12-2014

Aminoglycoside-Induced Otoneurotoxicity: Analysis of Inner Hair Cell Synaptic Plasticity Following Drug Exposure

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AMINOGLYCOSIDE-INDUCED OTONEUROTOXICITY: ANALYSIS OF INNER HAIR CELL SYNAPTIC PLASTICITY FOLLOWING DRUG EXPOSURE

by

Matthew M. Abernathy

A dissertation submitted to the Graduate College
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
Biological Sciences
Western Michigan University
December 2014

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Aminoglycoside antibiotics are powerful drugs for combating bacterial infections, but are limited in use due to their ototoxicity. This class of drug targets the auditory hair cells of the cochlea, causing cell death, which leads to a decline in auditory function. In spite of much research aimed at revealing a mechanism of damage, there are no co-therapies available to diminish the ototoxic liability of aminoglycosides. Existing research does show that there may be a neurodegenerative process that contributes to the observed toxicity. In an effort towards clarifying present understanding of issue, this dissertation project was conducted to characterize the neurodegenerative effects of kanamycin, a commonly used aminoglycoside antibiotic. Specifically we assessed the effects of kanamycin on inner hair cell ribbon synapse degeneration and plasticity in the guinea pig.

The project first identified the drug concentration and number of doses necessary to induce the desired effect, loss of inner hair cell ribbons. The kanamycin dosing regimen employed produced significant high frequency hearing loss with associated outer hair cell loss 14 days following the last administration. Although there was no inner hair cell loss at this time, treatment caused significant decreases in inner hair cell ribbons in the furthest basal regions of the cochlea. The next phase of the project examined how ribbon densities changed over time following kanamycin administration. Here, results
demonstrated that doses of kanamycin administered for 10 consecutive days generated significant reductions in ribbon densities acutely, followed thereafter by ribbon regeneration. The hearing deficits and outer hair cell losses, in addition the severity of the ribbon density changes were more severe with the increased dosage. Interestingly, the postsynaptic spiral ganglion neurons were not affected at 56 days from the last dose. The next project specifically looked at how kanamycin affected the inner hair cell ribbon and associated postsynaptic glutamate receptors. The results showed similar synaptic loss following dosing and confirmed that synaptic regeneration occurs over time. The final experiment in this series looked at how intra-aural gentamicin affects synapse densities acutely. The results indicate that intra-aural administration produces greater synaptic losses throughout the cochlea as compared to systemic administration. In conclusion, this project established the role of synaptopathic effects of aminoglycoside ototoxicity, and additionally illustrated the capacity for regeneration following the initial expression of such ototoxicity.
ACKNOWLEDGMENTS

I would like to thank everyone who has helped mentor and support me through my graduate career. I would like to first thank Drs. Eversole and Spitsbergen for enabling me to work through my graduate degrees in a non-traditional fashion. Both of you have played a critical role in my graduation by encouraging and leading me through my research. I would also like to thank Drs. Josef Miller and Richard Altschuler for providing the expert research guidance needed to succeed in my research. Dr. Altschuler I particularly appreciate the time you dedicated to helping me better understand how to process and analyze data to become a better scientist. Your efforts have largely contributed to the success of this dissertation project. I cannot fully express my gratitude for all that Dr. Baird has done for me during my graduate education. You have steered me in the right direction many times, and also been a source of support during my setbacks. Without your support and belief in me, I do not believe I could have achieved this goal. I have enjoyed you as a mentor and a friend, and look forward to working with you throughout my career. Lastly and most importantly, I would like to thank my wife. Tiffany you have never stopped believing in me. You were there for my successes but more importantly lifted me up during my failures. When I wanted to settle, you never let me quit, and for that I am forever thankful to you.

Matthew M. Abernathy
TABLE OF CONTENTS

ACKNOWLEDGEMENTS .................................................................................................................. ii

LIST OF TABLES .......................................................................................................................... vii

LIST OF FIGURES ....................................................................................................................... viii

CHAPTER

I. INTRODUCTION ...................................................................................................................... 1

   Physiology of Hearing ................................................................................................................. 1

   Aminoglycoside Ototoxicity ....................................................................................................... 3

   Impact of Ototoxicity on Neurodegeneration ........................................................................ 10

   Specific Aims ............................................................................................................................ 13

   References ............................................................................................................................... 14

II. CHARACTERIZATION OF INNER HAIR CELL RIBBON DENSITIES THROUGHOUT THE COCHLEA FOLLOWING 7 DAYS OF KANAMYCIN EXPOSURE .................................................................................................................. 29

   Introduction ............................................................................................................................. 29

   Methods ................................................................................................................................... 31

   Subjects .................................................................................................................................... 31

   Study Design ............................................................................................................................ 32

   Auditory Brainstem Response (ABR) Evaluations ............................................................ 32

   Tissue Processing ..................................................................................................................... 33

   Imaging and Analysis ............................................................................................................. 34
CHAPTER

Statistical Analyses……………………………………………………………………………….. 34
Results ……………………………………………………………………………………………… 35
Auditory Brainstem Response Evaluations……………………………………………………… 35
Auditory Hair Cell Analysis……………………………………………………………………….. 36
Inner Hair Cell Ribbon Density…………………………………………………………………… 37
Discussion………………………………………………………………………………………….. 41
References………………………………………………………………………………………... 43

III. EXPERIMENT 2: TIME COURSE EVALUATION OF INNER HAIR CELL RIBBON DENSITIES FOLLOWING 10 DAYS OF KANAMYCIN EXPOSURE……………………………………………………………………………………………………... 51
Introduction………………………………………………………………………………………… 51
Methods…………………………………………………………………………………………….. 52
Subjects…………………………………………………………………………………………….. 52
Study Design………………………………………………………………………………………… 53
Auditory Brainstem Response (ABR) Evaluations…………………………………………….. 53
Tissue Processing…………………………………………………………………………………… 54
Imaging and Analysis………………………………………………………………………………. 56
Statistical Analyses…………………………………………………………………………………. 57
Results……………………………………………………………………………………………… 57
Auditory Brainstem Response Evaluations……………………………………………………. 57
Auditory Hair Cell Analysis………………………………………………………………………. 60
<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inner Hair Cell Ribbon Density</td>
<td>64</td>
</tr>
<tr>
<td>Type I Spiral Ganglion Density Evaluations</td>
<td>69</td>
</tr>
<tr>
<td>Discussion</td>
<td>70</td>
</tr>
<tr>
<td>References</td>
<td>73</td>
</tr>
<tr>
<td>IV. EXPERIMENT 3: TIME COURSE CHARACTERIZATION OF THE EFFECTS OF KANAMYCIN ON AUDITORY HAIR CELL SYNAPSE PLASTICITY</td>
<td>77</td>
</tr>
<tr>
<td>Introduction</td>
<td>77</td>
</tr>
<tr>
<td>Methods</td>
<td>80</td>
</tr>
<tr>
<td>Subjects</td>
<td>80</td>
</tr>
<tr>
<td>Study Design</td>
<td>80</td>
</tr>
<tr>
<td>Auditory Brainstem Response (ABR) Evaluations</td>
<td>81</td>
</tr>
<tr>
<td>Tissue Processing</td>
<td>82</td>
</tr>
<tr>
<td>Imaging and Analysis</td>
<td>83</td>
</tr>
<tr>
<td>Statistical Analyses</td>
<td>83</td>
</tr>
<tr>
<td>Results</td>
<td>84</td>
</tr>
<tr>
<td>Auditory Brainstem Response Evaluations</td>
<td>84</td>
</tr>
<tr>
<td>Hair Cell Analysis</td>
<td>85</td>
</tr>
<tr>
<td>Inner Hair Cell Synapse Density</td>
<td>86</td>
</tr>
<tr>
<td>Discussion</td>
<td>90</td>
</tr>
<tr>
<td>References</td>
<td>94</td>
</tr>
</tbody>
</table>
Table of Contents - Continued

CHAPTER

V. EXPERIMENT 4: ACUTE CHARACTERIZATION OF INTRA-AURAL GENTAMICIN ON INNER HAIR CELL SYNAPSE PLASTICITY……… 101

Introduction................................................................. 101
Methods........................................................................... 103
Subjects.......................................................................... 103
Drug Formulations......................................................... 104
Surgical Approach........................................................... 104
Auditory Brainstem Response (ABR) Evaluations................. 105
Tissue Processing............................................................. 106
Imaging and Analysis....................................................... 107
Statistical Analyses.......................................................... 108
Results............................................................................ 109
Auditory Brainstem Response Evaluations............................ 109
Inner Hair Cell Synapse Density......................................... 111
Discussion....................................................................... 114
References....................................................................... 116

VI. DISSERTATION SUMMARY AND CONCLUSIONS..................... 120

References....................................................................... 125
LIST OF TABLES

1. Inner Hair Cell Ribbon Densities by Cochlear Region………………………… 40

2. Type I Spiral Ganglia Density Measurements from Midmodiolar Section of the Basal Cochlea by Interval……………………………………… 70
LIST OF FIGURES

1. Effects of kanamycin administration on auditory threshold............................... 36
2. Effects of kanamycin administration on hair cells........................................... 37
3. Effects of kanamycin administration on inner hair cell ribbon density................. 39
4. ABR threshold 1 day following kanamycin administration............................... 58
5. ABR threshold 14 days following kanamycin administration............................. 59
6. ABR threshold 56 days following kanamycin administration............................. 60
7. % missing hair cells 1 day following kanamycin administration.......................... 62
8. % missing hair cells 14 days following kanamycin administration .................... 63
9. % missing hair cells 56 days following kanamycin administration .................... 64
10. (10A) Inner hair cell ribbon density 1 day following kanamycin administration; (10B) image of control ribbon synapses; (10C) image of treated ribbon synapses......................................................... 66
11. (11A) Inner hair cell ribbon density 14 days following kanamycin administration; (11B) image of control ribbon synapses; (11C) image of treated ribbon synapses......................................................... 67
12. (12A) Inner hair cell ribbon density 56 days following kanamycin administration; (12B) image of control ribbon synapses; (12C) image of treated ribbon synapses......................................................... 68
13. Example midmodiolar section with labeled cochlear turns.............................. 69
14. Time course effects of kanamycin on ABR threshold...................................... 85
15. (15A) Average inner hair cell synapse density at 17.64 mm from apex by time interval; (15B) % missing hair cell loss at 17.64 mm from apex by time interval................................................................. 88
List of Figures - Continued

16. (16A) Average inner hair cell synapse density at 18.62 mm from apex by time interval; (16B) % missing hair cell loss at 18.62 mm from apex by time interval.................................................................................................................. 89

17. Representative 63X projection images of ribbon synapses from (17A) 1 day control, (17B) 14 day control, (17C) 56 day control, (17D) 1 day treated, (17E) 14 day treated, and (17F) 56 day treated tissues........................................... 90

18. Representative 63X projection images (x-z axis) from a (18A) day 14 control inner hair cell, (18B) day 14 treated inner hair cell, and (18C) day 58 treated inner hair cell from the 17.64 mm from apex........................................ 93

19. The average 32 kHz auditory threshold for the right (gentamicin-treated) ear and left (saline-treated) ear presented by time interval.......................................................... 109

20. % missing hair cells following direct middle ear administration of gentamicin. 111

21. (21A) Inner hair synapse density following direct middle ear administration of gentamicin Representative image of inner hair cells with associated ribbon synapses in (21A) control and (21B) treated tissues......................................................... 113
CHAPTER I

INTRODUCTION

Physiology of Hearing

Hearing is an important sense to possess as it facilitates communication, spatial orientation, and enables survival by detecting noises around us. Sound stimuli from our environment pass through the external ear into the middle ear where these are amplified by the tympanum and ossicles and transmitted into the inner ear (Møller, 2006). The cochlea of the inner ear is a fluid filled compartment which is separated into three chambers, the scala tympani, scala vestibuli, and scala media. The scala vestibuli and tympani are filled with a fluid call perilymph that is mainly characterized by high concentrations of sodium and low concentrations of potassium. The scala media is filled with endolymph which in contrast perilymph contains high levels of potassium and low levels of sodium. This ion gradient is actively maintained by sodium potassium pumps located in the stria vascularius (Roland and Rutka, 2004), and is an important feature allowing stimulus transduction.

The scala media contains a specialized organ called the organ of Corti. The organ of Corti contains thousands of specialized auditory sensory receptors (hair cells) that are differentiated by type according to their location and function. The outer hair cells are located in three organized rows along the basilar membrane, and act to help
direct active tuning of specific frequencies. The inner hair cells are organized in a single row along the inside of the basilar membrane and are responsible for ~95 of afferent neurotransmission (Pickles, 2012).

One conserved feature of all auditory hair cells (inner and outer) is the presence of stereocilia. As sound waves move into the cochlea, pressure waves are created in the perilymph which transmits shearing forces on the stereocilia. As the stereocilia are deflected, potassium ions enter the cell through mechanotransduction channels, ultimately causing cellular depolarization (Pickles, 2012). Depolarization of the outer hair cells causes the cells to change in length (Brownell, 1990). This electromotility is thought to enhance sensitivity of selected frequencies (Dallos and Evans, 1995). Each outer hair cell varies in length and is “tuned” to a specific frequency of vibration and thus enhances pressure waves of a specific frequency as they move through the cochlea while other adjacent outer hair cells help dampen off-frequency pressure waves (Canlon et al., 1988).

The outer hair cell enhanced motion of the basilar membrane causes the associated inner hair cell to depolarize, which in turn causes afferent neurotransmission. More specifically, the inner hair cell depolarization causes voltage-dependent calcium channels to open, and the influx of intracellular calcium facilitates neurotransmitter release (Brandt et al., 2003). The inner hair cell possesses ribbon structures at the base of the cell which maintain and hold stores of neurotransmitter filled vesicles (Schmidtz, 2009). The fusion and release of these vesicles is calcium dependent providing a mechanism for coordinated and sustained release in response to different intensity stimuli.
(Johnson et al., 2008). This function is critical in maintaining temporal resolution during normal hearing.

It has been shown that the afferent nervous system of the inner ear depends upon glutamateergic neurotransmission. Specifically, inner hair cells release glutamate into the active zone of the afferent processes of the Type I spiral ganglia cells (Coate and Kelly, 2013). These Type I spiral ganglia cells are only connected to the inner hair cells and are myelinated. Type II spiral ganglia cells are a minor part of the total population and innervate only the outer hair cells. The afferent processes of the Type I spiral ganglia cells are depolarized when glutamate binds to AMPA and kainate receptors on their membrane surface (Peppi et al., 2012; Ruel et al., 1999). These spiral ganglion neurons transmit chemical signals up through the cochlear nerve to the cochlear nucleus and via other neurons to higher order centers of the auditory pathway where sound is processed and interpreted.

**Aminoglycoside Ototoxicity**

Aminoglycoside antibiotics are valuable antimicrobial tools used for the treatment of gram-negative bacterial infections (Mingeot-Leclercq et al. 1999). The bactericidal effectiveness of these drugs has led to their use for the treatment of serious illnesses such as multi-drug resistant tuberculosis (ATS-CDC, 1993). In addition to their effectiveness in treating disease, these drugs are relatively inexpensive, and therefore are widely used in developing countries for treatment of less serious infections. The primary mechanism
of action of these compounds is to disrupt protein synthesis by means of binding to the 16S rRNA, which causes early cessation of translational activity, and/or translational misreading (Mingeot-Leclercq et al. 1999).

The dose and frequency of administration of these type drugs is limited by well documented oto- and nephro-toxicities (Dwivedi et al. 2009; Song et al. 1997; Jiang et al. 2005). Evidence of ototoxicity was clear early-on, as a paper published in 1959 reported that 80% of Kanamycin-treated tuberculosis patients had hearing loss that was not present before treatment (Brouet et al, 1959). Preventing these unwanted side-effects would enable less constringent use of these compounds, which may augment the spectrum of their clinical applications. Given the low cost of this class of anti-infective, this could have a major impact on health care costs in developed countries and positively impact quality of life in developing countries.

The ototoxicity associated with aminoglycoside administration is characterized by a loss of the auditory hair cells and interrelated/connected neural components. The events leading to the eventual loss of these cell types is characterized below in support of the specific aims of this project.

**Aminoglycoside Uptake in Hair Cells**

Aminoglycosides begin exerting toxic effects when they cross the blood-labyrinth barrier and enter the endolymph of the cochlea and vestibule. This process begins when aminoglycosides are administered systemically and the drug molecules travel through the blood stream to the capillaries of the stria vascularis (Dai and Steyger, 2008). Aminoglycosides exit the endothelial cells of the capillaries and then enter the
endolymph through the marginal cells of the intrastrial space beginning in the basal turn of the cochlea (Steyger and Karasawa, 2008; Dai and Steyger, 2008; Dai et al., 2006).

Once in the endolymph, the aminoglycoside molecules enter the inner and outer hair cells via three different mechanisms. First, the aminoglycoside molecules have been shown to enter and subsequently block the mechano-electric transducer (MET) channels on the stereocilia of the outer and inner hair cells (Marcotti et al., 2005; Kimitsuki et al., 2001; Steyger et al. 2003; Gale et al. 2001; Owens et al, 2009). A second mechanism of entry is believed to originate from the aminoglycoside molecules binding with specific receptors on the cell surface, thus promoting endocytosis (Hasino and Shero, 1995; Steyger et al. 2003). Once inside the hair cells, intracellular trafficking of aminoglycoside-filled endosomes results in distribution of the aminoglycosides molecules into lysosomes, the Golgi apparatus, mitochondria, the nucleus, and the endoplasmic reticulum. The third and final mechanism of entry originates from data suggesting that aminoglycosides enter hair cells via non-selective cation channels; specifically transient receptor potential channel subtype vallinoid (Karasawa et al., 2008; Li and Steyger, 2009). This type of channel entry is supported by data showing that TRPV channel blockers decrease auditory hair cell uptake of fluorescent aminoglycosides in a dose-dependent manner (Lee et al., 2013).

**Induction of Apoptosis by Aminoglycosides**

Once the drug enters the auditory hair cells, the aminoglycoside molecules induce hair cell apoptosis. The typical pathology associated with aminoglycosides begins with the outer hair cells of the basal turn being initially affected with damage progressing to
the inner hair cells, gradually along the cochlear length (Fee, 1980). There exists strong evidence that the events leading to apoptosis are attributable to the production of reactive oxygen species (ROS) (Song et al. 1997; Parlakpinar et al. 2004; Sha and Schacht 1999; Priuska and Schacht 1995; Wang et al., 2003). Once inside the cell, the aminoglycoside molecule binds with free iron to create a redox-active complex which catalyzes the formation of ROS (O2, H2O2, OH, and ONOO-) subsequently reducing proteins and phospholipids (Jiang et al. 2005, Chen et al. 2007; McFadden et al., 2003). Increased production of these free radicals is supported by studies showing increased production of cochlear xanthine oxidase, nitric oxide (NO) synthase II, and decrease levels of glutathione, glutathione reductase, and glutathione peroxidase following aminoglycoside exposure (Takumida et al. 1999, 2003; Rybak et al., 2000). Interestingly, the graded difference in aminoglycoside associated hair cell loss between the apex and basal regions of the cochlea has been attributed to the higher levels of glutathione in the apical hair cells (Sha et al. 2000). The pivotal role that ROS play in aminoglycoside ototoxicity is further supported by the ameliorating effects that free radical scavengers and iron chelators produce in the context of aminoglycoside challenge (Conlon et al., 1999; Wang et al., 2003; Chen et al., 2007; LePrell et al., 2007; McFadden et al., 2003; Song et al., 1997; Fetoni et al., 2003).

The production of excess ROS disrupts hair cell homeostatic functioning, leading to a stressed cellular environment. Evidence suggests that aminoglycoside-associated hair cell death, in part, originates from the phosphorylation of c-Jun N-terminal kinase (JNK), a stress-induced MAP kinase (Wang et al., 2003; Ylikoski et al., 2002). Stresses
associated with the activation of this type of MAP kinase include changes in temperature and pH, and more importantly increases in free radicals (Davis, 2000). Increases in reactive oxygen species following aminoglycoside administration have been well documented, and may act as the major triggering event leading to apoptosis (Forge and Schacht 2000; Sha and Schacht 1999, Lesniak et al. 2005). Following phosphorylation, the JNK kinase promotes differential regulation of the Bcl-2 family of genes, which consist of pro- and anti-apoptotic proteins (Cheng et al., 2005). The proapoptotic Bcl-2 proteins that have been shown to increase during hair cell death are Bax, Bak, Bid, Bad, and Bim (Cheng et al. 2005). Conversely, the anti-apoptotic proteins Bcl-2 and Bcl-XL have been shown to decrease following drug treatment (Cheng et al. 2005; Abi-Hachem et al., 2010). The balance of these proteins acts as the determining factor promoting or inhibiting apoptosis. When the protein balance shifts towards proapoptosis, the Bax protein forms pores in the mitochondrial membrane subsequently causing the release of cytochrome C and free radicals (Cheng et al. 2005; Van De Water et al., 2004). The release of these free radicals adds to the oxidative insult already generated by the presence of the aminoglycosides. Free cytochrome C binds with apoptotic protease activating factor (APAF-1), dATP, and procaspase 9 to form the apoptosome (Van De Water et al., 2004). The apoptosome initiates the cleavage of procaspase 9 to form activated caspase 9 (Eshraghi et al., 2007; Van De Water et al., 2004). Once activated, caspase 9 activates caspase 3 (Davis, 2000; Hu et al., 2008, Dehne et al. 2002). Caspase 3 is an effector caspase and is ultimately responsible for executing cell death. This caspase protein first acts to remove select apoptosis inhibitory proteins (IAPs) (Van De Water et
al., 2004). After removal of these select proteins, caspase 3 promotes cell death by degrading proteins such as poly (ADP-ribose) polymerase (PARP-1), DNA topoisomerase, and transducer/activator of transcription-1, which all involve cellular repair and protein synthesis (Jiang et al., 2006; Van De Water et al., 2004). Inactivation of these types of proteins results in nuclear condensation and fragmentation, which represents the final stages of apoptosis (Hu et al. 2008).

There is also evidence to suggest that ROS may additionally trigger cell death via caspase-independent mechanisms. As suggested above, aminoglycosides can enter the cell and create ROS, which subsequently attack cell and organelle membranes. In the case of the mitochondria, membrane disturbances can cause the release of apoptosis-inducing factor (AIF) and endonuclease G (Endo G) (Jiang et al., 2006). Endo G is a Dnase, which once released from the mitochondria can enter the nucleus and cause DNA fragmentation (Li et al., 2001). AIF acts similarly when released from the mitochondria as it translocates to the nucleus and causes chromatin condensation and DNA fragmentation (Cande et al., 2002). Other studies have indicated that the influx of calcium associated with aminoglycoside administration results in the activation of Calpains, a class of calcium dependent proteases (Momiyama et al., 2006; Jiang et al., 2006). Once activated, calpains can bind to lysosomal membranes and cause the release of cathepsins (Jiang et al., 2006). Cathepsins cleave PARP-1 (like caspase 3), which in turn initiates cell death (Syntichaki et al., 2002). The role of calpain activity in AGA associated hair cell death is supported by the prevention of hair cell loss in-vivo with administration of Leupeptin, a calpain inhibitor (Momiyama et al., 2006).
Interestingly beyond caspase-dependent and caspase-independent processes, there is a third postulated mechanism of cellular death (incorporating aspects of each of the above mechanisms) which involves reactive nitrogen species (RNS). Limited research exists about this pathway, but its contribution to the overall damage induced by aminoglycoside administration has been established by several studies. RNS production is believed to originate from excessive NMDA receptor activation (Segal et al., 1999; Basile et al., 1996; Takumida and Anniko, 2000). The polyamine-like structure of aminoglycosides has been shown to enable binding and subsequent activation of NMDA receptors (Pullan et al., 1992). In the presence of aminoglycosides, NDMA and non-NMDA (AMPA and Kainate) type receptors are activated which in turn facilitate massive Ca2+ entry into the auditory cell and spiral ganglion neurons (Takumida et al., 1999). It is proposed that the presence of high levels of Ca2+ induce the expression of nitrogen oxide synthase II (NOS II), which is capable of producing large quantities of nitrogen oxide (NO) (Takimuda et al., 1999; Takimuda and Anniko, 2000). NO quickly binds with superoxide molecules to form peroxynitrite (Takumida et al., 1999). Peroxynitrite is highly reactive and modifies proteins containing the amino acid tyrosine, e.g. actin and neurofilaments, and activates downstream cell death pathways (Takumida et al., 1999). There is evidence showing that prevention of peroxynitrite formation via administration of NMDA receptor antagonists and peroxynitrite scavengers is efficacious at reducing aminoglycoside ototoxicity (Takumida et al., 2001; Takumida and Anniko, 2000; Segal et al., 1999; Basile et al., 1999; Duan et al., 2000).
Impact of Ototoxicity on Neurodegeneration

There are multiple studies showing profound neurodegeneration of spiral ganglion neurons following aminoglycoside administration (Glueckert et al. 2008; Hong et al., 2006). Again, spiral ganglion cells are connected to the inner hair cells via afferent peripheral processes, and their central axonal process projects onto cells in the cochlear nucleus (McGuiness and Shepard, 2005; Spoendlin and Schrott, 1988; Nadol, 1988).

The loss of the spiral ganglion cells following aminoglycoside administration is secondary to the loss of the afferent peripheral processes and inner hair cell synapses, which has been shown to occur within 3 days of aminoglycoside/loop diuretic exposure (Duan et al., 2000; Glueckert et al., 2008). Spiral ganglion cells begin to significantly degenerate approximately 2 to 5 weeks following a standard 7-day treatment with an ototoxic dose of kanamycin (Jyung et al, 1989; Poirrier et al., 2010). Due to the length of time necessary to induce the loss of the spiral ganglion cells, it is generally believed that the degeneration of these cells is due to a lack of neurotrophic support associated with the loss of inner hair cells and/or damage to support cells (Duan et al., 2000; McFadden et al., 2004; Mattson and Furukawa, 1998). This hypothesis is supported by the rescue of spiral ganglion cells and peripheral processes by administration of various neurotrophic factors (Miller et al. 2007; McGuiness and Shepard 2005; Miller et al., 1997).

The neurodegeneration observed following many cochlear pathologies has been shown to originate with the loss of the inner hair cell synapse. The presynaptic inner hair cell ribbon is responsible for coordinated glutamate release onto afferent spiral ganglion
processes which is necessary for precise temporal resolution during audition. The inner hair cell ribbon is composed of multiple proteins that act as a scaffold for the glutamate filled vesicles (Zanazzi and Matthews, 2009). The activation of these ribbon structures has been linked to voltage gated L-type calcium channels, which are believed to enable sustained neurotransmitter release (Meyer et al., 2009). In the case of noise trauma, glutamate is released in large quantities, which then activates the postsynaptic NMDA receptors causing massive sodium and water entry into the afferent neurons (Puel et al., 1997). It has been reported that within 24 hours of noise trauma, over half of the ribbon structures and afferent processes are completely degenerated (Kujawa and Liberman, 2009).

Kujawa and Liberman (2009) reported that synapse ribbon structures decline in numbers following a noise insult, but the auditory deficits incurred are temporary as threshold sensitivity returns to baseline levels weeks after the insult. In their study, auditory function recovered and no hair cell losses were observed but the ribbons structures were lost permanently. At 1 and 2 years following the injury, the inner hair cells were found to be still intact, however, substantial losses in spiral ganglion cells were observed.

Similarly in terms of response to cochlear trauma, Chen et al. (2013) showed that inner hair cell ribbon synapse densities decrease acutely following repeated aminoglycoside administration in mice. There have been several other studies that have provided insights into the effects of aminoglycosides on inner hair cell neurotransmission. For instance, a recent study found that expression of the vesicle
fusion protein, Otoferlin, is up-regulated in mice during a standard course of aminoglycoside treatment (ShuNa et al., 2009). Otoferlin is an essential transmembrane protein found in inner hair cells that facilitates vesicle exocytosis, and when absent leads to severe deafness (Roux et al., 2006). Of particular importance is the fact that Otoferlin facilitates glutamate vesicle exocytosis in a calcium-dependent manner (Ramakrishnan et al., 2014). It has been suggested that in the presence of aminoglycosides, NDMA and non-NMDA (AMPA and Kainite) type receptors are activated which in turn facilitate massive Ca2+ entry into inner hair cells (Takumida et al, 1999). This hypothesis is supported by the sparing of inner and outer hair cells by administration of leupeptin, a calcium-activated protease inhibitor, in models of aminoglycoside ototoxicity (Ding et al., 2002; Momiyama et al., 2006). Together, these data suggest that aminoglycosides induce calcium entry into the inner hair cell via NMDA receptors, which in turn increase the activity/expression of Otoferlin.

Understanding the time course of effects of aminoglycosides on ribbon synapse densities in relation to inner hair cell and spiral ganglion loss, would provide critical insight into the long-term ramifications of aminoglycoside use on auditory neurotransmission. If found that aminoglycosides induce inner hair cell synapse density decreases with only a mild effect on hair cell viability, the hearing loss observed following use may involve a major neurodegenerative processes that has not been considered when developing treatments to prevent this unwanted side effect.
Specific Aims

The specific aim of this dissertation project was to understand the effects of kanamycin on inner hair cell synapse plasticity. It was the goal of the project to understand how different concentrations of the drug affect inner hair cell synapse densities. It was also a goal to understand if synaptic degeneration occurs overtime and how this affects spiral ganglion viability.

Specific Aim 1:
Test the hypothesis that kanamycin treatment will result in a dose-dependent decline in the number of synaptic structures, as represented by the presence of presynaptic ribbons.

Specific Aim 2:
Test the hypothesis that a low dose of kanamycin, e.g. a dose capable of inducing outer hair cell loss without inner hair cell loss, will cause a loss of synaptic structures without loss of the corresponding inner hair cells.

Specific Aim 3:
Test the hypothesis that inner hair cell synapse degeneration without inner hair cell death eventually leads to spiral ganglion cell death.

Specific Aim 4:
Test the hypothesis that the concentration of aminoglycosides in the perilymph of the cochlea impacts the timing and severity of synaptic loss observed.
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CHAPTER II

CHARACTERIZATION OF INNER HAIR CELL RIBBON DENSITIES THROUGHOUT THE COCHLEA FOLLOWING 7 DAYS OF KANAMYCIN EXPOSURE

Introduction

It has been well demonstrated that prolonged exposure to systemic aminoglycoside antibiotics can cause ototoxicity in many species. Given this type of compound’s potent bactericidal properties, much effort has been exerted to ameliorate this adverse side-effect. As indicated in the introduction to this dissertation, it is believed that aminoglycosides form metabolites that cause cellular stress, ultimately leading to apoptosis. Interestingly, there have been many different types of therapies developed with the aim of decreasing cellular stress, e.g. antioxidants, in efforts to reduce this ototoxicity (Le Prell et al., 2014; Ojano-Dirain et al., 2014; Fetoni et al., 2012; Wang et al., 2003). Although moderate hair cell sparing has been reported, these results further suggest that the mechanism is more complex than originally thought.

Aminoglycoside ototoxicity has been shown to have dose dependent properties with regard to the auditory functional deficits and hair cell toxicity (Montalbano et al., 2014; Gong et al., 2012; Maniu et al., 2011). The outer hair cells appear most sensitive to drug treatment followed by the inner hair cells (Schmitz et al., 2014; Ye et al., 2009). Interestingly, the outer hair cells within the base of the cochlea are more sensitive to aminoglycosides than the hair cells in the mid and apical regions, a finding which may be
related to endogenous mechanisms of stress handling (Choung et al., 2009; Wu et al., 2002; Sha et al., 2001). The inner hair cell is responsible for the majority of neurotransmission and it has been shown that once inner hair cells die, associated spiral ganglion cells degenerate (Safieddine et al., 2012; van Loon et al., 2013; Bae et al., 2008). It has been proposed that inner hair cells and cochlear support cells produce neurotrophins which maintain nerve viability, and therefore the loss of these cells contribute to secondary nerve degeneration (Yu et al., 2013; Miller et al., 1997).

There are other types of cochlear insults that produce ototoxicity and nerve degeneration similar to that observed with aminoglycosides. Acoustic overexposure, a mechanism of noise- induced cochlear damage, has been shown to cause robust hair cell loss and afferent neuron degeneration (Bielefeld et al., 2011; Park et al., 2013; Wang and Ren, 2012; Kujawa and Liberman, 2006). Kujawa and Liberman (2009) reported that synapse ribbon structures in mice decline in numbers following a noise insult, resulting in a temporary auditory threshold shift that resolved several days following the injury. Following this type of injury, the ribbon structures were lost without corresponding catastrophic change to the inner hair cell; the mechanism of this selective sub-cellular modification was thought to be excessive glutamate release. When assessed 1 and 2 years following the injury, the inner hair cells were found to be still intact, however, substantial losses in spiral ganglion cells were observed. Lin et al. (2011) confirmed that the same neural degeneration following noise-induced temporary threshold shifts occurs in the guinea pig.
No similar data exist characterizing the effects of nominal doses of AGAs on glutamate ribbons in the guinea pig. The similarity of cell-death pathways involved in noise- and aminoglycoside-induced pathology allow us to hypothesize that such a mechanism (e.g., low dose AGA treatment) will result in loss of synapses and spiral ganglion cells without the loss of inner hair cells. This study was conducted in an attempt to establish that presynaptic ribbon densities decrease in the guinea pig following aminoglycoside exposure, and to define the distribution of the decrease in inner hair cell ribbon density along the length of the cochlea. Our results indicate that high doses of aminoglycosides produce significant inner hair cell ribbon density decreases in the basal region of the guinea pig cochlea.

Methods

Subjects

All animal use and the study protocol were approved by the Institutional Animal Care and Use Committee at MPI Research Inc., a fully AALAC-accredited research facility. Twenty male albino Hartley guinea pigs (Charles River laboratories, MI) were placed on study at 8 weeks of age. The animals were single housed in wire mesh caging and provided environmental enrichments. All subjects were placed on ad libitum access to both food and water and weighed between 200 and 400 g at the initiation of the experiment. The room was temperature and humidity regulated with fluorescent lighting provided on a 12 hour light/dark cycle (0600h – 1800h). Clinical observations were
conducted twice daily by AALAS-accredited research technicians that monitored food and water access as well as the general health and wellbeing of the animals.

**Study Design**

Twenty guinea pigs were placed into treatment groups of 5 animals per group. The animals received subcutaneous (SC) injections of saline, or kanamycin at 100, 200, or 400 mg/kg once daily for 7 consecutive days. The animals were allowed a 14-day recovery period prior to euthanasia. Kanamycin sulfate (Sigma Aldrich, MO) was formulated as an 80 mg/ml solution in normal sterile saline USP (0.9% sodium chloride). Normal sterile saline USP (Sigma Aldrich, MO) and formulated kanamycin doses were administered subcutaneously at a volume equivalent of 5 ml/kg. The animals were allowed a 14-day recovery period prior to euthanasia.

**Auditory Brainstem Response (ABR) Evaluations**

All animals underwent bilateral auditory brainstem response (ABR) evaluations at 4, 10, and 20 kHz prior to being assigned to study, and on the day prior to euthanasia. The sound presentation was generated using an RZ6 multi I/O processor (TDT Technologies, FL). The animals were anesthetized with an intraperitoneal drug combination of 40 mg/kg ketamine hydrochloride (100 mg/ml) and 0.25 mg/kg of dexmedetomidine (0.50 mg/ml). Once a stable plane of anesthesia was confirmed as a loss of pain reflex, the animals were placed in the prone position within an electrically shielded sound attenuation chamber. Pin lead electrodes were placed to collect the ABR biopotential data. The lead configuration used to collect the electrophysiological data consisted of a positive lead under the target ear, a reference lead at the vertex of the skull,
and a ground lead under the contralateral ear. The terminal ends of the pin leads were connected to a Medusa preamplifier with a 20X gain and a 4-channel headstage (TDT Technologies, FL). The stimulus used to elicit the ABR waveform was a 15 ms pure tone pip with a 1 ms rise/fall time. The tone pips were delivered at a rate of 10 cycles per second through a MF-1 transducer (TDT Technologies, FL). The stimulus intensity was attenuated in 10 dB steps beginning at 80 dB SPL. The biopotential data were averaged across the 512 stimulus presentations for each intensity level. An auditory threshold was defined as the minimal sound pressure level showing a reliable waveform with recognizable elements of the auditory brainstem response.

Tissue Processing

The day following the final ABR evaluation, all animals were euthanized by anesthesia with intraperitoneal sodium pentobarbital to effect. Whole body intracardiac perfusion was performed by the application of 0.9% saline followed by 4% w/v paraformaldehyde. The left and right temporal bones from each animal were harvested. A small hole was made in the apex of each cochlea and the oval window was perforated to facilitate intrascalvar perfusion of 4% paraformaldehyde. The cochleae were then stored in a solution of 0.5% paraformaldehyde and placed in refrigerated conditions (2-8°C) until further processing.

The cochleae were dissected to reveal the organ of Corti. The organ of Corti was then fluorescently stained to identify the auditory hair cells and inner hair cell ribbons. For staining, the tissues were first placed in a blocking solution consisting of 1% goat serum (Invitrogen, CA) and 0.3% Triton X-100 in phosphate buffered saline (PBS). The
cochleae were then incubated overnight in a primary antibody solution of 1:100 mouse anti-CtBP2 antibody (BD Transduction Laboratories, CA) at 2 - 8°C. The cochleae were rinsed and then incubated for an hour in a secondary antibody solution containing 1:200 Alexa Fluor® 488 Goat anti-mouse IgG (H+L) antibody (Life Technologies, CA) and 1:200 Alexa Fluor® 568 phalloidin (Life Technologies, CA).

**Imaging and Analysis**

All tissue imaging was conducted using a Zeiss Axiovert 200M confocal microscope at 63X magnification. Using a photo reticule, the organs of Corti were separated into 0.98 mm segments beginning in the apex and z-stack images were captured for each segment. For image capture, z-stacks were created using a 0.12 μm step size. The images were deconvolved and saved as individual planes. Each plane per region was then imported into ImageJ v1.47t for 3D reconstruction. A 3D project was created for each region allowing 90 degree rotation at the x-axis of the 3D image. Using the cell counter plugin, the number of CtBP2 ribbons was manually counted at the base of each inner hair cell. The present and missing outer and inner hair cells were quantified within each of these regions to assess the severity of drug-induced damage. The CtBP2 ribbon density per inner hair cell was then calculated for each region by dividing the total ribbon count by the number of inner hair cells present in the region.

**Statistical Analyses**

Hair cell counts and inner hair cell ribbon densities were compared between groups using descriptive statistics (mean ± SD). Each group’s hair cell counts were
averaged into 0.98 sections. The inner hair cell ribbons were counted and a density measurement was calculated for each region. The densities (ribbons/hair cell) in each region were averaged within each animal and then each group. All group comparisons were conducted using an Analysis of Variance (ANOVA) procedure, followed by a Tukey post hoc test. All statistical analyses were performed using Graphpad Prism v4.00.

**Results**

**Auditory Brainstem Response Evaluations**

The difference between pretest and postdose thresholds were averaged bilaterally for each animal and group, and are presented in Figure 1. As expected with this type of ototoxin, the 400 mg/kg dose of kanamycin produced a significantly increased 20 kHz threshold of 38.10 ± 16.63 (p≤0.01). The thresholds for the 4 and 10 kHz frequencies within the 400 mg/kg dose group were unaffected, presenting with averages that were similar to pretest and control values. The 100 and 200 mg/kg kanamycin doses did not significantly affect any of the thresholds for the frequencies assessed on study.
Figure 1. Effects of kanamycin administration on auditory threshold.

Auditory Hair Cell Analysis

The hair cell histology data for each group are presented in Figure 2. The number of missing outer and inner auditory hair cells were averaged into 0.98 mm segments beginning in the apex of the cochlea and are presented as successive regions. The 100 and 200 mg/kg dose groups displayed limited to minor damage to the outer and inner hair cells throughout the cochlea. In contrast, the 400 mg/kg dose caused significant damage to the outer hair cells beginning in region 13 and continuing through region 17.
Additionally in the 400 mg/kg dose group, some minor inner hair cell damage was detected beginning in region 14 and continuing through region 17. The outer hair cell damage observed in this dose group tonotopically supported a sensorineural basis for the observed 20 kHz ABR threshold shift.

**Figure 2.** Effects of kanamycin administration on hair cells.

**Inner Hair Cell Ribbon Density**

The inner hair cell ribbon densities were calculated every 0.98 mm. The average ribbon density was analyzed in successive regions beginning in the apex. Analysis of the
inner hair cell ribbon density data revealed moderate density decreases within the lower basal regions of the 400 mg/kg dose group (see Figure 3). The 400 mg/kg dose group’s ribbon densities significantly decreased by 35% in region 16 and 36% in region 17 when compared to control and the 200 mg/kg dose group (p<0.05). Table 1 shows the average inner hair cell ribbon density for each region of the cochlea in each group. The densities from the control group showed minor variability throughout the entire cochlea. There were no significant differences in density observed in any part of the cochlea in the 100 and 200 mg/kg dose groups as compared to control.
Figure 3. Effects of kanamycin administration on inner hair cell ribbon density.
<table>
<thead>
<tr>
<th>Cochlear Region</th>
<th>Control</th>
<th>200 mg/kg</th>
<th>400 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20.64 ± 2.16</td>
<td>20.91 ± 3.09</td>
<td>17.50 ± 1.61</td>
</tr>
<tr>
<td>2</td>
<td>24.28 ± 6.01</td>
<td>22.62 ± 4.07</td>
<td>20.75 ± 2.48</td>
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<td>21.74 ± 2.19</td>
<td>21.09 ± 4.51</td>
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<td>22.44 ± 4.15</td>
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<tr>
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<td>20.43 ± 2.76</td>
</tr>
<tr>
<td>8</td>
<td>21.17 ± 2.59</td>
<td>22.75 ± 3.87</td>
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<td>9</td>
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<td>17.84 ± 0.00</td>
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</table>
Discussion

The results of this study show that high doses of kanamycin produce significant reductions in inner hair cell presynaptic ribbons within the basal region of the cochlea. The highest dose of kanamycin used on study produced high frequency hearing loss with associated tonotopically consistent outer hair cell loss and no inner hair cell loss. The lower doses of kanamycin tested produced no functional deficits, hair cell loss, or ribbon density decreases. Interestingly, the regions that produced the most significant decreases in ribbon densities were associated with robust outer hair cell losses.

Important aspects of this study are the dose concentration, number of doses, and recovery period selected for use. The 400 mg/kg dose of kanamycin was selected based on numerous articles documenting the capacity of this dose to produce profound hair and spiral ganglion cell loss (Arpini et al. 1979; Hangfu et al. 1992; Song et al. 1998; Brown et al. 1980; Toyoda and Tachibana 1978; Poirrier et al. 2010; Yamane et al. 1983; Ishii et al. 1968; Hawkins and Engstrom 1964; Dallos and Wang, 1974). The 100 and 200 mg/kg doses of kanamycin are half and quarter concentrations of the high dose, and were selected to produce minimal ototoxicity in an effort to mimic a benign noise exposure.

The number of doses and the timing of the study were specifically designed to elicit a maximal response in the auditory hair cells. Each drug dose was administered for seven consecutive days to reflect a clinically relevant treatment time course. It has been shown that systemically administered aminoglycosides distribute to the perilymph of the inner ear (Li and Steyger, 2011; Wang and Steyger, 2009, Imamura and Adams, 2003). The clearance kinetics of these drugs in the inner ear differ from the systemic circulation.
in that the drug persists much longer in the inner ear (Hiel et al., 1992). Given the persistence of drug in the inner ear, multiple days of dosing reliably causes the drug to accumulate and increase in concentration in the endolymph surrounding the auditory hair cells (Erdem et al., 2005). We allowed a 14 day recovery after dosing and prior to the terminal assessments as this approach has been shown to elicit reproducible hair cell toxicity putatively due to the increased time of exposure (Pavlidis et al., 2014).

Another critical design consideration for characterizing inner hair cell ribbon degeneration following aminoglycoside administration was the use of guinea pigs. The guinea pig was selected to assess the effects of aminoglycosides on inner hair cell ribbon densities because this model has been shown to be highly sensitive to ototoxic therapies (Shi et al., 2014; Kashio et al., 2012, Poirrier et al., 2010). The primary model used to evaluate the function and plasticity of the inner hair cell ribbon synapse following noise insults has been the mouse (Yuan, et al., 2014; Tong, et al., 2013; Brugeaud, et al., 2013). We chose not to use the mouse for this model as the mouse is resistant to ototoxic damage rendering it less suitable in the context of characterizing the mechanisms associated with aminoglycoside ototoxicity (Taylor, et al., 2008). In illustration of this point, i.e. studies using mice have induced mortality with high doses of aminoglycoside without causing cochlear lesions (Murillo-Cuesta, et al., 2009).

In conclusion, this study confirms that aminoglycosides produce decreases in inner hair cell ribbon densities without associated inner hair cell toxicity. The ribbon density decreases detected in regions 16 and 17 suggest that there may an acute neurotoxic effect of aminoglycoside exposure that could be targeted to produce enhanced
protective therapies. Future studies should be conducted to establish the time course of effects on inner hair cell ribbon densities and the impact on spiral ganglion viability. Additionally, the connection between outer hair cell losses and ribbon density decreases should be examined by inducing a more severe cochlear lesion.

References


CHAPTER III

EXPERIMENT 2: TIME COURSE EVALUATION OF INNER HAIR CELL RIBBON DENSITIES FOLLOWING 10 DAYS OF KANAMYCIN EXPOSURE

Introduction

Previously, we revealed that kanamycin administration produced significant decreases in presynaptic inner hair cell ribbons after seven days of administration. In this experiment, inner hair cell ribbon densities decreased specifically in basal regions associated with increased outer hair cell losses and minimal to no inner hair cell losses. Lower doses of kanamycin were administered on study, but they did not produce changes in ribbon densities or hair cell losses. This suggests that a level of hair cell toxicity must be achieved to induce ribbon density changes.

Type I spiral ganglion neurons are connected to inner hair cells and represent ~95 percent of the neuronal population of the ganglion (Coate and Kelly, 2013). These neurons are maintained by neurotrophins that are expressed from the inner hair cells and cochlear supports cells (Despres et al., 1994, Medd and Bianchi, 2000, Zilberstein et al., 2012). In models of aminoglycoside ototoxicity, auditory hair cell death occurs acutely following treatment and secondary spiral ganglion degeneration occurs weeks to months later (Jeong et al., 2010, Alam et al., 2007).
Models of noise-induced synaptopathy demonstrate that once inner hair cell ribbons are lost the spiral ganglion shows secondary degeneration months after the initial insult (Kujawa and Liberman, 2009, Lin et al., 2011). In these models, the degree of ribbon loss is highly correlated with the degree of spiral ganglion loss. These results suggest that our aminoglycoside model of presynaptic ribbon loss may produce similar spiral ganglion losses.

This study was conducted to determine if higher concentrations of kanamycin produce greater magnitude decreases in ribbon densities and or produce additional areas of damage/synaptic loss along the length of the cochlea (more apical regions). Additionally, the time course of changes in ribbon densities and spiral ganglion densities were assessed at multiple intervals following kanamycin administration. The results indicate that higher concentrations of kanamycin induce ribbon changes in larger areas of the cochlea with an associated greater magnitude of loss in each area. Interesting, the results indicate that these changes are transient and recover over time. The initial decreases in ribbon density produced no spiral ganglion degeneration at 56 days following kanamycin administration.

Methods

Subjects

All animal use and the study protocol were approved by the Institutional Animal Care and Use Committee at MPI Research Inc., a fully AALAC-accredited research facility. Forty five male albino Hartley guinea pigs (Charles River laboratories, MI) were placed on study at 6 weeks of age. The animals were single housed in wire mesh caging
and provided environmental enrichments. All subjects were placed on *ad libitum* access to both food and water and weighed between 250 and 400 g at the initiation of the experiment. The room was temperature and humidity regulated with fluorescent lighting provided on a 12 hour light/dark cycle (0600h – 1800h). Clinical observations were conducted twice daily by AALAS-accredited research technicians that monitored food and water access as well as the general health and wellbeing of the animals.

**Study Design**

Forty-five guinea pigs were placed into treatment groups of 5 animals per group. The animals received subcutaneous (SC) injections of saline, or kanamycin at 200 or 400 mg/kg once daily for 10 consecutive days. Designated animals from each treated group were euthanized at 1, 14, and 56 days following the final kanamycin dose. Kanamycin sulfate (Sigma Aldrich, MO) was formulated as an 80 mg/ml solution in normal sterile saline USP (0.9% sodium chloride). Normal sterile saline USP (Sigma Aldrich, MO) and formulated kanamycin doses were administered subcutaneously at a volume equivalent of 5 ml/kg.

**Auditory Brainstem Response (ABR) Evaluations**

All animals underwent bilateral auditory brainstem response (ABR) evaluations at 4, 8, 10, 16, and 20 kHz prior to being assigned to study, and on the day prior to euthanasia for each time interval. The sound presentation was generated using an RZ6 multi I/O processor (TDT Technologies, FL). The animals were anesthetized with an intraperitoneal drug combination of 40 mg/kg ketamine hydrochloride (100 mg/ml) and 0.25 mg/kg of dexmedetomidine (0.50 mg/ml). Once a stable plane of anesthesia was
confirmed as a lack of pain response, the animals were placed in the prone position within an electrically shielded sound attenuation chamber. Pin lead electrodes were placed to collect the ABR biopotential data. The lead configuration used to collect the electrophysiological data consisted of a positive lead under the target ear, a reference lead at the vertex of the skull, and a ground lead under the contralateral ear. The terminal ends of the pin leads were connected to a Medusa preamplifier with a 20X gain and a 4-channel headstage (TDT Technologies, FL). The stimulus used to elicit the ABR waveform was a 15 ms pure tone pip with a 1 ms rise/fall time. The tone pips were delivered at a rate of 10 cycles per second through a MF-1 transducer (TDT Technologies, FL). The stimulus intensity was attenuated in 10 dB steps beginning at 80 dB SPL. The biopotential data were averaged across the 512 stimulus presentations for each intensity level. An auditory threshold was established by determining the level of activity that was 5 dB higher than the sound pressure level that induced no recognizable elements comprising an ABR waveform.

**Tissue Processing**

The day following the final ABR evaluation, all animals were euthanized by carbon dioxide inhalation. Whole body intracardiac perfusion was performed by the application of 0.9% saline followed by 4% w/v paraformaldehyde. The left and right temporal bones from each animal were harvested.

The right temporal bones were placed directly in 0.5% paraformaldehyde and stored in refrigerated conditions (2 - 8°C) until further processing. A small hole was made in the apex of the left cochleae and the oval window was perforated to facilitate
intrascalar perfusion of 4% paraformaldehyde. The left cochleae were then stored in a solution of 0.5% paraformaldehyde and placed in refrigerated conditions (2 - 8°C) until further processing.

The left cochleae were dissected to reveal the organ of Corti. The organ of Corti was then fluorescently stained to identify the auditory hair cells and inner hair cell ribbons. For staining, the tissues were first placed in a blocking solution consisting of 1% goat serum (Invitrogen, CA) and 0.3% Triton X-100 in phosphate buffered saline (PBS). The cochleae were then incubated overnight in a primary antibody solution of 1:100 mouse anti-CtBP2 antibody (BD Transduction Laboratories, CA). The cochleae were rinsed and then incubated for an hour in a secondary antibody solution containing 1:200 Alexa Fluor® 488 Goat anti-mouse IgG (H+L) antibody (Life Technologies, CA) and 1:200 Alexa Fluor® 568 phalloidin (Life Technologies, CA).

The right temporal bone from each animal was placed in a 10% EDTA and decalcified in a tissue microwave (500W, 25°C) for 14-21 days, or until appropriately decalcified. Following decalcification, the temporal bones were dissected on a bias placing the cochlea on a parallel plane with the microtomy blade. The cochlea was mounted in paraffin and preliminarily sectioned until the mid modiolar region was reached. Once located, five 5 µm sections were serially obtained from each cochlea and then processed and stained using a standard haematoxylin and eosin staining procedure.
Imaging and Analysis

All tissue imaging for the left organ of Corti was conducted using a Zeiss Axiovert 200M confocal microscope at 63X magnification. Using a photo reticule, the left organs of Corti were separated into 0.98 mm segments beginning in the apex and z-stack images were captured for each segment. For image capture, z-stacks were created using a 0.2 µm step size. The images were deconvolved and saved as individual planes. Each plane per region was then imported into ImageJ v1.47t for 3D reconstruction. A 3D project was created for each region allowing 90 degree rotation at the x-axis of the 3D image. Using the cell counter plugin, the number of CtBP2 ribbons was manually counted at the base of each inner hair cell. The present and missing outer and inner hair cells were quantified within each of these regions to assess the severity of drug-induced damage. The CtBP2 ribbon density per inner hair cell was then calculated for each region.

The midmodiolar sections of the right cochlea were analyzed using Bioquant Osteo II imaging software. The software was used to collect images from seven regions of each cochlea (apical turn 2 (A2), mid turn 1 (M1), mid turn 2 (M2), and basal turn 1 (B1)) using a 20X objective. The software was then used to determine the area of each region by outlining the Rosenthal’s canal. Within the outlined region, each Type I spiral ganglia cell (corresponding to inner hair cell innervation) was counted using the criteria that each cell will be myelinated (presence of a sheath) and will also possess a nucleolus. Cell count and Rosenthal’s canal area were used to calculate spiral ganglion density.
Statistical Analyses

Hair cell counts and synapse densities were compared between groups using descriptive statistics (mean ± SD). Each group’s hair cell counts were averaged into 0.98 sections. The CtBP2 ribbons/inner hair cell densities in each region and spiral ganglion densities per cochlear turn were averaged for each group. All group comparisons were conducted using an Analysis of Variance (ANOVA) procedure, followed by Tukey post hoc tests. All statistical analyses were performed using Graphpad Prism v4.00.

Results

Auditory Brainstem Response Evaluations

At 1 day following the last day of kanamycin administration, there were no notable changes in threshold at any of the frequencies assessed (4, 8, 10, 16, and 20 kHz) in any dose group. After 14 days from the last day of kanamycin dosing, the average threshold for each frequency in the 400 mg/kg kanamycin dose group significantly increased to 71 ± 15.30 dB SPL at 4 kHz (p≤0.05), 56 ± 16.43 dB SPL at 8 kHz (p≤0.05), 68 ± 18.18 dB SPL at 10 kHz (p≤0.01), 54 ± 21.12 dB SPL at 16 kHz (p≤0.001), and 69 ± 15.42 dB SPL at 20 kHz (p≤0.001). There were no significant threshold differences detected between the 200 mg/kg dose group and controls at this time. The thresholds in the 400 mg/kg dose group continued to increase at day 56 presenting with averages of 79 ± 21.14 dB SPL at 4 kHz (p≤0.001), 70 ± 22.03 dB SPL at 8 kHz (p≤0.001), 78 ± 21.79 dB SPL at 10 kHz (p≤0.001), 70 ± 18.41 dB SPL at 16 kHz (p≤0.001), and 79 ± 10.48 dB SPL at 20 kHz (p≤0.001). Again, there were no significant threshold increases noted in
the 200 mg/kg dose group at this time. The ABR data from each time interval is
displayed in Figures 4, 5, and 6.

Figure 4. ABR threshold 1 day following kanamycin administration.
Figure 5. ABR threshold 14 days following kanamycin administration.
Figure 6. ABR threshold 56 days following kanamycin administration.

Auditory Hair Cell Analysis

The hair cell analyses for each dose group are presented in Figures 7, 8, and 9. The missing outer and inner auditory hair cells were averaged into 0.98 mm segments beginning in the apex of the cochlea, and are presented as successive regions. As shown in Figure 7, there were moderate increases in outer hair cell losses observed below region 18 and minor increases in inner hair cells below region 20 in the 400 mg/kg dose group. There were no other hair cell losses observed in the other dose groups at this time. At 14
days following the last dose, outer hair cell losses above 50% or more were noted in all regions beginning in region 11 through region 21 in the 400 mg/kg dose group. Again, there were only minor increases in inner hair cell losses noted in region 20. Interestingly, there was a large spike in outer hair cell losses observed in the 200 mg/kg dose group at region 21. The hair cell analysis from 56 days following the last dose presented with similar losses as those observed 14 days following the last kanamycin dose. The 400 mg/kg dose group had losses above 50% beginning in region 11 and ending at region 21. There were no other prominent losses in the other dose groups at this time interval.
Figure 7. % missing hair cells 1 day following kanamycin administration.
Figure 8. % missing hair cells 14 days following kanamycin administration.
Day 56 After the Last Kanamycin Dose
% Missing Hair Cells

Figure 9. % missing hair cells 56 days following kanamycin administration.

**Inner Hair Cell Ribbon Density**

The inner hair cell ribbon densities were determined in cochlear regions 16-21 (exactly 15.68, 16.66, 17.64, 18.24, 19.6, and 20.58 mm from apex, respectively). There was a significant decrease in ribbon density identified in region 19 of the 200 mg/kg dose group 1 day following the last dose (p≤0.05). Similarly, there were also significant decreases detected in regions 19 and 20 of the 400 mg/kg dose group at this time (p≤0.05). Interestingly, the ribbon densities recovered to control levels by 14 days following the last dose in both dose groups. This recovery was not maintained when
assessed 56 days from the last kanamycin dose, as significant ribbon density decreases were again identified in region 18 of the 400 mg/kg dose group. All inner hair cell ribbon density data are presented in Figures 10, 11, and 12.
Figure 10. (10A) Inner hair cell ribbon density 1 day following kanamycin administration; (10B) image of control ribbon synapses; (10C) image of treated ribbon synapses.
Figure 11. (11A) Inner hair cell ribbon density 14 days following kanamycin administration; (11B) image of control ribbon synapses; (11C) image of treated ribbon synapses.
Figure 12. (12A) Inner hair cell ribbon density 56 days following kanamycin administration; (12B) image of control ribbon synapses; (12C) image of treated ribbon synapses.
**Type I Spiral Ganglion Density Evaluations**

The average Type I spiral ganglia density for each cochlear turn evaluated in the 400 mg/kg kanamycin dose group is presented in table 2. Given that the ribbon density changes were isolated to the furthest basal regions of the cochlea in the left ears, the data generated from the basal turn of the midmodiolar sections from the right ears were clearly most relevant in terms of further characterizing kanamycin neurotoxicity. The Type I spiral ganglia density results showed that there were no differences in the average density measurements between any of the intervals assessed in the 400 mg/kg dose group.

![Image](image_url)  
**Figure 13.** Example midmodiolar section with labeled cochlear turns.
Table 2
Type I Spiral Ganglia Density Measurements from Mid-Modiolar Sections of the Basal Cochlea by Interval

<table>
<thead>
<tr>
<th>Interval</th>
<th>Cochlear Turn (cells/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A2</td>
</tr>
<tr>
<td>1 Day Following the Last 400 mg/kg Kanamycin Dose</td>
<td>1.54 ± 0.21</td>
</tr>
<tr>
<td>14 Days Following the Last 400 mg/kg Kanamycin Dose</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>56 Days Following the Last 400 mg/kg Kanamycin Dose</td>
<td>1.63 ± 0.14</td>
</tr>
</tbody>
</table>

Discussion

This study demonstrates that ten consecutive days of kanamycin administration produces profound outer hair cell losses with minimal cytotoxic effects on inner hair cells. The present results also indicate that presynaptic inner hair cell ribbons decrease initially following kanamycin administration, but then regenerate over time. There appears to be no corresponding effect on spiral ganglion cellular density over time which implies that ribbons may regenerate, restoring functional support to the type I neurons.

In this study, the number of kanamycin doses was increased from 7 to 10. This experimental design modification was made in order to produce greater hair cell toxicity, and also examine effects on inner hair cell ribbon densities. The increased number of doses produced severe outer hair cell loss through the mid and basal turns of the organ of Corti. Interestingly, this increased hair cell death, which is associated with exposure to high concentrations of drug, was accompanied by greater decreases in ribbon densities.
and also affected a greater length of the cochlear partition. These results suggest that in addition to OHC cytotoxicity, this regimen of drug exposure directly impacts the severity of the ribbon loss observed.

Recently, Lin et al. (2011) suggested that the use of ribbon markers alone to quantify synapse activity is insufficient, as cochlear insults can produce ribbons that do not display the normal association with the inner hair cell active zone and/or plasma membrane. These same ribbon anchorage issues have been observed in in-vitro models of glutamate toxicity (Wang and Green, 2011). The displaced ribbons do not have a postsynaptic connection and lead to underestimates of the number of lost synapses. For this reason, it has been suggested that synapse density values can only be accurately measured using dual markers of pre- and post-synaptic proteins. Future studies should examine the effect of aminoglycosides on inner hair cell - auditory neuron connections to determine if functional synapses are formed during the recovery observed in this study.

Although we only used a presynaptic marker to monitor inner hair cell synapse densities, these results suggest, that unlike noise-induced synaptopathy, aminoglycosides may produce a transient decrease in ribbon densities followed by a subsequent recovery in numbers. This in-vivo finding is rare in terms of synapse recovery as the only other demonstration of a recoverable synaptopathic injury has been induced by AMPA/kainite administration (Pujol and Puel, 1999). Direct inferences cannot be drawn between these models, however as the administration of AMPA/kainite acts directly on afferent neuronal glutamate receptors and not the presynaptic ribbons (Puel et al., 1995, Puel et
al., 1997). Nevertheless, the AMPA/kainite models provide support that in-vivo inner hair cell synapse re-innervation is possible.

In the noise-induced synaptopathy models, the loss of inner hair cell ribbons and postsynaptic connections lead to delayed spiral ganglion degeneration (Kujawa and Liberman, 2009, Kujawa and Liberman, 2006). The inner hair cells are largely unaffected in these experiments, which suggest that inner hair cell synaptic activity is critical to maintain spiral ganglion cell survival (Sergeyenko et al., 2013; Stamataki et al., 2006). The lack of secondary degeneration in the spiral ganglion in our study supports the possibility that functional connections were formed after expression of the initial insult.

The spiral ganglion begins to degenerate in the guinea pigs following aminoglycoside treatment only weeks after the initial insult, but continues to decline through approximately 12 months (Yamagata et al., 2004, Duan et al., 2000). Generally, aminoglycosides produce a lesion characterized by outer hair cell loss in the basal region of the cochlea which progresses apically, followed thereafter by inner hair cell death (Fee, 1980). The observed delay in inner hair cell loss may explain the time course of spiral ganglion degeneration, given the supportive role of inner hair cells in maintaining neuronal viability. In models of hair cell ablation, where both inner and outer hair cell death is rapidly induced by aminoglycosides and loop diuretics, spiral ganglion neurons begin to degenerate at an increased rate beginning as early as several weeks after loss of outer and inner hair cells (Agterberg et al., 2009, Tan and Shepherd, 2006, McFadden et al., 2004). Our study examined spiral ganglion densities out to 56 days following the
cochlear injury, showing no effects. However, longer term examinations should be conducted to assess continued term survival of these neurons and synaptic connections.

In conclusion, our results suggest that aminoglycosides produce significant inner hair cell ribbon loss in basal regions of the cochlea followed by a recovery of the ribbons. This implies that aminoglycosides produce a coverable synaptopathy. Future studies should be conducted to assess both the pre- and post-synaptic effects of aminoglycosides on inner hair cell – auditory neuron connections.

References


CHAPTER IV

EXPERIMENT 3: TIME COURSE CHARACTERIZATION OF THE EFFECTS OF KANAMYCIN ON AUDITORY HAIR CELL SYNAPSE PLASTICITY

Introduction

Synaptopathy following noise and other insults to the cochlea has been shown to alter auditory temporal precision and suprathreshold waveform magnitude (Moser et al., 2006). There are several types of potential synaptopathic insults in the cochlea and there appears to be a variable response of the inner hair cell to re-innervate following each type. Understanding the recoverability of synaptopathy following the different insults could provide insights into potential therapeutic interventions to recover auditory sensitivity.

Our laboratory has observed that ototoxic doses of kanamycin produce a transient decrease in inner hair cell ribbon density followed by recovery over time. The inner hair cells are spared during the ototoxic treatment, but severe outer hair cell losses are observed through the mid and basal turns of the cochlea. Though no postsynaptic markers were used in this experiment, the regenerated ribbons appeared to regain synaptic function as no spiral ganglion degeneration was observed 56 days following the last dose of kanamycin. Chen et al (2012) found similar inner hair cell synaptic loss in mice following gentamicin exposure. Although Chen et al. did not conduct quantitative
synapse measures months after the insult, no synaptic recovery was observed weeks after gentamicin administration. If the postsynaptic afferent connections do in fact re-innervate regenerated ribbons, these results would be contrary to what has been found in noise induced models of synaptopathy.

Kujawa and Liberman (2006, 2009) showed that a mild noise exposure, yielding a temporary threshold shift, could be followed by complete recovery of auditory sensitivity, but inner hair cell synapses were permanently reduced. The observed reduction of synapse number leads to a secondary degeneration of type-I spiral ganglion neurons (Sergeyenko et al., 2013; Stamataki et al., 2006). It has been suggested that moderate noise exposures preferentially affect high threshold, low spontaneous rate afferent fibers, which may contribute to reduced speech discrimination late in life where concomitant hair cell loss is not observed (Furman et al., 2013). This may be a species-specific anomaly, as other studies have shown that noise-induced synaptopathy in guinea pigs is recoverable, and does not show spiral ganglion degeneration months after the insult (Shi et al., 2013, Liu et al. 2012).

Synaptic regeneration has been shown to occur following in-vivo cochlear perfusions of α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and/or kainic acid with complete recovery of auditory function (d’Aldin et al, 1997; Pujol et al., 1999; Puel et al. 1994). This re-innervation has also been confirmed in in-vitro models of excitotoxicity. Wang and Green (2011) showed that direct administration of NMDA and kainic acid to organ of Corti explants resulted in a 99% reduction in inner hair cell synapses. Kainic acid administration caused presynaptic ribbon migration to perinuclear
locations within the inner hair cell, with postsynaptic terminal regeneration over time. These inner hair cell synapses regenerated following kainic acid exposure, but the recovery was quantitatively incomplete.

Building from our prior results showing inner hair cell ribbon regeneration, the current study was conducted to determine if both the pre- and post-synaptic elements of the inner hair cell synapse regenerate following administration of ototoxic doses of kanamycin. The use of co-localized pre- and post-synaptic markers to assess synaptic integrity provides a more precise evaluation of pathophysiology as cochlear insults appear to produce orphan ribbons which lack a postsynaptic connection (Yuan and Chi, 2014; Tong et al., 2013; Liberman et al., 2012; Lin et al., 2011). The current study therefore used co-appearance of CTBP2 (presynaptic ribbons) and GlurR2/3 (abundant afferent postsynaptic glutamate receptors) as markers for the inner hair cell synapses. The inner hair cell synapses were assessed in the basal regions of the cochlea at 17.6 and 18.6 mm from the apex, located in the 32 kHz frequency region (Viberg and Canlon, 2004).

The results supported the hypothesis that aminoglycoside treatment can induce loss of inner hair cell - auditory nerve synapses, and also that synaptic reconnection occurs in this model. Inner hair cell synapses significantly decreased 1 day following the last of 10 daily doses of kanamycin, with significant increases in both pre- and post-synaptic components observed 14 and 58 days following treatment termination.
Methods

Subjects

All animal use was approved by the Institutional Animal Care and Use Committee at MPI Research Inc., a fully AALAC-accredited research facility. Forty eight male albino Hartley guinea pigs (Charles River laboratories, MI) were placed on study at 8 weeks of age. The animals were single housed in plastic shoe box caging with sanitized wood chips and environmental enrichments. All subjects were placed on ad libitum access to both food and water and weighed between 250 and 500 g at the initiation of the experiment. The room was temperature and humidity regulated with fluorescent lighting provided on a 12 hour light/dark cycle (0600h – 1800h).

Study Design

Forty-eight guinea pigs were randomly placed into six treatment groups of 8 animals per group. Three treatment groups received a subcutaneous dose of saline, and the remaining 3 treatment groups received a subcutaneous dose of 400 mg/kg kanamycin sulfate. All groups received one dose daily for ten consecutive days. Kanamycin sulfate (Sigma Aldrich, MO) was formulated as an 80 mg/ml solution in normal sterile saline USP (0.9% sodium chloride). Normal sterile saline USP (Sigma Aldrich, MO) and formulated kanamycin doses were administered subcutaneously at a volume equivalent of 5 ml/kg. One kanamycin and one saline treated group were euthanized at one of three time intervals (1, 14, or 58 days following the termination of dosing).
**Auditory Brainstem Response (ABR) Evaluations**

Unilateral (left ear) ABR evaluations were conducted at 32 kHz prior to study initiation and prior to the designated terminal time interval. The sound presentation was generated using an RZ6 multi I/O processor (TDT Technologies, FL). The animals were anesthetized with an intraperitoneal drug combination of 40 mg/kg ketamine hydrochloride (100 mg/ml) and 0.25 mg/kg of dexmedetomidine (0.5 mg/ml). Once a stable plane of anesthesia was confirmed as a lack of pain response, the animals were placed in the prone position within an electrically shielded sound attenuation chamber. Pin lead electrodes were placed to collect the ABR biopotential data. The lead configuration used to collect the electrophysiological data consisted of a positive lead under the target ear, a reference lead at the vertex of the skull, and a ground lead under the contralateral ear. The terminal ends of the pin leads were connected to a Medusa preamplifier with a 20X gain and a 4-channel headstage (TDT Technologies, FL). The stimulus used to elicit the ABR waveform was a 5 ms pure tone pip with a 0.5 ms rise/fall time. The 32 kHz tone was delivered in a closed-field at a rate of 10.1 cycles per second through a MF-1 transducer (TDT Technologies, FL). The stimulus intensity was attenuated in 10 dB steps beginning at 80 dB SPL. The biopotential data were averaged across the 512 stimulus presentations for each intensity level. An auditory threshold was established by determining the level of activity that was 5 dB higher than the sound pressure level that induced no recognizable elements comprising an ABR waveform.
**Tissue Processing**

Animals were euthanized via carbon dioxide inhalation on the day following the final ABR evaluation. Whole body intracardiac perfusion of 0.9% saline followed by 4% w/v paraformaldehyde was conducted. The left temporal bone from each animal was harvested and intrascalar perfusion using 4% paraformaldehyde was carried out through a small hole in the apex of each cochlea and the oval window. The cochleae were then stored overnight in a solution of 0.5% paraformaldehyde at 2 - 8°C.

The otic capsule and overlying tissues were removed to reveal the organ of Corti. To label the presynaptic ribbons (CtBP2), postsynaptic glutamate receptors (GluR2/3), and the auditory hair cells (Phalloidin), the organs of Corti were first placed in a blocking solution consisting of 1% goat serum (Invitrogen, CA) and 0.3% Triton X-100 in phosphate buffered saline (PBS) with calcium and magnesium. This was followed by co-immunolabeling with a primary antibody solution comprised of 1:100 mouse anti-CtBP2 antibody (BD Transduction Laboratories, CA) and 1:100 rabbit anti-GluR2/3 polyclonal antibody (Millipore, MA) overnight at 2 - 8°C. Cochleae were then rinsed in phosphate buffered saline (PBS) with calcium and magnesium, and then incubated for an hour in a secondary antibody solution containing 1:200 Alexa Fluor® 488 Goat anti-mouse IgG (H+L) antibody (Life Technologies, CA), 1:200 Alexa Fluor® 568 goat anti-rabbit IgG (H+L) antibody (Life Technologies, CA), and 1:200 Alexa Fluor® 350 phalloidin (Life Technologies, CA).
**Imaging and Analysis**

The organ of Corti was microdissected in four surface preparations and viewed using a Zeiss Axiovert 200M confocal microscope at 63X magnification. The surface preparations were viewed in 0.98 mm segments beginning in the apex for the purposes of locational analysis using a photo reticule. The regions corresponding to 17.6 and 18.6 mm from apex were selected for confocal imaging. For image capture, z-stacks were created using a 0.4 µm step size. The images were deconvolved and saved as individual planes. Each plane per region was then imported into ImageJ v1.47t for 3D reconstruction. A 3D project was created for each region allowing 90 degree rotation at the x-axis of the 3D image. Using the cell counter plugin, the number of closely apposed or co-localized CtBP2 and GluR2/3 puncta were manually counted at the base of each inner hair cell in the region of interest using a digital zoom of 400X, and the mean synapse density per inner hair cell calculated for that region. The mean for each treatment group was determined, and inner hair cell synapse density averages were analyzed for significant differences between the 1, 14, and 58 day intervals. The number of present and missing outer and inner hair cells within the reticule length for each region was also determined.

**Statistical Analyses**

The ABR data, missing hair cell data, and inner hair cell synapse density measures are presented as the mean ± 1 S.D. The ABR data and inner hair cell synapse density averages were analyzed for significant differences between the 1, 14, and 58 days.
following treatment termination using one-way analysis of variance with Tukey post hoc
tests in Graphpad Prism v4.00.

Results

Auditory Brainstem Response Evaluations

The average pretest and terminal ABR threshold for each interval is shown in
Figure 14 as mean ± S.D. The average 32 kHz auditory threshold in the saline treated
group 1 day following treatment termination was 23.00 ± 5.88 dB SPL. The average
threshold of the kanamycin treated group at this time interval was significantly increased
to 47.88 ± 27.19 (p≤0.05). By 14 days from treatment termination, the average ABR
threshold continued to significantly increase to 72.50 ± 12.43 (p≤0.01) in the kanamycin–
treated group. The saline control group at this time point remained at baseline, with a
value of 21.38 ± 8.60. The ABR threshold increase plateaued and maintained at an
average of 73.50 ± 12.32 (p≤0.001) through Day 58 following treatment termination.
Again, the saline control group at this interval remained at a baseline level of 24.13 ±
8.79. These time course data indicate that 1 day following the termination of dosing the
cochlea is undergoing a process of dysfunctional changes that peak 14 days from
treatment termination, which is then maintained as reflected by the average Day 58 ABR
threshold.
Figure 14. Time course effects of kanamycin on ABR threshold.

Hair Cell Analysis

The 10-day dose regimen of 400 mg/kg kanamycin produced robust outer hair cell losses while largely sparing inner hair cells. The hair cell data evaluated within each region are presented in Figures 15 and 16. At 1 day following treatment termination,
there were no missing inner or outer hair cells in the 17.6 and 18.6 mm from apex regions of the saline control group. The kanamycin-treated group at this time interval presented with 50% missing outer hair cells within both the 17.6 and 18.6 mm from apex regions. The kanamycin treated inner hair cells at this time interval incurred either no, or minimal losses of 0 and 11% in the 17.6 and 18.6 mm from apex regions, respectively. At 14 days subsequent to treatment termination, there were minor outer hair cell and no inner hair cell losses observed in the saline control group. In contrast, there were outer hair cell losses of 86% observed within each region evaluated in the kanamycin-treated group at this interval. The inner hair cell losses characterized at this time were again either absent, or minimal at 0 and 4% 17.64 and 18.6 mm from apex regions, respectively. The severity of damage increased at 58 days in the kanamycin-treated group with 100% outer hair cell losses in both regions. The inner hair cell sparing was still present at this time with 1% losses observed in both regions assessed. Again, the saline control group 58 days following treatment termination presented with minimal outer, and no inner, hair cell losses within the 17.6 and 18.6 mm from apex regions.

**Inner Hair Cell Synapse Density**

Figures 15 and 16 show the inner hair cell synapse density changes for each time interval assessed. At 1 day after treatment termination, there was a significant ~50% reduction in synapse density observed in the 17.6 mm from apex region in the kanamycin-treated group as compared to the time-matched saline control (p≤0.001). The 18.6 mm from apex region in the kanamycin-treated group presented with an even greater ~65% reduction in synapse density compared to the saline control at this same time.
interval ($p \leq 0.001$). By 14 days from treatment termination, the kanamycin-induced synapse degeneration had recovered to 33 and 48% percent of the saline control values in the 17.6 mm and 18.6 mm from apex regions, respectively (both $p \leq 0.001$). This recovery effect continued through 58 days, with the kanamycin-treated group average density measures reaching minimal differences of only 17% relative to the control group at 17.6 mm from apex and 37% relative to the control group in the 18.6 mm from apex region ($p \leq 0.001$). In the region of interest 17.6 mm from apex, the number of labeled connections in the kanamycin treated group was no longer significantly reduced from the saline controls at 56 days following cessation of treatment.

Many of the CTBP2 immunostained ribbons 14 days following kanamycin treatment were qualitatively noted to be larger than those in the time matched control group, similar to what has been reported days following noise overstimulation (Lin et al., 2011; Kujawa and Liberman, 2009). Interestingly this was less evident in the 1 day and 58 day termination post treatment groups.
Figure 15. (15A) Average inner hair cell synapse density at 17.64 mm from apex by time interval; (15B) % missing hair cell loss at 17.64 mm from apex by time interval.
Figure 16. (16A) Average inner hair cell synapse density at 18.62 mm from apex by time interval; (16B) % missing hair cell loss at 18.62 mm from apex by time interval.
Figure 17. Representative 63X projection images of ribbon synapses from (17A) 1 day control, (17B) 14 day control, (17C) 56 day control, (17D) 1 day treated, (17E) 14 day treated, and (17F) 56 day treated tissues.

Discussion

This study confirms previous results indicating that inner hair cell ribbons degenerate acutely and subsequently regenerate over time. The results of this study demonstrate that the regenerated inner hair cell ribbons form connections with postsynaptic afferent terminals. The magnitude of re-innervation observed in this study
is comparable to what has been reported with kainic acid and AMPA insult both in vivo (d'Aldin et al., 1997; Puel et al., 1995) and in vitro (Wang and Green, 2011). Both models of synaptic re-innervation are similar to the effects observed in guinea pigs after noise exposure, but are in contrast to what has been reported following noise exposure in mice, where synaptic connections are lost permanently (Kujawa and Liberman, 2009; Lin et al., 2011). There may be a variety of pathologic and genetic factor contributing to species differences in re-innervation phenomena. Obviously these differences in repair plasticity raise the important issue of which model, if either, represent the human condition.

Studies have shown that the neurotrophic factor NT-3 can play an important role in regulation of the synaptic connections during postnatal development (Wan et al., 2013; Sugawara et al., 2007) and regeneration following cochlear insults (Wang and Green, 2011). It has been shown that inner hair cells (Green et al., 2012) and supporting cells (Sugawara et al., 2007; Stankovic et al., 2004) secrete this neurotrophic factor, which helps maintain neuronal viability. Supporting cells have been shown to play a critical role in maintaining spiral ganglion health by also secreting other neurotrophic and protective factors (Sobkowicz et al., 2002, Stankovic et al., 2004, May et al. 2013, Monzack and Cunningham, 2013). Given their role in fostering synaptic regeneration, the key difference in re-innervation potential between noise exposure in mice and the other models may be the severity of damage induced to the inner hair cell supporting cell complex. Cochlear insults have been shown to cause damage in the form of free radical formation in supporting cells (Ladrech et al., 2013, Inai et al., 2012; Du et al., 2011), but
the insult may differentially affect supporting cell functions in each species, specifically the expression of growth and other regulatory and protective factors during the acute period following damage.

Our results illustrated greater regeneration and reconnection in the more apical of the two regions of interest assessed. This supports an NT-3 contribution to the observed re-innervation, as previous studies showed that NT-3 expression is greater apically in the cochlea, and that regeneration after auditory nerve sectioning is greater in the apex (Sugawara et al., 2005, 2007). At 14 days following the termination of kanamycin dosing, we observed many inner hair cell ribbons that were qualitatively larger in size as compared to the saline controls (see figure 18). This observation was not present at 58 days from the last dose. These same ribbon size changes have also been observed in the noise-induced models of synaptopathy, although the change was permanent in these noise models (Kujawa and Liberman et al., 2009).

This observation may provide insights into the processes involved in re-innervation. Sobkowicz et al. (1986) found an increase in the formation of presynaptic complexes, accumulations of electron-dense vesicles at the presynaptic membrane opposed to afferent nerve fibers, in denervated organ of Corti explants. In a process described as reactive synaptogenesis, denervated cultures exhibit dendritic filapodia that interact with the postsynaptic plasma membrane of inner hair cells (Sobkowicz et al., 1998). Within 24 hours, inner hair cell ribbons preferentially migrate to these filapodia forming large groupings of ribbons. Over time, the filapodia deteriorate and the large ribbon groupings are no longer present, only leaving behind sparse inner hair cell
synapses. The large ribbons observed at day 14 after dosing may represent a stage of reactive synaptogenesis where large groupings of ribbons associate around neuronal filapodia.

![Image](image.png)

**Figure 18.** Representative 63X projection images (x-z axis) from a (18A) day 14 control inner hair cell, (18B) day 14 treated inner hair cell, and (18C) day 58 treated inner hair cell from the 17.64 mm from apex.

There have been two recent studies that also examined the effects of aminoglycoside administration on inner hair cell – auditory nerve synapses. Chen et al. (2012) showed a pattern of declining numbers of inner hair cell – auditory nerve synapses in the cochlea of mice using three alternating days of gentamicin administration. Inner hair cell synapse densities were assessed on each dose day and indeed decreased with each successive dose. This is consistent with our results where a large decrease was seen one day subsequent to the 10th day of dosing. Chen et al (2012) did not look at later
periods so there was no opportunity to see the re-innervation that we observed. Liu et al. (2013) employed a 14-day dose regimen of gentamicin in mice, and contrary to our results and those of Chen et al. (2012) found that inner hair cell synapse densities increased over control levels during the dosing period, peaking on the 7th dose and then returning to control levels by the 14th dose. We did not assess inner hair cell – auditory neuron synapse densities during the dosing period, however the decrease we found 1 day after cessation would suggest quite different effects in our model. It is possible that there are species differences, as mice have been shown to be relatively resistant to aminoglycoside ototoxicity (Poirrier et al., 2010; Blakley et al., 2008; Taylor, et al., 2008). However, Chen et al (2012) also used the mouse model, and reported similar decreases to present findings in guinea pig.

In conclusion, our data show that kanamycin administration produces inner hair cell synapse degeneration with subsequent regeneration. These findings may provide a platform for the understanding of synaptogenesis in the adult cochlea. In addition, this model also may provide insights into developing treatments aimed at reducing synaptopathy in patients with hearing loss.

References


CHAPTER V

EXPERIMENT 4: ACUTE CHARACTERIZATION OF INTRA-aural GENTAMICIN ON INNER HAIR CELL SYNAPSE PLASTICITY

Introduction

Gentamicin is an aminoglycoside antibiotic historically used for the treatment of gram negative bacterial infections (Mingeot-Leclercq, et al., 1999). Much like kanamycin, gentamicin is oto- and nephrotoxic (Rybak and Ramkumar, 2007). Within the class of aminoglycoside antibiotics, each compound appears to induce varying levels of ototoxicity (Kotecha and Richardson, 1994). Despite this undesirable toxicity, gentamicin is now commonly used as a secondary treatment for multi-drug resistant tuberculosis and also in cases of severe infection (ATS-CDC, 1993).

Beyond systemic use as an antibacterial agent, gentamicin has been used for many years as a treatment for Meniere’s disease (Silverstein et al., 2010, Bodmer et al., 2007). The treatment of Meniere’s disease requires that gentamicin is injected through the tympanum into the middle ear (Silverstein et al., 1999). The ototoxic properties of gentamicin are exploited to ablate vestibular hair cells which in turn cause a reduction in the vestibular dysfunction observed in this disease (Assimakopoulos and Patrikakos, 2003).
During systemic administration of aminoglycosides, the drug distributes to the inner ear, but peak concentrations are variable due to metabolism and clearance from the systemic circulation (Tran Ba Huy, et al., 1986; Brummett, et al., 1978). Direct administration of these types of drugs to the round window allows for a more controlled delivery to the inner ear. One side-effect of transtympanic injections of gentamicin for Meniere’s disease is high frequency hearing loss (Möller et al., 1988, Wu and Minor, 2003).

We recently showed that systemic administration of aminoglycoside antibiotics produces significant synaptopathy in the basal regions of the cochlea at 17.6 and 18.6 mm from apex following treatment. Our previous results also suggested there is a dose-dependence in the inner hair cell ribbon loss observed. As both of these prior studies were systemic administrations of kanamycin, this brings into question how other types of aminoglycosides may affect inner hair cell synapse densities acutely, and also if doses of intra-aural aminoglycosides will produce more dramatic changes in synapse densities throughout the cochlea.

The current study was conducted to address both of these questions to develop a better understanding of aminoglycoside antibiotics mechanisms of ototoxicity and potential for regenerative processes. Gentamicin was selected for evaluation as it is commonly used intra-aurally and systemically in clinical practice. Additionally, the test subjects were surgically implanted with bilateral middle ear catheters to reduce systemic distribution and to achieve high drug perilymph concentrations by direct round window
delivery. Our results show that gentamicin produces acute inner hair cell synapse density reductions following administration similar to kanamycin. Furthermore, direct administration of gentamicin to the middle ear produced profound inner hair cell synapse loss beginning at 15.7 mm from apex of the cochlea without an associated inner hair cell loss.

Methods

Subjects

All animal use and the study protocol were approved by the Institutional Animal Care and Use Committee at MPI Research Inc., a fully AALAC-accredited research facility. Five female albino Hartley guinea pigs (Charles River laboratories, MI) were placed on study at 8 weeks of age. The animals were single housed, in plastic shoe box caging with sanitized wood chips and environmental enrichments. All subjects were placed on *ad libitum* access to both food and water and weighed between 250 and 500 g at the initiation of the experiment. The room was temperature and humidity regulated with fluorescent lighting provided on a 12 hour light/dark cycle (0600h – 1800h). Clinical observations were conducted twice daily by AALAS-accredited research technicians that monitored food and water access as well as the general health and wellbeing of the animals.
**Drug Formulations**

A 50 µl dose of a 200 mg/ml solution of gentamicin sulfate (Sigma Aldrich, MO) formulated in normal sterile saline USP (0.9% sodium chloride) was administered through a catheter into the right middle ear cavity of each animal. As a control, 50 µl of normal sterile saline USP (Sigma Aldrich, MO) was also administered through an additional catheter into the left middle ear of each animal.

**Surgical Approach**

All animals were instrumented with bilateral middle ear catheters using standard sterile surgical procedures to facilitate direct administration of gentamicin. To place the catheters, the animals were anesthetized with intraperitoneal dexmedetomidine (0.5 mg/kg; 0.5 mg/ml) and xylazine (10 mg/kg; 100 mg/ml, MWI Veterinary Supply, ID). A midline skin incision was made on the dorsum of the head from approximately the medial canthus caudally and then continuing post-auricularly to the base of each ear. Several stainless steel screws were placed in the skull to anchor and stabilize each catheter. A small hole was drilled slightly lower than the midpoint of the bulla of each ear, and a 22 gauge sterile catheter with an attachable injection port was inserted. The catheters were secured within the hole with Carboxylate cement (Henry Schein Animal Health, OH). The catheters were then further secured to the skull screws with fine gauge surgical steel wire. Methyl methacrylate (Henry Schein Animal Health, OH) was then used to encase
the catheters and skull screws. The animals were allowed 7 to 10 days recovery prior to placement on study and subsequent receipt of any drug or experimental treatments.

**Auditory Brainstem Response (ABR) Evaluations**

All animals underwent bilateral ABR evaluations at 32 kHz prior to study initiation (at least 14 days post-surgery), and at 3 and 24 hours following dosing. The sound presentation was generated using an RZ6 multi I/O processor (TDT Technologies, FL). The animals were anesthetized with an intraperitoneal drug combination of 40 mg/kg ketamine hydrochloride (100 mg/ml) and 0.25 mg/kg of dexmedetomidine (0.5 mg/ml). Once a stable plane of anesthesia was confirmed as a lack of pain response, the animals were placed in the prone position within an electrically shielded sound attenuation chamber. Pin lead wires were placed to collect the ABR biopotential data. The lead configuration used to collect the electrophysiological data consisted of a positive lead under the target ear, a reference lead at the vertex of the skull, and a ground lead under the contralateral ear. The terminal ends of the pin leads were connected to a Medusa preamplifier with a 20X gain and a 4-channel headstage (TDT Technologies, FL). The stimulus used to elicit the ABR waveform was a 15 ms pure tone pip with a 1 ms rise/fall time. The 32 kHz tone was delivered at a rate of 10 cycles per second through a MF-1 transducer (TDT Technologies, FL). The stimulus intensity was attenuated in 10 dB steps beginning at 80 dB SPL. The biopotential data were averaged across the 512 stimulus presentations for each intensity level. An auditory threshold was
established by determining the level of activity that was 5 dB higher than the sound pressure level that induced no recognizable elements comprising an ABR waveform.

**Tissue Processing**

The day following the final ABR evaluation, all animals were euthanized via carbon dioxide inhalation. Whole body intracardiac perfusion was performed by the application of 0.9% saline followed by 4% w/v paraformaldehyde. The temporal bones from each animal were harvested. A small hole was made in the apex of each cochlea and the oval window was perforated to facilitate intrascalar perfusion of 0.5% paraformaldehyde. The cochleae were then stored in a solution of 0.5% paraformaldehyde and placed in refrigerated conditions (2 - 8°C) overnight.

The cochleae were dissected to reveal the organ of Corti. To label the presynaptic ribbons, postsynaptic glutamate receptors, and the auditory hair cells, the organ of Corti were first placed in a blocking solution consisting of 1% goat serum (Invitrogen, CA) and 0.3% Triton X-100 in phosphate buffered saline (PBS) with calcium and magnesium. The cochleae were then incubated overnight in a primary antibody solution comprised of 1:100 mouse anti.CtBP2 antibody (BD Transduction Laboratories, CA) and 1:100 rabbit anti-GluR2/3 polyclonal antibody (Millipore, MA). After rinsing, the cochleae were then incubated for an hour in a secondary antibody solution containing 1:200 Alexa Fluor® 488 Goat anti-mouse IgG (H+L) antibody (Life Technologies, CA), 1:200 Alexa Fluor®
568 goat anti-rabbit IgG (H+L) antibody (Life Technologies, CA), and 1:200 Alexa Fluor® 568 phalloidin (Life Technologies, CA).

**Imaging and Analysis**

All fixed and stained tissue slide imaging was conducted using a Zeiss Axiovert 200M confocal microscope at 63X magnification. The organ of Corti was first evaluated to quantify the proportion of missing hair cells. The missing outer and inner hair cells were assessed in 0.14 mm segments using a reticule, beginning in the apex and moving toward the basal turn. Hair cell counts were then entered into Cytogram v3.0.6 to generate average left and right ear cytocochleograms.

The organs of Corti were separated into 0.98 mm segments for the purposes of locational analysis. The regions corresponding to 6.86, 15.68, 16.66, 17.64, 18.24, and 19.6 mm from apex were selected for confocal imaging based on historical data revealing drug sensitivity within these regions. For image capture, z-stacks were created using a 0.4 µm step size. The images were deconvolved and saved as individual planes. Each plane per region was then imported into ImageJ v1.47t for 3D reconstruction. A 3D project was created for each region allowing 90 degree rotation at the x-axis of the 3D image. Using the cell counter plugin, the number of co-localized CtBP2 and GluR2/3 puncta, each representing a functional synapse, were manually counted at the base of each inner hair cell. A synapse density per inner hair cell was then calculated for each region.
**Statistical Analyses**

The ABR data and ribbon density measures are presented as mean ± S.D. The ABR data averages were analyzed for significant differences between pretest and the 3 and 24 hours intervals using repeated measure analysis of variance with Tukey post hoc tests in Graphpad Prism v4.00. The inner hair cell synapse density measurements for each region were compared between the control and gentamicin treated ears using student t-tests in Graphpad Prism v4.00.
Results

Auditory Brainstem Response Evaluations

As shown in figure 19, the 32 kHz mean pretest threshold for the left ear was 21.25 ± 7.50 dB SPL. Three hours after administration of 50 µL of saline, the mean threshold in the left ear increased to 33.75 ± 18.87 dB SPL. This increased threshold was attributed to the presence of fluid in the middle ear as the average threshold returned to 21.25 ± 6.29 dB SPL 24 hours following administration.
Figure 19. The average 32 kHz auditory threshold for the right (gentamicin-treated) ear and left (saline-treated) ear presented by time interval.

The 32 kHz mean pretest threshold in the right ear was 26.25 ± 7.50 dB SPL. Three hours following the administration of the 40 mg dose of gentamicin, the mean right ear threshold significantly increased to 53.75 ± 14.36 dB SPL. By 24 hours, this threshold shift further increased in the right ear to 75.00 ± 9.13 dB SPL indicating the presence of gentamicin induced sensorineural hearing loss.

Cytocochleogram Analysis

The average hair cell data for each group is presented in figure 20. The hair cell analysis demonstrated that saline control injections had no effect on the viability of the outer or inner hair cells throughout the entire organ of Corti. Gentamicin, however, produced a robust cytotoxic effect on outer hair cells engendering losses of 40% 10 mm from apex, and increasing to 100% beginning 14 mm from apex. Inner hair cell losses were minimal throughout the organs of Corti until a point approximately 19 mm from apex where the percentage of hair cell loss increased above 40%. Interestingly, in the region 4 mm to 8 mm from apex, outer hair cell losses were below 20%.
Figure 20. % missing hair cells following direct middle administration of gentamicin.

**Inner Hair Cell Synapse Density**

The average inner hair cell synapse densities from selected regions of the organ of Corti collected from the left saline control treated ear 24 hours subsequent to administration:
Distance from Apex (mm)  Synapses per Hair Cell
6.86  14.57 ± 1.55
15.68  17.27 ± 3.09
16.66  14.83 ± 3.91
17.64  14.65 ± 1.56
18.62  13.25 ± 1.93
19.60  12.84 ± 1.73

The average inner hair cell synapse densities from selected regions of the organ of Corti collected from the right gentamicin treated ear 24 hours subsequent to administration:

Distance from Apex (mm)  Synapses per Hair Cell
6.86  12.54 ± 3.35
15.68  4.10 ± 2.23
16.66  4.38 ± 3.61
17.64  4.56 ± 2.28
18.62  3.07 ± 0.92
19.60  3.09 ± 0.41

There were no significant differences in the average synapse density between the saline and gentamicin treated ears as evaluated 6.86 mm from apex region. As shown in figure 2, the gentamicin treatment significantly decreased the synapse density by 76% in the 15.68 mm region (p=0.0001), 70% in the 16.66 mm region (p=0.001), 69% in the 17.64 mm region (p=0.00004), 77% in the 18.24 mm region (p=0.00003), and 76% in the 19.6 mm region (p=0.005). By comparing the synaptic densities of the corresponding
hair cell data in these regions to the hair cell and synaptic density in the 6.86 mm region, it appears that the decreases in inner hair cell synaptic densities occurred in regions that have greater outer hair cell damage. Interestingly moderate to no inner hair cell losses were detected.

**Figure 21.** (21A) Inner hair synapse density following direct middle ear administration of gentamicin Representative image of inner hair cells with associated ribbon synapses in (21A) control and (21B) treated tissues.
Discussion

The results of this study show that intra-ural gentamicin produces severe inner hair cell synaptopathy acutely following dosing. While there is severe auditory dysfunction and outer hair cell loss incurred, there is only minimal inner hair cell loss observed. The synaptopathy following intra-ural gentamicin began at 15.6 mm from apex, not-surprisingly indicating greater neurotoxicity compared to systemic kanamycin, which showed effects beginning at 17.6 mm from apex. These results suggest that compounds within the aminoglycoside class of compounds are synaptotoxic in the inner ear, and also that intra-ural administration of these compounds may allow greater drug exposure and subsequently affect a larger area within the cochlea.

The use of middle ear catheters to directly administer drug formulations to the round window allows for a higher concentration of drug exposure within the perilymph as compared to systemic routes. Administration by systemic routes leads to variable concentrations of aminoglycosides within the cochlea due to metabolic and distributional confounds, which subsequently produces corresponding local variability in neuronal viability. Employment of the transtympanic route in this study allowed for collection of data towards a comprehensive and definitive modeling of the acute oto-neurotoxic effects of gentamicin.

We reported that high doses of systemic kanamycin produce decreases in inner hair cell synapse densities in the lower basal regions of the cochlea. Intra-ural
gentamicin produced a greater quantitative reduction in synapse density, and also affected more of the apical regions of the cochlea. This suggests that the inner hair cell synapse losses observed are directly correlated to the effective drug exposure within the inner ear. The inner hair cells in both studies were largely unaffected by the drug treatment, implying that these drugs alter the inner hair cell function or directly causes the deterioration of the ribbon synapse. The ribbon synapse decreases observed arise largely in the basal region of the cochlea where severe outer hair cell losses occur. It has been shown that the hair cells in the basal region of the cochlea are more sensitive to drug treatment as a result of decreased endogenous mechanisms for handling cellular stress (Sha et al., 2001).

The synaptopathy observed in this study is relevant for patients being treated for Meniere’s disease. Most studies conducted on gentamicin-treated Meniere’s patients report that hearing loss is observed in the minority of patients if the treatment is monitored closely (Silverstein et al., 2010). Kujawa and Liberman (2009) found that noise exposures producing temporary threshold increases induced permanent inner hair cell synapse degeneration and long term spiral ganglion loss. Although threshold sensitivity is preserved, supra-threshold wave I amplitude is drastically reduced, impacting long term selective auditory attention (Bharadwaj et al., 2014, Furman et al., 2013, Demanez et al., 2003). We have shown that aminoglycoside-induced synaptopathy is at least partially recoverable, but the long term ramifications on spiral ganglion
degeneration have not been assessed. If these here to fare considered “safe” doses of gentamicin are producing recoverable synaptopathic insults, the long term effects may mimic those same supra-threshold hearing deficits observed with noise exposures.

In conclusion, this study confirmed that aminoglycosides produce significant decreases in inner hair cell synapses acutely following intra-aural administration, which is variably recoverable. Additionally, a clinically relevant, 10 mg dose produced more widespread synaptic damage in the cochlea than that observed with systemic aminoglycoside administration. These results suggest that caution should be use when using aminoglycoside treatments via the transtympanic route as the long term repercussions of synaptic loss have not be evaluated and may contribute to long term hearing deficits.

References


CHAPTER VI

DISSEPTION SUMMARY AND CONCLUSIONS

The aims of this dissertation project were to elucidate the effects of aminoglycosides on synaptic plasticity. We were first able to determine that high systemically administered doses of kanamycin produce significant effects on inner hair cell ribbon densities in the base of the cochlea. Building upon these results, we determined that the observed reduction in inner hair cell ribbon density occurred only acutely followed by a period of apparent partial recovery. Up to this point in the project, we had analyzed only presynaptic structures; therefore the next project examined both pre- and post-synaptic structures and the effect of kanamycin on their co-localization. These experimental results clearly confirmed that aminoglycoside therapy produced a partially recoverable synaptopathic insult. Our final experiment looked at how direct administration of aminoglycosides to the middle ear affected synapse plasticity. This experiment showed that direct administration of aminoglycosides produced significantly more synaptic degeneration and also affected more of the cochlea as compared to systemic administration. Of the greatest potential significance was the discovery that inner hair cell ribbon synapse degeneration is recoverable.

These results have several implications with regard to enhancing preventative therapies to prevent aminoglycoside-induced hearing loss in humans. Identification of
the effects of aminoglycoside antibiotics on synapse degeneration supports the role of excitotoxicity as a mechanism associated with hair cell death. Substantial work will be required to establish the proper NMDA antagonist compound and relative efficacious dose concentration that will provide sufficient protection. In all likelihood, a combination therapy consisting of antioxidants and NMDA antagonists will be required to provide complete protection from aminoglycoside damage. One challenge in designing a co-therapy to be administered along with an aminoglycoside will be that the patient will have compromised kidney function. The kidney is critical for drug metabolism and thus compromised function may consequently exacerbate damage as aminoglycoside concentrations will be maintained at high concentrations for a longer duration. In spite of these challenges, our results provide insight into the mechanisms involved in aminoglycoside ototoxicity and could help direct future protective therapies.

The finding that kanamycin-induced synaptopathy is recoverable is impactful and significant in the field of otolaryngology. As reiterated multiple times in this dissertation, the finding that synaptopathies are recoverable in guinea pigs provides hope that the same effect may be observed in humans. Before any clinical advances can occur, additional research must be conducted to better understand the processes that are involved in this regenerative process. Additionally, the function and sustainability of these new synapses must be determined to understand the implications of inducing re-innervation in patient populations.
Understanding why regeneration occurs in guinea pigs and not mice would provide the necessary information on how to approach inducing re-innervation as a therapy. Future research should focus on quantifying the expression of various nerve growth factors, such as NT-3 and BDNF, during the re-innervation process. It has been shown that these specific growth factors help induce synaptogenesis following cochlear trauma (Wang and Green, 2011). Studies should focus on the endogenous expression of these nerve growth factors in the cochlea of mice and guinea pigs to understand if there is a differential expression in each species. A differential level of endogenous nerve growth factors, with higher levels being expressed in guinea pigs, would strongly support why re-innervation occurs in the guinea pig and not in mice. It was recently shown that mice genetically modified to overexpress NT-3 were capable of regenerating synapses following a mild noise exposure (Wan et al., 2014). This recent finding further supports that expression of these nerve growth factors is critical for re-innervation in the cochlea. Using the aminoglycoside model of synaptic re-innervation, it should also be determined if NT-3 expression is upregulated immediately following kanamycin administration during re-innervation. These data would support the development of an exogenous NT-3 therapy to promote synaptogenesis following cochlear trauma.

Future research should also focus on characterizing auditory function following synaptic re-innervation. Liu et al. (2013) found that temporal resolution, as defined by the ability to process multiple click stimuli during ABR evaluations, was not restored in
spite of recovery of auditory threshold and synaptic re-innervation. Other research has shown that mild noise overexposure produces transient threshold increases with associated synaptic loss, but in recovery the supra-threshold neural amplitude of the ABR wave I, associated with cochlear neurotransmission, is significantly reduced (Kujawa and Liberman, 2009). This effect has been further examined showing that the high threshold, low spontaneous rate subset of afferent cochlear neurons is highly susceptible to noise exposure (Furman et al., 2013). The afferent dendrites have been shown to have different afferent neuron populations that are activated by different intensity levels (Kantardzhieva et al., 2013, Liberman et al., 2011). If there appears to be a sub-population of afferent neurons that are sensitive to noise, it should also be determined if there are a sub-population of afferent neurons that are sensitive to aminoglycoside treatment.

The aminoglycoside-induced synaptopathy model is challenging to assess neural function via ABR due to the severe outer hair cell losses observed following treatment. The outer hair cells provide amplification of sound waves within the cochlea and therefore a reduction in the suprathreshold wave I amplitude may be due to a loss of hair cell function and not correlated with a loss of synaptic connections. An alternative method to assess the impact of re-innervation of temporal resolution and wave I neural amplitude would be to use an electrically evoked auditory brainstem response (eABR). The use of eABRs following aminoglycoside treatment would allow for the monitoring of inner hair cell neurotransmission during re-innervation. eABRs are used clinically to
determine residual auditory nerve function in patients looking to receive a cochlear prosthetic device.

To determine if sub-populations of the afferent neurons are affected by kanamycin treatment, single unit nerve fiber recordings would need to be conducted following treatment. This is consistent with the procedures that were used to determine the subpopulation of afferent nerve fibers that were most sensitive to noise overexposure. Characterization of afferent nerve fiber sensitivity following kanamycin administration would provide insight into potential contributing factors to the hearing loss observed following aminoglycoside ototoxicity. Furthermore, this data may help to determine if there is a sub-population of afferent nerve fibers, such as the high threshold fiber, that are sensitive to cochlear damage in general. If found that a sub-population of afferent neurons is most vulnerable to cochlear trauma, identification of the cause could help provide a therapeutic target to reduce neurodegeneration.

Future research should also be directed at evaluating the long term effects of synaptic loss and regeneration in the guinea pig. In the mouse it has been shown that a cochlear injury resulting in synaptic loss results in a secondary effect of spiral ganglion degeneration beginning approximately 1 year from the initial insult (Kujawa and Liberman, 2009). The same effect was not observed in the guinea pig following synaptic loss as assessed at 6 months and 1 year following injury (Liu et al., 2013; Lin et al., 2011). The lack of this effect could be explained by the relative differences in life span.
which could contribute to the difference in timing of secondary degeneration, or may also be explained by the presence of synaptic re-innervation which would maintain spiral ganglion neuron viability. Therefore conducting a study using kanamycin-induced synaptopathy in guinea pigs that assessed auditory function, synaptic density, and spiral ganglion degeneration over a 2 year time-course would provide clarity on the long-term implications of synaptic re-innervation on restoration of auditory function. This data could provide insight into approaches to help restore auditory function in patients with long term hearing dysfunction.

References


