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Role of alpha synuclein in noise induced hearing loss

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**ROLE OF ALPHA SYNUCLEIN IN NOISE INDUCED HEARING
LOSS**

by

Melissa Ellen Mooney

**A Capstone Project
submitted in partial fulfillment of the
requirements for the degree of:**

Doctor of Audiology

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Program in Audiology and Communication Sciences**

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Abstract: The recent discovery of the contribution of alpha synuclein in the auditory system prompted further investigation of its functional role. Auditory brainstem response (ABR) and gap detection testing were completed on wild-type and transgenic M83 mice to assess the role of alpha synuclein in noise-induced hearing loss and central auditory function.

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ABBREVIATIONS

A53T	point mutation in alpha synuclein
ABR	Auditory Brainstem Response
APD	Auditory Processing Disorder
BCSG-1	breast cancer specific gene-1
CSP α	cysteine-string protein-alpha
dB (SPL)	decibels (sound pressure level)
GABA	gamma amino butyric acid
Hz	hertz
kHz	kilohertz
NIHL	noise-induced hearing loss
TTS	temporary threshold shift
PTS	permanent threshold shift

INTRODUCTION AND REVIEW OF THE LITERATURE

Sensitivity of the Auditory System

The mammalian cochlea is both a structurally and functionally complex organ, so it's not surprising that only a portion of the physiologic mechanisms that act to enhance auditory system sensitivity have been discovered. Much is known about the mechanical amplification processes that are essential for enhanced sensitivity and sharp frequency selectivity. Less information is available pertaining to the actual event of signal transduction between the hair cell and the cochleovestibular nerve that carries the auditory signal to the brain.

We know that the external ear aids in sound localization and increases sound pressure levels through resonance. There are several mechanisms in the middle ear that are known to enhance auditory sensitivity. As an impedance matching device, the middle ear functions to increase force and pressure (energy) in the transmission of sound waves. The tensor tympani and stapedius muscles act to stiffen the ossicular chain, which augments the perception of higher frequency sounds. This action fights the upward spread of masking phenomenon. The acoustic reflex in the middle ear, which functions as a natural amplitude compressor, is known to aid in speech discrimination above 90 dB.

The cochlear amplifier is a functional process controlled by outer hair cell motility. It reflects how outer hair cells actively respond to acoustic stimulation and contribute to physiologic auditory enhancement. Through appropriately timed electromechanical feedback, outer hair cells are believed to amplify and sharpen

frequency tuning (Russell et al., 2003). Hearing becomes degraded when the outer hair cells are damaged.

As sound travels up the auditory pathway, the medial superior olive and the lateral superior olive use timing and intensity, respectively, to enhance differences in stimulation. Detection of interaural intensity differences and the preservation and transmission of timing information in the auditory brainstem nuclei is crucial for sound localization (Saul et al., 2008). The expression of voltage-gated potassium channels facilitates prompt responses to stimuli, which improves neural synchronization (Joris et al., 1994). As sound descends along the auditory pathway, the olivocochlear bundle functions to improve signal detection in noise, as well as protect the cochlea from mechanical damage and over stimulation.

There are several neural codes that allow a finely tuned auditory system. The place code suggests that the activity of a neuron is correlated to the location of the hair cell to which it is connected. Auditory nerve fibers are narrowly tuned to provide highly specific frequency information. The temporal code emphasizes that stimulus information is relayed by the rate of neuronal discharges. This release, or action potential, is the required neuronal response for signal transmission. Neurons are thought to be functionally organized by phase locking and firing to specific parts of a cyclic stimulus. When the phase locking abilities of a group of neurons is summed, stimulus frequencies can be more completely described. The maintenance of high-frequency timing information is crucial for decoding complex sounds, such as speech recognition (Oertel, 1999).

Normal auditory processing is necessary for complex listening, learning, and communication. As discussed earlier, the central auditory nervous system allows for sound localization using temporal and amplitude cues, as well as pitch and tone judgment (Katz, 2002). An auditory processing disorder (APD) occurs when there is a deficiency in one or more of the following: sound localization, temporal aspects of audition, auditory performance with competing acoustic signals, and auditory performance with degraded acoustic signals (ASHA, 1996). Individuals with auditory processing disorders have trouble interpreting acoustic input.

Glutamate, stored in pre-synaptic vesicles, is the primary excitatory neurotransmitter of the auditory system. Metabolism, vesicular transport, synaptic re-uptake and its effect on post-synaptic receptors can all play roles in glutamate toxicity (Tadros et al., 2007). Changes of glutamate, serotonin, and gamma amino butyric acid (GABA) have been associated with age and/or hearing loss in the mouse inferior colliculus (Tadros et al., 2007). It has been proposed that gene-regulated modifications of these neurotransmitter receptors are linked to age-related changes in complex sound processing. The dysfunction and changed regulation of the excitatory/inhibitory neurotransmitter pathways are considered key roles in the complex sound processing deficits associated with age related hearing loss, or presbycusis (Christensen et al., 2009).

The auditory cortex is the site of significant functional changes in genetic expression correlated with presbycusis. This disorder occurs due to an accumulation of auditory stressors superimposed on a genetically regulated aging process. Presbycusis begins with reduced sensitivity at high frequencies. Auditory processing abilities decay and sound localization becomes impaired. Different types of presbycusis involve a loss

of outer hair cells, the degeneration of stria vascularis, a reduction of spiral ganglion cells and/or reduction in synaptic activity in the central auditory pathway. Noise exposure is the number one risk factor for presbycusis.

Noise Induced Hearing Loss

According to the National Institute for Occupational Safety and Health (2001), permanent noise-induced hearing loss (NIHL) is the chief health hazard posed by occupational and recreational situations. Of the 30 million cases of hearing loss in the United States, one third are related to noise exposure. NIHL involves the irreparable loss of afferent neurons and cochlear hair cells which leads to auditory threshold deterioration (reviews: Slepecky, 1986 and Saunders et al., 1991). NIHL characteristically involves high frequencies (3-8 kHz).

The mechanisms of NIHL are similar to the types of presbycusis described earlier. Intense noise can cause spiral ganglion cell and hair cell (outer more than inner) degeneration and loss (Clark and Bohne, 1999). Excitotoxicity mechanisms lead to metabolic exhaustion and neuronal damage. Physical trauma can cause injury to the tectorial membrane, basilar membrane, and stereocilia. Noise can act as a mechanical stressor by overstretching inner ear tissues. The tectorial membrane may no longer be properly anchored to create the shearing force necessary for stereocilia deflection, and the organ of Corti may separate from the basilar membrane. If noise is too intense, it can also lead to vascular constriction, which reduces blood flow in the stria vascularis.

Whether noise is harmful depends on its intensity, frequency, duration, and individual susceptibility. In the case of this study, a temporary threshold shift (TTS) classifies transient hair cell dysfunction 24-48 hours after exposure and is not a stable

auditory threshold loss. A permanent threshold shift (PTS) is irreversible and the hearing loss is considered stable 14 days post noise exposure. Recent studies have explored cellular occurrences that can lead to sensory cell loss, including calcium dysregulation (Le Prell et al., 2006) and oxidative stress (Evans and Halliwell, 1999).

Synuclein Proteins

From the preceding discussion, synuclein proteins might be of interest for hearing because of their function in synaptic regulation and vesicle transport. Synucleins are therefore likely involved in the synaptic activity of the auditory system. Synucleins are small, soluble, proteins that are principally expressed in neurons and some varieties of cancer. This lipid membrane binding family consists of at least three isoforms: alpha, beta, and gamma synuclein. Over the past two decades, synuclein research has been resplendent with intriguing hypotheses, stimulating conclusions, and controversial information (Surguchov, 2008).

Maroteaux and colleagues (1988) coined the term “synuclein” after finding these proteins in both neural nuclear envelopes and pre-synaptic terminals. Alpha synuclein was initially isolated from cholinergic vesicle preparations of the electric organ of the ray *Torpedo californica* (Surguchov, 2008). Alpha synuclein was then recognized as a protein involved in synaptic plasticity in song learning in the zebra finch (George et al., 1995). Several years later a second member of the synuclein family, Beta synuclein, was discovered in the bovine brain. Gamma synuclein, the most recent isoform identified in mammalian species, was identified independently in several laboratories and termed breast cancer specific gene 1 (Surguchov, 2008).

Synuclein Localization

Alpha, beta, and gamma synucleins are localized in various areas within the peripheral and central nervous systems. Alpha and beta synuclein are primarily found at pre-synaptic nerve terminals in the central nervous system. Alpha and beta synuclein, abundantly expressed and developmentally regulated in the central nervous system, are upregulated by growth factors (Surguchov, 2008). In contrast, gamma synuclein is predominantly localized in the peripheral nervous system and has a different pattern of expression and regulation.

In 1992, Mori and colleagues localized alpha, beta, and gamma synucleins through immunohistochemistry. Alpha and beta are located in the same neurons, but in separate subregions. Alpha synuclein is primarily associated with synaptic terminals, while beta synuclein is found more extensively in the cell body. Alpha synuclein is found in areas of the brain where continual synaptic changes occur, such as the olfactory bulb, deeper layers of the cerebral cortex, and pontine nucleus (Clayton & George, 1998). It is most concentrated in the caudate nucleus, substantia nigra, putamen and ventral pallidus, basal ganglion, and catecholaminergic regions in the midbrain (Li et al., 2002). Beta synuclein has been discovered more widely and uniformly in the central nervous system, including the granular cell layer of the olfactory bulb, layer IV of the cerebral cortex, CA3 of the hippocampus, basal ganglia, and thalamic reticular nuclei. Gamma synuclein resides mainly in neuronal cytosol of the peripheral nervous system and epidermis, as well as the central nervous system (Norris et al., 2004). This protein is found in both cholinergic and catecholaminergic regions of the midbrain. Gamma synuclein is most

concentrated at the locus coeruleus in the brainstem and the nuclei of most cranial nerves (Li et al., 2002).

Suggested Physiologic Roles

The functional roles of synuclein proteins are often assumed by their location. Little is definitively understood about the cellular roles of synuclein proteins. Alpha synuclein is speculated to play a role in vesicle transportation, maintenance of synaptic vesicle pools, and signal-induced cytoskeletal regulation. Beta synuclein plays a proposed role in vesicular transport and sperm cell meiosis.

Information pertaining to synuclein function mainly stems from what is known about alpha synuclein proteins. The flexibility in alpha synuclein formation is based on an influential environment, due to its amino acid structural patterns (Sidhu et al., 2004). At a physiological level, alpha synuclein can function in vesicle synthesis and synaptic neurotransmitter release. When alpha synuclein is over expressed, it can also result in inappropriate deployment of these functions at the synapse (Surguchov, 2008).

Alpha synuclein proteins have several proposed functions. One proposed function is that of a *regenerator*. Quilty et al. (2003) reported that both alpha and beta synuclein gather around damaged neuronal terminals. Fan et al. (2006) reported that an increased expression of beta synuclein yields reduced alpha synuclein expression. Alpha and beta synuclein proteins seem to be inversely regulated. Beta synuclein proteins may help to balance alpha synucleins and block them from accumulating (Adamczyk, Solecka & Strosznajder, 2005). Alpha synuclein is also thought to play a role in the regeneration of epithelial basal cells and receptor neurons in the olfactory system (Duda et al., 1999).

Another proposed role of alpha synuclein is that of a *synaptic regulator*. The protein sustains synaptic flow by regulating vesicle flow in neuronal pre-synaptic terminals. In 2002, Cabin et al. found decreased levels of synapsin, a protein that regulates synaptic neurotransmitter release, in alpha synuclein knockout mice (relative to wild-type littermates). This discovery suggests that alpha synuclein is necessary for optimal synaptic maintenance.

In 2004, Sidhu and colleagues reported that the alpha synuclein plays a functional role in the modulation of dopamine transporter function. This suggests that alpha synuclein has a responsibility in regulating the synaptic integrity of dopamine. Interference in alpha-synuclein's ability to perform this function can produce abnormal cellular dopamine content. This can result in neurodegeneration of the synaptic nerve terminals. While alpha synuclein is not a toxic protein, but it can transform into a toxic molecule with the company of oxidized dopamine (Sidhu et al., 2004).

Alpha synuclein has been shown to function as a normal regulatory protein by binding to and reducing the activity of tyrosine hydroxylase, a rate-limiting enzyme involved in dopamine biosynthesis (Perez and Hastings, 2004). High doses of proteasomal inhibition are known to be toxic. In 2004, Snyder and colleagues reported that alpha synuclein acts as a molecular chaperone to regulate proteasomal inhibition. However, Sawada et al. (2004) proposed that low level proteasomal inhibition might be protective.

A recently hypothesized third role for alpha synuclein is that of a *neuroprotector*. A molecular chaperone protects cells by facilitating degradation of injured proteins. In 2001, Chandra and colleagues found that transgenic expression of alpha synuclein

eradicates the fatality and neurodegeneration caused by deletion of the molecular chaperone cysteine-string protein-alpha (CSP α). CSP α is another synaptic vesicle protein that functions to prevent the growth of potentially toxic molecules at nerve terminals. Mice lacking CSP α without transgenic alpha synuclein develop progressive, eventually lethal neurodegeneration (Fernandez-Chacon et al., 2004). A loss of endogenous synuclein action accelerated degeneration of pre-synaptic nerve terminals in these mice (Chandra et al., 2005). CSP α -deficient mice expressing transgenic alpha synuclein have a much greater survival rate. Thus, it appears that up regulation of alpha synuclein compensates for the loss of CSP α activity to suppress pre-synaptic deterioration. In these mice, alpha synuclein acts by a downstream mechanism entailing phospholipid binding, which suggests that it operates to protect nerve terminals from injury (Chandra, 2004).

Apoptotic proteins, when associated with alpha synucleins, show reduced expression and activity. Alpha synuclein acts as a mitochondrial protector by blocking activity and expression of the Parkinson's Disease-inducing neurotoxins (Sawada et al. 2004). However, according to Sidhu and colleagues (2004), this protective action might be dependent upon the level of cell differentiation, or may change with maturation.

Pathologic Roles of Synucleins

Both alpha and beta synuclein are most recognized for their role in protein aggregations that are the hallmark of Alzheimer's Disease and Parkinson's Disease. The "synucleinopathies" are a collection of neurodegenerative diseases with similar pathologic lesions and aggregations of synuclein proteins including Parkinson's disease, Alzheimer's disease, Huntington's disease, and dementia with lewy bodies. Synuclein

aggregation can directly damage the ubiquitin proteasome system and trigger apoptosis (Snyder et al., 2005).

Gamma synucleins typically serve the same functions as alpha and beta, but to a lesser extent (Quilty et al., 2003). Gamma synuclein is also known as breast cancer specific gene-1 (BCSG-1) and has several functions. Gamma synuclein stimulates the ligand-dependent transcriptional action of estrogen receptor- α in cultured breast cancer cells, stimulates cell proliferation, and up-regulates matrix metalloproteases, which are enzymes implicated in tumorigenesis and neurodegeneration (Snyder et al., 2004). In 2004, Snyder and colleagues reported an association between gamma synuclein and the cellular response to oxidative stress.

Gamma synuclein proteins may be uniquely involved in cytoskeletal preservation (Norris et al., 2004). Gamma synuclein also has the capability to block JNK signaling, which is commonly correlated with induction of apoptosis (Pan et al., 2002). In 2003, Ninkina and colleagues reported that gamma synuclein is not crucial for motor and peripheral sensory neuron function, regardless of high expression levels.

In 1996, Polymeropoulos and colleagues identified a locus for early onset Parkinson's disease, an autosomal dominant trait found in a small number of families, was mapped to the same region of chromosome 4 as the human alpha synuclein gene. PARK1 is a point mutation in alpha synuclein (A53T) associated with familial Parkinson's disease development. Patients with the A53T mutation show signs of neuronal death, gliosis, and extensive lewy bodies in the brain (Spira et al., 2001).

Parkinson's disease is a synucleinopathy of unknown etiology that causes a progressive and debilitating movement disorder. Its hallmark symptoms are all

suggestive of a loss of dopaminergic neurons and dysfunction in the substantia nigra pars compacta, where lewy bodies are typically located (Hardy et al., 2006). Wild-type alpha synuclein, found aggregated in the form of amyloid fibrils, is the most abundantly found component in these proteinaceous plaques (Sung and Eliezer, 2007).

The key to why alpha synuclein accumulates as protein deposits in synucleinopathies may lie in defective axonal transport (Surguchov, 2008). In several neurodegenerative disorders, axonal transport breaks down. Alpha synuclein is transported along axons from the cell body to synaptic terminals. Flawed alpha synuclein transport leads to a local over expression of the protein in the perikaryon, since it does not properly leave the cell body (Surguchov, 2008). An over expression of alpha synuclein can change its typical cellular localization and affect its folding and/or association with other proteins. This increase in alpha synuclein's local concentration may lead to its accumulation as lewy bodies (Surguchov, 2008).

Role of Synucleins in Sensory Systems

The involvement of synuclein proteins in sensory systems has raised exciting questions and there is still much to discover. It has been suggested that synuclein proteins play fundamental roles in the sensory systems, including vision and audition. Most of the literature describes synuclein location and function in the brain, but very little information is available pertaining to these sensory systems. Surguchov and colleagues (2001) reported that synucleins are expressed in the optic nerve and retina. Pathologies associated with retinal dystrophy, such as macular degeneration, retinitis pigmentosa, and

glaucoma have a common cellular pathway that eventually leads to photoreceptor death and visual loss.

In the cochlea, alpha and beta synuclein are expressed in spiral ganglion cells, primarily in the nuclear region (Surgucheva, 2006). Alpha synuclein is expressed throughout the entire central nervous system and is most plentiful in catecholaminergic regions (Akil et al., 2008). Alpha synuclein's presence in the cochlea was suspected, since Eybalin (1993) reported the function of cholinergic neuronal signaling in the organ of Corti. In 2008, Akil and colleagues studied synucleins in the mammalian organ of Corti and discovered that the proteins are expressed in the efferent synapse of outer hair cell base (alpha and gamma), spiral ganglion (beta), inner spiral bundle (gamma), and stria vascularis (alpha and beta). Synucleins must play a role in normal auditory function, as they are localized to the efferent cholinergic neuronal auditory system.

Adamczyk and Strosznajder (2006) reported an association between alpha synuclein and the voltage-gated calcium flux, a functional component of outer hair cell efferent signaling. The identification of synucleins at the outer hair cell base and efferent synapse suggest that these proteins are a part of the long-term, sustained release of acetylcholine in the efferent auditory system. Akil et al. (2008) proposed that because of the predominant localization of synucleins in the efferent neuronal system of the inner ear, the proteins might play a role in susceptibility to noise-induced hearing loss.

In 2002, Gates and colleagues proposed that central auditory speech-processing deficits might be an early manifestation of Alzheimer's disease. In addition to the debilitating symptoms of any neurodegenerative disorder, hearing loss can have a synergistic effect in leading sufferers to social isolation, withdrawal, and even depression.

Even with normal audiometric thresholds, individuals with central auditory processing deficits often have difficulty comprehending speech in unfavorable listening situations. P300, an electrophysiologic measure of central auditory processing, is sensitive to the degree of cognitive degeneration in synucleinopathies. Gerschlager and colleagues (2001) reported that P300 latencies were prolonged in Parkinson's Disease patients. Artieda et al. (1992) reported a limitation in the auditory temporal processing ability to detect very short silent intervals (gap detection) in patients with Parkinson's Disease. Perhaps an abundance of alpha synuclein could facilitate this process, as its neuroprotective role in the auditory system has been hypothesized. However, too much of this protein could cause aggregated clumps that might add to the impairment. Very few studies like these are available, which leaves much left to discover about central auditory processing and neurodegenerative disorders like Parkinson's Disease.

Present Study

From the foregoing, it might be predicted that disruption of synuclein function or location might impact both peripheral and central auditory function. In our laboratory, the functional role of synucleins in audition was recently studied. Synuclein isoforms were primarily localized in spiral ganglion neurons, hair cells, and the stria vascularis. It was shown that mice with targeted deletions of the gene that codes for alpha-synuclein exhibit elevated auditory brainstem response thresholds, suggesting a role for the protein in normal hearing mechanisms. Mice lacking alpha synuclein exhibit a high frequency hearing loss, which proves that alpha synuclein is essential for maintaining hearing sensitivity (unpublished data).

The neuropathologic basis for auditory processing disorders remains poorly understood (Saul et al., 2008). Although many mouse models of sensorineural hearing loss that involve the peripheral auditory system have been described, few studies have described mutations associated with retrocochlear hearing deficits. The current study seeks to determine the role that alpha-synuclein plays in noise-induced hearing loss and central auditory processing. Since alpha synuclein aggregation is stimulated by agents such as age, environmental toxins, and possibly noise, changes were monitored that might occur in the accumulation of synuclein proteins with exposure to noise in mice. Among possible outcomes, we questioned whether additional alpha synuclein might promote an increase in hearing sensitivity, or in susceptibility to noise induced damage. If, as suspected, alpha-synuclein facilitates synaptic vesicle release then alpha knockout mice may be protected from noise exposure and alpha over-expressers would suffer enhanced damage. Conversely, mice over-expressing alpha-synuclein may also exhibit improper aggregation of this protein which actually may reduce hearing sensitivity and thus limit noise-induced changes in hearing thresholds. If susceptibility to NIHL is reduced, this study may find a therapeutic value of alpha synuclein. If we discover changes in the localization or depletion of alpha synuclein following noise exposure, this may imply a compensatory mechanism to produce more alpha synuclein when the protein is used.

Electrophysiology: Adaptation and Recovery.

Adaptation is the reduction in a neural response due to previous stimulation. Recovery from adaptation refers to the process by which the auditory nervous system returns to a baseline level of function following stimulation. Adaptation and recovery may be examined with a variety of psychoacoustic paradigms including forward masking, gap detection, and decrement detection. In psychophysics, changes in threshold due to prior stimulation are examined as measures of adaptation and recovery. Physiologic experiments examine the changes in firing rates of single neurons or changes in latency and amplitude of evoked potentials as quantifications of adaptation and recovery.

The modified gap detection paradigm has been used extensively in understanding age-related changes in the auditory brainstem. Due to the need for a more sensitive assessment, gap detection testing was an appropriate supplement to auditory brainstem response (ABR) measures. Boettcher et al. (1993) reported that older (36-month) gerbils, compared to young adult (6-8 month) gerbils, showed minimal changes in adaptation and recovery in the most peripheral wave of the ABR. In contrast, recovery from adaptation was significantly prolonged in more central waves of the ABR in the older gerbils. These results occurred in the absence of threshold elevations and were thus considered to be neural, rather than sensory, changes. Comparable results were reported in elderly human subjects with normal thresholds by Poth et al. (1999).

Changes in response of older mice have also been examined with the modified gap paradigm by Walton and colleagues (2006). This study reported that single neuron responses in the inferior colliculus are abnormal in older mice whereas more peripheral responses are normal. This data supports the argument that adaptation and recovery may

be abnormal in some subject groups, independently of peripheral sensitivity, and that such changes may be compared at different levels of the auditory brainstem to allow the identification of sites of abnormalities in neural processing.

The present study utilized a modified form of a gap detection paradigm to measure adaptation and recovery in the ABR. Two identical wide-band noises were presented within a 150-ms window in order to quantify the latency and amplitudes of the ABR to the onset of each noise stimulus. The time period between the offset of the first noise of a pair and the onset of the second noise of a pair was varied to determine recovery from adaptation. This pattern permits examination of the complete ABR waveform, ranging from the most peripheral wave (representing the auditory nerve bundle) to more central waves (including the olivary complex and the lateral lemniscus). This allows for examination of responses from different levels in the auditory brainstem and identification of differences in adaptation and recovery between the auditory periphery and more central regions of the brainstem.

MATERIALS AND METHODS

Animals

19 mice (8-11 weeks of age) were used in the present study. The M83 mouse has a gene inserted that codes for an over-expression of human alpha synuclein. There were seven M83 mice genotyped as homozygous for the α -synuclein-producing gene. Five M83 mice were genotyped heterozygous for the α -synuclein-producing gene. The control group was comprised of seven wild-type littermates. All mice used were taken from the same litters. All groups were roughly evenly balanced by gender. Mice were purchased directly from The Jackson Laboratory (JAX) or were derived from breeders purchased from JAX. Animal cages were stored in an approved facility at Washington University, and were kept on a 12/12-light/dark cycle. Food and water were available on an ad-lib basis.

Auditory Brainstem Response

The basic procedure involved a hearing assessment by auditory brainstem response (ABR) thresholds on M83 homozygous, heterozygous, and wild type mice. Threshold sensitivity was evaluated by ABR prior to treatment to obtain baseline thresholds, again 24-48 hours post noise exposure to assess any temporary threshold shift, and again 14 days post noise exposure to measure any permanent threshold shift.

Tucker-Davis Technologies (TDT) System II hardware and BioSig 33 software were used. Calibration occurred prior to any recordings. Animals were anesthetized with a solution of ketamine and xylazine (80/15 mg/kg, i.p.). Subdermal needle electrodes

were placed in the mid-back (ground), medial to the pinna of the tested ear (active), and at the vertex (reference). Body temperature and heart rate were monitored throughout testing using a rectal probe, and body temperature was maintained at $37.5 \pm 1.0^{\circ}\text{C}$ using an isothermal pad. The right ear of each mouse was stimulated with 5 msec tonebursts (1000 repetitions, 20/second, 1.0 msec rise time) at frequencies of 5, 10, 20, 28.3, and 40 kHz while the left ear was compressed. The test was then repeated in the left ear of each mouse while the right was compressed. Filter settings were 100 to 10,000 Hz and speaker distance was 7 cm. The first wave of the ABR is thought to be generated by early auditory nerve and cochlear activity and is the most robust wave of the mouse ABR (Zheng et al., 1999). Therefore, thresholds were observed as the lowest level that the first negative peak (wave I) could be identified using the following bracketing technique: increase attenuation 10 dB following a positive response and decrease attenuation 5 dB following a negative response.

Anesthesia for ABR recording was well-tolerated, as no animals were lost as a result. All mice used for evaluation had baseline thresholds within normal limits. ABR thresholds were also conducted on 19 additional mice, but this data was excluded from statistical analysis due to a between-ear threshold difference of more than fifteen percent between ears at any one frequency.

Noise Exposure

The three genotypes of mice were exposed one time to fifteen minutes of broadband noise (8,000-16,000 Hz) at 110 dB SPL. This procedure occurred 48 hours post baseline ABR. Groups of two animals were placed in a wire cage mounted on a

pedestal inserted into a B&K 3921 turntable. To ensure a uniform sound field, the cage was suspended between four Motorola KSN1020 A piezo ceramic speakers at 0, 90, 180, and 360 degrees azimuth in a single-walled sound-proof booth with foam treatment (Industrial Acoustics, Bronx, NY). Noise was produced and filtered with General Radio 1310 generators and Krohn-Hite 3550 filters, respectively. The overall noise level was checked at various points in the cage using a B&K 4135 1/4 inch microphone in combination with a B&K 2231 sound level meter. The cage was rotated at approximately 0.013 Hz throughout the duration of the exposure in order to achieve as consistent an exposure as possible. No attempt was made to provide food or water throughout the exposure.

Gap Detection

The stimuli were two-octave-wide bands of noise centered at 20 kHz with a 12 dB/octave roll off. Each stimulus lasted 50 ms with rise-fall times nominally equal to 0 ms. Although very short rise-fall times are known to result in spectral splatter, the spectral width of the noise stimuli were considered to compensate for influences of spectral splatter. The stimuli were designed using with TDT SigGen software and generated with a TDT RP 2.1 D/A (100 kHz sampling rate). Signals were routed from the D/A to a PA5 digital attenuator, an earphone buffer, and an ES-1 earphone. Monaural presentation (free-field) to the right ear was used for all experiments. The presentation level was 70 dB SPL, calibrated with an ACO Pacific microphone.

Stimuli were presented in pairs, as shown in Figure 5. Each stimulus window consisting of two identical noise stimuli, with a silent period (“gap”) of 4-32 ms

separating the offset of the first stimulus of a pair from the onset of the second stimulus of a pair. The total duration of each stimulus window was 150 ms regardless of the gap duration. A silent period of 5 ms began each stimulus window. The ABR was collected for the entire 150-ms window. Each stimulus window was repeated every 500 ms.

The ABR waveform for the first and second noises were analyzed in terms of amplitude and latency for waves 1, 1a, 2, 3, 4, and 5. Figure 6B shows ABR waveforms elicited by a stimulus pair with gap duration of 16 ms. Figure 6A shows an expanded waveform elicited by the first noise of a pair. The peak latency of each wave is marked; as are the amplitude measures for waves 1 and 4. Waveform morphology in the mouse is not standardized in the literature and the waveform labels are limited to this study. Similarly, there is little information correlating waveform morphology to structure in the mouse and thus any such correlations in this paper are extrapolated from data on the cat and gerbil (Melcher et al., 1996).

Wave 1 is presumed to represent activity from the distal section of the auditory nerve and wave 1A may represent activity of auditory nerve fibers as they leave the cochlea. The waves tend to have a short inter-peak latency and for the purposes of this paper are not considered to be elicited by two separate nerve bundles. Based on data from other species (and not yet proven in the mouse), wave 2 represents activity of neurons in the cochlear nucleus, wave 3 represents activity in the superior olivary complex, and wave 4 represents activity of the lateral lemniscus.

Statistical Analysis

To investigate the possible function of alpha synuclein in noise-induced hearing loss, comparison of mean values of ABR thresholds and gap detection were conducted between homozygous, heterozygous, and wild-type M83 mice was performed with analysis of variance (ANOVA) with Bonferroni correction. A Holm-Sidak multiple comparisons significance was defined as a p value less than 0.05. This test was used to identify differences at specific frequencies.

RESULTS

Baseline auditory thresholds

Figure 1 shows baseline auditory thresholds by genotype. There was no obvious phenotype—physical or behavioral—observed in the transgenic mice due to over expression of α -synuclein gene relative to wild type littermates. Two-Way Repeated Measures ANOVA revealed no significant interaction between genotype and frequency for baseline ABR thresholds, $F = 0.403$ $p = 0.916$. The Holm-Sidak multiple comparisons analysis did not reveal a significant effect of genotype at any individual frequency tested.

Temporary threshold shifts

Figure 2 shows temporary auditory threshold shifts by genotype. A Two-Way Repeated Measures ANOVA revealed no significant interaction between genotype and frequency for temporary shift ABR thresholds, $F = 0.0546$ $p = 1.0$. The Holm-Sidak multiple comparisons analysis did not reveal a significant effect of genotype at any individual frequency tested.

Permanent threshold shifts

Figure 3 shows permanent auditory threshold shifts by genotype. A Two-Way Repeated Measures ANOVA (one factor repetition) revealed no significant interaction between genotype and frequency for permanent shift ABR thresholds, $F = 0.20$ $p = 0.990$. The Holm-Sidak multiple comparisons analysis did not reveal a significant effect of genotype at any individual frequency tested.

In completing post mortem histological examinations on the mice tested in the study, it was found that the general cochlear architecture was sound.

Adaptation and recovery: Effect of genotype on latency

Figure 7 shows mean latency shifts for wild type, heterozygous, and homozygous animals. Each wave is shown in a separate panel. At a gap duration of 4 ms, the heterozygous and homozygous groups had smaller latency shifts than the wild type group, for each wave. For example, for wave 1, the average shift at 4 ms was 0.8 ms for the wild type group and less than 0.4 ms for the heterozygous and homozygous groups. For wave 2, the latency shift for the wild type group was 0.83 ms at 4 ms, whereas the shift was approximately 0.6 ms for the heterozygous and homozygous groups. Overall, the differences between the wild type group and the two M83 groups ranged from approximately 0.25 to 0.5 ms across waves for the 4 ms gap duration.

In contrast with results at the 4-ms gap duration, differences between groups were less consistent for longer gap durations. For waves 1, 1A, and 2, wild type subjects had larger latency shifts than M83 groups at both 4 and 8 ms, but shifts were similar across all three groups at 16 and 32 ms gaps. Waves 3-5 did not show consistent trends across groups at gap durations of 8-32 ms.

DISCUSSION

The purpose of the current study was to determine the role that alpha-synuclein plays in noise-induced hearing loss and central auditory processing. It was hypothesized that over expression of alpha synuclein would lead to an increase in hearing sensitivity or increased susceptibility to noise induced damage. If, as suspected, alpha-synuclein facilitates synaptic vesicle release, then alpha over-expressers would suffer enhanced damage. Our ABR data revealed that alteration of alpha synuclein does not have any effect on the susceptibility to noise damage. However, our gap detection data implies a facilitation of synaptic transmission between hair cells and afferent nerve terminal. This facilitation appears to enhance central auditory function, as suggested by the resetting speed demonstrated by the detection of the second noise of the gap. This suggests that the alpha synuclein protein can play an advantageous role in central auditory function. The mechanisms that control of synaptic events, such as a modulation of alpha synuclein, may be a way overcoming the deficits seen in central auditory processing disorders.

Limitations of the present study

One shortcoming of this experiment was the small sample size. It is likely that with a much larger subject population for gap detection testing, an effect may be found for waves 2 and 3. Also relative to gap detection, it is difficult to maintain exact placement of the electrodes across mice. This may account for some of the variability between mice.

The mice used in this study come from a mixed background: B6 and C3H. We chose this mouse for study because it shares the background of knockout and transgenic models

we hoped to test to support the present findings. The use of this mouse may have impacted our results by increasing variability, but the mice were all used prior to the age where we could see hearing loss in a C57 background.

Clinical Implications and Future Studies

The Gap Detection paradigm provides evidence for a specific role of alpha synuclein in the central auditory pathway. Alpha synuclein facilitates transmission between the hair cell and the afferent nerve terminal. The present study documents support for the use of gap detection as a feasible measure of assessing differences in hearing sensitivity that are not readily apparent in ABR threshold differences. Mouse gap detection studies can be used in the future to better elucidate the mechanisms of central auditory processing. Further research should assess alpha and beta synuclein knockouts in gap detection testing. These investigations, especially if waves 2 and 3 are assessed, may provide a more extensive look into the central auditory pathway.

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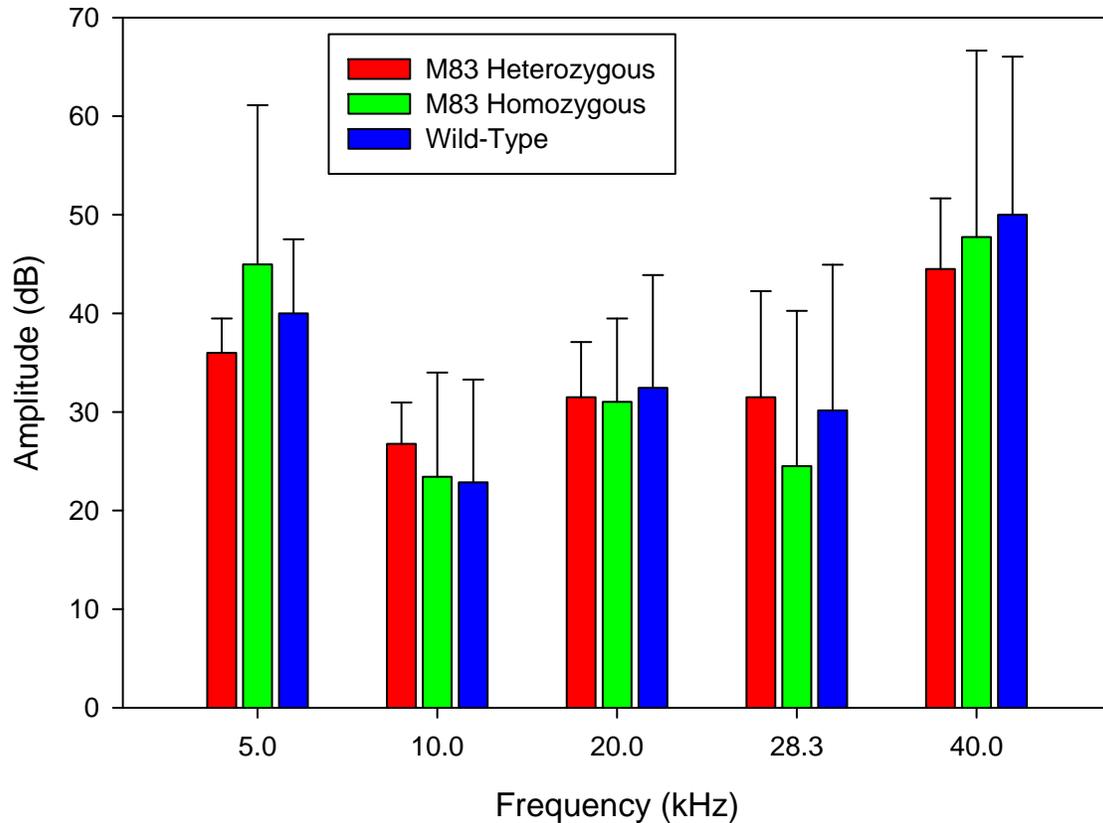
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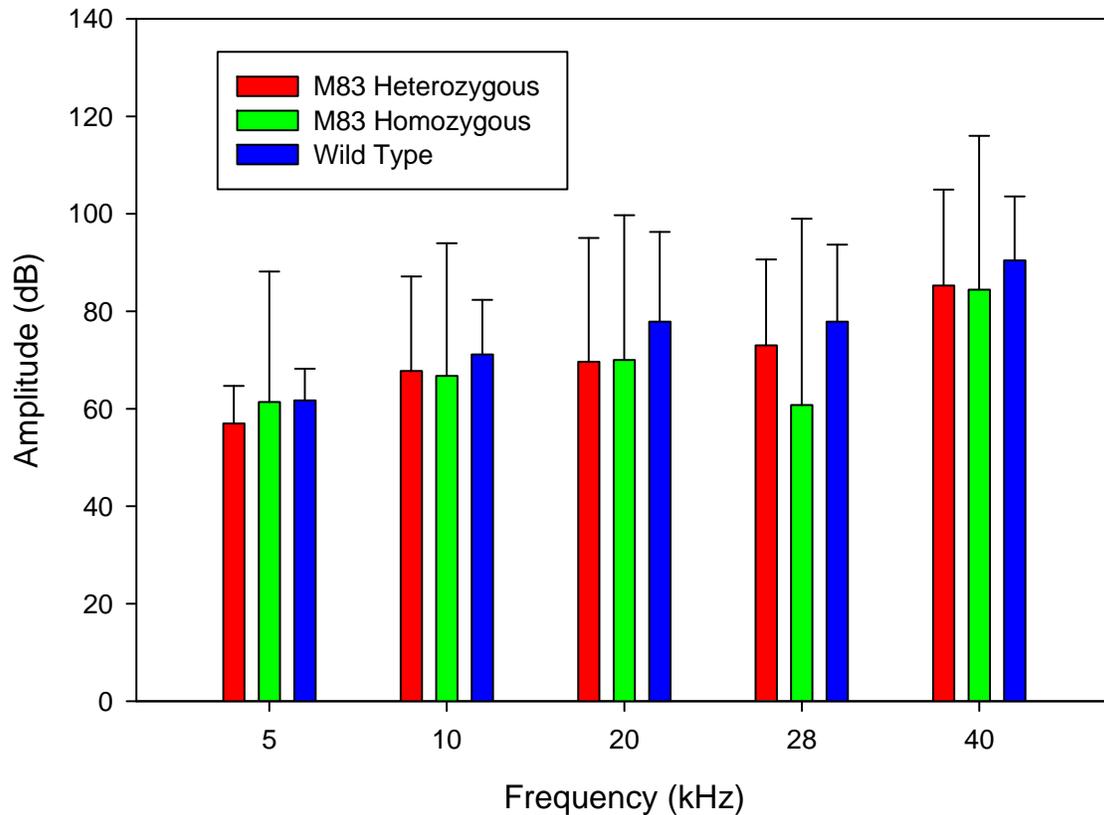
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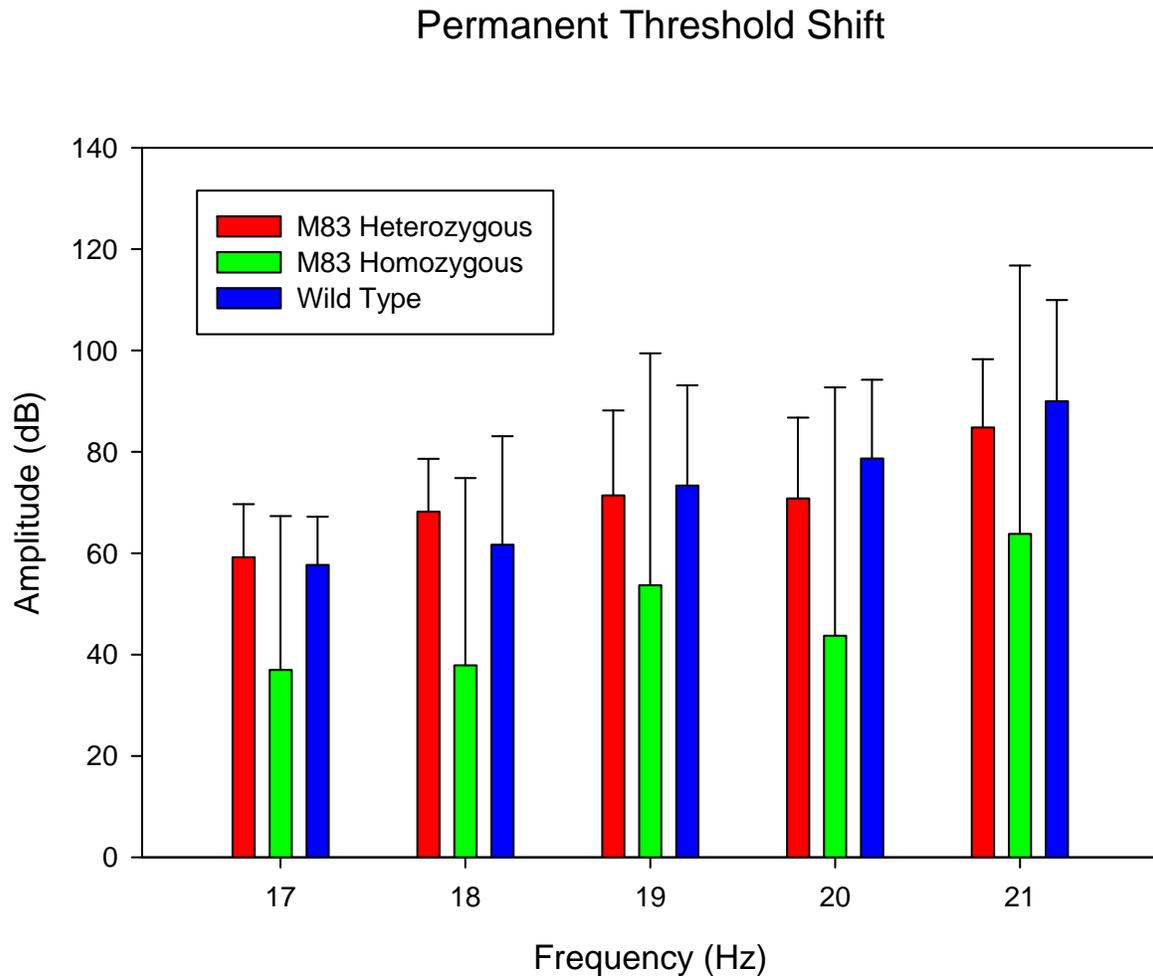
APPENDIX

Figure 1.**Baseline Auditory Thresholds****Figure 1: Baseline Auditory Thresholds.**

This is a bar graph of the baseline ABR thresholds for M83 heterozygous, M83 homozygous, and their wild-type counterpart mice. On the x-axis is frequency in kHz and the y-axis is ABR thresholds in dB. The red bar represents the heterozygous mice, the green bar represents the homozygous mice, and the blue bar represents the wild-type mice. In this graph, you can see that the baseline auditory thresholds for M83 mice are not significantly different from the wild-type mice.

Figure 2.**Temporary Threshold Shifts****Figure 2: Temporary Threshold Shifts.**

This is a bar graph of the temporary threshold shifts for M83 homozygous, M83 heterozygous, and their wild-type counterpart mice. On the x-axis is frequency in kHz and the y-axis is ABR thresholds in dB. The red bar represents the homozygous mice, the green bar represents the heterozygous mice, and the blue bar represents the wild-type mice. In this graph, you can see that the temporary threshold shifts for M83 mice are not significantly different from the wild-type mice.

Figure 3.**Figure 3: Permanent Thresholds Shifts.**

This is a bar graph of the permanent threshold shifts for M83 homozygous, M83 heterozygous, and their wild-type counterpart mice. On the x-axis is frequency in kHz and the y-axis is ABR thresholds in dB. The red bar represents the homozygous mice, the green bar represents the heterozygous mice, and the blue bar represents the wild-type mice. In this graph, you can see that the permanent threshold shifts for M83 mice are not significantly different from the wild-type mice.

Figure 4.

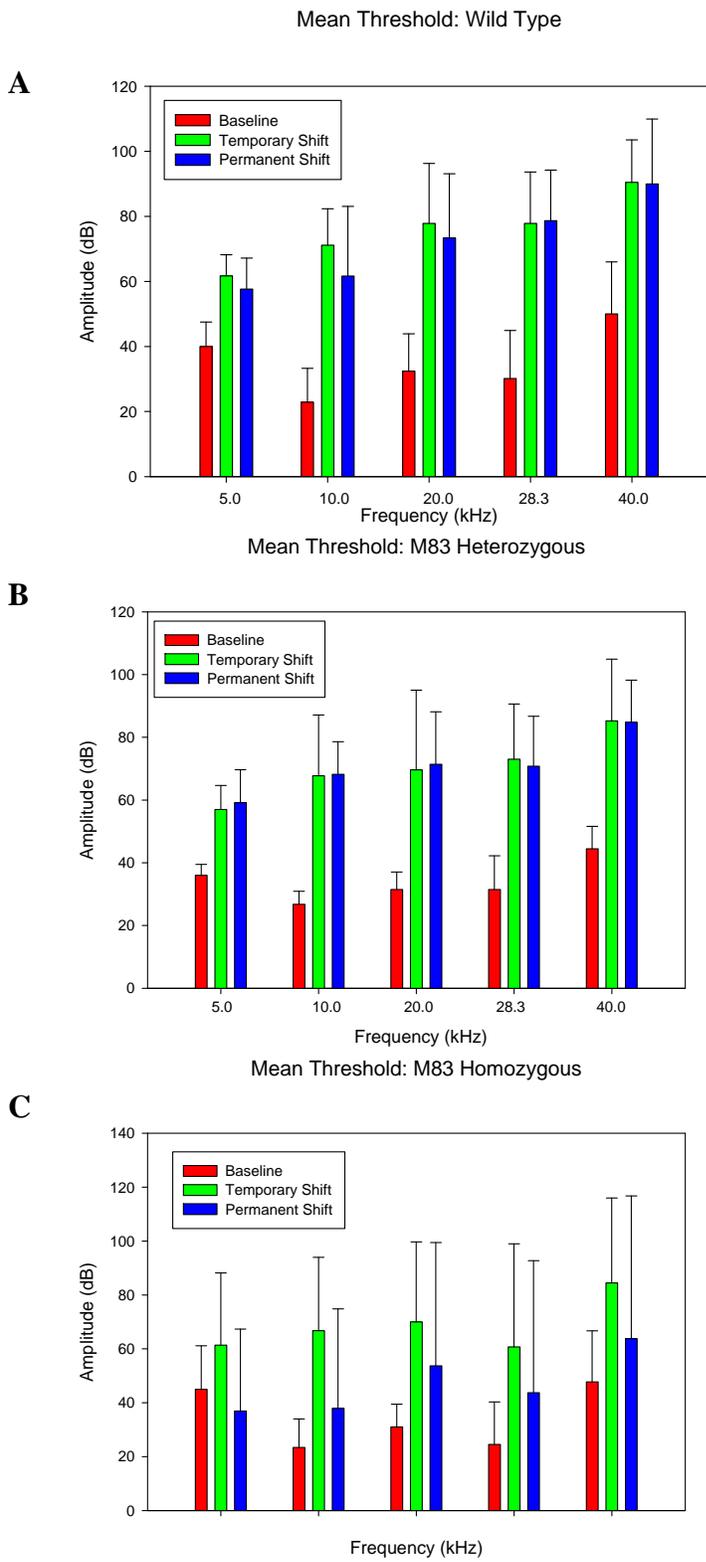
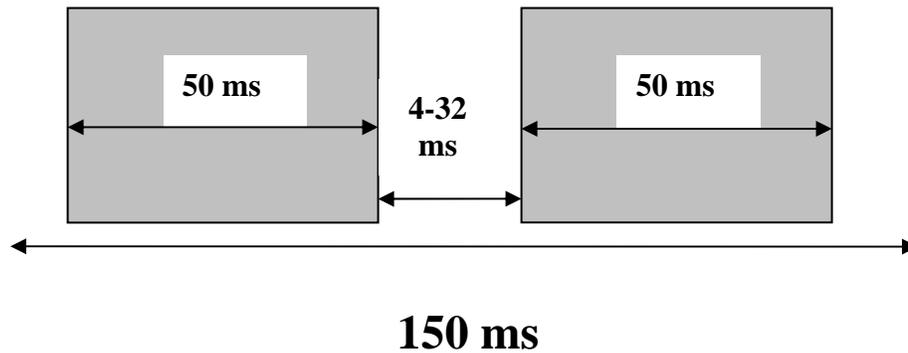


Figure 4: Mean ABR thresholds by genotype.

This is a bar graph of the mean ABR thresholds for wild-type, M83 heterozygous, and M83 homozygous mice. On the x-axis is frequency in kHz and the y-axis is ABR thresholds in dB. The red bar represents the baseline threshold, the green bar represents the temporary threshold shift, and the blue bar represents the permanent threshold shift.

Figure 5.**Figure 5: Gap detection stimulus.**

This is a diagram of a stimulus pair. Each stimulus cycle of 150 ms consisted of two identical wide-band noises (two-octave band width) with nominal rise-fall times of 0 ms. A 5-ms silent period began each cycle. The gap duration (time between the offset of the first noise and onset of the second noise) varied from 4-32 ms. The full cycle was repeated every 500 ms.

Figure 6.

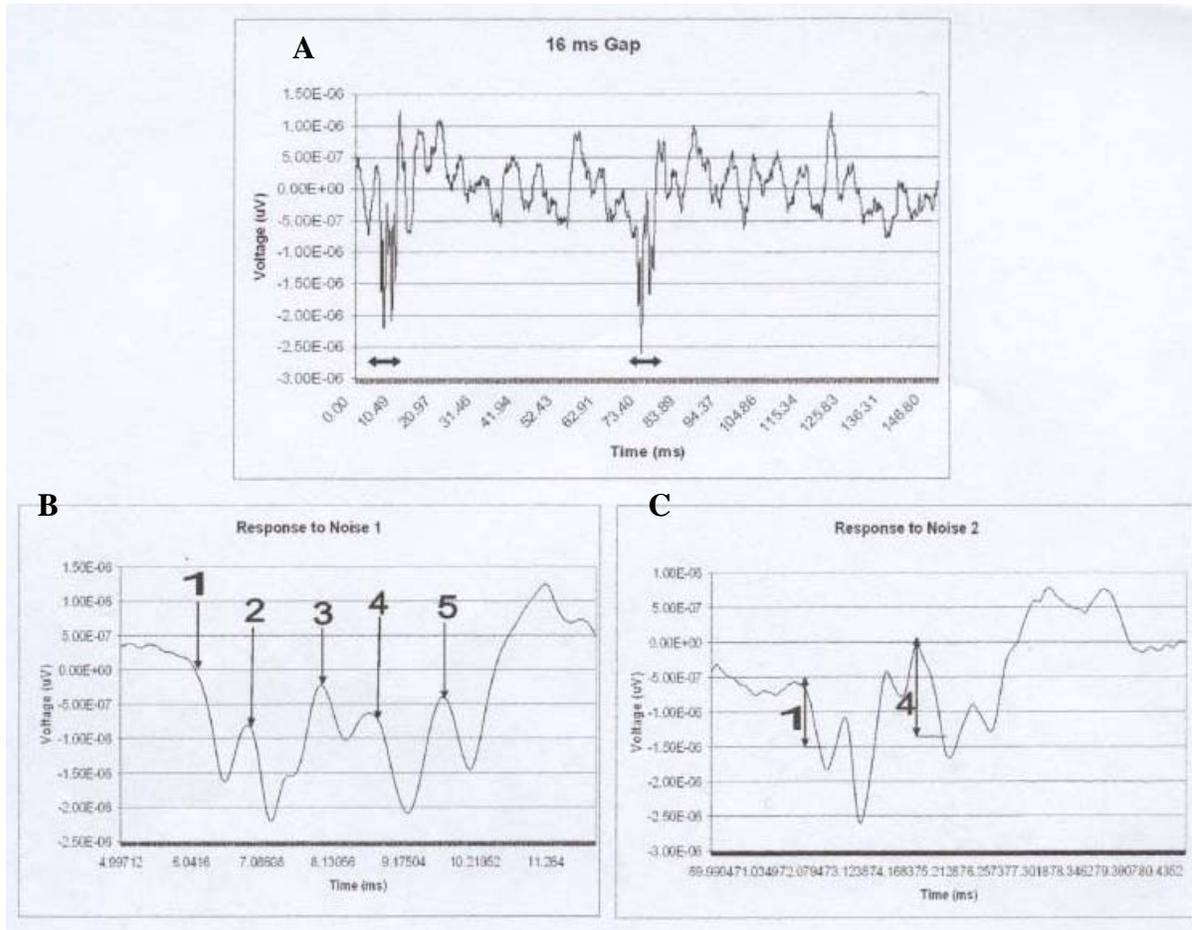


Figure 6: Gap detection noise response.

Section A shows a picture of the waveform of responses for a stimulus pair with a gap of 16 ms. Section B depicts the waveform showing the ABR to the first noise in the stimulus pair. Section C displays how the amplitude ratio was calculated.

Figure 7.

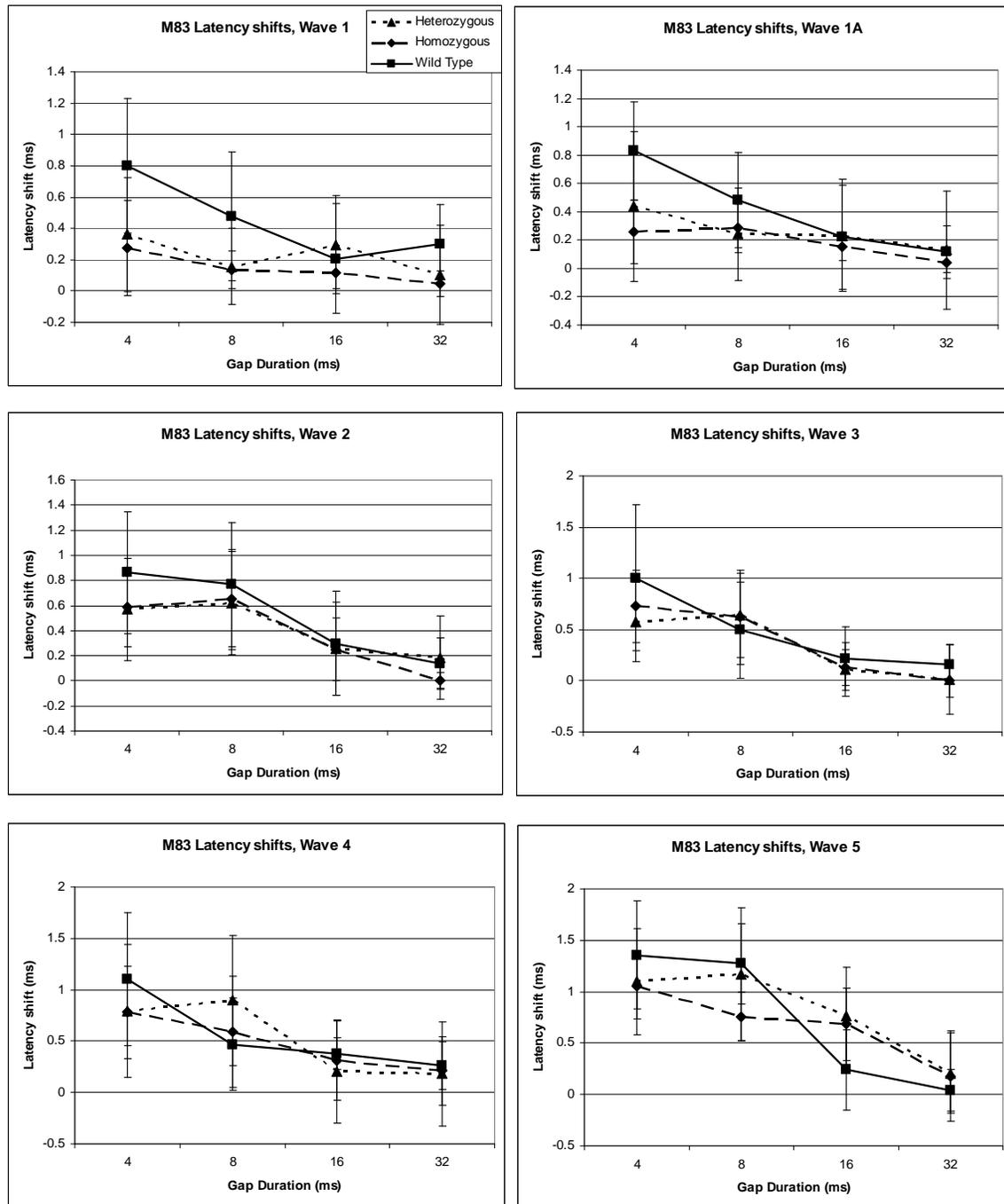


Figure 7: Gap detection latency shifts for each group.

These are line graphs of the mean latency shifts for each group of mice, plotted as a function of gap duration. Each panel shows a separate ABR wave. On the x-axis is gap duration in ms and the y-axis is latency in ms. The solid black line with squares

represents the wild-type mice, the solid black line with diamonds represents the M83 homozygous mice, and the dotted line represents the M83 heterozygous mice.