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Role of synuclein proteins in auditory function

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ROLE OF SYNUCLEIN PROTEINS IN AUDITORY FUNCTION

by

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***Abstract:** The recent discovery of synuclein proteins in peripheral auditory tissues has prompted a closer examination of the role of these proteins in hearing. In the present study, auditory brainstem response thresholds of synuclein knockout mice are compared to wild type mice.*

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Abstract

Alpha, beta and gamma-synuclein are small soluble proteins produced by three different genes and are expressed primarily in the pre-synaptic terminals of neural tissues in certain tumors. Although the normal function of these proteins is not definitively known, their recent association with human neurodegenerative disorders has focused a great deal of interest on them. In a recent pilot study to examine the localization of these proteins in rodent sensory systems, we found synuclein isoforms in several auditory tissues, including spiral ganglion neurons, hair cells and stria vascularis. To assess the functional role of these proteins in hearing, we examined auditory brainstem response thresholds of mice with targeted deletions of alpha- and gamma-synuclein proteins. Mice with targeted deletion of alpha-synuclein or both alpha- and gamma-synuclein exhibited increased thresholds compared to wild type littermates at higher frequencies tested. Data collected in this study could provide new information about the role of synuclein proteins in the auditory system as well as other tissues.

Role of Synuclein Proteins in Auditory Function

Introduction

Synuclein Proteins

Synuclein proteins are a family of small, heat-stable soluble proteins, found primarily in neurons. There are three different types of synuclein proteins: alpha (α), beta (β), and gamma (γ) (Clayton & George, 1998). The gene for α-synuclein is found on chromosome 4, the gene for β-synuclein is found on 5, and the gene for γ-synuclein proteins is found on chromosome 10 (Jellinger, 2003).

Synuclein proteins are composed of 120-140 amino acids, arranged in almost the same repeating pattern for 93 residues. Mice and rats share identical synuclein composition for the first 93 residues, and human synuclein proteins vary from these rodents only by two residues (Clayton & George, 1998).

The first published description and terming of "synuclein" proteins occurred in 1988 by Maroteaux et al. It was named as such due to findings of "synucleins" in both pre-synaptic terminals and neuronal nuclear envelopes. This was the discovery of α -synuclein proteins. Around this time, these proteins were discovered in multiple labs with many different names being assigned. Within ten years following α -synuclein discovery, beta and gamma were also characterized. (Clayton & George, 1998; Lavedan, 1998)

Localization

Alpha-, beta- and gamma-synucleins are localized in different areas within the central and peripheral nervous systems. All three are found in high quantities in the human brain

(Lavedan, 1998), although specific localization reports in the current literature are varied and often contradictory.

Alpha-synuclein proteins are found primarily in the pre-synaptic terminals of developed neurons throughout the central nervous system. During fetal development, at about 15-18 weeks gestation, α-synucleins migrate from neuronal cell bodies to the terminals. (Norris et al., 2004) The most concentrated areas with α-synucleins are the caudate nucleus, substantia nigra, putamen and ventral pallidus, basil ganglion catecholaminergic regions in the midbrain. It is important to note in particular that the substantia nigra is the production source of dopaminergic neurons, and therefore the main source of Parkinson's disease. Alpha-synucleins are also found in high levels in the locus coerulus and vagus nuclei of the brainstem, and preganglionic sympathetic nuclei and laminae I and II in the spinal cord (Li, Jensen & Dahlstrom, 2002).

Beta-synuclein proteins are found more uniformly throughout the brain, and in some skeletal muscles. Similar to α-synuclein proteins, β-synucleins also originate at cell bodies and relocate to pre-synaptic nerve terminals at 17-20 weeks gestation (Norris et al., 2004). Beta-synucleins are located in somatic cholinergic regions—that is, around neurons that use the neurotransmitter, acetylcholine. In particular, β-synucleins occupy the nuclei of cranial nerves, including the oculomotor, facial, hypoglossal, accessory and ambiguous (Li, Jensen & Dahlstrom, 2002).

The gamma synuclein protein is localized primarily in the cytosol of neurons in the peripheral nervous system and in epidermis, although it is also found in the brain and spinal cord. (Norris et al., 2004) Gamma-synucleins reside in both cholinergic and catecholaminergic regions. Notable locations of high concentration of γ-synuclein are the locus coeruleus in the brainstem, the terminals and soma of Edinger-Westphal nuclei (of the oculomotor nerve), and the

nuclei of most cranial nerves. In the spinal cord, like α-synuclein, γ-synuclein proteins are found in laminae I and II as well as preganglionic sympathetic nuclei (Li, Jensen & Dahlstrom, 2002).

In mammals and birds, α- and β-synuclein proteins are found in highest concentration in the hippocampus, cortex, olifactory bulb and nuclei—areas of the brain where continual synaptic modification occurs (Clayton & George, 1998).

Suggested Roles in Normal Physiology

Little is known definitively about the cellular functions of synuclein proteins. Beta and γ-synucleins in particular are yet to be understood. Beta-synuclein proteins may help to balance α-synucleins and block them from accumulating (Adamczyk, Solecka & Strosznajder, 2005). In general, γ-synucleins often serve the same functions as α- and β-, only to a lesser extent (Quilty et al., 2003). Uniquely, γ-synuclein proteins may be involved in cytoskeletal preservation (Norris et al., 2004).

Many conclusions about the role of synucleins are made based on their localization. As previously stated, both α- and β-synuclein are present in neuronal pre-synaptic terminals; this in itself provides information about potential physiology. George et al. (1995) studied the role of synucleins in the song development of songbirds. During the month-long period of their critical development, synuclein proteins are abundant in song control nucleus (IMAN) terminals, after which time the quantity of synucleins decreases significantly. This study supports the belief that α- and β-synuclein proteins are important for neuronal plasticity, as the proteins are only present while terminals restructure and new synaptic connections are made (Clayton & George, 1998).

The most information on synucleins is derived from α-synuclein proteins. There are many proposed functions of α-synuclein proteins. The amino acid structural patterns of α-

synuclein proteins allow for versatility in its formation based on an influential environment (Sidhu et al., 2004).

One role of synuclein proteins is that of a *regenerator*. Duda et al (1999) studied cells within the olfactory system and asserted that α-synucleins were involved in the constant regeneration of epithelial basal cells and receptor neurons (Norris et al., 2004). Both α- and β-synuclein (and γ- to a lesser extent) were found to gather around neuronal terminals that have been damaged (Quilty et al., 2003).

A second proposed role is that of a synaptic *regulator*. Alpha-synucleins maintain synaptic flow by regulating the passage of vesicles in neuronal pre-synaptic terminals. Cabin et al (2002) examined α-synuclein knockout mice and found problematic synaptic transmission was occurring; both docked and distal vesicles were weakened with over-stimulation (Norris et al., 2004). Cabin et al. (2002) also found in α-synuclein knockout mice that, relative to wild-type littermates, there were decreased levels of synapsin, a protein which regulates synaptic neurotransmitter release. This implies that synucleins are essential for synaptic maintenance and vesicle recycling (Sidhu et al., 2004). Alpha-synuclein proteins also help to maintain homeostasis and plasticity in neuronal synapses. Specifically, α-synuclein proteins help to maintain the homeostasis of dopamine levels; should this role fail to be fulfilled due to mutation, neurodegeneration could occur, as we will discuss in the following section (Sidhu et al., 2004).

Alpha-synucleins may also act as a *protector*. In fact, through the interaction with α-synucleins, apoptotic proteins are reduced in activity and expression. Jensen et al. found that α-synuclein proteins directly protect mitochondria by blocking MPP+ and rotenone, neurotoxins that inhibit dopaminergic and mitochondreal function and contribute to Parkinson's. However, this blocking protection by synucleins does not occur for all cells, as seen with differentiated

dopaminergic neurons. This could mean that the protective capability of synucleins is dependent upon level of cell differentiation, or perhaps this ability changes with age (Sidhu et al., 2004). Kholodilov et al. (1999) also induced apoptosis with dopaminergic neurons, and found α-synuclein expressed specifically in surviving, protected neurons, rather than those that do not survive (Sidhu et al., 2004).

A final role of α-synucleins is that of a *molecular chaperone*. Ostrerova et al (1999) showed that α-synuclein and chaperone protein 14-3-3 share similar sequencing and may also bind together and serve similar protein-folding functions. Souza et al (2000) determined that all three types of synucleins could serve as molecular chaperones *if* they were without mutation (Norris et al., 2004). At the end of α-synucleins is an acidic carboxyl-terminal, made of glutamate and aspartate residues, which help with the ability to serve as a chaperone (Sidhu et al., 2004).

Synuclein Pathology

There are a number of ways in which synuclein proteins can become pathological. These include: aggregation due to mutation of the proteins, oxidative stress, over-expression of synuclein proteins and possibly aging (Volles & Lansbury, 2007; Norris et al., 2004; Adamczyk, Solecka & Strosznajder, 2005; Kaplan, Ratner & Haas, 2003). However, Xu et al. (2002) stated that over-expression does not always lead to pathology. They found that, while over-expression of α-synuclein is toxic for dopaminergic neurons, it might serve as a protectant to other types of neurons.

Uniquely, γ-synucleins have been linked to breast cancer. That is, γ-synucleins are present in much greater quantities in advanced stages of breast cancer tissue than in non-

cancerous breast tissues. It is thought, therefore, that they may contribute to cancer progression (Norris et al., 2004).

Three known α-synuclein mutations have been linked to familial Parkinson's disease. These protein mutations are called A30P, A53T and E46K. The α-synuclein mutation, A30P, hinders membrane binding, A53T may cause apoptosis of dopaminergic neurons (Kaplan, Ratner & Haas, 2003) and E46K may lead to more aggregates than the other two mutants (Pandey, Schmidt & Galvin, 2006).

Because they are believed to play important roles in neuronal regulation and protection, mutations of synucleins will naturally have disastrous effects. The term "Synucleinopathies" describes neurodegenerative disorders involving aggregates of α-synuclein abnormalities. The proteins accumulate or are misfolded, become unsoluable and can form Lewy bodies, the histological hallmark of Parkinson's disease, Down's syndrome, Alzheimer's disease and other cytoplasmic inclusions (Jellinger, 2003). The link between Parkinson's and α-synuclein proteins has been a highly discussed topic for the last several years, following the discovery of the causative mutations mentioned above.

Braak et al. (2003) found that synuclein pathology sometimes originates toward the back of the brainstem, then moves to limbic system and other regions within the cortex, and years later to the substantia nigra of the midbrain. Because α-synucleins have been found to regulate the amount of dopamine made, mutation greatly disrupts regulation. Down's syndrome and Alzheimer's disease are commonly developed due to α-synuclein aggregates within the amygdala of the limbic system (Galvin, 2006).

Aging has been found to be another possible cause of synuclein pathology. There are significant changes in α-synuclein localization that occur in the brain due to aging. Namely,

immunoreactivity and expression of α-synuclein proteins in the cerebellum significantly reduces with age. In addition, immunoreactivity in the cortex and hippocampus significantly reduced in aged rats, yet expression of the gene was unaffected in these regions. There was also more oxidative stress present. The mobility of α-synuclein proteins through axons became significantly slower in aged rats. This slowed transmission makes synucleins more vulnerable to mutation and aggregation. There was not an significant aging effect noted for β-synuclein proteins. (Adamczyk, Solecka & Strosznajder, 2005).

Localization in Sensory Systems

Recent discoveries by Surgucheva, Faddis and Surguchov (2007) have indicated that synuclein proteins are also localized within both the visual and auditory systems.

Gamma-synuclein were discovered to be abundant in the lens of rat embryos, with the quantity decreasing over the course of 42 days. In addition, γ-synucleins are localized in cells of the cornea, retina, and in photoreceptors.

Within the cochlea of the auditory system, both α- and β-synuclein proteins were found in hair cells. Large amounts of γ-synucleins were found specifically in outer hair cells and β-synuclein proteins were found in spiral ganglion cells. Synucleins were also discovered in the stria vascularis (Surgucheva et al., personal communication).

Present Study

The known presence of synuclein proteins within the auditory system led to the investigation of the role they play within the auditory system. Are synucleins essential for the maintenance and protection of cells within the auditory system, as they are in many parts of the

brain? Using transgenic mice, the current study examined if the absence of the gene producing synuclein proteins influenced hearing sensitivity of mice.

Materials & Methods

Animals

Thirty-two juvenile mice (eight-weeks of age) were used in the present study. There were six genotyped with a knockout of the α-synuclein-producing gene, and seven γ-synuclein protein knockout mice. There were twelve double-knockout mice, lacking the gene for both α- and γ-synuclein proteins. The control group was comprised of six wild-type mice. Both the synuclein knockout and the wild type mice were taken from the same litters.

Male: female breeding pairs that were both heterozygous for a specific deleted synuclein gene were used to produce litters of mixed genotypes. By breeding in this manner, both true knockouts and wild type mice could be pulled from the same litters to help avoid differences due to genetic and environmental influences. However, too few such pairs were actually available to perform this type of comparison. ABR thresholds were also conducted on some heterozygous littermates but the heterozygous genotypes were not included in the statistical analysis due to the small sample size. 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.

Methods

Auditory Brainstem Response (ABR) Recordings were collected once for each mouse, using the right ear. Using an anesthetic solution (80mg/kg ketamine, 15 mg/kg zylazine, IP), the animals were sedated at least ten minutes prior to performing ABR recordings.

In a sound-proof booth, the animals were placed on a thermostatically-controlled heating pad that, in conjunction with a rectal probe (Yellow Springs Instruments Model 73A), regulated their temperature at 37.5 ±1 °C. Three platinum needle electrodes (Grass) were inserted in the animals; the active probe was inserted vertically behind the ear, the reference was inserted horizontally in the coronal plane between the eyes, and the ground was inserted horizontally in the back. The electrodes were hooked up to a Grass P15 differential amplifier (100-10,000 Hz, x100), which led to a custom amplifier that provided an additional gain of x1,000. A Cambridge Electronic Design µ1401 and SIGNAL™ and custom signal averaging software on a 120MHz Pentium PC digitized the information at 30kHz and were used to conduct and analyze the recordings.

A Wavetek Model 148 oscillator produced the sine wave stimuli, and a custom electronic switch shaped it to 5ms total duration, with 1ms rise/fall times. A Crown D150A power amplifier amplified the stimulus. The amplified tone burst signal was presented 1,000 times at 20 signals per second, using a KSN1020A piezo ceramic speaker, placed 10 cm laterally away from the rodent's patent right pinna. Evoked threshold measures were recorded at 5, 10, 20, 28.3 and 40 kHz, using increments no less than 5dB. Calibration was done with a B&K 4135 ¼ inch microphone at the aforementioned distance from the speaker.

Data Analysis

To investigate the possible auditory function of synuclein-proteins, as measured by Auditory Brainstem Response thresholds, a two-way Analysis of Variance (ANOVA) was across frequency and genotype—wild type, α-synuclein knockout, γ-synuclein knockout, and double knockout. To test the level of deletion possibly needed to influence hearing function, the

Auditory Brainstem Response thresholds of heterozygous littermates were also taken and analyzed.

Results

There was no obvious phenotype—physical or behavioral—observed in the transgenic mice due to deletion of the γ-or α-synuclein gene relative to wild type littermates.

A Two-Way Repeated Measures ANOVA (one factor repetition) revealed a significant main interaction between genotype and frequency, $F(12) = 2.19$, $p = 0.017$. A Holm-Sidak method analysis revealed a highly significant effect of genotype at 40 kHz when comparing α-synuclein knockout with wild type mice ($p = 0.000$) (See Figure 1). There was also a significant effect of genotype found at 40 kHz when comparing double-knockout mice with wild type mice ($p = 0.022$) (See Figure 3). Heterozygous mice did not significantly differ from wild type.

In completing post mortem histological examinations on the mice tested in the study, it was found that the general cochlear architecture was sound, but there was a possible reduction of spiral ganglion cell density in the base of the cochlea.

Discussion

Results of this study demonstrate a high frequency (40 kHz) hearing loss due to the deletion of the α-synuclein protein gene. Double knockout mice, lacking both alpha and gamma-synuclein proteins, were also found to have significantly elevated threshold at 40 k Hz. Gamma synuclein knockout mice did not demonstrate an increased threshold. Presumably the double knockout effect represents a functional deficit caused by the absent α-synuclein protein. It was

also found that the effect of the different genotypes is dependent upon the frequency tested—the interaction was statistically significant.

The present study is the first to demonstrate a sensory function and deficit associated with synuclein proteins. In previous investigations, even dopaminergic systems showed no deficits with the deletion of synuclein proteins; rather, deficits occurred due to synuclein mutation or over-expression. Results of this study revealed that the absence of synuclein proteins does have negative effects on hearing sensitivity, and therefore that synuclein proteins do play some role in auditory function.

In light of synuclein localization in the cochlea and significantly elevated high-frequency thresholds, there are a few roles that synuclein proteins could play in the auditory system. As previously stated, one of their main proposed functions in neurons is synaptic regulation and regeneration and vesicle transmission; therefore, synucleins are likely involved in synaptic activity in the auditory system as well. Synuclein proteins could also play a role in outer-hair cell motility.

One shortcoming of this experiment was the small sample size. It is likely that with a much larger test population, elevated thresholds of transgenic synuclein knockout mice would appear over more frequencies. Further histological exploration should take place with this larger sample size to understand the effect of the gene deletion on auditory structures with greater specificity.

Because there is believed to be a decline in synuclein expression with age (Adamczyk, Solecka & Strosznajder, 2005), another future study could investigate a possible connection between synuclein proteins and age-related hearing loss. In addition, due to the protective role of synucleins, a future study could test the outcome of noise exposure on mice with a synuclein gene deletion.

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Figure Legend

Figure 1. Auditory Brainstem Response Thresholds Of Alpha-Synuclein Knockout Mice. This is a line graph of the ABR thresholds for α-synuclein KO mice and their wild-type counterparts. On the x-axis is frequency in kHz and the y-axis is ABR thresholds in dB. The solid black line represents the wild-type mice and the green dotted lines represent the α-KO mice. The red dotted line indicates the heterozygous data, used for comparison. In this graph, you can see that the thresholds of α-knockout mice are consistently above the wild type, although it is only a significantly elevated threshold at 40kHz.

Figure 2: Auditory Brainstem Response Thresholds of Gamma-Synuclein Knockout Mice. This is a line graph of the ABR thresholds for γ-synuclein KO mice and their wild-type counterparts. On the x-axis is frequency in kHz and the y-axis is ABR thresholds in dB. The solid black line represents the wild-type mice and the green dotted lines represent the γ-KO mice. The red dotted line indicates the heterozygous data, used for comparison. The graph suggests that there is a difference in γ-KO thresholds, but a two-way ANOVA indicates that this difference is not significant.

Figure 3: Auditory Brainstem Response Thresholds of Alpha/Gamma-Synuclein Double Knockout Mice. This is a graph of the ABR thresholds for α- and γ-synuclein double KO mice and their wild type littermates. At 40kHz, there is a significant effect of genotype compared to the wild-type mice. The significant threshold elevation seen on this graph is thought to be an effect of the deletion of α-synuclein rather than γ, since γ-synuclein single knockout mice were not found to have significant threshold differences.

Figure 1

Auditory Brainstem Response Thresholds
Of Alpha-Synuclein Knockout Mice

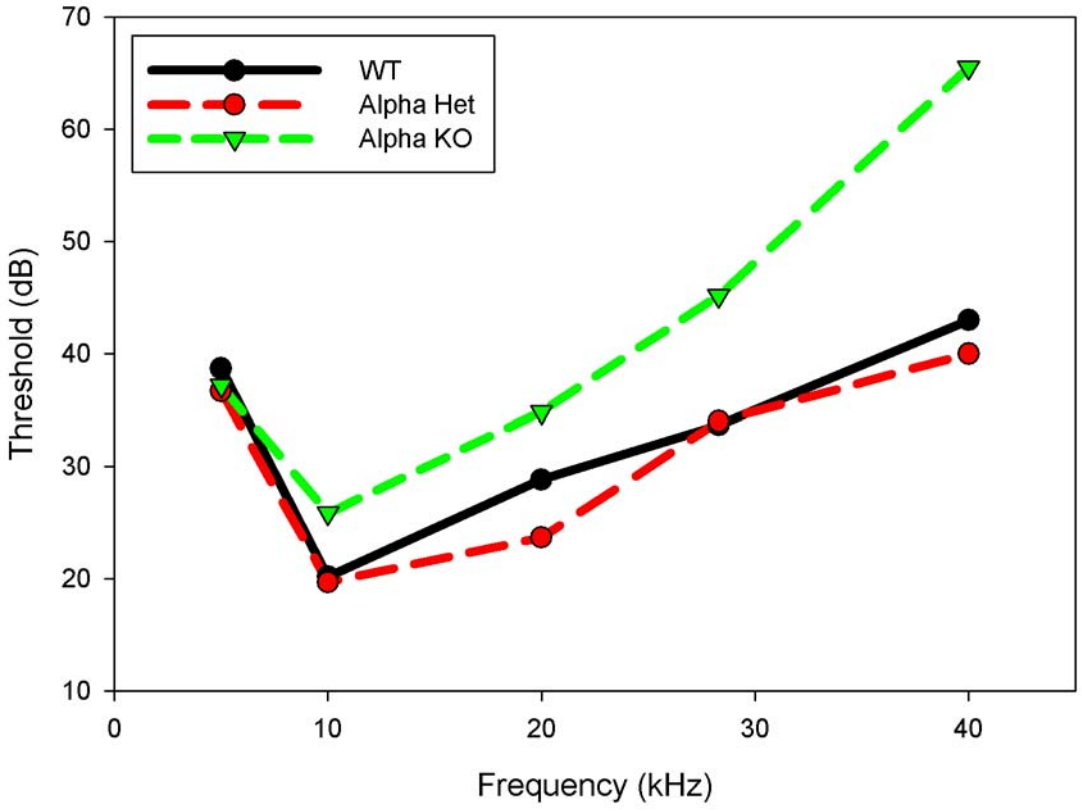

Figure 2

Auditory Brainstem Response Thresholds of Gamma-Synuclein Knockout Mice

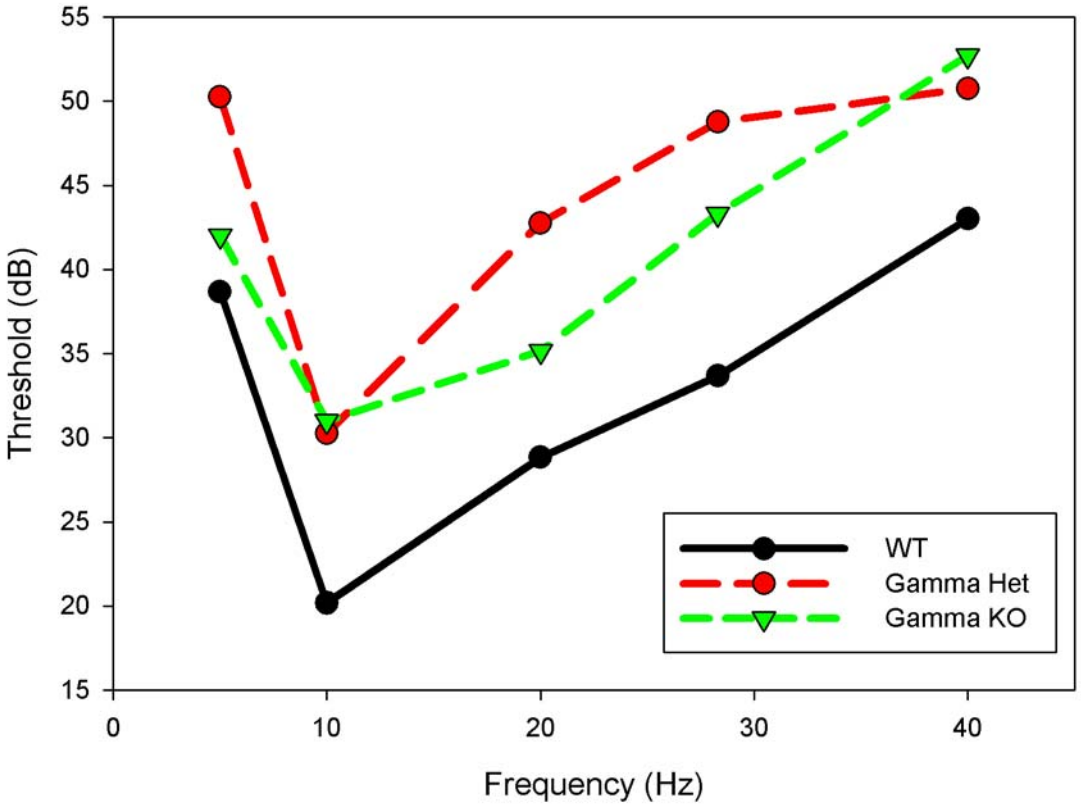

Figure 3

Auditory Brainstem Response Thresholds of Alpha/Gamma-Synuclein Double Knockout Mice