., what remains after main effects and interactions between genotype, test time, and gender are accounted for) for any two repeated measures is the same across the experimental lifespan. The autoregressive structure postulates that the correlation is stronger between time points that are closer together, and weaker between time points that are farther apart. Both the auto-regressive and compound symmetry approaches assume that un-modeled characteristics have a similar effect for multiple measures, but auto-regression allows for these relationships to change over time (e.g., a mouse with 'good' hearing at p20 is likely to still have good hearing at p25, but this may be less likely by p65). Fit statistics for both auto-regressive and compound symmetry models were generated to determine how strongly the data appear to correlate with a given model, and the fit was better for the auto-regressive approach. This confirms the logical assumption that the auto-regressive model is more appropriate than the compound symmetry model for this dataset.

DPOAE level and ABR (click and tone pip) threshold, suprathreshold latency (absolute and interpeak), and amplitude data were evaluated in separate analyses. For all dependent variables, the assumed model was:

Y = intercept + Gender + Genotype (Homozygous/Non-Homozygous) + Time + all interactions

Preliminary analyses revealed no significant differences in measures of hearing (DPOAE level, ABR threshold, latency, amplitude) between heterozygous carriers (+/-) and wildtype (+/+) littermates. Therefore, these data were pooled to increase power. The non-mutant mouse (+/-, +/+) group will be referred to in this document as *control animals* for comparison to mutant (-/-) data.

Pitfalls. Following collection of nearly three-quarters of the data an unexplainable shift in 8k Hz threshold data was observed. Specifically, thresholds across animals seemed notably lower (better) than those collected previously. Ultimately, it was discovered that another NIH investigator using the shared equipment uncovered a filter box and changed the setting from "High Pass" to "Direct." Until this point, all data had been collected using the "High Pass" filter setting. This was not the appropriate filter setting for an 8k Hz stimulus, and the presence of the filter was not apparent to the investigative team.

A discussion with the manufacturer (IHS) of the effects of the change in filter setting revealed that 8k Hz data collected with the filter on (High Pass) results in a loss of stimulus intensity; in other words, 8k Hz thresholds collected with the filter on (High Pass) will be higher (poorer) than those collected with the filter off (Direct). The manufacturer is unaware of any negative effect of having the filter on when collecting 16k and 32k Hz data, and in fact recommends this setting when using these stimuli.

Data collected during the time between the noticeable shift in thresholds and the discovery of the filter box (approximately two weeks) were discarded. To account for a

possible discrepancy in data collected with different filter settings, it was decided (via personal communication with SGS, CB, and KK) that all remaining 8k Hz data would be collected twice: once with the filter turned on (High Pass), and once with the filter turned off (Direct). The aim was to develop a correction factor for all previous data collected *a posteriori*.

Following completion of data collection, 8k Hz data were analyzed to determine if the effect of the filter box was consistent between genotypes and across hearing thresholds. A consistent finding would suggest that a correction factor could be applied to data collected with the Direct filter setting. However, results revealed an effect of genotype; the difference between the High Pass and Direct settings is approximately 4 dB larger for wildtype (+/+) than for homozygous (-/-) mice and the effect varied depending on the threshold for hearing. Therefore, no correction factor was applied and all 8k Hz data reported for this study represent those data collected with the filter on High Pass.

Results

Twenty mice from each experimental (genotype) group were evaluated starting at p20 every five days until p65, for a total of 60 mice with 10 longitudinal data points. Sporadic data points are missing due to uncontrollable circumstance (e.g., problems with sedation); in total, 575 test sessions occurred. Demographic data, including genotype, are included in Figure 28. Animal weight (grams) as a function of age is plotted in Figure 29. As expected, mutant (-/-) mice weighed less than control animals (-/+, +/+) across the experimental lifespan. While control animals continued to gain weight, mutant mice reached their peak weight at approximately p40 after which they maintained and eventually lost weight.

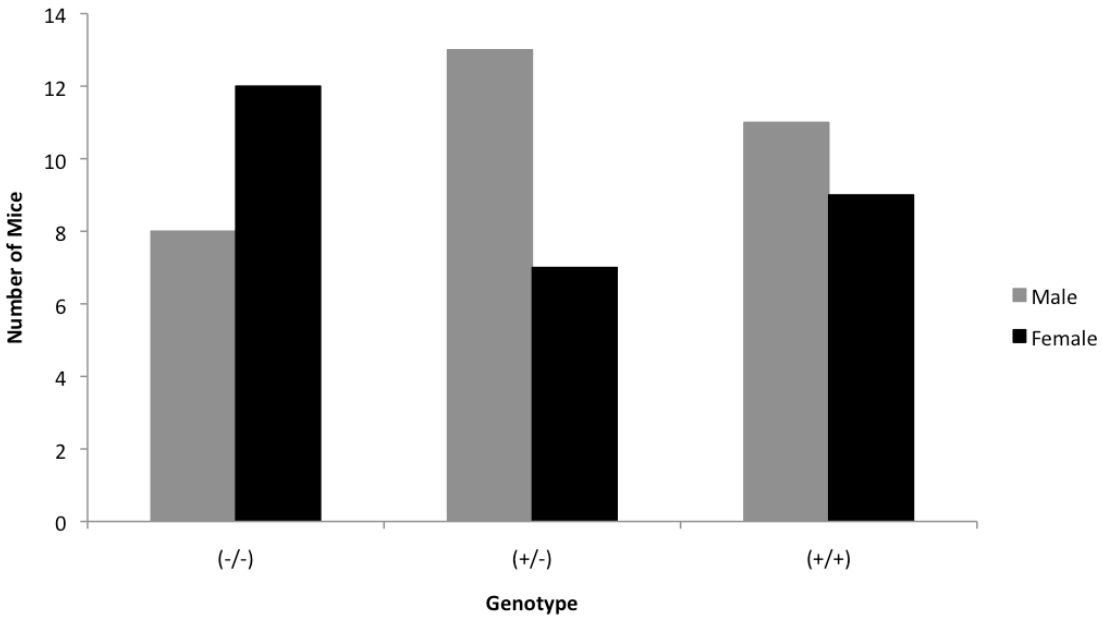


Figure 28. Number of mice by genotype and gender for homozygous (-/-), heterozygous (+/-), and wildtype (+/+) *NPC1* littermates.

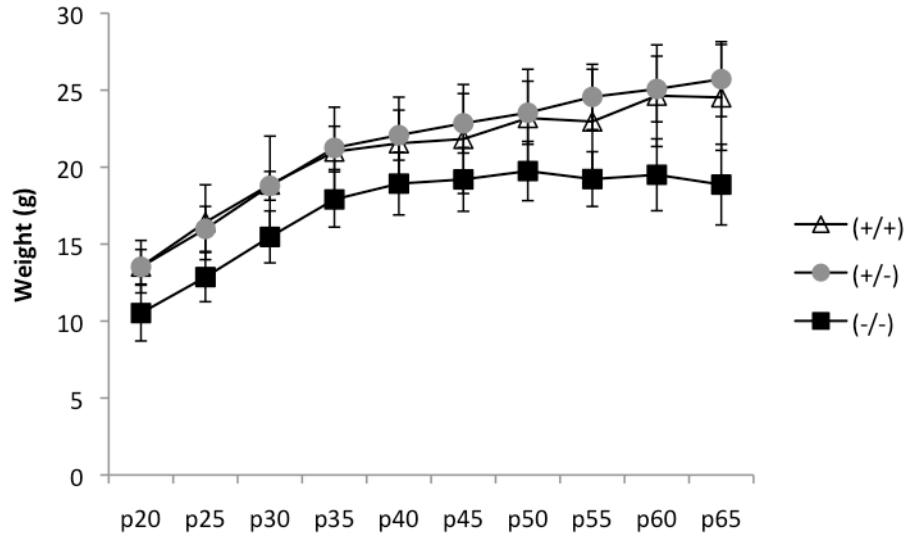


Figure 29. Mean (SD) weight for homozygous (-/-), heterozygous (+/-), and wildtype (+/+) *NPC1* littermates across the experimental lifespan.

DPOAE Findings. Mean (SD) DPOAE level and noise floor for mutant and control animals are presented in Figure 30. Both groups had relatively robust signal-to-noise ratios (SNR) across the experimental life span with the exception of the lowest frequency tested (5297 Hz) in the mutant group at p20; however, the SNR at this frequency was noted to improve by p65.

Statistical results of the repeated measures model for DPOAE level for the mutant and control groups are presented in Table X. This table includes data and statistical results displayed in three columns: A, B, and C. Column A shows estimates of DPOAE level (dB SPL) by frequency for mutant (-/-) and control animals (+/- +/+) at p20, and the effect of disease (genotype) at that point in time (F-statistic, degrees of freedom, p-value). At p20 mutant animals have significantly lower DPOAE levels than control animals at all test frequencies except for 10641 Hz (p=0.05) and 7547 Hz (p=0.13). This is not a true main effect as it is conceived in a typical ANOVA, which is time-invariant, but rather an effect of disease 'within' a value of time (e.g., p20). Column B shows the estimates of progression in DPOAE level (dB) for mutant and control animals per five-day test session; positive values indicate an increase in DPOAE level, and negative values indicate a decrease in DPOAE level every five days. Statistical results examining the significance of the progression within genotype (first two columns within Column B), as well as the significance of genotype on progression of DPOAE level (last two columns within Column B) are shown. It is noted that while there is a significant progression of DPOAE level for the control group at each frequency and for the mutant group at the lower frequencies, there is not a significant difference between the two groups in DPOAE level progression at any frequency. Column C shows estimates of DPOAE level (dB

SPL) for mutant and control animals at p65, and the effect of disease at that point in time. Estimates provided for p20 and p65 in columns A and C, respectively, are drawn from the repeated measures model that includes the entire data set at all time points to provide an observation at the beginning and end of the experimental lifespan. These do not represent simple analyses (e.g., t-tests) between observations at test time one and test time 10. Column C indicates that at p65, mutant animals continue to have significantly lower DPOAE levels than control animals at all test frequencies.

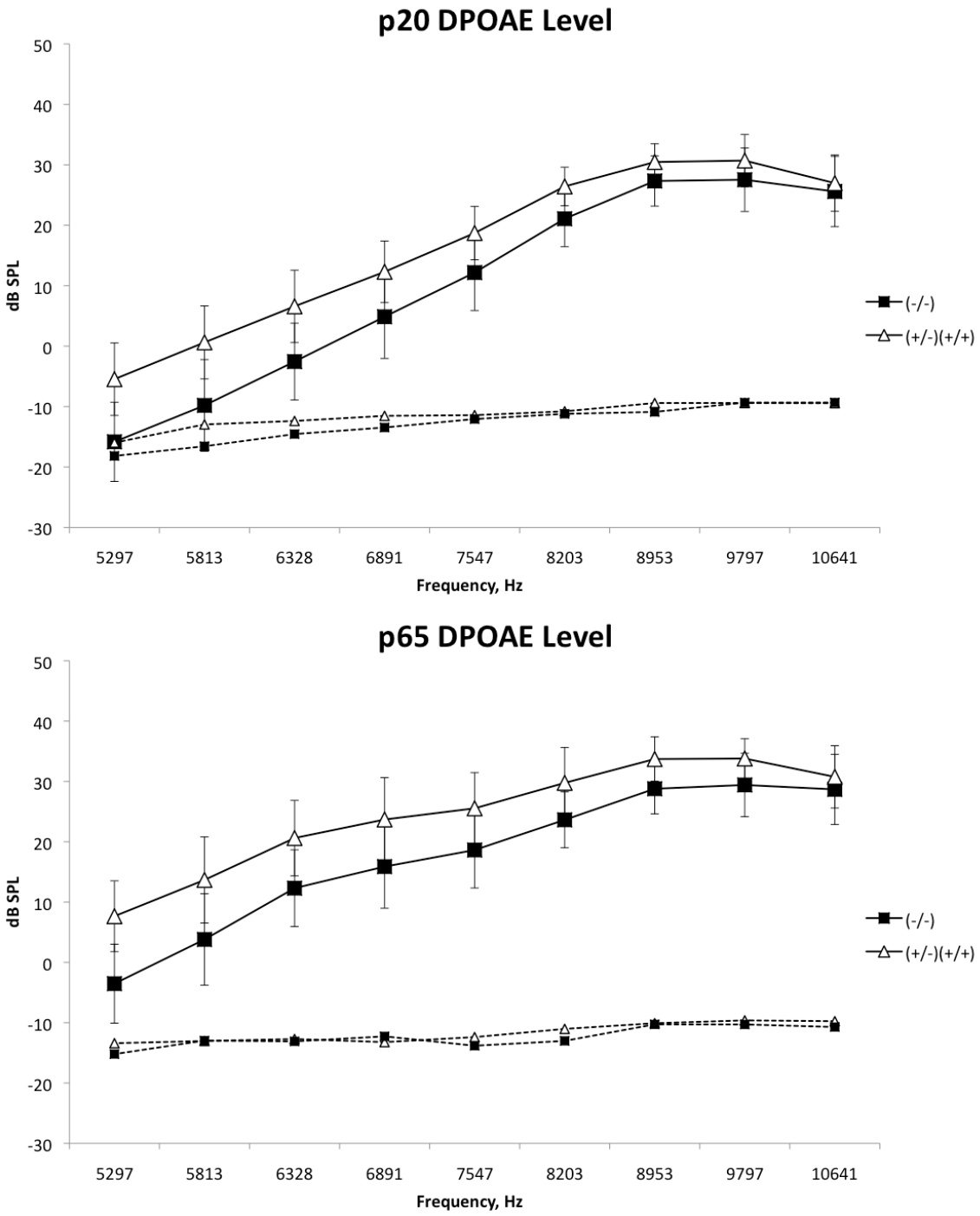


Figure 30. Mean (SD) DPOAE level (solid lines) for mutant (-/-) and control (+/-, +/+) animals at p20 (top panel) and p65 (bottom panel). The noise floor is also plotted (dotted lines).

Table X. Results of repeated measures analyses for DPOAE level (dB SPL) at p20 and p65, and DPOAE level progression data (dB).

	(A) p20 DPOAE level				(B) DPOAE level progression/5 days				(C) p65 DPOAE Level			
Hz	-/-	+/- +/+	F (df, df)	p value	-/- (p value)	+/- +/+ (p value)	F (df, df)	p value	-/-	+/- +/+	F (df, df)	p value
10641	27.2	28.9	4.01 (1, 56)	0.0500	0.196 (0.0960)	0.306 (0.0003)**	0.58 (1, 500)	0.4478	29.0	31.6	10.34 (1, 56)	0.0022**
9797	29.5	32.2	13.28 (1, 56)	0.0006**	0.621 (0.5514)	0.246 (0.0011)**	2.05 (1, 500)	0.1528	30.1	34.4	35.21 (1, 56)	<.0001**
8953	28.7	31.8	16.42 (1, 56)	0.0002**	0.080 (0.4479)	0.276 (0.0003)**	2.25 (1, 500)	0.1344	29.4	34.3	40.64 (1, 56)	<.0001**
8203	22.8	28.9	32.14 (1, 56)	<.0001**	0.219 (0.0834)	0.297 (0.0012)**	0.25 (1, 500)	0.6187	24.7	30.6	41.68 (1, 56)	<.0001**
7547	19.3	21.8	2.43 (1, 56)	0.1250	-0.007 (0.9749)	0.497 (0.0047)**	2.87 (1, 500)	0.0908	19.2	26.3	19.61 (1, 56)	<.0001**
6891	7.6	15.1	34.99 (1, 56)	<.0001**	1.179 (<.0001)**	1.083 (<.0001)**	0.19 (1, 500)	0.6625	18.3	24.8	27.43 (1, 56)	<.0001**
6328	1.0	9.8	44.05 (1, 56)	<.0001**	1.535 (<.0001)**	1.354 (<.0001)**	0.61 (1, 500)	0.4369	14.8	22.0	29.43 (1, 56)	<.0001**
5813	-6.5	3.8	57.22 (1, 56)	<.0001**	1.478 (<.0001)**	1.264 (<.0001)**	0.83 (1, 500)	0.3615	6.8	15.1	37.85 (1, 56)	<.0001**
5297	-12.7	-2.1	67.73 (1, 56)	<.0001)**	1.300 (<.0001)**	1.265 (<.0001)**	0.02 (1, 500)	0.8750	-1.0	9.3	64.00 (1, 56)	<.0001**

* significant at p<.05 level
** significant at p<.01 level
Note. (df, df), degrees of freedom numerator, degrees of freedom denominator.

Table XI evaluates the effect of disease, as well as the relationship between gender and test-time for DPOAE level data, separately for male and female mice. This is not a typical examination of main and interaction effects of gender, disease, and time, but rather tests the effects of interest (disease and disease*time) at all levels of the blocking variable gender. Exploring differences between male mice and female mice simply identifies differences between mice, but does not identify effects of disease. Therefore, these comparisons were established to observe the effect of disease within gender; specifically, the effect of disease within male mice and the effect of disease within female mice. Table XI is also displayed in three columns: A, B, and C. Column A shows the statistical comparison between mice with (-/-) and without (+/- +/+) disease (F-statistic, degrees of freedom, p-value) for females and males at p20. Column B compares the progression of DPOAE level every five days in mice with and without disease for females and males. Column C shows the comparison between mice with and without disease for females and males at p65.

There is no consistent difference in the effect of disease within genders observed on DPOAE level across the experimental lifespan. That is, when significant effects of disease between mutant animals and control animals are observed (Table X), these occur for both female and male mice at most test frequencies across the experimental lifespan. An exception to this is DPOAE level at p20 at 8953 Hz, and DPOAE progression and level at p65 at 7547 Hz, where the effect of disease was only observed in male mice.

Table XI. Results of repeated measures analyses examining effects of disease within gender and test time for DPOAE level.

Comparisons are between female and male mice with (-/-) and without (+/- +/+) disease.

	(A) p20 DPOAE Level				(B) DPOAE level progression/5 days				(C) p65 DPOAE Level			
	Female		Male		Female		Male		Female		Male	
Hz	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value	F (df, df)	P value	F (df, df)	p value	F (df, df)	p value
10641	0.78 (1, 56)	0.3805	3.65 (1, 56)	0.0612	1.05 (1, 500)	0.3064	0.01 (1, 500)	0.9337	6.23 (1, 56)	0.0155*	4.27 (1, 56)	0.0434*
9797	4.89 (1, 56)	0.0311*	8.51 (1, 56)	0.0051**	1.29 (1, 500)	0.2558	0.80 (1, 500)	0.3704	15.97 (1, 56)	0.0002**	19.26 (1, 56)	<.0001**
8953	2.53 (1, 56)	0.1170	16.38 (1, 56)	0.0002**	1.74 (1, 500)	0.1882	0.67 (1, 500)	0.4118	12.99 (1, 56)	0.0007**	28.76 (1, 56)	<.0001**
8203	5.27 (1, 56)	0.0255*	31.36 (1, 56)	<.0001**	0.51 (1, 500)	0.4750	0.00 (1, 500)	0.9889	11.45 (1, 56)	0.0013**	32.29 (1, 56)	<.0001**
7547	2.75 (1, 56)	0.1027	0.34 (1, 56)	0.5594	0.00 (1, 500)	0.9818	5.50 (1, 500)	0.0194*	2.57 (1, 56)	0.1146	21.03 (1, 56)	<.0001**
6891	9.53 (1, 56)	0.0031**	27.06 (1, 56)	<.0001**	0.37 (1, 500)	0.5456	0.00 (1, 500)	0.9739	4.45 (1, 56)	0.0394*	27.15 (1, 56)	<.0001**
6328	15.44 (1, 56)	0.0002**	29.23 (1, 56)	<.0001**	0.57 (1, 500)	0.4507	0.13 (1, 500)	0.7192	7.36 (1, 56)	0.0089**	23.99 (1, 56)	<.0001**
5813	22.19 (1, 56)	<.0001**	35.37 (1, 56)	<.0001**	1.01 (1, 500)	0.3165	0.10 (1, 500)	0.7541	9.69 (1, 56)	0.0029**	30.47 (1, 56)	<.0001**
5297	25.99 (1, 56)	<.0001**	42.19 (1, 56)	<.0001**	0.25 (1, 500)	0.6197	0.06 (1, 500)	0.8039	18.23 (1, 56)	<.0001**	48.59 (1, 56)	<.0001**

* significant at p<.05 level

** significant at p<.01 level

Note. (df, df), degrees of freedom numerator, degrees of freedom denominator.

ABR Threshold Findings. ABR threshold data at p20 and p65 for all genotypes for click, 8k, 16k, and 32k Hz stimuli are shown in Figure 31. Results of initial repeated measures modeling revealed that the mutant group exhibited higher (poorer) thresholds than control animals at p20 for 16k and 32k Hz, and at p65 for all stimuli. Because no significant differences in ABR thresholds were observed between heterozygous (+/-) and wildtype (+/+) animals, their data were collapsed for additional analyses. Results of follow-up repeated measures modeling (two experimental groups: collapsed control group and mutant group) for ABR threshold comparing the mutant and control groups are presented in Table XII. For a detailed explanation of table formatting, refer to DPOAE level results described on pages 116 and 117. Threshold data are provided in dB SPL. Positive progression estimates (dB) indicate an increase in threshold and negative values indicate a decrease in threshold every five days.

At p20 (Table XII, Column A), mutant animals have significantly poorer hearing than control animals at 16k Hz and 32k Hz. By p65, mutant mice have significantly poorer hearing as measured by the ABR threshold across all test stimuli compared to control animals. Mutant animals exhibit a significant progression in ABR click threshold at a rate of 0.32 dB/ 5 days with no significant change in hearing among the control animal group over the time span of the experiment. For 8k Hz and 16k Hz stimuli, thresholds of the mutant animals become progressively poorer at rates of .4 and .7 dB/5 days, respectively, with no significant change in threshold in control animals. At 32k Hz, mutant animals do not exhibit progression in their hearing thresholds, but control animals do at a rate of 3.6 dB/5days. Despite this progression in the non-mutant group, mutant animals continue to have significantly poorer hearing at 32k Hz for all test times

compared to control animals. The progression observed in ABR stimuli by genotype is shown in Figures 32 and 33. Results of the repeated measures modeling examining differences between groups at each test interval are provided in Tables XIII, XIV, XV, and XVI (click, 8k, 16k, and 32k Hz analyses, respectively). The progression in threshold of mutant animals observed for click and 8k Hz ABR stimuli results in significant differences between mutant and control animals at p35, which continues through the remainder of the experimental lifespan. ABR thresholds at 16k and 32k Hz in the mutant group were significantly poorer than control animals at all test intervals.

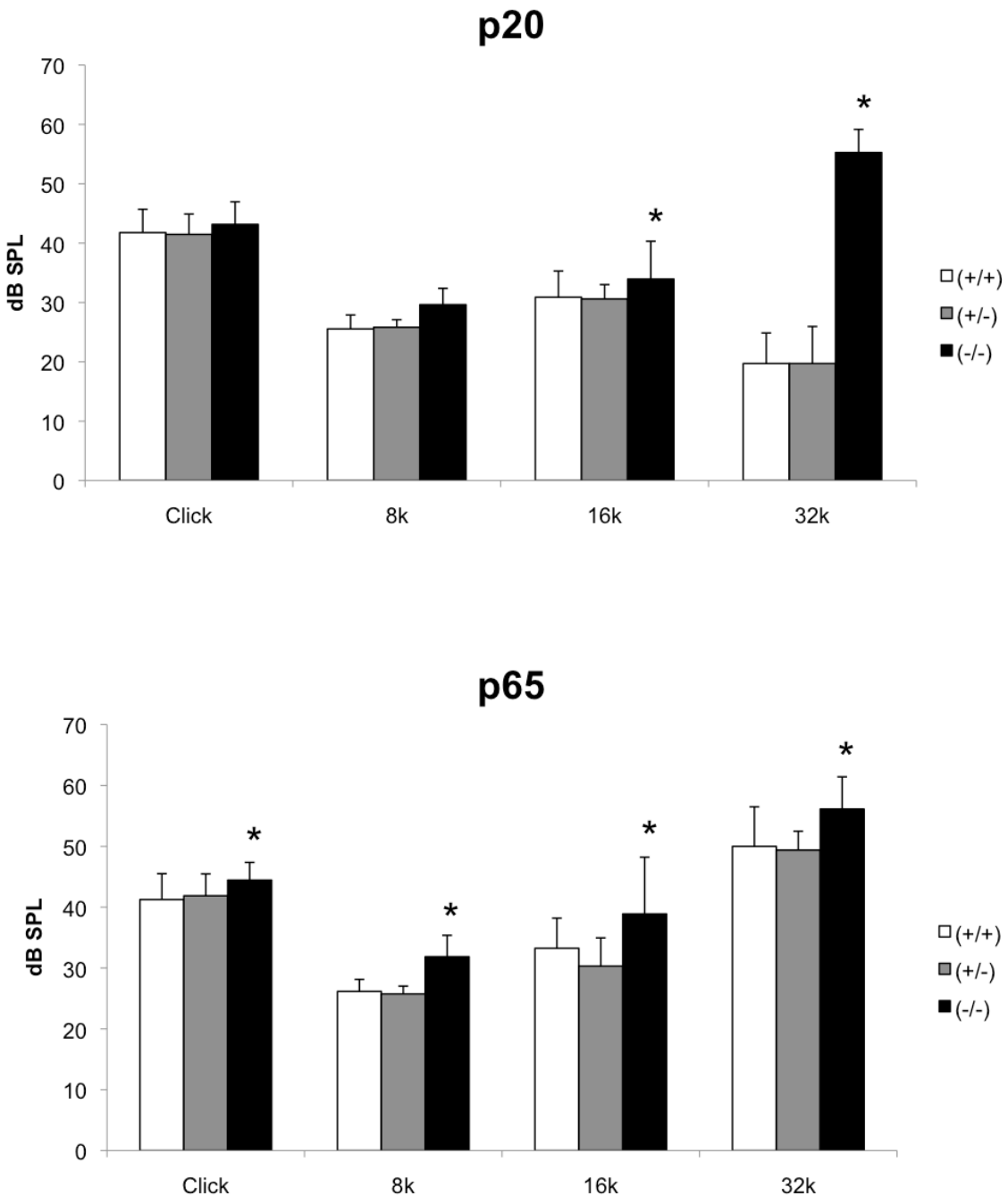


Figure 31. Mean (SD) ABR thresholds for homozygous (-/-), heterozygous (+/-), and wildtype (+/+) *NPC1* littermates at p20 (top panel) and p65 (bottom panel).

* $p < .05$, (-/-) significantly different from control animals (+/-, +/+).

Table XII. Results of repeated measures analyses for ABR threshold (dB SPL) at p20 and p65, and ABR threshold progression data (dB).

	(A) p20 Threshold				(B) Threshold progression/5 days				(C) p65 Threshold			
	-/-	+/- +/+	F (df, df)	p value	-/- (p value)	+/- +/+ (p value)	F (df, df)	p value	-/-	+/- +/+	F (df, df)	p value
Click	42.55	42.21	0.15 (1,56)	0.6981	0.32 (0.0124)*	-0.05 (0.6003)	5.48 (1, 502)	0.0196*	45.40	41.78	18.15 (1, 56)	<.0001**
8k	46.52	46.33	0.03 (1, 56)	0.8529	0.43 (0.0040)**	-0.01 (0.9512)	5.66 (1, 501)	0.0177*	50.41	46.27	16.94 (1, 56)	0.0001**
16k	33.39	29.83	6.80 (1, 56)	0.0117*	0.73 (0.0002)**	0.21 (0.143)	4.74 (1, 501)	0.0299*	39.96	31.66	36.98 (1, 56)	<.0001**
32k	54.15	17.87	520.06 (1, 56)	<.0001**	0.27 (0.2328)	3.59 (<.0001)**	138.95 (1, 500)	<.0001**	56.60	50.21	16.26 (1, 56)	0.0002**

* significant at p<.05 level
** significant at p<.01 level
Note. (df, df), degrees of freedom numerator, degrees of freedom denominator.

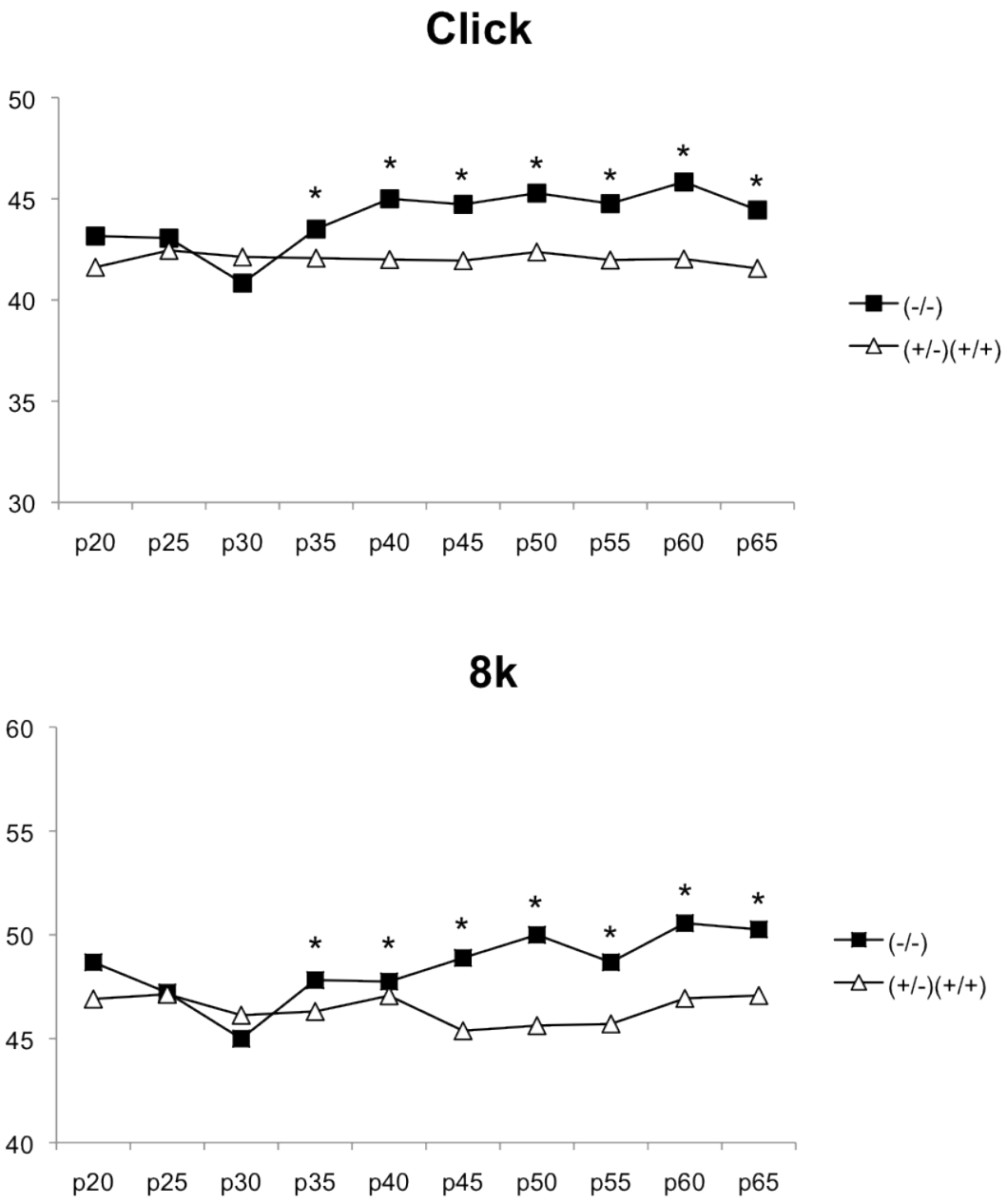


Figure 32. Mean ABR threshold data for click (top panel) and 8k Hz (bottom panel) stimuli for mutant (-/-) and control (+/-, +/+) animals. *p < .05.

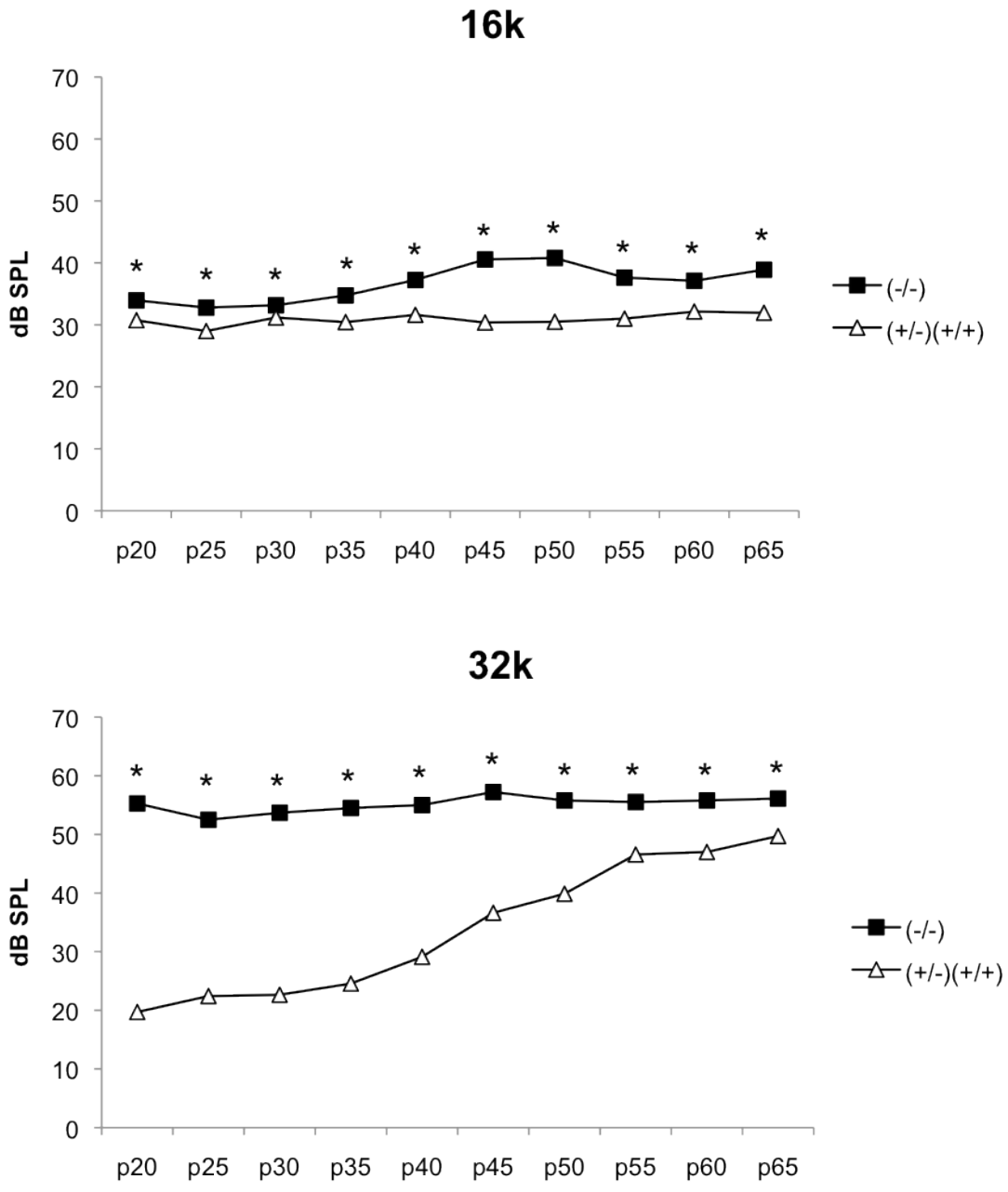


Figure 33. Mean ABR threshold data for 16k (top panel) and 32k Hz (bottom panel) stimuli for mutant (-/-) and control (+/-, +/+) animals. *p < .05.

Table XII. Results of repeated measures analysis by test time for click ABR threshold.

	-/- Estimate	+/- +/+ Estimate	F (df, df)	p value
p20	42.55	42.21	0.15 (1, 56)	0.6981
p25	42.86	42.17	0.92 (1, 56)	0.3428
p30	43.18	42.12	2.93 (1, 56)	0.0923
p35	43.50	42.07	7.10 (1, 56)	0.0101*
p40	43.81	42.02	13.51 (1, 56)	0.0005**
p45	44.13	41.97	19.62 (1, 56)	<.0001**
p50	44.45	41.93	22.31 (1, 56)	<.0001**
p55	44.76	41.88	21.83 (1, 56)	<.0001**
p60	45.08	41.83	20.07 (1, 56)	<.0001**
p65	45.40	41.78	18.15 (1, 56)	<.0001**

* significant at p<.05 level

** significant at p<.01 level

Note. (df, df), degrees of freedom numerator, degrees of freedom denominator.

Table XIV. Results of repeated measures analysis by test time for 8k Hz ABR threshold.

	-/- Estimate	+/- +/+ Estimate	F (df, df)	p value
p20	46.52	46.33	0.03 (1, 56)	0.8529
p25	46.95	46.33	0.53 (1, 56)	0.4705
p30	47.38	46.32	2.11 (1, 56)	0.1523
p35	47.82	46.31	5.62 (1, 56)	0.0212*
p40	48.25	46.31	11.32 (1, 56)	0.0014**
p45	48.68	46.30	17.05 (1, 56)	0.0001**
p50	49.12	46.29	19.89 (1, 56)	<.0001**
p55	49.55	46.29	19.84 (1, 56)	<.0001**
p60	49.98	46.28	18.51 (1, 56)	<.0001**
p65	50.41	46.27	16.94 (1, 56)	0.0001**

* significant at p<.05 level

** significant at p<.01 level

Note. (df, df), degrees of freedom numerator, degrees of freedom denominator.

Table XV. Results of repeated measures analysis by test time for 16k Hz ABR threshold.

	-/- Estimate	+/- +/+ Estimate	F (df, df)	p value
p20	33.39	29.83	6.80 (1, 56)	0.0117*
p25	34.12	30.04	11.93 (1, 56)	0.0011**
p30	34.85	30.24	20.25 (1, 56)	<.0001**
p35	35.58	30.45	32.32 (1, 56)	<.0001**
p40	36.31	30.66	45.85 (1, 56)	<.0001**
p45	37.04	30.86	54.83 (1, 56)	<.0001**
p50	37.78	31.07	55.39 (1, 56)	<.0001**
p55	38.50	31.27	50.12 (1, 56)	<.0001**
p60	39.23	31.48	43.26 (1, 56)	<.0001**
p65	39.96	31.67	36.98 (1, 56)	<.0001**

* significant at p<.05 level

** significant at p<.01 level

Note. (df, df), degrees of freedom numerator, degrees of freedom denominator.

Table XVI. Results of repeated measures analysis by test time for 32k Hz ABR threshold.

	-/- Estimate	+/- +/+ Estimate	F (df, df)	p value
p20	54.15	17.87	520.06 (1, 56)	<.0001**
p25	54.42	21.46	573.28 (1, 56)	<.0001**
p30	54.69	25.06	619. 70 (1, 56)	<.0001**
p35	54.97	28.65	630.33 (1, 56)	<.0001**
p40	55.24	32.24	563.36 (1, 56)	<.0001**
p45	55.51	35.83	413. 05 (1, 56)	<.0001**
p50	55.79	39.43	244.44 (1, 56)	<.0001**
p55	56.06	43.02	120.52 (1, 56)	<.0001**
p60	56.33	46.61	50.11 (1, 56)	<.0001**
p65	56.61	50.21	16.26 (1, 56)	0.0002**

* significant at p<.05 level

** significant at p<.01 level

Note. (df, df), degrees of freedom numerator, degrees of freedom denominator.

There was no consistent effect of disease on gender distinguishing male from female mice for the majority of the ABR threshold data (Table XVII). For 16k Hz stimuli at p20, and for click stimuli progression, there is an effect of disease observed in female mice but not in male mice. The reverse is true for 8k and 16k progression data. Otherwise, both male and female mice exhibited effects of disease.

Table XVII. Results of repeated measures analyses examining effects of disease within gender and test time for ABR threshold.

Comparisons are between female and male mice with (-/-) and without (+/- +/+) disease.

	(A) p20 Threshold				(B) Threshold progression/5 days				(C) p65 Threshold			
	Female		Male		Female		Male		Female		Male	
	F (df, df)	p value	F (df, df)	P value	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value
Click	0.02 (1, 56)	0.8777	0.15 (1, 56)	0.6993	4.13 (1, 502)	0.0427*	1.70 (1, 502)	0.1923	12.19 (1, 56)	0.0009**	6.56 (1, 56)	0.0131*
8k	1.44 (1, 56)	0.2357	0.74 (1, 56)	0.3949	0.75 (1, 501)	0.3856	5.99 (1, 502)	0.0147*	6.84 (1, 56)	0.0114*	10.19 (1, 56)	0.0023**
16k	8.66 (1, 56)	0.0047**	0.69 (1, 56)	0.4106	0.90 (1, 501)	0.3438	4.39 (1, 501)	0.0367*	19.54 (1, 56)	<.0001**	17.55 (1, 56)	<.0001**
32k	281.76 (1, 56)	<.0001**	241.40 (1, 56)	<.0001**	78.56 (1, 500)	<.0001**	61.63 (1, 501)	<.0001**	6.60 (1, 56)	0.0129*	9.73 1, 56)	0.0029**

* significant at p<.05 level

** significant at p<.01 level

Note. (df, df), degrees of freedom numerator, degrees of freedom denominator.

ABR Latency Findings. Results of repeated measures modeling for ABR absolute and interpeak latency data are included in Table XVIII (click), Table XIX (8k Hz), Table XX (16k Hz), and Table XXI (32 Hz). See pages 116 and 117 for a detailed description of table formatting. Latency data are provided in ms. Positive progression estimates indicate an increase in latency, and negative values indicate a decrease in latency every five days.

For the click stimulus (Table XVIII), there were no significant differences in latency at p20 between mutant and control animals, and latency data from mutant animals did not change significantly over time, with the exception of a slight increase (.005 ms/5 days) in latency at wave I. Conversely, control animals exhibited a significant decrease in latency across all variables. In general, the effect was greater for later occurring waves. Observation of a decrease in ABR absolute latency early in life is typically associated with maturational changes (e.g., myelination of the auditory nerve). This shortening in the latency of control animals resulted in mutant mice having significantly longer latencies than control animals by p65 across all variables.

A similar pattern as the one observed for click latency data was seen for other ABR stimuli (Tables XIX, XX, and XXI). That is, very few differences in latency existed between control and mutant animals at p20. No difference was observed at 16k Hz, and of the six absolute latencies that were different between groups at 8k and 32k Hz, all but one (wave II, 8k Hz) revealed that mutant mice had longer latencies than control animals. The more robust finding, however, was that for all ABR stimuli, control animals underwent a significant decrease in latency across the experimental lifespan in all but three variables (waves I and II at 32k Hz; wave II at 16k Hz). Again, the effect was

generally greater for later occurring waves. Mutant mice, however, underwent far fewer significant changes in latency over this time period, and most changes involved a prolongation in the absolute latency of early waves, resulting in a decrease in interpeak latency. The result of these progressive shifts in latency was that mutant mice had significantly longer latencies than control animals for all but three (29/32) ABR latency variables at p65.

ABR mean (SD) absolute latency data as a function of ABR wave component are shown separately for the mutant and control groups at p20 and p65 for 8k, 16k, and 32k Hz in Figures 34 through 36. In general, these figures show a decrease in latency, especially for the later waves, for the control group at p65 compared to p20. An example of the significant changes in latency observed over time, including a larger effect for later occurring waves, is shown for click data in Figure 37.

Table XVIII. Results of repeated measures analyses for ABR latency (ms) at p20 and p65, and ABR latency progression data (dB) for click stimuli.

	(A) p20 Latency				(B) Latency progression/5 days				(C) p65 Latency			
Click	-/-	+/- +/+	F (df, df)	p value	-/- (p value)	+/- +/+ (p value)	F (df, df)	p value	-/-	+/- +/+	F (df, df)	p value
I	1.37	1.37	0.16 (1, 56)	0.6918	0.005 (0.01)*	-0.004 (0.005)**	12.93 (1, 493)	0.8004	1.42	1.33	41.00 (1, 56)	<.0001**
II	2.14	2.20	2.07 (1, 56)	0.1562	-0.003 (0.6334)	-0.030 (<.0001)**	14.27 (1, 493)	0.0002**	2.11	1.91	23.32 (1, 56)	<.0001**
III	3.23	3.17	0.94 (1, 56)	0.3360	-0.000 (0.9679)	-0.022 (0.0024)**	3.08 (1, 492)	0.0797	3.23	2.97	15.16 (1, 56)	0.0003**
IV	4.21	4.18	0.22 (1, 56)	0.6408	0.0003 (0.9743)	-0.039 (<.0001)**	9.79 (1, 493)	0.0019**	4.21	3.83	33.27 (1, 56)	<.0001**
V	5.10	5.02	0.97 (1, 56)	0.3289	-0.008 (0.5299)	-0.064 (<.0001)**	14.32 (1, 493)	0.0002**	5.03	4.44	53.89 (1, 56)	<.0001**
I-III	1.86	1.80	0.81 (1, 56)	0.3171	-0.005 (0.5575)	-0.017 (0.0105)*	1.06 (1, 492)	0.3039	1.81	1.64	6.89 (1, 56)	<.0001**
III-V	1.87	1.83	0.28 (1, 56)	0.6340	-0.008 (0.4806)	-0.040 (<.0001)**	5.92 (1, 492)	0.0153*	1.80	1.47	21.74 (1, 56)	<.0001**
I-V	3.73	3.65	0.96 (1, 56)	0.3310	-0.012 (0.2593)	-0.059 (<.0001)**	10.54 (1, 493)	0.0012**	3.61	3.12	41.58 (1, 56)	<.0001**

* significant at p<.05 level
** significant at p<.01 level
Note. (df, df), degrees of freedom numerator, degrees of freedom denominator.

Table XIX. Results of repeated measures analyses for ABR latency (ms) at p20 and p65, and ABR latency progression data (dB) for 8k Hz stimuli.

	(A) p20 Latency				(B) Latency progression/5 days				(C) p65 Latency			
8k	-/-	+/- +/+	F (df, df)	p value	-/- (p value)	+/- +/+ (p value)	F (df, df)	p value	-/-	+/- +/+	F (df, df)	p value
I	1.77	1.76	0.15 (1, 56)	0.7035	-0.001 (0.7526)	-0.015 (<.0001)**	22.85 (1, 492)	<.0001**	1.75	1.63	55.08 (1, 56)	<.0001**
II	2.50	2.64	12.13 (1, 56)	0.0010**	-0.004 (0.4987)	-0.039 (<.0001)**	23.03 (1, 492)	<.0001**	2.46	2.29	19.67 (1, 56)	<.0001**
III	3.63	3.55	4.36 (1, 56)	0.0413*	-0.001 (0.8905)	-0.037 (<.0001)**	25.90 (1, 492)	<.0001**	3.63	3.22	114.03 (1, 56)	<.0001**
IV	4.67	4.65	0.29 (1, 56)	0.5927	0.002 (0.7954)	-0.043 (<.0001)**	24.80 (1, 492)	<.0001**	4.69	4.26	80.16 (1, 56)	<.0001**
V	5.71	5.52	8.51 (1, 56)	0.0051**	-0.024 (0.0132)*	-0.065 (<.0001)**	11.50 (1, 491)	0.0008**	5.49	4.93	74.25 (1, 56)	<.0001**
I-III	1.88	1.79	5.68 (1, 56)	0.0206*	-.001 (0.9220)	-0.023 (<.0001)**	11.77 (1, 492)	0.0007**	1.87	1.59	67.49 (1, 56)	<.0001**
III-V	2.06	1.95	3.04 (1, 56)	0.0868	-0.022 (0.0150)*	-0.027 (<.0001)**	0.30 (1, 491)	0.5859	1.86	1.70	7.22 (1, 56)	0.0095**
I-V	3.94	3.75	10.24 (1, 56)	0.0023**	-0.023 (0.0099)**	-0.050 (<.0001)**	6.05 (1, 491)	0.0143*	3.74	3.30	54.45 (1, 56)	<.0001**

* significant at p<.05 level
** significant at p<.01 level
Note. (df, df), degrees of freedom numerator, degrees of freedom denominator.

Table XX. Results of repeated measures analyses for ABR latency (ms) at p20 and p65, and ABR latency progression data (dB) for 16k Hz stimuli.

	(A) p20 Latency				(B) Latency progression/5 days				(C) p65 Latency			
16k	-/-	+/- +/+	F (df, df)	p value	-/- (p value)	+/- +/+ (p value)	F (df, df)	p value	-/-	+/- +/+	F (df, df)	p value
I	1.55	1.55	0.03 (1, 56)	0.8637	0.008 (0.0006)**	-.003 (0.0434)*	16 (1, 491)	<.0001**	1.62	1.52	44.48 (1, 56)	<.0001**
II	2.35	2.37	0.23 (1, 56)	0.6326	-0.002 (0.6794)	-0.004 (0.3027)	0.07 (1, 491)	0.7895	2.33	2.33	0.00 (1, 56)	0.9655
III	3.44	3.40	0.52 (1, 56)	0.4727	-0.015 (0.1126)	-0.020 (0.0036)**	0.17 (1, 491)	0.6774	3.31	3.23	2.02 (1, 56)	0.1610
IV	4.52	4.42	2.37 (1, 56)	0.1290	-0.014 (0.1569)	-0.041 (<.0001)**	4.89 (1, 491)	0.0275*	4.40	4.05	27.09 (1, 56)	<.0001**
V	5.37	5.33	0.42 (1, 56)	0.5206	-0.013 (0.2423)	-0.063 (<.0001)**	14.48 (1, 491)	0.0002**	5.26	4.76	47.32 (1, 56)	<.0001**
I-III	1.92	1.85	1.05 (1, 56)	0.3098	-0.023 (0.0241)*	-0.017 (0.0107)*	0.12 (1, 491)	0.7332	1.73	1.70	0.23 (1, 56)	0.6355
III-V	1.91	1.91	0.00 (1, 56)	0.9876	0.005 (0.6210)	-0.041 (<.0001)**	12.95 (1, 491)	0.0004**	1.96	1.55	35.79 (1, 56)	<.0001**
I-V	3.82	3.77	0.43 (1, 56)	0.5155	-0.020 (0.0480)*	-0.059 (<.0001)**	10.17 (1, 491)	0.0015**	3.64	3.24	35.03 (1, 56)	<.0001**

* significant at p<.05 level
** significant at p<.01 level
Note. (df, df), degrees of freedom numerator, degrees of freedom denominator.

Table XXI. Results of repeated measures analyses for ABR latency (ms) at p20 and p65, and ABR latency progression data (dB) for 32k Hz stimuli.

	(A) p20 Latency				(B) Latency progression/5 days				(C) p65 Latency			
32k	-/-	+/- +/+	F (df, df)	p value	-/- (p value)	+/- +/+ (p value)	F (df, df)	p value	-/-	+/- +/+	F (df, df)	p value
I	1.56	1.48	14.72 (1, 56)	0.0003**	0.021 (<.0001)**	0.009 (0.0001)**	7.98 (1, 490)	0.0049**	1.75	1.56	73.76 (1, 56)	<.0001**
II	2.32	2.33	0.02 (1, 56)	0.8807	0.014 (0.0027)**	0.001 (0.8125)	5.33 (1, 490)	0.0213*	2.54	2.33	13.86 (1, 56)	0.0005**
III	3.41	3.38	0.67 (1, 56)	0.4150	0.010 (0.1100)	-0.018 (<.0001)**	13.05 (1, 490)	0.0003**	3.50	3.21	48.48 (1, 56)	<.0001**
IV	4.49	4.38	4.22 (1, 56)	0.0447*	0.011 (0.1772)	-0.032 (<.0001)**	17.86 (1, 489)	<.0001**	4.59	4.08	84.84 (1, 56)	<.0001**
V	5.46	5.29	7.04	0.0104*	-0.003 (0.7237)	-0.044 (<.0001)**	12.47 (1, 490)	0.0005**	5.43	4.89	73.77 (1, 56)	<.0001**
I-III	1.84	1.90	2.93 (1, 56)	0.0924	-0.010 (0.0498)*	-0.028 (<.0001)**	7.82 (1, 490)	0.0054**	1.75	1.64	9.07 (1, 56)	0.0039**
III-V	2.04	1.91	5.55 (1, 56)	0.0220*	-0.011 (0.1501)	-0.024 (<.0001)**	1.55 (1, 490)	0.2131	1.93	1.69	20.03 (1, 56)	<.0001**
I-V	3.89	3.81	1.72 (1, 56)	0.1953	-0.024 (0.0070)**	-0.053 (<.0001)**	7.07 (1, 490)	0.0081**	3.67	3.33	33.45 (1, 56)	<.0001**

* significant at p<.05 level
** significant at p<.01 level
Note. (df, df), degrees of freedom numerator, degrees of freedom denominator.

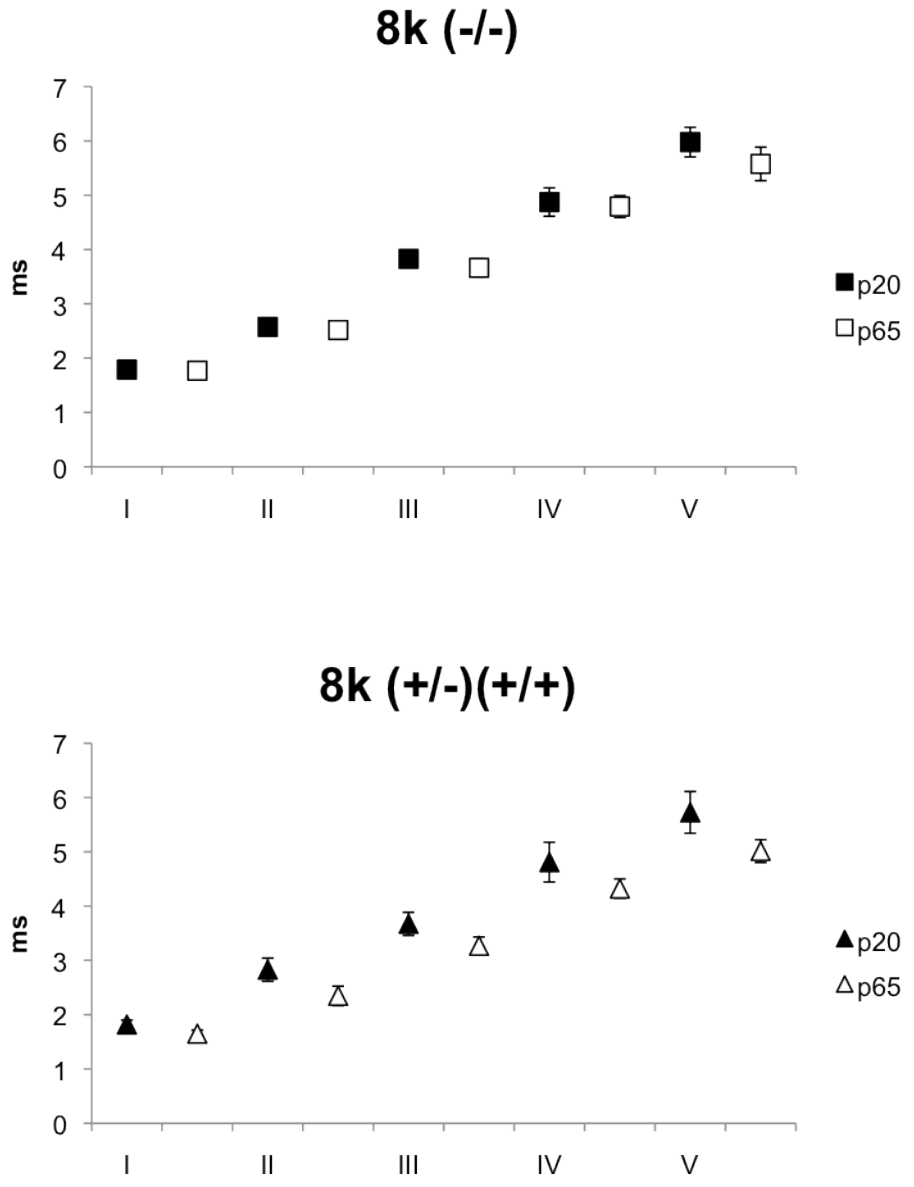


Figure 34. ABR mean (SD) absolute latency data at 8k Hz for mutant (top panel) and control (bottom panel) animals at p20 and p65.

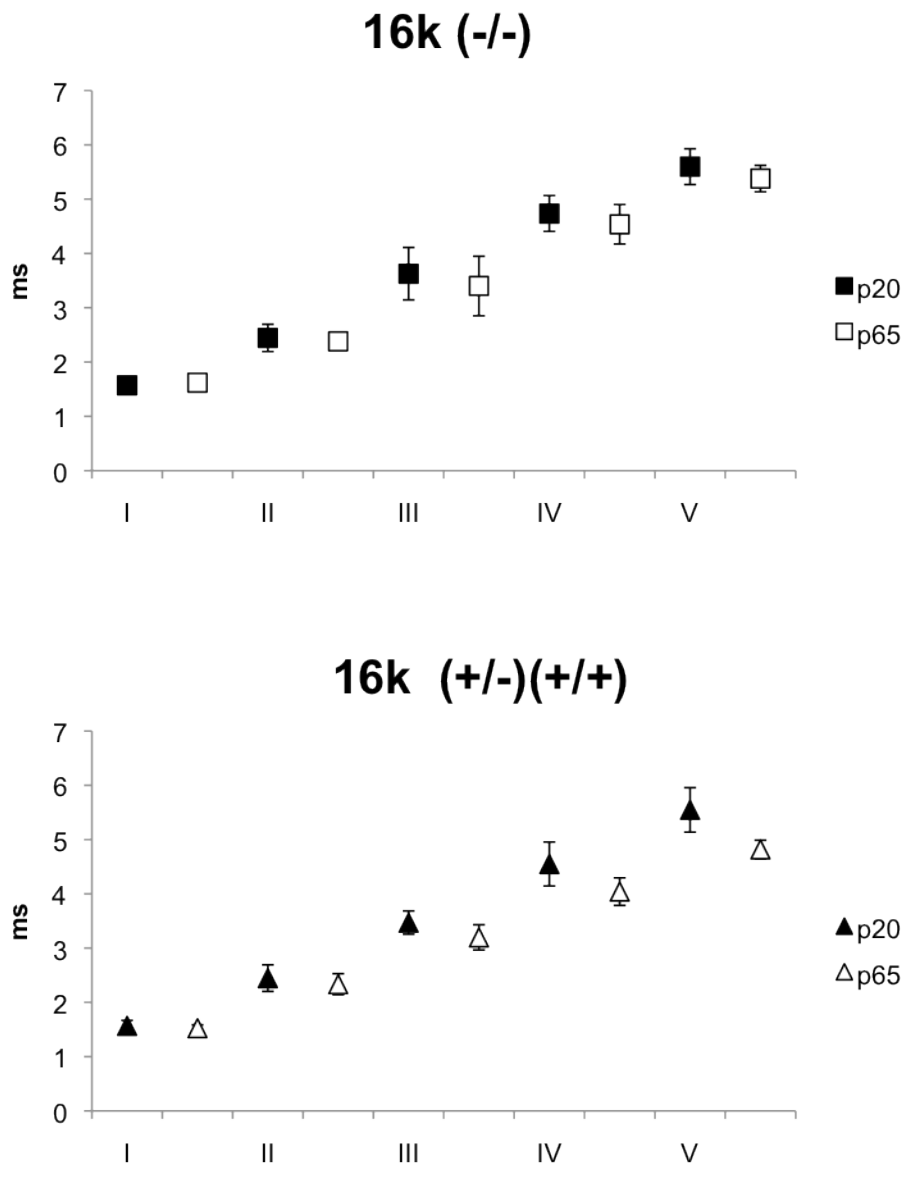


Figure 35. ABR mean (SD) absolute latency data at 16 kHz for mutant (top panel) and control (bottom panel) animals at p20 and p65.

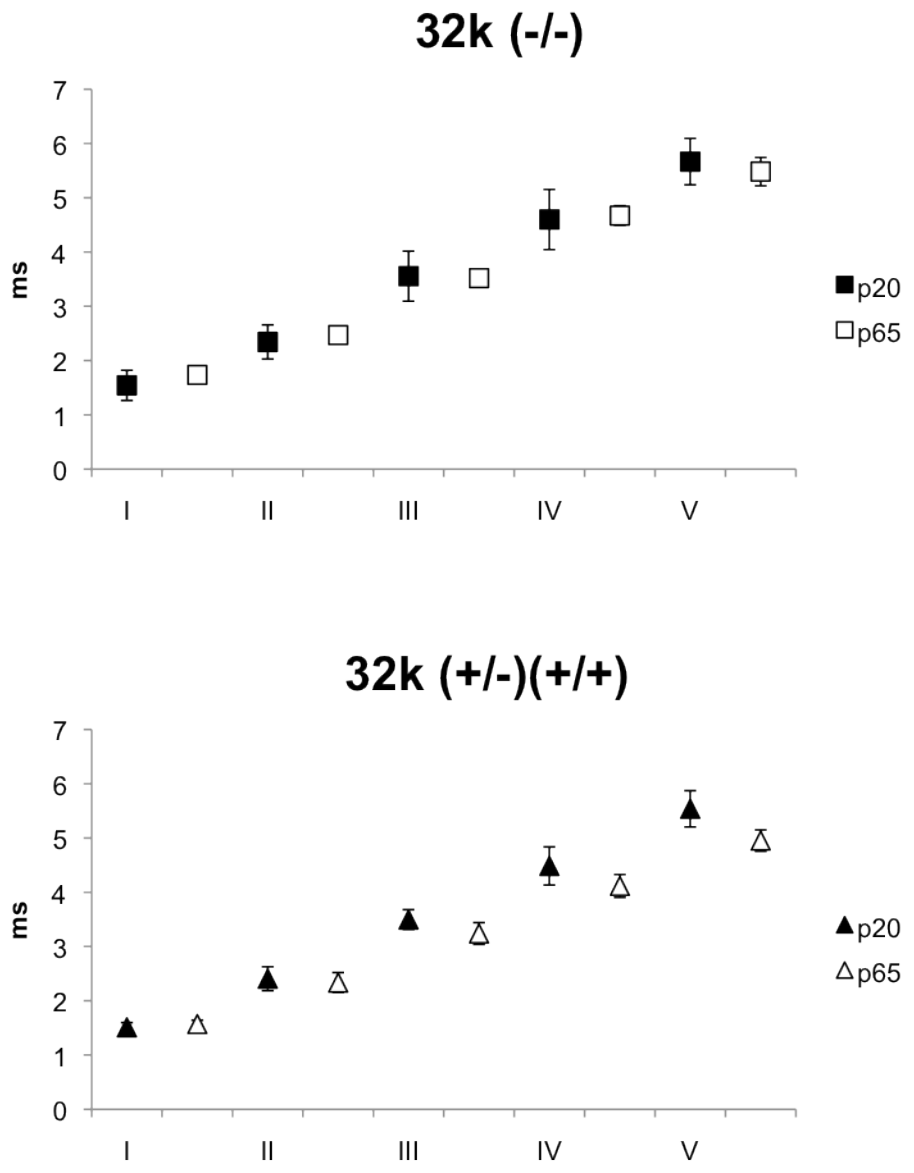


Figure 36. ABR mean (SD) absolute latency data at 32 kHz for mutant (top panel) and control (bottom panel) animals at p20 and p65.

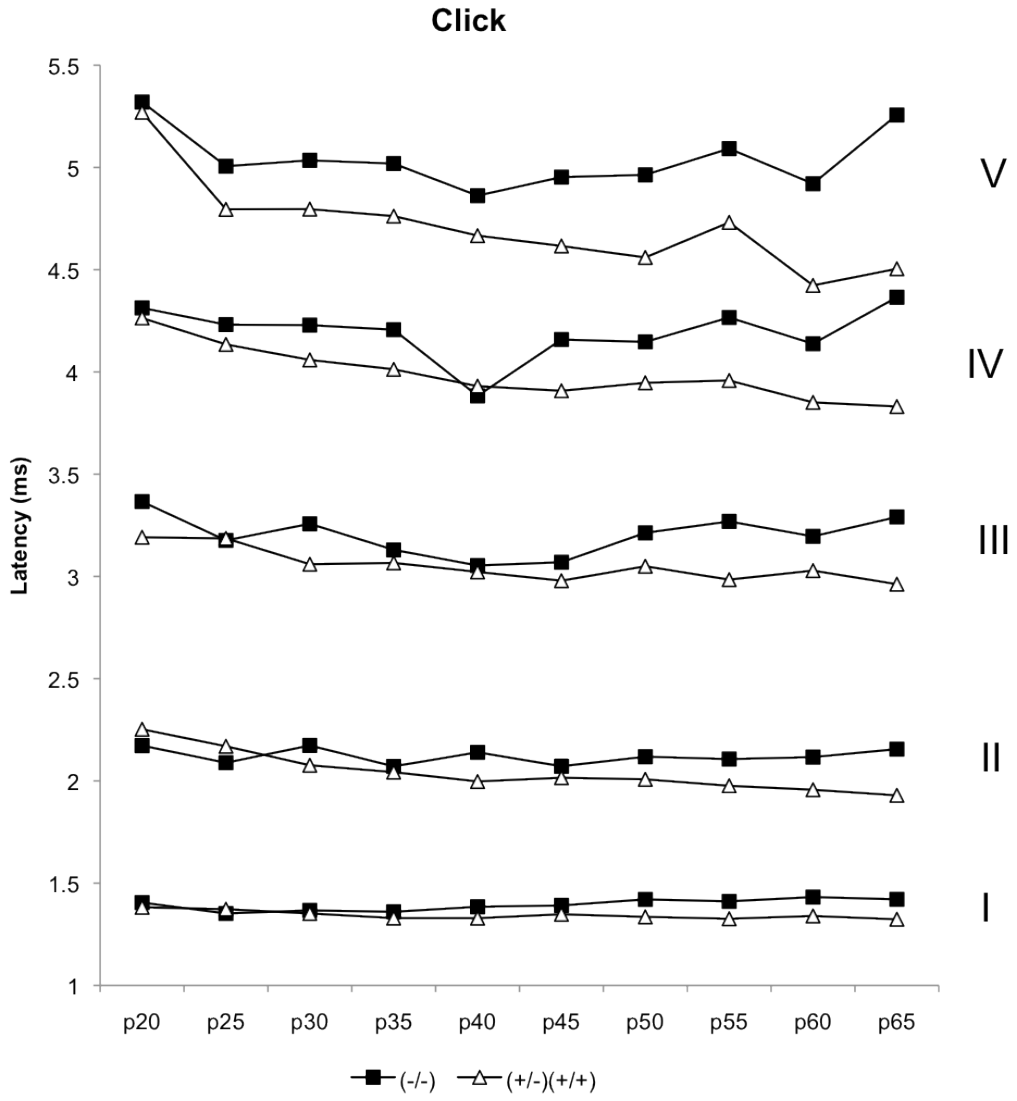


Figure 37. ABR mean latency data for mutant (-/-) and control (+/-, +/+) animals across the experimental lifetime for component waves I-V.

Effects of disease within gender were not observed consistently across waveform absolute or interpeak latency variables (Tables XXII, XXIII, XXIV, XXV); however, sporadic effects of disease within gender were identified. For the click stimulus (Table XXII), effects of disease within gender were observed for progression of waves III and IV and interpeak III-V, and at p65 for wave III and interpeak I-III. Effects of disease within gender were observed for 8k Hz stimuli (Table XXIII) at p20 for wave V, for progression of wave V and interpeak I-III and I-V, and at p65 for interpeak I-III. For 16k Hz stimuli (Table XXIV), effects of disease within gender were observed at p65 for wave III. Effects of disease within gender were observed for 32k Hz stimuli (Table XXV) at p20 for waves IV and V and interpeak III-V, for progression of waves I and II, and at p65 for wave II and interpeak I-III. Of the 18 variables at which an effect of disease was observed within one gender and not the other, 17 of these were identified effects of disease in male mice.

Table XXII. Results of repeated measures analyses examining effects of disease within gender and test time for ABR latency for click stimuli. Comparisons are between female and male mice with (-/-) and without (+/- +/+) disease.

	(A) p20 Latency				(B) Latency progression/5 days				(C) p65 Latency			
Click	Female		Male		Female		Male		Female		Male	
	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value
I	0.17 (1, 56)	0.6839	0.03 (1, 56)	0.8711	2.83 (1, 493)	0.0934	11.25 (1, 493)	0.0009**	10.26 (1, 56)	0.0022**	33.49 (1, 56)	<.0001**
II	2.67 (1, 56)	0.1081	0.20 (1, 56)	0.6593	7.66 (1, 493)	0.0058**	6.67 (1, 493)	0.0101*	8.68 (1, 56)	0.0047**	14.91 (1, 56)	0.0003**
III	0.13 (1, 56)	0.7208	0.98 (1, 56)	0.3258	0.33 (1, 492)	0.5672	8.68 (1, 492)	0.0034**	0.34 (1, 56)	0.5608	35.04 (1, 56)	<.0001**
IV	0.84 (1, 56)	0.3645	2.25 (1, 56)	0.1396	3.60 (1, 493)	0.0582	6.30 (1, 493)	0.0124*	5.17 (1, 56)	0.0268*	33.59 (1, 56)	<.0001**
V	0.05 (1, 56)	0.8302	2.40 (1, 56)	0.1271	5.65 (1, 493)	0.0178*	8.75 (1, 493)	0.0032**	14.07 (1, 56)	0.0004**	43.05 (1, 56)	<.0001**
I-III	0.07 (1, 56)	0.7962	0.98 (1, 56)	0.3270	1.00 (1, 492)	0.3171	5.53 (1, 492)	0.0190*	1.96 (1, 56)	0.1674	24.44 (1, 56)	<.0001**
III-V	0.11 (1, 56)	0.7440	1.06 (1, 56)	0.3079	9.26 (1, 492)	0.0025**	0.23 (1, 492)	00.6340	23.08 (1, 56)	<.0001**	3.48 (1, 56)	0.0672
I-V	0.07 (1, 56)	0.7861	2.55 (1, 56)	0.1159	4.58 (1, 493)	0.0329*	5.98 (1, 493)	0.0148*	10.91 (1, 56)	0.0017**	33.13 (1, 56)	<.0001**

* significant at p<.05 level
** significant at p<.01 level
Note. (df, df), degrees of freedom numerator, degrees of freedom denominator.

Table XXIII. Results of repeated measures analyses examining effects of disease within gender and test time for ABR latency for an 8k Hz stimulus. Comparisons are between female and male mice with (-/-) and without (+/- +/+) disease.

	(A) p20 Latency				(B) Latency progression/5 days				(C) p65 Latency			
8k	Female		Male		Female		Male		Female		Male	
	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value
I	1.13 (1, 56)	0.2933	0.21 (1, 56)	0.6449	10.61 (1, 492)	0.0012**	12.25 (1, 492)	0.0005**	17.92 (1, 56)	<.0001**	38.58 (1, 56)	<.0001**
II	5.05 (1, 56)	0.0286*	7.11 (1, 56)	0.0100*	7.14 (1, 492)	0.0078**	16.59 (1, 492)	<.0001**	4.66 (1, 56)	0.0352*	16.51 (1, 56)	0.0002**
III	2.42 (1, 56)	0.1253	1.97 (1, 56)	0.1655	9.75 (1, 492)	0.0019**	16.41 (1, 492)	<.0001**	46.15 (1, 56)	<.0001**	68.46 (1, 56)	<.0001**
IV	0.01 (1, 56)	0.9260	0.42 (1, 56)	0.5187	10.35 (1, 492)	0.0014**	14.53 (1, 492)	0.0002**	30.27 (1, 56)	<.0001**	50.73 (1, 56)	<.0001**
V	3.62 (1, 56)	0.0623	4.90 (1, 56)	0.0309*	3.58 (1, 491)	0.0592	8.27 (1, 491)	0.0042**	25.40 (1, 56)	<.0001**	50.34 (1, 56)	<.0001**
I-III	4.73 (1, 56)	0.0338*	1.51 (1, 56)	0.2245	3.72 (1, 492)	0.0545	8.40 (1, 492)	0.0039**	29.53 (1, 56)	<.0001**	38.05 (1, 56)	<.0001**
III-V	1.10 (1, 56)	0.2997	1.98 (1, 56)	0.1651	0.01 (1, 491)	0.9310	0.44 (1, 491)	0.5060	1.43 (1, 56)	0.2368	6.59 (1, 56)	0.0129*
I-V	5.25 (1, 56)	0.0258*	5.02 (1, 56)	0.0290*	1.55 (1, 491)	0.2132	4.85 (1, 491)	0.0282*	19.17 (1, 56)	<.0001**	36.20 (1, 56)	<.0001**

* significant at p<.05 level
** significant at p<.01 level
Note. (df, df), degrees of freedom numerator, degrees of freedom denominator.

Table XXIV. Results of repeated measures analyses examining effects of disease within gender and test time for ABR latency for a 16k Hz stimulus. Comparisons are between female and male mice with (-/-) and without (+/- +/+) disease.

	(A) p20 Latency				(B) Latency progression/5 days				(C) p65 Latency			
16k	Female		Male		Female		Male		Female		Male	
	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value
I	0.00 (1, 56)	0.9489	0.09 (1, 56)	0.7681	5.75 (1, 491)	0.0169*	10.48 (1, 491)	0.0013**	14.57 (1, 56)	0.0003**	31.02 (1, 56)	<.0001**
II	0.01 (1, 56)	0.9352	0.54 (1, 56)	0.4649	0.41 (1, 491)	0.5230	0.94 (1, 491)	0.3322	0.95 (1, 56)	0.3328	0.75 (1, 56)	0.3899
III	0.05 (1, 56)	0.8194	0.60 (1, 56)	0.4410	0.08 (1, 491)	0.7758	0.71 (1, 491)	0.4008	0.06 (1, 56)	0.8118	4.77 (1, 56)	0.0332*
IV	0.57 (1, 56)	0.4552	1.98 (1, 56)	0.1649	1.37 (1, 491)	0.2423	3.74 (1, 491)	0.0536	7.19 (1, 56)	0.0096**	21.42 (1, 56)	<.0001**
V	0.10 (1, 56)	0.7509	0.34 (1, 56)	0.5596	6.83 (1, 491)	0.0093**	7.65 (1, 491)	0.0059**	21.00 (1, 56)	<.0001**	26.36 (1, 56)	<.0001**
I-III	0.27 (1, 56)	0.6087	0.85 (1, 56)	0.3605	0.60 (1, 491)	0.4401	0.07 (1, 491)	0.7955	0.56 (1, 56)	0.4584	1.86 (1, 56)	0.1782
III-V	0.04 (1, 56)	0.8517	0.04 (1, 56)	0.8439	8.58 (1, 491)	0.0036**	4.77 (1, 491)	0.0294*	25.59 (1, 56)	<.0001**	11.89 (1, 56)	0.0011**
I-V	0.14 (1, 56)	0.7081	0.30 (1, 56)	0.5884	5.06 (1, 491)	0.0249*	5.11 (1, 491)	0.0242*	16.63 (1, 56)	0.0001**	18.40 (1, 56)	<.0001**

* significant at p<.05 level
** significant at p<.01 level
Note. (df, df), degrees of freedom numerator, degrees of freedom denominator.

Table XXV. Results of repeated measures analyses examining effects of disease within gender and test time for ABR latency for a 32k Hz stimulus. Comparisons are between female and male mice with (-/-) and without (+/- +/+) disease.

	(A) p20 Latency				(B) Latency progression/5 days				(C) p65 Latency			
32k	Female		Male		Female		Male		Female		Male	
	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value
I	7.11 (1, 56)	0.0100*	7.61 (1, 56)	0.0078**	1.07 (1, 490)	0.3018	8.46 (1, 490)	0.0038**	19.39 (1, 56)	<.0001**	58.69 (1, 56)	<.0001**
II	0.01 (1, 56)	0.9306	0.02 (1, 56)	0.9012	0.40 (1, 490)	0.5292	6.67 (1, 490)	0.0101*	0.93 (1, 56)	0.3383	17.78 (1, 56)	<.0001**
III	0.00 (1, 56)	0.9986	1.28 (1, 56)	0.2627	4.68 (1, 490)	0.0310*	8.57 (1, 490)	0.0036**	13.38 (1, 56)	0.0006**	37.58 (1, 56)	<.0001**
IV	0.12 (1, 56)	0.7304	6.22 (1, 56)	0.0156*	8.47 (1, 490)	0.0038**	9.39 (1, 490)	0.0023**	27.50 (1, 56)	<.0001**	59.64 (1, 56)	<.0001**
V	1.19 (1, 56)	0.2797	6.84 (1, 56)	0.0114*	7.00 (1, 490)	0.0084**	5.56 (1, 490)	0.0188*	30.36 (1, 56)	<.0001**	43.73 (1, 56)	<.0001**
I-III	3.26 (1, 56)	0.0765	0.42 (1, 56)	0.5187	4.20 (1, 490)	0.0409*	3.65 (1, 490)	0.0567	2.71 (1, 56)	0.1054	6.71 (1, 56)	0.0122*
III-V	1.40 (1, 56)	0.2409	4.49 (1, 56)	0.0385*	1.89 (1, 490)	0.1698	0.17 (1, 490)	0.6776	12.18 (1, 56)	0.0009**	8.17 (1, 56)	0.0060**
I-V	0.02 (1, 56)	0.8981	2.82 (1, 56)	0.0989	5.72 (1, 490)	0.0172*	1.96 (1, 490)	0.1623	17.00 (1, 56)	0.0001**	16.49 (1, 56)	0.0002**

* significant at p<.05 level

** significant at p<.01 level

Note. (df, df), degrees of freedom numerator, degrees of freedom denominator.

ABR Amplitude Findings. Results of repeated measures modeling for ABR amplitude data are included in table XXVI (click), table XXVII (8k Hz), table XXVIII (16k Hz), and table XXIX (32 Hz). See pages 116 and 117 for a detailed description of table formatting. Amplitude data are provided in microvolts. Positive progression estimates indicate an increase in amplitude, and negative values indicate a decrease in amplitude every five days.

For click and 8k Hz stimuli, both mutant and control animals exhibit progressive decrease in amplitude across most component peaks of the ABR over the experimental time period. While there are sporadic waveforms that are significantly different in amplitude between the two groups at both p20 and p65, a clear pattern is not apparent. That is, mutant mice have both smaller and larger amplitudes than control animals for various components of the ABR waveform. At 16k and 32k Hz, mutant and control animals both show a progressive decline in amplitude (i.e., decline in synchronized neural activity) over the experimental lifespan, but at each of these frequencies, mutant mice have smaller amplitude waveforms at most peaks both at p20 and p65. Notably, 16k and 32k Hz are the test frequencies with the greatest difference in hearing (ABR thresholds) between groups.

Table XXVI. Results of repeated measures analyses for ABR amplitude (μV) at p20 and p65, and ABR amplitude progression data (μV) for click stimuli.

	(A) p20 Amplitude				(B) Amplitude progression/5 days				(C) p65 Amplitude			
Click	-/-	+/- +/+	F (df, df)	p value	-/- (p value)	+/- +/+ (p value)	F (df, df)	p value	-/-	+/- +/+	F (df, df)	p value
I	3.48	4.22	8.95 (1, 56)	0.0041**	-0.178 (<.0001)**	-0.312 (<.0001)**	9.05 (1, 493)	0.0028**	1.88	1.42	3.46 (1, 56)	0.0682
II	1.31	0.85	16.32 (1, 56)	0.0002**	-0.044 (0.0109)*	-0.001 (0.9702)	4.19 (1, 492)	0.0412*	0.92	0.84	0.45 (1, 56)	0.5051
III	0.68	0.83	2.62 (1, 56)	0.1109	-0.036 (0.0068)**	-0.011 (0.2546)	2.34 (1, 491)	0.1265	0.36	0.74	17.58 (1, 56)	<.0001**
IV	0.83	0.71	1.70 (1, 56)	0.1977	-0.013 (0.3425)	-0.024 (0.0166)*	0.41 (1, 490)	0.5226	0.71	0.49	5.84 (1, 56)	0.0189*
V	1.09	1.11	0.07 (1, 56)	0.7992	-0.037 (0.0027)**	-0.029 (0.0013)**	0.30 (1, 492)	0.5826	0.76	0.85	1.41 (1, 56)	0.2408

* significant at p<.05 level
** significant at p<.01 level
Note. (df, df), degrees of freedom numerator, degrees of freedom denominator.

Table XXVIII. Results of repeated measures analyses for ABR amplitude (μV) at p20 and p65, and ABR amplitude progression data (μV) for 8k Hz stimuli.

	(A) p20 Amplitude				(B) Amplitude progression/5 days				(C) p65 Amplitude			
8k	-/-	+/- +/+	F (df, df)	p value	-/- (p value)	+/- +/+ (p value)	F (df, df)	p value	-/-	+/- +/+	F (df, df)	p value
I	3.76	3.76	0.00 (1, 56)	0.9770	-0.216 (<.0001)**	-0.286 (<.0001)**	2.53 (1, 492)	0.1120	1.82	1.18	6.72 (1, 56)	0.0122*
II	1.22	0.76	19.01 (1, 56)	<.0001**	-0.021 (0.1805)	-0.001 (0.9307)	1.06 (1, 485)	0.3028	1.03	0.75	7.31 (1, 56)	0.0090**
III	0.65	0.82	3.14 (1, 56)	0.0819	-0.046 (0.0010)**	-0.021 (0.0382)*	2.17 (1, 486)	0.1410	0.23	0.63	17.98 (1, 56)	<.0001**
IV	1.08	0.61	36.70 (1, 56)	<.0001**	-0.054 (<.0001)**	-0.016 (0.0495)*	7.06 (1, 487)	0.0081**	0.59	0.46	2.93 (1, 56)	0.0927
V	0.91	0.88	0.27 (1, 56)	0.6036	-0.046 (<.0001)**	-0.047 (<.0001)**	0.00 (1, 491)	0.9602	0.50	0.46	0.38 (1, 56)	0.5384

* significant at p<.05 level
** significant at p<.01 level
Note. (df, df), degrees of freedom numerator, degrees of freedom denominator.

Table XXX. Results of repeated measures analyses for ABR amplitude (μV) at p20 and p65, and ABR amplitude progression data (μV) for 16k Hz stimuli.

	(A) p20 Amplitude				(B) Amplitude progression/5 days				(C) p65 Amplitude			
16k	-/-	+/- +/+	F (df, df)	p value	-/- (p value)	+/- +/+ (p value)	F (df, df)	p value	-/-	+/- +/+	F (df, df)	p value
I	3.37	5.00	37.51 (1, 56)	<.0001**	-0.191 (<.0001)**	-0.369 (<.0001)**	14.04 (1, 490)	0.0002**	1.65	1.68	0.01 (1, 56)	0.9114
II	1.27	2.06	34.80 (1, 56)	<.0001**	-0.070 (0.0004)**	-0.128 (<.0001)**	5.56 (1, 490)	0.0188*	0.64	0.92	4.32 (1, 56)	0.0423*
III	0.63	0.85	5.98 (1, 56)	0.0177*	-0.021 (0.1181)	-0.025 (0.0075)**	0.09 (1, 486)	0.7697	0.44	0.62	4.03 (1, 56)	0.0494*
IV	0.63	0.64	0.00 (1, 56)	0.9708	-0.007 (0.5403)	-0.011 (0.1535)	0.11 (1, 490)	0.7351	0.57	0.53	0.29 (1, 56)	0.5964
V	1.00	1.14	3.10 (1, 56)	0.0836	-0.044 (0.0005)**	-0049 (<.0001)**	0.13 (1, 491)	0.7165	0.60	0.70	1.40 (1, 56)	0.2424

* significant at p<.05 level
** significant at p<.01 level
Note. (df, df), degrees of freedom numerator, degrees of freedom denominator.

Table XXXII. Results of repeated measures analyses for ABR amplitude (μV) at p20 and p65, and ABR amplitude progression data (μV) for 32k Hz stimuli.

	(A) p20 Amplitude				(B) Amplitude progression/5 days				(C) p65 Amplitude			
32k	-/-	+/- +/+	F (df, df)	p value	-/- (p value)	+/- +/+ (p value)	F (df, df)	p value	-/-	+/- +/+	F (df, df)	p value
I	2.05	3.77	84.39 (1, 56)	<.0001**	-0.141 (<.0001)**	-0.244 (<.0001)**	9.32 (1, 489)	0.0024**	0.78	1.57	18.30 (1, 56)	<.0001**
II	1.38	2.05	29.88 (1, 56)	<.0001**	-0.051 (0.0058)**	-0.089 (<.0001)**	2.75 (1, 489)	0.0976	0.92	1.25	7.68 (1, 56)	0.0076**
III	0.70	0.76	0.53 (1, 56)	0.4717	-0.034 (0.0056)**	-0.020 (0.0206)*	0.81 (1, 487)	0.3695	0.40	0.58	5.08 (1, 56)	0.0281*
IV	0.63	0.62	0.02 (1, 56)	0.8868	-0.017 (0.0693)	-0.010 (0.1588)	0.42 (1, 489)	0.5152	0.47	0.53	0.93 (1, 56)	0.3396
V	0.69	0.97	16.40 (1, 56)	0.0002**	-0.020 (0.0633)	-0.039 (<.0001)**	2.23 (1, 490)	0.1361	0.51	0.62	2.56 (1, 56)	0.1150

* significant at p<.05 level
** significant at p<.01 level
Note. (df, df), degrees of freedom numerator, degrees of freedom denominator.

Similar to the latency data, effects of disease within gender were not consistently observed across waveform amplitude variables. Sporadic effects of disease within gender were identified. For the click stimulus (Table XXX) effects of disease within gender were observed at p20 for waves I and III, for progression of wave I, and at p65 for wave IV. Effects of disease within gender were observed for 8k Hz stimuli (Table XXXI) for progression of wave IV, and at p65 for wave II. For 16k Hz stimuli (Table XXXII), effects of disease within gender were observed at p20 for wave III, and at p65 for waves II and III. Effects of disease within gender for 32k Hz stimuli (Table XXXIII) were observed at p65 for wave II. Of the 10 variables at which an effect of disease was observed within one gender and not the other, 9 of these were identified effects of disease in female mice.

Table XXX. Results of repeated measures analyses examining effects of disease within gender and test time for ABR amplitude for click stimuli. Comparisons are between female and male mice with (-/-) and without (+/- +/+) disease.

	(A) p20 Amplitude				(B) Amplitude progression/5 days				(C) p65 Amplitude			
Click	Female		Male		Female		Male		Female		Male	
	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value
I	6.61 (1, 56)	0.0128*	2.88 (1, 56)	0.0954	5.77 (1, 493)	0.0167*	3.50 (1, 493)	0.0619	1.71 (1, 56)	0.1965	1.75 (1, 56)	0.1912
II	6.65 (1, 56)	0.0126*	9.73 (1, 56)	0.0029**	1.85 (1, 492)	0.1743	2.34 (1, 492)	0.1264	0.11 (1, 56)	0.7443	0.38 (1, 56)	0.5418
III	7.52 (1, 56)	0.0082**	0.11 (1, 56)	0.7372	0.26 (1, 491)	0.6091	2.62 (1, 491)	0.1062	12.94 (1, 56)	0.0007**	5.60 (1, 56)	0.0214*
IV	0.03 (1, 56)	0.8699	2.68 (1, 56)	0.1073	0.49 (1, 490)	0.4834	0.05 (1, 490)	0.8281	1.84 (1, 56)	0.1809	4.19 (1, 56)	0.0453*
V	0.08 (1, 56)	0.7769	0.01 (1, 56)	0.9340	0.08 (1, 492)	0.7832	1.02 (1, 492)	0.3131	0.03 (1, 56)	0.8592	3.27 (1, 56)	0.0759

* significant at p<.05 level
** significant at p<.01 level
Note. (df, df), degrees of freedom numerator, degrees of freedom denominator.

Table XXXI. Results of repeated measures analyses examining effects of disease within gender and test time for ABR amplitude for an 8k Hz stimulus. Comparisons are between female and male mice with (-/-) and without (+/- +/+) disease.

	(A) p20 Amplitude				(B) Amplitude progression/5 days				(C) p65 Amplitude			
8k	Female		Male		Female		Male		Female		Male	
	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value
I	0.12 (1, 56)	0.7349	0.08 (1, 56)	0.7384	0.86 (1, 492)	0.3544	1.72 (1, 492)	0.1899	3.33 (1, 56)	0.0736	3.39 (1, 56)	0.0707
II	11.51 (1, 56)	0.0013**	7.82 (1, 56)	0.0071**	0.67 (1, 485)	0.4152	0.42 (1, 485)	0.5167	4.25 (1, 56)	0.0438*	3.14 (1, 56)	0.0819
III	2.23 (1, 56)	0.1406	1.06 (1, 56)	0.3083	1.39 (1, 486)	0.2395	0.84 (1, 486)	0.3598	11.85 (1, 56)	0.0011**	6.64 (1, 56)	0.0126*
IV	16.80 (1, 56)	0.0001**	19.91 (1, 56)	<.0001**	5.02 (1, 487)	0.0255*	2.37 (1, 487)	0.1241	0.15 (1, 56)	0.7038	4.00 (1, 56)	0.0505
V	0.14 (1, 56)	0.7118	0.14 (1, 56)	0.7145	0.28 (1, 491)	0.5962	0.18 (, 491)	0.6679	1.62 (1, 56)	0.2084	0.13 (1, 56)	0.7241

* significant at p<.05 level
** significant at p<.01 level
Note. (df, df), degrees of freedom numerator, degrees of freedom denominator.

Table XXXII. Results of repeated measures analyses examining effects of disease within gender and test time for ABR amplitude for 16k Hz stimulus. Comparisons are between female and male mice with (-/-) and without (+/- +/+) disease.

	(A) p20 Amplitude				(B) Amplitude progression/5 days				(C) p65 Amplitude			
16k	Female		Male		Female		Male		Female		Male	
	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value
I	18.62 (1, 56)	<.0001**	18.91 (1, 56)	<.0001*	5.17 (1, 490)	0.0234*	9.03 (1, 490)	0.0028**	0.44 (1, 56)	0.5112	0.22 (1, 56)	0.6389
II	26.27 (1, 56)	<.0001**	10.77 (1, 56)	0.0018**	3.48 (1, 490)	0.0628	2.20 (1, 490)	0.1390	4.35 (1, 56)	0.0415*	0.79 (1, 56)	0.3793
III	4.37 (1, 56)	0.0412*	1.93 (1, 56)	0.1698	0.00 (1, 486)	0.9817	0.15 (1, 486)	0.7031	4.36 (1, 56)	0.0414*	0.62 (1, 56)	0.4342
IV	0.63 (1, 56)	0.4306	0.49 (1, 56)	0.4871	0.78 (1, 490)	0.3763	0.14 (1, 490)	0.7097	0.47 (1, 56)	0.4942	0.01 (1, 56)	0.9331
V	0.98 (1, 56)	0.3258	2.21 (1, 56)	0.1426	0.38 (1, 491)	0.5378	0.01 (1, 491)	0.9331	0.00 (1, 56)	0.9704	2.78 (1, 56)	0.1013

* significant at p<.05 level
** significant at p<.01 level
Note. (df, df), degrees of freedom numerator, degrees of freedom denominator.

Table XXXIII. Results of repeated measures analyses examining effects of disease within gender and test time for ABR amplitude for 32k Hz stimulus. Comparisons are between female and male mice with (-/-) and without (+/- +/+) disease.

	(A) p20 Amplitude				(B) Amplitude progression/5 days				(C) p65 Amplitude			
32k	Female		Male		Female		Male		Female		Male	
	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value	F (df, df)	p value
I	48.73 (1, 56)	<.0001**	36.54 (1, 56)	<.0001**	4.46 (1, 489)	0.0352*	4.86 (1, 489)	0.0279*	12.75 (1, 56)	0.0007**	6.29 (1, 56)	0.0150*
II	27.50 (1, 56)	<.0001**	6.61 (1, 56)	0.0128*	2.29 (1, 489)	0.1312	0.73 (1, 489)	0.3932	7.68 (1, 56)	0.0075**	1.41 (1, 56)	0.2395
III	0.23 (1, 56)	0.6325	0.29 (1, 56)	0.5896	0.80 (1, 487)	0.3730	0.15 (1, 487)	0.6948	3.96 (1, 56)	0.0515	1.49 (1, 56)	0.2279
IV	0.78 (1, 56)	0.3810	1.06 (1, 56)	0.3071	0.11 (1, 489)	0.7386	1.48 (1, 489)	0.2250	0.11 (1, 56)	0.7415	1.03 (1, 56)	0.3149
V	10.61 (1, 56)	0.0019**	6.22 (1, 56)	0.0156*	2.05 (1, 490)	0.1530	0.49 (1, 490)	0.4840	0.79 (1, 56)	0.3778	1.86 (1, 56)	0.1781

* significant at p<.05 level
** significant at p<.01 level
Note. (df, df), degrees of freedom numerator, degrees of freedom denominator.

Summary and Discussion

Mutant NPC mice were found to have evidence of peripheral and retrocochlear auditory dysfunction when compared to findings from control animal littermates. DPOAE findings support a cochlear contribution to the hearing loss. At p20, mutant animals had significantly lower DPOAE levels compared to control animals at all but two test frequencies. Because of limitations of the equipment, DPOAEs could only be measured up to 10641 Hz, which is lower in frequency than the hearing loss exhibited by ABR measures of threshold. This significant difference in DPOAE level persisted across the experimental lifespan such that by p65 mutant mice continued to have lower level 2f1-f2 distortion products.

Both groups demonstrated progressive increases in DPOAE level from p20 to p65, although the effect was larger and across a broader frequency range for control animals. Such an increase in DPOAE level with age suggests continued postnatal maturation occurring in the cochlea through at least p65 in this background strain. Although no developmental data have been published on the BALB/c mouse at this early age, a progressive increase in DPOAE level has been reported in the C57BL/6J background strain from p9, at which point no DPOAEs could be recorded, through p28 where they reach adult-like levels (Narui, et al., 2009). Similar postnatal development in DPOAE level has been reported in the rat and gerbil (e.g., Lenoir & Puel, 1987; Norton, Bargones, & Rubel, 1991). In the C57BL/6J strain, development occurred first and was greatest for lower frequencies collected (8k and 20k Hz), although the

investigators did not test at frequencies below 8k Hz. Similarly, the developmental changes observed in both the mutant and control animals in the current study were larger in the lower frequencies (< 7k Hz).

When considering the tonotopic nature of the cochlea, these data indicate the basal region may be more developmentally stable in control animals in this background strain, and that medial and apical regions are susceptible to developmental changes in either the mechanical properties of the basilar membrane and/or the electromotility of the outer hair cells during postnatal development (Long & Tubis, 1988; Brown, McDowell, & Forge, 1989). That mutant mice did not show as much increase in DPOAE level in low frequencies, and no increase in the higher frequencies, provides support that NPC disease pathogenesis may disrupt the maturational processes of the developing auditory system in mice. While DPOAE data support a cochlear site of lesion, histology of the inner ears of these animals will be necessary to confirm this assertion.

High frequency hearing loss is present in mutant *NPC1* (-/-) mice by postnatal day 20 (Figure 31). Specifically, ABR thresholds are significantly elevated at 16k Hz and 32 kHz. This is weeks before diseased mice become otherwise overtly affected by exhibiting neurological symptoms (e.g., ataxia, tremor, dystonia). While it was hypothesized that mutant mice would have high frequency hearing loss, given the progressive nature of the disease, it was expected that the hearing loss would become evident at a later point in the lifespan. The observation of significant hearing loss in all of the mutant animals within the first three weeks of life suggests either premature deterioration of the

auditory system or developmental dysfunction. Longitudinal data confirm that the hearing loss is progressive in mutant mice at all test stimuli (click, 8k, 16k Hz) except for 32k Hz, where they have hearing loss present as early as p20.

Non-mutant control animals (+/-, +/+) underwent a progressive, age-related decline in ABR threshold at 32k Hz, but not at lower test frequencies. The BALB/c background strain is known to undergo age-related decline in hearing as early as p50. Willott, et al. (1998) noted an elevation in 24k Hz ABR thresholds of BALB/c mice compared to a C57 strain as early as 50 days of age. The investigators speculated that even at this early age BALB/c mice do to not appear to hear high frequencies well, although they did not test above 24k Hz. The current data suggest that age-related decline in high frequency hearing (32k Hz) can begin much earlier than what was previously reported in BALB/c mice.

It was hypothesized that mutant animals would exhibit evidence of retrocochlear pathology on their ABR findings, much like humans with NPC. Furthermore, because the human analysis that sought to uncover progressive deterioration in the ABR was inconclusive, perhaps, in part, because of too short an experimental window, it was anticipated that these progressive changes could be observed in mutant mice.

The ABR latency analysis and comparison between mutant and control animals revealed no significant differences in absolute and interpeak latencies for click and 16k Hz stimuli at p20. For those ABR latencies at 8k and 32k Hz where there was a significant difference in latency observed, mutant mice almost always (8/9 variables) had longer latencies than control animals. While differences in

early components of the waveform (e.g., waves, I, II, and III) can be explained by hearing loss in mutant mice, prolongation in later waves (e.g., wave V) and interpeak latencies (e.g., III-V) cannot be explained by peripheral hearing, and suggests retrocochlear involvement. In general, interpeak latency components of the ABR are unaffected by pure conductive and cochlear hearing loss, and are thought to reflect a delay in brainstem transmission time through the auditory brainstem.

Mutant mice had significantly longer ABR absolute and interpeak latencies than control animals by p65. Given the progressive nature of the disease, it was hypothesized that mutant animals would undergo a significant prolongation in ABR latencies, perhaps in part because of changes in peripheral hearing, but also because of retrocochlear deterioration. The difference in latency between groups at p65 was not a reflection of a prolongation in latencies for the mutant group, however, but rather a significant shortening in control-animal absolute and interpeak latency. In general, ABR peak latencies of the mutant animals remained stable and, in the few cases where they did change significantly, most involved prolongation of absolute latencies of early peaks and a concomitant decrease in interpeak latencies.

Developmental changes in ABR latency in mice have not been reported previously in the literature. While the onset of the ABR (thresholds) has been documented in C57BL/6J mice (Narui, et al., 2009), and saturates at approximately 14 days, it is not clear what, if any, developmental changes may occur in individual peak latencies. It would appear from these data that in the

BALB/c background strain, postnatal maturational effects on the ABR do occur, and that some aspect of NPC pathogenesis halts this development in affected mice.

Postnatal maturation of the ABR has been well documented in other mammals, including humans. In the human model, ABRs exhibit changes during at least the first 18 months of life (e.g., Gorga et al., 1987, 1989), which justifies different clinical normative data for this population (for review see Hall, 1992). At birth, the ABR waveform is incomplete, typically missing waves II and IV and sometimes wave III, and characterized by prolonged interpeak latencies (I-III, III-V, I-V). During the first two years of life, as additional component peaks emerge, waves III and V become progressively shorter in latency. Evidence suggests that in humans, the peripheral auditory system matures faster than the central nervous system (e.g., Montandon, Cao, Engel, & Grajew, 1979), which explains why wave I is more prominent in newborns than adults. Prolongations in interpeak latency are purported to reflect an immature central nervous system, specifically incomplete nerve myelinization, reduced axon diameter, and immature synaptic function (e.g., Schwartz, et al., 1989) all of which are believed to develop postnatally.

It would appear that a similar maturation is taking place in the auditory system of the BALB/c mouse where almost every component peak of the ABR exhibited significant progressive shortening across the experimental lifespan. That the maturation effect was larger for later-occurring waves is supported by the knowledge that, in humans, the peripheral system matures faster than the central

auditory nervous system. In general, however, this effect was absent in mutant animals, lending further support to a disruption in the development of the auditory system in diseased mice. ABR abnormalities reported here are consistent with the histological auditory brainstem pathology identified in 8-week-old NPC mutant mice by Luan et al. (2008). Specifically, the investigators documented lower neural density in the cochlear nucleus and a proliferation of astrocytes in the inferior colliculus and medial geniculate nucleus of diseased animals. In order to definitively link the abnormal histology reported by these investigators with the auditory dysfunction shown in the current data, future studies examining histology and auditory function in the same animals are necessary.

ABR amplitude findings showed no evidence of a clear effect of disease in the lower test frequencies, but did reveal smaller amplitudes in mutant mice, primarily in the early ABR peaks, at 16k and 32k Hz, which is consistent with the documented high frequency hearing loss. Amplitude of the ABR depends on both the number of neurons activated by an incoming stimulus, and their synchronization when firing. Cochlear hearing loss, especially in the high frequencies, is known to affect both aspects of neural activation and is often reflected by decreased amplitude in the early components of the ABR in humans (e.g., Picton, Woods, Baribeau-Braun, & Healey, 1977). The ABR amplitude data presented here also reflects evidence of maturational change in the waveform. Both murine groups demonstrated significant decline in waveform amplitude across the experimental lifespan for all stimuli.

Effects of gender on DPOAE and ABR findings were not significant in most analyses, although the few effects that were identified in DPOAE level and ABR latency progression implicated a greater effect of disease on male mice than female mice. Gender differences are not considered part of the phenotype for NPC, however Võikar, et al. (2002) observed that in several measures of cognitive function and motor impairment, male NPC (-/-) mice were more affected than females. Similar gender differences have been reported elsewhere in other neurodegenerative mouse models with cerebellar involvement in which female mutant mice were less affected neurologically and showed less deterioration in their neuroanatomy than their male counterparts (Doulazmi, et al, 1999; Henderson, et al., 2000; Ogura, Matsumoto, Mikoshiba, 2001). Anecdotally, the female mutant mice in the current study showed more exploratory activity and less weight loss near the end of the experimental lifespan than male mice, but the minimal effects observed on hearing are not sufficiently robust to confirm an overall effect of gender.

CHAPTER 4: COMPREHENSIVE DISCUSSION AND SUMMARY

The two experiments described in this document used a comparative, translational approach to evaluate comprehensively the auditory phenotype associated with NPC. Although limited literature suggested that hearing loss may be related to the disease, the degree, progression, and site of lesion associated with the possible hearing loss in NPC have not been reported. In the current study, overall findings confirm widespread auditory dysfunction is part of the disease process of NPC in both humans and the (BALB/cNctr-*Npc1*^{m1N}/J) mouse model. This has valuable implications for researchers seeking to understand better the natural history of the disease, and clinicians and families who aim to provide the best quality of care for their patients and loved ones.

Results of experiment one confirm a pervasive high frequency hearing loss that is sensorineural in nature in a large proportion of patients with NPC. It is not possible to determine the precise onset of hearing loss in the human population, although data from the current study suggest it may vary. Nonetheless, this was a predominately pediatric population in whom high frequency hearing loss was common. High frequency hearing loss in children, in the absence of noise exposure, can result from several non-hereditary etiologies, including, but not limited to: ototoxicity, toxoplasmosis, congenital syphilis, Rh-incompatibility, cytomegalovirus, and other herpes simplex viruses. While hearing loss in a child is unexpected, it is unlikely that early onset high frequency hearing loss alone can serve as an early indication of NPC. The combination of hearing loss and hepatic dysfunction may steer the observant clinician toward a lysosomal disorder (e.g.,

mucopolysaccharidosis) and more awareness on the auditory manifestations associated with NPC may result in its inclusion in this list. However, this association is further confounded by evidence that not all patients with NPC in this study exhibited hearing loss.

While the heterogeneity and small size of the sample made it difficult to identify variables, such as gender and age at disease onset, that may contribute to hearing loss in NPC, results among patients were consistent sufficiently to point to a mixed (cochlear and retrocochlear) site of lesion in most individuals, with some patients exhibiting a profile for auditory neuropathy spectrum disorder (ANSD). However, not all patients exhibited the same degree of impairment or site of lesion suggesting a possible confounding influence of genetic and environmental modifiers. There are over 240 disease-causing mutations associated with NPC (Runz, 2009). This fact alone limits significantly the ability to determine phenotype-genotype correlations in a human population with NPC; however, it is probable that such molecular heterogeneity contributes to the large variability in auditory function observed in the current study.

Cross-sectional data, in combination with several case examples from individuals who provided longitudinal behavioral data, support a progressive decline in hearing in at least some individuals. These data represent the first conclusive report of progressive auditory dysfunction in patients with NPC and suggest the auditory system is vulnerable to disease-related pathological insult across the lifetime.

Five late-onset cases of the disease were evaluated separately because of their unique audiological profiles. All of these patients presented with hearing loss, and in at least three cases, hearing loss was a premonitory symptom of the disease. Although the sample size is small, in conjunction with other reports of hearing loss as an early manifestation of this variant (Sévin, et al., 2007), this finding seems sufficiently robust to conclude that NPC should be considered in the differential diagnosis of all patients with subtle neurological involvement (e.g., learning delay) and idiopathic hearing loss.

Auditory dysfunction has been reported in several other lysosomal storage diseases that affect children, including Gaucher disease type 3 (Bamiou, et al., 2001), Fabry disease (Palla, et al., 2007; Ries, et al., 2007), mucopolysaccharidoses (e.g., Schachern, et al., 2007) and Pompe disease (Capelle, et al., 2010); however none of these disorders affects the auditory system in ways similar to NPC. With the exception of limited evidence for a mixed site of lesion in Pompe disease, the phenotypes associated with these disorders do not include robust evidence for cochlear and retrocochlear involvement such as that which has been described in the current study on NPC. The auditory system may have a useful role in distinguishing NPC from other lysosomal storage diseases early in the diagnostic process before cytological and biochemical assessment can occur.

Results of experiment two identify for the first time auditory dysfunction in the mouse model for NPC disease. High frequency hearing loss is present at least as early as p20, and thresholds progressively deteriorate for all test stimuli

(click, 8k, 16k, 32k Hz) across the experimental lifespan to p65. DPOAE data support a cochlear contribution to the hearing loss, although future histology will be necessary to confirm this hypothesis. Maturational change in control animals was observed for both DPOAE 2f1-f2 level and for ABR latencies, which progressively increased and shortened, respectively. These DPOAE and ABR changes typically did not take place in mutant mice, or occurred to a significantly smaller degree, suggesting a halt in the developmental processes of the auditory system in diseased animals.

The principal biochemical defect in NPC is an over-accumulation of exogenous and unesterified cholesterol throughout the body, most notably in the central nervous system and visceral organs. The relationship between over-accumulation of cholesterol and hearing has been examined, but remains unclear. In humans, inconclusive evidence has been offered to suggest a correlation between elevated blood cholesterol and triglyceride levels (hyperlipidemia) with sensorineural hearing loss (e.g., Evans, et al., 2006). Detrimental effects from hyperlipidemia on DPOAE amplitude (Preyer, Baisch, Bless, & Gummer, 2001) and pure-tone thresholds have also been reported, with speculation that the effect may be greater at the basal and high frequency test region where the cochlea is more susceptible to ischemic change (Cunningham & Goetzinger, 1974). Similar harmful outcomes of hyperlipidemia on hearing and cochlear morphology have been shown in mice (Guo, Zhang, Du, Nair, & Yoo, 2005) and guinea pigs, although short-term dietary changes (influx or reduction in high-fat diet) have not been shown to affect hearing (Evans, et al., 2006). Dietary control of cholesterol

intake and the use of pharmacologic statins to reduce the production of cholesterol have not been effective in treating NPC. It is unlikely the underlying cause of hearing loss associated with hyperlipidemia and NPC is the same, but it does implicate a role for cholesterol and other lipids in the auditory system, and suggests their function may be widespread and complex.

Cholesterol is a critical component in regulating mammalian plasma membrane properties such as lateral mobility, permeability, and stiffness (Organ & Raphael, 2009) among other important functions. Within the cochlea, cholesterol regulates lipid composition, mobility, and stiffness of the lateral walls of the outer hair cells (Evans, et al., 2006). Rajagopalan, et al. (2007) showed that changes in cochlear cholesterol levels modulated the amplitude of DPOAEs in mice. By altering cholesterol in the outer hair cell wall, the investigators were able to show a relationship between membrane cholesterol levels and the membrane protein prestin, which regulates motility of the sensory cells, thereby indirectly affecting outer hair cell tuning. When cholesterol was depleted in the outer hair cells, hearing loss was evident by a reduction in DPOAE level. Adding cholesterol back to the cells initially improved hearing, but was followed by a decrease in hair cell electromotility and DPOAE level. The investigators showed also a decrease in the amount of membrane cholesterol during maturation of the OHCs, and they concluded this normal reduction during the process of maturation helps to tune the sensory cells to function maximally. Levic and Yamoah (2011) observed a similar maturational effect from cholesterol within cochlear outer hair cells. Specifically, cholesterol was a critical component in determining the

magnitude of voltage-gated potassium currents within outer hair cells, and this effect was only important in developing but not mature cells.

These recent reports highlighting the critical role of cholesterol in developing hair cells may explain why DPOAEs from mutant NPC mice in the current study did not achieve levels consistent with their control littermates during the maturation process. It is possible disease-related alterations in the processing of cellular cholesterol prohibit the normal reduction in cholesterol necessary for cells to completely mature. As unesterified cholesterol, sphingolipids, and gangliosides accrue within cells throughout the disease process, further detrimental effects on cellular structure and function are likely, and may manifest as progressive declines in auditory function. This hypothesis applies to both cochlear cells, and lipid-dense areas of neural tissue, including the myelinated auditory nerve, that may account for both the cochlear and retrocochlear dysfunction observed in all NPC mice and most humans in the current study.

The auditory phenotype observed in humans with NPC and mutant mice is relatively consistent, although not completely parallel. All mutant mice exhibited early-onset high-frequency hearing loss, cochlear and retrocochlear dysfunction, evidence of a disruption in cochlear and auditory brainstem maturation, and progression of hearing loss. High frequency hearing loss was common in humans with NPC, as was a mixed (cochlear and retrocochlear) site of lesion. Progressive hearing loss also was evident in some individuals; however, not all patients with NPC exhibited auditory dysfunction. This variability between a heterogeneous, outbred, human population and a homogeneous, environmentally controlled,

inbred species is not surprising. That auditory dysfunction was consistently observed in all NPC mice and a majority of patients with NPC leaves little doubt, however, that NPC pathology can detrimentally affect the auditory system.

High frequency hearing loss is an early-onset manifestation of the disease in NPC mice that occurs weeks prior to other observable neurological symptoms (e.g., ataxia). It is unclear when hearing loss in humans with NPC firsts manifests. This is a difficult question to answer, in part because of the variability in the auditory phenotype observed, and also because many patients (23 in the current study) were unaware of their hearing loss, suggesting the hearing loss was present for some time and simply undetected. It seems likely that the auditory system can serve as a useful clinical marker for disease onset and progression in the mouse model of NPC where a robust phenotype was observed. The combination of auditory data, such as those reported in the current study, and auditory histology will have important implications for understanding better the pathophysiology of the disease and the role of *NPC1* in the auditory system. The early onset of the auditory phenotype and evidence for disruption of auditory maturation provides new insights into understanding disease behavior and progression, and offers a useful tool for evaluating the efficacy of future therapeutic interventions. The BALB/cNctr-*Npc1*^{m1N}/J strain of mice has been described as a model for severe human NPC disease, and alternate murine models are under development (Madra & Sturley, 2010). The current study supports exploration of auditory function in any future models of NPC disease.

Data presented here represent the largest cohort of patients with NPC evaluated comprehensively for auditory dysfunction, and for the first time explore auditory manifestations in the (BALB/cNctr-*Npc1*^{m1N}/J) mouse model. Taken together, these data implicate the pathological processes of NPC in the manifestation of hearing loss and auditory dysfunction. It is important that clinicians and researchers be aware of the involvement of the auditory system, which has historically has been an overlooked component of the disorder, as hearing loss in some patients will affect daily communication. Patients with NPC should be routinely monitored for hearing loss throughout their lives from the time of diagnosis. Clinicians should be aware of the obstacles that may exist in obtaining comprehensive behavioral evaluations in some patients with NPC and the value of electrophysiological or other objective measures of auditory system integrity in the evaluation of hearing. Emphasis should be placed on the collection of high-frequency information, as even minimal hearing loss in this region can have deleterious effects on social and academic progress. Families should be counseled regarding the implications for communication involved with cochlear hearing loss and disorders of the auditory nerve, most notably difficulty listening in background noise and in the absence of visual cues.

While the data presented here are comprehensive, there are several questions that remain. In the human population with NPC, will ABR data ultimately show progressive deterioration and, if so, what is the time course of such change? It is unlikely, given the heterogeneity of the sample and the large variation in patient ability for participation in longitudinal behavioral monitoring,

that the auditory system will serve independently as a clinical marker, for example, in future therapeutic trials. However, enough data have been presented here to suggest that the absence of hearing loss or the lack of progression in some patients may well indicate a potential therapeutic benefit from intervention in some patients with NPC. It seems more likely, however, that auditory function in the mouse model will serve sooner as a guide for intervention efficacy. To elucidate further the pathogenesis of the findings presented here and perhaps understand better the effect of potential treatments, histology of the cochlear and auditory brainstem pathways will be a necessary component of future work with the mouse model.

List of Appendices

Appendix A

Glossary: All definitions were obtained from Stedman's Medical Dictionary, 27th Edition, Baltimore.

Ascites: Abnormal accumulation of fluid in the abdomen

Anamnestic: 1. Assisting the memory 2. Relating to the medical history of a patient

Astrocyte: One of the large neuroglia cells of the nervous tissue. SEE ALSO neuroglia

Ataxia: An inability to coordinate muscle activity during voluntary movement; most often due to disorders of the cerebellum or the posterior columns of the spinal cord; may involve the limbs, head, or trunk

Cataplexy: A transient attack of extreme generalized weakness, often precipitated by an emotional response, such as surprise, fear, or anger; one component of the narcolepsy quadrad

Cholestasis: An arrest in the flow of bile; c. due to obstruction of bile ducts is accompanied by formation of plugs of inspissated bile in the small ducts, canaliculi in the liver, and elevation of serum direct bilirubin and some enzymes.

Complementation: Interaction between two genetic units, one or both of which are defective, permitting the organism containing these units to function normally, whereas it could not do so if either unit were absent.

Cytological: Relating to cytology

Cytology: They study of the anatomy, physiology, pathology, and chemistry of the cell. SYN Cellular biology, cytobiology

Dysarthria: A disturbance of speech due to emotional stress, to brain injury, or to paralysis, incoordination, or spasticity of the muscles used for speaking

Dysphagia: Difficulty in swallowing

Dystonia: A state of abnormal (either hypo- or hyper-) tonicity in any of the tissues resulting in impairment of voluntary movement

Esterification: The process of forming an ester, as in the reaction of ethanol and acetic acid to form ethyl acetate

Ganglioside: A glycosphingolipid chemically similar to cerebrosides but containing one or more sialic acid residues; found principally in nerve tissue, spleen, and thymus

Hepatosplenomegaly: Enlargement of the liver and spleen

Hypotonia: A condition in which there is diminution or loss of muscular tonicity

Lipid: "Fat-soluble" an operational term describing a solubility characteristic, not a chemical substance, i.e., denoting substances extracted from animal or vegetable cells by nonpolar solvents; included in the heterogeneous collection of materials thus extractable are fatty acids, glycerides and glyceryl ethers, phospholipids, sphingolipids, long-chain alcohols and waxes, terpenes, steroids, and "fat-soluble" vitamins such as A, D, and E

Lysosome: A cytoplasmic membrane-bound vesicle measuring 5-8 nm and containing a wide variety of glycoprotein hydrolytic enzymes active at an acid pH; serves to digest exogenous material, such as bacteria, as well as effete organelles of the cells

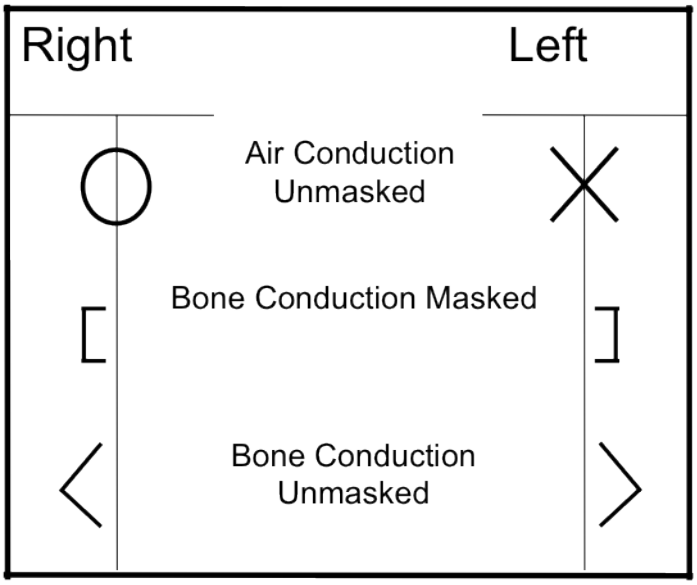
Neuroglia: Non-neuronal cellular elements of the central and peripheral nervous system; formerly believed to be merely supporting cells but now thought to have important metabolic functions, since they are invariably interposed between neurons and the blood vessels supplying the nervous system. In central nervous tissue they include oligodendroglia cells, astrocytes, ependymal cells, and microglia cells. The satellite cells of ganglia and the neurolemmal or Schwann cells around peripheral nerve fibers can be interpreted as the oligodendroglia cells of the peripheral nervous system

Pleiotropy: Production by a single mutant gene of apparently unrelated multiple effects at the clinical or phenotypic level

Sphingolipid: Any lipid containing a long-chain base like that of sphingosine; a constituent of nerve tissue

Unesterified: see esterification

Appendix B


Right
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Air Conduction
Unmasked
Bone Conduction Masked
Bone Conduction
Unmasked

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