Histological and functional aspects of Alpha-synuclein in neurodegeneration

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Abstract: Alpha-synuclein has been implicated in the cellular mechanisms that control auditory sensitivity. In other systems it can also confer protection against cellular injury. Auditory brainstem response thresholds and immunohistochemistry were used to assess the ability of alpha-synuclein to protect against oxidative damage to the cochlea.
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TABLE OF CONTENTS

Acknowledgements ii
List of Tables and Figures 2
Abbreviations 3
Introduction 4
Materials and Methods 14
Results 19
Discussion 21
References 25
LISTS OF FIGURES AND TABLES

FIGURE 1: Baseline Auditory Thresholds 30
FIGURE 2: Overall Threshold Shift 31
FIGURE 3: Overall Threshold Change 32
FIGURE 4: Synuclein Localization in the Normal Mouse organ of Corti 33
FIGURE 5: Immunofluorescence in the M83 Transgenic Mouse 34
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABR</td>
<td>auditory brainstem response</td>
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<tr>
<td>Ala53Thr</td>
<td>point mutation in alpha synuclein</td>
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<tr>
<td>ANOVA</td>
<td>analyses of variance</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<tr>
<td>dB (SPL)</td>
<td>decibels (sound pressure level)</td>
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<tr>
<td>EP</td>
<td>endocochlear potential</td>
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<tr>
<td>Hz</td>
<td>hertz</td>
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<td>IHC</td>
<td>inner hair cell</td>
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<td>i.p.</td>
<td>Intraperitoneal</td>
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<td>kHz</td>
<td>kilohertz</td>
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<tr>
<td>KO</td>
<td>knock-out</td>
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<tr>
<td>MPTP</td>
<td>1-methyl-4phenyl-1,2,3,6-tetrahydropyridine</td>
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<tr>
<td>OHC</td>
<td>outer hair cells</td>
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<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>SNHL</td>
<td>sensorineural hearing loss</td>
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<td>wt</td>
<td>wild type</td>
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INTRODUCTION AND REVIEW OF THE LITERATURE

Auditory Physiology and Cochlear Mechanics

The cochlea is a structurally and functionally complex organ. Sound is funneled through outer ear and transmitted through the middle ear to the cochlear fluids where the final effect is to stimulate, appropriately, the sensory hair cells of the cochlea. The mammalian cochlea contains two classes of hair cells arranged in rows along the organ of Corti. Hair cells are neuroepithelial cells (Ashmore, 2008). Only a portion of the physiologic mechanisms used to enhance the auditory systems sensitivity have been correctly identified. The presence of two different classes of hair cells, the inner hair cell (IHC) and outer hair cell (OHC), in the organ of Corti were appreciated nearly one hundred years ago, yet the function of these cells have only become apparent in recent years. There are approximately 3,500 IHC and 11,000 OHC in each human cochlea (Ashmore, 2008). Anatomical studies indicate that the majority of the nerve fibers carrying information to the brain are in contact with IHC. This infers that most of the information from our acoustic world reaches the brain by means of the IHC (Alfred, 2006). We are aware that the cochlear amplifier is a functional process which has been determined to enhance the frequency selectivity of the cochlea due to a source of mechanical energy which is controlled by OHC motility. This amplifier contributes to physiologic auditory enhancement due to the OHC actively responding to acoustic stimulation. Hearing is known to be degraded when the OHC are damaged, likely due to the fact that the cochlear amplifier refines the sensitivity selectivity of the mechanical vibrations of the cochlea (Alfred, 2006) yet less is known about the
signal transduction between hair cells and the cochleovestibular nerve that carries the auditory signal from the cochlea to the brain.

**Synuclein Proteins**

Synuclein proteins are small, soluble proteins expressed primarily in neural tissues (Surguchov, 2008). The first synuclein was identified and named by Maroteaux, *et al.* (1988). Maroteaux and colleagues discovered synuclein proteins in neural nuclear envelopes as well as pre-synaptic terminals (Maroteaux, *et al.*, 1988 and Surguchov, 2008). The synuclein family of lipid binding proteins includes three known proteins: α-synuclein, β-synuclein, and γ-synuclein. Alpha and beta synuclein proteins are primarily localized to the brain, while γ-synuclein is localized more broadly, in tissues including brain, peripheral nerve, retina, and several tumors. Gamma-synuclein is a known marker for breast cancer (Ji *et al.*, 1997).

Alpha-synuclein has an easily changeable conformation. This configuration is altered considerably upon lipid binding and interactions with other ligands (Surguchov, 2008; Perrin, 2000). Alpha-synuclein also has many molecular isoforms that are generated as a result of different posttranslational modifications, alternative splicing, truncation, aggregation states and binding to other proteins and ligands. These isoforms have different features including conformation and localization (Surguchov, 2008). Due to these features, synucleins tend to transition from one form to another with changing properties. These properties depend on the physiological conditions of the cells and tissues where synucleins are expressed and make it increasingly difficult to define the protein’s function.

The majority of papers devoted to the synucleins admittedly indicate that the normal physiologic function of α-synuclein remains unknown (Surguchov, 2008). However, copious
research articles have suggested possible functions of the protein and list multiple processes which synucleins are involved in. These possible functions include synaptic plasticity, maintenance of the synaptic vesicle pool, and chaperone activity (Surguchov, 2008). Therefore, while the functional role of $\alpha$-synuclein may not be clearly defined, it is related with the maintenance of synaptic vesicle pools and also plays a role in several other cellular processes.

As previously stated, synuclein proteins are necessary for synaptic regulation and vesicle transport. Due to their function in synaptic regulation and vesicle transport, synuclein proteins are therefore likely involved in the synaptic activity of the auditory system. It was recently found that mice with targeted deletions of the gene that codes for alpha-synuclein exhibit elevated auditory brainstem response thresholds, suggesting a role for this protein in normal hearing mechanisms.

Over the past two decades, synuclein research has been highlighted with intriguing hypotheses, stimulating conclusions, and controversial information. Polymeropoulos, et al. (1997) were amongst the first to indicate that $\alpha$-synuclein is genetically and neuropathologically linked to Parkinson’s disease.

**Localization of Synucleins in the Cochlea**

As previously mentioned, synucleins have been localized to the presynaptic nerve terminals and nucleus. There is also research supporting the axonal transport of synuclein to the nerve terminals and the association of synuclein with synaptic vesicles (Surguchov, 2008).

Alpha-synuclein has commonly been localized to the efferent cholinergic neural auditory system. This is suggestive that alpha-synuclein plays a role in normal auditory function and in
turn, insinuates that this synuclein plays a role in auditory neurodegenerative disorders (Akil, *et al.*, 2008).

In 2008, Akil, *et al.* localized the three synuclein isoforms, alpha, beta, and gamma, within the mouse cochlea. Alpha-synuclein was localized predominantly to the base of IHC and OHC as well as the efferent tunnel crossing fibers. Knowing that alpha-synuclein localizes prominently to the neuronal system within the cochlea, it is essential to study the hair cell synapse.

**Role of Paraquat in Free Radical Damage**

Oxidative stress and free radicals are known to induce metabolic damage to structures, including the auditory system. Paraquat herbicide has been known to cause oxidative stress and the generation of free radicals. Paraquat has also been linked to neurodegenerative disorders, possibly due to the interaction between paraquat and the dopamine system (Thiruchelvam, *et al.*, 2000). Paraquat has been identified as a known inducer of apoptosis in neural cells (Melchiorri, *et al.*, 1998)

Paraquat is a toxic herbicide used primarily in third world countries that produces high levels of superoxide (Nicotera, *et al.*, 2004 and Bielefeld, 2005). Superoxide is the product of the one-electron reduction of dioxygen, and has been identified as a contributing factor to cochlear pathology from sources including noise and ototoxic drugs. Ototoxicity appears to be mediated primarily by the superoxide radical. The exact function of the superoxide radical in regards to cochlear damage is unclear (Nicotera, *et al.*, 2004) but the excess production of superoxide radical is sufficient to cause cochlear damage and hair cell loss.
Paraquat has been identified as causing oxidative stress (Nicotera, *et al.*, 2004). Oxidative stress has been proposed as a common factor in the etiology of sensorineural hearing loss (SNHL). By increasing the concentration of paraquat to a system, which can easily navigate through cell membranes, there is a systematic decrease in the number of hair cells. Nicotera, *et al.* identified that exposing in vitro samples of cochlear cultures to paraquat for 24 hours resulted in the destruction of OHC and IHC in a dose dependent fashion where the number of hair cells decreased in parallel with increasing concentration of paraquat. Contrary to most ototoxic drugs, there was no evidence that paraquat destroyed OHC before IHC. This is a similar pathological result to a SNHL.

Bielefeld, *et al.* (2005) performed inferior colliculus evoked potential testing on paraquat affected systems. Their results indicated similar findings in that inferior colliculus evoked potentials increase in threshold in a dose dependent manner. The paraquat treatment lead to a change in hearing threshold across the tested frequency range of 500Hz, 10kHz, 20kHz, 40kHz, and 80kHz. These results imply that paraquat affected cells from base to apex. A dose response to 10mM induced the greatest permanent threshold shift with mean shifts of 30-45dB. Ten millimolars of paraquat induced the greatest OHC and IHC loss with more damage occurring at the basal end than the apical end.

In 1996, Clerici and Yang first indicated that generators of superoxide, such as paraquat, induced significant shifts in the compound action potential threshold when introduced into the perilymph (Bielefeld, *et al.*, 2005). The electron transport chain in the mitochondria is thought to be a major source of intracellular superoxide. Hair cells are known to be highly demanding of energy and consuming of large amounts of oxygen (Bielefeld, *et al.*, 2005). Paraquat would
increase the demand for oxygen and tax the mitochondria causing more superoxide to be produced.

**Synucleins and Parkinson’s disease**

Due to the localization of alpha-synuclein and the ability of the protein to perform as a synaptic regulator, it has been suggested to play a role in neural regulation as well. Parkinson’s disease is a common neurodegenerative disorder characterized by the loss of dopaminergic neurons in the nigrostriatal pathway and the formation of intraneuronal inclusions called Lewy bodies. Alpha-synuclein is a major component found in Lewy bodies in Parkinson’s disease patients (Li, *et al.*, 2005) and alpha-synuclein mutations have been associated with clinical and pathological Parkinsonism in rare autosomal dominant familial cases (Polymeropoulos, *et al.*, 1997) Therefore, alpha-synuclein aggregation may be the cause of Lewy body formation and neurodegeneration (Manning-Bogt, 2001).

Upregulation of alpha-synuclein occurred when interactions between alpha-synuclein and paraquat *in vitro* studies were performed. (Manning-Bogt, 2001) Microscopic examination showed enhanced immunoreactivity in the midbrain of paraquat treated animals as well as a robust alpha-synuclein staining in the substantia nigra pars compacta after being exposed to the herbicide paraquat. (Manning-Bogt, 2001)

**Endocochlear Potential**

The endocochlear potential (EP) is an electrochemical potential found in the inner ear. The EP is a positive voltage of 80 - 100 mV seen in the endolymphatic space of the cochlea relative to surrounding spaces. It is highly dependent on metabolism and ion transport. In the
event of anoxia, an occluded blood supply or treatment with ion transport inhibitors such as ethacrynic acid or furosemide, the EP rapidly falls and becomes negative. Paraquat induced oxidative stress, which causes the generation of free radicals, would result in a metabolic change if introduced to the inner ear. The stria vascularis, which is thought to be responsible for maintaining the EP is susceptible to metabolic damage. Therefore, it is possible that by inducing metabolic changes within the inner ear, which would affect the stria vascularis, the EP may be subsequently altered as well.

**Synucleins in Vesicle Recovery at Ribbon Synapses**

Inner hair cells in the cochlea possess a unique synaptic specialization, a synaptic ribbon. Alpha-synuclein likely plays a regulatory role at the synaptic base of the synaptic ribbon. The electron-dense synaptic ribbon, or the synaptic body, of the hair cell afferent synapse has been recognized for decades and its known precision to code the temporal fine structure of acoustic stimuli is unparalleled. (George, n.d.)

Cochlear afferent neurons are faced with the challenge of encoding sounds of different intensities and durations. For this, they must rely on synaptic mechanisms that allow a high degree of temporal fidelity, yet can sustain a high level of neurotransmitter release. The hair cell afferent synapse’s capacity for sustained signal has been attributed to the presence of the synaptic vesicles (Nouvian, *et al.*, 2006).

Both directly-attached vesicles, referred to as docked vesicles, and nearby undocked vesicles constitute a pool of synaptic vesicles that could sustain seconds of exocytosis at most. With stimulation exhausting the number of docked vesicles, it is assumed that ongoing spontaneous and sustained driven release of hair cells requires mechanisms for rapid and
efficient replenishment of the readily releasable pool of vesicles (Fuchs, et al., 2003). Both physiological and anatomical evidence implicate alpha-synuclein in the regulation of synaptic vesicle populations within the presynaptic terminal.

Murphy, et al. (2000) examined the presynaptic morphology of cultured hippocampal neurons in which alpha-synuclein expression was partially reduced by treatment with antisense oligonucleotides. The numbers of docked vesicles were unchanged by antisense treatment. However, there was a significant decrease in the distal or undocked pool of synaptic vesicles in the alpha-synuclein depleted cells versus controls.

Studies using gene knock-out (KO) technology in mice also support a role for alpha-synuclein regulation of synaptic vesicle populations. Alpha-synuclein KO mice (Cabin, et al., 2002) displayed 50% fewer undocked vesicles in hippocampal synapses than wild type animals, while the numbers of docked vesicles remained unchanged. These mice had normal electrophysiological responses to stimuli expected to deplete only docked vesicles, but showed impaired responses to prolonged repetitive stimuli, which deplete both docked and undocked vesicles. Alpha-synuclein KO mice show impaired synaptic responses to stimulation designed to deplete docked vesicles as well as reserve pool vesicles (George, n.d.). This was interpreted to mean that replenishment of vesicles by the reserve population was slower. Therefore alpha-synuclein may be required for the maintenance of a ‘reserve’ subset of presynaptic vesicles.

As previously stated, the presence of alpha-synuclein likely aids in the maintenance of synaptic vesicles, researchers have investigated the over-expression of alpha-synuclein in synaptic regulation. Alpha-synuclein mice were not found to differ from wild-type animals in numbers of dopaminergic neurons in the substantia nigra (Abeliovich, et al., 2000). This is a surprising result, given the known association of human alpha-synuclein mutations with
nigrostriatal degeneration. The transgenic mice did exhibit more rapid recovery of dopamine released in a paired-stimulus paradigm. The authors conclude that alpha-synuclein negatively regulates dopamine neurotransmission, perhaps by recycling of the readily releasable (docked) pool of presynaptic vesicles.

In summary, evidence indicates that alpha-synuclein has a fundamental role in the maintenance and availability of synaptic vesicle pools in the presynaptic terminal. One might therefore reasonably expect that transgenic mice that over-produce alpha-synuclein would have a greater number of docked vesicles, and might more readily replenish the readily releasable pool of vesicles. This would ensure continued signal transmission during prolonged sounds.

**Alpha Synuclein and Neuroprotection**

While it has been suggested that alpha-synuclein is likely to play a role in neurodegenerative processes that underlie Parkinson’s disease (Polymeropoulos, *et al.*, 1997), some studies indicate a neuroprotective role of alpha-synuclein. In support of this hypothesis, *in vitro* findings have indicated that incubations of recombinant alpha synuclein in the presence of paraquat resulted in a dramatic acceleration of protein fibrillation. Mice that were exposed to either paraquat or Parkinson-inducing toxicant MPTP exhibited an up-regulation of alpha-synuclein that appeared to be a part of a neuronal response to toxic insults (Manning-Bogt, 2001). Moreover, while mice over-expressing alpha-synuclein displayed paraquat-induced protein aggregates, they were completely protected against neurodegeneration (Manning-Bogt, *et al.*, 2003). Using mice with transgenic expression of human wild-type versus Ala53Thr mutant alpha-synuclein, there was no indication of a significant difference between paraquat neurotoxicity in either mouse with alpha-synuclein over-expression. Collectively, these findings
dissociate toxicant-induced alpha-synuclein deposition and neurodegeneration, and argue that alpha-synuclein plays a protective role against toxic insults.

**Present Study**

The present study sought to evaluate the effect of alpha-synuclein on paraquat toxicity. We designed a protocol to evaluate whether paraquat induced oxidative stress is capable of causing a significant loss of auditory function. We also tested whether over-expression of alpha-synuclein is protective against oxidative damage in the cochlea. Protection was measured by changes in auditory brainstem response (ABR) threshold.
MATERIALS AND METHODS

Animals and paraquat administration

Genetically altered mice provide useful models for auditory neuroscience research and serve as highly effective models for assessing the function of the synuclein proteins in the auditory system. (Willot, et al., 1996) Two separate groups of mice were considered to evaluate the effects of paraquat and alpha-synuclein. A control group was comprised of six wild-type (wt) CBA/CaJ littermate animals. Three control animals received paraquat injections while three control animals received no treatment. A second group of twelve M83 transgenic C57-BL/6 mice; a sixth generation backcrossed onto a CBA/CaJ strain, and their wt littermates were studied to evaluate the effects of alpha-synuclein on oxidative damage in the cochlea. The M83 transgenic mice were bred with a gene for human alpha-synuclein inserted in order to produce excess amounts of this protein. All twelve animals, nine M83 transgenic animals and three wt littermates, received paraquat injections. Mice were purchased directly from The Jackson Laboratory (JAX) or were derived from breeders purchased from JAX. Prior to procedures and between test times, the animals were housed in a quiet colony in an approved facility at Washington University. They were kept on a 12/12-light/dark cycle. Food and water were available on an ad-lib basis.

Paraquat dichloride (Sigma) was dissolved in saline to a final concentration of 1mg/ml. Mice received intraperitoneal (i.p.) injections of paraquat dichloride hydrate twice a week for four consecutive weeks. Hearing sensitivity was assessed weekly for the duration of the injections. Animals were killed by cervical dislocation following eighth injection
**Electrophysiological Assessment**

To test hearing sensitivity, electrophysiologic evaluations were assessed by evoked ABR thresholds performed on M83 homozygous, heterozygous, and wild type mice. Threshold sensitivity was evaluated by ABR prior to paraquat treatment in order to obtain baseline hearing sensitivity thresholds. Animals were tested weekly following i.p. injections of paraquat (10mg/kg). Testing was performed at four time points; one week post 2 injections, two weeks post 4 injections, 3 weeks post 6 injection, and four weeks post 8 injections. Pre-exposure thresholds were subtracted from the four week measurements to calculate threshold change at each frequency time point.

Tucker-Davis Technologies (TDT) System II hardware and BioSig 32 software were used. Calibration occurred prior to each recording session. Animals were intraperitoneally injected with a solution of ketamine and xylazine (80/15 mg/kg) to achieve a deep plane of anesthesia. Subdermal needle electrodes were placed in the mid-back (ground), medial to the pinna of the tested ear (active), and at the vertex (reference). Animal’s body temperature and heart rate were monitored throughout testing by using a rectal probe. Body temperatures were maintained at 37.5 ± 1.0°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. Filter settings were 100 to 10,000 Hz and speaker distance was 7 cm. ABR waveforms were recorded in 5dB (SPL) intervals and thresholds were identified by visual inspection. 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) Thresholds
were observed as the lowest level that the first peak (wave I) was visualized. All mice used for evaluation had baseline thresholds within normal limits.

**Histological Assessment**

**Tissue Preparation**

Localizing the synuclein protein provided helpful cues in speculating the functional role the synuclein family plays in the cochlear structures. Following the final physiological measurements, animals were sacrificed by cervical dislocation. Cochlear structures were dissected and tissues were preserved using transcardial or round window perfusions to conserve pertinent cochlear structures. Cochlear structures were then embedded in paraffin and sectioned in the midmodiolar plane.

**Immunohistochemistry**

Paraffin sections were washed in xylene and ethanol solutions for deparaffination procedures. Prior to staining, all sections were pre-incubated in normal goat blocking serum for 30 minutes at room temperature. For oncomodulin immunohistochemistry, sections were incubated in oncomodulin primary solution (rabbit anti ocomodulin 1:500) for 4 hours before being rinsed in PBS solution 3 times for 5 minutes. Secondary antibody goat anti rabbit Alexa fluorescence 546 (1:200) was then applied to sections which were incubated for 30 minutes. Sections were washed in PBS solution 2 times for 5 minutes.

For alpha-synuclein immunofluorescence staining, Sections were incubated for 4 hours in primary guinea pig anti alpha-synuclein antibody (Abcam 16784) solution (1:2,000). After
washing sections in PBS solution 3 times for 5 minutes, sections were incubated with the corresponding secondary antibody, goat anti guinea pig (Molecular Probes 1:250) for 30 minutes. Sections were washed in PBS solution 2 times for 5 minutes prior to cover-slipping the sections for observation.

For beta-synuclein, sections were incubated in primary rabbit anti beta-synuclein (Epotomics 1537Y 1:200) for 4 hours. Sections were washed in PBS solution 3 times for 5 minutes prior to being incubated with corresponding goat anti rabbit secondary (Alexa fluorescence 488 1:250). Sections were rinsed in PBS solution 2 times for 5 minutes prior to cover-slipping for observation.

Gamma-synuclein immunofluorescence was performed by applying primary goat anti-gamma-synuclein antibody (Santa Cruz 10698 1:150) for 4 hours. Sections were then washed in PBS solution 3 times for 5 minutes and incubated with corresponding rabbit anti goat secondary (Alexa fluorescence 594 1:250). Sections were washed in PBS solution 2 times for 5 minutes prior to cover-slipping for observation.

For all samples, alpha-, beta-, and gamma-synuclein, slides treated with the same technique but without incubation of the primary antibody were used as a control. Slides were observed using a Zeiss confocal microscope.

**Statistical Analyses**

In order to assess the possible function of α-synuclein, a comparison of mean values of ABR threshold changes were compared between M83 transgenic mice and wt M83 mice was performed with two-way analyses of variance (ANOVA) with Bonferroni correction. A Holm-
Sidak multiple comparisons significance was defined as a p value less than 0.001. This test was used to identify differences at specific frequencies amongst different genotypes.
RESULTS

_Electrophysiology_

_Baseline Auditory Thresholds_

Figure 1 indicates baseline auditory thresholds of the M83 transgenic animals and their wt littermates. Electrophysiological responses for initial hearing sensitivity revealed there to be no obvious phenotype, either physical or behavioral, observed in the transgenic alpha-synuclein mouse relative to wt littermates.

_Threshold Changes_

Figure 2 represents the results of the mean threshold shift for the control study used to confirm paraquat toxicity. A mean threshold for all six wt control CBA/CaJ mice indicate indicates a baseline threshold of hearing sensitivity for these wt animals. Three animals received no treatment, and three animals received eight paraquat injections, two times a week for four weeks. A Two-Way Repeated Measures ANOVA revealed a significant interaction between treatment and frequency (p< 0.001). The effect of treatment within frequency was significant at all frequencies tested.

Figure 3 represents the overall threshold change for M83 transgenic animals and their wt counterparts. All twelve animals received paraquat injections twice a week for four consecutive weeks. Overall threshold change was then calculated by subtracting pre-exposure baseline thresholds from the four week threshold measurements at each frequency tested. A Two-Way
Repeated Measures ANOVA revealed no significant interaction between treatment and frequency for threshold shifts across the range of frequencies tested. The Holm-Sidak multiple comparisons analysis did not reveal a significant effect of genotype at any individual frequency tested.

**Immunohistochemistry**

In completing post mortem histological examinations on the mice tested in the study, it was found that the general cochlear architecture was sound. Immunohistological analysis of wt and control mice indicated the presence of alpha-synuclein in the murine cochlea. Triple labeling immunofluorescence was performed to determine the location of the synuclein isoforms within the inner ear. As seen in Figure 4, OHC are labeled in blue by a calcium binding protein, oncomodulin. Supporting Dieter’s cells are labeled with antibodies to gamma-synuclein as seen in burgundy. Alpha-synuclein, labeled in green, was consistently concentrated in the synaptic base of OHC and IHC between oncomodulin positive OHCs and gamma synuclein positive Dieter’s cells. Alpha-synuclein was also localized to the efferent tunnel-crossing fibers and beta-synuclein was localized primarily to the spiral ganglion (data not shown).

**Immunofluorescence in the M83 Transgenic Mouse**

Immunofluorescence within the M83 alpha-synuclein transgenic mouse model indicated alpha-synuclein expression primarily in the base of OHCs, weakly at the base of IHC, and strongly in the stria vascularis as seen in Figure 5. Immunofluorescence within the wt mouse model indicated the presence of alpha-synuclein at or near the base of OHCs, weakly at the base of IHCs, and noticeably absent in the stria vascularis.
DISCUSSION

Research is needed to assess the functional and histological role of the alpha-synuclein protein in hopes of identifying the role of this synuclein isoform in neural function and neurodegenerative diseases. The recent identification of the synuclein proteins within the auditory neuronal system raises interesting questions about their functional role and possible pathological or protective attributes within the inner ear.

Initial research identified and outlined the ribbon synapse surrounded by both docked and undocked synaptic vesicles. These ribbon synapses are located within the mammalian inner ear, and are specifically associated with hair cells in the organ of Corti. Prior work by other groups provided evidence that alpha-synuclein, which has been identified as a synaptic regulator, has an effect on the number of undocked synaptic vesicles available to the ribbon synapse. The number of undocked synaptic vesicles decreases when the expression of alpha-synuclein was reduced. Therefore the undocked synaptic vesicles, which are necessary to replenish the docked vesicles as they are fired in response to stimulus, are less efficient at replacing the docked vesicles and achieving the accurate response to stimulus which is necessary to preserve hearing sensitivity. Mice with a decrease in alpha-synuclein expression had normal electrophysiological responses to stimuli expected to deplete only docked vesicles, but impaired responses to prolonged repetitive stimuli, which depletes both docked and undocked vesicles.

We confirmed the presence of the three synuclein isoforms within the mammalian organ of Corti. Alpha-synuclein profiles were localized at or near the base of both IHC and OHC. By locating this synuclein protein to the synaptic terminals, we can further support the hypothesis
that alpha-synuclein functions as a regulator of synaptic transmissions between sensory hair cells and spiral ganglion neurons.

Immunofluorescence in the M83 transgenic mouse model indicated an up-regulation in alpha-synuclein expression in the stria vascularis compared to wt littermates. Endocochlear potential testing was performed on several M83 transgenic mice to evaluate a potential effect of this upregulation. The EP values measured fell in the normal range for C57/B6 mice. However, these measures were surprisingly variable. We intend to repeat the EP measurements once our mouse model is fully backcrossed to a CBA background.

Electrophysiological results indicated that by introducing paraquat herbicide to the auditory system, the system would be put in oxidative stress and, as a result, hearing threshold sensitivity increased at all frequencies tested. Knowing that paraquat induced oxidative stress caused a functional loss of hearing; paraquat was introduced to the murine model over-expressing alpha-synuclein. The over expression of alpha-synuclein was hypothesized to have a protective effect on the neurodegenerative result of paraquat. Our ABR data in the M83 transgenic and wt animals did not indicate a significant change in threshold; however M83 transgenic animal’s thresholds consistently remained closer to baseline thresholds when compared to their wt littermates over the majority of frequencies tested.

We can infer then that paraquat does cause oxidative stress leading to a loss of auditory function and augmented levels of alpha-synuclein appear to play a protective role in synaptic preservation. It is possible that functional ABR’s may not be specific enough to identify this protective role. Further testing should be conducted to evaluate the potential protection against the loss of auditory sensitivity due to alpha-synuclein over-expression.
This neuroprotective effect is at odds with findings in other experimental systems that have indicated a relationship between alpha-synuclein and neuronal injury. However there has been evidence that increased levels of alpha-synuclein do not necessarily lead to neurotoxicity due to the fact that AS over-expression in transgenic mice does not consistently induce neuronal damage. (Matsuoka, et al., 2001)

In agreement with our results, there have been in vitro findings that AS may play a protective role when faced with apoptotic stimuli. (Hashimoto, 2002). Our current results and previous data indicate that the toxic consequences of alpha-synuclein expression may vary significantly. The role pf alpha-synuclein must therefore be considered relative to specific tissues.

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 paraquat injection and electrophysiologic assessment, a significant effect may be seen across the range of frequencies tested.

The mice used in this study come from a mixed background. Currently we are crossing a C57-BL/6 mouse to a CBA/CaJ background. This may account for some of the variability between mice.

Clinical Implications and Future Studies

This current study was limited to the evaluation of hearing sensitivity alone. Future testing using the M83 transgenic mouse model and paraquat herbicide should include additional functional testing in order to identify a specific mechanism or site of protection. A battery of testing would be more appropriate. This battery should include EP testing to further examine the
effects of up-regulation within the stria vascularis of the M83 transgenic mouse as well as distortion product otoacoustic emissions testing to evaluate the effects of the protein on the cochlear amplifier. Gap Detection testing should be performed to evaluate the IHC and central auditory pathway, and finally additional ABR testing with an increased number of M83 transgenic animals and their wt littermates is appropriate once the mice are fully backcrossed onto a CBA/CaJ background. A histological evaluation including hair cell counts to evaluate damage would be necessary for the correlation of these functional tests. This battery of testing would serve to identify the site of the alpha synuclein neuroprotection that was suggested in this study.
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APPENDIX

FIGURE 1.

Figure 1- Baseline Auditory Thresholds: This is a line graph representing the mean ABR baseline threshold for M83 transgenic and their wild-type littermates. On the x-axis is frequency in kHz and the y-axis represents amplitude in dB SPL. The blue triangles represent the M83 transgenic animal’s mean baseline hearing threshold at each frequency tested. The red boxes represent the wt animal’s mean baseline hearing threshold at each frequency tested. This graph depicts that no obvious phenotype, either physical or behavioral, was observed in the transgenic alpha-synuclein mouse relative to wt littermates and these mice do not differ in baseline sensitivity.
Figure 2 – Overall Threshold Shift: This is a line graph indicating the threshold change of the control CBA/CaJ group of animals. On the x-axis are frequencies tested in kHz. The y-axis indicates ABR threshold in dB SPL. The blue circles represent the mean baseline thresholds for all control animals. The red squares represent the ABR thresholds of mice that did not receive paraquat injections. The green triangles represent the ABR thresholds of animals who received the paraquat protocol of 8 injections over a 4 week time interval. A Two-Way repeated Measures ANOVA revealed a significant interaction between genotype and frequency, p < 0.001 (0.0000441). Genotype within frequency was significant at all frequencies tested.
Figure 3 – Overall Threshold Change: This is a bar graph indicating the overall threshold change for M83 transgenic animals and their wt counterpart mice. On the x-axis is frequency in kHz and the y-axis is ABR thresholds change in dB SPL. Threshold change was calculated by subtracting pre-exposure thresholds from the four week threshold measurements at each frequency tested. The blue bar represents the M83 transgenic animals, and the red bar represents their wild-type counterparts. By looking at this graph, it seems as though the M83 homozygous mice have better thresholds, but this difference is not statistically significant.
FIGURE 4.

Figure 4 – Synuclein localization in the Normal Mouse organ of Corti: This is an example of triple label immunofluorescence localizing each of the synuclein isoforms. In this photo the outer hair cells have been labeled with the calcium binding protein, oncomodulin, in blue. Dieter’s cells are labeled with antibodies to gamma-synuclein as seen in burgundy. Alpha-synuclein profiles, presumably synaptic terminals, are labeled green and appear to lie near the base of IHCs and OHCs, between oncomodulin-positive OHCs and gamma-synuclein-positive Dieter’s cells. The phase contrast photograph is shown at the right for comparison.
Figure 5 – Immunofluorescence in the M83 Transgenic Mouse: This is an example of alpha-synuclein immunofluorescence localizing in an M83 transgenic animal. In this photo alpha-synuclein, labeled in green, is expressed primarily in the synaptic base of the outer hair cells as well as in the stria vascularis. Immunofluorescence within the wild type mouse model indicates the presence of alpha-synuclein, in green, at or near the base of outer hair cells and the absence of alpha-synuclein in the stria vascularis. OHC have been labeled in red for landmark purposes. There is an obvious up-regulation of alpha-synuclein in the stria vascularis of the M83 transgenic mouse model.