UNDERSTANDING THE UNDERLYING MECHANISMS OF NOISE-INDUCED HEARING LOSS: A MOLECULAR AND PHYSIOLOGICAL APPROACH

Cherylea Jane Browne

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DECLARATION

I, Cherylea Jane Browne, hereby declare in accordance with the bylaws of the University of Western Sydney that all work in this thesis is entirely my own unless otherwise acknowledged. This thesis was completed during my enrolment with the Department of Anatomy and Cell Biology, from February 2009 to September 2012, under the supervision of Dr Carl Parsons and Professor John Morley.

This work has not been previously presented for any other degree or qualification, at this or any other university.

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# List of Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>ABR</td>
<td>Auditory Brainstem Response</td>
</tr>
<tr>
<td>AC</td>
<td>Auditory Cortex</td>
</tr>
<tr>
<td>ACEC</td>
<td>Animal Care and Ethics Committee</td>
</tr>
<tr>
<td>AES</td>
<td>Anterior Ectosylvian Sulcus</td>
</tr>
<tr>
<td>AIDS</td>
<td>Auto-immune Deficiency Syndrome</td>
</tr>
<tr>
<td>ALA</td>
<td>Alpha Lipoic Acid</td>
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<tr>
<td>ALCAR</td>
<td>Acetyl-L-Carnitine</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated Protein Kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<tr>
<td>AVCN</td>
<td>Anterior Ventral Cochlear Nucleus</td>
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<td>BAT</td>
<td>Brown Adipose Tissue</td>
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<tr>
<td>Calb1</td>
<td>Calbindin</td>
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<tr>
<td>CAP</td>
<td>Compound Action Potential</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium</td>
</tr>
<tr>
<td>CBP</td>
<td>Calcium Binding Protein</td>
</tr>
<tr>
<td>CD</td>
<td>Compact Disc</td>
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<tr>
<td>CL</td>
<td>Chemiluminescence</td>
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<td>CN</td>
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<tr>
<td>CN VIII</td>
<td>Vestibulocochlear Nerve (Cranial Nerve #7)</td>
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<tr>
<td>dB</td>
<td>Decibel</td>
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<tr>
<td>DCN</td>
<td>Dorsal Cochlear Nucleus</td>
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<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>Acronym</td>
<td>Full Form</td>
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<td>-----------------------------------------------</td>
</tr>
<tr>
<td>FRA</td>
<td>Frequency Response Area</td>
</tr>
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<td>GABA</td>
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<td>GABA_{\alpha1}</td>
<td>GABA A Receptor Subunit Alpha 1</td>
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<td>GAD-65</td>
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<td>GAP-43</td>
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<td>GLM</td>
<td>General Linear Model</td>
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<tr>
<td>GPx</td>
<td>Glutathione Peroxidase</td>
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<td>Glutathione</td>
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<td>IC</td>
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<td>Central Nucleus of the Inferior Colliculus</td>
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<td>Inner Hair Cell</td>
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<tr>
<td>ITI</td>
<td>Inter-trial Interval</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal Kinase</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Daltons</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>kHz</td>
<td>Kilo Hertz</td>
</tr>
<tr>
<td>LL</td>
<td>Lateral Lemniscus</td>
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<td>LSO</td>
<td>Lateral Superior Olive</td>
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<tr>
<td>LTP</td>
<td>Long Term Potentiation</td>
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<tr>
<td>mg</td>
<td>Milligram</td>
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<tr>
<td>MGB</td>
<td>Medial Geniculate Body</td>
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<tr>
<td>mm</td>
<td>Millimetre</td>
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<td>mM</td>
<td>Millimolar</td>
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xvii
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<td>MmAb</td>
<td>Mouse Monoclonal Antibody</td>
</tr>
<tr>
<td>MON&lt;sup&gt;N/SAT&lt;/sup&gt;</td>
<td>Monotonic (no saturation)</td>
</tr>
<tr>
<td>MON&lt;sup&gt;SAT&lt;/sup&gt;</td>
<td>Monotonic (with saturation)</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribo-Nucleic Acid</td>
</tr>
<tr>
<td>MSO</td>
<td>Medial Superior Olive</td>
</tr>
<tr>
<td>MTB</td>
<td>Medial Trapezoid Body</td>
</tr>
<tr>
<td>n</td>
<td>Number of subjects/animals</td>
</tr>
<tr>
<td>N.</td>
<td>Nuclei/Nucleus</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetyl Cysteine</td>
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<tr>
<td>NIHL</td>
<td>Noise Induced Hearing Loss</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-Aspartate</td>
</tr>
<tr>
<td>NR1</td>
<td>N-Methyl-D-Aspartate receptor subunit 1</td>
</tr>
<tr>
<td>NR2A-D</td>
<td>N-Methyl-D-Aspartate receptor subunit 2A-D</td>
</tr>
<tr>
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<td>Nitric Oxide</td>
</tr>
<tr>
<td>NON MON</td>
<td>Non Monotonic</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric Oxide Synthase</td>
</tr>
<tr>
<td>NSAIDS</td>
<td>Non-steroidal Anti-inflammatory Drugs</td>
</tr>
<tr>
<td>OHC</td>
<td>Outer Hair Cell</td>
</tr>
<tr>
<td>OTC</td>
<td>2-oxothiazolidine-4-carboxylate</td>
</tr>
<tr>
<td>PES</td>
<td>Posterior Ectosylvian Sulcus</td>
</tr>
<tr>
<td>PLD</td>
<td>Personal Listening Device</td>
</tr>
<tr>
<td>PTS</td>
<td>Permanent Threshold Shift</td>
</tr>
<tr>
<td>PVCN</td>
<td>Posterior Ventral Cochlear Nucleus</td>
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<tr>
<td>RLF</td>
<td>Rate Level Function</td>
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<tr>
<td>RMS</td>
<td>Root Mean Square</td>
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<td>RNS</td>
<td>Reactive Nitrogen Species</td>
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<td>Full Form</td>
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<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>RpAb</td>
<td>Rabbit Polyclonal Antibody</td>
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<tr>
<td>rpm</td>
<td>rotations per minute</td>
</tr>
<tr>
<td>SA</td>
<td>Spontaneous Activity</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SIP</td>
<td>Schedule-induced Polydipsia</td>
</tr>
<tr>
<td>SIV</td>
<td>Startle Index Value</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error Mean</td>
</tr>
<tr>
<td>SOC</td>
<td>Superior Olivary Complex</td>
</tr>
<tr>
<td>SPL</td>
<td>Sound Pressure Level</td>
</tr>
<tr>
<td>TAC</td>
<td>Total Antioxidant Capacity</td>
</tr>
<tr>
<td>TB</td>
<td>Trapezoid Body</td>
</tr>
<tr>
<td>TDT</td>
<td>Tucker-Davis Technologies</td>
</tr>
<tr>
<td>TTS</td>
<td>Temporary Threshold Shift</td>
</tr>
<tr>
<td>Ucp1</td>
<td>Uncoupling Protein 1</td>
</tr>
<tr>
<td>VCN</td>
<td>Ventral Cochlear Nucleus</td>
</tr>
<tr>
<td>Vmax</td>
<td>Maximum Startle Amplitude</td>
</tr>
<tr>
<td>WAT</td>
<td>White Adipose Tissue</td>
</tr>
<tr>
<td>WH &amp; S</td>
<td>Work Health and Safety</td>
</tr>
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</table>
doi:10.1371/journal.pone.0033272
PUBLISHED ABSTRACTS


NATIONAL AND INTERNATIONAL PRESENTATIONS

The 40th Annual Society for Neuroscience Conference. Chicago, IL, USA. October 2009. Poster presentation titled: The expression of GABA$_A$ receptor subunit $\alpha 1$ in rat Auditory Cortex, Inferior Colliculus and Dorsal Cochlear Nucleus following a noise-induced cochlear lesion.

The 30th Annual Australian Neuroscience Society Conference (joint meeting with Australian Physiological Society). Sydney, New South Wales, Australia. January 2010. Poster presentation titled: Tracking the expression of GABA$_A$ receptor $\alpha 1$ subunit in multiple nuclei of the auditory pathway following noise induced hearing loss.


The University of Western Sydney Student Conference. Sydney, New South Wales, Australia. December 2010. Oral presentation titled: Tracking the expression of GABA$_A$ receptor $\alpha 1$ subunit, GAD-67 and NMDA receptor 2A in rat auditory pathway after noise induced hearing loss.

8th Australasian Auditory Neuroscience Workshop. Auckland, New Zealand. January 2011. Poster presentation: Tracking the expression of GABA$_A$ receptor $\alpha 1$...
subunit, GAD-67, Calbindin and NMDA receptor 2A in rat auditory pathway after noise induced hearing loss.


**The 35th Midwinter Meeting for the Association for Research in Otolaryngology.** Baltimore, MD, USA. February 2011. Poster Presentation: Tracking the expression of GABA<sub>A</sub> receptor subunit α1, Glutamic-Acid Decarboxylase-67, N-Methyl-D-Aspartate receptor subunit 2A in rat auditory pathway following noise-induced hearing loss.

**Australian National University Kioloa Neuroscience Colloquium.** Kioloa, New South Wales, Australia. March 2011. Oral presentation titled: The mechanisms of tinnitus: a molecular and physiological approach


**The 36th Midwinter Meeting for the Association for Research in Otolaryngology.** San Diego, CA, USA. February 2012. Poster Presentation: Alpha Lipoic Acid and Noise Induced Hearing Loss: A Prevention and Treatment Trial.
ABSTRACT

The main purpose of this thesis was to investigate the physiological, molecular and behavioural consequences of unilateral noise-induced hearing loss. In particular, how these properties develop in the month following noise-induced hearing loss. These investigations have implications for the generation of associated acoustic disorders, such as tinnitus and possibly hyperacusis. Tinnitus is the sensation of a sound when no external sound source is present. It is estimated to affect 10-17% of adults worldwide. There are many ways in which tinnitus can be generated; however noise-induced hearing loss is the most common cause and is increasingly apparent in individuals across all age groups. Most individuals in today’s society are exposed to damaging levels of noise on a regular basis; at the workplace, at concerts and the use of personal listening devices. It is understood that tinnitus is a disorder of the auditory system, but the neural mechanisms remain unknown. Excessive levels of noise can cause damage to the cochlea, which can result in premature deafness and noise-induced hearing loss. This damage causes a range of changes in the auditory pathway. Changes observed include plasticity of tonotopic representation, changes in auditory neuron physiology, changes in the pattern of spontaneous activity and changes in the balance of excitatory and inhibitory transmitter systems. Moreover, damage to the cochlea frequently results in tinnitus. This suggests that one or more of these changes may be involved in tinnitus generation.

This project used an established rat model of tinnitus to investigate its underlying neural mechanism(s). I have focussed on the time-course of changes in the auditory system following damage to the cochlea with the aim of elucidating which brain regions may be involved in the generation of tinnitus. In vivo electrophysiological recordings were
performed to investigate changes in neuronal excitability and spontaneous activity in the Auditory Cortex, Inferior Colliculus and the Dorsal Cochlear Nucleus. Protein analysis was performed to quantify the levels of excitatory, inhibitory and neuroplasticity-related proteins in the ipsilateral and contralateral Auditory Cortex, Inferior Colliculus and Dorsal Cochlear Nucleus. Behavioural techniques were utilised to track the tinnitus spectrum of noise-exposed animals. Finally, a potentially therapeutic agent, Alpha Lipoic Acid, was administered to noise-exposed animals in an attempt to protect against or treat noise-induced hearing loss.

In conclusion, this thesis has provided an in depth analysis of the time-course changes that occur in the auditory pathway after noise-induced hearing loss. This investigation has shown that noise-induced hearing loss leads to significant physiological and molecular disruptions in the auditory pathway of rats over time. Significant fluctuations were observed in neuronal excitability and spontaneous activity in the Auditory Cortex, Inferior Colliculus and Dorsal Cochlear Nucleus immediately after noise-induced hearing loss and continued over time. The results of the physiological study suggest that the low frequency region of the Auditory Cortex may be the first auditory site that is involved in the generation of tinnitus or contributes to the acute tinnitus spectrum, and that the high frequency region of the Auditory Cortex, and the Inferior Colliculus and Dorsal Cochlear Nucleus are involved in the perception of the chronic tinnitus spectrum. In the molecular study, protein expression was also observed to change immediately after noise-induced hearing loss and continued to fluctuate over the time-course. The characteristic decrease of inhibition that previous researchers have observed after noise-induced hearing loss may not necessarily be due to significant increases of excitatory neurotransmission-related proteins; rather a significant decrease of inhibitory-neurotransmission related proteins which lead to an overall increase in excitation. In
addition, this study has shown that the perception of tinnitus, and possibly hyperacusis, changes over time. The results of the behavioural study highlighted the variations of the perceived frequency of tinnitus across time-points and in individual animals, but also highlighted the similarities in chronic tinnitus frequencies. After noise-induced hearing loss, a small subset of animals experienced tinnitus, which was an initial broad tinnitus spectrum, with specific frequencies greater than others (high and low) and followed by a narrowing to specific frequencies that were the same as the greatest initial frequencies (high and low). A small subset of animals exhibited hyperacusis-like responses, which may reflect mechanism whereby the auditory system compensates for the loss of hearing by increasing central gain. Finally, the therapeutic intervention study showed that the administration of Alpha Lipoic Acid did not effectively protect or treat the effects of noise-induced hearing loss. My findings suggest that Alpha Lipoic Acid may not be an effective pre or post-treatment for moderate NIHL. Animals pre-treated with Alpha Lipoic Acid experienced the most significant hearing losses after noise-induced hearing loss and may suggest that long durations of treatment and higher doses of Alpha Lipoic Acid may play a role in cochlear toxicity, which leads to hair cell vulnerability.

This thesis has investigated the one month time-course changes of molecular, physiological and behavioural changes in the young adult rat after NIHL. This investigation has presented novel findings that significant time-dependent changes occur in the auditory pathway after NIHL. The physiological changes over the one month time-course reflect a constant compensatory mechanism between excitability and SA; the molecular changes indicate fluctuations in excitatory and inhibitory neurotransmission-related systems over the one month time-course and the behavioural manifestations of tinnitus highlight the variability of animals’ perceptions after NIHL. This study has proposed that the Day 8 time-point may be a crucial period after NIHL,
whereby the auditory system attains a state of partial recovery. Interpreting these mechanisms will be crucial for understanding the generation and development of acoustic disorders such as tinnitus and hyperacusis, and potentially the development of a therapeutic intervention.
CHAPTER ONE

GENERAL

INTRODUCTION
1.1: NOISE-INDUCED HEARING LOSS

Noise induced hearing loss (NIHL) is defined as a hearing impairment arising from exposure to excessive noise (Smith, 1998). Symptoms may include gradual loss of hearing, decrease of hearing sensitivity and the development of acoustic disorders such as tinnitus and hyperacusis. Tinnitus is defined as the ‘perception of sound or noise originating from the ears or head in the absence of an evident external stimulus’ (Eggermont, 1990) (see section 1.4), and hyperacusis is defined as the unusual tolerance to common environmental sounds that are neither threatening nor uncomfortably loud to a typical person’ (Vernon, 1987, Klein et al., 1990) (see section 1.5). It is estimated that 1 in 10 people worldwide has some form of NIHL (Mahboubi et al., 2012). Noise exposure is the most common preventable cause of sensorineural hearing loss in the world (Rabinowitz, 2000).

Most people are frequently exposed to damaging levels of noise (see section 1.1.1) in the workplace and at entertainment venues. Furthermore, the increasing use of personal listening devices (PLD) such as mp3 players, radios and CD (compact disc) players, is becoming a major concern (Kurmis and Apps, 2007, Peng et al., 2007, Kumar et al., 2009, Figueiredo et al., 2011). Currently, there is no effective treatment for NIHL (Lynch and Kil, 2005). Most of the efforts to reduce NIHL have concentrated on reducing risks from occupational noise exposure in adults, such as the often mandatory use of ear plugs or ear muffs at constructions sites. However, many studies have reported an increasing trend of NIHL in children and adolescents. For example, 40% of students between the ages of 16 and 25 years have audiological evidence of NIHL (Lees et al., 1985). Also, 9-12.5% of school aged children (6-19 years) were reported to have noise-induced threshold shifts (Blair, 1996, Niskar et al., 2001).
Unfortunately, there appears to be a lack of education concerning hearing protection for young people. The only available information is via hearing awareness websites directed at educating parents, such as ‘It’s a Noisy Planet’: “The National Institute on Deafness and Other Communication Disorders’ sponsors ‘It’s a Noisy Planet. Protect Their Hearing’. This national public education campaign is designed to increase awareness among parents of children ages 8 to 12 about the causes and prevention of NIHL. With this information, parents and other adults can encourage children to adopt healthy hearing habits before and during the time that they develop listening, leisure and working habits” (sourced from http://www.noisyplanet.nidcd.nih.gov).

Despite the web-based hearing conservation campaigns, most young people are unaware of NIHL or how they can prevent it. Recently, a survey was conducted on the MTV Web site, in which only 16% of teens and young adults who responded reported that they had heard, read, or seen any information on NIHL (Chung et al., 2005). Even when young people understand the risk of NIHL, they do not always follow through by adopting habits that protect their hearing. These habits are simple, such as turning down the volume of PLD or wearing earplugs or earmuffs in noisy environments. One study of American college students found that even among those who knew about NIHL, almost three-quarters had never worn hearing protectors (Crandell et al., 2004). It is clear that NIHL and the associated acoustic disorders will become a major health issue in the general population of the near future.

1.1.1: LEVELS OF ENVIRONMENTAL NOISE

The levels of various environmental noise is summarised in Table 1.1 The Australian Work Health and Safety (WH&S) Laws provide guidelines (Table 1.2) which demonstrate the combinations of various noise levels and the associated length of time a
person without hearing protectors can be exposed before the standard is exceeded. It is clear that it is not difficult to exceed the WH&S standards: for example a person listening to a PLD (all full volume) for more than 15 minutes, a person attending a sporting event or music concert for more than 57 seconds, etc.

**TABLE 1.1: LEVELS OF NOISE EXHIBITED BY VARIOUS ENVIRONMENTAL SOUNDS.**

<table>
<thead>
<tr>
<th>Noise Level (dB)</th>
<th>Noise Type</th>
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<tr>
<td>20</td>
<td>- Leaves rustling</td>
</tr>
<tr>
<td>30</td>
<td>- Whisper</td>
</tr>
<tr>
<td>40</td>
<td>- Quiet library</td>
</tr>
<tr>
<td>50</td>
<td>- Moderate rainfall</td>
</tr>
<tr>
<td>60</td>
<td>- Normal conversation</td>
</tr>
<tr>
<td></td>
<td>- Dishwashers</td>
</tr>
<tr>
<td>70</td>
<td>- Traffic</td>
</tr>
<tr>
<td></td>
<td>- Vacuums</td>
</tr>
<tr>
<td>80</td>
<td>- Alarm clocks</td>
</tr>
<tr>
<td>90</td>
<td>- Lawnmowers</td>
</tr>
<tr>
<td></td>
<td>- Power tools</td>
</tr>
<tr>
<td></td>
<td>- Blenders</td>
</tr>
<tr>
<td></td>
<td>- Hair dryers</td>
</tr>
<tr>
<td>100</td>
<td>- PLD (at full volume)</td>
</tr>
<tr>
<td>110</td>
<td>- Music Concerts</td>
</tr>
<tr>
<td></td>
<td>- Car horns</td>
</tr>
<tr>
<td></td>
<td>- Sporting events</td>
</tr>
<tr>
<td>120</td>
<td>- Jet planes (during takeoff)</td>
</tr>
<tr>
<td>130</td>
<td>- Jackhammers</td>
</tr>
<tr>
<td></td>
<td>- Ambulances</td>
</tr>
<tr>
<td>140</td>
<td>- Fireworks</td>
</tr>
<tr>
<td></td>
<td>- Gun shots</td>
</tr>
<tr>
<td></td>
<td>- Custom car stereos (at full volume)</td>
</tr>
</tbody>
</table>

(Sourced from American Academy of Audiology website; www.HowsYourHearing.org)
TABLE 1.2: AUSTRALIAN WH&S NOISE EXPOSURE GUIDELINES.

This table demonstrates the combinations of various noise levels and the associated length of time a person without hearing protectors can be exposed before standards are exceeded.

<table>
<thead>
<tr>
<th>Noise Level (dB)</th>
<th>Exposure Time</th>
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<td>80</td>
<td>16 hours</td>
</tr>
<tr>
<td>82</td>
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<td>85</td>
<td>8 hours</td>
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<td>100</td>
<td>15 minutes</td>
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<td>7.5 minutes</td>
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<td>3.8 minutes</td>
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<td>109</td>
<td>1.9 minutes</td>
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<td>57 seconds</td>
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<td>115</td>
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<td>118</td>
<td>14.4 seconds</td>
</tr>
<tr>
<td>121</td>
<td>7.2 seconds</td>
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<td>124</td>
<td>3.6 seconds</td>
</tr>
<tr>
<td>127</td>
<td>1.8 seconds</td>
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<tr>
<td>130</td>
<td>0.9 seconds</td>
</tr>
</tbody>
</table>

(Sourced from WH&S government ‘Managing Noise and Preventing Hearing Loss at Work’; www.safeworkaustralia.gov.au)
1.1.2: THE CONSEQUENCES OF NOISE-INDUCED HEARING LOSS

1.1.2.1: SOCIAL

Depending on the severity of the condition, people with NIHL may be greatly handicapped socially. The first signs of NIHL can be observed in the typical 4-6 kHz “notch” observed on audiograms (McBride and Williams, 2001) indicating a loss of hearing ability in the middle of the frequency range of human voices. This hearing loss in most cases increases with continued noise exposure. The consequences of hearing impairment include inability to interpret speech sounds, often resulting in a reduced ability to communicate, economic and educational disadvantages, social isolation and difficulties within home, work and school life (Weinstein and Ventry, 1982, Strawbridge et al., 2000, Arlinger, 2003, Lieu, 2004).

NIHL is a significant social and public health problem in Australia and the world. In 2001/2002, the direct cost of workers’ compensation deafness claims was calculated to be just over $30 million in Australia alone (Morris, 2006).

1.1.2.2: PATHOLOGICAL

In animal models, exposure to damaging levels of noise is known to cause significant structural and physiological damage to the cochlea (Webster and Webster, 1981, Robertson, 1982, Morest and Bohne, 1983, Liberman and Dodds, 1987, Bauer et al., 2007, Bauer et al., 2008, Mulders et al., 2011). There is now ample evidence in animal studies that cochlear damage causes substantial neuroplasticity such as molecular and physiological changes throughout the peripheral and central auditory pathway. Alterations in the peripheral auditory pathway influence the progression of central changes due to reduced or altered input. These include plastic responses such as axonal
pruning (Potashner et al., 1997) and sprouting (Illing et al., 2005), trans-synaptic
degeneration and new synapse formation (Kim et al., 2004b), neurotransmitter and
receptor changes (see section 1.3), neuronal shrinkage and cell loss (Aarnisalo et al.,
2000) and changes in the tonotopic distribution of neurons within nuclei (Salvi et al.,
2000b, Irvine and Wright, 2005).

I have utilised a rat model of NIHL due to its similarities to the peripheral and central
auditory pathway of humans. The auditory system can be divided into ascending and
descending pathways, similar to other sensory nervous systems. Normally, descriptions
of the auditory pathway are based on results from animals such as cats, rats and
monkeys. Species differences regarding the anatomy and physiology of the auditory
nervous system are be taken into account when results of different species are compared.
In particular, the cortical regions have considerable differences between different
species. In the human primary auditory cortex, the high frequencies are represented
rostrally and the lower frequencies are represented caudally. The rat auditory pathway is
similar to the human auditory pathway concerning connections and structure, and share
some similarities in frequency representation (albeit differing sensitivities), making the
rat a suitable animal model for this research.
1.1.2.2.1: The Peripheral Auditory System

When a sound is presented the frequency waves vibrate the tympanic membrane. These vibrations are then translated to the cochlea. The organ of Corti (Fig. 1.1), which is located within the cochlea, is an assembly of supporting cells and inner and outer hair cells (IHCs and OHCs, respectively) that are supported by the basilar membrane. The basilar membrane is displaced by vibrations of the endolymph, which stimulates the hair cells by bending their stereociliary bundles against the tectorial membrane. Signals from each inner hair cell are relayed to the central auditory pathway via 10 to 20 afferent fibres of the cochlear portion of the Vestibulocochlear nerve (CN VIII).

![The Human Organ of Corti](source)

Figure 1.1: The Human Organ of Corti (sourced from (Fettiplace and Hackney, 2006))

Acoustic trauma is known to produce multiple types of structural damage to the peripheral auditory system. The most vulnerable cells in the cochlea are the IHCs and OHCs respectively. During and after noise exposure, the hair cells are affected by the generation of oxidants, such as Reactive Nitrogen Species (RNS) and Reactive Oxygen Species (ROS). After noise exposure the hair cells develop stereocilia defects, migrating nuclei, swollen cell bodies and, eventually, a high level of cell death, which
consequently leads to a loss in hearing sensitivity (Liberman and Dodds, 1987). In most mammals, IHCs and OHCs cannot regenerate. This loss of IHCs and OHCs causes degeneration of CN VIII fibres, reflected in losses of spiral ganglion cells (Kujawa and Liberman, 2006) and morphologic changes in the ascending neural pathways (Morest and Bohne, 1983).

It is presumed that this peripheral damage influences changes in the central auditory pathway due to altered neural input (Noreña and Eggermont, 2003). These resulting central changes are thought to be involved in the perception of acoustic disorders such as tinnitus and hyperacusis, which are commonly associated with NIHL.

Figure 1.2: Electromicrographs of the Rat Organ of Corti. This figure highlights the IHCs (blue *) and OHCs (red *) before (A) and after (B) excessive noise exposure (taken from http://newsroom.hei.org/pr/hri/photo.aspx?fid=138451&id=E0C32378).
1.1.2.2.2: The Central Auditory System

This study primarily focused on the changes that occur after NIHL at various levels of the central auditory system, the brainstem, midbrain and cortex. This section describes the ascending auditory pathway focusing on the function of the Dorsal Cochlear Nucleus (DCN), Inferior Colliculus (IC) and Auditory Cortex (AC) (highlighted in Fig. 1.3).

Figure 1.3: The Central Auditory Pathway of the Rat (Caspary et al., 2008). This figure highlights the classical ascending auditory pathway. Legend: Medial Geniculate Body = MGB, Primary Auditory Cortex = AC, Inferior Colliculus = IC, Dorsal Cochlear Nucleus = DCN, Posterior Ventral Cochlear Nucleus = PVCN, Lateral Superior Olive = LSO, Medial Superior Olive = MSO and Medial Trapezoid Body = MTB.
Brainstem

All auditory nerve fibres from the cochlea form their first synapses at the ipsilateral Cochlear Nucleus (CN) complex. The CN complex consists of the DCN and the Ventral Cochlear Nucleus (VCN), which is subdivided into two divisions: the anterior VCN (AVCN) and the posterior VCN (PVCN). Within the CN complex, this study focused primarily on the DCN. The DCN is a cortex-like structure on the dorsolateral surface of the brainstem. There exists tonotopic organisation in both divisions of the CN. In the rat DCN, low frequency information is located ventrolaterally and high frequency information is located dorsomedially (Kaltenbach and Lazor, 1991). The DCN has efferent and afferent interactions with many neural parts of the auditory system, as summarised in Table 1.3. Previous animal studies have shown that the DCN also has many non-auditory connections (Itoh et al., 1987, Weinberg and Rustioni, 1987, Wright and Ryugo, 1996, Zhou and Shore, 2004, Haenggeli et al., 2005) (see section 1.1.2.2.2.1).

Axons from the cells in each of the three divisions of the CN cross the midline in three striae. Some of the fibres (or their collaterals) make synaptic connections with cells in the nuclei of the Superior Olivary Complex (SOC). The fibres of these three striae join and form the Lateral Lemniscus (LL), which ascends to the contralateral IC. Some fibres of the LL make synaptic contact with the nuclei of the LL. In addition, some fibres from the PVCN and AVCN do not cross the midline and project to the ipsilateral IC.

Midbrain

The IC is the principal midbrain nucleus of the auditory pathway and receives input from several peripheral brainstem nuclei in the auditory pathway, as well as inputs from the AC. The IC has three sub-nuclei; although, not all are involved in hearing (see section 1.1.2.2.2.1). It is divided into the Central Nucleus (ICC), the Dorsal Cortex (ICd) and the
External Cortex (ICx); however, the role of the ICC is exclusively auditory (Aitkin et al., 1994) and in fact is essential for normal hearing (Jenkins and Masterton, 1982). The IC integrates acoustic information from many auditory nuclei and relays the processed information to higher centres and is thought to be involved in the integration of all sound location data (Bock and Webster, 1974, Binns et al., 1992). It has a complex array of afferent, efferent and intrinsic connections, these are summarised in Table 1.3. The IC may have the most diverse connections of any auditory structure. Most of these connections are topographic; they are thought to be related to processes embedded in the tonotopic arrangement of characteristic frequency in the ICC.

Cortex

Fibres from the IC project to the ventral part of the thalamic nucleus, the Medial Geniculate Body (MGB). The information is then relayed to the AC. The AC is the region of the brain that is responsible for the perception of auditory information, including identifying and segregating auditory "objects" and sound localisation (Price et al., 1992, Weinberg, 1997, Ahissar et al., 2001, Tramo et al., 2002). In humans it is located on the superior temporal gyrus in the temporal lobe. The primary AC is surrounded by a belt of secondary fields and more lateral parabelt fields at a third level of processing which include the processing of auditory spatial information and the processing of auditory patterns, including communication sounds and speech (Rauschecker, 1998). The belt areas of the auditory cortex receive more diffuse input from the belt areas of the medial geniculate complex and therefore are less precise in their tonotopic organisation (Kaas and Hackett, 2000). The primary AC has a topographical map of the cochlea. The tonotopic maps are different in every animal though some share similarities. In the human, high frequencies are represented rostrally and low frequencies are represented caudally. Likewise, in the rat high frequencies are
represented rostrally and low frequencies are represented caudally (Fig. 1.4). The AC has efferent and afferent interactions with many parts of the auditory system. These are summarised in Table 1.3. Other studies have shown that the primary AC also has many non-auditory connections (Bizley et al., 2007, Kayser et al., 2009, Musacchia and Schroeder, 2009) (see section 1.1.2.2.2.1).

Figure 1.4: Tonotopic Map of Rat Primary Auditory Cortex (sourced from (Kenet et al., 2007)).

1.1.2.2.2.1: OTHER SENSORY MODALITIES INVOLVED IN TINNITUS

There is increasing evidence to suggest that the neural activity underlying tinnitus involves abnormal interactions between multiple sensory modalities, sensorimotor systems, neurocognitive networks and the brain pathways involved in processing emotional reactions (Cacace, 2003). The non-auditory connections in the AC, IC and DCN have been shown to be involved in somatosensory system interactions of tinnitus. Activation of the somatosensory system (particularly the trigeminal nerve) has been found to affect the loudness and character of tinnitus (Itoh et al., 1987, Møller et al., 1992, Cacace et al., 1994, Cacace et al., 1999a, Cacace et al., 1999b, Shore et al., 2000). The IC has connections with the amygdala, which is thought to process inputs from various sensory modalities (auditory, somatosensory, visual, olfactory, gustatory) (Aggleton et al., 1986). Craniocervical modulation of tinnitus has also been observed; it is thought to involve the non-auditory connections of the DCN (Levine, 1999).
TABLE 1.3: SUMMARY OF AFFERENT AND EFFERENT AUDITORY PROJECTIONS AND INTRINSIC CONNECTIONS IN THE DORSAL COCHLEAR NUCLEUS, INFERIOR COLICULUS AND THE AUDITORY CORTEX.

<table>
<thead>
<tr>
<th>Region</th>
<th>Afferent</th>
<th>Efferent</th>
<th>Intrinsic</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCN</td>
<td>- IC</td>
<td>- IC</td>
<td>-IC</td>
</tr>
<tr>
<td></td>
<td>- Dorsal N. of the LL</td>
<td>- Medial Geniculate N.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Ventral N. of the LL</td>
<td>- Anterior VCN</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Periolivary N.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- AC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Cochlea (Via Auditory Nerve)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- VCN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC</td>
<td>- DCN</td>
<td>- Dorsal N. of the LL</td>
<td>- Intracollicular</td>
</tr>
<tr>
<td></td>
<td>- Anterior VCN</td>
<td>- Intermediate N. of the LL</td>
<td>- Commissural</td>
</tr>
<tr>
<td></td>
<td>- Posteroventral CN</td>
<td>- Ventral N. of the LL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Medial Superior Olivary N.</td>
<td>- DCN</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Lateral Superior Olivary N.</td>
<td>- Anteroventral CN</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Medial N. of the TB</td>
<td>- Posterior VCN</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Lateral N. of the TB</td>
<td>- Superior Olivary Complex</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Ventromedial N. of the TB</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Superior Paraolivary N.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Dorsal N. of the LL</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Ventral N. of the LL</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- AC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td>- N. of the LL</td>
<td>- DCN</td>
<td>- Commissural</td>
</tr>
<tr>
<td></td>
<td>- IC</td>
<td>- IC</td>
<td>- Corticocortical</td>
</tr>
<tr>
<td></td>
<td>- Ventral N. of the MGB</td>
<td>- Pontine N.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Ventral N. of the MGB</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: IC = Inferior Colliculus, N = Nucleus/Nuclei, LL = Lateral Lemniscus, AC = Auditory Cortex, VCN = Ventral Cochlear Nucleus, DCN = Dorsal Cochlear Nucleus, CN = Cochlear Nucleus, TB = Trapezoid Body and MGB = Medial Geniculate Body.
1.1.2.2.3: Changes in the Central Auditory Pathway after NIHL

This project has focused on the molecular and physiological changes in the DCN, IC and AC (highlighted in Fig. 1.3) following NIHL. There is abundant research into changes in these areas after NIHL. In the CN, there are the following plastic responses: changes in neurotransmission, transmitter receptor function, the expression of ion conductance channels, and anatomical readjustments in the number or density of functioning synapses (Kaltenbach et al., 2005). Reorganisation of the tonotopic map in the IC of adult rats has also been observed. This reorganisation shows expansions of frequency representations across the IC. At the single cell level, Frequency Response Areas (FRA) also show significant increases in their threshold and spontaneous activity following acoustic trauma (Izquierdo et al., 2008a). There is also abundant research pertaining to the changes in the AC, particularly the change in tonotopic representation. Robertson and Irvine (1989) examined the immediate effects of unilateral cochlear lesions on the topographical map of frequency in the AC of guinea pigs. They found shifts in characteristic frequencies toward frequencies spared by the lesion (Robertson and Irvine, 1989). It has also been shown that a deprived area of AC becomes responsive to sensory inputs formerly represented only within the cortical sectors surrounding those representing lesioned input sources. This is due to tonotopic reorganisation and by the expansion of receptive fields (Buonomano and Merzenich, 1998).

There are many afferent, efferent and intrinsic connections between the different levels of the central auditory system and surrounding structures (Table 1.3). The afferent, efferent and intrinsic connections demonstrate the interrelatedness between the DCN, IC and AC. Therefore, it is reasonable to theorise that if one or more of the main auditory nuclei demonstrated a maladaptive change, all or most of the auditory nuclei would also be affected.
1.2: PHYSIOLOGICAL BASIS OF NOISE-INDUCED HEARING LOSS

There is now ample evidence that cochlear damage causes significant physiological changes throughout the auditory system. These include changes in spontaneous activity, changes in cell excitability, and changes in the tonotopic distribution of neurons within nuclei.

1.2.1: SPONTANEOUS ACTIVITY AND CELL EXCITABILITY

The definition of Spontaneous Activity (SA) in the nervous system is neural activity in the absence of intentional acoustic stimulation (Willott et al., 1988). SA is important during development (Moody, 1998), as it has shown to be involved in axon outgrowth (Holliday and Spitzer, 1990, Gu et al., 1994), pruning of synaptic connections (O'Leary et al., 1994) and maturation of neuronal signalling properties (Holliday and Spitzer, 1993). SA in the developing auditory system is involved in the refinement and maintenance of tonotopic representation (Leao et al., 2006). A previous study showed that supporting cells in the developing rat cochlea spontaneously release Adenosine Triphosphate (ATP), which causes nearby IHCs to depolarise and release glutamate, triggering discrete bursts of action potentials in primary auditory neurons (Tritsch et al., 2007). After acoustic trauma, changes in SA have been observed in the DCN (Kaltenbach et al., 1998, Kaltenbach et al., 2000, Brozoski et al., 2002, Chang et al., 2002, Imig and Durham, 2005, Finlayson and Kaltenbach, 2009), IC (Robertson et al., Imig and Durham, 2005, Bauer et al., 2008, Dong et al., 2009a, Mulders and Robertson, 2009, Dong et al., 2010a, Mulders et al., 2011) and in the AC (Noreña and Eggermont, 2003, Seki and Eggermont, 2003a, Noreña et al., 2010). It is clear that acoustic trauma stimuli at varying durations, frequencies and sound pressure levels (SPL), cause changes in SA in many animal models (Table 1.4). Protein expression is thought to underlie
Alterations in SA are also believed to be connected to the reorganisation of tonotopic maps following intense noise exposure. Tonotopic reorganisation has been shown in numerous studies (Robertson and Irvine, 1989, Kaltenbach et al., 1992, Harrison et al., 1998, Irvine and Wright, 2005, Izquierdo et al., 2008a). The AC, IC and DCN have been implicated as possible sites for the generation of tinnitus-producing signals owing to their tendency to become hyperactive following exposure to tinnitus-inducing agents such as intense sound (Kaltenbach et al., 2005) and this hyperactivity may be the first signs of tinnitus. Due to the vast array of connections in the auditory pathway (Table 1.3), it is possible that changes in the AC, IC and DCN could influence changes in other auditory sites/nuclei.

Stimulus-driven activity is neural activity that is evoked by a stimulus (Lakatos et al., 2005). Changes in stimulus-driven activity can be a sign of changes in the excitability state of the auditory system, which has been observed after NIHL. Reductions in stimulus-driven activity have been observed in the AC after acoustic trauma (Tan et al., 2007). It has been proposed that reductions in stimulus-driven activity may lead to an increase in SA, as a compensatory mechanism (Schaette and Kempter, 2006). This suggests that there is a complementary relationship between SA and cell excitability, and may also indicate that both of these aberrant neural responses cause or contribute to the generation of acoustic disorders after NIHL.
## TABLE 1.4: PREVIOUS STUDIES INVESTIGATING THE CHANGES IN SPONTANEOUS ACTIVITY AND CELLULAR EXCITABILITY AFTER ACOUSTIC TRAUMA

<table>
<thead>
<tr>
<th>Region</th>
<th>Animal</th>
<th>Acoustic Trauma</th>
<th>Observation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCN</td>
<td>Rat</td>
<td>10 kHz tone at 130 dB for 4 hours</td>
<td>↓ regular SA</td>
<td>(Chang et al., 2002)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↑ irregular SA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hamster</td>
<td>10 kHz tone at 115 dB for 4 hours</td>
<td>↑ SA</td>
<td>(Finlayson and Kaltenbach, 2009)</td>
</tr>
<tr>
<td></td>
<td>Chinchilla</td>
<td>4 kHz tone at 80 dB for 5 days</td>
<td>↑ SA</td>
<td>(Brozoski et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>15–20 kHz at 90 dB/octave for 1 hour</td>
<td>↑ SA</td>
<td>(Imig and Durham, 2005)</td>
</tr>
<tr>
<td></td>
<td>Hamster</td>
<td>10 kHz tone at 125-130 dB for 4 hours</td>
<td>↑ SA</td>
<td>(Kaltenbach et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>Hamster and Rat</td>
<td>10 kHz tone at 125-130 dB for 4 hours</td>
<td>↑ SA</td>
<td>(Kaltenbach et al., 2000)</td>
</tr>
<tr>
<td>IC</td>
<td>Guinea Pig</td>
<td>10 kHz tone at 124 dB for 1 hour</td>
<td>↑ SA</td>
<td>(Mulders and Robertson, 2009)</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>15–20 kHz noise at 90 dB/octave for 1 hour</td>
<td>↑ SA</td>
<td>(Imig and Durham, 2005)</td>
</tr>
<tr>
<td></td>
<td>Chinchilla</td>
<td>4 kHz tone at 85 dB for 1 hour</td>
<td>↑ SA</td>
<td>(Bauer et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>Guinea Pig</td>
<td>mechanical cochlear lesion</td>
<td>↑ SA</td>
<td>(Dong et al., 2009a)</td>
</tr>
<tr>
<td></td>
<td>Guinea Pig</td>
<td>10 kHz tone at 124 dB for 1 hour</td>
<td>↑ SA</td>
<td>(Dong et al., 2010a)</td>
</tr>
<tr>
<td>AC</td>
<td>Cat</td>
<td>6 kHz tone at 115 dB for 2 hours</td>
<td>↑ SA</td>
<td>(Seki and Eggermont, 2003a)</td>
</tr>
<tr>
<td></td>
<td>Cat</td>
<td>5 or 6 kHz tone at 120 dB for 1 hour</td>
<td>↑ SA</td>
<td>(Noreña et al., 2003)</td>
</tr>
<tr>
<td></td>
<td>Kitten</td>
<td>6 kHz tone 126 dB for 1 hour</td>
<td>↑ SA</td>
<td>(Eggermont and Komiya, 2000)</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>16 kHz noise at 115 dB for 1 hour</td>
<td>↓ excitability</td>
<td>(Tan et al., 2007)</td>
</tr>
</tbody>
</table>
1.2.2: Tonotopic Reorganisation

One change that has been well documented is the alteration of tonotopic maps in the AC following cochlear damage induced by acoustic trauma (see section 1.1.2.2.3). In most cases, the acoustic trauma leads to deafferentation which causes regions of the cortex to become deprived of normal input. Studies in monkeys, cats and rodents show that a loss of input to the AC from acoustic trauma leads to areas of missing frequencies (deprived neurons) in the cortical tonotopic map (Salvi et al., 2000a, Moller, 2006). It has been shown that neurons within the deprived regions show increased SA and increased neural synchrony compared with their less affected neighbours (Eggermont and Komiya, 2000, Noreña et al., 2003). It has also been shown that these deprived neurons become responsive to frequencies adjacent to the cortical regions that represent the frequency range where hearing was damaged. This results in an enlarged representation of sounds in the undamaged regions of the edge of the frequency region that is deprived of input (Rajan et al., 1993) (for example see Fig. 1.5).

![Figure 1.5: Normal and reorganized tonotopic maps in cat primary auditory cortex. The characteristic frequency at each recording site is colour-coded and overlaid on a photograph of the cortical surface for a control cat (A) and a cat with a noise induced hearing loss (B) (Eggermont and Roberts, 2004). Legend: AES = Anterior Ectosylvian Sulcus; PES = Posterior Ectosylvian Sulcus.](image-url)
The functional significance of this reorganisation is not clear, but it may help individuals adjust to a hearing loss and more effectively use the remaining inputs (Kaas et al., 2001). A human study by Moore and Vinay (2009) showed that subjects who acquired a dead region at high frequencies within the cochlea (high frequency hearing loss), which possibly led to cortical reorganisation, were associated with having a better ability to process information at low frequencies for frequency discrimination, improved amplitude-modulation detection and consonant identification (Moore and Vinay, 2009). However, a study by Brown et al (2004) showed that the frequency discrimination in reorganised areas of the cat AC had no detectable advantages, even though more cortical areas were devoted to a particular frequency (Brown et al., 2004).

Tonotopic reorganisation after noise trauma has been also been observed in the IC and DCN, to a lesser extent than the AC (Table 1.5).
Thus, it is clear that previous researchers have observed significant changes in SA, cell excitability, and tonotopic reorganisation in the auditory pathway after NIHL (Table 1.4 and 1.5). However, there are no investigations into these changes over time. In addition, there are no studies that have investigated multiple auditory sites at one time after NIHL. This will be the first investigation to record from multiple sites in the auditory pathway simultaneously after NIHL. This study may provide insight into the physiological changes occurring in each auditory centre after NIHL and may indicate from where the change is driven. In this study, I have focused on the changes in SA and cell excitability in auditory neurons after NIHL. I have attempted to correlate the physiological changes with changes in protein expression and determine whether these changes cause or contribute to the perception of tinnitus.

**TABLE 1.5: PREVIOUS STUDIES INVESTIGATING THE CHANGES IN TONOTOPIC ORGANISATION AFTER ACOUSTIC TRAUMA**

<table>
<thead>
<tr>
<th>Region</th>
<th>Animal</th>
<th>Acoustic Trauma</th>
<th>Observation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCN</td>
<td>Hamster</td>
<td>10 kHz tone at 120-126 dB for 0.5-6 hours</td>
<td>Tonotopic changes</td>
<td>(Kaltenbach et al., 1992)</td>
</tr>
<tr>
<td>IC</td>
<td>Rat</td>
<td>5-8 kHz tone at 110–121 dB for 3.3–16 hours</td>
<td>Tonotopic changes</td>
<td>(Izquierdo et al., 2008b)</td>
</tr>
<tr>
<td></td>
<td>Guinea Pig</td>
<td>10 kHz tone at 124 dB for 1 hour</td>
<td>Tonotopic changes</td>
<td>(Dong et al., 2010b)</td>
</tr>
<tr>
<td>AC</td>
<td>Cat</td>
<td>6 kHz tone at 115 dB for 2 hours</td>
<td>Tonotopic changes</td>
<td>(Seki and Eggermont, 2003b)</td>
</tr>
<tr>
<td></td>
<td>Guinea Pig</td>
<td>Mechanical lesion</td>
<td>Tonotopic changes</td>
<td>(Robertson and Irvine, 1989)</td>
</tr>
<tr>
<td></td>
<td>Cat</td>
<td>5 or 6 kHz tone at 120 dB for 1 hour</td>
<td>Tonotopic changes</td>
<td>(Noreña et al., 2003)</td>
</tr>
<tr>
<td></td>
<td>Kitten</td>
<td>6 kHz tone 126 dB for 1 hour</td>
<td>Tonotopic changes</td>
<td>(Eggermont and Komiya, 2000)</td>
</tr>
</tbody>
</table>
1.3: Molecular Consequences of Noise-Induced Hearing Loss

Research has shown that excessive noise exposure has a profound effect on the up- and down-regulation of important neurotransmitters, receptors and regulatory enzymes in the auditory pathway (Kaltenbach et al., 2005). These molecular changes can directly influence cellular physiology and may lead to premature hearing loss and acoustic disorders, such as tinnitus and hyperacusis. In this study, I was interested in tracking the level of expression of important molecular markers over time after NIHL. This study will function as an accompaniment to the electrophysiological study (Chapter 3) of this thesis, to determine whether molecular changes are directly related to physiological changes in the auditory system after NIHL. There are many molecular markers that are involved in regulating excitatory and inhibitory processes and that are involved during neuroplasticity. This study focused on selected molecular markers, including excitatory, inhibitory and neuroplasticity markers.

1.3.1: Proteins that Influence Changes in Neurotransmission

Genes and proteins that influence such processes as the synthesis, release, re-uptake/breakdown of neurotransmitters, and the dynamics of transmitter vesicles can be altered after cochlear damage. These changes have been observed at all levels of the auditory pathway. Investigation into the balance of excitatory and inhibitory transmitter systems is of particular interest, as these systems are thought to correlate with the changes in SA and cell excitability which is observed after noise trauma (see section 1.2.1).
1.3.1.1: **Excitatory Markers**

The N-Methyl-D-Aspartate (NMDA) receptor is the most abundant excitatory receptor that is expressed throughout the entire mature nervous system. The NMDA receptor is a glutamate-gated cation-specific ion channel that plays a key role in synaptic plasticity, including learning and memory (Collingridge and Lester, 1989). There are many different receptor subunits NMDA receptor subunit 1 and NMDA receptor subunit 2A-D (NR1 and NR2A-D, respectively); the subunit composition of NMDA receptors establishes differences of individual receptor properties. The NR1 subunits can form a homomeric receptor (Moriyoshi et al., 1991), whereas the NR2 subunits can only form heteromeric receptors in association with NR1. Association of NR2 subunits with NR1 increases NMDA receptor current (Monyer et al., 1994). This project focused on the NMDA receptor subunit 2A (NR2A), due to its fast kinetics and its ability to modify channel properties (Carroll and Zukin, 2002). The NR2A subunit has been studied extensively in regards to its involvement in visual plasticity (Roberts and Ramoa, 1999, Chen et al., 2000, Murphy et al., 2005, Cho et al., 2009). For example, Roberts and Ramoa observed an increase in the expression of NR2A subunits during the onset of ocular dominance plasticity in the ferret (Roberts and Ramoa, 1999). The role that NR2A plays in visual plasticity may also occur in auditory plasticity. A limited number of studies have investigated the effects of NIHL on the expression of NR2A. One study by Marionowski and colleagues (2000), found a decrease in the expression of NR2A mRNA after ototoxic exposure in IC and CN of young rats (Marianowski et al., 2000b). It has also been shown that injury induced activation of NR2A containing NMDA receptors functions as a pro-survival signal (DeRidder et al., 2006).
The NR2A subunit has been chosen for this study, due to its major role in the excitatory transmitter system, its involvement in synaptic plasticity and pro-survival signalling, and its fast kinetics and ability to modify channel properties.

1.3.1.2: Inhibitory Markers

The Gamma-Amino Butyric Acid (GABA) receptor is the most abundant inhibitory neurotransmitter receptor that is expressed throughout the entire nervous system and is responsible for most of the physiological activities of GABA. The GABA<sub>A</sub> receptor is a ligand-gated ion channel that mediates synaptic inhibition. There are many receptor subunits that form heteromeric GABA<sub>A</sub> receptor types; these include six α, three β, three γ and one δ subunit/s. I have chosen to focus on the GABA<sub>A</sub>α1 subunit, as the main GABA<sub>A</sub> receptor subtype contains the α1 subunit (α1β2γ2; 43% of all GABA<sub>A</sub> receptors in the rat brain) (McKernan and Whiting, 1996). Synaptic inhibition mediated by GABA is involved in both short and long term regulation of neuronal excitability and in fast synaptic inhibition (Angelotti and MacDonald, 1993). Previous studies have shown that after acoustic trauma, the expression of GABA<sub>A</sub>α1 decreases in the IC and DCN (Dong et al., 2009a, Dong et al., 2010a). The GABA<sub>A</sub>α1 subunit will be investigated in this study, due to its major role in the inhibitory transmitter system, its abundance in the central nervous system, and its ability to conduct fast synaptic inhibition.

Glutamic Acid Decarboxylase (GAD) is an enzyme that converts intracellular glutamate into the neurotransmitter GABA (Erlander et al., 1991). There are two isoforms in the mammalian brain, GAD-65 (molecular weight: 65 kilo Daltons (kDa)) and GAD-67 (molecular weight: 67 kDa). I have focused on GAD-67, as it is responsible for the majority of GABA production in the rat central nervous system (Asada et al., 1997). A decrease in the expression of GAD-67 can lead to reduced levels of GABA, which has
been previously observed after acoustic trauma (Yang et al., 1998, Milbrandt et al., 2000) and therefore can ultimately lead to an overall decrease in inhibition. GAD-67 will be investigated in this study due to its major role in the inhibitory transmitter system by producing the majority of GABA in the central nervous system.

Glycine and its several receptors are also involved in fast inhibitory neurotransmission throughout the central nervous system, especially within the midbrain, brainstem and spinal cord (Peyret et al., 1987, Sanes et al., 1987, van den Pol and Gorcs, 1988, Saransaari and Oja, 2009, Hossaini et al., 2012). GABA and glycine, and their receptors have been shown to be co-localised in various parts of the central nervous system, and has been suggested that the two neurotransmitters are co-released and therefore are involved in co-transmission (Kolston et al., 1992, Maxwell et al., 1995, Todd et al., 1996, Crook et al., 2006). Glycine receptors have been shown to decrease after unilateral mechanical cochlear lesions in the cochlear nucleus (Dong et al., 2009a) and glycine has been shown to significantly fluctuate after unilateral ossicle removal and unilateral cochlear ablation in brainstem auditory nuclei (Suneja et al., 1998a). Though not investigated in this thesis, the changes in glycine and the glycine receptors may play an important role in the aberrant physiological changes that occur in the auditory pathway after NIHL.

1.3.1.3: Other Markers Indicative of Increased Excitation and Plasticity

Calbindin (Calb1) is a Calcium Binding Protein (CBP) that is characteristically expressed in several auditory nuclei and is thought to serve as a buffer, protecting cells against toxic levels of calcium, which is observed during cell hyperexcitability. It has been postulated that Calb1 has a neuroprotective effect against excitotoxic damage caused by disruption of intracellular calcium homeostasis (Mattson et al., 1991, Iacopino
et al., 1992). In excitotoxicity, elevations of Ca\(^{2+}\) (calcium) progress to an irreversible loss of Ca\(^{2+}\) homeostasis and neuronal death. Previous studies have found that Calb1 increases in the auditory pathway immediately after acoustic trauma (Idrizbegovic et al., 1998, Forster and Illing, 2000). Calb1 will be investigated in this study, due to its role as a calcium buffer and presence during heightened levels of activity.

Growth-Associated Protein 43 kDa (GAP-43) is a protein that is located in the growth cones of neurons (Meiri et al., 1986). The expression of GAP-43 has been shown to increase during heightened levels of neuroplasticity. Previous research indicates that GAP-43 also plays a key role in axonal guidance (Benowitz and Perrone-Bizzozero, 1991, Benowitz and Routtenberg, 1997). In addition, GAP-43 (-/-) mice show defects in path-finding of retinal ganglion cell axons across the optic chiasm (Strittmatter et al., 1995). Over-expression of GAP-43 leads to formation of new synapses and enhanced sprouting after injury whereas reduced sprouting is found with a mutant GAP-43 that cannot be phosphorylated by Protein Kinase C. This control of sprouting implies that GAP-43 is an intrinsic determinant of the neurons’ growth state. GAP-43 has been shown to increase after cochleotomy; in the cochlear nucleus (Illing and Reisch, 2006) and in other auditory structures (Illing et al., 1997, Michler and Illing, 2002). GAP-43 has been chosen to be investigated in this study, due to its role in neuronal path-finding and its presence during heightened levels of plasticity.

Previous researchers have investigated excitatory, inhibitory and plasticity marker expression in the auditory pathway after noise trauma (Table 1.6). Despite the extensive research into this area, there is no investigation into the changes of these proteins after NIHL over time. In addition, this is the first investigation into the molecular changes that occur in the AC after NIHL. This is the first study that has investigated the effect of NIHL on multiple regions of the auditory pathway in the same animal; this has enabled
me to assess relative changes of protein expression within animals to attempt to understand the mechanisms that underlie NIHL. In this study I have focused on selected molecular markers/proteins; GABA\textsubscript{A}\textsubscript{α1}, GAD-67, NR2A, Calb1 and GAP-43. I have attempted to correlate changes in their expression with changes in cellular physiology and determine whether these changes cause or contribute to the perception of tinnitus.

**TABLE 1.6: MOLECULAR CHANGES OBSERVED AFTER ACOUSTIC TRAUMA**

<table>
<thead>
<tr>
<th>Region</th>
<th>Animal</th>
<th>Acoustic Trauma</th>
<th>Observation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC</td>
<td>Rat</td>
<td>Cochleotomy</td>
<td>No change in NR2A</td>
<td>(Argence et al., 2006)</td>
</tr>
<tr>
<td>IC, CN</td>
<td>Rat</td>
<td>Aminoglycoside</td>
<td>↓ NR2A</td>
<td>(Marianowski et al., 2000a)</td>
</tr>
<tr>
<td>IC, DCN</td>
<td>Guinea Pig</td>
<td>Mechanical cochlear lesion</td>
<td>↓ GABA\textsubscript{A}\textsubscript{α1}</td>
<td>(Dong et al., 2009b)</td>
</tr>
<tr>
<td>IC</td>
<td>Rat</td>
<td>10 kHz at 115 dB for 2 hours</td>
<td>↑ GABA</td>
<td>(Tan, Ruttiger et al. 2007)</td>
</tr>
<tr>
<td>IC, CN</td>
<td>Guinea Pig</td>
<td>Mechanical cochlear lesion</td>
<td>↓ GAD-67</td>
<td>(Dong et al., 2009b)</td>
</tr>
<tr>
<td>IC</td>
<td>Rat</td>
<td>12 kHz at 106 dB for 10 hours</td>
<td>↓ GAD-67 ↓ GABA release ↑ GABA\textsubscript{A}\textsubscript{α1}</td>
<td>(Milbrandt et al., 2000)</td>
</tr>
<tr>
<td>DCN</td>
<td>Rat</td>
<td>Cochleotomy</td>
<td>↑ Calb1</td>
<td>(Forster and Illing, 2000)</td>
</tr>
<tr>
<td>DCN</td>
<td>Mouse</td>
<td>6-12 kHz at 80 dB or 103 dB for 2 hours</td>
<td>↑ Calb1</td>
<td>(Idrizbegovic et al., 1998)</td>
</tr>
<tr>
<td>IC</td>
<td>Rat</td>
<td>Click stimuli at 130 dB for 30 minutes</td>
<td>↑ GAP-43</td>
<td>(Michler and Illing, 2002)</td>
</tr>
</tbody>
</table>
1.4: TINNITUS

Tinnitus is defined as the ‘perception of sound or noise originating from the ears or head in the absence of an evident external stimulus’ (Eggermont, 1990). Among the variety of descriptions attributed to this condition include a ringing, roaring, buzzing, chirping, humming, or clicking sound. The sound may be continuous in nature, vibratory (or non-vibratory), low pitched, high-pitched, multi-pitched, or pulsatile, and is frequently accompanied by hearing loss. This percept may be localised to one ear, both ears, or somewhere in the head (Heller, 2003).

Tinnitus is clinically heterogeneous, reflecting multiple aetiologies, and its complexity is related to its biological and psychological components. Many factors contribute to the challenge of studying tinnitus. Tinnitus occurs across a broad range of ages, with different rates of onset, variable severity and progression and a variety of putative events, including acoustic trauma, ototoxicity and age related hearing loss (Ahmad and Seidman, 2004). Because tinnitus is the perception of a sound, it should be noted that tinnitus is not a disease, but rather a symptom.

Tinnitus is estimated to affect 10-17% of the world’s population (Di Pietro et al., 2007). In a subset of the sufferers, tinnitus presents a debilitating problem, excluding them from normal life and, in some cases, causing such distress that they consider committing suicide (Jastreboff and Hazell, 1993).

1.4.1: CAUSES

Tinnitus may be either objective or subjective. This distinction is relevant in terms of both aetiology and treatment. Objective tinnitus is rare and refers to a sound that is also appreciated by an independent examiner, often by auscultation (Carlin et al., 1997). It
can be generated, for example by rhythmic muscular spasms (e.g. palatal myoclonus) and turbulent blood flow. Subjective tinnitus, the predominant form, is experienced solely by the patient (Eggermont, 1990). This type of tinnitus has a complex origin and extensive differential diagnosis, and is often complicated by psychological factors.

Table 1.7 outlines the various causes of objective and subjective tinnitus; however subjective tinnitus is the focus of this project. Subjective tinnitus may result from an abnormality or dysfunction at any level of the auditory system.

**TABLE 1.7: CAUSES OF OBJECTIVE AND SUBJECTIVE TINNITUS**

<table>
<thead>
<tr>
<th>Objective Tinnitus</th>
<th>Subjective Tinnitus</th>
<th>Traumatic</th>
<th>Drugs (adverse effects)</th>
<th>Miscellaneous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vascular (pulsatile tinnitus resulting from turbulent blood flow)</td>
<td>Otological</td>
<td>NIHL</td>
<td>Salicylate, Non-steroidal anti-inflammatory drugs (NSAIDS), loop diuretics, aminoglycoside and many others</td>
<td></td>
</tr>
</tbody>
</table>
| • Arteriosclerosis  
• Aneurysms  
• Arterio-venous malformations  
• Benign intracranial hypertension  
• Carotid stenosis  
• Glomus tumours  
• Hydrocephalus  
• Hyperdynamic states (anaemia, pregnancy, hyperthyroidism)  
• Vascular loops  
• Venous anomalies | • Infectious  
  • Middle ear effusion  
  • Otitis externa  
• Neoplastic  
  • Cholesteatoma  
  • Meningioma  
  • Osteoma/exostosis  
• Vestibular schwannoma  
• Labyrinthine  
  • Meniere’s disease  
  • NIHL  
  • Perilymphatic fistula  
  • Presbyacusis | • Head trauma  
• Ossicular discontinuity  
• Tympanic membrane perforation  
• Neck injuries | | • Temporomandibular joint disorders  
• Nutritional deficiencies (zinc, iron, magnesium, cyanocobalamin [vitamin B12])  
• Metabolic disturbances  
• Dietary (salt, caffeine, alcohol, simple sugars, monosodium glutamate and other food additives)  
• Depression |
| Muscular (contractions resulting in clicking, vibrational sound) | Neurological | Other | | |
| • Palatal myoclonus  
• Middle ear muscle myoclonus (tensor tympani, stapedius) | • Multiple sclerosis  
• Migraine  
• Seizure disorders  
• Stress | • Impacted cerumen  
• Otosclerosis | | |
| Miscellaneous | | | | |
| • Patulous Eustachian tube | | | | |

(sourced from (Ahmad and Seidman, 2004))
1.4.2: **Mechanisms of Tinnitus Involving the Central Auditory Pathway**

Despite the widespread investigation into the physiological basis of the generation of tinnitus, the underlying mechanism/s remains unknown. The theories of tinnitus generation and development include the effects of cochlea damage (peripheral nervous system) to the changes occurring in auditory processing (central nervous system). This study focused on the consequences of peripheral damage on the central nervous system, as in many cases subjective tinnitus originates in the central auditory system or in brain structures related to the central auditory system. This is supported by the fact that tinnitus persists in patients with acoustic neuroma after transection of the auditory nerve (House and Brackmann, 1981, Matthies and Samii, 1997).

It is known that NIHL causes changes (either up- or down-regulations) in gene expression in the central auditory pathway (which affects specific functions that control the level of excitability of the cell), changes in SA and the reorganisation of tonotopic maps (see section 1.2 and 1.3). It is also known that damage to the cochlea frequently results in tinnitus (Brozoski et al., 2002, Heffner and Harrington, 2002, Bauer, 2003, Bauer et al., 2007, Bauer et al., 2008). This suggests that one or more of these changes are involved in the generation or perception of tinnitus.

**Mechanisms of Tinnitus Involving the Dorsal Cochlear Nucleus**

The DCN has been shown to become hyperactive immediately after cochlear trauma. These immediate changes are thought to trigger persistent pathological neuroplastic changes distributed across more than one level of the auditory system (Saunders, 2007). Kaltenbach and colleagues hypothesised that the major functions that are most likely impacted in the DCN which contribute to tinnitus include: changes in neurotransmission,
changes in transmitter receptor function, and changes in the expression of ion conductance channels and anatomical readjustments in the number or density of functioning synapses (Kaltenbach et al., 2005).

Mechanisms of Tinnitus Involving the Inferior Colliculus

The IC has been suggested as a possible tinnitus generator due to the significant changes that occur on the molecular level (see section 1.3) and its ability to become hyperactive after NIHL (see section 1.2.1). The IC has been identified to have extensive connections throughout the brain/brainstem (see section 1.1.2.2.2). A possible mechanism of tinnitus involving the IC could be that the molecular and physiological changes that occur within the IC after NIHL are capable of affecting multiple sites throughout the central nervous system.

Mechanisms of Tinnitus Involving the Auditory Cortex

Numerous animal studies have demonstrated that after auditory damage, altered neural activity and processing occurs in the primary and secondary AC (see section 1.2). Therefore, a possible mechanism(s) of tinnitus involving the AC could be that increased SA and changes in the pattern of neural activity (such as bursting and tonotopic reorganisation, which leads to enhanced synchrony) contribute to the perception of tinnitus.
1.4.3: ANIMAL MODELS OF TINNITUS

An animal model of a disease or pathology is used to mimic the disease/pathology experimentally. In animal models of tinnitus, there are pharmacological and trauma-induced models. However, one of the main issues of using animal models is the ambiguous and subjective nature concerning whether an animal does in fact develop the perception of tinnitus. There have been several studies into confirming the behavioural manifestations of tinnitus in animals, which require intense pre-training and high animal numbers (Falk and Tang, 1972, Jastreboff et al., 1988, Lobarinas and Falk, 1998, Bauer et al., 1999, Heffner and Harrington, 2002, Guitton et al., 2003, Ruttiger et al., 2003).

1.4.3.1: CONFIRMING THE PRESENCE OF TINNITUS

In order to have an effective animal model of tinnitus, the presence of tinnitus needs to be confirmed. There are many studies that have formulated methods to verify the presence of tinnitus in animals including: Pavlovian shock-induced lick suppression techniques in water-deprived rats (Jastreboff et al., 1988, Heffner and Harrington, 2002), food-restricted rats paired with foot shock (Bauer et al., 1999), shock avoidance conditioning procedures (Guitton et al., 2003), positive reinforcement techniques (Ruttiger et al., 2003) and combined shock avoidance conditioning with schedule-induced polydipsia (Falk and Tang, 1972, Lobarinas and Falk, 1998).

These methods are effective in determining if an animal perceives tinnitus, but are limited as they cannot indicate the tinnitus spectrum being perceived. In addition, these previous methods are time consuming in regards to the prior training of the animals. A recent method published by Turner and colleagues appears to be able to identify the range of the perceived tone/s and is relatively fast and concise without the need for prior training (Turner et al., 2006).
Benefits of Turner’s gap detection technique include (a) food or water deprivation is not necessary; (b) no training, learning, memory, or motivational demands are placed on the animal; (c) the startle neural circuit is well known and its modulation using background sounds has been studied extensively; and (d) testing can be done quickly in a single 40-min session, allowing rapid assessment of acute manipulations. The test is based on the ability of the acoustic startle reflex to be reduced by a preceding signal/stimulus, in this case a silent gap in an otherwise constant acoustic background. Gaps and other pre-startle stimuli (pre-pulse inhibition) have been previously used in laboratory animals and humans as an audiometric tool to quantify features of the acoustic environment that a subject can detect. Salient pre-startle stimuli, including gaps, inhibit the startle response to a degree. The main hypothesis of Turners study suggests that when an animal effectively detects a silent gap embedded in an acoustic background, its response to a startle stimulus will be reduced in magnitude. Subsequently, when the background sound in which the gap is embedded is similar to an animal’s tinnitus, the animal will be unable to detect the silent gap and its response to a startle stimulus will be not be attenuated. Thus, it should respond in a similar manner as if there was no gap embedded in the background sound at all. Turner and colleagues confirmed that rats with independent evidence of tinnitus at 10 kHz (validated against food/foot shock study), demonstrated difficulty detecting a silent gap in a 10 kHz background, and thus substantiated the technique.

There are two time-course studies that track the changes of gap detection over time (Longenecker and Galazyuk, 2011, Turner et al., 2012). Longenecker and Galazyuk (2011) exposed mice to a narrow-band noise centred at 16 kHz (4–22 kHz) at 116 dB (SPL) unilaterally for 1 hour. They tested gap detection on 1, 3, 14, 21, 28, 35, 42, 49, 56 and 84 days after acoustic trauma on the same group of animals. They found that 12
out of 14 of the sound-exposed mice developed behavioural signs of tinnitus (86%). On the day following exposure, all mice demonstrated signs of acute tinnitus over the entire range of sound frequencies used for testing (10–31 kHz). The tinnitus spectrum of these animals varied over time. 2–3 months after the acoustic trauma, a behavioural evidence of tinnitus was evident only at a narrow frequency range (20–31 kHz) representing a presumed chronic condition. Turner and colleagues (2012) exposed mice to a narrow-band noise centred on 16 kHz at 116 dB (SPL) unilaterally for 1 hour. They tested gap detection in the same group of animals on days 1, 3-4 and 7-8, then weekly for the first 12 weeks, and then monthly until 7 months post-exposure. Turner and colleagues also showed that the tinnitus-presenting mice exhibited gap-detection deficits at frequencies higher than 16 kHz (frequency of acoustic trauma stimulus) over time (Turner et al., 2012).

To date, there has been no investigation into the time-course of tinnitus perception in rats. My investigations centre on identifying the specific perception of tinnitus following NIHL, with the aim of understanding the changes in perception of tinnitus. Understanding the development of tinnitus after acoustic exposure is of utmost importance for the development of treatments for tinnitus. This study also enables me to compare the molecular (Chapter 4) and physiological (Chapter 3) changes that occur within these same animals to their perception of tinnitus.
1.5: Hyperacusis

Hyperacusis is defined as the ‘unusual tolerance to common environmental sounds that are neither threatening nor uncomfortably loud to a typical person’ (Vernon, 1987, Klein et al., 1990). The implication is that the experience can be evoked by sounds of low intensity and that sounds in general, rather than specific sounds, are problematic (Baguley, 2003). Little is known about the aetiology of hyperacusis, other than that it may be closely related to the aetiology of tinnitus and may share a common underlying pathophysiology (Nelson and Chen, 2004). The coincidence of tinnitus complaints and of experiences of hyperacusis has been widely noted among patients attending tinnitus clinics with a primary complaint of tinnitus; the prevalence of hyperacusis being between 40-80% (Jastreboff and Jastreboff, 2000, Dauman and Bouscau-Faure, 2005). Hyperacusis with concurrent tinnitus has been linked to tinnitus-inducing agents in animal studies (Parrish and Turner, 2008) and NIHL (Turner et al., 2012).
1.6: CURRENT TREATMENTS FOR NOISE-INDUCED HEARING LOSS

For decades, a vast amount of research has gone into developing treatments for the reduction, prevention and attenuation of NIHL. However despite the extensive research, there are currently no Food and Drug Administration (FDA) approved drugs for the treatment or reduction of NIHL in humans (Lynch and Kil, 2005). Experimental evidence suggests that free radicals, generated in the cochlea (Kopke et al., 1999), play a significant role in NIHL, through damaging the OHC of the inner ear and reacting with lipids, proteins and nucleotides within the cochlea (Seidman et al., 1993, Yamane et al., 1995b, Yamashita et al., 2005). Therefore much focus has been placed on antioxidant agents in NIHL, to ascertain whether they are effective in attenuating and preventing NIHL, by behaving as free radical scavengers (Seidman et al., 1993, Lynch and Kil, 2005, Yamashita et al., 2005, Le Prell et al., 2007).

The main groups of compounds that have been shown to prevent NIHL in animal studies include antioxidants, antioxidant enzymes, calcineurin inhibitors, diuretics, glucocorticoids, growth factors, iron chelators, Jun N-terminal Kinase (JNK) inhibitors, magnesium, NMDA antagonists and Nitric Oxide Synthase (NOS) inhibitors. The compounds that were tabulated in Lynch and Kil’s review paper (2005) were successful in reducing temporary threshold shifts (TTS) and/or reducing permanent threshold shifts (PTS) and exhibited protective effects when administered prior to acoustic trauma and up to three days post-acoustic trauma. A total of eleven groups of antioxidant compounds and two groups of antioxidant enzymes have been identified to prevent NIHL (Lynch and Kil, 2005) (Table 1.8). Antioxidants such as glutathione (GSH), D-methionine, ebselen, resveratrol, ascorbic acid and coenzyme Q10, are examples of antioxidants/antioxidant enzymes that have attenuated NIHL in animal models when
taken prior to noise trauma (Oishi and Schacht, 2011). There is potential for developing post-NIHL treatments as it has been shown that free radicals can have a delayed onset of generation, up to seven to ten days after acoustic trauma (Yamashita et al., 2004). However, there are limited antioxidants that are found to be successful in post-treating NIHL. In a study conducted by Yamashita et al. (2005), the administration of both salicylate (a tinnitus-inducing agent) and trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid, a lipo-soluble derivative of vitamin E) were found to have a number of benefits such as significantly reducing hearing deficits, reduced hair cell damage and a reduction in free radicals. Administration prior and up to three days after noise trauma, showed significant beneficial effects on hearing thresholds.

**TABLE 1.8: SUMMARY OF COMPOUNDS TESTED TO PREVENT NIHL**

<table>
<thead>
<tr>
<th>Class</th>
<th>Compound</th>
<th>Effective dose</th>
<th>ROA</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antioxidants</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSH produgs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allopurinol</td>
<td>50–100 mg/kg</td>
<td>i.p.</td>
<td></td>
<td>TTS reduction</td>
</tr>
<tr>
<td>ALCAR</td>
<td>100 mg/kg</td>
<td>i.p.</td>
<td></td>
<td>PTS reduction; limited study</td>
</tr>
<tr>
<td>Edavarone</td>
<td>17 mM</td>
<td>local</td>
<td></td>
<td>PTS reduction</td>
</tr>
<tr>
<td>Lipic acid</td>
<td>50–200 mg/kg</td>
<td>i.p., p.o.</td>
<td></td>
<td>TTS and PTS reduction</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>430 µg/kg</td>
<td>p.o.</td>
<td></td>
<td>TTS and PTS reduction; limited study; extensive pretreatment</td>
</tr>
<tr>
<td>R-PIA</td>
<td>50 mM</td>
<td>local</td>
<td></td>
<td>PTS reduction; limited study</td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>10–50 mg/kg</td>
<td>i.p.</td>
<td></td>
<td>TTS and PTS reduction</td>
</tr>
<tr>
<td>Methionine</td>
<td>200 mg/kg</td>
<td>i.p.</td>
<td></td>
<td>PTS reduction</td>
</tr>
<tr>
<td>Monoethylster</td>
<td>50–150 mM</td>
<td>local</td>
<td></td>
<td>TTS and PTS reduction</td>
</tr>
<tr>
<td>NAC</td>
<td>325 mg/kg</td>
<td>i.p.</td>
<td></td>
<td>TTS and PTS reduction; Ph-III NIHL completed</td>
</tr>
<tr>
<td>OTC</td>
<td>735 mg/kg</td>
<td>i.p.</td>
<td></td>
<td>Limited PTS protection</td>
</tr>
<tr>
<td>Antioxidant enzymes GPX</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ebselen/SP1-1005</td>
<td>4–30 mg/kg</td>
<td>p.o.</td>
<td></td>
<td>TTS and PTS reduction; acute stroke studies halted; NIHL Ph II/III upcoming</td>
</tr>
<tr>
<td>SOD</td>
<td>SOD–PEG</td>
<td>2000 µg</td>
<td>l.m.</td>
<td>Limited study; TTS reduction; potential for SOD paradox</td>
</tr>
<tr>
<td>Calcineurin inhibitors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>10 µg/ml</td>
<td>local</td>
<td></td>
<td>TTS and PTS reduction; limited study</td>
</tr>
<tr>
<td>FK506</td>
<td>1–10 µg/ml</td>
<td>local</td>
<td></td>
<td>TTS and PTS reduction; limited study</td>
</tr>
<tr>
<td>Diuretics</td>
<td>Mannitol</td>
<td>15 mg/kg</td>
<td>i.p.</td>
<td>Limited study; PTS reduction</td>
</tr>
<tr>
<td>Glucocorticoids</td>
<td>Dexamethasone</td>
<td>100 ng/ml</td>
<td>local</td>
<td>Limited study; PTS reduction is U-shaped</td>
</tr>
<tr>
<td>Growth factors</td>
<td>aFGF</td>
<td>1000 ng/ml</td>
<td>local</td>
<td>PTS reduction; limited study</td>
</tr>
<tr>
<td></td>
<td>GDNF</td>
<td>100 ng/ml</td>
<td>local</td>
<td>PTS reduction; higher dose was ototoxic</td>
</tr>
<tr>
<td>Iron chelators</td>
<td>Deferoxamine</td>
<td>100 mg/kg</td>
<td>s.c.</td>
<td>Limited study; PTS reduction; clinically observed ototoxicity</td>
</tr>
<tr>
<td>JNK Inhibitors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CEP-1347</td>
<td>1 mg/kg</td>
<td>s.c.</td>
<td></td>
<td>PTS reduction, limited study; Parkinson’s Disease studies halted</td>
</tr>
<tr>
<td>D-JNKI-1</td>
<td>1–100 µM</td>
<td>local</td>
<td></td>
<td>TTS and PTS reduction</td>
</tr>
<tr>
<td>Magnesium</td>
<td>Mg</td>
<td>4 g in humans</td>
<td>p.o.</td>
<td>TTS and PTS reduction; efficacy correlates with Mg deficiency versus treatment</td>
</tr>
<tr>
<td>NMDA antagonists</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbamathione</td>
<td>5.6 mg/kg</td>
<td>i.p.</td>
<td></td>
<td>PTS reduction; limited study</td>
</tr>
<tr>
<td>Carboxerine</td>
<td>1.6–12.8 mg/ml</td>
<td>local</td>
<td></td>
<td>PTS reduction; transient block of sound transduction</td>
</tr>
<tr>
<td>MK-801</td>
<td>1 mg/kg</td>
<td>i.p.</td>
<td></td>
<td>PTS reduction; limited study</td>
</tr>
<tr>
<td>PD 174494</td>
<td>10 mg/kg</td>
<td>i.p.</td>
<td></td>
<td>Limited PTS protection</td>
</tr>
<tr>
<td>NOS inhibitors</td>
<td>L-NAME</td>
<td>1 mg/kg</td>
<td>i.p.</td>
<td>Limited study; some ototoxicity seen at higher frequencies</td>
</tr>
</tbody>
</table>

(sourced from review by (Lynch and Kil, 2005))
**1.6.1: Alpha Lipoic Acid**

Alpha Lipoic Acid (ALA) is a powerful lipophilic antioxidant free radical scavenger that binds to hydroxyl radicals, hypochlorous acid, nitric oxide (NO), peroxynitrite, hydrogen peroxide and singlet oxygen. It also chelates iron, copper and other transition metals (Whiteman et al., 1996) and in doing so, protects against mitochondrial dysfunction (Conlon et al., 1999).

ALA is a naturally occurring dithiol compound which exhibits strong antioxidant properties and has been found to have numerous pharmacotherapeutic properties (Biewenga et al., 1997). ALA can be derived from dietary sources such as organ meats (heart, kidney, liver) and to a lesser degree fruits and vegetables. It has been used since the 1990’s as a dietary supplement in the form of a capsule/tablet and is readily absorbed in the gut (Conlon et al., 1999, Shay et al., 2009) and undergoes significant hepatic first-pass metabolism (Teichert et al., 2003). ALA has been used as a therapeutic agent for the treatment of several medical conditions including myocardial and cerebral ischemia-reperfusion injury, heavy-metal poisoning, radiation damage, diabetes, neurodegenerative disease and AIDS (Auto-immune Deficiency Syndrome) (Packer et al., 1995).

Recent studies with ALA have shown promising results for the treatment of hearing loss, which demonstrated that this compound may be a potential treatment for attenuating NIHL. In previous studies performed in vitro and in vivo, ALA was observed to increase intracellular GSH levels up to 70%, which makes it ideal for the treatment of NIHL, as GSH is an important natural cochlear protectant (Hoffman et al., 1988). Other ideal properties include good penetration across the blood brain barrier and low toxicity in animal studies (Conlon et al., 1999, Cremer et al., 2006b, a).
In recent studies the therapeutic effects of ALA have been investigated in animals with hearing loss and was found to have beneficial outcomes (Conlon et al., 1999, Rybak et al., 1999, Seidman, 2000, Husain et al., 2005, Le and Keithley, 2007). A study performed in rats with aminoglycoside-induced cochlear damage, showed that those receiving ALA in combination with amikacin demonstrated a reduced elevation of compound action potential (CAP) thresholds, compared to animals receiving amikacin alone (Conlon et al., 1999). Other studies have shown similar results of hearing recovery with administration of ALA, when hearing loss was induced through ototoxic drugs such as carboplatin (Husain et al., 2005) and in the aging inner ear where animals fed with antioxidant-rich diets had shown reduced cochlear degeneration (Seidman, 2000, Le and Keithley, 2007). Investigations into the effect of ALA on NIHL is limited to one study (Diao et al., 2003). Diao and colleagues treated rats with ALA prior to the acoustic trauma and found that ABR thresholds of the saline treated animals were significantly increased compared to the ALA treated animals. They also found that ALA treated animals had lower levels of NO in the cochlear tissue and had a higher total antioxidant capacity (TAC) in the cochlea. Daio and colleagues did not look at the effects of ALA treatment after the acoustic trauma on ABR thresholds.

Thus, the antioxidant properties of ALA, along with its ability to cross the blood brain barrier, and its minimal toxicity, make ALA an ideal compound to study for NIHL. As free radicals have been shown to form in the inner ear for up to ten days after noise trauma (Yamashita et al., 2004), the strong antioxidant potency of ALA could be used as a novel therapy for post-treatment of NIHL. Furthermore, the observed effect that ALA has on intracellular GSH production suggests that it may have a beneficial and protective effect on the cochlea and reduce age-associated deterioration in auditory sensitivity and cochlear function (Conlon et al., 1999, Seidman, 2000).
1.7: HYPOTHESES AND AIMS

Previous studies have observed significant changes in the primary auditory pathway after NIHL, however, the research has been conducted on many different species (i.e. rats, guinea pigs, chinchillas, mice, monkeys) and have utilised a variety of acoustic trauma methods. Comparing results from each investigation to another can be ineffective due to the vast amount of variables. Despite the extensive research into this field, not one of these studies have tracked the electrophysiological, molecular or behavioural changes over a 32-day time-period to analyse the development of changes in the auditory system after NIHL.

My project is therefore novel in its approach, as I have investigated the time-course of changes in the rat after NIHL; comparing the electrophysiological changes, behavioural manifestations of tinnitus and the level of protein expression in animals that have experienced the same set of test conditions. I have also investigated the effects of a therapeutic agent, ALA, on the prevention and treatment of NIHL.

1.7.1: HYPOTHESES

It was hypothesised that after NIHL, I would observe:

- significant changes in the excitability of auditory neurons in response to noise stimuli;

- significant changes in the SA of auditory neurons;

- significant changes in the expression of receptors and markers, involved in regulating excitatory and inhibitory transmission, in the auditory pathway;
- significant changes in the expression of receptors and markers, characteristic of neuroplasticity, in the auditory pathway;

- correlations with the electrophysiological changes and the changes in the expression of receptors and markers of interest; and

- significant benefits of ALA administration on the development of NIHL in animals treated before and after NIHL.

1.7.2: AIMS

My aim was to track the changes that occur in the central auditory pathway after NIHL over the period of a month, in order to determine whether there is an opportunity for therapeutic intervention.

I have:

- performed in vivo electrophysiological recordings in the AC, IC and DCN to assess the changes that have occurred in auditory neurons after NIHL;

- utilised western blot techniques to quantify the expression of various receptors and molecular markers over a one month time course after NIHL;

- measured the perception of tinnitus in noise-exposed rats using the gap-detection behavioural technique;

- compared electrophysiological recordings over the same time course, with behavioural and western blot observations; and

- tested a therapeutic intervention, ALA, based on previous results.
CHAPTER TWO

GENERAL RESEARCH METHODS
This Chapter outlines general research methods for each study. Any additional information or variations such as electrophysiological recordings, western blot methods, behavioural techniques, therapeutic intervention and specific statistical analyses will be noted in the appropriate methods section of each study.

All experimental procedures in this study were approved by the University of Western Sydney Animal Care and Ethics Committee (ACEC). This study conformed to the National Health and Medical Research Council code of practice for the care and use of animals for scientific purposes; every effort was made to reduce the number of animals used.

My basic method was to use a loud sound to induce a partial hearing loss in rats, referred to as the Noise-Induced Tinnitus Model (Bauer and Brozoski, 2001). This is a standard model used throughout the world (Heffner and Harrington, 2002, Bauer, 2003, Turner et al., 2006, Longenecker and Galazyuk, 2011, Brozoski et al., 2012, Turner et al., 2012).

With this noise-induced model of tinnitus I investigated the physiological characteristics of neurons in the AC, IC and DCN by simultaneously recording extracellular activity using multichannel microelectrodes inserted into those specific regions. I investigated the molecular changes by utilising western blot methods and thereby quantifying the level of specific markers/proteins involved in regulating cell activity. I also have attempted to confirm tinnitus behaviourally in noise-exposed animals. With these measures I investigated any correlations between physiological, molecular and behavioural changes that occur in rats after NIHL. Finally I have evaluated a potential prevention and/or treatment for NIHL and tinnitus.
2.1: ANIMALS

Male Long Evans rats were obtained from Monash Animal Services (Monash University, Melbourne, VIC). Rats were also bred on site, using stock from the same supplier (Monash Animal Services). Animals were housed in double-decker, individually ventilated cages (Techniplast, Italy). These cages were made from polycarbonate which complies with the NSW Department of Primary Industries standards. The dimensions of these cages were 405 millimetres (mm) x 250 mm x 185 mm (length x width x height), which equals a total of 501cm$^2$ floor area. Up to two adult rats were housed per cage (manufacturers’ recommendations) in single sex groups, with a minimum of one per cage; however, single housing was avoided whenever possible. Litter mates were housed together and were monitored by The University of Western Sydney School of Medicine Animal Facility Manager. A standard laboratory rodent diet was accessible using food hoppers, and water was provided in sanitised bottles. Environmental enrichment was provided in each cage such as small boxes, toilet rolls and tissue paper. Experiments were performed on rats at the age of 11-14 weeks (young adults). The animals used in the Electrophysiological (Chapter 3), Molecular (Chapter 4) and Lipoic (Chapter 6) studies and were not the same animals.

2.2: ANAESTHESIA AND RECOVERY

Each rat was anaesthetised via an intraperitoneal injection of ketamine/medetomidine (75 milligrams (mg)/ kilogram (kg); 0.5 mg/kg). To reverse the effects of the anaesthesia following the procedure, a subcutaneous injection of atipamezole (1 mg/kg) was administered. Animals were monitored until they had fully recovered, and after recovery they were returned to the animal house.
2.3: ACOUSTIC TRAUMA

Each rat was anaesthetised as described in section 2.2. The animal was then placed on a feedback-controlled heating pad with a rectal probe to maintain a constant body temperature of 37°C (Physitemp, Clifton, NJ; Catalogue #TCAT 2LV Controller). A small tube, 3 mm inner diameter and originating from a speaker (CTS Powerline, Piezo Electric, Woburn, MA), was tightly and securely placed in the animals left ear canal. Rats were unilaterally exposed to a 16 kilo hertz (kHz) band pass (1/10th octave (115 dB SPL)) noise for 1 hour. The right ear canal remained unblocked during the acoustic trauma. The power spectrum of the acoustic stimulus is pictured below (Fig. 2.1). Control animals were not exposed to the acoustic trauma, but were exposed to a SHAM acoustic trauma procedure; they underwent the same protocol as the exposed rats, in regards to anaesthesia, auditory brainstem response testing (see section 2.4), and were placed in a sound proof room for 1 hour next to the acoustic trauma speaker. The speaker was not turned on during this period. The effect of the anaesthesia was reversed following the procedure, as described in section 2.2.

![Energy distribution of the noise used in the experiment.](image-url)

Figure 2.1: Energy distribution of the noise used in the experiment.
2.4: Auditory Brainstem Response Audiograms

Auditory Brainstem Response (ABR) audiograms provide a reasonably accurate estimate of an animal's hearing sensitivity. It was essential that the animals had sufficient hearing prior to any experimental manipulations.

The ABR test was used to evaluate the function of the Vestibulocochlear (CN VIII) and the ascending pathways of the central auditory system (Moller, 1985). The test involved the placement of sub-dermal electrodes on the scalp. The electrodes recorded the auditory evoked potentials that were produced by the auditory pathway in response to tones. The resulting recording was a series of waves that were generated as a result of activity in various structures in the auditory pathway. These waves, labelled with roman numerals, occur in the first 10 milliseconds after onset of an auditory stimulus (Jewett et al., 1970, Jewett and Williston, 1971) (Fig. 2.2).

The auditory structures that generate the ABR are as follows (Moller, 1985):

- **Wave I** – the peripheral region of CN VIII
- **Wave II** – the central region of CN VIII
- **Wave III** – the Cochlear Nucleus
- **Wave IV** – the Superior Olivary Nucleus/Lateral Lemniscus
- **Wave V** – the Lateral Lemniscus/Inferior Colliculus

![Figure 2.2: ABR waveform indicating the different waves from I-V.](image)

Chapter Two: General Research Methods
An ABR test was performed on an animal before and after any procedure on both ipsilateral and contralateral auditory pathways (left and right ears). The experiment was performed within a sound proof room. The animal was anaesthetised as above (see section 2.2) and placed on a heating pad with a rectal probe to maintain a constant body temperature of 37°C. Tucker Davis Technologies (TDT) system 3 Software (OpenEx) and hardware were used to generate stimuli and to acquire data. Stimuli were generated using an RX6 multifunction processor with a sampling rate of 100 kHz. Stimuli were attenuated using TDT PA5 attenuators and transduced using TDT EC1 electrostatic speakers. The sound was presented to the animal via a small tube (3.5 mm in diameter) originating from a speaker (TDT EC1), placed tightly and securely in the ear canal. Animals were presented with tones at 1, 2, 4, 8, 16 and 32 kHz, at a range of 0-90 dB SPL (90 dB was the maximum that the software could deliver). Tone bursts were 3 ms in duration, presented every 17 ms. Each frequency/SPL combination was presented 500 times sequentially from lowest to highest. The resulting evoked potentials were measured using stainless steel electrodes placed subdermally; behind the left ear (active), behind the right ear (active), in the midline 2.5 cm anterior to the intra-aural axis (reference), and in the rear leg (ground). Waveforms were averaged by the program and plotted in Open Explorer (TDT). An ABR test plot (Fig. 2.3) was provided which indicated the average waveform for each SPL/frequency combination. At the conclusion of the experiment, the electrodes were removed and cleaned. The effect of the anaesthesia was reversed following the procedure, as described in section 2.2.
The audiogram of the animal was determined by identifying the lowest SPL that elicited a response (clear I-V waveforms) for each frequency tested (Fig. 2.3).

![Figure 2.3: ABR test plot of a control animal. White boxes highlight the hearing threshold SPL (dB (decibel)) for each frequency tested which was the animal’s audiogram.](image)

2.4.1: STATISTICAL ANALYSES

Statistical analyses were performed using SPSS (IBM Corporation, Somers, NY). Thresholds were evaluated for each animal, respectively. The absolute hearing thresholds from the pre and post ABR audiograms (specifically the SPLs) of each group were compared at individual frequencies, but not across frequencies, using a two tailed student’s t-test with a p value of <0.05 considered significant. Control and post acoustic trauma ABR threshold shifts were compared at individual frequencies, but not across frequencies, using a one-way analysis of variance (ANOVA) and Tukey’s post-hoc multiple comparison tests with a p value of < 0.05 considered significant. If no response was evoked at a SPL of 90 dB, a SPL threshold of 90 dB was assigned.
2.5: STIMULUS CALIBRATION

All calibration procedures were performed with the output of the speakers passing through the ear bars, which were coupled to the microphone with flexible tubing.

Pure Tone Stimuli

A calibration curve was obtained using TDT SigCal. An ACO Pacific 7017 microphone and PS9200 power supply were used to record the stimuli.

Noise Stimuli

Noise bursts were measured using a Brüel & Kjaer 2610 measuring amplifier and a 4191 microphone. The output voltage was adjusted to achieve the desired noise level.

2.6: EUTHANASIA FOR TERMINAL EXPERIMENTS

The rats were under anaesthesia as described in section 2.2 and were painlessly euthanased by decapitation.
CHAPTER THREE

STUDY ONE

Tracking the Physiological Characteristics of Auditory Neurons following NIHL
3.1: **INTRODUCTION**

Acoustic trauma is known to produce physiological and molecular changes in the peripheral and central auditory pathway, which can lead to alterations in the physiological characteristics of auditory neurons. It is presumed that damage to the peripheral auditory system influences changes in the central auditory pathway due to altered neural input (Noreña and Eggermont, 2003). This can lead to changes in SA, stimulus-driven activity and tonotopic representation (refer to section 1.2). Changes in tonotopic representation has been observed in numerous studies that used excessive noise to induce a hearing loss (Robertson and Irvine, 1989, Kaltenbach et al., 1992, Harrison et al., 1998, Rauschecker, 1999, Syka, 2002, Irvine and Wright, 2005, Izquierdo et al., 2008a). Previous studies revealed a significant decline in peripheral neural input following noise exposure (Salvi, 1982), thereby depriving central auditory regions of their normal input. It is this deprivation that is believed to result in the expansion of frequency representations within the AC, in which the deprived neurons become responsive to frequencies adjacent to the cortical regions that represent the frequency range where hearing was damaged. This type of frequency reorganisation has been observed in the somatosensory system appearing immediately after the amputation of a digit on a forelimb (Calford and Tweedale, 1988). The mechanisms of tonotopic reorganisation are not clearly understood. However, it is thought that noise-induced SA and hyperactivity/hyperexcitability are related to this auditory plasticity.

Neurons that release more than one action potential per second in the absence of intentional acoustic stimulation are defined as neurons with SA (Willott et al., 1988). After acoustic trauma, changes in SA have been reported in various parts of the auditory system (refer to section 1.2). Protein expression and synthesis are thought to underlie
changes in SA (refer to section 1.3) by altering the excitatory and/or inhibitory capacity of auditory neurons, and thereby leading to hyperactivation of the auditory system (Weisz et al., 2007a). Previous research has shown that after acoustic trauma, SA increases in the AC (Eggermont and Komiya, 2000, Noreña and Eggermont, 2003, Noreña et al., 2003, Seki and Eggermont, 2003a), the IC (Wang et al., 2002, Imig and Durham, 2005, Ma et al., 2006, Bauer et al., 2008, Izquierdo et al., 2008a, Mulders et al., 2011), and in the DCN (Kaltenbach et al., 1998, Kaltenbach et al., 2000, Brozoski et al., 2002, Chang et al., 2002, Finlayson and Kaltenbach, 2009), and these changes have frequently been associated with the incidence of tinnitus (Kaltenbach et al., 2000, Komiya and Eggermont, 2000, Brozoski et al., 2002, Imig and Durham, 2005, Bauer et al., 2008, Dong et al., 2010a). Thereby, the AC, IC and DCN have been implicated as possible sites for the generation of tinnitus, owing to their tendency to become hyperactive following exposure to acoustic trauma and other tinnitus-inducing agents (Brozoski et al., 2002, Chang et al., 2002, Imig and Durham, 2005, Bauer et al., 2008).

Stimulus-driven activity is neural activity that is evoked by a stimulus (Lakatos et al., 2005). Changes in stimulus-driven activity can be a sign of a change in the excitability state of the auditory system, which has been observed after NIHL. Reductions in stimulus-driven activity have been observed in the AC after NIHL (Tan et al., 2007). It has been proposed that reductions in stimulus-driven activity may lead to an increase in SA, as a compensatory mechanism (Schaette and Kempter, 2006). This suggests that there is a complementary relationship between SA and stimulus-driven activity and may suggest that both of these aberrant neural responses cause or contribute to the generation of acoustic disorders after NIHL.

As mentioned above, many investigations have centred on SA and stimulus-driven activity in the central auditory pathway following acoustic exposure. To date, there has
been no investigation into the SA and stimulus-driven activity that occurs in the AC, IC and DCN in the same animal. There are also limited investigations into the development of these changes over time. The majority of previous research investigated the immediate changes of SA, with limited investigations sampling various time-points after acoustic exposure. This investigation centres on the SA and stimulus-driven activity changes in the contralateral AC, contralateral IC and ipsilateral DCN within the same animal following NIHL, with the aim of determining which brain region/s may be involved in the generation of auditory disorders by assessing the relative SA and excitability changes over time.

Understanding the development of SA and excitability changes that occur after NIHL is of utmost importance for understanding the underlying mechanisms and/or generation of tinnitus, and for the development of treatments for auditory disorders.
3.2: Hypothesis and Aims

3.2.1: Hypothesis

It was hypothesised that NIHL would lead to immediate changes in SA and stimulus-driven activity in the AC, IC and DCN and that these changes would persist over time.

3.2.2: Aims

My aim was to track the development of physiological changes that occurred in the central auditory pathway after NIHL over the period of a month, in order contribute to the understanding of the underlying mechanisms of tinnitus. My aim was also to identify a possible site of generation of tinnitus and to understand how fluctuations in SA and excitability could cause or contribute to the perception of tinnitus. This work was performed to complement the molecular (Chapter 4) and behavioural (Chapter 5) studies of this thesis.

Accordingly, I performed in vivo multi-unit neuron recordings to assess the SA and stimulus-driven activity changes that occurred after NIHL at various time-points (two hours, and four, eight, sixteen and thirty-two days after NIHL).
3.3: RESEARCH METHOD

3.3.1: ANIMALS AND TREATMENT GROUPS

Rats were housed and maintained as previously described in section 2.1. Fifteen rats aged between 11-14 weeks (250-350g) were randomly assigned to six groups used to assess the effect of NIHL on the physiological characteristics of auditory neurons in the contralateral AC, contralateral IC and ipsilateral DCN. These groups were a control group (n = 3), Day 0 (n = 2), Day 4 (n = 2), Day 8 (n = 3), Day 16 (n = 3) and Day 32 (n = 2) post-acoustic trauma group. The experimental procedures were approved by the Animal Ethics Committee of the University of Western Sydney (ACEC #6670) and conformed to the Australian code of practice for the care and use of animals for scientific purposes.

3.3.2: ACOUSTIC TRAUMA AND AUDITORY BRAINSTEM RESPONSE TESTS

Animals were exposed to the acoustic trauma (see section 2.3) and ABR tests were performed (see section 2.4) before and after the acoustic trauma, and once before the electrophysiological experiment. Statistical analyses were performed as described in section 2.4.1.

3.3.3: ELECTROPHYSIOLOGICAL RECORDING

The animal was anaesthetised according to section 2.2 and placed on a heating pad with a rectal probe to maintain a constant body temperature of 37°C. After the initial dose, the depth of general anaesthesia was monitored using the flexor withdrawal reflex, concurrently with body temperature, whisker movement, and respiration rate. A pulse oximeter (MouseOx; Starr Life Sciences Corp., Oakmont, PA) was connected to the
animal’s foot, which measured heart rate, blood pressure and blood oxygen levels. If at any time the depth of anaesthesia was too shallow, the anaesthetic was increased with supplementary doses. At the time of anaesthetic induction, animals were given an intraperitoneal injection of dexamethasone (0.15 mg/kg) to reduce cerebral oedema and a subcutaneous injection of atropine sulphate (0.1 mg/kg) to reduce mucosal secretions and lessen the chance of respiratory difficulty during anaesthesia. These doses were repeated every twelve hours. Body fluids were maintained with subcutaneous injections of Hartmann’s solution (compound sodium lactate) warmed to body temperature. Animals were placed in a stereotaxic head-holder fitted with hollow ear-bars to allow sound delivery.

3.3.3.1: Surgery and Locating Auditory Sites

A 2cm incision was made in the midline of the scalp that was retracted to reveal the skull. The fascia on the skull and the right temporalis muscle was removed. Three craniotomies were made with a dental drill and burr to reveal the dura mater. The AC craniotomy was drilled in the right squamosal bone, 2-8 mm post-bregma. The IC craniotomy was drilled from the midline to the lateral border of the right parietal bone. The DCN craniotomy was drilled from the midline to the lateral border of the left interparietal bone. The dura mater was carefully removed prior to electrode penetration and each craniotomy was covered with agar to prevent drying of the brain/brainstem.

Prior to penetration, the coordinates of the IC and DCN were determined using atlases of sagittal sections of the rat brain (Paxinos, 2007). Bregma has been shown to be a reliable landmark to use in locating these brain regions (Doron et al., 2002, Paxinos, 2007, Smith et al., 2012). Using medial/lateral, dorsal/ventral and anterior/posterior coordinates, the angle of penetration was determined. Prior to the DCN electrode penetration, the left
lateral part of the cerebellum was removed to reveal the superior surface of the DCN. The electrode for the DCN was rostrally angled between 20° and 30°, positioned 3.2-3.6 mm lateral and 13-14 mm posterior to bregma, and was inserted between 3000µm and 4000µm ventrally from the dura. Electrode placement was indicated by the onset of robust noise-evoked activity.

The electrode for the IC was caudally angled between 5° and 15°, positioned 1.90-2.10 mm lateral and 7-8 mm posterior to bregma, and was inserted between 4000µm and 5000µm ventrally from the cortical surface. Electrode placement was indicated by the onset of robust noise-evoked activity with short spike latencies, this was done to maintain consistency with other studies (Seluakumaran et al., 2008, Dong et al., 2009a, Mulders and Robertson, 2009, Dong et al., 2010a, Dong et al., 2010b, Mulders et al., 2011).

The three AC penetrations (high, medium and low frequency regions) were placed 5.9-6.1 mm lateral and between 2.5 mm and 6 mm posterior from bregma, the electrodes were inserted between 1000µm-3000µm ventrally from the cortical surface (Fig. 3.1). Electrode placement was indicated by the onset of robust noise-evoked activity with short and precise spike latencies with low thresholds. This was done to maintain consistency with other studies (Brugge and Merzenich, 1973, Merzenich et al., 1975, Stiebler et al., 1997, Kilgard and Merzenich, 1999, Doron et al., 2002). The final position of each electrode penetration also depended on the presence of large vessels, which were avoided at all times.
3.3.3.2: IN VIVO BRAIN/BRAINSTEM RECORDING

Tucker-Davis Technologies (TDT) system 3 Software (OpenEx) and hardware were used to generate stimuli and to acquire data. Stimuli were generated using a TDT RX6 multifunction processor with a sampling rate of 100 kHz. Stimuli were attenuated using TDT PA5 attenuators and transduced using TDT EC1 electrostatic speakers. The sound was presented to the animal via a small tube (3.5 mm in diameter) and originating from a speaker (Tucker Davis Technologies Eci Piezo Electric Woburn, MA) placed tightly and securely in the ear canal. Animals were presented with white noise over a SPL range of 0-80 dB. Stimulus duration was 50 ms with a sweep period of 900 ms. There were 50 repetitions which resulted in a 6.8 minute recording. Stimulus-driven activity was recorded over this period for each electrode penetration. SA was collected over a similar time period for each electrode penetration. The resulting evoked potentials were
measured using platinum electrodes with iridium recording sites (NeuroNexus technologies, Ann Arbor, MI), positioned with micromanipulators (Narashige). Recording electrodes consisted of a 64-channel electrode used for the AC (8 shanks, 8 recording points/shank), a 32-channel electrode used for the IC (1 shank, 32 recording points/shank), and a 32-channel electrode used for the DCN (4 shanks, 8 recording points/shank). The electrodes were connected to a low impedance head stage (RA4LI (TDT)), which connected to a preamplifier (RA16PA16 (TDT). The agar was removed prior to electrode insertion. Once the auditory neurons were located, each craniotomy was covered with agar to prevent drying, once the craniotomies were covered completely the recording commenced. Due to the length of the AC (3-3.5 mm wide), three penetrations were performed to record from all frequency areas. Each penetration spanned 1.4 mm. Therefore each penetration was separated and analysed depending on the frequency region it was placed in: be it the high, medium or low frequency region. At the conclusion of the experiment, animals were euthanased according to section 2.5.

3.3.3.3: Statistical Analyses

Spike thresholds were set directly before each recording using a fixed voltage window discriminator; spikes had to exceed the threshold before they were recorded. The RMS threshold level was set to 3.5-4.5.

There were a few factors that may have contributed to the variability of results. Firstly, there was a difference in hearing losses and recovery experienced by individual animals after NIHL. To overcome this, the animals were analysed within their groups (time-points) and were also analysed separately. Secondly, the anaesthesia state of the animals may have also influenced variability of the results. In order to avoid this, the anaesthetic was regularly administered to the animal to ensure a deep anaesthesia state, and the
animals’ vitals were monitored during the experiment (refer to section 3.3.3). Lastly, electrode impedance may have also played a role in the variability of results and thus was addressed by using the same electrodes for each animal.

3.3.3.3.1: Stimulus-driven activity

Multi-unit recordings were made in the AC, IC and DCN. The animals were presented with white noise which ranged from 0 dB-90 dB (SPL) in 10 dB (SPL) steps. The microelectrodes recorded multi-unit spikes, which are the action potentials of a collection of neurons. A spike count histogram represents the number of spikes counted on one channel on a recording site at one SPL. The period of the histogram was 100 ms. For the IC and DCN recordings, the spike counts of all recording sites were averaged at each SPL (0 dB- 90 dB). This resulted in ten individual numbers (for each SPL) for both the IC and DCN separately. This was converted to a spike rate (spikes/second). For AC recordings, the spike counts of the recording sites on individual shanks (eight in total) were averaged for each SPL, which resulted in eight individual numbers for the AC for each SPL. These averages were also converted into a spike rate. These spike rates were used to assess the excitability of the regions in response to a specific SPL.

The spike rates for each SPL were plotted on a graph (refer to Fig. 3.2). The spike rate at any given SPL was divided by the spike rate at 0 dB (this served as an internal control), and a ratio which indicated relative activity was thus produced. This ratio will be referred to as the excitability index. An excitability index less than 1 indicated that the relative excitability of the neurons was less than baseline values (less excitable), an index equal to 1 indicated that the relative excitability was the same as baseline values (no difference in excitability), and an index greater than 1 indicated that the relative excitability was greater than baseline values (more excitable). Accordingly, a higher excitability index indicated a greater excitability of the neurons.
When the noise stimulus was presented to the animal at low SPLs, there was generally a low spike rate. The point when the spike count started to increase indicated that the cells were responding to the noise stimulus and can also be referred to as the threshold response. When the threshold response occurs, the SPL being presented will be referred to as the ‘inflection point’. Once the inflection point was identified, the SPL that was the midpoint between the inflection point SPL and the maximum SPL tested (90 dB SPL) or at the point when the response saturated, this was used for analysis. In this way, the average excitability index value from the midpoint was used as an indicator of excitability.

**Figure 3.2: Example of a Spike Count Chart for the Inferior Colliculus, indicating inflection point and midpoint.**

For each DCN and IC recording, data were compared between different regions within the group and between the groups. For each AC recording, the data was compared to the different regions of the AC (high, medium and low frequency regions) to identify whether there were region specific effects of the NIHL. Then the average of the three frequency regions was compared between the different regions within the animal and between the groups.
Statistical analyses were performed using SPSS (IBM Corporation, Somers, NY). For multiple comparisons of the AC stimulus-driven activity data (low, medium and high frequency regions), a one-way ANOVA and Games-Howell post-hoc multiple comparison test was used with a p value of <0.05 considered significant. Independent samples t-tests were used to analyse the IC and DCN data due to the low animal numbers. A p value of <0.05 was considered significant.

3.3.3.1.1: RESPONSE PROFILES OF INDIVIDUAL RECORDING SITES

When analysing the stimulus-driven responses on individual recording channels, it was evident that the neurons shared common response profiles. Figure 3.3 depicts examples of each observed stimulus-driven response profile. The most common response profiles observed in this study were two types of monotonic responses: monotonic responses that had sharp increases followed by saturation ($\text{MON}_{\text{SAT}}$) and monotonic responses that increased progressively but did not saturate ($\text{MON}_{\text{N/SAT}}$). However, the $\text{MON}_{\text{N/SAT}}$ may have saturated if the SPL of the noise stimulus was higher. Thus, it is conceivable that they are the same as the $\text{MON}_{\text{SAT}}$ responses. However, the rate level function slopes (to be discussed) vary between the two types of monotonic responses. For this reason, the two monotonic responses are thought to be unique. Another response profile was evident in the later time-points and was not observed in the control animals. These responses had sharp increases (Slope 1) followed by decrease in response, which was followed by a slower increase (Slope 2); these were classified as non-monotonic (NON MON) responses. Such responses have been identified in auditory neurons in response to noise stimuli (Rouiller et al., 1983). Some channels were non-responsive. This may be due to the channels themselves functioning sub-optimally, or it could be that the neuron population surrounding a particular channel was not responding to the stimulus.
Figure 3.3: Representative $\text{MON}^\text{SAT}$, $\text{MON}^\text{N/SAT}$ and NON MON Responses
3.3.3.1.2: Rate Level Function Slopes

The spikes recorded in each experiment were normalised to the maximum spike value recorded per channel, during the whole recording period. The spikes recorded were then presented as a percentage of the maximum response. The slope of the line indicates the input/output or the rate level function (RLF) of the stimulus-driven responses.

To obtain the function of the slope, I used the slope of the regression line equation (Equation 1). The slope of the regression line returns the slope of the linear regression line through data points in known ‘y’ values and known ‘x’ values. The slope is the vertical distance divided by the horizontal distance between any two points on the line, which is the rate of change along the regression line.

$$b = \frac{\sum(x - \bar{x})(y - \bar{y})}{\sum(x - \bar{x})^2}$$

Equation 1. The equation for the slope of the regression. Where x and y are the sample means AVERAGE (known ‘x’ values) and AVERAGE (known ‘y’ values).

The values entered into the equation for the MON^{SAT} responses were from the inflection point to the point of saturation. The values entered into the equation for the MON^{NSAT} responses were from the inflection point to the highest SPL response recorded. The NON MON responses were analysed differently, as there were two inflection points with varying slopes. In these cases, the values entered into the equation were from the first inflection point to the peak of the slope (Slope 1) and from the second inflection point to the highest SPL response recorded (Slope 2).

In this study, the RLF slopes were used as an indicator of excitability for the neuronal population/s exhibiting MON^{SAT}, MON^{NSAT} and NON MON responses. A greater RLF slope value indicated that less stimulus input was required to lead to an activity output;
Therefore the neurons were more excitable. A lower RLF slope value indicated that more stimulus input was required to lead to an activity output; therefore the neurons were less excitable.

The RLF slope values for the $\text{MON}^{\text{SAT}}$ and $\text{MON}^{\text{N/SAT}}$ stimulus-driven response profiles were averaged separately within each region/time-point. These averages were then compared within the region/time-points using a one-way ANOVA and Tukey’s test for post hoc analysis with a p value of $<0.05$ considered significant. This demonstrated the changes in RLF slopes within each region over time per stimulus-driven response profile when compared to control. The RLF slopes of the $\text{MON}^{\text{SAT}}$ and $\text{MON}^{\text{N/SAT}}$ responses were compared within each region using an independent samples t-test, to determine any differences between the $\text{MON}^{\text{SAT}}$ and $\text{MON}^{\text{N/SAT}}$ responses within each region per time-point.

The NON MON responses were present in the Day 16 and Day 32 animals, therefore these results required separate analyses. In addition, the presence of two slopes also required another form of analysis. A paired t-test was used to compare Slope 1 and Slope 2, to analyse the difference in RLF slopes within each region within a group. A repeated measures ANOVA with a Tukey’s post hoc test was used to analyse the differences between Slope 1 and Slope 2 across the regions per time-point with a p value of $<0.05$ considered significant.

3.3.3.3.2: SPONTANEOUS ACTIVITY

Multi-unit spikes of raw SA were counted over 5.8 minutes in the absence of any stimulus (Fig. 3.4). The resulting number of spikes were converted into a spike rate/second. These spike rates were used to assess the level of SA in each region. The spike rate data that was collected in the stimulus-driven activity experiment in the same
region at 0 dB (no stimulus present) was used as the baseline for the SA data. This was used as an internal control. The DCN spike rates for all recording channels were averaged. The average spike rate was normalised by subtracting the average spike rate at 0 dB (from the same region) from the stimulus-driven activity recording. The resulting DCN spike rate was compared between different groups. The IC spike rates for all recording channels were averaged. The average spike rate was normalised by subtracting the average spike rate at 0 dB (from the same region) from the stimulus-driven activity recording. The resulting IC spike rate was compared between different groups. The AC spike rates for the recording channels in each frequency region (low, medium and high) were averaged. The average spike rate was normalised by subtracting the average spike rate at 0 dB (from the same region) from the stimulus-driven activity recording. The resulting AC spike rate was compared between each frequency region so as to identify whether there were frequency region specific changes in the AC. The values were then compared between different groups. Statistical analyses were performed using SPSS (IBM Corporation, Somers, NY). For multiple comparisons of the data, a one-way ANOVA and a Games-Howell post-hoc multiple comparison test were used wherein a p value of <0.05 was considered significant.

Figure 3.4: Example of Raw Spontaneous Activity from the DCN of a control animal.
3.4: RESULTS

Due to the small number of animals used in each group, the results of this study may have been subject to interanimal variation. All attempts were made to ensure comparable experimental conditions to produce homogeneity between the animals at different time-points.

3.4.1: AUDITORY BRAINSTEM RESPONSE AUDIOGRAMS

The ABR audiograms indicated significant threshold shifts following NIHL. Figure 3.5A-F presents the mean ABR audiograms of all animals obtained prior to NIHL and at 0, 4, 8, 16 and 32 days after NIHL. All values in the figures are expressed as mean ± standard error mean (SEM).

The control group showed a normal ABR audiogram (Fig. 3.5A), which was expected as the animals were not exposed to the acoustic trauma stimulus. The Day 0, 4, 8, 16 and 32 groups showed a significant threshold increase across most frequencies (Fig. 3.5). The one-way ANOVAs determined that the threshold shifts were significantly different between time-points; at 1 kHz, F (5, 14) = 6.562, p = 0.008; at 2 kHz, F (5, 14) = 5.632, p = 0.013; at 4 kHz, F (5, 14) = 10.140, p = 0.002; at 8 kHz, F (5, 14) = 9.967, p = 0.002; at 16 kHz, F (5, 14) = 71.153, p<0.001; and at 32 kHz, F (5, 14) = 87.592, p<0.001.

A Tukey’s post hoc test was used to compare the ABR threshold shifts of the control animals, at each frequency, to the threshold shifts of the other groups. The results of these tests are presented in Table 3.1. The lesioned (ipsilateral) ear indicated significant threshold shifts across most frequencies immediately after NIHL; this immediate hearing loss was also observed in Chapter 4 of this thesis (see section 4.4.1: Fig. 4.2B).
Threshold shifts were increased at all frequencies in the Day 4 and Day 8 groups. In the Day 16 and Day 32 group, high frequencies were elevated (8-32 kHz), whilst the lower frequencies were not significantly different to the pre-lesion values due to the high variability. Table 3.1 presents the threshold shifts in dB SPL/frequency/group with p values. The ABR audiograms of the contralateral ear are presented in Table 3.2 and indicated no significant changes in threshold shifts.
Figure 3.5: ABR Audiograms of absolute thresholds of ipsilateral (lesioned) ear. A one-way ANOVA with Tukey’s test was used to compare threshold shifts between frequencies of: (A) control animals, (B) 0 days post-lesion, (C) 4 days post-lesion, (D) 8 days post-lesion, (E) 16 days post-lesion, and (F) 32 days post-lesion recordings. Results are presented as the mean ± SEM (SPL (dB)), *p<0.05.
### TABLE 3.1: POST ACOUSTIC TRAUMA THRESHOLD SHIFTS IN ALL EXPOSURE GROUPS (IPSILATERAL/LESIONED EAR). A ONE-WAY ANOVA WITH TUKEY’S TEST WERE USED TO COMPARE THRESHOLD SHIFTS BETWEEN FREQUENCIES OF EACH TIME-POINT.

<table>
<thead>
<tr>
<th>Group</th>
<th>Frequency</th>
<th>1 kHz</th>
<th>2 kHz</th>
<th>4 kHz</th>
<th>8 kHz</th>
<th>16 kHz</th>
<th>32 kHz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0 (n = 2)</td>
<td>50 dB (p = 0.056)</td>
<td>50 dB (p = 0.056)</td>
<td>47.5 dB (p = 0.023)</td>
<td>72.5 dB (p = 0.002)</td>
<td>75 dB (p&lt;0.001)</td>
<td>65 dB (p&lt;0.001)</td>
<td></td>
</tr>
<tr>
<td>Day 4 (n = 2)</td>
<td>62.5 dB (p = 0.017)</td>
<td>57.5 dB (p = 0.027)</td>
<td>65 dB (p = 0.003)</td>
<td>60 dB (p = 0.007)</td>
<td>80 dB (p&lt;0.001)</td>
<td>85 dB (p&lt;0.001)</td>
<td></td>
</tr>
<tr>
<td>Day 8 (n = 3)</td>
<td>48.3 dB (p = 0.038)</td>
<td>46.6 dB (p = 0.046)</td>
<td>56.6 dB (p = 0.004)</td>
<td>60 dB (p = 0.003)</td>
<td>56.6 dB (p&lt;0.001)</td>
<td>70 dB (p&lt;0.001)</td>
<td></td>
</tr>
<tr>
<td>Day 16 (n = 3)</td>
<td>8.3 dB (p = 0.984)</td>
<td>11.6 dB (p = 0.936)</td>
<td>16.6 dB (p = 0.616)</td>
<td>48.3 dB (p = 0.014)</td>
<td>56.6 dB (p&lt;0.001)</td>
<td>68.3 dB (p&lt;0.001)</td>
<td></td>
</tr>
<tr>
<td>Day 32 (n = 2)</td>
<td>21.1 dB (p = 0.151)</td>
<td>22.2 dB (p = 0.151)</td>
<td>21.3 dB (p = 0.08)</td>
<td>39.4 dB (p = 0.039)</td>
<td>63.8 dB (p&lt;0.001)</td>
<td>67.7 dB (p&lt;0.001)</td>
<td></td>
</tr>
</tbody>
</table>

### TABLE 3.2: POST ACOUSTIC TRAUMA THRESHOLD SHIFTS IN ALL EXPOSURE GROUPS (CONTRALATERAL/NON-LESIONED EAR). A ONE-WAY ANOVA WITH TUKEY’S TEST WERE USED TO COMPARE THRESHOLD SHIFTS BETWEEN FREQUENCIES OF EACH TIME-POINT.

<table>
<thead>
<tr>
<th>Group</th>
<th>Frequency</th>
<th>1 kHz</th>
<th>2 kHz</th>
<th>4 kHz</th>
<th>8 kHz</th>
<th>16 kHz</th>
<th>32 kHz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0 (n = 2)</td>
<td>0 dB (p = 1.0)</td>
<td>-2.5 dB (p = 0.98)</td>
<td>-2.5 dB (p = 0.952)</td>
<td>-2.5 dB (p = 0.997)</td>
<td>0 dB (p = 1.0)</td>
<td>2.5 dB (p = 0.989)</td>
<td></td>
</tr>
<tr>
<td>Day 4 (n = 2)</td>
<td>15 dB (p = 0.35)</td>
<td>5 dB (p = 0.748)</td>
<td>5 dB (p = 0.575)</td>
<td>-10 dB (p = 0.527)</td>
<td>7.5 dB (p = 0.391)</td>
<td>2.5 dB (p = 0.989)</td>
<td></td>
</tr>
<tr>
<td>Day 8 (n = 3)</td>
<td>1.6 dB (p = 1.0)</td>
<td>1.6 dB (p = 0.995)</td>
<td>1.6 dB (p = 0.986)</td>
<td>1.6 dB (p = 0.999)</td>
<td>1.6 dB (p = 0.995)</td>
<td>5 dB (p = 0.765)</td>
<td></td>
</tr>
<tr>
<td>Day 16 (n = 3)</td>
<td>3.3 dB (p = 0.993)</td>
<td>-1.6 dB (p = 0.995)</td>
<td>-3.3 dB (p = 0.803)</td>
<td>1.6 dB (p = 0.999)</td>
<td>-1.6 dB (p = 0.995)</td>
<td>-3.3 dB (p = 0.941)</td>
<td></td>
</tr>
<tr>
<td>Day 32 (n = 2)</td>
<td>0 dB (p = 1.0)</td>
<td>5 dB (p = 0.748)</td>
<td>0 dB (p = 1.0)</td>
<td>0 dB (p = 1.0)</td>
<td>0 dB (p = 1.0)</td>
<td>0 dB (p = 1.0)</td>
<td></td>
</tr>
</tbody>
</table>
3.4.2: STIMULUS-DRIVEN ACTIVITY

3.4.2.1: OVERALL STIMULUS-DRIVEN ACTIVITY CHANGES IN THE AUDITORY PATHWAY

This section will describe the excitability changes in the AC, IC and DCN over the thirty-two days. When consolidating the excitability indexes of all three auditory regions it was evident that NIHL has a significant effect on the overall excitability of the auditory system over time (Fig. 3.6). A one-way ANOVA was performed, which compared the excitability indexes from all time-points and showed that there was a significant difference between time-points, F (5, 29) = 3.609, p = 0.014. A Tukey’s post hoc test was used compare the control excitability index value to the subsequent time-points. This was done to analyse the change of excitability indexes in the auditory pathway over time. The Tukey’s test showed that significant decreases in the excitability index was observed at Day 4 (p = 0.021) and at Day 16 (p = 0.047) when compared to control. Decreases were also observed at Day 0, Day 8 and Day 32, although statistical significance was not reached.

![Figure 3.6: Excitability Indexes of the three regions averaged (which includes the low, medium and high frequency region of the AC, and the IC and DCN) over 32 days. Results are presented as the mean ± SEM excitability index. A one-way ANOVA with Tukey’s test was used to compare the overall excitability index of the control animals to the overall excitability index at each time-point, *p<0.05.](image-url)
Consolidating the data in this way may mask significant changes that may be occurring in the areas investigated. Section 3.4.2.2 highlights the changes in each time-point/group occurring in the AC (high, medium and low frequency regions), the IC and the DCN, and also presents the changes that occurred in the AC, IC and DCN of individual animals per time-point/group.

3.4.2.2: Stimulus-Driven Excitability Changes in the Auditory Cortex, Inferior Colliculus and Dorsal Cochlear Nucleus

This section will present the results of the excitability changes in the high, medium and low frequency region of the AC, IC and DCN over time. One-way ANOVAs were performed on each region, which compared the excitability indexes from each time-point. A Tukey’s post hoc test was used to compare the control excitability index value to the excitability index value of subsequent time-points. This was done to analyse the change of excitability indexes in the high, medium and low frequency region of the AC, IC and DCN over time, separately. A p value of less than 0.05 was considered significant.

3.4.2.2.1: The Auditory Cortex

The results from the ANOVA showed that there was a significant difference between time-points, F (5, 29) = 3.609, p = 0.014. The Tukey’s test showed that the excitability index of the high frequency region increased immediately after NIHL; however, this did not reach statistical significance. On Day 4, the excitability index was decreased significantly compared to control (p<0.001) and on Day 8, Day 16 and Day 32, the excitability index had returned to normal levels (Fig. 3.7A).

The medium frequency region excitability index was decreased compared to control at Day 0 (p = 0.003) and Day 16 (p = 0.031). At Day 32, the excitability index of the
medium frequency region was increased compared to control (p = 0.049) (Fig. 3.7B). The medium frequency region appeared to be the least affected up to sixteen days after NIHL. This finding was unusual, as this was the region that would have been most affected by the acoustic trauma stimulus (noise centred at 16 kHz). On Day 32, the medium frequency region of the AC was the only region within the AC that exhibited an increase in excitability index. Except that at Day 32 the medium frequency region was the only region to show higher activity.

The low frequency region excitability index was decreased compared to control at Day 0 (p = 0.004), Day 4 (p = 0.003), Day 8 (p = 0.002) and at Day 16 (p = 0.011). The low frequency region excitability index returned to near-normal levels by Day 32 (Fig. 3.7C). This was similar to changes that occurred in the IC (see section 3.4.2.2.2) and in the DCN (see section 3.4.2.2.3); which included a high baseline excitability index followed by an immediate decrease after NIHL followed by a slight increase by Day 32.
Figure 3.7: Excitability Indexes of the (A) high, (B) medium and (C) low frequency regions of the AC over 32 days. Results are presented as the mean ± SEM excitability index. A one-way ANOVA with a Tukey’s test was used to compare the excitability index of the control animals to the excitability indexes of the animals at subsequent time-points, *p<0.05 and **p<0.001.
When comparing the excitability indexes from each frequency region of the AC at each time-point, it was evident that each frequency region of the AC responds in a different manner to one another (Fig. 3.8). A one-way ANOVA was used to compare the excitability indexes of the three frequency regions at each time-point. A Tukey’s post hoc test was used to analyse the differences between the frequency regions at each time-point.

The ANOVA determined that there was a significant difference between the excitability indexes of the frequency regions in the control animals, $F(2, 39) = 43.694, p<0.001$. The Tukey’s test showed that the low frequency region had a higher excitability index compared to the medium ($p<0.001$) and high ($p<0.001$) frequency regions. In addition, the excitability index in the high frequency region of the AC was higher than the medium frequency region ($p = 0.039$).

In the Day 0 animals, there was a significant difference between the excitability indexes of the frequency regions, $F(2, 27) = 15.619, p<0.001$. The Tukey’s test showed that the excitability index in the high frequency region was significantly higher than the medium ($p<0.001$) and low ($p<0.001$) frequency regions.

The ANOVA determined that there was a significant difference between the excitability indexes of the frequency regions in the Day 4 animals, $F(2, 26) = 5.483, p = 0.011$. The Tukey’s test showed that the excitability index in medium frequency region was significantly higher than the low frequency region ($p = 0.008$).

In the Day 8 animals, significant differences were observed between the excitability indexes of the frequency regions, $F(2, 36) = 4.931, p = 0.013$. The Tukey’s test determined that the excitability index of the high frequency region was significantly higher than the low frequency region ($p = 0.01$).
The ANOVA showed that there was a significant difference between the excitability indexes of the frequency regions in the Day 16 animals, $F(2, 36) = 13.107$, $p<0.001$. The Tukey’s test determined that the excitability index in the medium frequency region was significantly lower than the high ($p<0.001$) and low ($p = 0.001$) frequency regions.

No significant differences between the excitability indexes of the frequency regions were observed in the Day 32 animals, $F(2, 23) = 1.968$, $p = 0.165$.

Figure 3.8: Excitability indexes of the high, medium and low frequency regions of the AC over 32 days. Results are presented as the mean ± SEM excitability index. A one-way ANOVA with a Tukey’s test was used to compare the excitability index of each frequency region at each time-point, *$p<0.05$ and **$p<0.001$. 
3.4.2.2.2: THE INFERIOR COLICULUS

The IC (Fig. 3.9) had the highest excitability index baseline in the control animals, when compared to the AC (see section 3.4.2.2.1) and the DCN (see section 3.4.2.2.3). After NIHL, an immediate decrease in the excitability index at Day 0 (p = 0.044) was observed. A general reduction in the excitability index was observed over time reaching significance at Day 16 (p = 0.029). The changes in the IC over the thirty-two days were very similar to the changes that occurred in the DCN (see section 3.4.2.2.3) and in the low frequency region of the AC (see section 3.4.2.2.1), which included a high baseline excitability index followed by an immediate decrease after NIHL followed by a slight increase by Day 32.

![Figure 3.9: Excitability Index of the IC over 32 days. Results are presented as the mean ± SEM excitability index. A one-way ANOVA with a Tukey’s test was used to compare the excitability index of the control animals to the excitability indexes of the animals at subsequent time-points, *p<0.05.](image-url)
3.4.2.2.3: The Dorsal Cochlear Nucleus

The DCN (Fig. 3.10) had a high excitability index baseline in the control animals, similar to the low frequency region of the AC (see section 3.4.2.2.1) and the IC (see section 3.4.2.2.2). After NIHL, an immediate decrease was observed in the excitability index at Day 0 (p<0.001), which remained decreased at Day 4 (p = 0.006), Day 8 (p = 0.02) and Day 16 (p = 0.003). At Day 32 the excitability index returned to near normal levels. The changes in the DCN over the thirty-two days were very similar to the changes that occurred in the IC (see section 3.4.2.2.2) and in the low frequency region of the AC (see section 3.4.2.2.1), which included a high baseline excitability index followed by an immediate decrease after NIHL followed by a slight increase by Day 32.

![Figure 3.10: Excitability index of the DCN over 32 days. Results are presented as the mean ± SEM excitability index. A one-way ANOVA with a Tukey’s test was used to compare the excitability index of the control animals to the excitability indexes of the animals at subsequent time-points, *p<0.05.](image-url)
3.4.2.3: Stimulus-driven Activity Changes in the Auditory Cortex, Inferior Colliculus and Dorsal Cochlear Nucleus in All Animals

This section will summarise the overall stimulus-driven response profiles (types identified and % of total recording channels), inflection points (threshold responses) and the RLF input/output slope data (gain function of neural responses) for the AC, IC and DCN for all animals over time.

Two representative animals have been analysed and are presented in Appendix 1. The Appendix will present the individual ABR audiograms, stimulus-driven response profiles (types identified and percentage of total recording channels), inflection points (threshold responses) and the RLF input/output slope data (gain function of neural responses) for the AC, IC and DCN of two individual animals. The data of a Day 16 and Day 32 animal will be presented as a representation of the whole experimental group.
3.4.2.3.1: Percentage of Stimulus Driven Responses in Auditory Cortex, Inferior Colliculus and Dorsal Cochlear Nucleus of all Animals Across Time

All response types were identified in the high frequency region of the AC (Fig. 3.11). The $\text{MON}^{\text{NSAT}}$ and $\text{MON}^{\text{SAT}}$ responses were at similar levels in the control animals. Immediately after NIHL (Day 0), $\text{MON}^{\text{SAT}}$ responses were not identified and there was a low level of $\text{MON}^{\text{NSAT}}$ responses. There was a high level of non-responsive channels. In the Day 4 group, the $\text{MON}^{\text{NSAT}}$ responses were increased compared to the Day 0 group and there was a reduction in non-responsive channels. In the Day 8 group, $\text{MON}^{\text{SAT}}$ responses were present at similar levels to the $\text{MON}^{\text{NSAT}}$ responses. The Day 16 animals did not exhibit any responses; all channels were non-responsive. In the Day 32 group, all responses were identified. $\text{MON}^{\text{SAT}}$ responses were similar to the control levels of $\text{MON}^{\text{SAT}}$ responses and NON MON and $\text{MON}^{\text{NSAT}}$ responses were at similar levels.

**Figure 3.11:** The percentage of identified stimulus-driven responses in the high frequency region of the AC over 32 days.
In the medium frequency region of the AC (Fig. 3.12), the MON\textsuperscript{SAT} responses were the most abundant when compared to the MON\textsuperscript{N/SAT} responses. A small percentage of channels were non-responsive. Immediately after NIHL (Day 0), the MON\textsuperscript{SAT} responses were not identified; there was a high level of non-responsive channels and a reduced amount of MON\textsuperscript{N/SAT} responses when compared to the Day 0 group. The Day 4 animals almost had equal levels of MON\textsuperscript{N/SAT} responses and non-responsive channels. The Day 8 group was similar to the Day 4 group, with the addition of MON\textsuperscript{N/SAT} responses. At Day 16, there was a decrease in MON\textsuperscript{N/SAT} responses compared to Day 8 and an increase in non-responsive channels. NON MON responses were present and were more abundant than the MON\textsuperscript{N/SAT} and MON\textsuperscript{SAT} responses. The Day 32 group was similar to the Day 16 group; however had an increase in non-responsive channels.

**MEDIUM AC**

![Graph showing percentage of identified stimulus-driven responses in the medium frequency region of the AC over 32 days.](image)

*Figure 3.12: The percentage of identified stimulus-driven responses in the medium frequency region of the AC over 32 days.*

All response profiles were identified in the low frequency region of the AC (Fig. 3.13). The control animals exhibited MON\textsuperscript{N/SAT} and MON\textsuperscript{SAT} responses, the MON\textsuperscript{N/SAT}
responses were more abundant than the MON\textsuperscript{SAT} responses. Non-responsive channels were also evident. Immediately after NIHL (Day 0), MON\textsuperscript{SAT} responses were not identified. There was a high level of non-responsive channels and a decreased amount of MON\textsuperscript{N/SAT} responses when compared to control. The Day 0 and Day 4 groups showed a slow decrease in MON\textsuperscript{N/SAT} responses over time and concurrently a slow increase of non-responsive channels over time. The Day 8 group showed a low level of MON\textsuperscript{SAT} responses and a high level of non-responsive channels. The Day 16 group exhibited a small amount of MON\textsuperscript{SAT} responses, MON\textsuperscript{N/SAT} responses and NON MON responses, a high level of non-responsive channels were present. At Day 32, the non-responsive channels were at a similar level to that of the Day 16 animals. The MON\textsuperscript{SAT} responses were lower than the Day 16 animals; however there was an increase in MON\textsuperscript{N/SAT} responses. NON MON responses were also present, but were at a lower level than the Day 16 group.

![LOW AC](image)

Figure 3.13: The percentage of identified stimulus-driven responses in the low frequency region of the AC over 32 days.
All three response types were observed in the IC (Fig. 3.14). In the control animals there was almost an equal amount of $\text{MON}^\text{N/SAT}$ and $\text{MON}^\text{SAT}$ responses. Immediately after NIHL (Day 0), the $\text{MON}^\text{SAT}$ responses were not identified, there was an increase in $\text{MON}^\text{N/SAT}$ responses, and there was a small percentage of non-responsive channels. On Day 4, there were increased levels of non-responsive channels and a decreased amount of $\text{MON}^\text{N/SAT}$ responses. On Day 8, a small amount of $\text{MON}^\text{SAT}$ responses were present and there were equal levels of $\text{MON}^\text{N/SAT}$ responses and non-responsive channels. The Day 16 animals exhibited NON MON responses at similar levels to the $\text{MON}^\text{SAT}$ responses. The $\text{MON}^\text{N/SAT}$ responses were higher than the Day 8 group and the non-responsive channels had decreased. The Day 32 group exhibited an increase in non-responsive channels and a decrease in $\text{MON}^\text{N/SAT}$ responses compared to Day 16. NON MON responses were identified and were at similar levels to the Day 16 group.

Figure 3.14: The percentage of identified stimulus-driven responses in the IC over 32 days.
MON\textsuperscript{N/SAT} responses were identified in the DCN (Fig. 3.15). Immediately after NIHL (Day 0), the level of MON\textsuperscript{N/SAT} responses slightly decreased compared to control and non-responsive channels were evident. On Day 4, the MON\textsuperscript{N/SAT} responses decreased substantially and a marked increase in non-responsive channels was observed. This was a similar finding in the IC. From Day 8 to Day 32, a slow increase in MON\textsuperscript{N/SAT} responses was observed over time and concurrently a reduction in non-responsive channels was observed.

**Figure 3.15:** The percentage of identified stimulus-driven responses in the DCN 32 days.
3.4.2.3.2: Inflection Points of Stimulus Driven Responses in Auditory Cortex, Inferior Colliculus and Dorsal Cochlear Nucleus of All Animals Across Time

This section presents the inflection points of the MON$^{\text{SAT}}$ and MON$^{\text{N/SAT}}$ responses in each region over the 32-day time course. A one-way ANOVA was performed and determined whether there was a significant difference between inflection points at different time-points. A Tukey’s test was used to compare the inflection points of the control animals to the inflection points of the animals at the subsequent time-points, the results are presented in Table 3.3. The data analysis showed that the inflection points of the MON$^{\text{SAT}}$ responses changed significantly after NIHL.

In the high frequency region of the AC, the inflection points of the MON$^{\text{SAT}}$ responses were significantly different between groups, $F (2, 107) = 1218.458$, $p<0.001$. The Tukey’s test showed that there was a significant increase at Day 8 when compared to control. In addition, there was a significant decrease at Day 32 when compared to control. The one-way ANOVA showed that in the medium frequency region of the AC, the inflection points of the MON$^{\text{SAT}}$ responses were significantly different between groups, $F (3, 101) = 10.519$, $p<0.001$. The Tukey’s test showed that the inflection point at Day 8 was significantly higher than the inflection point at control; there was also a significant decrease observed at Day 32 when compared to control. The one-way ANOVA determined that in the low frequency region of the AC, the inflection points of the MON$^{\text{SAT}}$ responses were significantly different between groups, $F (3, 78) = 336.463$, $p<0.001$. The Tukey’s test indicated that there were significant decreases observed at Day 16 and Day 32. The one-way ANOVA showed that in the IC, the inflection points of the MON$^{\text{SAT}}$ responses were significantly different between groups, $F (3, 62) = 63.995$, $p<0.001$. The Tukey’s test determined that there was an increase of inflection
point at Day 8 and Day 16 when compared to control; there was also a decrease at Day 32. This decrease at the later time-points was a common finding in the inflection points of the MON\textsuperscript{SAT} responses. This could suggest that after NIHL MON\textsuperscript{SAT} responses become more sensitive to noise stimuli.

**TABLE 3.3: THE INFLECTION POINTS FOR MON\textsuperscript{SAT} RESPONSES PER REGION OVER 32 DAYS. A ONE-WAY ANOVA WITH A TUKEY’S TEST WAS USED TO COMPARE THE CONTROL INFLECTION POINTS WITH INFLECTION POINTS OF SUBSEQUENT TIME-POINTS**

<table>
<thead>
<tr>
<th>MON\textsuperscript{SAT} Region</th>
<th>Region</th>
<th>High AC</th>
<th>Med AC</th>
<th>Low AC</th>
<th>IC</th>
<th>DCN</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control (n = 3)</strong></td>
<td></td>
<td>47.8 ± 0.6</td>
<td>42.8 ± 1.8</td>
<td>43.3 ± 1.1</td>
<td>37.0 ± 0.8</td>
<td></td>
</tr>
<tr>
<td><strong>Day 0 (n = 2)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Day 4 (n = 2)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Day 8 (n = 3)</strong></td>
<td></td>
<td>70.0 ± 0.0 (p&lt;0.001)</td>
<td>61.1 ± 1.1 (p&lt;0.001)</td>
<td>70.0 ± 0.0 (p&lt;0.001)</td>
<td>70.0 ± 0.0 (p&lt;0.001)</td>
<td></td>
</tr>
<tr>
<td><strong>Day 16 (n = 3)</strong></td>
<td></td>
<td>44.2 ± 2.9 (p = 0.989)</td>
<td>36.3 ± 2.2 (p&lt;0.001)</td>
<td>45.7 ± 1.7 (p = 0.001)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Day 32 (n = 2)</strong></td>
<td></td>
<td>21.5 ± 0.6 (p&lt;0.001)</td>
<td>22.9 ± 1.8 (p = 0.002)</td>
<td>20.0 ± 0.0 (p&lt;0.001)</td>
<td>24.0 ± 4.0 (p&lt;0.001)</td>
<td></td>
</tr>
</tbody>
</table>

The data analysis showed that the inflection points of the MON\textsuperscript{NSAT} responses changed significantly after NIHL, the results of the Tukey’s test are presented in Table 3.4.

In the high frequency region of the AC, the inflection points of the MON\textsuperscript{NSAT} responses were significantly different between groups, F (4, 124) = 47.316, p<0.001. The Tukey’s test showed that there was a significant increase in the inflection points at all time-points except Day 16 when compared to control. The one-way ANOVA determined that in the medium frequency region of the AC, the inflection points of the MON\textsuperscript{NSAT} responses were significantly different between groups, F (5, 233) = 66.934, p<0.001. The Tukey’s test showed that there was a significant increase in inflection points at Day 0, Day 4, Day 8, and Day 16 when compared to control. The one-way ANOVA determined that in
the low frequency region of the AC, the inflection points of the MON\textsuperscript{NSAT} responses were significantly different between groups, F (4, 116) = 37.664, p<0.001. The Tukey’s test showed that there was a significant increase in inflection points at all time-points (when MON\textsuperscript{NSAT} responses were present) when compared to control. In the IC, the inflection points of the MON\textsuperscript{NSAT} responses were significantly different between groups, F (5, 215) = 72.067, p<0.001. The Tukey’s test showed that there was a significant increase in inflection points at Day 0, Day 4, Day 8, and Day 16 when compared to control. The one-way ANOVA determined that in the DCN, the inflection points of the MON\textsuperscript{NSAT} responses were significantly different between groups, F (5, 248) = 51.651, p<0.001. The Tukey’s test showed that there was a significant increase in inflection points at Day 0, Day 4, and Day 8 when compared to control. There was also a significant decrease in inflection point at Day 32 when compared to control; this was a unique finding in the MON\textsuperscript{NSAT} responses, as all of the inflection points identified in each frequency region of the AC and the IC were significantly higher than control values.

**TABLE 3.4: THE INFLECTION POINTS FOR MON\textsuperscript{NSAT} RESPONSES PER REGION OVER 32 DAYS. A ONE-WAY ANOVA WITH A TUKEY’S TEST WAS USED TO COMPARE THE CONTROL INFLECTION POINTS WITH INFLECTION POINTS OF SUBSEQUENT TIME-POINTS**

<table>
<thead>
<tr>
<th>MON\textsuperscript{NSAT}</th>
<th>Region</th>
<th>High AC</th>
<th>Med AC</th>
<th>Low AC</th>
<th>IC</th>
<th>DCN</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time-point</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n = 3)</td>
<td></td>
<td>56.0 ± 1.5</td>
<td>46.6 ± 1.5</td>
<td>46.8 ± 1.4</td>
<td>53.3 ± 1.2</td>
<td>52.7 ± 1.1</td>
</tr>
<tr>
<td>Day 0 (n = 2)</td>
<td></td>
<td>80.0 ± 0.0 (p&lt;0.001)</td>
<td>72.8 ± 1.3 (p&lt;0.001)</td>
<td>75.3 ± 0.9 (p&lt;0.001)</td>
<td>89.4 ± 0.3 (p&lt;0.001)</td>
<td>77.9 ± 1.5 (p&lt;0.001)</td>
</tr>
<tr>
<td>Day 4 (n = 2)</td>
<td></td>
<td>78.8 ± 0.6 (p&lt;0.001)</td>
<td>80.0 ± 0.0 (p&lt;0.001)</td>
<td>80.0 ± 0.0 (p&lt;0.001)</td>
<td>80.0 ± 0.0 (p&lt;0.001)</td>
<td>80.0 ± 0.0 (p&lt;0.001)</td>
</tr>
<tr>
<td>Day 8 (n = 3)</td>
<td></td>
<td>70.4 ± 0.6 (p&lt;0.001)</td>
<td>67.6 ± 0.5 (p&lt;0.001)</td>
<td></td>
<td>70.6 ± 0.6 (p&lt;0.001)</td>
<td>70.0 ± 0.0 (p&lt;0.001)</td>
</tr>
<tr>
<td>Day 16 (n = 3)</td>
<td></td>
<td></td>
<td></td>
<td>59.6 ± 2.7 (p&lt;0.001)</td>
<td>55.9 ± 3.8 (p = 0.015)</td>
<td>61.3 ± 1.9 (p &lt; 0.011)</td>
</tr>
<tr>
<td>Day 32 (n = 2)</td>
<td></td>
<td>67.0 ± 1.5 (p&lt;0.001)</td>
<td>50.0 ± 11.3 (p = 0.945)</td>
<td>68.7 ± 1.3 (p&lt;0.001)</td>
<td>55.9 ± 5.5 (p&lt;0.001)</td>
<td>45.2 ± 2.7 (p = 0.028)</td>
</tr>
</tbody>
</table>

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**CHAPTER THREE: STUDY ONE**
3.4.2.3.3: TIME COURSE CHANGES OF STIMULUS-DRIVEN ACTIVITY
This section will present the results of the MON\textsuperscript{N/SAT}, MON\textsuperscript{SAT} and NON MON RLF slope changes in the AC, IC and DCN after NIHL. The data presented in the figures is the average RLF slope value ± SEM (Fig. 3.16-3.20).

Auditory Cortex (High Frequency Region)
MON\textsuperscript{N/SAT} responses were identified in the high frequency region of the AC in the control animals and in the Day 0, Day 4, Day 8 and Day 32 animals (Fig. 3.16A). MON\textsuperscript{N/SAT} responses were not present in the Day 16 animals. A one-way ANOVA was used to compare the RLF slope values at each time-point and showed that there was a significant difference of MON\textsuperscript{N/SAT} RLF slope values between time-points, $F(4, 124) = 9.850$, $p<0.001$. A Tukey’s post hoc test was used for multiple comparisons of RLF slope values between each time-point. This was done to analyse the changes in RLF slope values of MON\textsuperscript{N/SAT} responses over time in the high frequency region of the AC. The Tukey’s test showed that on Day 0, there was a significant increase in the RLF slope when compared to control ($p<0.001$). An increase was observed at Day 4 ($p<0.001$) and at Day 8 and Day 32, the RLF slopes were at near control levels.

MON\textsuperscript{SAT} responses were identified in the high frequency region of the AC in the control animals and in the Day 8 and Day 32 animals (Fig. 3.16B). The responses were not present in the Day 0, Day 4 and Day 16 animals. The ANOVA showed that there was a significant difference of MON\textsuperscript{SAT} RLF slope values between time-points, $F(2, 89) = 26.213$, $p<0.001$. The Tukey’s test showed that on Day 8, the MON\textsuperscript{SAT} responses had a significantly higher RLF slope compared to control ($p<0.001$). The MON\textsuperscript{SAT} responses were also present in the Day 16 animals; however the RLF slope values were near control levels. A significant increase in RLF slope value was observed at Day 32 when compared to control ($p = 0.05$).
When comparing the MON\textsuperscript{SAT} and MON\textsuperscript{N/SAT} responses at each time-point, it was evident that each response type differed in RLF slope values in the high frequency region of the AC (Fig. 3.16C). An independent samples t-test was used to compare the MON\textsuperscript{N/SAT} RLF slope values and the MON\textsuperscript{SAT} RLF slope values at each time-point, where both responses were present. This was done to analyse the differences between the RLF slope values of MON\textsuperscript{N/SAT} and MON\textsuperscript{SAT} responses at each time-point. The results of the t-tests showed that in the control animals the MON\textsuperscript{N/SAT} RLF slope values were significantly higher than the MON\textsuperscript{SAT} RLF slope values (p<0.001). This was also observed in the IC. Comparisons were not made at Day 0 and Day 4 as the MON\textsuperscript{SAT} responses were not present, which was also observed in the IC. At Day 8, the MON\textsuperscript{SAT} RLF slope values were significantly higher than the MON\textsuperscript{N/SAT} RLF slope values (p<0.001). Comparisons were not made at Day 16 as both MON\textsuperscript{SAT} and MON\textsuperscript{N/SAT} responses were not present. By Day 32, the MON\textsuperscript{SAT} and MON\textsuperscript{N/SAT} RLF slope values were at similar levels. This suggests that in the high frequency region of the AC, the MON\textsuperscript{SAT} and MON\textsuperscript{N/SAT} RLF slope values are typically opposite each other before and after NIHL. For example when MON\textsuperscript{SAT} RLF slope values were high, the MON\textsuperscript{N/SAT} RLF slope values were low and vice versa.
Figure 3.16: The RLF Slopes of (A) MON\textsuperscript{N/SAT} and (B) MON\textsuperscript{SAT} responses in the high frequency region of the AC over 32 days. A one-way ANOVA with a Tukey's test was used to compare the RLF slope of the control groups to the RLF slopes of each time-point, *p<0.05 and **p<0.001. An independent samples t-test was used to compare the RLF slopes of (C) MON\textsuperscript{N/SAT} and MON\textsuperscript{SAT} responses, *p<0.05 and **p<0.001.
Auditory Cortex (Medium Frequency Region)

MON^{N/SAT} responses were identified in the medium frequency region of the AC at each time-point throughout the 32-day time course (Fig. 3.17A). The ANOVA showed that there was a significant difference of MON^{N/SAT} RLF slope values between time-points, F(5, 233) = 33.069, p<0.001. The Tukey’s test showed that at Day 0, there was a significant increase in the RLF slope when compared to control (p<0.001). An increase was observed at Day 4 (p<0.001). At Day 8 and Day 32, the RLF slopes were close to control levels.

MON^{SAT} responses were identified in the medium frequency region of the AC in the control animals and in the Day 8, Day 16, and Day 32 animals (Fig. 3.17B). The responses were not identified in Day 0 and Day 4 animals. The ANOVA showed that there was a significant difference of MON^{N/SAT} RLF slope values between time-points, F(3, 101) = 7.64, p<0.001. The Tukey’s test showed that on Day 8 and Day 16, the RLF slope values were similar to control levels. A significant increase in the RLF slope values was observed at Day 32 when compared to control (p<0.001).

When comparing the RLF slope values of each response at each time-point, it was evident that the neuronal populations exhibiting the MON^{SAT} and the MON^{N/SAT} responses respond differently to each other in the high frequency region of the AC (Fig. 3.17C). An independent samples t-test was used to compare the MON^{N/SAT} RLF slope values and the MON^{SAT} RLF slope values at each time-point, where both responses were present. The results of the t-tests showed that in the control animals the MON^{SAT} RLF slope values were significantly higher than the MON^{N/SAT} RLF slope values (p<0.001). This was in contrast to the observation in the IC and high frequency region of the AC. Comparisons were not made at Day 0 and Day 4 as the MON^{SAT} responses were not
present, which was also observed in the IC and in the high frequency region of the AC. At Day 8, the MON\textsuperscript{N/SAT} RLF slope values were significantly higher than the MON\textsuperscript{SAT} RLF slope values (p<0.001). Comparisons were not made at Day 16 as neither MON\textsuperscript{SAT} and MON\textsuperscript{N/SAT} responses were present. There were no significant differences between the MON\textsuperscript{SAT} and MON\textsuperscript{N/SAT} RLF slope values as they were at similar levels. At Day 32, the MON\textsuperscript{SAT} RLF slope values were significantly higher than the MON\textsuperscript{N/SAT} RLF slope values (p = 0.015). This suggests that in the medium frequency region of the AC, the MON\textsuperscript{SAT} and MON\textsuperscript{N/SAT} RLF slope values are for the most part opposite each other before and after NIHL. For example when MON\textsuperscript{SAT} RLF slope values were high, the MON\textsuperscript{N/SAT} RLF slope values were low and vice versa.
Figure 3.17: The RLF Slopes of (A) MON^{N/SAT} and (B) MON^{SAT} responses in the medium frequency region of the AC over 32 days. A one-way ANOVA with a Tukey’s test was used to compare the RLF slope of the control groups to the RLF slopes of each time-point, *p<0.05 and **p<0.001. An independent samples t-test was used to compare the RLF slopes of (C) MON^{N/SAT} and MON^{SAT} responses, *p<0.05 and **p<0.001.
Auditory Cortex (Low Frequency Region)

MON^{N/SAT} responses were identified in the low frequency region of the AC in the control animals and in Day 0, Day 4, Day 16, and Day 32 animals (Fig. 3.18A). MON^{N/SAT} responses were not present in the Day 8 animals. The ANOVA showed that there was a significant difference of MON^{N/SAT} RLF slope values between time-points, F(4, 147) = 17.198, p<0.001. The Tukey’s test showed that at Day 0 there was a significant increase in the RLF slope when compared to control (p<0.001). An increase was observed at Day 4 (p = 0.002) as well. At Day 16 and Day 32, the RLF slopes values were at near control levels.

MON^{SAT} responses were identified in the low frequency region of the AC in the control animals and in the Day 8, 16 and 32 animals (Fig. 3.18B). The ANOVA showed that there was no significant difference of MON^{SAT} RLF slope values between time-points, F(3, 47) = 0.837, p = 0.481. Immediately after NIHL (Day 0) and at Day 4, MON^{SAT} responses were not present. On Day 8 and Day 16, MON^{SAT} responses were present and the RLF slope values were similar to control levels. An increase in the RLF slope was observed at Day 32 when compared to control, however this did not reach statistical significance.

When comparing the RLF slope values of each response at each time-point, it was evident that the neuronal populations exhibiting the MON^{SAT} and MON^{N/SAT} responses responded similarly in the low frequency region of the AC (Fig. 3.18C). An independent samples t-test was used to compare the MON^{N/SAT} RLF slope values and the MON^{SAT} RLF slope values at each time-point, where both responses were present. This was done to analyse the differences between the RLF slope values of MON^{N/SAT} and MON^{SAT} responses at each time-point. The results of the t-tests showed that in the control animals
the MON\textsuperscript{SAT} RLF slope values were similar to the MON\textsuperscript{N/SAT} responses. This was different to the observations in the IC and high and medium frequency region of the AC, where one RLF slope value was significantly higher than the other. Comparisons were not made at Day 0 and Day 4 as the MON\textsuperscript{SAT} responses were not present, which was also observed in the IC and in the high and medium frequency region of the AC. There were no significant differences between the MON\textsuperscript{N/SAT} and MON\textsuperscript{SAT} RLF slope values at Day 8, Day 16 or Day 32. This suggests that in the low frequency region of the AC, the MON\textsuperscript{SAT} and MON\textsuperscript{N/SAT} RLF slope values remain similar to each other before and after NIHL.
Figure 3.18: The RLF Slopes of (A) $\text{MON}^\text{N/SAT}$ and (B) $\text{MON}^\text{SAT}$ responses in the low frequency region of the AC over 32 days. A one-way ANOVA with a Tukey’s test was used to compare the RLF slope of the control groups to the RLF slopes of each time-point, $^*p<0.05$ and $^{**}p<0.001$. An independent samples t-test was used to compare the RLF slopes of (C) $\text{MON}^\text{N/SAT}$ and $\text{MON}^\text{SAT}$ responses, $^*p<0.05$. 

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CHAPTER THREE: STUDY ONE
Inferior Colliculus

MON\textsuperscript{NSAT} responses were identified in the IC at each time-point throughout the 32-day time course (Fig. 3.19A). A one-way ANOVA was used to compare the RLF slope values at each time-point and showed that there was a significant difference of MON\textsuperscript{NSAT} RLF slope values between time-points, $F (5, 218) = 45.894$, $p<0.001$. A Tukey’s post hoc test was used for multiple comparisons of RLF slope values between each time-point. This was done to analyse the changes in RLF slope values of MON\textsuperscript{NSAT} responses over time in the IC. The Tukey’s test showed that at Day 0, there was a significant increase in the RLF slope when compared to control ($p<0.001$). An increase was also observed at Day 4 and Day 8 (both $p<0.001$). The RLF slope values at Day 16 and Day 32 were at near control levels.

MON\textsuperscript{SAT} responses were identified in the IC in the control animals and in the Day 8, Day 16 and Day 32 animals (Fig. 3.19B). The responses were not identified in the Day 0 and Day 4 animals. A one-way ANOVA was used to compare the RLF slope values at each time-point and showed that there was a significant difference of MON\textsuperscript{SAT} RLF slope values between time-points, $F (3, 62) = 68.266$, $p<0.001$. A Tukey’s post hoc test was used for multiple comparisons of RLF slope values between each time-point. This was done to analyse the changes in RLF slope values of MON\textsuperscript{SAT} responses over time in the IC. The Tukey’s test showed that on Day 8, the MON\textsuperscript{SAT} RLF slope values were not significantly different to control values. The MON\textsuperscript{SAT} responses were also present in the Day 16 animals, but the RLF slope values were near control levels. A significant increase in RLF slope values was observed at Day 32 when compared to control RLF slope values ($p<0.001$).

When comparing the MON\textsuperscript{SAT} and MON\textsuperscript{NSAT} responses at each time-point, it was evident that each response type differed in RLF slope values (Fig. 3.19C). An
independent samples t-test was used to compare the $\text{MON}^{\text{N/SAT}}$ RLF slope values and the $\text{MON}^{\text{SAT}}$ RLF slope values at each time-point, where both responses were present. This was done to analyse the differences between the RLF slope values of $\text{MON}^{\text{N/SAT}}$ and $\text{MON}^{\text{SAT}}$ responses at each time-point. The results of the t-tests showed that in the control animals the $\text{MON}^{\text{N/SAT}}$ RLF slope values were significantly higher than the $\text{MON}^{\text{SAT}}$ RLF slope values ($p = 0.02$). Comparisons were not made at Day 0 and Day 4 as the $\text{MON}^{\text{SAT}}$ responses were not present. At Day 8, the $\text{MON}^{\text{N/SAT}}$ RLF slope values were significantly higher than the $\text{MON}^{\text{SAT}}$ RLF slope values ($p < 0.001$). On Day 16, both $\text{MON}^{\text{SAT}}$ and $\text{MON}^{\text{N/SAT}}$ RLF slope values were at similar levels. At Day 32, the $\text{MON}^{\text{SAT}}$ RLF slope values were significantly greater than the $\text{MON}^{\text{N/SAT}}$ RLF slope values ($p < 0.001$). This suggests that in the IC, the RLF slope values in the $\text{MON}^{\text{SAT}}$ and $\text{MON}^{\text{N/SAT}}$ responses are opposite each other before and after NIHL. For example when $\text{MON}^{\text{SAT}}$ RLF slope values were high, the $\text{MON}^{\text{N/SAT}}$ RLF slope values were low and vice versa.
Figure 3.19: The RLF Slopes of (A) MON\textsuperscript{N/SAT} and (B) MON\textsuperscript{SAT} responses in the IC over 32 days. A one-way ANOVA with a Tukey’s test was used to compare the RLF slope of the control groups to the RLF slopes of each time-point, *p<0.05 and **p<0.001. An independent samples t-test was used to compare the RLF slopes of (C) MON\textsuperscript{N/SAT} and MON\textsuperscript{SAT} responses, *p<0.05 and **p<0.001.
Dorsal Cochlear Nucleus

MON\textsuperscript{N/SAT} responses were identified in the DCN at each time-point throughout the 32-day time course (Fig. 3.20). A one-way ANOVA was used to compare the RLF slope values at each time-point and showed that there was a difference in RLF slope values between time-points, $F (5, 184) = 15.792, p<0.001$. A Tukey’s post hoc test was used for multiple comparisons of RLF slope values between each time-point. This was done to analyse the changes in RLF slope values of the MON\textsuperscript{N/SAT} responses over time in the DCN. The Tukey’s test showed that there was no difference in the RLF slope in the Day 0 group when compared to control. At Day 4, a decrease in RLF slope values was observed; however, this was not significant due to high variability. At Day 8, an increase in RLF slope values was observed ($p<0.001$) when compared to control. At Day 16 and Day 32, decreases in RLF slope values were observed ($p = 0.015$ and $p>0.001$, respectively). MON\textsuperscript{SAT} responses were not present in the DCN across all time-points.

![Figure 3.20: The RLF Slope of MON\textsuperscript{N/SAT} responses in the DCN over 32 days. A one-way ANOVA with a Tukey’s test was used to compare the RLF slope of the control groups to the RLF slopes of each time-point, *p<0.05 and **p<0.001.](image-url)
Non-Monotonic Responses

NON MON responses were only observed in the medium and low frequency region of the AC and in the IC at Day 16 (n = 2) and in the high, medium and low frequency regions of the AC and in the IC at Day 32 (n = 1).

A repeated measures two-way ANOVA was performed on this data, with group (time-point) and region as the independent variables and slope (Slope 1/Slope 2) as the repeated measure (Fig. 3.21A-B).

Between-subject effects analysis indicated that there was a main effect of region across all of the animals, $F(3, 179) = 19.833, p<0.001$. The high frequency region of the AC (2.6) had a significantly lower average RLF slope compared to the IC (3.9) ($p<0.001$), the medium (3.5) ($p<0.001$) and low (4.6) ($p<0.001$) frequency regions of the AC. This indicated that the neuronal population that exhibited the NON MON responses in the high frequency region of the AC were less excitable than in the other tested regions. In addition, the average RLF slope in the low frequency region of the AC was significantly higher than the average RLF slopes in the IC (3.9) ($p = 0.017$) and the high (2.6) ($p<0.001$) and medium (3.5) ($p<0.001$) frequency regions of the AC. This suggests that the neurons that exhibited the NON MON responses in the low frequency region of the AC were more excitable than in the other tested regions.

There was no significant difference in the overall slopes (Slope 1/Slope 2) when comparing both time-points (Day 16 and Day 32), $F(1, 179) = 1.677, p = 0.197$. The within-subjects effects analysis determined that there was a main effect of slope, $F(1, 179) = 47.978, p<0.001$. Overall, Slope 1 (4.39) was significantly higher than Slope 2 (2.68) ($p<0.001$), which could suggest that there was an inhibitory influence affecting Slope 2.
Figure 3.21: The RLF Slope values for Slope 1 and Slope 2 of (A) NON MON responses in the Day 16 animals (n = 2) and (B) NON MON responses in the Day 32 animal (n = 1) in various regions.
3.4.3: SPONTANEOUS ACTIVITY

This section will present the results of the SA changes in the AC, IC and DCN after NIHL. The data presented in the figures are the average spike rates ± SEM (spikes/second) (Fig. 3.22-3.24). A one-way ANOVA was used to compare the spike rate of the control group to each spike rate per time-point/group in each frequency region of the AC (Fig. 3.22). A Tukey’s post hoc test was used for multiple comparisons of spike rate between each time-point. This was done to analyse the changes in spike rate over time in each frequency region of the AC. A one-way ANOVA was used to compare the spike rate of the three frequency regions of the AC at each time-point (Fig. 3.23). A Tukey’s post hoc test was used for the multiple comparisons of spike rate between each frequency region. This was done to analyse the spike rate within each time-point between each frequency region of the AC. For the data in Figure 3.24, a one-way ANOVA was used to compare the control spike rate to each spike rate per time-point in the IC and DCN. A Tukey’s post hoc test was used for multiple comparisons of spike rate between each time-point. This was done to analyse the changes in spike rate over time in the IC and DCN. For all analyses, a p value less than 0.05 was considered significant.

3.4.3.1: SPIKE RATE CHANGES IN THE AUDITORY CORTEX OVER TIME

In the high frequency region (Fig. 3.22A), the one-way ANOVA determined that there were significant differences in spike rate between time-points, F (5, 762) = 51.255, p<0.001. The Tukey’s post hoc test was performed and showed the following results. A decrease in spike rate was observed immediately after NIHL (Day 0) when compared to control (p<0.001). The spike rate in the Day 4 group increased slightly compared to Day 0, however, was still lower than control (p = 0.031). The Day 8 rats showed a reduction in spike rate (p<0.001). On Day 16, the spike rate levels were significantly higher than control.
the spike rate level of controls (p<0.001). On Day 32, the spike rate levels were
decreased compared to the spike rate levels of the control group (p = 0.003). Overall
there were significant fluctuations in spike rate over time after NIHL.

In the medium frequency region (Fig. 3.22B), the one-way ANOVA determined that
there were significant differences in spike rate between time-points, F (5, 636) = 29.426,
p<0.001. The Tukey’s post hoc was performed and showed the following results. A
decrease in spike rate was observed immediately after NIHL (Day 0) when compared to
control (p<0.001). The spike rate at Day 4 increased slightly compared to Day 0,
however, was still lower than control (p<0.001). The Day 8 rats showed a decrease in
spike rate compared with control (p<0.001). On Day 16, the spike rate levels were again
decreased compared to control (p<0.001). By Day 32, the spike rate levels were
decreased compared to control (p<0.001). Overall, the medium frequency region
experienced significant decreases in spike rate over time after NIHL.

In the low frequency region (Fig. 3.22C), the one-way ANOVA determined that there
were significant differences in spike rate between time-points, F (5, 634) = 73.403,
p<0.001. The Tukey’s post hoc was performed and determined statistically significant
differences between the spike rates of control animals to the spike rate of the animals at
the subsequent time-points. The spike rate at Day 4 increased when compared to control
(p<0.001). The Day 8 rats showed a significant increase in spike rate compared to
control (p<0.001). On Day 16, the spike rate levels were similar to control levels. By
Day 32, the spike rate levels were again increased compared to control (p = 0.007).
Overall, there were significant increases in spike rate over time after NIHL.
Figure 3.22: Spontaneous Activity in (A) high, (B) medium and (C) low frequency regions of the AC over 32 days. Results are presented as the average spike rate ± SEM (spikes/second). A one-way ANOVA with a Tukey’s post hoc test was used to compare control to each time-points, *p<0.05 and **p<0.001.
3.4.3.2: Spike Rate Differences in the Auditory Cortex at Each Time-point

When comparing the three frequency regions of the AC at each time-point, it was clear that each region responded significantly differently to one another after NIHL (Fig. 3.23).

In the control animals, the one-way ANOVA determined that there were significant differences in spike rate between frequency regions, $F(2, 382) = 21.480$, $p<0.001$. The Tukey’s post hoc test showed that the low frequency region had the lowest spike rate compared to the high ($p<0.001$) and medium ($p<0.001$) frequency regions.

In the Day 0 animals, the one-way ANOVA determined that there were significant differences between frequency regions, $F(2, 317) = 37.846$, $p<0.001$. The Tukey’s post hoc showed that the spike rate of the medium frequency region was significantly higher than the spike rate of the high ($p = 0.001$) and low ($p<0.001$) frequency regions.

At Day 4, the one-way ANOVA determined that there were significant differences in spike rate between frequency regions, $F(2, 317) = 4.213$, $p = 0.016$. The Tukey’s post hoc showed that the spike rate of the high frequency region was significantly higher than the medium frequency region ($p = 0.012$).

On Day 8, the one-way ANOVA determined that there were significant differences in spike rate between frequency regions, $F(2, 317) = 172.981$, $p<0.001$. The Tukey’s post hoc showed that the spike rate of the low frequency was higher than the high ($p<0.001$) and medium ($p<0.001$) frequency regions.

By Day 16, the one-way ANOVA determined that there were significant differences in spike rate between frequency regions, $F(2, 316) = 207.268$, $p<0.001$. The Tukey’s post
hoc showed that the spike rate of the high frequency region was significantly increased compared to the medium (p<0.001) and low (p<0.001) frequency regions. In addition, the medium frequency region was higher than the low frequency region (p<0.001).

On Day 32 the one-way ANOVA determined that there were significant differences in spike rate between frequency regions, F (2, 380) = 14.584, p<0.001. The Tukey’s post hoc showed that the spike rate of the high frequency region was higher than the medium (p<0.001) and low (p<0.001) frequency regions.

![Figure 3.23: Spontaneous Activity in high (red bar), medium (orange bar) and low (blue bar) frequency regions of the AC over 32 days. Results are presented as the mean ± SEM (spikes/second). A one-way ANOVA with a Tukey’s test was used to compare the three regions per time-point, *p<0.05 and **p<0.001.](image)

### 3.4.3.3: Spike Rate Changes in the Inferior Colliculus and the Dorsal Cochlear Nucleus Over Time

The analyses showed that there were significant changes in spike rate over time in both the IC and DCN (Fig. 3.24). The IC and DCN shared a similar profile of spike rate changes over time. In the IC (Fig. 3.24A), the one-way ANOVA determined that there were significant differences in spike rate between time-points, F (5, 474) = 43.620, p<0.001. The Tukey’s post hoc showed that there were no changes in spike rate after
NIHL on Day 0 and Day 4. On Day 8, there was an increase in spike rate when compared to control (p = 0.048). The spike rate was also increased at Day 16 (p<0.001) and on Day 32 (p<0.001) when compared to control values.

In the DCN (Fig. 3.24B), the one-way ANOVA determined that there were significant differences in spike rate between time-points, F (5, 474) = 10.315, p<0.001. The Tukey’s post hoc showed that there were no changes in spike rate after NIHL on Day 0 and Day 4. On Day 8 and Day 16, there was an increase in spike rate when compared to control (p = 0.042 and p<0.001, respectively). On Day 32, the spike rate was slightly less than the Day 16 spike rate levels; though it was still higher than control values it did not reach statistical significance.

Figure 3.24: Spontaneous Activity in the (A) IC and (B) DCN over 32 days. Results are presented as the mean spikes/second ± SEM. A one-way ANOVA with a Tukey’s test was used to compare control to each time-point, *p<0.05 and **p<0.001.
3.5: DISCUSSION

The level of stimulus-driven activity and SA has been used as a measure of neural excitability and hyperactivity, respectively, in the auditory system. I have quantified and measured the stimulus-driven activity and SA in the contralateral AC, contralateral IC and ipsilateral DCN of the young adult rat up to 32 days following NIHL. This study has demonstrated that NIHL significantly alters the normal levels of SA and stimulus-driven activity in the auditory pathway of the rat over time. My findings support previous investigations that changes in excitability and SA occur after NIHL. These changes may serve as part of the underlying mechanisms that leads to acoustic disorders following NIHL.

The Effect of NIHL on Hearing Thresholds

The degree of hearing loss produced by exposure to a sound is dependent on the loudness of the sound and the duration of the exposure. TTS, which are non-permanent shifts in hearing thresholds, may be observed immediately following exposure (Puel et al., 1998, Syka and Rybalko, 2000, Bauer, 2003), and if the sound is loud enough it may produce permanent elevations in thresholds resulting in PTS (Clark, 1991).

The hearing loss produced by the stimulus used in this experiment can be characterised by significant threshold shifts across all frequencies and all time-points. The threshold shifts ranged from 48-75 dB at Day 0, 60-85 dB at Day 4, 47-70 dB at Day 8, 8-68 dB at Day 16 and 21-68 dB at Day 32. The threshold shifts for all frequencies immediately increased after NIHL (Day 0) and remained elevated until Day 8. At Day 16, the hearing thresholds of the lower frequencies showed slight recovery compared to the Day 8 group, with only the hearing thresholds at 8 kHz, 16 kHz and 32 kHz remaining elevated. At Day 32, the lower frequencies remained recovered, with only 16 kHz and
32 kHz elevated. This indicated that the animals sustained a permanent threshold shift in the higher frequency region, which was expected due to the frequency of the acoustic trauma stimulus used.

The acoustic trauma stimulus used in this study caused a significant increase in hearing thresholds across most frequencies for the duration of the study. Others who have used a similar acoustic trauma treatment have shown mixed results. Turner and colleagues used the same strain of rats and reported that after 4 months recovery thresholds remained elevated for all frequencies tested (except 10 kHz and 32 kHz) (Turner et al., 2006). Additionally, Bauer reported that threshold shifts remained elevated 7 months after being exposed to a 16 kHz octave band noise at 105 dB for 1 hour (Bauer, 2003). It should be noted that the maximum hearing loss in the present study occurred at 16 kHz and 32 kHz and at those frequencies the losses were similar to those of comparable studies (Bauer, 2003, Turner et al., 2006, Wang et al., 2009).

Electrophysiology

The current study utilised multiple multi-unit recordings to assess the physiological changes in the auditory pathway after NIHL. This discussion attempts to draw parallels with single-unit recording studies. Though not completely analogous, clear similarities are evident between the two methods. It also must be noted that the precise location of the auditory sites could not be confirmed in this study, as CF maps and histological confirmation were not achieved. In this study, electrode placement was determined using stereotaxic coordinates in relation to bregma, and by stimulation of the auditory centres with broadband noise.

Electrophysiological results demonstrated that the AC, IC and DCN in the adult Long Evans rat exhibited significant changes in neuronal activity, as measured by stimulus-
driven activity and SA after NIHL. These fluctuations in stimulus-driven activity and SA may play a role in the development or generation of tinnitus, the reorganisation of tonotopic maps and may be a result of changes previously shown in excitatory and inhibitory transmitter systems.

The Effect of NIHL on Stimulus-Driven Activity

After NIHL, the auditory pathway exhibited an overall decrease in stimulus-driven activity. This was most likely the result of auditory nerve deafferentation. It has been shown that acoustic over-exposure (which in most cases leads to NIHL) causes decreases in SA and excitability of the auditory nerve fibres (Liberman and Kiang, 1978, Liberman and Dodds, 1984). However, this decrease in excitability could also be the result of a protective mechanism that functions in response to acoustic insults, to prevent overstimulation and excitotoxicity in the auditory pathway. Though there was an overall decrease in excitability, some fluctuations were observed over time, which may reflect a constant compensatory mechanism that attempts to rebalance neural activity. Interestingly, the low frequency region of the AC, the IC and the DCN shared a similar pattern in the fluctuations of excitability. This may suggest that after NIHL, the low frequency region of the AC, the IC, and the DCN respond in a similar manner. This could be due to a top-down effect of the AC on the IC and DCN.

After NIHL, there was an overall decrease in excitability in the AC (when combining the frequency regions). This is in agreement with the study by Tan and colleagues (Tan et al., 2007) that used a pure-tone acoustic trauma (10 kHz tone at 109 dB SPL for 2 hours). Tan and colleagues chronically implanted two female Wistar rats with electrodes in the AC and reported an overall reduction in stimulus-driven activity after acoustic trauma. The stimulus-driven activity of one rat recovered to near control levels by four days and the other rat recovered to near control levels by seven days after the acoustic trauma.
trauma (Tan et al., 2007). Overall, an immediate decrease was observed in the stimulus-driven activity in the AC, but returned to control levels by Day 32. This shows that Tan’s animals recovered more rapidly than the animals in the current study. Different methodologies were utilised in each study and may have contributed to the differing results; e.g. acoustic trauma methods used (10 kHz tone at 109 dB SPL for 2 hours vs. 16 kHz noise at 115 dB for 1 hour), number of animals used and frequency regions of the AC investigated.

When analysing the three frequency regions separately, it was clear that there were significant differences between each frequency region of the AC after NIHL. This was most likely due to how the acoustic trauma stimulus affected different parts of the AC, due to the spectrum of the acoustic trauma stimulus. As previously mentioned, the low frequency region responded similarly to the DCN and IC. The medium frequency region, where the frequency of the acoustic trauma stimulus was centred, exhibited slight fluctuations above and below the baseline levels. The fact that this region did not exhibit considerable changes in stimulus-driven activity within the first two weeks may suggest that the medium frequency region was deafferented and therefore its excitability was lower due to the lack of functional inputs. However, excitability increased at the end of the time-course, which may indicate that the excitability of the medium frequency region has a delayed response to NIHL or may indicate that the region was recovering from the deafferentation. The high frequency region of the AC responded differently to the other regions tested in this study, with an initial sharp increase in excitability followed by a return to control levels. This could suggest that the high frequency region has an immediate transient response to acoustic insults, but returns to normal function quite rapidly.
It is known that acoustic exposure causes decreases of stimulus-driven activity and SA of auditory nerve fibres (Liberman and Kiang, 1978, Rajan et al., 1993, Norena et al., 2002, Noreña and Eggermont, 2003, Noreña et al., 2003) and this could be responsible for the overall decreases observed in the stimulus-driven activity in the AC, IC and DCN in this study. Schaette and Kempter (2006) proposed that a compensatory mechanism would trigger increases of activity in the auditory pathway and the increases in SA would elevate above control levels to compensate for lost stimulus-driven activity. This model predicts that SA would be increased the most in tonotopic regions sustaining the largest decreases of active input (Schaette and Kempter, 2006).

Rate Level (Gain) Function Slopes

This is the first study to investigate the time-course of changes in multi-unit gain functions (as an indicator of cell excitability) of neuronal populations in the AC, IC and DCN of the same animal. MON\textsuperscript{N/SAT} and MON\textsuperscript{SAT} responses were the most abundant responses observed in this study. Generally, the results indicated that MON\textsuperscript{N/SAT} responses had steeper RLF slopes (higher gain function) with higher thresholds, whilst the MON\textsuperscript{SAT} responses had more gradual RLF slopes (lower gain functions) with lower thresholds. Essentially this means that the neuronal population that exhibited MON\textsuperscript{N/SAT} responses were more excitable (once they were activated). However, they required a higher threshold to activate firing and the neuronal population that exhibited MON\textsuperscript{SAT} responses were less excitable (once they were activated) and required a lower threshold to activate firing. Both response types were identified in the IC and all regions of the AC across all time-points, which is in agreement with other studies that have identified similar response profiles (Aitkin, 1991, Watkins and Barbour, 2011b). The DCN only exhibited MON\textsuperscript{N/SAT} responses. This stands in contrast to other studies that have identified MON\textsuperscript{SAT} responses in the DCN (Ding and Voigt, 1997, Ding et al., 1999, ...
Spirou et al., 1999). Since MON$^{\text{SAT}}$ responses were not identified in the DCN of any animal throughout the whole time-course, the method utilised for locating the DCN in this study may have led to repeated recordings from a population of neurons that do not exhibit MON$^{\text{SAT}}$ responses.

In the high frequency region of the AC, the MON$^{\text{N/SAT}}$ slope values increased immediately and returned to control levels by Day 8, suggesting that there may be some partial recovery that occurs in the high frequency region of the AC around the Day 8 time-point. This was similar to the excitability findings in the same region. The MON$^{\text{SAT}}$ responses were present in the control animals but were not identified immediately after the NIHL yet were present on Day 8, further suggesting that there may be some partial recovery that occurs in the high frequency region of the AC around the Day 8 time-point. For a second time, when the MON$^{\text{SAT}}$ responses were present the RLF slope values were either higher or lower than the MON$^{\text{N/SAT}}$ slopes values, in most cases.

In the medium frequency region of the AC, the MON$^{\text{N/SAT}}$ slope values increased immediately and up to Day 8. The MON$^{\text{SAT}}$ responses were present in the control animals but were not identified after NIHL, yet reappeared at Day 8, suggesting that there may be some partial recovery at this time-point. The MON$^{\text{SAT}}$ responses were identified at Day 16 at control levels and were found to be increased at Day 32. This was similar to the MON$^{\text{SAT}}$ responses in the IC and the high frequency region of the AC. This could suggest that neuronal populations exhibiting the MON$^{\text{SAT}}$ responses in the IC and the high frequency region of the AC are non-responsive for a short period after NIHL, however become responsive over time.

In the low frequency region of the AC, the MON$^{\text{N/SAT}}$ slopes increased immediately and remained high until Day 4. The MON$^{\text{SAT}}$ responses were present in the control animals
but were not identified after the NIHL, however reappeared at Day 8, which again suggests a partial recovery after NIHL. The MON$^\text{SAT}$ responses were identified at Day 16 and Day 32. This was similar to the MON$^\text{SAT}$ responses in the IC and in the high and medium frequency region of the AC. When both responses were present, the RLF slope values did not differ between each response type. This may be due to the high animal variability or may suggest that the low frequency region was the least affected by the acoustic trauma stimulus, which would support the excitability findings previously discussed.

The IC MON$^\text{N/SAT}$ slope values increased immediately and remained elevated until eight days after NIHL. This suggests that the neuronal population that exhibited MON$^\text{N/SAT}$ responses in the IC increased in excitability after NIHL. The MON$^\text{SAT}$ responses were present in the control animals but were not identified after NIHL. However, they reappeared at Day 8, suggesting that there may be some partial recovery that occurs in the IC around the Day 8 time-point. MON$^\text{SAT}$ responses were also identified at Day 16 at control levels and were found to be significantly increased at Day 32. This may suggest that neuronal populations that exhibit MON$^\text{SAT}$ responses in the IC become hyperexcitable over time.

After NIHL, the DCN MON$^\text{N/SAT}$ slope values did not change immediately; an increase in slope value was observed at Day 8. This suggests that the neuronal population/s in the DCN did not exhibit changes in excitability until one week after NIHL, which is similar to the SA observations (to be discussed) There was a subsequent decrease at Day 16, suggesting a peak of hyperexcitability followed by a decrease. This may be due to molecular changes that are known to occur after NIHL in the DCN (Chapter 4).
In the IC and medium frequency region of the AC, when the MON\textsuperscript{SAT} responses were present, the RLF slope values appeared to be opposite to that of the RLF slope values of the MON\textsuperscript{NSAT} responses. For example, when MON\textsuperscript{SAT} slope values were high, the MON\textsuperscript{NSAT} slope values were low and vice versa; except at Day 16, when both responses had a similar RLF slope. A similar result was found in the high frequency region with the only variation being that the RLF slopes of both responses were at similar levels on Day 32, instead of Day 16. This suggests that there is a compensatory relationship between the two MON\textsuperscript{NSAT} and MON\textsuperscript{SAT} responses. This was not observed in the low frequency region of the AC.

NON MON responses were identified at Day 16 and Day 32. This could suggest that a mechanism occurred between the Day 8 and Day 16 time-point, which led to the presence of the NON MON responses. The NON MON responses observed in this study were unique; they could not be characterised as typical NON MON responses. A typical NON MON response begins with an increase, which leads to a peak and then a subsequent decrease. The typical NON MON response profile resembles a bell curve (Parham et al., 2000, Watkins and Barbour, 2011a). However, in this study the NON MON responses did not continue decreasing after the initial peak but rather increased again. A typical NON MON response is controlled by inhibitory inputs, which results in a downward slope after the peak (Caspary et al., 1987, Nelken and Young, 1994). As the NON MON responses in this study do not resemble typical NON MON responses, an explanation could be that the inhibitory influences were not dominant enough to cause a complete decrease in stimulus-driven activity. This could be caused by a decrease in inhibitory transmitters or an increase in excitatory transmitters, which has been observed in the auditory pathway after NIHL (Chapter 4). In all cases, Slope 1 had a higher RLF gain compared to Slope 2. Between the slopes was a marked reduction in RLF; this may
suggest that the neurons producing the responses were subject to additional inhibitory input. However, the slope did not completely return to 0 dB SPL levels, which is commonly seen in typical non-monotonic RLFs. Instead, there was another increase in the RLF slope, which continued to increase and did not saturate. The continuation of RLF Slope 2 may suggest an inhibitory influence. The emergence of NON MON responses in the AC, IC and DCN at Days 16 and 32 may reflect an attempt to increase inhibition. This may be in response to the changes of SA that were observed in these regions, as the major increases in SA were observed between Day 8 and Day 32 (to be discussed). It may be possible that the emergence of NON MON responses was related to the levels of SA in the auditory system.

Another explanation is that the NON MON responses may not be one neuronal population responding to the noise-stimulus. Since these were multi-unit recordings, it may be possible that two populations of neurons were responding to the stimuli. The first slope (Slope 1) may be the response of neurons that have a lower threshold and the second slope (Slope 2) may be the response of neurons that have a higher threshold. However, the fact that all of the second slopes had less gain compared to the first slopes may validate an inhibitory influence on one neuronal population. Also, the transition between the two slopes was smooth, which supports the theory that it was one neuronal population responding to the noise stimulus. If it were two separate neuronal populations responding to the stimulus, it would be expected that the transition between the two slopes would show more fluctuations or a plateau.

The Effect of NIHL on Spontaneous Activity

Several in vivo investigations have examined the effects of acoustic trauma on SA levels in the AC, IC and DCN. Most investigations have utilised single-unit recordings to analyse spike rate, with limited investigations performing multi-unit recordings. This
investigation has used multi-unit recordings to analyse the changes in spike rate over time. Though it is known that there are differences between single-unit and multi-unit recordings (Zeitler et al., 2006), I propose that single-unit studies can be made comparable to this study by looking at relative changes in SA; i.e. the fold-change, which can be a relative fold increase or decrease. For example; if an average spike rate is generated by the spike rate range (e.g. 40-100 spikes/second equates to an average of 70 spikes/second), the fold change can be obtained by dividing the control spike rate by the spike rate after the acoustic insult. In this way, the relative changes that occur can be effectively compared between single and multi-unit recordings. The results of this multi-unit investigation indicated that the acoustic trauma stimulus that was utilised caused significant changes in SA.

In the AC, Komiya and Eggermont (2000) demonstrated in a multi-unit recording cat study, that control levels of SA, where the mean firing rate (± standard deviation (SD)) of units whose CFs were above 6.5 kHz was 1.43 ± 1.17 (spikes/second) and for those whose CFs were <6.5 kHz it was 1.36 ± 1.28 (spikes/second). In exposed animals the mean firing rate (± SD) of units in reorganised areas was 2.33 ± 2.43 (spikes/second) (CFs were >6.5 kHz), which is a relative 1.713-fold increase. The mean firing rate of units in normal areas was 1.36 ± 1.54 (spikes/second) (CFs were <6.5 kHz); which is to say there was no fold change (Komiya and Eggermont, 2000). Though the frequency regions where the recording took place were not the same as in this study, it is clear that the control SA levels recorded were much higher in the high and medium frequency region, compared to Komiya and Eggermont’s study. In this study, the SA in the high frequency region was 15-17 spikes/second and after NIHL, increased to levels as high as 28-32 spikes/second (Day 16), which is a relative 1.875-fold increase. This increase in fold change is very similar to the increase in fold change of the spike rate in the
reorganised region of the AC found in Komiya and Eggermont’s study. This may suggest that the high frequency region of the AC was reorganised after NIHL. The SA in the medium frequency region was 13-16 spikes/second and after NIHL decreased to 3-6 spikes/second; a 0.31-fold decrease. This may be due to the deafferentation in this region. However, in the low frequency region used in the current study (which is analogous to the region Komiya and Eggermont recorded from, i.e. <6.5 kHz), the SA was 2-3 spikes/second and increased to 17-18 spikes/second at eight days after NIHL, which is a 7-fold increase. Komiya and Eggermont’s results showed that there was no change in spike rate in the low frequency region. The chief difference between the two studies is that Komiya and Eggermont’s study used cats and this study used rats. There may be species differences in the baseline levels of SA. In addition, the acoustic trauma stimulus differed between the two studies; a 6 kHz tone at 126 dB (SPL) for 1 hour compared to 16 kHz noise at 115 dB (SPL) for 1 hour. Another disparity is that this current study recorded from animals up to thirty-two days after acoustic trauma, whereas Komiya and Eggermont’s study was conducted on animals 7-16 weeks after acoustic trauma.

In the IC, Mulders and colleagues demonstrated in the guinea pig that control levels of SA were generally below 7 spikes/second while in exposed animals, activity at 2-3 weeks after acoustic trauma was clearly elevated (15-23 spikes/second), which is a fold increase of 2.71 (Mulders and Robertson, 2009, Mulders et al., 2011). Manzoor and colleagues demonstrated in hamsters that the control levels of SA were below 3 spikes/second and in exposed animals SA was elevated (15-20 spikes/second at 2-3 weeks), which is a fold increase of 5.83 (Manzoor et al., 2012a). In this study, control levels were below 4 spikes/second and the exposed animals showed clearly elevated
levels (16-18 spikes/second between 2-3 weeks), which is a fold increase of 4.25. This suggests that the findings of this study are consistent with other SA findings in the IC.

In the DCN, Manzoor and colleagues demonstrated that control levels of SA were generally ranging between 20-70 spikes/second, whilst exposed animals showed SA levels between 10-150 spikes/second (Manzoor et al., 2012b). Another study by Zhang and Kaltenbach (1998) demonstrated that control Sprague Dawley rats exhibited spike rates of 40-100 spikes/second and exposed animals showed a spike rate generally around 90-300 spikes/second (Zhang and Kaltenbach, 1998). Though the results of Manzoor's and Zhang's studies seem to be in contradiction to the results of the current study, there is however a similarity in the fold changes. In Manzoor’s study, the DCN control spike rate was generally 4-6 spikes/second and the exposed animals exhibited spike rates generally ranging between 10-14 spikes/second. The fold change for Manzoor’s hamsters was a 1.8-fold increase after acoustic trauma, whilst the fold change for Zhang's rats was a 2.8-fold increase. Meanwhile, the fold change in the current study was 2.4. This indicated that the animals of this study exhibited the same relative increases in SA after acoustic trauma to the previous studies conducted by Manzoor and Zhang.

In the AC, the three frequency regions responded differently after NIHL. In the high frequency region, an overall decrease in SA was observed, except for a significant increase at Day 16. The low frequency region generally had an increase in SA after NIHL. However, the medium frequency region exhibited an overall decrease in SA. This may be explained by the spectrum of the lesion stimulus as it would be centred in the medium frequency region of the AC, which would result in a lack of input as the region was deafferented. However, this does not support Schaette and Kempter's (2006) model that predicts that SA would be increased the most in tonotopic regions sustaining the largest decreases of active input. The responses in the high and low frequency region
may suggest that these regions were compensating for the loss of input into the medium frequency region. An explanation for the increase in SA in the surrounding areas could be that there was a loss of surround inhibition. Loss of surround inhibition can be the result of previously inhibited cortical inputs being “unmasked” after peripheral damage. This has been observed in the visual system (Gilbert and Wiesel, 1992, Calford et al., 1999), somatosensory system (Rasmusson and Turnbull, 1983, Calford and Tweedale, 1991a, Calford and Tweedale, 1991b) and auditory system (Robertson and Irvine, 1989, Rajan et al., 1993, Willott et al., 1993, Rajan, 1998, Eggermont and Komiya, 2000). Previous studies have shown that auditory cortical neurons receive various inputs from broad frequency bands (Wang et al., 2000, Norena and Eggermont, 2002) and it has been hypothesised that intracortical inhibition serves to inhibit or “mask” some of these inputs (Phillips and Hall, 1992, Rajan, 1998, Wang et al., 2000, Norena and Eggermont, 2002). After an acoustic trauma, regions in the cortex with the peripheral damage are hypothesised to provide a reduced amount of inhibition to neighbouring frequency regions (Salvi et al., 2000a, Wang et al., 2002). As a consequence, this release from intracortical inhibition “unmasks” excitatory inputs (Robertson and Irvine, 1989, Rajan et al., 1993, Willott et al., 1993, Eggermont and Komiya, 2000). Previously, Rajan reported that unilateral limited cochlear damage with a threshold shift of <25 dB caused a loss of functional surround inhibition in the cortex in adult cats, without reorganisation of the tonotopic map (Rajan, 1998). However, increased spontaneous firing rates have been found in AC regions with tonotopic reorganisation induced by exposure to loud pure tones (Eggermont and Komiya, 2000, Komiya and Eggermont, 2000). This could suggest that the lesioned region (medium frequency region) does not undergo tonotopic reorganisation immediately after NIHL, but rather that it occurs in the surrounding regions (high and low frequency) first. Tonotopic reorganisation may begin to occur in the lesioned area one month after NIHL, which was when SA was found to be increased.
The increase of SA occurred at Day 4 in the low frequency region and at Day 16 in the high frequency region. This could suggest that tonotopic reorganisation starts in the low frequency region as early as four days and sixteen days in the high frequency region. Alternatively, it could suggest that the high and low frequency regions experience increases in SA due to the loss of functional surround inhibition without necessarily resulting in tonotopic reorganisation.

In the IC, the spike rate was not affected immediately after NIHL. The first significant increase was observed between Day 4 and 8. These findings support previous research into the development of SA in the IC after NIHL. Dong and colleagues (2009) reported that SA was not observed immediately after acoustic trauma, however was identified one week after a mechanical lesion (Dong et al., 2009a). Mulders and Robertson (2009) showed that SA appeared one week after an acoustic trauma (Mulders and Robertson, 2009). Other studies from the Robertson laboratory, recorded SA from guinea-pigs immediately after, and two weeks after acoustic trauma. These studies showed that SA was not present immediately, however was evident in the two week recovery animals (Dong et al., 2010a, Mulders et al., 2011).

In the DCN, the spike rate was also not affected immediately after NIHL. The first significant increase was observed between Day 4 and 8. This supports a previous study that showed that SA emerges between two and five days after acoustic exposure in the DCN (Kaltenbach et al., 2000). Other studies have also shown that SA does not develop in the DCN immediately after acoustic trauma (Kaltenbach et al., 1998, Brozoski et al., 2002, Finlayson and Kaltenbach, 2009, Pilati et al., 2012). After NIHL, the rats showed a 1.7-2.4-fold increase in SA. This supports previous work by Zhang and Kaltenbach (1998) that observed a 1.4–2.4-fold increase in the DCN of rats following tone exposure (Zhang and Kaltenbach, 1998).
In this study, it was clear that the IC and DCN had similar SA activity profiles over time. It is known that the IC and DCN share extensive connections between each other (Osen, 1972, Beyerl, 1978, Adams, 1979, Ryugo et al., 1981, Oliver, 1984, Alibardi, 2000). A recent time-course study highlighted the relationship between the IC and DCN with SA (Manzoor et al., 2012b). Manzoor and colleagues (2012) showed that hamsters exhibited increases of SA in the IC and DCN over thirty-six days after acoustic exposure. These increases were higher at each time-point, which was also observed in this study in the IC and DCN, as they showed a similar profile in their SA increases. In another recent study, Manzoor and colleagues (2012) exposed animals to an acoustic trauma and measured the SA in the IC before and after the DCN was ablated (either by thermocautery, aspiration or freezing) (Manzoor et al., 2012a). Manzoor and colleagues demonstrated that after DCN ablation the SA levels in the IC returned to control levels. This implies that the SA in the IC is dependent on inputs of the DCN.

Significance of Time-Course Changes

To date, there are limited investigations into the time-course of changes that occur in the auditory pathway after NIHL. This is a novel time-course investigation into the changes of neuronal excitability and SA in the contralateral AC, IC and ipsilateral DCN in the same animals after NIHL. Fig. 3.25 provides a summary of the electrophysiological results from the AC, IC and DCN over the time-course.

The IC and DCN responded to NIHL similarly over time. This is most likely due to their extensive connections and the dependence of the IC on DCN inputs. After the initial decrease of stimulus-driven activity, both regions maintained low levels of activity followed by a slight increase at the end of the time-course. In addition, the neuronal population that exhibited MON$^{\text{SAT}}$ responses were responding to lower thresholds. This could suggest that neuronal populations that exhibit the MON$^{\text{SAT}}$ responses become
more sensitive to noise stimuli in order to increase stimulus-driven activity after NIHL. This increased sensitivity may have resulted in the slight increase of stimulus-driven activity that was observed. The increase observed in the DCN cannot be explained by an increased sensitivity of the neuronal populations exhibiting the MON\textsuperscript{SAT} responses, as MON\textsuperscript{SAT} responses were not identified in the DCN. However, it is clear that the neuronal population exhibiting MON\textsuperscript{N/SAT} responses in the DCN increased in sensitivity.

The SA remained at control levels after NIHL and only started to increase at Day 8. It then peaked at Day 16 and continued through to Day 32, which is supported by previous investigations that also show a delayed increase of SA in the IC and DCN (Kaltenbach et al., 1998, Brozoski et al., 2002, Dong et al., 2009a, Finlayson and Kaltenbach, 2009, Mulders and Robertson, 2009, Dong et al., 2010a, Mulders et al., 2011, Pilati et al., 2012). In the IC, the MON\textsuperscript{SAT} responses re-emerged at Day 8 and the NON MON responses were also identified in the Day 16 animals. The levels of stimulus-driven activity and SA over time could indicate that a compensatory mechanism occurs to increase excitability. This supports Schaette and Kempter’s model (2006) that a compensatory mechanism would trigger increases of activity in the auditory pathway and the increases in SA would elevate above normal levels to compensate for lost stimulus-driven activity (Schaette and Kempter, 2006). This delayed increase in SA may be responsible for the delayed development of the chronic tinnitus spectrum, which was observed in Chapter 5 of this thesis. This suggests that the changes of excitability and SA and the IC and DCN may be responsible for the behavioural manifestations of chronic tinnitus.

The excitability and SA varied between the three regions of the AC after NIHL. After NIHL, the low frequency region exhibited a significant decrease in stimulus-driven activity that started to increase at Day 16 (compared to Day 8) and continued to Day 32,
which was similar to the findings in the IC and DCN. The slight increase observed at Day 16, when compared to Day 8, was accompanied by the re-emergence of $\text{MON}^{\text{SAT}}$ responses. This could suggest that the re-emergence of $\text{MON}^{\text{SAT}}$ responses indicate some form of hearing recovery or partial recovery of the auditory system. In addition, the neuronal population that exhibited $\text{MON}^{\text{SAT}}$ responses were responding to lower thresholds. This suggests that $\text{MON}^{\text{SAT}}$ responses become more sensitive to noise stimuli in order to increase stimulus-driven activity after NIHL. This increased sensitivity may have resulted in the slight increase of stimulus-driven activity that was observed. However, the SA did not increase gradually over time, like in the IC and DCN. This could suggest that the compensatory mechanism proposed by Schaette and Kempter (2006) may occur in the low frequency region of the AC before it occurs in the IC and DCN. This might also suggest that there is a top-down effect from the low frequency region to the IC and DCN, which leads to the development of SA after NIHL. In addition, this early increase in SA in the low frequency region of the AC may contribute to the immediate behavioural manifestations of tinnitus, which were observed in Chapter 5 of this thesis and in individuals who reported experiencing tinnitus immediately after acoustic trauma (Widen and Erlandsson, 2004, Shargorodsky et al., 2010). At Day 16, the SA was at similar levels to that of the control animals and at the same time-point, NON MON responses were present. This could indicate that the auditory system attempts to decrease SA levels by exhibiting an inhibitory influence which may have led to the decrease of SA (when compared to Day 8) and this was possibly confirmed by the presence of NON MON responses. NON MON responses were also present at Day 32; there was also a slight increase in SA, however not a high as the SA levels observed at Day 4 and Day 8.
After NIHL, the excitability and SA in the medium frequency region of the AC only showed an overall decrease over time, with the exception of the stimulus-driven activity increasing compared to control on Day 32. This was most likely due to the deafferentation experienced by this region. However, Schaette and Kempter’s (2006) model predicted that SA would be increased the most in tonotopic regions sustaining the largest decreases of active input. The findings of this study were in disagreement with this prediction. Undoubtedly the medium frequency region was deafferented to some extent, as the acoustic trauma stimulus was centred at 16 kHz. However, the reason for the lack of substantial changes in stimulus-driven activity and SA is uncertain. The re-emergence of MON\textsuperscript{SAT} responses at Day 8 and the presence of NON MON responses at Day 16 did not appear to affect the levels of SA or stimulus-driven activity. However, the neuronal population that exhibited MON\textsuperscript{SAT} responses were responding to lower thresholds. This was a similar finding to the other regions of the AC and the IC. This could suggest that neurons exhibiting MON\textsuperscript{SAT} responses become more sensitive to noise stimuli in order to increase excitability after NIHL. This increased sensitivity may have resulted in the slight increase of stimulus-driven activity that was observed in all regions of the AC and in the IC. In addition, NON MON responses were identified and may suggest a mechanism to inhibit the increase in SA. The increase observed in the stimulus-driven activity at Day 32 could suggest that the medium frequency region of the AC has a delayed response to NIHL and may play a role in the development of the perception of chronic tinnitus frequencies.

The high frequency region of the AC was the only region that showed an increase in stimulus-driven activity immediately after NIHL. This indicated that the neurons within the high frequency region become more excitable, whereas the other regions become less excitable. This may signify that the neurons experienced a loss of surround
inhibition, which increased its excitability compared to the other regions. Consequently, the high frequency region of the AC may be the first region where acoustic disorders, such as hyperacusis and tinnitus, are generated after NIHL. In addition, the neuronal populations that exhibited MON\textsuperscript{SAT} responses were responding to lower thresholds. This could suggest that remaining functional neurons become more sensitive to noise stimuli in order to increase stimulus-driven activity after NIHL. This increased sensitivity may have resulted in the slight increase of stimulus-driven activity that was observed. When comparing the levels of SA to the level of stimulus-driven activity, a complementary relationship was observed. Immediately after NIHL there was a decrease in SA, and decrease was also evident at Day 4 and Day 8. This could be a result of the loss of input from the hearing loss or may be due to the decreases of stimulus-driven activity and SA of auditory nerve fibres after acoustic trauma (Liberman and Kiang, 1978, Rajan et al., 1993, Norena et al., 2002, Noreña and Eggermont, 2003, Noreña et al., 2003). However, at Day 16 a significant increase in SA was observed. This increase occurred around the same time that the stimulus-driven activity was at control levels. This could suggest an over-compensatory mechanism in the high frequency region of the AC at Day 16. This could also contribute to the development of the chronic tinnitus spectrum. At Day 32, the SA levels were decreased (compared to Day 16). NON MON responses were identified and could suggest a heightened inhibitory influence in the auditory system.
## Summary of Electrophysiological Results

<table>
<thead>
<tr>
<th></th>
<th>DAY 0</th>
<th>DAY 4</th>
<th>DAY 8</th>
<th>DAY 16</th>
<th>DAY 32</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>↓ SA</td>
<td>↑ RLF (MON N/SAT)</td>
<td>↑ EXCIT</td>
<td>↑ RLF (MON N/SAT)</td>
<td>↑ SA</td>
</tr>
<tr>
<td></td>
<td>↑ EXCIT</td>
<td>↑ RLF (MON N/SAT)</td>
<td>↓ SA</td>
<td>↓ EXCIT</td>
<td>↓ SA</td>
</tr>
<tr>
<td>H</td>
<td>↑ RLF (MON N/SAT)</td>
<td>↑ RLF (MON N/SAT)</td>
<td>↓ SA</td>
<td>↓ EXCIT</td>
<td>↓ SA</td>
</tr>
<tr>
<td></td>
<td>↓ SA</td>
<td>↑ RLF (MON N/SAT)</td>
<td>↑ EXCIT</td>
<td>↑ RLF (MON N/SAT)</td>
<td>↑ SA</td>
</tr>
<tr>
<td>M</td>
<td>↓ SA</td>
<td>↓ EXCIT</td>
<td>↑ RLF (MON N/SAT)</td>
<td>↓ SA</td>
<td>↓ EXCIT</td>
</tr>
<tr>
<td></td>
<td>↑ RLF (MON N/SAT)</td>
<td>↑ RLF (MON N/SAT)</td>
<td>↓ SA</td>
<td>↓ EXCIT</td>
<td>↓ SA</td>
</tr>
<tr>
<td>L</td>
<td>↓ EXCIT</td>
<td>↑ RLF (MON N/SAT)</td>
<td>↑ RLF (MON N/SAT)</td>
<td>↓ EXCIT</td>
<td>↑ SA</td>
</tr>
<tr>
<td></td>
<td>↑ RLF (MON N/SAT)</td>
<td>↑ RLF (MON N/SAT)</td>
<td>↓ SA</td>
<td>↓ EXCIT</td>
<td>↓ SA</td>
</tr>
</tbody>
</table>

**IC**

|       | ↓ EXCIT  | ↑ RLF (MON N/SAT) | ↑ SA    | ↓ EXCIT | ↓ EXCIT |
|       | ↑ RLF (MON N/SAT) | ↑ RLF (MON N/SAT) | ↓ SA    | ↓ EXCIT | ↓ EXCIT |
|       | ↑ RLF (MON N/SAT) | ↑ RLF (MON N/SAT) | ↓ SA    | ↓ EXCIT | ↓ EXCIT |
|       | ↑ RLF (MON N/SAT) | ↑ RLF (MON N/SAT) | ↓ SA    | ↓ EXCIT | ↓ EXCIT |

**DCN**

|       | ↓ EXCIT  | ↑ RLF (MON N/SAT) | ↑ SA    | ↓ EXCIT | ↓ EXCIT |
|       | ↑ RLF (MON N/SAT) | ↑ RLF (MON N/SAT) | ↓ SA    | ↓ EXCIT | ↓ EXCIT |
|       | ↑ RLF (MON N/SAT) | ↑ RLF (MON N/SAT) | ↓ SA    | ↓ EXCIT | ↓ EXCIT |
|       | ↑ RLF (MON N/SAT) | ↑ RLF (MON N/SAT) | ↓ SA    | ↓ EXCIT | ↓ EXCIT |

Figure 3.25: Summary of Electrophysiological results in the three frequency regions of the AC, IC and DCN over 32 days.

In summary, this study provides the first investigation into the 32-day time-course of stimulus-driven activity and SA in the contralateral AC, IC and ipsilateral DCN utilising a rat model of unilateral NIHL. I have investigated the development of these physiological changes and have gained a deeper understanding of the underlying mechanisms of NIHL. I have shown for the first time that NIHL leads to changes in stimulus-driven activity and SA immediately and over time after NIHL in the rat. This study suggests that animals at the Day 8 time-point experience some form of partial recovery after NIHL. Changes in the excitability and SA may correlate with the molecular changes that occur in the auditory pathway after NIHL (Chapter 4), and also the associated behavioural manifestations of tinnitus and hyperacusis-like responses that occur after NIHL (Chapter 5). The resulting changes may contribute to the generation of acoustic disorders. The results of this study suggest that the low frequency region of the AC may be the first auditory site that is involved in the generation of tinnitus, or contributes to the acute tinnitus spectrum, and that the high frequency region of the AC and the IC and DCN are involved in the perception of the chronic tinnitus spectrum.
CHAPTER FOUR

STUDY TWO

Tracking the Expression of Excitatory and Inhibitory Neurotransmission-Related and Neuroplasticity-Related Proteins following NIHL
4.1: INTRODUCTION

Noise exposure causes a range of changes throughout the auditory pathway including an imbalance of excitatory and inhibitory transmitter systems and changes in the expression of neuroplasticity markers (Marianowski et al., 2000b, Milbrandt et al., 2000, Zettel et al., 2003, Alvarado et al., 2005, Tan et al., 2007, Dong et al., 2009a). It is known that intense noise exposure leads to TTS in hearing, but also that in some instances permanent loss occurs and this may be accompanied by auditory disorders such as tinnitus and hyperacusis in humans (Axelsson and Sandh, 1985). This suggests that these molecular changes may be involved in causing auditory disorders, although the mechanisms and site/s of generation have yet to be identified.

This study focused on selected excitatory and inhibitory neurotransmission-related proteins, and on neuroplasticity-related proteins in the auditory pathway up to one month following NIHL. The central nervous system relies on a fine balance of excitatory and inhibitory inputs (Sun et al., 2010) and an imbalance may have significant physiological implications in the auditory system (refer to section 1.3). An imbalance may influence the reorganisation of tonotopic maps and could lead to changes in SA, which has been previously reported after acoustic trauma (Robertson and Irvine, 1989, Kaltenbach et al., 1992, Harrison et al., 1998, Chang et al., 2002, Noreña and Eggermont, 2003, Seki and Eggermont, 2003a, Mulders and Robertson, 2009, Dong et al., 2010a). Many studies have reported changes in expression of excitatory and inhibitory neurotransmission-related proteins and the level of neuroplasticity-related proteins in the auditory pathway after acoustic trauma. Changes have been observed in the NR2A messenger ribo-nucleic acid (mRNA) (Marianowski et al., 2000b), GABA (Milbrandt et al., 2000, Tan et al., 2007), GABA\textsubscript{A}\textsuperscript{1} (Dong et al., 2009a), GAD-67 (Milbrandt et al., 2000, Dong et al.,
2009a), Calb1 (Idrizbegovic et al., 1999), and GAP-43 (Illding et al., 1997). However, the findings of these studies vary significantly from one another. This may be due to the different species used, age of the animals, method of acoustic trauma, method of quantification, and the differing time-points for assessment of the protein changes.

As mentioned previously, many investigations have centred on changes in the central auditory pathway following acoustic exposure. Clearly partial or total hearing loss results in significant changes throughout the auditory pathway that cannot be explained by peripheral loss alone. This statement is supported by the fact that tinnitus persists in patients with acoustic neuroma after transection of the auditory nerve (House and Brackmann, 1981, Matthies and Samii, 1997). The AC, IC, and the DCN have each been investigated as sites where hearing related disorders may be generated after acoustic exposure. The AC has been shown to be vulnerable to acoustic exposure through the development of tonotopic reorganisation (Robertson and Irvine, 1989, Kaltenbach et al., 1992, Harrison et al., 1998, Irvine and Wright, 2005, Izquierdo et al., 2008a). The IC has been reported to be functionally and neurochemically altered after acoustic trauma (Milbrandt et al., 2000, Alvarado et al., 2005, Izquierdo et al., 2008a). The DCN has also been implicated as a possible site for the generation of tinnitus-producing signals owing to its tendency to become hyperactive following exposure to tinnitus inducing agents such as intense sound and cisplatin (Kaltenbach et al., 2005).

This study has attempted to find the possible site of generation of acoustic disorders after NIHL. There are limited investigations into the development of molecular changes over time, the majority of studies sample time-points before and immediately after acoustic exposure, with few investigations systematically sampling time-points after acoustic exposure. This investigation centres on the protein changes in the contralateral and ipsilateral AC, IC and DCN within the same animals following NIHL, with the aim of
determining which brain regions may be involved in the generation of auditory disorders by assessing the relative changes in protein expression over time. Understanding the development of changes that occur after NIHL is of utmost importance for the generation and progress treatments for auditory disorders.
4.2: HYPOTHESIS AND AIMS

4.2.1: HYPOTHESIS

It was hypothesised that NIHL would lead to an increase in excitatory and neuroplasticity marker expression and a decrease in inhibitory marker expression in the AC, IC and DCN over time.

4.2.2: AIMS

My aim was to track the molecular changes that occurred in the central auditory pathway after NIHL over the period of one month, in order to determine if there were detectable fluctuations in excitatory and inhibitory markers.

Accordingly, I tracked the expression of excitatory and inhibitory neurotransmission-related proteins and the expression of neuroplasticity-related proteins in the contralateral and ipsilateral AC, IC, and DCN over 32 days after NIHL.
4.3: RESEARCH METHOD

4.3.1: ANIMALS AND TREATMENT GROUPS

Rats were housed and maintained as previously described in section 2.1. Thirty rats aged between 11-14 weeks (250-350g) were randomly assigned to six groups used to assess the effect of NIHL on the expression of excitatory and inhibitory neurotransmission-related proteins and of neuroplasticity-related proteins in the contralateral and ipsilateral AC, IC and DCN. These groups were a control group (n = 5), Day 0 (n = 5), Day 4 (n = 5), Day 8 (n = 5), Day 16 (n = 5) and Day 32 (n = 5) post-acoustic trauma group. The experimental procedures were approved by the Animal Ethics Committee of the University of Western Sydney (ACEC #6670) and conformed to the Australian code of practice for the care and use of animals for scientific purposes – 7th Edition. The animals used in this study are the same animals used in the behavioural study of this thesis (Study Three: Chapter Five).

4.3.2: ACOUSTIC-trauma AND AUDITORY BRAINSTEM RESPONSE TESTS

Animals were exposed to the acoustic trauma (see section 2.3) and ABR tests (see section 2.4) were performed before and after the acoustic trauma (to confirm whether the rat received adequate NIHL) and once before the western blot experiments (either at 0, 4, 8, 16 or 32 days after NIHL). Statistical analyses were performed as described in section 2.4.1.
4.3.3: Western Blotting for NMDA Receptor Subunit 2A, Calbindin-D-28 kDa, GABA$_\alpha$1 Receptor $\alpha$1 Subunit, Glutamic-Acid Decarboxylase 67 and Growth Associated Protein-43 Protein

The western blot is an analytical technique used to detect specific proteins in a given sample of tissue homogenate. It allowed the effective quantification of molecular marker/protein expression after NIHL in this study.

Rats were anaesthetised according to section 2.2. Five animals from each of the six exposure groups and their respective controls (30 in total) were decapitated at 0, 4, 8, 16 or 32 days after NIHL. Rats were decapitated at the atlas/axis junction. An incision in the scalp was made from the nasal bone to the occipital bone and the scalp was retracted to reveal the skull. Using ronjours, the occipital bone was carefully peeled off to reveal the cerebellum. The skull was removed to an adequate extent for the brain to be extracted. The brain was immediately placed in ice-cold saline solution. The brain was transferred into a plastic weigh boat and covered in foil, and then snap-frozen in liquid nitrogen for 30 seconds. The brain was stored in a -80°C freezer until the experiment proceeded.

At the time of the experiment, the brains were semi-thawed in 3mL of protease inhibitor cocktail (Sigma-Aldrich Inc. Catalogue #8340), and the contralateral and ipsilateral AC, IC and DCN were removed. Regions were identified in accordance with a rat atlas (Paxinos and Watson, 2005). Each tissue sample was placed in 500µl of protease inhibitor cocktail. All tissue samples were kept on ice (4°C) throughout the experiment unless otherwise specified. Tissues were homogenised with protease inhibitor cocktail with a hand held tissue grinder (Pellet Pestle Motor, Kontes, Vineland, NJ). The homogenate was passed through a 27 gauge syringe needle to further shear the tissue. It was then was centrifuged for 10 minutes at 1000 rotations per minute (rpm) and the
supernatant was collected. The supernatant was then centrifuged for 20 minutes at 12000 rpm and the pellet then collected. The pellet was resuspended in protease inhibitor cocktail, 100µL (AC and IC) and 50µL (DCN), and passed through a 30 gauge syringe needle. 5µl was removed from each sample and a protein assay (Bio-Rad, Hercules, CA) was performed to determine the protein concentration per ml. 2x sample buffer was added to each sample; 100µL (AC and IC) and 50µL (DCN). Each well was loaded with 40µg of protein and samples were run for 30 minutes at 60V and 90 minutes at 120V on 10% SDS-PAGE gel. The gel was transferred overnight at 30 mA for a minimum of 17 hours. The gel was transferred onto 0.2µm pore size nitrocellulose membrane (Sigma-Aldrich, St Louis, MO).

The resulting membranes were blocked with 5% skim milk (to prevent non-specific binding) on a rotator for 1 hour. The membrane was probed with a primary antibody multiplex diluted in 3% skim milk for 2 hours, which consisted of GABA_Aα1 (Chemicon, Temecula, CA; Catalogue #06-868) at a concentration of 1:4000, GAD-67 (Santa Cruz Biotechnologies; Catalogue #28376) at a concentration of 1:400, NR2A (Abcam, Cambridge, MA; Catalogue #14596-50) at a concentration of 1:3000, Calb1 (Abcam, Cambridge, MA; Catalogue #9481-500) at a concentration of 1:150, and GAP-43 (Abcam, Cambridge, MA; Catalogue #50608) at a concentration of 1:400. Table 4.1 outlines the antibodies used in this study. The membrane was washed repeatedly and probed with secondary antibodies, goat anti-rabbit (Sigma-Aldrich, St Louis, MO; Catalogue #4914) at a concentration of 1:1000, and goat anti-mouse (Sigma-Aldrich, St Louis, MO; Catalogue #4416) at a concentration of 1:750 for 1 hour. The membrane was incubated for 5 minutes with a Chemiluminescence (CL) substrate, Supersignal West Pico (Pierce, Rockford, IL; Catalogue #34087) and Supersignal West Femto (Pierce, Rockford, IL; Catalogue #34094), when the signal was low. The membranes were
placed on CL exposure films (Pierce, Rockford, IL; Catalogue #34090) for varying times depending on the signal.

**TABLE 4.1: DESCRIPTION OF COMMERCIAL ANTIBODIES USED IN THIS STUDY. LEGEND: RpAb = RABBIT POLYCLONAL ANTIBODY AND MmAb = MOUSE MONOCLONAL ANTIBODY**

<table>
<thead>
<tr>
<th>Target Antigen/ Molecular Weight</th>
<th>Type</th>
<th>Raised Against</th>
<th>Conc. used</th>
<th>Catalogue # and Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA(_{\alpha1})/ 52 kDa</td>
<td>RpAb</td>
<td>A synthetic peptide derived from human GABA(_{\alpha1}) protein</td>
<td>1:4000</td>
<td>AB65269 Abcam Ltd</td>
</tr>
<tr>
<td>GAP-43/ 43 kDa</td>
<td>RpAb</td>
<td>HPLC purified GAP-43 from neonatal rat forebrain membranes</td>
<td>1:400</td>
<td>AB50608 Abcam Ltd</td>
</tr>
<tr>
<td>NR2A / 170 kDa</td>
<td>RpAb</td>
<td>Fusion protein of mouse origin corresponding to the major cytoplasmic domain of the NR2A protein</td>
<td>1:3000</td>
<td>AB14596 Abcam Ltd</td>
</tr>
<tr>
<td>Calb1/ 28 kDa</td>
<td>MmAb</td>
<td>Purified Calb1 from chicken gut</td>
<td>1:200</td>
<td>AB9481 Abcam Ltd</td>
</tr>
<tr>
<td>GAD-67/ 67 kDa</td>
<td>MmAb</td>
<td>Amino acids 1-101 of GAD-67 of human origin</td>
<td>1:500</td>
<td>SC-28376 Santa Cruz Inc</td>
</tr>
</tbody>
</table>

The films were processed in an x-ray film processor (Agfa CP-1000). The films were scanned in a LAS-4000 imaging system (Fujifilm Life Science, USA) and images were digitised (Fig. 4.1). The images were analysed using Multi-Gauge Analysis Software Version 2.0 (Fujifilm Life Science, USA), which quantified the level of density in each band selected compared to the background. Deep Purple Total Protein Stain (GE Healthcare Life Sciences; Catalogue #RPN6305) was used to standardise results.
Figure 4.1: Example of Digitised Image of CL exposed X-ray film. Lanes were (from left to right); control (SHAM), Day 0, Day 4, Day 8, Day 16, Day 32 and two controls (non-auditory brain tissue). The numbers on the right indicate the molecular weight in kDa. The top bands are the NR2A protein (170 kDa), the middle bands are the GAD-67 protein (67 kDa) and the bottom bands are the GABA\textsubscript{A}\textalpha1 (52 kDa).

4.3.3.1: Statistical Analyses

Statistical analyses were performed using SPSS (IBM Corporation, Somers, NY). For multiple comparisons of the western blot data, a one-way ANOVA and Tukey’s post-hoc multiple comparison test were used with a p value of <0.05 considered significant.
4.4: RESULTS

4.4.1: AUDITORY BRAINSTEM RESPONSE AUDIOGRAMS

ABR audiograms indicated significant threshold shifts following NIHL. Figure 4.2A-F presents the mean ABR audiograms of all animals obtained prior to and 0, 4, 8, 16 and 32 days after NIHL. All values in the figures are expressed as mean ± SEM.

The control SHAM trauma group showed no difference at any frequency (Fig. 4.2A), which was expected as the animals were not exposed to the acoustic trauma stimulus. The Day 0 (Fig. 4.2B), Day 4 (Fig. 4.2C), Day 8 (Fig. 4.2D), Day 16 (Fig. 4.2E) and Day 32 (Fig. 4.2F) groups showed significant threshold increases across most frequencies. Individual one-way ANOVAs showed that there were differences between time-points in threshold shifts at 1 kHz (F (5, 29) = 5.659, p = 0.001), 2 kHz (F (5, 29) = 7.003, p<0.001), 4 kHz (F (5, 29) = 9.724, p<0.001), 8 kHz (F (5, 29) = 6.768, p<0.001), 16 kHz (F (5, 29) = 9.515, p<0.001) and 32 kHz (F (5, 29) = 12.930, p<0.001).

A Tukey’s post hoc test was used to compare the ABR threshold shifts of the control animals, at each frequency, to the threshold shifts of the other groups. Table 4.2 presents the threshold shifts in dB SPL/frequency/group with p values. The ABR audiograms of the contralateral ear are presented in Table 4.3 and indicated no change in threshold shifts. The lesioned (ipsilateral) ear indicated threshold shifts immediately after NIHL; this was also observed in Chapter 3 of this thesis (section 3.4.1: Fig. 3.5B). These increases were observed in the Day 4 group, which was also similar to the Chapter 3 study (section 3.4.1: Fig. 3.5C). In the Day 8 group, the high frequencies were elevated (16 kHz and 32 kHz), whilst the lower frequencies were not significantly different from the pre-lesion values due to the high variability. In the Day 16 group, most threshold shifts were increased, except for 1 kHz. In the Day 32 group, threshold shifts across all frequencies were increased, similar to the Day 0 and 4 groups.
Figure 4.2: ABR Audiograms of absolute thresholds of ipsilateral (lesioned) ear. A one-way ANOVA with a Tukey’s test was used to comparing pre-lesion recordings to: (A) post-lesion (SHAM), (B) 0 days post-lesion, (C) 4 days post-lesion, (D) 8 days post-lesion, (E) 16 days post-lesion, and (F) 32 days post-lesion recordings. Results are presented as the mean ± SEM (SPL (dB)), *p<0.05.
TABLE 4.2: POST ACOUSTIC TRAUMA THRESHOLD SHIFTS IN ALL EXPOSURE GROUPS (IPSILATERAL/LESIONED EAR) (N = 5/GROUP). A ONE-WAY ANOVA WITH A TUKEY’S TEST WERE USED TO COMPARE THE CONTROL THRESHOLD SHIFTS TO THE DIFFERENT TIME-POINTS. SIGNIFICANT P VALUES ARE IN BOLD.

<table>
<thead>
<tr>
<th>Group</th>
<th>Frequency</th>
<th>1 kHz</th>
<th>2 kHz</th>
<th>4 kHz</th>
<th>8 kHz</th>
<th>16 kHz</th>
<th>32 kHz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control  (SHAM)</td>
<td></td>
<td>1 dB</td>
<td>3 dB</td>
<td>0 dB</td>
<td>2 dB</td>
<td>1 dB</td>
<td>2 dB</td>
</tr>
<tr>
<td>Day 0</td>
<td></td>
<td>50 dB</td>
<td>53 dB</td>
<td>53 dB</td>
<td>64 dB</td>
<td>73 dB</td>
<td>65 dB</td>
</tr>
<tr>
<td>Day 4</td>
<td></td>
<td>41 dB</td>
<td>47 dB</td>
<td>50 dB</td>
<td>53 dB</td>
<td>60 dB</td>
<td>62 dB</td>
</tr>
<tr>
<td>Day 8</td>
<td></td>
<td>17 dB</td>
<td>17 dB</td>
<td>18 dB</td>
<td>37 dB</td>
<td>52 dB</td>
<td>39 dB</td>
</tr>
<tr>
<td>Day 16</td>
<td></td>
<td>36 dB</td>
<td>39 dB</td>
<td>36 dB</td>
<td>52 dB</td>
<td>65 dB</td>
<td>67 dB</td>
</tr>
<tr>
<td>Day 32</td>
<td></td>
<td>47 dB</td>
<td>54 dB</td>
<td>55 dB</td>
<td>55 dB</td>
<td>65 dB</td>
<td>71 dB</td>
</tr>
</tbody>
</table>

TABLE 4.3: POST ACOUSTIC TRAUMA THRESHOLD SHIFTS IN ALL EXPOSURE GROUPS (CONTRALATERAL/NON-LESIONED EAR) (N = 5/GROUP). A ONE-WAY ANOVA WITH A TUKEY’S TEST WERE USED TO COMPARE THE CONTROL THRESHOLD SHIFTS TO THE DIFFERENT TIME-POINTS. SIGNIFICANT P VALUES ARE IN BOLD.

<table>
<thead>
<tr>
<th>Group</th>
<th>Frequency</th>
<th>1 kHz</th>
<th>2 kHz</th>
<th>4 kHz</th>
<th>8 kHz</th>
<th>16 kHz</th>
<th>32 kHz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control  (SHAM)</td>
<td></td>
<td>5 dB</td>
<td>2 dB</td>
<td>2 dB</td>
<td>2 dB</td>
<td>6 dB</td>
<td>1 dB</td>
</tr>
<tr>
<td>Day 0</td>
<td></td>
<td>-3 dB</td>
<td>-1 dB</td>
<td>3 dB</td>
<td>3 dB</td>
<td>2 dB</td>
<td>0 dB</td>
</tr>
<tr>
<td>Day 4</td>
<td></td>
<td>-4 dB</td>
<td>-3 dB</td>
<td>-7 dB</td>
<td>-5 dB</td>
<td>-3 dB</td>
<td>-3 dB</td>
</tr>
<tr>
<td>Day 8</td>
<td></td>
<td>-13 dB</td>
<td>9 dB</td>
<td>3 dB</td>
<td>1 dB</td>
<td>7 dB</td>
<td>-2 dB</td>
</tr>
<tr>
<td>Day 16</td>
<td></td>
<td>-1 dB</td>
<td>0 dB</td>
<td>1 dB</td>
<td>1 dB</td>
<td>2 dB</td>
<td>-1 dB</td>
</tr>
<tr>
<td>Day 32</td>
<td></td>
<td>-4 dB</td>
<td>-4 dB</td>
<td>0 dB</td>
<td>-2 dB</td>
<td>-1 dB</td>
<td>-3 dB</td>
</tr>
</tbody>
</table>
4.4.2: PROTEIN EXPRESSION IN DOMINANT AUDITORY PATHWAY

(CONTRALATERAL AC, CONTRALATERAL IC AND IPSILATERAL DCN)

Figure 4.3 shows the pattern of protein expression in contralateral AC, contralateral IC
and ipsilateral DCN, which is the dominant auditory pathway with respect to the
lesioned ear. Tukey’s post-hoc analysis showed statistically significant changes in the
dominant auditory pathway compared with the control animals and between time-points
and these are summarised in the following sections. Figure 4.5 summarises the
significant changes in protein expression in the dominant and non dominant auditory
pathway.

4.4.2.1: EXCITATORY NEUROTRANSMISSION RELATED PROTEINS

Contralateral AC (Fig. 4.3A)

The one-way ANOVA determined that there was a significant difference in the overall
expression of NR2A between time-points, $F(5, 25) = 2.983, p = 0.036$. However, the
Tukey’s test did not show any specific differences between

time-points. There was no

significant difference in Calb1 expression between time-points, $F(5, 26) = 0.863, p =

0.522$.

Contralateral IC (Fig. 4.3B)

The one-way ANOVA showed that there was a significant difference in NR2A
expression between time-points, $F(5, 27) = 4.143, p = 0.008$. The Tukey’s test
determined that the expression was decreased by 44% at Day 32 when compared to Day

0 ($p = 0.02$). There was no significant difference in Calb1 expression between time-

points, $F(5, 27) = 1.993, p = 0.124$. 
Ipsilateral DCN (Fig. 4.3C)

No significant differences were observed in NR2A expression between time-points, F (5, 27) = 0.429, p = 0.823. The one-way ANOVA determined that there was a significant difference in Calb1 expression between time-points, F (5, 27) = 4.508, p = 0.007. The Tukey’s test showed that the expression was decreased by 50% at Day 0 compared to control (p = 0.026) and expression was increased by 118% at Day 32, when compared to Day 0 (p = 0.012).

4.4.2.2: Inhibitory Neurotransmission Related Proteins

Contralateral AC (Fig. 4.3A)

The one-way ANOVA determined that GABAₐ₁ expression was significantly different between time-points, F (5, 27) = 4.154, p = 0.008. The Tukey’s test showed that expression was decreased by 44% at Day 16 (p = 0.016) and by 51% at Day 32 (p = 0.009) compared to controls. There was no significant difference in GAD-67 expression between time-points, F (5, 27) = 2.279, p = 0.082.

Contralateral IC (Fig. 4.3B)

The one-way ANOVA determined that there was a significant difference in GABAₐ₁ expression between time-points, F (5, 27) = 2.798, p = 0.045. However, the Tukey’s test did not determine specific differences between time-points. The one-way ANOVA showed that GAD-67 expression was significantly different between time-points, F (5, 27) = 2.914, p = 0.039. The Tukey’s test determined that the expression was decreased by 45% at Day 16 when compared to Day 0 (p = 0.030).
Ipsilateral DCN (Fig. 4.3C)

A significant difference in GABA_α1 expression between time-points was determined by the one-way ANOVA, \( F(5, 27) = 2.886, p = 0.04 \). However, the Tukey’s test did not show specific differences between the time-points. The one-way ANOVA determined that there was a significant difference in GAD-67 expression between time-points, \( F(5, 27) = 3.573, p = 0.016 \). The Tukey’s test showed that the expression was decreased at Day 4 when compared to control (\( p = 0.038 \)).

4.4.2.3: Neuroplasticity related proteins

Contralateral AC

After NIHL, there was no significant difference in GAP-43 expression between time-points, \( F(5, 27) = 1.570, p = 0.216 \).

Contralateral IC

The one-way ANOVA determined that there was no significant difference in GAP-43 expression between time-points, \( F(5, 27) = 1.838, p = 0.153 \).

Ipsilateral DCN

The one-way ANOVA showed that there was no significant difference in GAP-43 expression between time-points, \( F(5, 27) = 0.154, p = 0.976 \).
Figure 4.3: Expression of proteins of interest after NIHL in dominant auditory pathway: (A) contralateral AC, (B) contralateral IC and (C) ipsilateral DCN. A one-way ANOVA with a Tukey’s test was used to compare control levels to each time-point. Results presented are the mean ± SEM of density units expressed as a % compared to controls. NOTE: only protein expression that reached statistical significance (full lines) or indicates trends (dotted lines) are plotted on the graphs, *p<0.05
4.4.3: Protein Expression in Non-Dominant Auditory Pathway
(Ipsilateral AC, Ipsilateral IC and Contralateral DCN)

Figure 4.4 shows the pattern of protein expression in ipsilateral AC, ipsilateral IC and contralateral DCN, which is the non-dominant auditory pathway with respect to the lesioned ear. Tukey’s post-hoc analysis showed statistically significant changes in the dominant auditory pathway compared to the control animals and between groups and these are summarised as follows. Figure 4.5 summarises the significant changes in protein expression in the dominant and non dominant auditory pathway.

4.4.3.1: Excitatory Neurotransmission Related Proteins

Ipsilateral AC (Fig. 4.4A)

There was no significant difference in NR2A expression between time-points, $F(5, 27) = 1.120$, $p = 0.385$. The one-way ANOVA determined that there was a significant difference in Calb1 expression between time-points, $F(5, 27) = 3.997$, $p = 0.012$. The Tukey’s test showed that the expression was increased by 117% at Day 16 when compared to Day 0 ($p = 0.047$).

Ipsilateral IC

The one-way ANOVA showed that there was no significant difference in NR2A expression between time-points, $F(5, 27) = 1.472$, $p = 0.239$. The one-way ANOVA determined that there was no significant difference in Calb1 expression between time-points, $F(5, 26) = 1.030$, $p = 0.428$.

Contralateral DCN (Fig. 4.4C)

There was no significant difference in NR2A expression between time-points, $F(5, 25) = 0.705$, $p = 0.626$. The one-way ANOVA determined that there was a significant
difference in Calb1 expression between time-points, \( F (5, 25) = 3.284, p = 0.025 \). The Tukey’s test showed that there was a 45% decrease at Day 8 when compared to Day 0 (\( p = 0.016 \)).

4.4.3.2: Inhibitory Neurotransmission Related Proteins

Ipsilateral AC (Fig. 4.4A)

The one-way ANOVA determined that there was a significant difference in GABA\(_{A}\alpha1\) expression between time-points, \( F (5, 26) = 3.375, p = 0.022 \). The Tukey’s test showed that the expression was decreased by 19% at Day 4 (\( p = 0.034 \)) and 35% at Day 32 (\( p = 0.026 \)) when compared to control. The one-way ANOVA determined that there was a significant difference in GAD-67 expression between time-points, \( F (5, 27) = 3.139, p = 0.03 \). The Tukey’s test showed that the expression was decreased by 39% at Day 0 (\( p = 0.017 \)) when compared to control.

Ipsilateral IC (Fig. 4.4B)

The one-way ANOVA showed that there was no significant difference in GABA\(_{A}\alpha1\) expression between time-points, \( F (5, 26) = 1.402, p = 0.264 \). A significant difference in GAD-67 expression between time-points was determined by the one-way ANOVA, \( F (5, 25) = 3.310, p = 0.024 \). The Tukey’s test showed a 48% decrease in expression at Day 4 when compared to control (\( p = 0.018 \)) and an 86% increase at Day 32 when compared Day 4 (\( p = 0.048 \)).

Contralateral DCN (Fig. 4.4C)

There was no significant difference in GABA\(_{A}\alpha1\) expression between time-points, \( F (5, 27) = 1.842, p = 0.158 \). The one-way ANOVA determined that there was a significant difference in GAD-67 expression between time-points, \( F (5, 26) = 3.004, p = 0.034 \). The
Tukey’s test showed that there was a significant decrease in expression at Day 8 (p = 0.048) and Day 32 (p = 0.049) when compared to Day 0.

4.4.3.3: Neuroplasticity Related Proteins

Ipsilateral AC (Fig. 4.4A)

The one-way ANOVA determined that there was a significant difference in GAP-43 expression between time-points, $F (5, 27) = 5.017$, $p = 0.04$. The Tukey’s test determined that the expression was decreased by 58-68% at Day 0 ($p = 0.024$), Day 4 ($p = 0.010$), Day 8 ($p = 0.007$) and Day 16 ($p = 0.019$), when compared to control.

Ipsilateral IC

There was no significant difference in GAP-43 expression between time-points, $F (5, 27) = 0.089$, $p = 0.993$.

Contralateral DCN

The one-way ANOVA determined that there was no significant difference in GAP-43 expression between time-points, $F (5, 25) = 0.320$, $p = 0.893$. 

Figure 4.4: Expression of proteins of interest after NIHL in non-dominant auditory pathway: (A) ipsilateral AC, (B) ipsilateral IC and (C) contralateral DCN. A one-way ANOVA with a Tukey’s test was used to compare control levels to each time-point. Results presented are the mean ± SEM of density units expressed as a % compared to controls. NOTE: only protein expression that reached statistical significance are plotted on the graphs, *p<0.05.
<table>
<thead>
<tr>
<th></th>
<th>DAY 0</th>
<th>DAY 4</th>
<th>DAY 8</th>
<th>DAY 16</th>
<th>DAY 32</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>Dom.</td>
<td></td>
<td></td>
<td>↓ GABA&lt;sub&gt;A&lt;/sub&gt;R</td>
<td>↓ GABA&lt;sub&gt;A&lt;/sub&gt;R</td>
</tr>
<tr>
<td></td>
<td>N/Dom.</td>
<td>↓ GAD-67</td>
<td>↓ GABA&lt;sub&gt;A&lt;/sub&gt;R</td>
<td>↓ GAP-43</td>
<td>↓ GABA&lt;sub&gt;A&lt;/sub&gt;R</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↓ GAP-43</td>
<td>↓ GAP-43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC</td>
<td>Dom.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N/Dom.</td>
<td></td>
<td>↓ GAD-67</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCN</td>
<td>Dom.</td>
<td>↓ CALB</td>
<td>↓ GAD-67</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N/Dom.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.5: Summary of significant molecular changes in the dominant (Dom.) and non-dominant (N/Dom) AC, IC and DCN.
4.5: DISCUSSION

I have quantified the protein expression of GABA\textsubscript{A}α1, GAD-67, NR2A, Calb1 and GAP-43 in the ipsilateral and contralateral AC, IC and DCN of the young adult rat up to 32 days following NIHL. I have demonstrated that NIHL significantly alters the normal levels of excitatory and inhibitory neurotransmission-related proteins and neuroplasticity-related proteins in the auditory pathway of the rat over time. These findings suggest that these changes in protein expression may serve as part of the molecular mechanism underlying significant physiological changes that lead to acoustic disorders following NIHL.

The Effect of NIHL on Hearing Thresholds

As observed in the previous chapter, the hearing loss produced by the stimulus used in this study can be characterised by significant threshold shifts across all frequencies and all time-points. The threshold shifts ranged from 49-74 dB at Day 0, 46-67 dB at Day 4, 18-48 dB at Day 8, 31-65 dB at Day 16 and 47-67 dB at Day 32. It is interesting to note the gradual reduction of hearing thresholds over the first eight days. The observation in the Day 8 group suggests that some hearing function had recovered after NIHL. However, this recovery was not sustained at the later time-points (Day 16 and Day 32 groups) as the hearing thresholds began to increase to levels similar to that of the immediate threshold increases of the Day 0 group. This was not seen in Chapter 3. Although the animals were exposed to the same acoustic trauma stimulus, the animals in Chapter 3 did not have increased threshold shifts across all frequencies at the Day 32 time-point. Instead, the animals had significant threshold increases at the higher frequencies, which were expected due to the frequency of the acoustic trauma stimulus used in this study. This finding highlights the variability of the NIHL used in this study.
The acoustic trauma stimulus used caused a significant increase in hearing thresholds for the duration of this study. Others who have used a similar acoustic trauma treatment have shown mixed results. Turner and colleagues used the same strain of rats and reported that after 4 months recovery thresholds remained elevated for all frequencies tested (except 10 kHz and 32 kHz) (Turner et al., 2006). Additionally, Bauer (2003) reported that threshold shifts remained elevated 7 months after being exposed to a 16 kHz octave band noise at 105 dB for 1 hour (Bauer, 2003). In contrast, Wang and Caspary (2009), using Fisher Brown Norway rats, observed a full recovery by 16 weeks post-exposure (Wang et al., 2009). It should be noted that the maximum hearing loss in the present study occurred at 16 kHz and 32 kHz and at those frequencies the losses were greater than those of comparable studies (Bauer, 2003, Turner et al., 2006, Wang et al., 2009).

The initial threshold shifts may be a result of peripheral damage to the auditory system. The immediate changes that occur in IHCs and OHCs of the cochlea are quite variable (see (Nordmann et al., 2000) for extensive review). It is known that IHCs and OHCs cannot regenerate in humans and most mammals (Edge and Chen, 2008). The observation of the Day 8 group recovery may suggest a combination of central and peripheral events playing a role in the temporary recovery of hearing function after NIHL. It is not in disagreement with findings which suggest that peripheral recovery can occur, followed by central degenerative changes (Kim et al., 2004a, Lin et al., 2011) or changes in excitatory transmission (Muly et al., 2004).

The Effects of NIHL on Protein Expression

Western blot data demonstrated that all proteins of interest were expressed in the contralateral and ipsilateral AC, IC and DCN of the adult Long Evans rat. Fluctuations in the expression of excitatory and inhibitory neurotransmission-related proteins may
play a role in excitotoxicity, decreased neuronal survival, and the overall decrease of inhibitory neurotransmission. A significant decrease in neuroplasticity-related proteins may play a role in reorganisation of tonotopic maps, which has been shown to occur after acoustic trauma (Robertson and Irvine, 1989, Kaltenbach et al., 1992, Seki and Eggermont, 2003a, Izquierdo et al., 2008a). These changes could ultimately lead to the development of acoustic disorders, such as tinnitus and hyperacusis.

The Effect of NIHL on Excitatory Neurotransmission-Related Proteins

In this study, it was found that the expression of the CBP, Calb1, decreased after NIHL in the ipsilateral DCN and AC, and returned to normal levels over time. It has been postulated that Calb1 has a neuroprotective effect against excitotoxic damage caused by disruption of intracellular calcium homeostasis (Mattson et al., 1991, Iacopino et al., 1992). A possible reason for the decrease in Calb1 could be that the production of Calb1 was disrupted and therefore its lack of expression could be responsible for excitotoxic damage occurs in the auditory system after NIHL. Excitotoxicity in the cochlea and central auditory pathway has been researched extensively and is postulated to be a major contributor to the development of acoustic disorders (Pujol and Puel, 1999, Muly et al., 2004). Indeed, Muly and colleagues have reported evidence of excitotoxic damage following unilateral acoustic trauma in the chinchilla (Muly et al., 2004). In excitotoxicity, elevations of Ca\(^{2+}\) progress to an irreversible loss of Ca\(^{2+}\) homeostasis and neuronal death. In this study, a reduction of Calb1 in the ipsilateral AC and DCN could suggest that a dysfunction had occurred in the production of Calb1 that would affect its function as a cytoplasmic calcium buffer, thereby no longer being able to protect cells against excitotoxic damage. However, it is known that there are other CBPs such as Calretinin and Parvalbumin, which carry out similar functions to Calb1 (Heizmann, 1992). Thus it would be important to investigate the changes of other CBPs after NIHL.
as Calb1 levels were not increased. It has been shown that Calretinin and Parvalbumin protein levels increase in mice that were exposed to repeated periods of noise trauma (Arun, 2007). The immediate decrease of Calb1 in the ipsilateral DCN and AC that was observed in this investigation has not been shown in other studies. The expression of Calb1, in the contralateral DCN, slightly increased immediately after NIHL but returned to control levels over time. These contralateral DCN findings support previous studies that have found that Calb1 increases in the DCN immediately after acoustic trauma (Idrizbegovic et al., 1998, Forster and Illing, 2000).

The other excitatory neurotransmission-related protein that was investigated was NR2A. The expression of NR2A protein had a tendency to decrease over the month following the trauma in the contralateral IC. NMDA receptors containing the NR2A subunit display fast kinetics, and NR2A is the most widespread NMDA receptor subunit in the mature brain (Monyer et al., 1994). This decrease could suggest that the auditory system attempts to compensate for increased excitation by down-regulating the receptors. These results support previous research by Marionowski and colleagues (2000) who found a decrease in the expression of NR2A mRNA after ototoxic exposure in the IC and CN of young rats (Marianowski et al., 2000b). A decrease in the expression of NR2A in the contralateral IC could also suggest a reduction in neuronal survival and subsequently neuronal death, because injury induced activation of NR2A containing NMDA receptors functions as a pro-survival signal (DeRidder et al., 2006). A decrease in neuronal survival after acoustic trauma has been shown in numerous studies. An increase of apoptotic markers was observed in the CN after severe acoustic trauma (Coordes et al., 2011). Other studies have observed an increased in cell death and degeneration after acoustic trauma in the CN (Watanabe et al., 1992, Groschel et al.,
The protein levels of the CBP, Calb1, suggest that there may be fluctuations of excitation in central auditory structures after NIHL. The slight decrease observed in the protein levels of NR2A suggests that it may not have a direct effect on excitatory changes, but may have an effect on neuronal survival of auditory neurons.

The Effect of NIHL on Inhibitory Neurotransmission-Related Proteins

This is the first study that has investigated the effect of NIHL on the expression of $\text{GABA}_A\alpha_1$ in the ipsilateral and contralateral AC. After NIHL, the expression of $\text{GABA}_A\alpha_1$ decreased over time, firstly in the ipsilateral AC and followed by the contralateral AC. Previous studies have shown that the expression of $\text{GABA}_A\alpha_1$ also decreases in other regions of the auditory pathway, such as the IC and DCN (Dong et al., 2009a, Dong et al., 2010a). This down-regulation could result in a significant decrease in inhibition in these auditory areas on both ipsilateral and contralateral sides. A consequence of the reduction of inhibitory processes is that increased excitation may occur in the AC after NIHL, resulting in an altered pattern of SA in the AC as shown in Chapter 3. Indeed, changes in SA and neuronal excitability have been observed across the auditory pathway after acoustic trauma, specifically in DCN (Kaltenbach et al., 2005) (Chang et al., 2002), the IC (Dong et al., 2009a, Mulders and Robertson, 2009, Dong et al., 2010a) and the AC (Noreña and Eggermont, 2003, Seki and Eggermont, 2003a).

After unilateral NIHL, the expression of GAD-67 increased slightly in the contralateral IC and DCN, followed by a return to normal levels over time. A decrease was observed immediately in the ipsilateral AC and within the first week in the ipsilateral IC, however
the levels of GAD-67 in both regions returned to normal levels over time. Slight fluctuations were observed in the ipsilateral DCN followed by a return to control levels over time. GAD-67 is an enzyme that converts intracellular glutamate into the neurotransmitter GABA (Erlander et al., 1991). A decreased expression of GAD-67 may lead to reduced levels of GABA, which has been previously observed after acoustic trauma (Yang et al., 1998, Milbrandt et al., 2000), and therefore can ultimately lead to an overall reduction of inhibition. The change in the expression of GAD-67 was the most widespread finding in this study, and suggests that a disruption in inhibitory transmission plays an important role in the development of acoustic disorders.

The decreases observed in the protein levels of the GABAₐ₁ receptor subunit, and the GABA synthesis enzyme GAD-67, supports the theory of Dong and colleagues (2010) that both pre- and post-synaptic mechanisms contribute to deafness-related loss of inhibition (Dong et al., 2010b). The possibility that presynaptic GABA synthesis may diminish in the auditory pathway after ipsilateral partial deafness is supported by a study showing a loss of GABA positive neurons in the CN of aged rats with hearing loss (Yang et al., 1998) and decreased GABA release in the IC (Milbrandt et al., 2000). It has been demonstrated that GABA release and binding, as well as GAD-67 expression, are down-regulated in the IC after hearing loss (Suneja et al., 1998b, Caspary et al., 1999, Mossop et al., 2000). The down-regulation of GABAₐ₁ and GAD-67 would most likely result in an overall decrease in neuronal inhibition in the auditory pathway. This could lead to increases in SA, neuronal synchrony and excitability, which have been observed across the auditory pathway after acoustic trauma in Study One (Chapter 3) of this thesis and previous studies (Chang et al., 2002, Seki and Eggermont, 2003a, Bauer et al., 2008, Mulders and Robertson, 2009). These changes can lead to prolonged synchronous firing, which has been observed in the AC after acoustic trauma (Noreña
and Eggermont, 2003, Seki and Eggermont, 2003a). This has been shown to result in use-dependent synaptic modifications such as Long Term Potentiation (LTP). LTP is thought to promote the increase of synaptic strength and this may lead to the development of auditory disorders such as tinnitus (Weisz et al., 2007b).

Neuroplasticity-related proteins

The expression of GAP-43 has been shown to increase during heightened levels of neuroplasticity, as it is located in the growth cones of neurons (Meiri et al., 1986). Previous research indicates that GAP-43 also plays a key role in axonal guidance (Benowitz and Perrone-Bizzozero, 1991, Benowitz and Routtenberg, 1997). In addition, GAP-43 (-/-) mice show defects in path finding of retinal ganglion cell axons across the optic chiasm (Strittmatter et al., 1995). Over expression of GAP-43 leads to formation of new synapses and enhanced sprouting after injury whereas reduced sprouting is found with a mutant GAP-43 that cannot be phosphorylated by Protein Kinase C. This control of sprouting implies that GAP-43 is an intrinsic determinant of the neurons growth state. A significant decrease was observed in the overall expression of GAP-43 in the ipsilateral AC. This may have an impact on anatomical reorganisation of tonotopic maps after NIHL, as previous studies suggest that reduced GAP-43 expression may alter the fine-tuning of a cortical map through a combination of path-finding and synaptic plasticity mechanisms (McIlvain et al., 2003).

Significance of Time-Course Changes

To date, there are limited investigations into the time-course of changes that occur in the auditory pathway after NIHL. This is the first study that has investigated changes in the ipsilateral and contralateral AC, IC, and DCN in the same animals over time.

Samples were taken as early as 30 minutes after NIHL (Day 0). At this time-point
significant changes were observed in the expression of Calb1 (within the ipsilateral DCN), in GAD-67 (within the ipsilateral AC), and in GAP-43, (within the ipsilateral AC). This observation indicated that these proteins act immediately after NIHL and may suggest a role in an immediate active fine-tuning process.

Significant changes in both contralateral and ipsilateral DCN involved the expression of GAD-67 and Calb1. Over the time-course, GAD-67 and Calb1 were very closely related, both exhibiting fluctuations at similar time-points. As Calb1 is an indicator of excitation and GAD-67 is involved in synthesising GABA, it may suggest that there is a connection between these proteins that acts to balance excitatory and inhibitory actions in the DCN normally and after an acoustic insult. These fluctuations over time may indicate that the nervous system continually attempts to rebalance the changes that were occurring in response to altered input. For example, when the Calb1 increased, GAD-67 increased in order to produce more GABA and when Calb1 decreased, GAD-67 also decreased due to the reduced demand for GABA. A previous study has shown that neurons in the olfactory bulbs of mice co-express Calb1 and GAD-67 (Parrish-Aungst et al., 2007). However there is no study that has investigated the co-expression of these two proteins over time. It is not known the extent to which the levels of Calb1 and GAD-67 relate to each other over time.

The contralateral AC and IC showed similar significant decreases and near-significant trends in the levels of NR2A, GAD-67 and GABA_α1. This may indicate that the AC and IC contralateral to the lesioned ear respond in a similar fashion. The overall potential of the cells would be excitatory which as previously discussed can lead to LTP and may facilitate synaptic plasticity. It would be beneficial to research into the tonotopic changes of the AC and IC to identify whether these protein changes correlate to cortical reorganisation and increases of SA over time.
The ipsilateral IC experienced a decrease in the expression of GAD-67 within the first week after NIHL. The decrease in the GAD-67 may indicate a malfunction, which would lead to decreased levels of GABA that in turn would result in increased excitation. However, there were no significant changes in the expression of excitatory neurotransmission related proteins in the ipsilateral IC across the time-course. There have been no other reports of the ipsilateral IC changing significantly after acoustic trauma.

Many significant changes were observed in the ipsilateral AC. This included changes in GAD-67, GABA\(\alpha_1\) and GAP-43 expression. It was observed that GAD-67 and GABA\(\alpha_1\) protein levels were closely related over the time-course. This was expected, as both of these proteins work together in maintaining the balance of excitation and inhibition. The decrease in GAP-43 may suggest that the ipsilateral AC experienced maladaptive plastic changes, due to the neuronal path-finding properties of GAP-43. The findings in the ipsilateral AC, however significant, were unexpected as they were occurring in the contralateral auditory pathway (relative to the exposed ear). This may suggest that mechanisms from the contralateral auditory pathway may play a role in functional compensation after NIHL. This has not been shown in the AC, however bilateral molecular changes have been shown in the IC after unilateral cochlea trauma (Dong et al., 2009a).

In summary, this study provides the first 32-day time-course investigation of protein expression of NR2A, Calb1, GABA\(\alpha\)1, GAD-67, and GAP-43 in the contralateral and ipsilateral AC, IC, and DCN utilising a rat model of unilateral NIHL. I have investigated the development of these protein changes in order to gain a deeper understanding of the underlying mechanisms of NIHL. Gaining additional knowledge about the underlying mechanisms of NIHL is crucial for the development of therapeutic intervention.
My findings provide evidence that NIHL significantly affects the balance of excitatory and inhibitory neurotransmission-related proteins and neuroplasticity-related proteins in the rat auditory pathway. These results suggest that the characteristic decrease of inhibition that previous researchers have observed after acoustic trauma may not necessarily be due to significant increases of excitatory neurotransmission-related proteins; rather, a significant decrease of inhibitory-neurotransmission related proteins may have led to an overall increase in excitation. This does not support the original hypothesis.

In addition to confirming changes in excitatory and inhibitory-neurotransmission related protein expression, I have shown for the first time that mechanisms from the contralateral auditory pathway may play a role in functional compensation after NIHL. Changes in the ipsilateral and contralateral AC, IC, and DCN may reflect an attempt to balance excitatory and inhibitory transmission following noise-induced hearing loss. The resulting imbalances may contribute to the generation of acoustic disorders.
CHAPTER FIVE

STUDY THREE

Tracking the Perception of Tinnitus

using the Turner Gap Detection

Method following NIHL
5.1: INTRODUCTION

Research into the perception of tinnitus in humans is complicated by the fact that the experience of tinnitus is subjective. Although humans can report and provide interpretations of their perceptions, a number of variables arise from the bias of reports, the ability to describe their experience, and the individual’s aetiology of tinnitus. Therefore, a highly desirable alternative is utilising an animal model. Several researchers have developed behavioural tests to confirm the presence of tinnitus in animal models; however, these tests involve intense pre-training and produce limited results concerning the frequency spectrum of tinnitus that is perceived. Therefore, it is important to have a behavioural test that is fast and effective in confirming the presence of tinnitus in animals.

In humans, it is understood that the perception of tinnitus is highly variable; patients have reported experiencing differences in frequency and loudness on a daily basis (Penner, 1983, Stouffer and Tyler, 1990, Hiller and Goebel, 2006). It has also been shown that animals experience similar fluctuations in the perception of tinnitus (Longenecker and Galazyuk, 2011). Therefore, it is also important to have a behavioural test that can confirm what frequency is being perceived or what frequencies are contributing to the tinnitus spectrum in the animal. An effective behavioural test will allow a greater understanding of the underlying mechanism/s of tinnitus in animal models by allowing researchers to confirm the presence of tinnitus and to evaluate animal perception.

There are several studies that have investigated the behavioural manifestations of tinnitus in animal models (Jastreboff et al., 1988, Bauer et al., 1999, Heffner and...
Jastreboff and colleagues were the first to develop a behavioural test of tinnitus. Their study was based on a Pavlovian shock-induced lick suppression technique in water-deprived rats (Jastreboff et al., 1988). In their method, water-deprived rats were allowed to lick for water when a background sound was present; however, during randomly presented quiet intervals, a foot shock was given if the animal licked for water. Conditioned animals learned to lick for water when background sound was present and to suppress licking during quiet intervals. After training to standard performance, a tinnitus-inducing treatment, salicylate, was given to the experimental group, but not to the control group. During the post-treatment period, lick suppression was re-measured over five to ten days. Tinnitus was assumed to be present if animals continued to lick for water compared to the control group that suppressed licking due to the lack of background sound (or perception of tinnitus).

Variations were made to the Jastreboff lick suppression technique (Heffner and Harrington, 2002), so that water-deprived animals could avoid foot shock if they cease drinking during the quiet intervals. The presence or absence of tinnitus was assessed by comparing the rate of extinction in experimental compared to control groups. This procedure was used to assess tinnitus in hamsters exposed to high-intensity noise of different durations. Animals exposed for 1 hour or less did not show evidence of tinnitus, whereas those exposed for 2-4 hours exhibited signs of tinnitus. The abovementioned lick suppression techniques were not adapted to long term assessment and could only obtain acute measurements.
Lobarinas and colleagues developed an automated avoidance technique (Lobarinas et al., 2004) that could be used to assess tinnitus in individual animals. The behavioural response did not extinguish, thereby allowing tinnitus to be measured over extended periods of time. The technique combined shock avoidance conditioning with Schedule-Induced Polydipsia (SIP) (Falk and Tang, 1972, Lobarinas and Falk, 1998). Polydipsia, which refers to the excess intake of fluid, is induced by delivering a small amount of food at regularly spaced time intervals to food-restricted animals. Although the animals were not water deprived, the planned delivery of food pellets generated a high rate of licking for water between pellets. The SIP-induced licking was placed under acoustic control using a shock avoidance paradigm. Licking was allowed to occur in quiet, but licking was suppressed in the presence of sound by pairing sound with foot shock. During the quiet interval, salicylate treated animals suppressed their responses just as they were trained to do when a real sound was present, which was interpreted as evidence of tinnitus.

In other experiments, food-restricted rats were trained to press a bar for food in the presence of white noise and to suppress this response during quiet intervals that were paired with foot shock (Bauer et al., 1999). This approach also allowed for the long-term assessment of tinnitus. At random intervals, a test tone was substituted for white noise without shock. When the stimuli were test tones, the salicylate-treated group suppressed less than the control group; the explanation for these results was that the salicylate-treated subjects perceived the tones as ‘noisy’ due to presence of tinnitus and therefore suppressed less than controls. Whilst effective, the major disadvantage of this technique was that the animals needed to be trained for eight weeks before testing.

A behavioural test was needed that did not involve extensive training. Also during this time, the European Union Animal Welfare Policy was amended to include that research
animals required a means of escaping foot-shock. A behavioural technique was published that utilised ‘false positive’ responses occurring during a quiet interval to infer the presence or absence of tinnitus with only one week training required. The approach utilised a shock avoidance conditioning procedure in which rats learned to climb a pole during the presentation of a sound to avoid foot shock. Animals could remain on the cage floor during quiet intervals since the shock was turned off (Guitton et al., 2003). During salicylate treatment, rats climbed the pole (false positive) during quiet periods, which was interpreted as evidence of tinnitus.

Similarly, another approach employed a positive reinforcement technique by associating a fluid reward with the presence of a background sound. Consequently, the liquid feeder would not dispense the reward/liquid when there was an absence of sound (Ruttiger et al., 2003). This approach did not require ‘foot shock’ in the methodology. Salicylate was used to induce temporary tinnitus and significantly increased the rats’ feeder access activity during periods of silence, indicating a tinnitus experience. The auditory perception was estimated to be equivalent to a white noise sound of about 30 dB SPL.

The above methods provide an indication of a tinnitus percept in animals. However, they cannot indicate what frequency spectrum is being perceived, only assessing the one frequency being used. In addition, these previous methods are time consuming in regards to the prior training of the animals. A recent method published by Turner and colleagues, appears capable in identifying the frequency spectrum (able to test more than one frequency per test) of the tinnitus, and is relatively fast and concise, without the need for prior training (Turner et al., 2006).

The Turner Gap Detection method intends to provide rapid tinnitus screening in rats and small animals. Benefits of the gap detection technique include: (a) food or water
deprivation is not necessary; (b) no training, learning, memory, or motivational demands are placed on the animal; (c) the startle neural circuit is well known and its modulation using background sounds has been studied extensively; and (d) testing can be done quickly in a single 40-min session, allowing rapid assessment of acute manipulations.

The test is based on the ability of the acoustic startle reflex to be reduced by a preceding signal/stimulus, in this case a silent gap in an otherwise constant acoustic background. Gaps and other pre-startle stimuli (pre-pulse inhibition) have been previously used in laboratory animals and humans as an audiometric tool to quantify features of the acoustic environment that a subject can detect (Fitch et al., 2008, Li et al., 2009). More salient pre-startle stimuli, including gaps, inhibit the startle response to a greater degree than less salient stimuli (Swerdlow et al., 2004). The main hypothesis of Turner’s study was that when an animal effectively detects a silent gap embedded in an acoustic background, its subsequent response to a startle stimulus would be reduced in its magnitude. When the background sound, in which the gap is embedded, is relatively similar to an animal’s tinnitus, poorer detection of the silent gap should occur. Turner confirmed that rats with independent evidence of tinnitus (validated against food/foot shock study) at 10 kHz, compared with controls, demonstrated difficulty detecting a silent gap in a 10 kHz background. Hence, Turner’s gap detection method appears to be a fast effective test that can provide long-term assessment of animals without extensive pre-training required.

There are two time-course studies that track the changes of gap detection over time (Longenecker and Galazyuk, 2011, Turner et al., 2012). Longenecker and Galazyuk exposed mice to a narrow-band noise centred on 16 kHz (4–22 kHz) at 116 dB (SPL) unilaterally for 1 hour. They tested gap detection on 1, 3, 14, 21, 28, 35, 42, 49, 56 and 84 days after acoustic trauma on the same group of animals. It was shown that 86% of
the mice exhibited deficits in gap detection after being exposed to the acoustic trauma at all of the individual frequencies tested. It was also observed that the tinnitus spectrum of individual mice varied over time, with the majority of the tinnitus-presenting mice showing gap-detection deficits at frequencies higher than 16 kHz (acoustic trauma frequency) over time. Turner and colleagues (2012) exposed mice to a narrow-band noise centred on 16 kHz at 116 dB (SPL) unilaterally for 1 hour. They tested gap detection in the same group of animals on day 1, 3-4 and 7-8, and then weekly for the first 12 weeks, and then monthly until 7 months post-exposure. Turner and colleagues also showed that the tinnitus-presenting mice exhibited gap-detection deficits at frequencies higher than 16 kHz (acoustic trauma frequency) over time (Turner et al., 2012).

There has been no investigation into the time-course of tinnitus perception in rats after NIHL. Although there have been two studies performed on mice, by Longenecker and Galazyuk (2011) and Turner (2012), species differences may be present when compared to the rat. My investigations centre on identifying the specific perception of tinnitus following NIHL, with the aim of understanding the changes in perception of tinnitus and also to confirm that the animal model of NIHL I have utilised can effectively produce tinnitus in rats. If tinnitus can be confirmed in these animals, I may infer that the animals in the western blot (Chapter 4) and the electrophysiological (Chapter 3) studies of this thesis will also develop tinnitus and therefore the results of the other studies may be directly linked to the development of tinnitus. Understanding the development of tinnitus after acoustic exposure is of utmost importance for the development of treatments for tinnitus.
5.2: HYPOTHESIS AND AIMS

5.2.1: HYPOTHESIS

It was hypothesised that following NIHL in rats, behavioural manifestations of tinnitus will be evident and these manifestations will vary across animals and across time.

5.2.2: AIMS

My aim was to assess the time-dependent changes of the tinnitus perception in rats after the NIHL method I have utilised throughout the thesis. This study aims to complement the electrophysiological (Chapter 3) and molecular (Chapter 4) studies of this thesis.
5.3: RESEARCH METHOD

5.3.1: ANIMALS AND TREATMENT GROUPS

Rats were housed and maintained as previously described in section 2.1. Thirty rats were randomly assigned to six groups used to assess the effect of NIHL on the development of the experience of tinnitus. The groups were a control group (n = 5), Day 0 (n = 5), Day 4 (n = 5), Day 8 (n = 5), Day 16 (n = 5) and Day 32 (n = 5) post-acoustic trauma group. The animals used in this study were the same as used in the Molecular Study (Chapter 4). The experimental procedures were approved by the Animal Ethics Committee of the University of Western Sydney (ACEC #6258). The animals used in this study are the same animals used in the molecular study of this thesis (Study Two: Chapter Four).

5.3.2: ACOUSTIC TRAUMA AND AUDITORY BRAINSTEM RESPONSE TESTS

Animals were exposed to the acoustic trauma (see section 2.3) and ABR tests (see section 2.4) were performed before and after the acoustic trauma and once after the gap detection test. Statistical analysis was performed as described in section 2.3.1.

5.3.3: TURNER GAP DETECTION TEST

The methods of this study differ slightly from those of Longenecker and Galazyuk, and Turner; this study behaviourally tested groups of animals at each time-point, rather than one group of animals tested over time at each time-point.

Testing was performed using the gap detection test developed by Turner et al., (2006). Testing was conducted using SD Instruments SR-Lab Startle Response System hardware and software (San Diego Instruments, Inc). The startle response test chambers were
placed in a sound-attenuated room. The animal was awake and placed into a vented Perspex cylinder that rested on a support base connected to an accelerometer. Background noise stimuli were generated with TDT OpenEx software, which also triggered the startle stimulus. The background and startle stimulus was presented through a speaker (CTS Powerline, Piezo Electric, Woburn, MA) located in the ceiling of the testing chamber. Background signals comprised of narrow band noise centred at 4 kHz, 10 kHz, 16 kHz or 24 kHz. The background signals were not randomised over the test conditions, and were presented in the same sequence between groups. The four test conditions were run sequentially, each lasting approximately 10 minutes. Rats remained in the chambers between tests. Test stimuli were calibrated to 60 dB-SPL peak levels with a cloth model rat and a measuring amplifier (Model Type 2610, Brüel & Kjær). With the background noise turned off, the baseline noise levels in the chambers were between 32-40 dB. Each test consisted of 26 trials presented with a 14 seconds variable inter-trial interval (ITI). Each session began with a 3 minute acclimation period followed by two trials consisting of an abrupt startle eliciting noise burst (115 dB SPL, 20 ms duration) which served to habituate the startle response to a more stable base line. Data from the two initial trials were not used in the gap detection analysis, as the responses were typically larger than the remaining trial responses. The remainder of the session consisted of 12 startle-only trials and 12 gap + startle trials, which are identical to startle-only trials, except for the inserted gap. These were presented in a pseudorandom order. Gaps began 100 ms before the startle stimulus, were 50 ms in duration and were shaped with a 0.1 ms rise/fall gate (refer to Fig. 5.1). For the gap detection test, the protocol was intended to provide a 20-30\% reduction in an animals’ startle response amplitude. This would be caused by the preceding gap of silence in an otherwise continuous background sound, similar to other gap detection studies (Turner et al., 2006, Kraus et al., 2010, Longenecker and Galazyuk, 2011) (refer to Fig. 5.2).
Figure 5.1: Schematic Diagram of Turner Gap Detection Protocol.

Figure 5.2: Example of two startle responses in a Gap + Startle trial. Pre-animals are hypothesised to have an attenuated startle when presented with a preceding gap (black line) and Post-animals are hypothesised to have a greater response as the animals are unable to detect the gap (indicating the perception of tinnitus).
5.3.3.1: Statistical Analyses

The maximum startle amplitude (Vmax) was used for analysing the animals startle reflex to gap detection. The highest and lowest Vmax value was removed from each frequency tested (two of ten trials) to reduce variability within an individual animal’s responses (Jeskey and Willott, 2000, Turner et al., 2006, Longenecker and Galazyuk, 2011). The individual Vmax of the gap + startle trials were divided by the average of the startle only trials (Equation 1.) (Longenecker and Galazyuk, 2012).

\[
\frac{V_{\text{max}} (\text{Gap} + \text{Startle Trial})}{\text{Average } V_{\text{max}} (\text{Startle only Trials})} = SIV
\]

Equation 1. (Longenecker and Galazyuk, 2012)

The resulting number was a ratio; this will be referred to as the startle index value (SIV). A ratio of 1 means that the animal does not detect a gap whereas ratio values lower than 1 indicated that the gap has been detected. Longenecker and Galazyuk’s equation is a variation from Turner’s suppression ratio equation (mean response in gap trials/mean responses in startle-only trials). This was done to yield as many ratios as there are gap trials after outliers are removed from an individual animal. The mean and standard error from these ratios are calculated. Statistical analysis was performed using SPSS (IBM Corporation, Somers, NY). Three tests were performed on the SIVs; for all tests, a p value of <0.05 was considered significant.

Firstly, all frequencies (4 kHz, 10 kHz, 16 kHz and 24 kHz) in all of the animals per time-point (n = 5) were pooled and the pre and post-acoustic trauma values were compared using a general linear model (GLM) repeated measures test. If significant differences were present, the Tukey’s post-hoc analysis test was used to determine the
differences between SIVs at each time-point. These tests were performed to observe the overall change in post-acoustic trauma SIVs over time when compared to control and whether the overall pre and post acoustic trauma SIVs differed at each time-point.

Secondly, the SIVs of each frequency were averaged between the five animals. The groups of animals remained separated by time-point. The pre and post SIVs for each frequency were compared in individual groups (time-points) using a GLM repeated measures test and a Tukey’s post hoc test. These tests were performed to evaluate any frequency specific changes in each group (time-point).

Thirdly, the animals were treated as individual cases. The pre and post SIVs were compared to each frequency using a two-way ANOVA. If significant differences were present, pair-wise comparisons were made. These tests were performed to observe if there were any frequency specific changes in each animal.

Correlation coefficient analysis was also performed. The post SIVs and the post ABR thresholds per frequency test were analysed in each group. The SIVs were obtained at background frequencies of 4 kHz, 10 kHz, 16 kHz and 24 kHz, whereas the ABR test tested the animals hearing at 1 kHz, 2 kHz, 4 kHz, 8 kHz, 16 kHz and 32 kHz. The 4 kHz and 16 kHz post SIVs and the ABR absolute thresholds could be paired. Conversely, the 10 kHz and 24 kHz post SIVs did not have an exact ABR frequency match. Thus, the absolute thresholds at 8 kHz and 24 kHz had to be interpolated based on the absolute thresholds of the surrounding frequencies tested. A correlation value near 1 indicated a strong correlation between two variables (post SIV/post ABR hearing threshold) and a correlation value closer to 0 indicated that there was a weak correlation between the two variables. The correlation coefficient analysis was performed using Microsoft Office Excel 2007 (Microsoft Corporation).
5.4: RESULTS

5.4.1: AUDITORY BRAINSTEM RESPONSE AUDIOGRAMS

ABR audiograms indicated significant threshold shifts following NIHL. Figure 5.3A-F presents the mean ABR audiograms of all animals obtained prior to the acoustic trauma and at Day 0 (Fig. 5.3B), Day 4 (Fig. 5.3C), Day 8 (Fig. 5.3D), Day 16 (Fig. 5.3E) and Day 32 (Fig. 5.3F) post-acoustic trauma. All values in the figures are expressed as mean ± SEM.

The control SHAM trauma group showed no significant difference across all frequencies (Fig. 5.3A), which was expected, as the animals were not exposed to the acoustic trauma stimulus. The 0 (Fig. 5.3B), 4 (Fig. 5.3C), 8 (Fig. 5.3D), 16 (Fig. 5.3E) and 32 (Fig. 5.3F) day groups showed a significant threshold increase across most frequencies. The one-way ANOVAs determined that the threshold shifts were significantly different between time-points at 1 kHz (F (5, 29) = 5.659, p = 0.001), 2 kHz were (F (5, 29) = 7.003, p<0.001), 4 kHz (F (5, 29) = 9.724, p<0.001), 8 kHz (F (5, 29) = 6.768, p<0.001), 16 kHz (F (5, 29) = 9.515, p<0.001) and at 24 kHz (F (5, 29) = 12.930, p<0.001).

A Tukey’s post hoc test was used to compare the ABR threshold shifts of the control animals, at each frequency, to the threshold shifts of the other groups. The results of these tests are presented in Table 5.1 and 5.2. The lesioned (ipsilateral) ear indicated significant threshold shifts immediately after NIHL; this was also observed in Chapter 3 of this thesis (see section 3.4.1: Fig. 3.5B). These increases were observed in the Day 4 group, which was also similar to the Chapter 3 study (see section 3.4.1: Fig. 3.5C). In the Day 8 group, the high frequencies were elevated (16 kHz and 32 kHz), whilst the lower frequencies were not significantly different from the pre-lesion values due to the high variability. In the Day 16 group, threshold shifts were increased, except for 1 kHz, which was insignificant due to variability. In the Day 32 group, threshold shifts across all
frequencies were significantly increased, similar to the Day 0 and Day 4 groups. The increase that was observed in the Day 32 group was unique and was not observed in other studies of this thesis. Table 5.1 presents the threshold shifts in dB SPL/frequency/group with p values. The ABR audiograms of the contralateral (non lesioned) ear are presented in Table 5.2 and indicated no significant changes in threshold shifts. It was imperative that the animals’ had one ear with normal hearing, as the gap detection performance would be affected in an animal had hearing loss in both ears. It has been shown that in control animals, blocking one ear results in no difference in gap detection performance compared to both ears unblocked (Turner et al., 2006). The individual ABR audiograms are compared to the gap detection results in section 5.4.2.3.
Figure 5.3: ABR Audiograms of absolute thresholds of ipsilateral (lesioned) ear. A one-way ANOVA and Tukey’s post-hoc multiple comparison test was used to compare the pre-lesion recordings to: (A) post-lesion (SHAM), (B) 0 days post-lesion, (C) 4 days post-lesion, (D) 8 days post-lesion, (E) 16 days post-lesion, and (F) 32 days post-lesion recordings. Results are presented as the mean ± SEM (SPL (dB)), *p<0.05.
TABLE 5.1: POST ACOUSTIC TRAUMA THRESHOLD SHIFTS IN ALL EXPOSURE GROUPS (IPSILATERAL/LESIONED EAR) (N = 5/GROUP). A ONE-WAY ANOVA AND A TUKEY’S TEST WERE USED TO COMPARISON THE PRE-LESION THRESHOLDS TO THE THRESHOLDS OF THE SUBSEQUENT TIME-POINTS. SIGNIFICANT P VALUES ARE IN BOLD.

<table>
<thead>
<tr>
<th>Group</th>
<th>Frequency</th>
<th>1 kHz</th>
<th>2 kHz</th>
<th>4 kHz</th>
<th>8 kHz</th>
<th>16 kHz</th>
<th>32 kHz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (SHAM)</td>
<td>1 dB</td>
<td>3 dB</td>
<td>0 dB</td>
<td>2 dB</td>
<td>1 dB</td>
<td>2 dB</td>
<td>2 dB</td>
</tr>
<tr>
<td></td>
<td>(p = 0.9)</td>
<td>(p = 0.6)</td>
<td>(p = 1.0)</td>
<td>(p = 0.6)</td>
<td>(p = 0.8)</td>
<td>(p = 0.8)</td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>50 dB</td>
<td>53 dB</td>
<td>53 dB</td>
<td>64 dB</td>
<td>73 dB</td>
<td>65 dB</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(p = 0.003)</td>
<td>(p = 0.002)</td>
<td>(p &lt; 0.001)</td>
<td>(p &lt; 0.001)</td>
<td>(p &lt; 0.001)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 4</td>
<td>41 dB</td>
<td>47 dB</td>
<td>50 dB</td>
<td>53 dB</td>
<td>60 dB</td>
<td>62 dB</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(p = 0.019)</td>
<td>(p = 0.007)</td>
<td>(p = 0.001)</td>
<td>(p = 0.004)</td>
<td>(p = 0.001)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 8</td>
<td>17 dB</td>
<td>17 dB</td>
<td>18 dB</td>
<td>37 dB</td>
<td>52 dB</td>
<td>39 dB</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(p = 0.723)</td>
<td>(p = 0.807)</td>
<td>(p = 0.491)</td>
<td>(p = 0.076)</td>
<td>(p = 0.003)</td>
<td>(p = 0.018)</td>
<td></td>
</tr>
<tr>
<td>Day 16</td>
<td>36 dB</td>
<td>39 dB</td>
<td>36 dB</td>
<td>52 dB</td>
<td>65 dB</td>
<td>67 dB</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(p = 0.052)</td>
<td>(p = 0.038)</td>
<td>(p = 0.017)</td>
<td>(p = 0.005)</td>
<td>(p &lt; 0.001)</td>
<td>(p &lt; 0.001)</td>
<td></td>
</tr>
<tr>
<td>Day 32</td>
<td>47 dB</td>
<td>54 dB</td>
<td>55 dB</td>
<td>55 dB</td>
<td>65 dB</td>
<td>71 dB</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(p = 0.006)</td>
<td>(p = 0.002)</td>
<td>(p &lt; 0.001)</td>
<td>(p = 0.002)</td>
<td>(p &lt; 0.001)</td>
<td>(p &lt; 0.001)</td>
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TABLE 5.2: POST ACOUSTIC TRAUMA THRESHOLD SHIFTS IN ALL EXPOSURE GROUPS (CONTRALATERAL/NON-LESIONED EAR) (N = 5/GROUP). A ONE-WAY ANOVA AND A TUKEY’S TEST WERE USED TO COMPARISON THE PRE-LESION THRESHOLDS TO THE THRESHOLDS OF THE SUBSEQUENT TIME-POINTS. SIGNIFICANT P VALUES ARE IN BOLD.

<table>
<thead>
<tr>
<th>Group</th>
<th>Frequency</th>
<th>1 kHz</th>
<th>2 kHz</th>
<th>4 kHz</th>
<th>8 kHz</th>
<th>16 kHz</th>
<th>32 kHz</th>
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<tr>
<td></td>
<td>(p = 0.8)</td>
<td>(p = 0.6)</td>
<td>(p = 1.0)</td>
<td>(p = 0.6)</td>
<td>(p = 0.8)</td>
<td>(p = 1.0)</td>
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<tr>
<td>Day 0</td>
<td>-3 dB</td>
<td>-1 dB</td>
<td>3 dB</td>
<td>3 dB</td>
<td>2 dB</td>
<td>0 dB</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(p = 0.8)</td>
<td>(p = 1.0)</td>
<td>(p = 1.0)</td>
<td>(p = 1.0)</td>
<td>(p = 0.9)</td>
<td>(p = 1.0)</td>
<td></td>
</tr>
<tr>
<td>Day 4</td>
<td>-4 dB</td>
<td>-3 dB</td>
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<td>-5 dB</td>
<td>-3 dB</td>
<td>-3 dB</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(p = 0.7)</td>
<td>(p = 0.9)</td>
<td>(p = 0.5)</td>
<td>(p = 0.6)</td>
<td>(p = 0.3)</td>
<td>(p = 1.0)</td>
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<tr>
<td>Day 8</td>
<td>-13 dB</td>
<td>9 dB</td>
<td>3 dB</td>
<td>1 dB</td>
<td>7 dB</td>
<td>-2 dB</td>
<td></td>
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<tr>
<td></td>
<td>(p = 0.8)</td>
<td>(p = 0.8)</td>
<td>(p = 1.0)</td>
<td>(p = 1.0)</td>
<td>(p = 1.0)</td>
<td>(p = 1.0)</td>
<td></td>
</tr>
<tr>
<td>Day 16</td>
<td>-1 dB</td>
<td>0 dB</td>
<td>1 dB</td>
<td>1 dB</td>
<td>2 dB</td>
<td>-1 dB</td>
<td></td>
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<tr>
<td></td>
<td>(p = 1.0)</td>
<td>(p = 1.0)</td>
<td>(p = 1.0)</td>
<td>(p = 1.0)</td>
<td>(p = 0.9)</td>
<td>(p = 1.0)</td>
<td></td>
</tr>
<tr>
<td>Day 32</td>
<td>-4 dB</td>
<td>-4 dB</td>
<td>0 dB</td>
<td>-2 dB</td>
<td>-1 dB</td>
<td>-3 dB</td>
<td></td>
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<tr>
<td></td>
<td>(p = 0.7)</td>
<td>(p = 0.9)</td>
<td>(p = 1.0)</td>
<td>(p = 0.9)</td>
<td>(p = 0.5)</td>
<td>(p = 1.0)</td>
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5.4.2: GAP DETECTION PERFORMANCE IN CONTROL AND EXPOSED RATS

Comparisons between pre and post acoustic SIVs were used to determine if tinnitus was present in rats after NIHL. I observed that 48% of rats had deficits in gap detection after NIHL. Due to animal variability, the data are presented in three ways; firstly, averaging all frequencies of all animals within one group (Fig. 5.4A-B); secondly, averaging individual frequency responses in each group (Fig. 5.5A-F); and, thirdly, treating each animal as an individual case (Fig. 5.6-5.11).

5.4.2.1: OVERALL GAP DETECTION PERFORMANCE IN CONTROL AND EXPOSED RATS

When consolidating all frequencies of the five animals within each time-point (Fig. 5.4), it was observed that post-acoustic trauma SIVs changed over time compared to the SHAM controls. A repeated measures ANOVA was performed on this data, with group (time-point) as the independent variable and test (pre/post) as the repeated measure.

The average SIV for each time-point shows that as a group, the animals effectively detected the gap before NIHL (Fig. 5.4). Between subject effects analysis indicated that there was a main effect of time across all of the animals, F (5, 114) = 3.264, p = 0.009. The average SIVs for the Day 0 animals (1.110) was significantly higher than control (0.814) (p = 0.019) and Day 32 (0.776) (p = 0.005) animals. There was a significant effect of test (pre/post), F (1, 114) = 5.738, p = 0.018, the overall pre-test values (0.8316) were significantly lower than the overall post-test values (0.9416), indicating that the startle responses were significantly lower in the gap detection test before the acoustic trauma procedure. In addition, there was a significant interaction between the factors test (pre/post) and time, F (5, 114) = 4.384, p = 0.001. This was most likely due to the large increases in SIVs observed in the Day 0 animals (Fig. 5.5B).
The pre SIVs of each time-point ranged between 0.68-0.89, which was an 11-32% reduction in startle response amplitude caused by the preceding gap of silence in an otherwise continuous background sound. The original protocol was to ensure that there was a 20-30% reduction in startle response amplitude. However, due to high animal variability, some animals performed optimally and other animals’ responses were substandard.

Overall, this data suggests that Day 0 animals exhibited worse gap detection when compared to control SHAM animals and that by Day 4 and throughout to Day 32, the gap detection almost returned to control levels. However, pooling the data in such a way may result in significant changes within each group/time-point being overlooked. Therefore, the next set of results presents the changes of individual frequencies per group/time-point.

![Figure 5.4](image_url)

**Figure 5.4:** The Average SIV across all frequencies of the five animals per time-point. This figure presents the Pre- (light grey bars) and Post- (dark grey bars) acoustic trauma SIVs for each time-point. All values in the figures are expressed as mean SIV ± SEM. A repeated measures ANOVA was used to compare the pre/post SIVs between each time-point, *p<0.05.
5.4.2.2: GAP DETECTION PERFORMANCE FOR INDIVIDUAL FREQUENCIES IN EACH GROUP

A repeated measures ANOVA and a Tukey’s post hoc test were performed on this data set, with frequency as the independent variable and test (pre/post) as the repeated measure.

The average SIV for each frequency in the SHAM control group shows that as a group, the animals effectively detected the gap across all frequencies (Fig. 5.5A). Between subject effects analysis indicated that there was no main effect of frequency in the SHAM control group, $F (3, 16) = 0.239, p = 0.868$. Within subjects effects analysis indicated that there was a significant main effect of test (pre/post), $F (1, 16) = 4.988, p = 0.04$; the overall pre-test value (0.8789) was significantly higher than the overall post-test value (0.7485), indicating that the startle responses were significantly lower in the gap detection test after the SHAM lesion procedure. There were no significant interactions between the factors test (pre/post) and frequency, $F (3, 16) = 0.377, p = 0.799$.

The Day 0 group exhibited increases in SIV across all frequencies after NIHL (Fig. 5.5B). Between subject effects analysis indicated that there was no main effect of frequency in the Day 0 group, $F (3, 16) = 0.703, p = 0.564$. Within subjects effects analysis indicated that there was significant main effect of test (pre/post), $F (1, 16) = 2.707, p = 0.035$; the overall post SIV (1.3697) was higher than the overall pre SIV (0.8494), indicating that the startle responses were significantly higher in the gap detection test after the acoustic procedure. There were no significant interactions between the factors test (pre/post) and frequency, $F (3, 16) = 0.243, p = 0.865$. 
The Day 4 group showed no changes in SIV after NIHL across most frequencies (Fig. 5.5C) and was at similar levels to that of the SHAM control animals; however, at 24 kHz a decrease was observed in post SIVs. Between subject effects analysis indicated that there was no main effect of frequency in the Day 4 group, $F(3, 16) = 0.876, p = 0.474$. Within subjects effects analysis indicated that there was no main effect of test (pre/post), $F(1, 16) = 0.897, p = 0.358$. There were no significant interactions between the factors test (pre/post) and frequency, $F(3, 16) = 0.415, p = 0.744$.

No significant changes were observed in the Day 8 group after NIHL (Fig. 5.5D). Between subject effects analysis indicated that there was no main effect of frequency in the Day 8 group, $F(3, 16) = 0.156, p = 0.924$. Within subjects effects analysis indicated that there was no main effect of test (pre/post), $F(1, 16) = 0.715, p = 0.410$. There were no significant interactions between the factors test (pre/post) and frequency, $F(3, 16) = 0.336, p = 0.800$.

The Day 16 group exhibited an increase at 24 kHz, whilst the remaining frequencies were similar to control levels (Fig. 5.5E). Between subject effects analysis indicated that there was no main effect of frequency in the Day 16 group, $F(3, 16) = 0.299, p = 0.825$. Within subjects effects analysis indicated that there was no main effect of test (pre/post), $F(1, 16) = 0.717, p = 0.410$. There were no significant interactions between the factors test (pre/post) and frequency, $F(3, 16) = 0.972, p = 0.430$.

The Day 32 groups exhibited increases at 4 kHz and 24 kHz (Fig. 5.5F), while the other frequencies were at control levels. Between subject effects analysis indicated that there was no main effect of frequency in the Day 32 group, $F(3, 16) = 1.172, p = 0.351$. Within subjects effects analysis indicated that there was a main effect of test (pre/post), $F(1, 16) = 4.558, p = 0.049$; the overall post value (0.8840) was higher than the pre
value (0.6697), indicating that the startle responses were significantly higher in the gap detection test after the acoustic procedure. There was no significant interactions between the factors test (pre/post) and frequency, $F(3, 16) = 0.853, p = 0.485$.

Overall, these results suggest that the control SHAM animals effectively detected the gap at each frequency tested. The Day 0 group exhibited poor gap detection across all frequencies tested. These differences did not appear to be frequency specific. The Day 4 groups exhibited good gap detection at 16 kHz and 24 kHz and there were no differences between pre and post SIVs at 4 kHz and 10 kHz, which may indicate that the increases observed at Day 0, were transient. The Day 8 group exhibited good gap detection across most frequencies tested, except at 10 kHz. The Day 16 group exhibited good gap detection across most frequencies tested, except at 24 kHz. At Day 32, the animals exhibited good gap detection at 10 kHz and 16 kHz, whilst they exhibited poor gap detection at 4 kHz and 24 kHz. Even though the acoustic trauma stimulus used in this study was centred on 16 kHz, these results highlight that across all time-points 16 kHz was unaffected.

The pre SIVs at each time-point indicated that the animal within each group exhibited good gap detection before NIHL (Fig. 5.5).

The high variability between the animals within each group resulted in many changes between pre and post SIVs; therefore not reaching significance. It has been previously shown that not all animals present with evidence of tinnitus after an acoustic insult (Kraus et al., 2010, Longenecker and Galazyuk, 2011, Middleton et al., 2011). Thus it was important to analyse animals as individual cases. Longenecker and Galazyuk (2011) utilised this approach when analysing the changes in SIV after acoustic trauma in mice.
Figure 5.5: The Average SIV for each frequency in each group. Light grey bars indicate the pre-acoustic trauma SIVs and dark grey bars indicated the post-acoustic trauma SIVs. All values in the figures are expressed as mean SIV ± SEM. A repeated measures ANOVA was used to compare the pre/post SIVs between each frequency.

**Chapter Five: Study Three**
5.4.2.3: GAP DETECTION PERFORMANCE IN INDIVIDUAL CONTROL AND EXPOSED RATS

The individual startle response results are presented in Figures 5.6 – 5.11. These figures present the pre and post SIVs for each animal at each frequency tested, and the pre/post ABR audiograms. A two-way ANOVA and pair-wise comparison was performed on this data, with frequency as the independent variable and pre/post SIVs as the two dependent variables. Only significant changes are reported in this section. For the complete analyses reports see Appendix 2.

SHAM Control Animals

Figure 5.6 presents the mean SIV of individual animals obtained prior to (light grey bars) and immediately after (dark grey bars) SHAM acoustic trauma. It also presents the pre-acoustic trauma (blue dotted line) and post-SHAM acoustic trauma (black dotted line) ABR audiogram. No increases were observed in the mean SIV after SHAM acoustic trauma. Two out of five control animals show optimal suppression of their startle response when a gap was introduced, before and after the SHAM acoustic trauma (Fig. 5.6A-B), as the SIV was less than 1 at all times. Two out of the five animals exhibited a decrease in SIV after the second test (Fig. 5.6D-E). Also, the analysis demonstrated that there was no trend between frequencies across animals within this group.

In the second SHAM control animal (Fig. 5.6B), the overall pre/post SIVs at 24 kHz were lower than 4 kHz (p<0.001), 10 kHz (p = 0.007) and 16 kHz (p<0.001). Specifically, the pre SIVs at 4 kHz were higher than 10 kHz (p = 0.017), 16 kHz (p = 0.012) and 24 kHz (p<0.001), suggesting that before the second test the animal had better gap detection at 10 kHz, 16 kHz and 24 kHz compared to 4 kHz. In addition, the post SIVs at 24 kHz were lower than 4 kHz (p<0.001), 10 kHz (p = 0.001) and 16 kHz
(p<0.001), suggesting that after the second test the animal had better gap detection at 24 kHz compared to 4 kHz, 10 kHz and 16 kHz. In the third SHAM control animal (Fig. 5.6C) the overall pre/post SIVs at 24 kHz were higher than 4 kHz (p = 0.010) and 16 kHz (p = 0.001). The pre SIVs at 10 kHz were lower than 24 kHz (p = 0.019), suggesting that before the second test the animal had better gap detection at 10 kHz compared to 24 kHz. In addition, the post SIVs at 4 kHz were lower than 10 kHz (p = 0.001) and 24 kHz (p = 0.002) and 16 kHz was lower than 10 kHz (p<0.001) and 24 kHz (p<0.001). This suggests that after the second test the animal had better gap detection at 4 kHz and 16 kHz when compared to 10 kHz and 24 kHz. In the fourth SHAM control animal (Fig. 5.6D) there was a decrease in SIV at 10 kHz (p = 0.01) after acoustic trauma. In the fifth animal (Fig. 5.6E) there was a decrease in SIV at 24 kHz (p = 0.011) after acoustic trauma. The pre SIVs at 10 kHz were lower than 24 kHz (p = 0.009), suggesting that after the second test the animal had better gap detection at 10 kHz compared to 24 kHz.

This decrease in SIV, which was observed in two of the animals, suggests a better gap detection after the second test. This could be explained by a practice effect, which would suggest that the animals were able to detect the gap prior to the startle stimulus and thus would startle less in response to a startle + gap trial. However, the gap-detection test is a reflex test and has been shown to be unaffected by the practice effect (Turner et al., 2012).

When comparing the behavioural responses to the individual ABR audiogram, there appeared to be no relationship between these two measures (Fig. 5.6F). Correlation analysis was performed on the SIVs for each animal per frequency. The correlation coefficients for each frequency were 0.142 (4 kHz), 0.48 (10 kHz), 0.089 (16 kHz) and 0.123 (24 kHz). This indicated that under normal hearing conditions, gap detection was
not affected by absolute hearing levels. In other words, at frequencies when thresholds were relatively high, as typically seen at 4 kHz, gap detection was no different to that observed at frequencies where hearing sensitivity was greater.
Figure 5.6: The Pre and Post SHAM acoustic trauma SIVs at 4 kHz, 10 kHz, 16 kHz and 24 kHz for five individual control animals (A-E). A two-way ANOVA and pair-wise comparisons were used to analyse the data. Expressed as mean SIV ± SEM, *p<0.05 and **p<0.001. The animals’ pre and post SHAM acoustic trauma ABR audiogram is superimposed, indicating the threshold response per frequency tested. Expressed as raw threshold value (SPL(dB)). (F) The correlation between post SIVs and post ABR absolute thresholds for each frequency tested.
Day 0 Animals

Three out of the five Day 0 animals exhibited significant increases in the SIV at various frequencies immediately after NIHL. This highlights the variability between animals; there was no trend between frequencies across animals within the same group. Figure 5.7 presents the mean SIV of individual animals obtained prior to (light grey bars) and immediately after (dark grey bars) acoustic trauma (Day 0). It also presents the pre-acoustic trauma (blue dotted line) and post-acoustic trauma (black dotted line) ABR audiogram. The gap detection test was performed on the animals within one hour of being exposed to the acoustic trauma and being subject to an ABR recording. This was done seeing as the animals were required to be completely recovered from the anaesthesia prior to testing. The results of this study showed that these animals all experienced severe hearing loss (Fig. 5.7, black dotted line).

In the first Day 0 animal (Fig. 5.7A) there was a significant increase of SIV at 4 kHz (p<0.001), 10 kHz (p = 0.012) and 24 kHz (p = 0.002) after acoustic trauma. This suggests worsening gap detection immediately after the acoustic trauma. The pre/post SIVs at 4 kHz were significantly higher than the pre/post SIVs at 10 kHz (p<0.001), 16 kHz (p<0.001) and 24 kHz (p = 0.001). The post SIVs at 4 kHz were higher than 10 kHz (p<0.001), 16 kHz (p<0.001) and 24 kHz (p<0.001), suggesting that after acoustic trauma the animal had poor gap detection at 4 kHz compared to 10 kHz, 16 kHz and 32 kHz. In the second Day 0 animal (Fig. 5.7B) there was a significant increase of SIV at 16 kHz (p = 0.002) and 24 kHz (p = 0.033) after acoustic trauma. This suggests worsening gap detection at the higher frequencies. The pre SIVs at 16 kHz were lower than 4 kHz (p = 0.046) and 10 kHz (p = 0.044), suggesting that before the acoustic trauma the animal had better gap detection at 16 kHz compared to 4 kHz and 10 kHz. In addition, the post SIVs at 4 kHz were lower than 10 kHz (p = 0.003) and 16 kHz (p =
suggesting that after acoustic trauma the animal had better gap detection at 4 kHz compared to 10 kHz and 16 kHz. In the third Day 0 animal (Fig. 5.7C) the post SIVs at 10 kHz were lower than 4 kHz (p = 0.04) and 16 kHz (p = 0.021), suggesting that after acoustic trauma the animal had better gap detection at 10 kHz when compared to 4 kHz and 16 kHz. In the fourth Day 0 animal (Fig. 5.7D) there was a significant increase in SIV at 10 kHz (p<0.001) and 24 kHz (p = 0.005) after acoustic trauma. This also suggests worsening gap detection. The overall pre/post SIVs at 16 kHz were significantly lower than the pre/post SIVs at 10 kHz (p = 0.001) and 24 kHz (p = 0.018). The post SIVs at 10 kHz were higher than 4 kHz (p<0.001) and 16 kHz (p<0.001). Also, the SIVs at 24 kHz were higher than 4 kHz (p = 0.016) and 16 kHz (p = 0.003), suggesting that after acoustic trauma the animal had poor gap detection at 10 kHz and 24 kHz compared to 4 kHz and 16 kHz. In the fifth Day 0 animal (Fig. 5.7E) the pre SIVs at 24 kHz were lower than 10 kHz (p = 0.008) and 16 kHz (p = 0.003), suggesting that before acoustic trauma the animal had better gap detection at 24 kHz compared to 10 kHz and 16 kHz. In addition, the post SIVs at 16 kHz were lower than 10 kHz (p = 0.013) and 24 kHz (p = 0.028), suggesting that after acoustic trauma the animal had better gap detection at 16 kHz compared to 10 kHz and 24 kHz.

Correlation analysis was performed on the SIVs for each animal per frequency and was compared to the individual ABR audiogram (Fig. 5.7F). The correlation coefficients for each frequency were 0.047 (4 kHz), -0.692 (10 kHz), 0.283 (16 kHz) and -0.350 (24 kHz). This indicated that there was no systematic relationship between hearing levels and gap detection immediately after acoustic trauma. The contralateral (non-lesioned) ear may have played a role in the animals’ gap detection, as the hearing thresholds in that ear were normal.
Figure 5.7: The Pre and Post acoustic trauma SIVs at 4 kHz, 10 kHz, 16 kHz and 24 kHz for five individual Day 0 animals (A-E). A two-way ANOVA and pair-wise comparisons were used to analyse the data. Expressed as mean SIV ± SEM, *p<0.05 and **p<0.001. The animals’ pre and post acoustic trauma ABR audiogram is superimposed, indicating the threshold response per frequency tested. Expressed as raw threshold value (SPL (dB)). (F) The correlation between post SIV and post ABR absolute thresholds for each frequency tested.
Day 4 Animals

One out of the five Day 4 animals exhibited significant deficits in gap detection after NIHL. Figure 5.8 presents the mean SIV of individual animals obtained prior to (light grey bars) and four days after (dark grey bars) acoustic trauma. It also presents the pre-acoustic trauma (blue dotted line) and four days post acoustic trauma (black dotted line) ABR audiogram. One animal exhibited a significant increase of SIV at 4 kHz (p<0.001) and 10 kHz (p = 0.004) (Fig. 5.8E), which indicated that the animal was startling more in the gap + startle trials. The remaining four animals did not exhibit significant increases in SIV, but some exhibited significant decreases (Fig. 5.8A-B and E). One of these decreases was due to unusually high pre acoustic trauma values (Fig. 5.8E). For the remaining decreases, this suggests that the animals’ gap detection was improving or that their hearing may have become hypersensitive four days after acoustic trauma. This highlights the variability between animals; there was no trend between frequencies across animals within the group.

In the first Day 4 animal (Fig. 5.8A) there was a significant decrease of SIV at 4 kHz (p = 0.022) four days after acoustic trauma, suggesting an improvement in gap detection. In the second Day 4 animal (Fig. 5.8B) there was a significant decrease in SIV at 4 kHz (p = 0.008) and 10 kHz (p<0.001) four days after acoustic trauma, which also suggests an improvement in gap detection. The overall pre/post SIVs at 4 kHz were significantly higher than the pre/post SIVs at 16 kHz (p = 0.003) and 24 kHz (p<0.001). Also, the overall pre/post SIVs 10 kHz were significantly higher than 24 kHz (p = 0.041). In the fourth Day 4 animal (Fig. 5.8D) the overall pre/post SIVs at 10 kHz were significantly higher than the pre/post SIVs at 24 kHz (p = 0.004). In the fifth Day 4 animal (Fig. 5.8E) there was a significant decrease in SIV at 24 kHz (p = 0.044) and a significant increase in SIV at 4 kHz (p<0.001) and 10 kHz (p = 0.004) four days after acoustic trauma. This
suggests that this particular animal exhibited a worsening and an improvement in gap detection. The pre SIVs at 24 kHz were higher than 4 kHz \((p = 0.015)\) and 10 kHz \((p = 0.018)\), suggesting that before acoustic trauma the animal had poor gap detection at 24 kHz compared to 4 kHz and 10 kHz. In addition, the post SIVs at 4 kHz were higher than 16 kHz \((p<0.001)\) and 24 kHz \((p<0.001)\) and 10 kHz were higher than 16 kHz \((p = 0.007)\) and 24 kHz \((p = 0.011)\), suggesting that after acoustic trauma the animal had better gap detection at 16 kHz and 24 kHz compared 4 kHz and 10 kHz.

When comparing the behavioural responses to the individual ABR audiogram, there appeared to be a correlation between the increases/decreases in hearing thresholds with the increases/decreases in post SIVs at various frequencies (Fig. 5.8F). The ABR audiogram of the second animal shows a slight hearing recovery at 8 kHz and a decrease in post SIVs was observed at 4 kHz and 10 kHz (Fig. 5.8B). The ABR audiogram of the fourth animal shows a slight hearing recovery at 8 kHz and 16 kHz and a decrease in post SIVs was observed at 24 kHz (Fig. 5.8D). This could suggest that the improvement in hearing may contribute to the improvement in gap detection. However, the fifth animal experienced a slight hearing recovery at 1-8 kHz; however the post SIVs were significantly worse than the pre SIV (Fig. 5.8E).

Correlation analysis was performed on the SIVs for each animal per frequency (Fig. 5.8F). Though it appeared that some correlations could be made between the ABR thresholds, the correlation coefficients indicated that there was no systematic relationship between hearing levels and gap detection four days after acoustic trauma. The correlation coefficients for each frequency were -0.938 (4 kHz), 0.223 (10 kHz), 0.020 (16 kHz) and 0.707 (24 kHz). The contralateral (non-lesioned) ear may have also played a role in the animals’ gap detection, as the hearing thresholds were normal.
Figure 5.8: The Pre and Post acoustic trauma SIVs at 4 kHz, 10 kHz, 16 kHz and 24 kHz for five individual Day 4 animals (A-E). A two-way ANOVA and pair-wise comparisons were used to analyse the data. Expressed as mean SIV ± SEM, *p<0.05 and **p<0.001. The animals’ pre and 4 days post acoustic trauma ABR audiogram is superimposed, indicating the threshold response per frequency tested. Expressed as raw threshold value (SPL(dB)). (F) The correlation between post SIV and post ABR absolute thresholds for each frequency tested.
Day 8 Animals

The Day 8 animals did not exhibit specific deficits or improvements in gap detection after NIHL. Figure 5.9 presents the mean SIV of individual animals obtained prior to (light grey bars) and eight days after (dark grey bars) acoustic trauma. It also presents the pre-acoustic trauma (blue dotted line) and eight days post acoustic trauma (black dotted line) ABR audiogram.

In the second Day 8 animal (Fig. 5.9B) the pre SIVs at 4 kHz were lower than 24 kHz ($p = 0.03$), suggesting that before the acoustic trauma the animal had better gap detection at 4 kHz compared to 24 kHz. In addition, post SIVs at 24 kHz were lower than 4 kHz ($p = 0.04$) and 10 kHz ($p = 0.002$) suggesting that after acoustic trauma the animal had better gap detection at 24 kHz compared to 4 kHz and 10 kHz. In the third Day 8 animal (Fig. 5.9C) the pre SIVs at 24 kHz were lower than 4 kHz ($p = 0.037$) and 16 kHz ($p = 0.005$), suggesting that before acoustic trauma the animal had better gap detection at 24 kHz when compared to 4 kHz and 16 kHz. In addition, the post SIVs at 4 kHz were lower than 10 kHz ($p = 0.002$) and 24 kHz ($p = 0.048$), suggesting that after acoustic trauma the animal had better gap detection at 4 kHz compared to 10 and 24 kHz. Also, the SIVs at 10 kHz were lower than 16 kHz ($p = 0.005$), suggesting that after acoustic trauma the animal had better gap detection at 10 kHz compared to 16 kHz.

When comparing the behavioural responses to the individual ABR audiogram, there appeared to be no relationship between the two measures. Correlation analysis was performed on the SIVs for each animal per frequency (Fig. 5.9F). The correlation coefficients for each frequency were -0.540 (4 kHz), -0.217 (10 kHz), 0.220 (16 kHz) and 0.190 (24 kHz). This indicated that there was no systematic relationship between hearing levels and gap detection eight days after acoustic trauma.
Figure 5.9: The Pre and Post acoustic trauma SIVs at 4 kHz, 10 kHz, 16 kHz and 24 kHz for five individual Day 8 animals (A-E). A two-way ANOVA and pair-wise comparisons were used to analyse the data. Expressed as mean SIV ± SEM, *p<0.05 and **p<0.001. The animals’ pre and 8 days post acoustic trauma ABR audiogram is superimposed, indicating the threshold response per frequency tested. Expressed as raw threshold value (SPL (dB)). (F) The correlation between post SIV and post ABR absolute thresholds for each frequency tested.
Day 16 Animals

None of the five Day 16 animals exhibited significant deficits in gap detection after NIHL. Figure 5.10 presents the mean SIV of individual animals obtained prior to (light grey bars) and sixteen days after (dark grey bars) acoustic trauma. It also presents the pre-acoustic trauma (blue dotted line) and sixteen days post acoustic trauma (black dotted line) ABR audiogram.

In the first Day 16 animal (Fig. 5.10A) the overall pre/post SIVs at 4 kHz were significantly higher than the pre/post SIVs at 24 kHz (p = 0.025). In the second Day 16 animal (Fig. 5.10B) there was a significant reduction in SIV at 16 kHz (p<0.001) and 24 kHz (p = 0.008). These significant decreases cannot be attributed to high pre acoustic trauma SIVs, as pre values were normal. This suggests better gap detection sixteen days after acoustic exposure. The overall pre/post SIVs at 24 kHz were significantly lower than the pre/post SIVs at 4 kHz (p = 0.043) and 10 kHz (p = 0.036). The post SIVs at 4 kHz were higher than 16 kHz (p = 0.001) and 24 kHz (p = 0.001) and 10 kHz were higher than 16 kHz (p = 0.002) and 24 kHz (p = 0.002). This suggests that after acoustic trauma, the animal had poor gap detection at 4 kHz and 10 kHz compared to 16 kHz and 24 kHz. In the fourth Day 16 animal (Fig. 5.10D) the overall pre/post SIVs at 24 kHz were significantly higher than the pre/post SIVs at 4 kHz (p = 0.037). The post SIVs at 4 kHz were lower than 16 kHz (p = 0.004) and 24 kHz (p = 0.002), suggesting that after acoustic trauma the animal had better gap detection at 4 kHz compared to 16 kHz and 24 kHz. Also the SIVs at 10 kHz were lower than 24 kHz (p = 0.031), suggesting that after acoustic trauma the animal had better gap detection at 10 kHz compared to 24 kHz. In the fifth Day 16 animal (Fig. 5.10E) the overall pre/post SIVs at 24 kHz were significantly higher than the pre/post SIVs at 4 kHz (p = 0.005).
When comparing the behavioural responses to the individual ABR audiogram, there appeared to be no relationship between these two measures. Correlation analysis was performed on the SIVs for each animal per frequency (Fig. 5.10F). The correlation coefficients for each frequency were -0.590 (4 kHz), 0.016 (10 kHz), 0.283 (16 kHz) and 0.065 (24 kHz). This indicated that there was no systematic relationship between hearing levels and gap detection sixteen days after acoustic trauma. The contralateral (non-lesioned) ear may have also played a role in the animals’ gap detection, as the hearing thresholds were normal. In other words, at frequencies when thresholds were relatively high, as typically seen at 4 kHz, gap detection was no different to that observed at frequencies where hearing sensitivity was greater.
Figure 5.10: The Pre and Post acoustic trauma SIVs at 4 kHz, 10 kHz, 16 kHz and 24 kHz for five individual Day 16 animals (A-E). A two-way ANOVA and pair-wise comparisons were used to analyse the data. Expressed as mean SIV ± SEM, *p<0.05 and **p<0.001. The animals’ pre and 16 days post acoustic trauma ABR audiogram is superimposed, indicating the threshold response per frequency tested. Expressed as raw threshold value (SPL (dB)). (F) The correlation between post SIV and post ABR absolute thresholds for each frequency tested.
Day 32 Animals

Three of the Day 32 animals exhibited significant deficits in gap detection after NIHL. Figure 5.11 presents the mean SIV of individual animals obtained prior to (light grey bars) and thirty-two days after (dark grey bars) acoustic trauma. It also presents the pre-acoustic trauma (blue dotted line) and thirty-two days post acoustic trauma (black dotted line) ABR audiogram.

In the first Day 32 animal (Fig. 5.11A) there was a significant increase of SIV at 4 kHz (p = 0.023) and 24 kHz (p = 0.017). This suggests worsening gap detection. The overall pre/post SIVs at 4 kHz were significantly higher than the pre/post SIVs at 10 kHz (p = 0.027). In the second Day 32 animal (Fig. 5.11B) there was a significant increase of SIV at 24 kHz (p<0.001). This also suggests worsening gap detection. The pre/post SIVs at 24 kHz were significantly higher than the pre/post SIVs at 16 kHz (p = 0.015). The post SIVs at 24 kHz were higher than 4 kHz (p = 0.001), 10 kHz (p = 0.003) and 16 kHz (p<0.001), suggesting that after acoustic trauma the animal had poor gap detection at 24 kHz compared to 4 kHz, 10 kHz and 16 kHz. In the third animal (Fig. 5.11C) there was a significant increase of SIV at 4 kHz (p = 0.037) and 24 kHz (p<0.001). This suggests worsening gap detection at the same frequencies as the first and second Day 32 animals. The overall pre/post SIVs at 24 kHz were significantly higher than the pre/post SIVs at 10 kHz (p = 0.018) and 16 kHz (p<0.001). The post SIVs at 4 kHz were higher than 10 kHz (p = 0.028) and 16 kHz (p = 0.002), suggesting that after acoustic trauma the animal had poor gap detection at 4 kHz when compared to 10 kHz and 16 kHz. Also 24 kHz was higher than 4 kHz (p = 0.045), 10 kHz (p<0.001) and 16 kHz (p<0.001), suggesting that after acoustic trauma the animal had poor gap detection at 24 kHz when compared to 4 kHz, 10 kHz and 16 kHz. In the fourth Day 32 animal (Fig. 5.11D) the overall pre/post SIVs at 4 kHz were significantly higher than the pre/post SIVs at 10 kHz (p =
0.001), 16 kHz (p = 0.001) and 24 kHz (p = 0.024). The post SIVs at 4 kHz were higher than 10 kHz (p<0.001), 16 kHz (p<0.001) and 24 kHz (p = 0.001), suggesting that after acoustic trauma the animal had poor gap detection at 4 kHz compared to 10 kHz, 16 kHz and 24 kHz. Also 24 kHz was higher than 10 kHz (p = 0.016) and 16 kHz (p = 0.046), suggesting that after acoustic trauma the animal had poor gap detection at 24 kHz compared to 10 kHz and 16 kHz. In the fifth Day 32 animal (Fig. 5.11E) the post SIVs at 4 kHz were lower than 10 kHz (p = 0.002), 16 kHz (p = 0.034) and 24 kHz (p = 0.003), suggesting that after acoustic trauma the animal had better gap detection at 4 kHz compared to 10 kHz, 16 kHz and 24 kHz. This highlights the variability between animals; there was no trend between frequencies across animals within the same group.

When comparing the behavioural responses to the individual ABR audiogram, there appeared to be a correlation between the hearing thresholds and the post SIVs at various frequencies (Fig. 5.11A-D). In the first four animals, there were significant gap detection deficits in the same frequency regions that have high thresholds. The fourth animal showed better gap detection performance in the frequency region that had slight hearing recovery (Fig. 5.11D). This could suggest that hearing thresholds contribute to gap detection performance at Day 32. Correlation analysis was performed on the SIVs for each animal per frequency (Fig. 5.11F). The correlation coefficients for each frequency were -0.310 (4 kHz), -0.310 (10 kHz), 0.198 (16 kHz) and -0.528 (24 kHz). This indicated that there was no systematic relationship between hearing levels and gap detection thirty-two days after acoustic trauma. The contralateral (non-lesioned) ear may have also played a role in the animals’ gap detection, as the hearing thresholds were normal.
Figure 5.11: The Pre and Post acoustic trauma SIVs at 4 kHz, 10 kHz, 16 kHz and 24 kHz for five individual Day 32 animals (A-E). A two-way ANOVA and pair-wise comparisons were used to analyse the data. Expressed as mean SIV ± SEM, *p<0.05 and **p<0.001. The animals’ pre and 32 days post acoustic trauma ABR audiogram is superimposed, indicating the threshold response per frequency tested. Expressed as raw threshold value (SPL (dB)). (F) The correlation between post SIV and post ABR absolute thresholds for each frequency tested.
Hyperacusis-like Responses

Hyperacusis (see Chapter 1, section 1.5) is defined as the ‘unusual tolerance to common environmental sounds that are neither threatening nor uncomfortably loud to a typical person’ (Vernon, 1987, Klein et al., 1990). The implication is that the experience can be evoked by sounds of low intensity and that sounds in general, rather than specific sounds, are problematic (Baguley, 2003).

Four out of the twenty-five rats that were exposed to the acoustic trauma exhibited significant decreases in the post-acoustic trauma SIVs when compared to the pre-acoustic trauma SIVs. Table 5.3 shows each group with each of the background frequencies used in the gap-detection test. The numbers represent individual animals (1-4); this was carried out due to two animals that exhibited a hyperacusis-like response at more than one frequency. The decrease in SIV that was observed in one out of the four animals was attributed to having unusually high pre-acoustic trauma SIVs (ratio >1). The number for that animal in the table is in bold and italicised. This suggests that only three rats exhibited hyperacusis-like responses. The grey boxes indicate where no decreases in SIVs were observed and the white boxes indicate where increases were observed in SIVs.

| TABLE 5.3: ANIMALS THAT EXHIBITED HYPERACUSIS-LIKE RESPONSES AFTER NIHL |
|---------------------------|----------------|----------------|----------------|
| Group                    | Background Frequency |              |              |              |
|                          | 4 kHz | 10 kHz | 16 kHz | 24 kHz |
| Day 0                    |        |        |        |        |
| Day 4                    | (1)   | (2)   |        |        |
| Day 8                    |        |        |        |        |
| Day 16                   |        |        | (4)    | (4)    |
| Day 32                   |        |        |        |        |

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CHAPTER FIVE: STUDY THREE
When comparing the gap-detection SIVs and the post ABR audiograms of the four animals that exhibited hyperacusis-like responses there was no correlation between hearing loss or recovery and gap detection response. Four of the rats that exhibited hyperacusis-like responses exhibited a slight recovery of hearing at a similar frequency where gap detection had improved (Fig. 5.8B, Fig. 5.8E and Fig. 5.10B). The remaining rat did not show any recovery of hearing at a similar frequency where gap detection had improved (Fig. 5.8A).
5.5: DISCUSSION

This is the first time-course study to investigate the perception of tinnitus in rats after NIHL utilising the gap detection test (Turner et al., 2006). I have measured the startle responses of young adult rats up to thirty-two days following NIHL. This study has demonstrated that rats experience behavioural manifestations of tinnitus, and possibly hyperacusis, at various frequencies immediately and up to thirty-two days after NIHL.

The Effect of NIHL on Gap Detection Performance

The findings of this study suggests that the acoustic trauma stimulus utilised in this thesis leads to behavioural manifestations of tinnitus in rats at different frequencies than that of the acoustic trauma spectrum and that the perceived frequencies change over time. There was no evident systematic change in frequency. This investigation was consistent with other studies in that most of the control animals indicated good gap-induced inhibition of the startle response (ratios <1) (Turner et al., 2006, Wang et al., 2009, Kraus et al., 2010, Longenecker and Galazyuk, 2011, Turner et al., 2012). In addition, 28% of the noise-exposed animals developed behavioural signs of tinnitus after NIHL, which was unusually low compared to 56% (12 kHz noise at 126 dB SPL for 2 hours) (Kraus et al., 2010), 50% (16 kHz noise at 116 dB SPL for 45 minutes) (Middleton et al., 2011) and 86% (16 kHz noise at 116 dB for 1 hour) (Longenecker and Galazyuk, 2011) in other studies. This highlights the variability of animal responses between studies (e.g. 50% vs. 86%) and that not all animals exposed to an acoustic trauma develop tinnitus. This is similar to the human condition, as not all people with NIHL develop tinnitus (Konig et al., 2006). It has been shown in previous studies that the acoustic startle response is dependent on a few variables; species, gender and age of the animals used (Acri et al., 1995, Lehmann et al., 1999, Hince and Martin-Iverson,
2005). This could suggest that the experimental differences between the studies discussed and the current study may explain the variability in results.

After identifying that only 28% of the noise-exposed rats developed tinnitus, it was clear that the investigation produced less than optimal results. Firstly, the original gap detection protocol was to ensure that there was a 20-30% reduction in startle response amplitude caused by the preceding gap of silence in an otherwise continuous background sound. Unfortunately, the animals in this study only exhibited an 11-32% reduction in startle response amplitude. Improvements could be made to this study by ensuring that all animals exhibit a 20-30% reduction in startle amplitude caused by the preceding gap of silence in an otherwise continuous background sound, before the animals were exposed to the acoustic trauma. In addition, if certain animals did not exhibit the standard reduction in startle amplitude they could be excluded from the study.

Though there were only a small number of animals in this study, it was shown that the rats experienced significant changes in their gap-detection performance and in the ABR tests immediately after the acoustic trauma, which is in agreement with other studies (Holt et al., 2010, Kraus et al., 2010, Longenecker and Galazyuk, 2011). Overall, the Day 0 group had the most significant increases in SIVs compared to the subsequent time-points. All frequencies tested showed significant increases in SIV. However, the majority of the animals performed poorly at 4 kHz and 24 kHz. Longenecker and Galazyuk (2011) exposed mice to a similar acoustic trauma (narrow band-noise; 16 kHz at 116 dB for 1 hour) and found that immediately after acoustic trauma all frequencies showed an increased SIV, however the greatest increases were observed at 20 kHz and 25 kHz (Longenecker and Galazyuk, 2011). This shows that the immediate effects of NIHL were not centred on the acoustic trauma spectrum itself (16 kHz in both studies) but had an effect on higher frequencies (in both studies) and, in the case of this study, on
low frequencies. However, it should be noted that Longenecker and Galazyuk (2011) did not test the gap-detection of their mice in background noise less than 10 kHz. This study is in agreement with other studies that have shown that the range of tinnitus spectrum shifts to other frequencies outside the acoustic trauma frequency range. For example; a 10 kHz tinnitus after a 16 kHz centred noise trauma (Turner et al., 2006), a 24 kHz and 32 kHz tinnitus after a 17 kHz centred noise trauma (Wang et al., 2009), a 10 kHz tinnitus after a 16 kHz centred noise trauma (Middleton et al., 2011), and a 24 kHz tinnitus after a 16 kHz centred noise trauma (Turner et al., 2012).

In the Day 0 animals, most of the SIV increases in individual animals were unusually high; some animals appeared to be startling more during gap + startle trials compared to the startle only trials. This could be due to a post-effect of the anaesthesia or TTS, as the Day 0 animals were behaviourally tested on the same day as the exposure to the acoustic trauma. However, animals were fully recovered and alert before further testing. A control for this would be the SHAM animals, as they underwent the experimental conditions as the Day 0 animals, except not being exposed to the acoustic trauma. The SHAM animals were anaesthetised and placed in the sound proof chamber for 1 hr without being exposed to the stimulus. Anaesthesia was reversed and were allowed to fully recover, and were behaviourally tested. These two groups showed very different results, this suggests that the anaesthesia did not play a role in the high post SIVs, however, the TTS may have.

The Day 4 and Day 32 groups, also showed unusually high SIV increases in individual animals; some animals appeared to be startling more during gap + startle trials compared to the startle only trials. This could not be attributed to the post-effects of the anaesthesia or TTS, like the Day 0 animals. This finding has not been reported in any study that has performed gap detection tests on animals immediately after acoustic trauma, though
informal conversations with colleagues at conferences has confirmed that excessively high SIVs are not as uncommon as suggested by the lack of reports of high SIVs in the literature. In these instances, the high SIVs are regarded as outliers and therefore not considered in the analysis. Another way that these researchers treat high SIVs in the data set is by setting a limit for the response values, i.e. only SIVs that were below 1.5 were used in analysis. Since these types of results have not been published, the reason why some animals exhibit unusually high SIVs after NIHL has not been previously discussed. However, although speculative, abnormally high SIVs could indicate hyperacusis, and the startle stimulus could in fact be painful/intolerable, with the gap in the background noise could serve as a warning that the startle stimulus would follow (A. Galazyuk, personal communication). This would result in the animals startling more in the gap + startle trials compared to the startle only trials, as the animals were “expecting” the startle stimulus and therefore the animals have a heightened startle response due to fear. This could be is analogous to fear potentiated acoustic startle, in which a cue that is associated with an aversive stimulus amplifies an acoustic startle response (Brown et al., 1951, Siegel, 1967, Davis and Astrachan, 1978, Davis, 1986, Davis et al., 1989). However, the fact that Turner’s Gap Detection Test is theoretically based on an animal’s reflex does not support this speculation.

The fear-potentiated startle paradigm has been regarded a useful system with which to analyse neural systems involved in fear and anxiety (Davis et al., 1993). Previous researchers have shown that it is possible to measure conditioned fear using the acoustic startle reflex, by which an increase in the amplitude of the startle reflex is observed in the presence of a cue previously paired with a shock (Brown et al., 1951, Siegel, 1967, Davis and Astrachan, 1978, Davis, 1986, Davis et al., 1989). In the current study, the preceding gap could have led to fear conditioning in the rats that exhibited unusually
high post SIVs. This could suggest that the acoustic trauma stimulus used in this study produced three types of behavioural manifestations/responses; behavioural manifestations of tinnitus (high post SIV compared to pre SIV), hyperacusis-like responses without fear and/or intolerance (low post SIV compared to pre SIV) and hyperacusis-like responses with fear and/or intolerance (unusually high post SIV compared to pre SIV). The two types of hyperacusis-like responses were exhibited by animals with moderate to severe hearing losses. No differences could be identified between the animals in each group (hyperacusis-like responses with fear/hyperacusis-like responses without fear). If such a scheme is correct, then it would suggest that the underlying mechanisms involved in hyperacusis-like responses differ across individual animals, and in some cases can lead to a fear-potentiated startle. Previous studies have shown that an infusion of an NMDA antagonist into the amygdala, achieves an extinction of the fear-potentiated startle in animals (Falls et al., 1992). This method could be utilised in future studies to control for the fear-potentiated startle.

Over the time course tested, the wide range of gap-detection deficits that were immediately observed after acoustic trauma appeared to become more frequency-specific. Longenecker and Galazyuk (2011) also observed that over time the immediate wide range of gap-detection deficits began to narrow to a more specific frequency range. In this study, the specific frequencies that showed significant deficits in gap detection at Day 32 were 4 kHz and 24 kHz, which were the same frequencies that changed most significantly immediately after NIHL. These frequencies may correspond to chronic tinnitus frequencies. This finding supports Longenecker and Galazyuk's study. Upon reviewing their gap-detection deficit results at thirty-five days after acoustic trauma (similar to maximum time-point of this study), it was evident that the mice experienced gap-detection deficits at 20 kHz and 24 kHz (chronic tinnitus frequencies), which were
the same as the most significant frequencies that changed immediately after acoustic trauma in their study. Turner and colleagues (2012) also showed that tinnitus was evident immediately after acoustic trauma in mice. The most affected frequencies were 20 kHz, 24 kHz and 32 kHz (acute tinnitus frequencies). However, there was a narrowing of the affected frequencies between 5-7 weeks after acoustic trauma (chronic tinnitus frequencies) (Turner et al., 2012). This suggests that the animals used in this study were developing tinnitus in the same way that mice do after NIHL, which is an initial broad tinnitus spectrum, with specific frequencies greater than others are (high and low) and is followed by a narrowing to specific frequencies that are the same as the greatest initial frequencies (high and low).

Overall, the Day 4, Day 8, and Day 16 groups showed no significant increases in overall SIV. When comparing the gap-detection performance with the ABR audiograms of the animals it appeared that there was some hearing recovery in the ABR audiograms (discussed in Chapter 4: section 4.5). This slight “recovery” in hearing thresholds may explain the better performance in gap-detection. However, when the individual frequencies for each group were analysed, it was clear that there were some deficits and improvements in gap-detection performance in the Day 4 and Day 16 animals. The gap-detection performance in the Day 4 and Day 16 groups had predominantly improved. Although the ABR audiograms show significant increases across most frequencies tested, the animals had better gap-detection performance after the acoustic trauma. Parrish and Turner (2008) also observed this in a similar study. They found that the gap-detection in rats that were exposed to a tinnitus-inducing agent became worse (tinnitus) and improved (hyperacusis) in various frequencies within the same animal. Parrish and Turner (2008) suggested that an improved gap-detection could be attributed to a hyperacusis-like phenomenon caused by an increase in central gain, which occurs after
using tinnitus-inducing agents on rats (Parrish and Turner, 2008). This suggestion was made as Parrish and Turner’s animals were subject to pre-pulse inhibition tests that confirmed whether the animals’ hearing was essentially improving. Turner and colleagues (2012) showed similar results to the current study. Turner and colleagues exposed mice to a similar acoustic stimulus used in this study and found that animals’ exhibited hyperacusis-like responses 2-3 weeks after acoustic trauma (Turner et al., 2012). In this study, the Day 8 group were the only animals that did not exhibit deficits (tinnitus) or improvements (hyperacusis) in gap detection, suggesting that the animals had recovered from the NIHL.

The animals used in this study exhibited two types of gap-detection responses: some that were typical of tinnitus perception and others that were typical of hyperacusis. A greater post-acoustic trauma SIV, compared to the pre-acoustic trauma SIV, was considered tinnitus-like. A smaller post-acoustic trauma SIV, compared to the pre-acoustic trauma SIV, was considered hyperacusis-like. Hyperacusis-like responses were not attributed to unusually high pre-acoustic trauma values; for example if an animal presented with a pre-acoustic trauma SIV >1. When looking at individual animal results in the Day 4 and Day 16 groups, behavioural manifestations of tinnitus and hyperacusis-like responses were produced, in some cases an individual animal presented with both types of responses. A coincidence of tinnitus complaint and of experiences of hyperacusis has been widely noted in humans. Among patients attending tinnitus clinics with a primary complaint of tinnitus, the prevalence of hyperacusis was between 40-80% (Jastreboff and Jastreboff, 2000, Dauman and Bouscau-Faure, 2005). For this reason, tinnitus and hyperacusis is thought to share a common underlying pathophysiology with tinnitus (Nelson and Chen, 2004). This study has shown that 28% of the exposed rats developed behavioural signs of tinnitus and that 16% of the rats developed hyperacusis-like
symptoms. In addition, 14% of the rats that developed tinnitus-like responses also developed hyperacusis-like manifestations. This demonstrates that the hearing loss induced in this investigation can lead to a tinnitus perception and hyperacusis-like responses in some cases. This study cannot confirm whether these animals specifically developed hyperacusis, since such a small percentage of animals exhibited hyperacusis-like responses. Therefore, more experiments would be needed to validate the hyperacusis-like findings in this study.

Tinnitus-like responses were present in animals that had severe hearing loss (Day 0, Day 16 and Day 32) and moderate hearing loss (Day 32). Hyperacusis-like responses were present in animals that had severe hearing loss (Day 4 and Day 32) and moderate hearing loss (Day 16). This is not uncommon as recent epidemiological studies have shown that human patients that experience tinnitus have varying degrees of hearing loss (moderate, severe or profound) or have no hearing loss at all (Konig et al., 2006, Nicolas-Puel et al., 2006, Martines et al., 2010). Likewise, patients that experience hyperacusis report having varying levels of hearing loss (Kähäri et al., 2003).

Although behavioural manifestations of tinnitus were observed in this study, the gap detection test produced highly variable results between and within animals. The significant reductions of post-SIVs compared to pre-SIVs in animals that were exposed to the acoustic trauma may be explained by a hyperacusis-like response. However, that same observation in the control animals that were not exposed to the acoustic trauma stimulus suggests that the test is variable when small animal numbers are used. In a recent study by Turner and colleagues (2012), three of the eight control animals exhibited an improved startle reflex after the SHAM acoustic trauma (Turner et al., 2012). It is conceivable that the practice effect may have played a role in the improvement of gap-detection. However, theoretically the practice effect should not
affect the results as the trials were presented pseudo-randomly to the animals. This pseudo-random presentation of startle only and gap + startle trials should result in reflex-only responses. Turner also showed that the startle reflex, and the animals’ inhibition to gaps, was present on the first trial and was remarkably robust over time and, because a reflex was used, extinction of a learned response was not a concern (Turner et al., 2012). Although previous studies have shown that the gap detection test is a reliable test to determine the behavioural manifestations of tinnitus (Turner et al., 2006, Kraus et al., 2010, Longenecker and Galazyuk, 2011, Middleton et al., 2011, Longenecker and Galazyuk, 2012, Turner et al., 2012). However, in this study the limited amount of animals used in this study may have limited the test’s effectiveness.

The ABR thresholds and post-SIVs showed no correlation, this may be due to the limit of the thresholds tested. A SPL of 90dB was the highest level of sound presented in the ABR, this may have produced a ceiling effect for the animals that had hearing thresholds higher than 90dB.

In summary, this study provides the first investigation into the 32-day time-course of tinnitus perception in rats utilising an animal model of unilateral NIHL. I have investigated the development of tinnitus perception changes, in order to gain a deeper understanding of the underlying mechanisms of NIHL. In addition, it has highlighted the variations of the perceived frequency of tinnitus across time-points and in individual animals, but also highlights the similarities in chronic tinnitus frequencies. This study confirms that the rat-model of NIHL I utilised throughout this thesis can produce tinnitus and potentially hyperacusis in a small group of animals. I have shown for the first time that NIHL leads to the perception of tinnitus immediately after NIHL and causes chronic tinnitus in the rat. Changes in the perceived tinnitus spectrum may correlate with molecular and physiological changes that occur in the auditory pathway after NIHL.
(Chapter 3 and 4). The hyperacusis-like responses may reflect a mechanism by the auditory system to compensate for the loss of hearing by increasing central gain. The resulting fluctuations that I have observed in the rat are analogous to those that occur in humans, as many tinnitus patients have reported the perception of varying frequencies. If the chronic tinnitus frequency/frequencies can be somewhat predicted based on the most significant immediate changes in gap-detection performance after NIHL, those particular frequencies can be focused on immediately in the aim of attenuating the development of chronic tinnitus.
CHAPTER SIX

STUDY FOUR

The Effects of Alpha Lipoic Acid on Hearing Thresholds Following NIHL
6.1: INTRODUCTION

Experimental evidence suggests that free radicals play a significant role in NIHL. Free radicals disrupt normal cellular processes by attacking a variety of components in cells. They can oxidise lipids and proteins, destroy or destabilise membranes, disrupt the balance of ions, interfere with cell signals and the homeostasis of intracellular calcium, attack DNA and disrupt protein synthesis, and damage DNA repair and transcription processes (Halliwell, 1989, Orrenius and Nicotera, 1994, Halliwell, 1996, Evans and Halliwell, 1999, Halliwell, 2001). In addition, free radicals trigger the generation of excitatory amino acids, leading to excitotoxicity in neurons (Pellegrini-Giampietro et al., 1988, McFadden et al., 2001). In the cochlea, increased levels of free radicals have been observed after acoustic exposure (Yamane et al., 1995a, Yamane et al., 1995b, Ohlemiller et al., 1999, Gao et al., 2011). It is thought that the increase of free radicals results in a hearing loss by damaging the IHCs and OHCs of the inner ear and reacting with lipids, proteins and nucleotides in the cochlea (Seidman et al., 1993, Yamane et al., 1995, Yamashita et al., 2005). This is supported by the fact that noise-induced TTS, PTS and hair cell losses can be reduced with pharmacological treatments that target free radicals or induce antioxidant activity (Seidman et al., 1993, Ohlemiller et al., 1999, Cassandro et al., 2003, Franze et al., 2003, Hight et al., 2003, Hou et al., 2003, Pourbakht and Yamasoba, 2003, Seidman et al., 2003, Lynch et al., 2004, Takemoto et al., 2004, Kopke et al., 2005, Lynch and Kil, 2005, Tanaka et al., 2005, Yamashita et al., 2005, Yamasoba et al., 2005, Campbell et al., 2007, Coleman et al., 2007, Le Prell et al., 2007, Campbell et al., 2011).

As a result of this evidence, much focus has been directed on the role of antioxidant agents in NIHL, to ascertain whether they are effective in attenuating and/or preventing
NIHL (Seidman et al., 1993, Lynch and Kil, 2005, Yamashita et al., 2005, Le Prell et al., 2007). Table 6.1 lists the antioxidant enzymes and compounds shown to attenuate NIHL.

**TABLE 6.1: ANTIOXIDANT ENZYMES AND COMPOUNDS SHOWN TO ATTENUATE NOISE-INDUCED HEARING LOSS**

<table>
<thead>
<tr>
<th>Antioxidant Enzymes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione Peroxidases (GPx)</td>
<td>(Ohlemiller et al., 1999, Pourbakht and Yamasoba, 2003, Lynch et al., 2004, Yamasoba et al., 2005)</td>
</tr>
<tr>
<td>Catalyses the ability of GSH to act as an antioxidant. It has been previously shown that GPx activity decreases after acoustic trauma. Mimics of GPx have been shown to attenuate NIHL when administered before an acoustic trauma.</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Antioxidant Compounds</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allopurinol</td>
<td>(Seidman et al., 1993, Cassandro et al., 2003, Franze et al., 2003)</td>
</tr>
<tr>
<td>Is an inhibitor of xanthine oxidase and a scavenger of free radicals, provides protection when given before and immediately after acoustic trauma.</td>
<td></td>
</tr>
<tr>
<td>Acetyl-L-carnitine (ALCAR)</td>
<td>(Coleman et al., 2007)</td>
</tr>
<tr>
<td>Is an antioxidant compound that maintains mitochondrial bio-energy and integrity, was found to attenuate NIHL when administered immediately and 4 hours after acoustic trauma.</td>
<td></td>
</tr>
<tr>
<td>Edavarone</td>
<td>(Takemoto et al., 2004, Tanaka et al., 2005)</td>
</tr>
<tr>
<td>Is a potent antioxidant and a scavenger of free radicals, has been shown to protect against NIHL when administered before and up to 9 hours after the acoustic trauma.</td>
<td></td>
</tr>
<tr>
<td>Resveratrol</td>
<td>(Seidman et al., 2003)</td>
</tr>
<tr>
<td>Is an antioxidant found in plants, particularly red grapes, was shown to attenuate NIHL in animals treated for seven weeks prior to acoustic trauma.</td>
<td></td>
</tr>
<tr>
<td>R-N6-phenyliso propyladenosine (R-PIA)</td>
<td>(Hight et al., 2003)</td>
</tr>
<tr>
<td>Is an antioxidant-enhancing drug, was shown to protect against NIHL when administered prior to acoustic trauma.</td>
<td></td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>(Hou et al., 2003, Yamashita et al., 2005)</td>
</tr>
<tr>
<td>Is a fat soluble antioxidant which exhibits vitamin E activity, was found to reduce NIHL in animals treated before and three days after acoustic trauma.</td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>(Kopke et al., 2005, Campbell et al., 2007, Campbell et al., 2011)</td>
</tr>
<tr>
<td>Is an amino acid that is involved in the production of GSH, was found to attenuate NIHL after treatment up to seven hours after acoustic trauma.</td>
<td></td>
</tr>
<tr>
<td>Monoethylster</td>
<td>(Hight et al., 2003)</td>
</tr>
<tr>
<td>Increases cellular GSH and is particularly effective in increasing the mitochondrial pool, was shown to protect against NIHL when administered prior to acoustic trauma.</td>
<td></td>
</tr>
<tr>
<td>N-Acetyl Cysteine (NAC)</td>
<td>(Kopke et al., 2000, Coleman et al., 2007)</td>
</tr>
<tr>
<td>Is a precursor in the formation of the antioxidant GSH in the body, was shown to protect against NIHL when administered prior to and up to 4 hours after acoustic trauma.</td>
<td></td>
</tr>
<tr>
<td>2-oxothiazolidine-4-carboxylate (OTC)</td>
<td>(Yamasoba et al., 1998)</td>
</tr>
<tr>
<td>Is a cysteine pro-drug that promotes rapid restoration of GSH (when GSH is acutely depleted), was shown to protect against NIHL when administered two hours before and immediately after noise trauma.</td>
<td></td>
</tr>
</tbody>
</table>
It is clear that the role of antioxidant agents in NIHL are effective in attenuating (when administered up to three days after acoustic exposure) and/or preventing NIHL (when administered immediately or even up to seven weeks before acoustic exposure).

There is potential for developing post-NIHL treatments, as it is known that free radicals can have a delayed onset of generation (Yamashita et al., 2004). Yamashita and colleagues performed an immunohistochemical study quantifying the expression of Nitrotyrosine (a marker for RNS formation) and 4-hydroxy-2-noneal (a marker for ROS formation) and observed a peak in their immunoreactivity up to seven to ten days post acoustic trauma. Thus, the delayed generation of free radicals provides a rationale for investigating NIHL in animals that are treated (for longer than one day) after the acoustic trauma.

This study focused on the effect of ALA on hearing threshold shifts up to two months following NIHL. Recent studies with ALA have shown promising results for the treatment of hearing loss, demonstrating that this compound may be a potential treatment for attenuating NIHL. ALA is a naturally occurring dithiol compound that exhibits strong antioxidant properties and has been found to have numerous pharmacotherapeutic properties (Biewenga et al., 1997). ALA has been used successfully as a therapeutic agent for the treatment of several medical conditions including myocardial and cerebral ischemia-reperfusion injury, heavy-metal poisoning, radiation damage, diabetes, neurodegenerative disease and AIDS (Packer et al., 1995). ALA is a powerful lipophilic antioxidant free radical scavenger that binds to hydroxyl radicals, hypochlorous acid, NO, peroxynitrite, hydrogen peroxide and singlet oxygen. It also chelates iron, copper, and other transition metals (Whiteman et al., 1996) and in doing so, protects against mitochondrial dysfunction (Conlon et al., 1999). GSH also plays an important role in the detoxification of free radicals (Bray and Taylor, 1993).
ALA helps to overcome oxidative stress by increasing the GSH concentration that in turn leads to increased detoxification of hydrogen peroxide (Malarkodi et al., 2003). Packer and colleagues (1995) reported that ALA regenerated the GSH pool by the reduction of oxidised GSH. In previous studies performed both in vitro and in vivo (Packer et al., 1995), ALA was observed to increase intracellular GSH levels, which makes it ideal for the treatment of NIHL, as GSH is an important natural cochlear protectant (Hoffman et al., 1988).

In recent studies, the therapeutic effects of ALA have been investigated in animals with drug-induced hearing loss (Conlon et al., 1999, Rybak et al., 1999, Husain et al., 2005) and age-related hearing loss (Seidman, 2000, Le and Keithley, 2007) and was found to have beneficial outcomes. However, investigations into the effect of ALA on NIHL are limited to one study (Diao et al., 2003). Daio observed that guinea pigs treated with ALA prior to acoustic exposure were protected from the acoustic trauma, and had less hearing loss compared to the animals treated with saline.

Thus, the antioxidant property of ALA, along with its ability to cross the blood brain barrier and its minimal toxicity, makes ALA an ideal candidate to test for its effectiveness in treating NIHL. As free radicals have been shown to form in the inner ear for up to ten days after noise trauma (Yamashita et al., 2004), the strong antioxidant potency of ALA could be used as a novel therapy for post-treatment of NIHL. Furthermore the observed effects that ALA has on intracellular GSH production suggest that it may have a beneficial and protective effect on the cochlea and reduce age associated deterioration in auditory sensitivity and cochlear function (Conlon et al., 1999, Seidman, 2000).
This study has investigated whether ALA can be a suitable pre or post-treatment for NIHL. This investigation centres on the time course of changes of hearing thresholds following NIHL to assess whether ALA is an effective preventative measure or a post-treatment for hearing loss that is associated with NIHL. To date, there has been no longitudinal investigation into the effects of ALA on hearing thresholds over time, the only study available measured the hearing threshold immediately after NIHL (Diao et al., 2003). I measured the hearing thresholds of animals’ treated with ALA before and after NIHL using ABR tests over two months. Understanding the development of changes that occur after NIHL is of utmost importance for the development of therapeutic interventions.
6.2: HYPOTHESIS AND AIMS

6.2.1: AIMS

My aim was to assess the effects of pre and post-treatment of ALA on the time-course of auditory thresholds after NIHL.

6.2.2: HYPOTHESIS

It was hypothesised that: 1) animals treated with ALA would exhibit less hearing loss than controls (saline treated), as determined by auditory thresholds and 2) the animals treated with ALA before the acoustic trauma would have a reduced hearing loss and/or improved recovery compared to animals treated with ALA after the acoustic trauma.
6.3: Research Method

6.3.1: Animals and Treatment Groups

Rats were housed and maintained as previously described in section 2.1. Sixteen rats were randomly assigned to four groups, used to assess the effect of ALA treatment on hearing thresholds following NIHL. These groups were:

- **Pre-ALA (n = 5)**; one week of daily subcutaneous ALA (pH neutralised) injections (50 mg/kg/day) + saline, followed by acoustic trauma,

- **Pre-saline (n = 5)**; one week of daily subcutaneous saline injections (of equal volumes), followed by acoustic trauma,

- **Post-ALA (n = 3)**; acoustic trauma followed by two weeks of daily subcutaneous ALA (pH neutralised) injections (50 mg/kg/day) + saline, and;

- **Post-saline (n = 3)**; acoustic trauma followed by two weeks of daily subcutaneous saline injections (of equal volumes).

Each animal was weighed before each experiment in order to calculate the dosage of ALA to be administered.

The experimental procedures were approved by the Animal Ethics Committee of the University of Western Sydney (ACEC #9201) and conformed to the Australian code of practice for the care and use of animals for scientific purposes.
6.3.2: ALA Stock Solution

ALA (R-S form) was obtained from Sigma-Aldrich (Catalogue #62320). ALA (500 mg) was weighed and resuspended in 4.5mL of Milli-Q water mixed with 1.7mL of 4 m NaOH. The solution was pH balanced (7.2-7.4) with 400µl-450µl of 32 m HCl. The solution was filter sterilised through a 0.22µm syringe filter and frozen in aliquots at -80ºC until used. On the day of the experiment, the frozen solution was thawed and diluted with equal parts saline prior to administration. ALA solution was administered by subcutaneous injection (50 mg/kg). Equal volume of normal saline solution was used as a vehicle control. Injection volume ranged from 0.3-0.5mL.

6.3.3: Acoustic Trauma and Auditory Brainstem Response Tests

Animals were exposed to a different acoustic trauma stimulus compared to previous studies in this thesis (see section 2.3). Rats were unilaterally exposed to a 16 kHz band pass (1/10th octave (112 dB SPL)) noise for 1 minute rather than 16 kHz band pass (1/10th octave (115 dB SPL)) noise for 1 hour. The rationale for exposing the rats to a shorter and lower SPL acoustic trauma for this study was to make the exposure duration more realistic. Exposure to a loud sound for long durations of time is more realistic for factory and road workers, and for individuals that attend music concerts, for example. However, most people that are exposed to sporadic excessive noise (without hearing protection) may remove themselves from the stimulus and therefore may only be exposed for shorter durations. The duration of 1 minute was used as according to the WH&S standards of noise exposure, Table 1.2 (see section 1.1.1); an acoustic exposure at 112 dB for 57 seconds is capable of causing noise damage. This has been shown in previous studies using a similar acoustic trauma stimulus: 115 dB for 1 minute (Patuzzi and Thompson, 1991) and 120 dB for 1 minute (Davis et al., 1950). A one-hour
exposure to a high SPL directly in the ear canal is an unrealistic exposure when studying a therapeutic intervention for a human experience. A shorter and lower SPL acoustic trauma stimulus is therefore a more practical exposure to what would occur in humans. ABR tests (see section 2.4) were performed before, and immediately after NIHL, and at 4, 8, 16 and 32 days after acoustic trauma, on both ipsilateral and contralateral ears. Separate ABR’s were generated through stimulation of the left and right ears.

6.3.4: STATISTICAL ANALYSES

6.3.4.1: ABR RAW THRESHOLDS
ABR raw threshold values were analysed using a GLM one-way ANOVA with repeated measures in SPSS. A Tukey’s test was used to compare the overall raw threshold values per frequency. Pair-wise comparison analyses were made to determine whether there was a difference in ABR raw thresholds between the pre-lesion values and the subsequent time-points. If there was a significant difference, an independent samples t-test was used to compare the ABR raw thresholds at each frequency between pre-lesion and the subsequent time-points.

6.3.4.2: ABR THRESHOLD SHIFTS
ABR threshold shifts were analysed using a GLM two-way ANOVA with repeated measures in SPSS. The factors were treatment and frequency, and repeated measures were the threshold shifts. Separate analyses were performed to compare Pre-ALA vs. Pre-saline, Post-ALA vs. Post-saline, Pre-ALA vs. Post-ALA, and Pre-saline vs. Post-saline groups.

6.3.4.3: ANIMAL WEIGHT
All values in the figures are expressed as mean weight ± SEM (g). Control and treatment weights were compared on individual days, but not across days, using a univariate GLM.
6.4: RESULTS

6.4.1: ABR AUDIOGRAMS

ABR thresholds for all animals in each treatment group were recorded prior to and immediately after the acoustic trauma, and at 4, 8, 16 and 32 days after acoustic trauma.

6.4.1.1: PRE-SALINE GROUP

This section presents the ABR thresholds for the animals treated with saline before the acoustic trauma via stimulation of the contralateral (non-lesioned) ear (Table 6.2) and the ipsilateral (lesioned) ear (Fig. 6.1 and Table 6.3). These animals served as controls.

The one-way ANOVA with repeated measures determined that there was no significant effect of acoustic trauma on the ABR thresholds of the contralateral (non-lesioned) ear. The between-subjects effects analysis showed that there was a main effect of frequency, \( F(5, 24) = 48.596, p < 0.001 \). The within-subjects effects analysis showed that there was no main effect of time-point, \( F(6, 144) = 7.686, p = 0.243 \). There also was no interaction between time-point and frequency, \( F(30, 144) = 1.032, p = 0.177 \). The Tukey’s test showed that overall the ABR thresholds at 1 kHz were significantly higher than the ABR thresholds at 4 kHz, 8 kHz, 16 kHz and 32 kHz (all \( p < 0.001 \)) and the ABR thresholds at 2 kHz were significantly higher than the ABR thresholds at 4 kHz, 8 kHz, 16 kHz and 32 kHz (all \( p < 0.001 \)). The ABR thresholds at 4 kHz were higher than 32 kHz (\( p = 0.014 \)). The ABR thresholds at 8 kHz were higher than 32 kHz (\( p = 0.003 \)).
TABLE 6.2: MEAN THRESHOLD VALUES ± SEM (SPL (dB)) OF ABR AUDIOGRAMS OF PRE-SALINE RATS (CONTRALATERAL/NON-LESIONED EAR) (N = 5). A ONE-WAY ANOVA WITH REPEATED MEASURES WAS USED TO COMPARE PRE-LESION VALUES TO DIFFERENT ABR TIME-POINTS (RECOVERY TIME-POINTS) AT THE SAME FREQUENCY.

<table>
<thead>
<tr>
<th>ABR Time-point</th>
<th>Frequency</th>
<th>1 kHz</th>
<th>2 kHz</th>
<th>4 kHz</th>
<th>8 kHz</th>
<th>16 kHz</th>
<th>32 kHz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-lesion</td>
<td>36 ± 5.1 dB</td>
<td>38 ± 3.7 dB</td>
<td>20 ± 3.1 dB</td>
<td>21 ± 1.0 dB</td>
<td>14 ± 2.4 dB</td>
<td>8 ± 1.2 dB</td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>41 ± 2.4 dB</td>
<td>44 ± 2.9 dB</td>
<td>22 ± 2.0 dB</td>
<td>26 ± 2.4 dB</td>
<td>22 ± 3.7 dB</td>
<td>10 ± 2.2 dB</td>
<td></td>
</tr>
<tr>
<td>Day 4</td>
<td>37 ± 2.0 dB</td>
<td>42 ± 1.2 dB</td>
<td>22 ± 2.0 dB</td>
<td>22 ± 2.0 dB</td>
<td>18 ± 4.5 dB</td>
<td>12 ± 2.0 dB</td>
<td></td>
</tr>
<tr>
<td>Day 8</td>
<td>40 ± 2.7 dB</td>
<td>41 ± 1.0 dB</td>
<td>22 ± 2.0 dB</td>
<td>22 ± 2.0 dB</td>
<td>20 ± 3.2 dB</td>
<td>12 ± 2.0 dB</td>
<td></td>
</tr>
<tr>
<td>Day 16</td>
<td>26 ± 2.4 dB</td>
<td>32 ± 3.7 dB</td>
<td>16 ± 2.4 dB</td>
<td>18 ± 2.0 dB</td>
<td>10 ± 3.0 dB</td>
<td>10 ± 3.0 dB</td>
<td></td>
</tr>
<tr>
<td>Day 32</td>
<td>41 ± 5.1 dB</td>
<td>45 ± 2.7 dB</td>
<td>18 ± 3.7 dB</td>
<td>20 ± 1.6 dB</td>
<td>11 ± 1.0 dB</td>
<td>12 ± 2.0 dB</td>
<td></td>
</tr>
<tr>
<td>Day 64</td>
<td>38 ± 6.6 dB</td>
<td>39 ± 6.4 dB</td>
<td>17 ± 2.0 dB</td>
<td>20 ± 1.6 dB</td>
<td>12 ± 2.0 dB</td>
<td>11 ± 1.9 dB</td>
<td></td>
</tr>
</tbody>
</table>

The one-way ANOVA with repeated measures determined that for the ipsilateral (lesioned) ear there was a significant effect of the acoustic trauma on the ABR thresholds. The between-subjects effects analysis showed that there was a main effect of frequency, F (5, 24) = 58.478, p<0.001. The within-subjects effects analysis showed that there was a main effect of time-point, F (2.724, 65.388) = 29.077, p<0.001. There also was a significant interaction between time-point and frequency, F (13.622, 65.388) = 2.491, p = 0.007. The Tukey’s test showed that overall the ABR thresholds at 1 kHz were significantly higher than the ABR thresholds at 4 kHz, 8 kHz, 16 kHz and 32 kHz (all p<0.001) and the ABR thresholds at 2 kHz were significantly higher than the ABR thresholds at 4 kHz, 8 kHz, 16 kHz and 32 kHz (all p<0.001). The ABR thresholds at 4 kHz were higher than 16 kHz (p = 0.001) and 32 kHz (p<0.001). The ABR thresholds at 8 kHz were higher than 32 kHz (p = 0.025). Pair-wise comparison analyses showed that the overall pre lesion ABR thresholds were significantly lower than the overall ABR thresholds at Day 0 (p<0.001), Day 4 (p<0.001), Day 8 (p<0.001), Day 32 (p = 0.001)
and Day 64 (p = 0.035). An independent samples t-test was used to determine the specific differences between ABR threshold shifts at each frequency. The results of these tests are presented in Table 6.2. The t-test showed that after acoustic trauma (Day 0); there was a significant increase in ABR thresholds at all frequencies. Similar results were observed on Day 4, when thresholds of most frequencies were significantly elevated. By Day 8, most thresholds had returned to control levels, except 1 kHz, which was elevated. Threshold shifts at all frequencies had returned to control levels by Day 16. At Day 32, thresholds for all frequencies were elevated except 32 kHz. By Day 64, 32 kHz was elevated, while the threshold shifts for the remaining frequencies returned to control levels. Figure 6.1 shows the mean ABR audiogram threshold ± SEM (SPL (dB)) for each time-point.
Figure 6.1: Mean ABR audiograms (raw values of threshold SPL (dB)) ± SEM of Pre-saline rats over 64 days (n = 5). Full coloured lines indicate various ABR time-points (or recovery time-points) (see figure legend). Dotted Black line indicates the pre-lesion ABR values. P values are presented in Table 6.3 below.

| TABLE 6.3: MEAN THRESHOLD VALUES ± SEM (SPL (dB)) OF ABR AUDIOGRAMS OF PRE-SALINE RATS (IPSILATERAL/LESIONED EAR) (N = 5). A ONE-WAY ANOVA WITH REPEATED MEASURES WAS USED TO COMPARE PRE-LESION VALUES TO DIFFERENT ABR TIME-POINTS (RECOVERY TIME-POINTS) AT THE SAME FREQUENCY, *P<0.05. IF NO SIGNIFICANCE WAS DETERMINED BY THE TUKEY’S TEST; FOLLOW UP T-TESTS WERE NOT PERFORMED AND HENCE THE ABSENCE OF P VALUES. |
|--------------------------|-----------------|----------------|----------------|----------------|----------------|-----------------
|                         | **1 kHz**       | **2 kHz**      | **4 kHz**      | **8 kHz**      | **16 kHz**     | **32 kHz**     |
| Pre-saline               |                 |                 |                 |                 |                 |                 |
| Pre-lesion               | 34 ± 2.5 dB     | 38 ± 4.0 dB     | 22 ± 2.0 dB     | 17 ± 2.0 dB     | 7 ± 1.2 dB      | 1 ± 0 dB       |
| Day 0                    | 51 ± 4.0 dB     | 56 ± 5.1 dB     | 32 ± 2.5 dB     | 29 ± 3.3 dB     | 39 ± 4.0 dB     | 40 ± 5.0 dB    |
| (p = 0.01)               | (p = 0.029)     | (p = 0.022)     | (p = 0.009)     | (p = 0.001)     | (p = 0.001)     |                 |
| Day 4                    | 44 ± 2.4 dB     | 47 ± 2.5 dB     | 28 ± 2.0 dB     | 28 ± 3.8 dB     | 17 ± 2.5 dB     | 21 ± 6.2 dB    |
| (p = 0.011)              | (p = 0.07)      | (p = 0.004)     | (p = 0.011)     | (p = 0.003)     | (p = 0.032)     |                 |
| Day 8                    | 52 ± 4.6 dB     | 53 ± 4.6 dB     | 27 ± 2 dB       | 21 ± 1.8 dB     | 11 ± 2.9 dB     | 11 ± 6 dB      |
| (p = 0.018)              | (p = 0.07)      | (p = 1.0)       | (p = 0.178)     | (p = 0.10)      | (p = 0.171)     | (p = 0.171)    |
| Day 16                   | 34 ± 6.8 dB     | 39 ± 7.1 dB     | 22 ± 2 dB       | 17 ± 3.7 dB     | 7 ± 2 dB        | 5 ± 0 dB       |
| Day 32                   | 52 ± 5.1 dB     | 55 ± 4.1 dB     | 34 ± 1.8 dB     | 26 ± 1.8 dB     | 13 ± 2 dB       | 9 ± 2.9 dB     |
| (p = 0.025)              | (p = 0.021)     | (p = 0.024)     | (p = 0.001)     | (p = 0.004)     | (p = 0.052)     | (p = 0.052)    |
| Day 64                   | 36 ± 5.8 dB     | 43 ± 4.6 dB     | 26 ± 1 dB       | 19 ± 2.4 dB     | 11 ± 2.9 dB     | 7 ± 2 dB       |
| (p = 0.784)              | (p = 0.43)      | (p = 0.178)     | (p = 0.621)     | (p = 0.099)     | (p = 0.04)      | (p = 0.04)     |
6.4.1.2: Pre-ALA Group

This section presents the ABR thresholds for the animals treated with ALA before the acoustic trauma via stimulation of the contralateral (non-lesioned) ear (Table 6.4) and the ipsilateral (lesioned) ear (Fig. 6.2 and Table 6.5).

The one-way ANOVA with repeated measures determined that there was no effect of acoustic trauma on the ABR thresholds of the contralateral ear (non-lesioned ear). The between-subjects effects analysis showed that there was a main effect of frequency, $F(5, 24) = 61.902$, $p<0.001$. The within-subjects effects analysis showed no main effect of time-point, $F(3.327, 79.837) = 2.492$, $p = 0.158$, and no interaction between time-point and frequency, $F(16.633, 79.837) = 1.468$, $p = 0.234$. The Tukey’s test showed that overall the ABR thresholds at 1 kHz were higher than the ABR thresholds at 4 kHz, 8 kHz, 16 kHz and 32 kHz (all $p<0.001$) and the ABR thresholds at 2 kHz were higher than the ABR thresholds at 4 kHz, 8 kHz, 16 kHz and 32 kHz (all $p<0.001$). In addition, the ABR thresholds at 4 kHz were higher than 16 kHz ($p<0.001$) and 32 kHz ($p = 0.001$) and the ABR thresholds at 8 kHz were higher than 16 kHz ($p = 0.044$).

TABLE 6.4: Mean Threshold Values ± SEM (SPL (dB)) of ABR Audiograms of Pre-ALA Rats (Contralateral/Non-Lesioned Ear) ($N=5$). A One-Way ANOVA with Repeated Measures Was Used to Compare Pre-Lesion Values to Different ABR Time-Points (Recovery Time-Points) at the Same Frequency.

<table>
<thead>
<tr>
<th>Pre-ALA</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 kHz</td>
</tr>
<tr>
<td>Pre-lesion</td>
<td>30 ± 0.0 dB</td>
</tr>
<tr>
<td>Day 0</td>
<td>33 ± 1.2 dB</td>
</tr>
<tr>
<td>Day 4</td>
<td>33 ± 2.0 dB</td>
</tr>
<tr>
<td>Day 8</td>
<td>34 ± 1.9 dB</td>
</tr>
<tr>
<td>Day 16</td>
<td>30 ± 2.7 dB</td>
</tr>
<tr>
<td>Day 32</td>
<td>34 ± 3.3 dB</td>
</tr>
<tr>
<td>Day 64</td>
<td>37 ± 5.1 dB</td>
</tr>
</tbody>
</table>
The one-way ANOVA with repeated measures determined that for the ipsilateral (lesioned) ear there was a significant effect of acoustic trauma on the ABR thresholds. The between-subjects effects analysis showed that there was a main effect of frequency, \( F(5, 24) = 12.096, p<0.001 \). The within-subjects effects analysis showed that there was a main effect of time-point, \( F(3.259, 78.221) = 95.979, p<0.001 \). There also was a significant interaction between time-point and frequency, \( F(16.296, 68.221) = 3.265, p<0.001 \). The Tukey’s test showed that overall the ABR thresholds at 1 kHz were significantly higher than the ABR thresholds at 16 kHz (\( p = 0.005 \)) and 32 kHz (\( p = 0.003 \)) and the ABR thresholds at 2 kHz were significantly higher than the ABR thresholds at 4 kHz, 8 kHz, 16 kHz and 32 kHz (all \( p<0.03 \)). Pair-wise comparison analyses showed that the overall pre-lesion ABR thresholds were significantly lower than the overall ABR thresholds at Day 0 (\( p<0.001 \)), Day 4 (\( p<0.001 \)) and Day 8 (\( p<0.001 \)). An independent samples t-test was used to determine the specific differences between ABR threshold shifts at each frequency that was shown to be significant by the pair-wise comparison analysis. The results of these tests are presented in Table 6.4. The t-test showed that after acoustic trauma (Day 0); there was a significant increase in ABR thresholds at all frequencies. On Day 4, the thresholds of most frequencies returned to control levels except for 32 kHz. On Day 8, the threshold shifts at 1 kHz and 2 kHz became elevated, while the threshold shifts at the remaining frequencies were at control levels. The increase in threshold shift in the lower frequency region was also observed at Day 8 in the Pre-saline animals. At Day 16, Day 32, and Day 64, the threshold shifts for all frequencies were at control levels. Figure 6.2 shows the mean ABR audiogram threshold SPL (dB) ± SEM for each time-point.
Figure 6.2: Mean ABR audiograms (raw values of threshold SPL (dB)) ± SEM of Pre-ALA rats over 64 days (n = 5). Full coloured lines indicate various ABR time-points (or recovery time-points) (see figure legend). Dotted Black line indicates the pre-lesion ABR values. P values are presented in Table 6.5 below.

TABLE 6.5: MEAN THRESHOLD VALUES ± SEM (SPL (dB)) OF ABR AUDIOGRAMS OF PRE-ALA RATS (IPSILATERAL/LESIONED EAR) (N = 5). A ONE-WAY ANOVA WITH REPEATED MEASURES WAS USED TO COMPARE PRE-LESION VALUES TO DIFFERENT ABR TIME-POINTS (RECOVERY TIME-POINTS) AT THE SAME FREQUENCY, *P<0.05. IF NO SIGNIFICANCE WAS DETERMINED BY THE TUKEY’S TEST; FOLLOW UP T-TESTS WERE NOT PERFORMED AND HENCE THE ABSENCE OF P VALUES.

<table>
<thead>
<tr>
<th>Pre-ALA</th>
<th>1 kHz</th>
<th>2 kHz</th>
<th>4 kHz</th>
<th>8 kHz</th>
<th>16 kHz</th>
<th>32 kHz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-lesion</td>
<td>30 ± 4.1 dB</td>
<td>39 ± 4.1 dB</td>
<td>21 ± 3.5 dB</td>
<td>28 ± 2.5 dB</td>
<td>7 ± 1.3 dB</td>
<td>5.8 ± 6 dB</td>
</tr>
<tr>
<td>Day 0</td>
<td>60 ± 2.4 dB</td>
<td>68 ± 2.5 dB</td>
<td>49 ± 2.0 dB</td>
<td>54 ± 3.8 dB</td>
<td>69 ± 5.2 dB</td>
<td>61 ± 3.2 dB</td>
</tr>
<tr>
<td>(p = 0.003)</td>
<td>(p&lt;0.001)</td>
<td>(p = 0.003)</td>
<td>(p = 0.006)</td>
<td>(p&lt;0.001)</td>
<td>(p&lt;0.001)</td>
<td></td>
</tr>
<tr>
<td>Day 4</td>
<td>38 ± 2.9 dB</td>
<td>45 ± 3.1 dB</td>
<td>25 ± 0 dB</td>
<td>28 ± 3.8 dB</td>
<td>19 ± 5.2 dB</td>
<td>23 ± 9.2 dB</td>
</tr>
<tr>
<td>(p = 0.056)</td>
<td>(p = 0.261)</td>
<td>(p = 0.242)</td>
<td>(p = 1.0)</td>
<td>(p = 0.1)</td>
<td>(p = 0.034)</td>
<td></td>
</tr>
<tr>
<td>Day 8</td>
<td>44 ± 3.8 dB</td>
<td>54 ± 4.3 dB</td>
<td>26 ± 4.3 dB</td>
<td>29 ± 1.3 dB</td>
<td>17 ± 9.5 dB</td>
<td>13 ± 10 dB</td>
</tr>
<tr>
<td>(p = 0.013)</td>
<td>(p = 0.046)</td>
<td>(p = 0.326)</td>
<td>(p = 0.374)</td>
<td>(p = 0.2)</td>
<td>(p = 0.09)</td>
<td></td>
</tr>
<tr>
<td>Day 16</td>
<td>31 ± 8.9 dB</td>
<td>40 ± 5.2 dB</td>
<td>26 ± 4.3 dB</td>
<td>29 ± 3.2 dB</td>
<td>11 ± 2.5 dB</td>
<td>11 ± 7.5 dB</td>
</tr>
<tr>
<td>Day 32</td>
<td>30 ± 5.0 dB</td>
<td>39 ± 2.4 dB</td>
<td>21 ± 4.0 dB</td>
<td>28 ± 5.0 dB</td>
<td>10 ± 3.8 dB</td>
<td>11 ± 7.5 dB</td>
</tr>
<tr>
<td>Day 64</td>
<td>31 ± 5.5 dB</td>
<td>39 ± 5.0 dB</td>
<td>21 ± 5.2 dB</td>
<td>28 ± 2.4 dB</td>
<td>9 ± 2.9 dB</td>
<td>11 ± 7.5 dB</td>
</tr>
</tbody>
</table>
6.4.1.3: Post-saline Group

This section presents the ABR thresholds for the animals treated with saline after the acoustic trauma via stimulation of the contralateral (non-lesioned) ear (Table 6.6) and the ipsilateral (lesioned) ear (Fig. 6.3 and Table 6.7).

The one-way ANOVA with repeated measures determined that there was no significant effect of acoustic trauma on the ABR thresholds of the contralateral (non-lesioned) ear. The between-subjects effects analysis showed that there was a main effect of frequency, $F (5, 12) = 24.812, p<0.001$. The within-subjects effects analysis showed that there was no main effect of time-point, $F (3.224, 38.693) = 2.675, p = 0.057$. However, there was an interaction between time-point and frequency, $F (16.122, 38.693) = 2.137, p = 0.027$. The Tukey’s test showed that overall the ABR thresholds at 1 kHz were significantly higher than the ABR thresholds at 4 kHz, 8 kHz, 16 kHz and 32 kHz (all $p<0.001$), and the ABR thresholds at 2 kHz were significantly higher than the ABR thresholds at 4 kHz, 8 kHz, 16 kHz and 32 kHz (all $p<0.001$). In addition, the ABR thresholds at 4 kHz was higher than 16 kHz ($p = 0.039$) and 32 kHz ($p = 0.046$). The pair-wise comparison analysis showed that there were no significant differences between the overall pre-lesion ABR thresholds compared to the subsequent time-points, therefore no follow up independent samples t-tests were performed.
TABLE 6.6: MEAN THRESHOLD VALUES ± SEM (SPL (dB)) OF ABR AUDIOGRAMS OF POST-SALINE RATS (CONTRALATERAL/NON-LESIONED EAR) (N = 5). A ONE-WAY ANOVA WITH REPEATED MEASURES WAS USED TO COMPARE PRE-LESION VALUES TO DIFFERENT ABR TIME-POINTS (RECOVERY TIME-POINTS) AT THE SAME FREQUENCY.

<table>
<thead>
<tr>
<th>Post-saline</th>
<th>Frequency (kHz)</th>
<th>1 kHz</th>
<th>2 kHz</th>
<th>4 kHz</th>
<th>8 kHz</th>
<th>16 kHz</th>
<th>32 kHz</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-lesion</td>
<td>43 ± 3.3 dB</td>
<td>45 ± 2.9 dB</td>
<td>23 ± 3.3 dB</td>
<td>20 ± 0.0 dB</td>
<td>8 ± 1.6 dB</td>
<td>10 ± 0.0 dB</td>
</tr>
<tr>
<td></td>
<td>Day 0</td>
<td>43 ± 3.3 dB</td>
<td>45 ± 2.9 dB</td>
<td>27 ± 3.3 dB</td>
<td>27 ± 3.3 dB</td>
<td>13 ± 3.3 dB</td>
<td>10 ± 5.5 dB</td>
</tr>
<tr>
<td></td>
<td>Day 4</td>
<td>43 ± 3.3 dB</td>
<td>43 ± 3.3 dB</td>
<td>23 ± 3.3 dB</td>
<td>18 ± 1.7 dB</td>
<td>13 ± 3.3 dB</td>
<td>15 ± 2.9 dB</td>
</tr>
<tr>
<td></td>
<td>Day 8</td>
<td>43 ± 3.3 dB</td>
<td>43 ± 3.3 dB</td>
<td>23 ± 3.3 dB</td>
<td>17 ± 3.3 dB</td>
<td>17 ± 3.3 dB</td>
<td>15 ± 2.9 dB</td>
</tr>
<tr>
<td></td>
<td>Day 16</td>
<td>30 ± 5.8 dB</td>
<td>37 ± 3.3 dB</td>
<td>20 ± 0.0 dB</td>
<td>22 ± 1.7 dB</td>
<td>17 ± 3.3 dB</td>
<td>20 ± 5.8 dB</td>
</tr>
<tr>
<td></td>
<td>Day 32</td>
<td>30 ± 5.8 dB</td>
<td>38 ± 4.4 dB</td>
<td>18 ± 1.7 dB</td>
<td>18 ± 1.7 dB</td>
<td>15 ± 2.9 dB</td>
<td>13 ± 3.3 dB</td>
</tr>
<tr>
<td></td>
<td>Day 64</td>
<td>35 ± 7.6 dB</td>
<td>38 ± 4.4 dB</td>
<td>18 ± 1.7 dB</td>
<td>18 ± 1.7 dB</td>
<td>15 ± 2.9 dB</td>
<td>13 ± 3.3 dB</td>
</tr>
</tbody>
</table>

The one-way ANOVA with repeated measures determined that for the ipsilateral (lesioned) ear there was a significant effect of acoustic trauma on the ABR thresholds. The between-subjects effects analysis showed that there was a main effect of frequency, F (5, 12) = 127.930, p<0.001. The within-subjects effects analysis showed that there was a main effect of time-point, F (6, 72) = 10.090, p<0.001. There also was a significant interaction between time-point and frequency, F (30, 72) = 1.819, p = 0.02. The Tukey’s test showed that overall the ABR thresholds at 1 kHz were significantly higher than the ABR thresholds at 4 kHz, 8 kHz, 16 kHz and 32 kHz (all p<0.001) and the ABR thresholds at 2 kHz were significantly higher than the ABR thresholds at 4 kHz, 8 kHz, 16 kHz and 32 kHz (all p<0.001). In addition, the thresholds at 4 kHz was higher than 16 kHz (p = 0.002) and 32 kHz (p<0.001) and the thresholds at 8 kHz was higher than 32 kHz (p<0.001). Pair-wise comparison analyses showed that the overall pre-lesion ABR thresholds were significantly lower than the overall ABR thresholds at Day 0 (p<0.001), Day 4 (p = 0.01) and Day 8 (p = 0.011), Day 32 (p = 0.012) and Day 64 (p = 0.001). An independent samples t-test was used to determine the specific differences between ABR
threshold shifts at each frequency that was shown to be significant by the pair-wise comparison analysis. The results of these tests are presented in Table 6.6. The t-test showed that after acoustic trauma (Day 0); there was a significant increase in ABR thresholds at 16 kHz. On Day 4, the threshold shifts of all frequencies returned to control levels. On Day 8, the threshold shifts for all frequencies remained at control levels. At Day 32, the threshold shifts for all frequencies remained at control levels except at 2 kHz, which was elevated. Threshold shifts of all frequencies returned to control levels by Day 64. Figure 6.3 shows the mean ABR audiogram threshold SPL (dB) ± SEM for each time-point.
Figure 6.3: Mean ABR audiograms (raw values of threshold SPL (dB)) ± SEM of Post-saline rats over 64 days (n = 3). Full coloured lines indicate various ABR time-points (or recovery time-points) (see figure legend). Dotted Black line indicates the pre-lesion ABR values. P values are presented in Table 6.7 below.

### TABLE 6.7: MEAN THRESHOLD VALUES ± SEM (SPL (dB)) OF ABR AUDIOGRAMS OF POST-SALINE RATS (IPSILATERAL/LESIONED EAR) (N = 3). A ONE-WAY ANOVA WITH REPEATED MEASURES WAS USED TO COMPARE PRE-LESION VALUES TO DIFFERENT ABR TIME-POINTS (RECOVERY TIME-POINTS) AT THE SAME FREQUENCY, *P<0.05. IF NO SIGNIFICANCE WAS DETERMINED BY THE TUKEY’S TEST; FOLLOW UP T-TESTS WERE NOT PERFORMED AND HENCE THE ABSENCE OF P VALUES.

<table>
<thead>
<tr>
<th>Post-saline</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 kHz</td>
</tr>
<tr>
<td>Pre-lesion</td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>53 ± 1.7 dB (p = 0.109)</td>
</tr>
<tr>
<td>Day 4</td>
<td>35 ± 2.9 dB (p = 0.826)</td>
</tr>
<tr>
<td>Day 8</td>
<td>37 ± 4.4 dB (p = 1.0)</td>
</tr>
<tr>
<td>Day 16</td>
<td>37 ± 4.4 dB (p = 1.0)</td>
</tr>
<tr>
<td>Day 32</td>
<td>47 ± 1.7 dB (p = 0.225)</td>
</tr>
<tr>
<td>Day 64</td>
<td>50 ± 2.9 dB (p = 0.184)</td>
</tr>
</tbody>
</table>
6.4.1.4: Post-ALA Group

This section presents the ABR thresholds for the animals treated with ALA after the acoustic trauma via stimulation of the contralateral (non-lesioned) ear (Table 6.8) and the ipsilateral (lesioned) ear (Fig. 6.4 and Table 6.9).

The one-way ANOVA with repeated measures determined that there was no significant effect of acoustic trauma on the ABR thresholds of the contralateral (non-lesioned) ear. The between-subjects effects analysis showed that there was a main effect of frequency, F (5, 12) = 6.672, p = 0.003. The within-subjects effects analysis showed that there was no main effect of time-point, F (2.867, 34.402) = 1.744, p = 0.178. There was no interaction between time-point and frequency, F (14.334, 34.402) = 2.137, p = 0.336. The Tukey’s test showed that overall the ABR thresholds at 1 kHz were significantly higher than the ABR thresholds at 16 kHz (all p = 0.016) and the ABR thresholds at 2 kHz were significantly higher than the ABR thresholds at 16 kHz (p = 0.007) and 32 kHz (p = 0.026).

<table>
<thead>
<tr>
<th>Post-ALA</th>
<th>Frequency</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1 kHz</td>
</tr>
<tr>
<td>Pre-lesion</td>
<td>30 ± 5.8 dB</td>
</tr>
<tr>
<td>Day 0</td>
<td>30 ± 5.0 dB</td>
</tr>
<tr>
<td>Day 4</td>
<td>32 ± 1.7 dB</td>
</tr>
<tr>
<td>Day 8</td>
<td>33 ± 6.7 dB</td>
</tr>
<tr>
<td>Day 16</td>
<td>30 ± 5.8 dB</td>
</tr>
<tr>
<td>Day 32</td>
<td>33 ± 6.7 dB</td>
</tr>
<tr>
<td>Day 64</td>
<td>33 ± 8.8 dB</td>
</tr>
</tbody>
</table>

**Table 6.8: Mean threshold values ± SEM (SPL (dB)) of ABR audiograms of post-ALA rats (contralateral/non-lesioned ear) (N = 3). A one-way ANOVA with repeated measures was used to compare pre-lesion values to different ABR time-points (recovery time-points) at the same frequency.**
The one-way ANOVA with repeated measures determined that for the ipsilateral (lesioned) ear there was a significant effect of acoustic trauma on the ABR thresholds. The between-subjects effects analysis showed that there was a main effect of frequency, $F(5, 12) = 58.087$, $p<0.001$. The within-subjects effects analysis showed that there was a main effect of time-point, $F(6, 72) = 11.308$, $p<0.001$. There also was a significant interaction between time-point and frequency, $F(30, 72) = 4.661$, $p<0.001$. The Tukey’s test showed that overall the ABR thresholds at 1 kHz were significantly higher than the ABR thresholds at 4 kHz, 8 kHz, 16 kHz and 32 kHz (all $p<0.001$) and the ABR thresholds at 2 kHz were significantly higher than the ABR thresholds at 4 kHz, 8 kHz, 16 kHz and 32 kHz (all $p<0.001$). In addition, the thresholds at 4 kHz were higher than 16 kHz ($p = 0.001$) and 32 kHz ($p<0.001$) and the thresholds at 8 kHz were higher than 16 kHz ($p = 0.001$) and 32 kHz ($p<0.001$). Pair-wise comparison analyses showed that the overall pre-lesion ABR thresholds were significantly lower than the overall ABR thresholds at Day 0 ($p<0.001$), Day 4 ($p = 0.004$) and Day 8 ($p = 0.035$), Day 32 ($p = 0.009$) and Day 64 ($p = 0.007$). An independent samples t-test was used to determine the specific differences between ABR threshold shifts at each frequency that were shown to be significant by the pair-wise comparison analysis. The results of these tests are presented in Table 6.8. The t-test showed that after acoustic trauma (Day 0); there was a significant increase in ABR thresholds at 16 kHz and 32 kHz, while the remaining frequencies remained unaffected. By Day 4, the threshold shifts for all frequencies returned to control levels and this continued throughout to Day 64. These results were similar to the Post-saline treated animals. Figure 6.4 shows the mean ABR audiogram threshold SPL (dB) ± SEM for each time-point.
Figure 6.4: Mean ABR audiograms (raw values of threshold SPL (dB)) ± SEM of Post-ALA rats over 64 days (n = 3). Full coloured lines indicate various ABR time-points (or recovery time-points) (see figure legend). Dotted Black line indicates the pre-lesion ABR values. P values are presented in Table 6.9 below.

TABLE 6.9: MEAN THRESHOLD VALUES ± SEM (SPL (dB)) OF ABR AUDIOGRAMS OF POST-ALA RATS (IPSILATERAL/LESIONED EAR) (N = 3). A ONE-WAY ANOVA WITH REPEATED MEASURES WAS USED TO COMPARE PRE-LESION VALUES TO DIFFERENT ABR TIME-POINTS (RECOVERY TIME-POINTS) AT THE SAME FREQUENCY, *P<0.05. IF NO SIGNIFICANCE WAS DETERMINED BY THE TUKEY’S TEST; FOLLOW UP T-TESTS WERE NOT PERFORMED AND HENCE THE ABSENCE OF P VALUES.

<table>
<thead>
<tr>
<th>Frequency</th>
<th>Post-ALA</th>
<th>1 kHz</th>
<th>2 kHz</th>
<th>4 kHz</th>
<th>8 kHz</th>
<th>16 kHz</th>
<th>32 kHz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-lesion</td>
<td></td>
<td>32 ± 5.0 dB</td>
<td>.38 ± 5 dB</td>
<td>20 ± 2.5 dB</td>
<td>20 ± 0 dB</td>
<td>6 ± 7 dB</td>
<td>2 ± 2 dB</td>
</tr>
<tr>
<td>Day 0</td>
<td></td>
<td>45 ± 2.5 dB (p = 0.157)</td>
<td>48 ± 10 dB (p = 0.438)</td>
<td>22 ± 0 dB (p = 0.742)</td>
<td>23 ± 2.5 dB (p = 0.2)</td>
<td>35 ± 2.5 dB (p = 0.033)</td>
<td>32 ± 5.0 dB (p = 0.01)</td>
</tr>
<tr>
<td>Day 4</td>
<td></td>
<td>28 ± 0 dB (p = 0.423)</td>
<td>38 ± 2.5 dB (p = 1.0)</td>
<td>32 ± 0 dB (p = 0.118)</td>
<td>28 ± 5 dB (p = 0.199)</td>
<td>17 ± 0 dB (p = 0.193)</td>
<td>8 ± 2.5 dB (p = 0.059)</td>
</tr>
<tr>
<td>Day 8</td>
<td></td>
<td>30 ± 2.5 dB (p = 0.423)</td>
<td>37 ± 5 dB (p = 0.667)</td>
<td>25 ± 10 dB (p = 0.678)</td>
<td>30 ± 2.5 dB (p = 0.07)</td>
<td>13 ± 5.0 dB (p = 0.316)</td>
<td>15 ± 5.0 dB (p = 0.091)</td>
</tr>
<tr>
<td>Day 16</td>
<td></td>
<td>42 ± 5.0 dB (p = 0.122)</td>
<td>42 ± 10 dB (p = 0.074)</td>
<td>22 ± 5.0 dB (p = 0.225)</td>
<td>20 ± 2.5 dB (p = 1.0)</td>
<td>5 ± 2.0 dB (p = 0.957)</td>
<td>4 ± 0 dB (p = 0.707)</td>
</tr>
<tr>
<td>Day 32</td>
<td></td>
<td>47 ± 5.0 dB (p = 0.094)</td>
<td>48 ± 0 dB (p = 0.208)</td>
<td>25 ± 0 dB (p = 0.225)</td>
<td>20 ± 2.5 dB (p = 1.0)</td>
<td>8 ± 2.5 dB (p = 0.582)</td>
<td>2 ± 0 dB (p = 1.0)</td>
</tr>
</tbody>
</table>

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Chapter Six: Study Four
6.4.1.5: Comparing ABR threshold shifts of Pre-ALA vs. Pre-saline groups

The comparison between the Pre-ALA and Pre-saline treated animals was made to assess the effect of the pre-treatment of ALA on threshold shifts after NIHL. These results show animals pre-treated with ALA experienced greater threshold shifts compared to their saline counterparts (Fig. 6.5A-F).

A GLM two-way ANOVA with repeated measures analysis determined that threshold shifts differed statistically significantly between days, $F(2.939, 141.049) = 99.104$, $p<0.001$. Day 0 had the highest threshold shifts (29.25 dB) when compared to Day 4 (9.43 dB), Day 8 (9.01 dB), Day 16 (1.85 dB), Day 32 (6.6 dB) and Day 64 (2.6 dB). There was a significant interaction between days and group, $F(2.939, 141.049) = 19.335$, $p<0.001$. A significant interaction between days and frequency, $F(14.693, 141.049) = 5.255$, $p<0.001$, was observed. There was no significant interaction between days, groups and frequency, $F(14.693, 141.049) = 0.518$, $p = 0.925$.

The between subjects analysis indicated that there was no main effect of group, $F(1, 48) = 0.109$, $p = 0.743$. There was a main effect of frequency, $F(5, 48) = 4.587$, $p = 0.002$. Post hoc tests (LSD) indicated that overall, the threshold shifts at 32 kHz were significantly higher than the threshold shifts at 1 kHz ($p = 0.031$), 2 kHz ($p = 0.024$), 4 kHz ($p = 0.001$) and 8 kHz ($p<0.001$). In addition, the threshold shifts at 16 kHz were significantly higher than the threshold shifts at 4 kHz ($p = 0.019$) and 8 kHz ($p = 0.005$). There was no interaction between group and frequency, $F(5, 48) = 0.917$, $p = 0.478$.

Immediately after acoustic trauma (Day 0), Pre-ALA animals showed greater threshold shifts than Pre-saline animals at 4 kHz, 8 kHz, 16 kHz and 32 kHz (Fig. 6.5A). On Day 4, Pre-ALA animals showed a lower threshold shift than Pre-saline animals at 8 kHz, while the threshold shifts at the other frequencies were similar between the two groups.
(Fig. 6.5B). On Day 8, there were no differences between the threshold shifts of Pre-ALA and Pre-saline animals (Fig. 6.5C); the threshold shifts for each frequency were similar between groups. On Day 16, there were no differences between the threshold shifts of Pre-ALA and Pre-saline animals, threshold shifts for each group had almost returned to control levels (Fig. 6.5D). On Day 32, the threshold shifts of the Pre-ALA animals were lower than those of the Pre-saline animals at 1 kHz and 2 kHz, while the threshold shifts at the remaining frequencies were similar between groups (Fig. 6.5E). On Day 64, threshold shifts almost returned to control levels, with no differences between Pre-ALA and Pre-saline groups (Fig. 6.5F).
Figure 6.5: The ABR Threshold Shifts (SPL (dB)) for each frequency. Recorded on (A) Day 0, (B) Day 4, (C) Day 8, (D) Day 16, (E) Day 32 and (F) Day 64 after acoustic trauma. A GLM two-way ANOVA with repeated measures analysis was used to compare the ABR threshold shifts of Pre-saline animals to Pre-ALA animals expressed as mean ± SEM.
6.4.1.6: Comparing ABR threshold shifts of Post-ALA vs. Post-saline groups

The comparison between the Post-ALA and Post-saline treated animals was made to assess the effect of the post-treatment of ALA on threshold shifts after NIHL. These results show animals post-treated with ALA experienced similar threshold shifts compared to their saline counterparts across frequencies and across time (Fig. 6.6A-F).

A GLM two-way ANOVA with repeated measures analysis determined that threshold shifts differed statistically significantly between days, F (5, 120) = 17.453, p<0.001. Day 0 had the highest threshold shifts (14.25 dB) when compared to Day 4 (5.77 dB), Day 8 (5.63 dB), Day 16 (1.44 dB), Day 32 (5.36 dB) and Day 64 (6.61 dB). There was no significant interaction between day and group, F (5, 120) = 0.640, p = 0.670. There was significant interaction between day and frequency, F (25, 120) = 6.429, p<0.001. There was no significant interaction between day, group and frequency, F (25, 120) = 0.708, p = 0.841. The between subjects analysis indicated that there was no main effect of group, F (1, 24) = 0.02, p = 0.969 and no main effect of frequency, F (5, 24) = 0.637, p = 0.673. There was significant interaction between group and frequency, F (5, 24) = 0.035, p = 0.999. At Day 0, both groups had similar hearing loss after the acoustic trauma (Fig. 6.6A). By Day 4, 1 kHz and 2 kHz in both groups had returned to control levels, while the threshold shifts at the remaining frequencies were higher than control levels and were similar between groups (Fig. 6.6B). This pattern was continued to Day 8 (Fig. 6.6C). On Day 16, both groups had almost returned to control levels (Fig. 6.6D). At Day 32 and Day 64, the threshold shifts for both groups were similar (Fig. 6.6E-F).
Figure 6.6: The ABR Threshold Shifts (SPL (dB)) for each frequency. Recorded on (A) Day 0, (B) Day 4, (C) Day 8, (D) Day 16, (E) Day 32 and (F) Day 64 after acoustic trauma. A GLM two-way ANOVA with repeated measures analysis was used to compare the ABR threshold shifts of Post-saline animals to Post-ALA animals expressed as mean ± SEM.
6.4.1.7: Comparing ABR Threshold Shifts of Pre-ALA vs. Post-ALA Groups

This comparison was made to highlight the threshold shift differences between Pre-ALA and Post-ALA treated animals after NIHL. These results show that pre-ALA animals experienced greater threshold shifts compared to post-ALA animals (Fig. 6.7A-F).

A GLM two-way ANOVA with repeated measures analysis determined that threshold shifts differed significantly between days, F (3.492, 125.714) = 69.916, p<0.001. Day 0 had the highest threshold shifts (29.41 dB) when compared to Day 4 (7.02 dB), Day 8 (7.43 dB), Day 16 (2.89 dB), Day 32 (2.91 dB) and Day 64 (3.0 dB). There were interactions between day and group, F (3.492, 125.714) = 23.195, p<0.001; between day and frequency, F (17.460, 125.714) = 4.813, p<0.001, and between day, group and frequency, F (17.460, 125.714) = 2.221, p = 0.006.

The between-subjects effect analysis indicated that there was no main effect of frequency, F (5, 36) = 2.449, p = 0.052. There was a main effect of group, F (1, 36) = 4.414, p = 0.043, which indicated that the Pre-ALA treated animals had an overall higher SIV (10.117) compared to Post-ALA treated animals (6.556) (p = 0.043). There was no interaction between group and frequency, F (5, 36) = 0.463, p = 0.801.

On Day 0, Pre-ALA animals showed greater threshold shifts than Post-ALA animals at 4 kHz, 8 kHz, 16 kHz and 32 kHz (Fig. 6.7A). At Day 4, there were no differences between the threshold shifts of Pre-ALA and Post-ALA animals, as both groups almost returned to control levels (Fig. 6.7B). On Day 8, Pre-ALA animals showed a greater threshold shift than Post-ALA animals at 1 kHz and 2 kHz and also on Day 8, Pre-ALA animals showed a lesser threshold shift than Post-ALA animals at 8 kHz (Fig. 6.7C). By Day 16, 32 and 64 there were no differences between the threshold shifts of Pre-ALA and Post-ALA animals, as the threshold shifts at most frequencies were near control levels (Fig. 6.7D-F).
Figure 6.7: The ABR Threshold Shifts (SPL (dB)) for each frequency. Recorded on (A) Day 0, (B) Day 4, (C) Day 8, (D) Day 16, (E) Day 32 and (F) Day 64 after acoustic trauma. A GLM two-way ANOVA with repeated measures analysis was used to compare the ABR threshold shifts of Pre-ALA animals to Post-ALA animals expressed as mean ± SEM.
6.4.1.8: Comparing ABR Threshold Shifts of Pre-saline vs. Post-saline Groups

This comparison was made to highlight the differences between Pre-saline and Post-saline treated animals in threshold shifts after NIHL. Results show Pre-saline animals experienced greater threshold shifts compared to the Post-saline (Fig. 6.8A-F).

A GLM two-way ANOVA with repeated measures analysis determined that threshold shifts were significantly different between days, $F (3.137, 112.919) = 24.923$, $p<0.001$. Day 0 had the highest threshold shifts ($18.58$ dB) when compared to Day 4 ($9.10$ dB), Day 8 ($8.06$ dB), Day 16 ($0.50$ dB), Day 32 ($9.35$ dB) and Day 64 ($5.21$ dB). There was a significant interaction between day and group, $F (3.137, 112.919) = 3.239$, $p = 0.023$. There was significant interaction between day and frequency, $F (15.683, 112.919) = 2.854$, $p = 0.001$. There was no significant interaction between day, group and frequency, $F (15.683, 112.919) = 1.210$, $p = 0.272$. The between-subjects effect analysis indicated that there was no main effect of group, $F (1, 36) = 3.589$, $p = 0.066$ and no main effect of frequency, $F (5, 36) = 1.337$, $p = 0.271$. There was no significant interaction between group and frequency, $F (1, 36) = 0.393$, $p = 0.851$. On Day 0, Pre-saline animals had a higher threshold shift at $8$ kHz compared to the Post-saline groups (Fig. 6.8A). By Day 4, the threshold shifts of both groups were similar (Fig. 6.8B). On Day 8, Pre-saline animals showed a greater threshold shift than Post-saline animals at $1$ kHz, while the threshold shifts of the other frequencies remained similar (Fig. 6.8C). By Day 16, the threshold shifts of all frequencies returned to control levels in both groups (Fig. 6.8D). At Day 32 and Day 64 there were no differences between the threshold shifts (Fig. 6.8D-F).
Figure 6.8: The ABR Threshold Shifts (SPL (dB)) for each frequency. Recorded on (A) Day 0, (B) Day 4, (C) Day 8, (D) Day 16, (E) Day 32 and (F) Day 64 after acoustic trauma. A GLM two-way ANOVA with repeated measures analysis was used to compare the ABR threshold shifts of Pre-saline animals to Post-saline animals expressed as mean ± SEM.
6.4.2: Animal Weight

Animals were weighed to determine the amount of ALA to be administered. Not only did this allow the correct dose to be delivered to each animal but also to monitor the animals’ weight for welfare reasons. The normal weight gain rate of a Long Evans rat in young adulthood is 3-4 grams per day (Evans et al., 2005). This data indicated that animals treated with ALA weighed significantly less than their saline counterparts over time. Figure 6.9A presents the mean weight expressed as a percentage (of initial animal weight) of all Pre-saline vs. Pre-ALA treated animals obtained on each day of injection (i) and ABR test. Figure 6.9B presents the mean weight expressed as a percentage (of initial animal weight) of all Post-saline vs. Post-ALA treated animals obtained on each day of injection and ABR test. The Pre-ALA treated animals weighed significantly less compared to the Pre-saline treated animals, on the 5th day (99% compared to 102% (p = 0.012)), 6th day (99% compared to 102% (p = 0.005)), 7th day/Day 0 ABR test (100% compared to 102% (p = 0.025)) and Day 4 ABR test (98% compared to 103% (p = 0.001)) of ALA administration and on Day 8 ABR test (100% compared to 104% (p = 0.002)) (Fig. 6.9A). The Post-ALA treated animals weighed significantly less compared to the Post-saline treated animals, on the 8th day (98% compared to 100% (p = 0.032)), 9th day/Day 8 ABR test (98% compared to 101% (p = 0.043)), 10th day (99% compared to 101% (p = 0.044)), 11th day (99% compared to 101% (p = 0.041)) and 12th day (99% compared to 102% (p = 0.019)) of ALA administration (Fig. 6.9B). Over the time course, both Pre- and Post-ALA treated animals gained less weight compared to their saline counterparts. However when ALA administration discontinued, the Post-ALA treated animals weighed significantly more compared to the Post-saline treated animals (127% compared to 120% (p = 0.003)) at Day 64 (Fig. 6.9B).
Figure 6.9: Mean Weight (expressed as a percentage of baseline) ± SEM of ALA treated rats (n = 8) vs. saline treated rats (n = 8). A univariate GLM was used to (A) Compare Pre-saline and Pre-ALA groups and (B) compare Post-saline and Post-ALA groups. Abbreviations; # (i) = day # of injection, *p<0.05.
6.5: DISCUSSION

This investigation has assessed whether ALA is an effective preventative measure or post-treatment for hearing loss that is associated with acoustic trauma and NIHL. In this study, I have induced a moderate NIHL in young adult rats, treated the animals with ALA or saline before or after the acoustic trauma, and recorded the ABR thresholds over a 64-day recovery period. I have demonstrated that animals pre-treated with ALA experience greater threshold shifts compared to animals pre-treated with saline, and that post-treatment of ALA has no effect on the ABR thresholds after NIHL. As an aside, this study has shown that animals administered with ALA before and after acoustic trauma show significant reduction in weight compared to their saline counterparts.

The Effect of Moderate NIHL on Hearing Thresholds

The degree of hearing loss produced by exposure to a sound is dependent on the volume of the sound and the duration of the exposure. TTS are hearing losses that recover within weeks to months following exposure to a loud sound and are usually observed immediately following exposure (Puel et al., 1998, Syka and Rybalko, 2000, Bauer, 2003). If the sound causes sufficient damage to the cochlea it may produce permanent elevations in thresholds resulting in PTS (Clark, 1991).

The acoustic trauma stimulus employed in this study caused an immediate increase in threshold shifts. On Day 0, the threshold shifts ranged from 10-39 dB in Pre-saline animals, 26-62 dB in the Pre-ALA animals, and 5-24 dB in the Post-saline animals and 2-30 dB in Post-ALA animals. It was clear that the acoustic trauma utilised in this study does not induce PTS, as the ABR threshold shifts of most animals returned to pre-exposure levels by Day 4. This observation at all Day 4 time-points suggests that most hearing function has recovered after NIHL. Some fluctuations in hearing threshold were
observed over time in some groups; however, from Day 4 through to Day 64 the threshold shifts were similar to pre-exposure levels. Thus, the acoustic trauma stimulus used in this study clearly causes TTS. This was in contrast to the acoustic trauma used in the other studies contributing to this thesis (16 kHz noise at 112 dB for 1 minute vs. 16 kHz noise at 115 dB for 1 hour), which induced PTS. The more moderate acoustic trauma stimulus used in this investigation was intended to mimic a more realistic experience of NIHL.

The initial threshold shifts observed in this investigation may be a result of mild damage to the peripheral auditory system. The immediate changes that occur in IHCs and OHCs of the cochlea are quite variable; see (Nordmann et al., 2000) for extensive review. Mild effects to the hair cell are found after moderate exposures of short duration and these appear to return to normal after a short period. Effects that have been observed include an increase in the amount of OHC smooth endoplasmic reticulum (Engstrom et al., 1970, Ward and Duvall, 1971, Bohne et al., 1973, Lim and Dunn, 1979) and a proliferation accompanied by disorganisation of the subsurface cisternae and Hensen's bodies (Slepecky and Chamberlain, 1982). The observation of the Day 4 recovery observed in most groups may suggest that the animals used in this study experienced “mild” damage to the hair cells that returned to normal by four days after the acoustic trauma.

Others who have used a similar acoustic trauma treatment measured hearing only once after the exposure (Davis et al., 1950, Patuzzi and Thompson, 1991); therefore the recovery of hearing in those studies is unknown. This study supports work by Davis (1950) and Pattuzzi (1991) that indicate that acoustic trauma stimuli of short durations (one minute) are capable of causing immediate hearing loss.
The Effect of ALA Treatment on Hearing Threshold Shifts

Pre-ALA Treatment

This study has shown that animals treated with ALA before moderate NIHL had significantly greater hearing losses compared to their saline counterparts immediately after acoustic trauma. This finding was unexpected since Pre-ALA treated animals have been shown to display reduced hearing losses compared to their saline counterparts (Diao et al., 2003). On Day 4, both groups returned to similar hearing threshold levels and the similarities in hearing threshold shifts continued throughout the sixty-four days after acoustic trauma, with some fluctuations of different frequencies. The only difference between Pre-ALA and Pre-saline treated animals was that Pre-ALA animals experienced a greater hearing loss initially when compared to their saline counterparts. This seems to suggest that ALA administered before acoustic trauma was not beneficial as a pre-treatment for noise exposure but instead detrimental. Previously, this has not been shown with ALA or other antioxidants. Investigations into the effect of ALA on NIHL are limited to just one study (Diao et al., 2003). Diao and colleagues exposed sixty guinea-pigs to a 4 kHz octave noise at 115 dB for 5 hours and measured the ABR of ALA treated (10 mg/kg/day- one day prior to acoustic trauma) animals compared to saline treated controls. Diao reported a significant reduction in ABR thresholds in the ALA treated animals compared to the saline treated animals. Diao observed that ALA treated animals had lower levels of NO in the cochlea tissue, had higher TAC in the cochlea serum and experienced a protective effect from the ALA. When comparing this study to Daio’s investigation, ALA appears to have an otoprotective effect when animals were treated with less ALA (10 mg/kg/day for one day vs. 50 mg/kg/day for seven days) and when the acoustic trauma was more excessive (4 kHz noise at 115 dB for 5 hours vs. 16 kHz noise at 112 dB for 1 minute). The higher dose and length of administration may have played a role in decreasing the effectiveness of ALA in preventing NIHL. Although
there are no animal studies that have reported ALA toxicity at 50 mg/kg/day, in vitro studies have shown that lipoate (the conjugate base of ALA) can be toxic to human lymphocytes (Sen et al., 1997). Sen’s study observed that the application of a high concentration (2 and 5 millimolar (mM)) of lipoate on lymphocytes caused cell shrinkage, GSH depletion and DNA fragmentation effects in vitro. The higher dose of ALA used in the present study may have depleted GSH levels in the hair cells, causing the Pre-ALA treated animals to be more vulnerable to acoustic trauma. Therefore, this could be a possible mechanism for the increased hearing loss in animals pre-treated with ALA, although GSH levels were not measured in the cochlea during ALA administration and thus this proposal is merely speculative.

In addition, the ALA may have accumulated in the animals that led to toxic levels. The no-observed-adverse-effect-level is considered to be 60 mg/kg/day for rats (Cremer et al., 2006a). Throughout the duration of the investigation, the pre-treated animals would have received a total of 350 mg/kg of ALA before acoustic exposure. This level may have cause toxicity within the cochlea, which could have led to an increase in vulnerable hair cells. However, the pre-treatment of ALA on its own did not affect normal hearing levels, as the non-lesioned ear had normal hearing before and after the acoustic trauma.

Post-ALA Treatment

In most instances, one cannot predict when an acoustic trauma event will occur. Therefore, a post-treatment for NIHL is desirable. This is the first study to investigate the effects of ALA in animals after moderate NIHL. Animals post-treated with ALA or saline did not show any significant differences over the two months after acoustic trauma. Other studies have shown that post-treatment of antioxidant compounds are effective in reducing the severity of NIHL. Allopurinol and OTC were effective when administered immediately after acoustic trauma (Seidman et al., 1993, Yamasoba et al.,
1998, Cassandro et al., 2003, Franze et al., 2003). Edavarone, NAC, Methionine and ALCAR were effective when administered up to between 4 and 9 hours after acoustic trauma, (Kopke et al., 2000, Takemoto et al., 2004, Kopke et al., 2005, Tanaka et al., 2005, Campbell et al., 2007, Coleman et al., 2007, Campbell et al., 2011). Alpha-tocopherol was effective in reducing ABR thresholds when administered three days after acoustic trauma (Hou et al., 2003, Yamashita et al., 2005). All of these studies used a severe acoustic trauma method. ALA has been shown to protect against oxidative damage by scavenging ROS and RNS (Whiteman et al., 1996). The moderate acoustic trauma stimulus used in this study may not have caused an increase of free radicals in the IHCs and OHCs. Therefore, no significant differences were observed in either post-treated groups, as there were no (or was a shortage of) free radicals for the ALA to scavenge. This suggests that either the acoustic trauma stimulus used in this study was not capable of causing cochlear damage or that post-treatment of ALA was ineffective in treating NIHL after moderate acoustic exposure.

Pre- vs. Post-ALA Treatment

This study has shown that animals treated with saline or ALA before the acoustic trauma results in greater TTS overall compared to animals treated with saline or ALA after the acoustic trauma. The Pre-ALA animals had significantly higher threshold shifts when compared to the Post-ALA animals, which was unexpected, as it has been shown that ALA protects against acoustic trauma. The Pre-saline animals also had significantly higher threshold shifts when compared to the Post-saline animals, which was also unexpected as both of those groups served as controls. However, the threshold shifts in both groups returned to near pre-lesion levels throughout the time course. The only variations between the groups were the administration of ALA/saline (before or after acoustic trauma), the duration of administration (seven or fourteen days) and the number
of animals in each group (five or three animals per group). The ABR tests for each group (Pre-saline, Pre-ALA, Post-saline and Post-ALA) were performed on different days, but all animals within those individual groups were tested on the same days (pre-lesion, Day 0, Day 4, Day 8, Day 16, Day 32 and Day 64). Variability of the results may have occurred due to the batch differences of the animals and the low group numbers.

ALA has been shown to be an effective pre-treatment for NIHL when used at a lower dose compared to the dose used this study and when the acoustic trauma stimulus was longer in duration. Therefore, a lower dose of pre- and post-ALA could be administered, and the animals could be exposed to a longer acoustic trauma stimulus. If a lower dose is given after a longer acoustic trauma, the post-effects of ALA may prove effective.

The Effect of ALA on Body Weight

A study by Kim and colleagues (2004) showed that ALA might act as an anti-obesity agent (Kim et al., 2004c). These authors reported that ALA reduced food intake and body weight gain in a dose dependent manner when administered to Sprague–Dawley rats via intraperitoneal injection and in their diet, respectively. The anti-obesity effect in the Kim study was observed at 3 dosage levels (approx. 0.25%, 0.5% and 1% wt./wt. in rat chow); the rats lost more weight the higher the ALA concentration in the food. The amount of ALA/rat chow consumed by the rats in each dosage group was not estimated or measured, nor was food-intake monitored. This reveals a major limitation in Kim’s study. Based on my calculations, the concentration of ALA consumed by the rats in Kim’s study was relatively high (0.25% = 170-200 mg/kg/day, 0.5% = 340-400 mg/kg/day and 1.0% = 680-800 mg/kg/day), based on the average weight (358 ± 6g) and daily food consumption (84 ± 4 kcal/day) of a normal Sprague-Dawley rat consuming a standard laboratory rat chow (3.3 kcal/g) (Evans et al., 2005). Kim showed that the weight loss due to ALA administration was not a result of systemic toxicity, induced
illness, or conditioned taste aversion. Instead, was involved in the central regulation of food intake and energy expenditure.

The findings of this investigation support Kim’s study in that ALA affects the weight gain of ALA treated animals. Kim found that after two days, animals that had ALA in their diet had decreased in weight compared to their saline counterparts. The rats used in this study experienced weight loss at day 5 in Pre-ALA animals (which were injected with ALA for seven days) and at day 8 in Post-ALA animals (which were injected with ALA for fourteen days). Kim also injected animals with 50 mg/kg/day, 75 mg/kg/day or 100 mg/kg/day of ALA to observe if food intake was affected. The study showed that animals injected with 50 mg/kg/day of ALA did not reduce their food intake, unlike the rats that received >75/mg/kg/day of ALA. Therefore, the weight loss observed in this study (also using 50 mg/kg/day) was most likely not due to a decrease in food intake but rather an increase in metabolic activity or energy expenditure. The difference in the onset of weight loss may be due to the concentration of ALA administered. This study, however, delivered a known, lower concentration via a subcutaneous injection, compared to Kim’s study that introduced the ALA into the rat chow at varying doses (170-800 mg/kg/day). The high doses would have influenced the food intake and therefore a less amount of chow consumed.

Kim also found that rats rapidly gained weight on termination of the treatment. Similar results were observed in this study: the body weights of the treated animals increased rapidly, to similar weights of the saline treated animals. The Pre-ALA weights always remained slightly under the Pre-saline weights up to Day 64. Whereas, the Post-ALA animals exceeded the weight of the Post-saline animals at Day 64. A reason for this difference could be that the Post-ALA animals received one week more of ALA.
injections compared to the Pre-ALA animals. This may suggest a rebound effect of the energy expenditure system after longer periods of ALA treatment.

This investigation has shown that rats injected with 50 mg/kg/day of ALA for a period of seven or fourteen days weigh significantly less than their saline counterparts do during the course of administration. These findings suggest that the weight loss observed in this study was due to increased energy expenditure in the animals and not a decrease in food intake.

In summary, this study provides the first 64-day recovery investigation of the effects of ALA administration before and after acoustic trauma utilising a rat model of unilateral NIHL. I have investigated the development of ABR threshold shifts, in order to gain a better understanding of ALA and NIHL. My findings suggest that ALA may not be an effective pre or post-treatment for moderate NIHL. Specifically, the initial hearing losses in the Pre-ALA treated animals may suggest that longer durations of treatment and higher doses of ALA may play a role in cochlear toxicity, which leads to hair cell vulnerability. However, more evidence is required to infer that ALA is indeed ototoxic at these levels. The similarities of the hearing threshold of both Post-ALA and Post-saline groups suggests that either post-treatment of ALA was ineffective in treating NIHL after moderate acoustic exposure, or that the acoustic trauma stimulus provided insufficient damage to produce increases in free radicals for the ALA to scavenge. As an aside, I have shown that the pre and post-administration of ALA significantly reduces weight in ALA-treated animals when compared to their saline counterparts, by increasing energy expenditure.
CHAPTER SEVEN

FINAL DISCUSSION
7.1: SUMMARY OF AIMS, HYPOTHESES AND FINDINGS

It has been previously shown that significant changes occur in the auditory pathway after NIHL. However, the research has been conducted on many different species and has utilised a variety of methodologies. Comparing results and observations from each investigation in most cases is unproductive due to the vast number of variables. This is the first study that has made an investigation into the changes that occur over the time course of a month in the rat following NIHL, comparing the electrophysiological changes, behavioural manifestations of tinnitus, and protein expression in animals that have experienced the same set of test conditions. In addition, I have also investigated the effects of a therapeutic agent, ALA, on the prevention and treatment of NIHL. The results of this study highlight the occurrence of significant changes in the physiological properties of neurons, protein expression, and manifestations of tinnitus and hyperacusis in the rat after NIHL, and that these changes vary over one month. This study indicates a novel finding in that the Day 8 time-point may be a significant period, as it appeared that the auditory system had partially recovered. However, the mechanisms involved in this recovery may have been maintained and reacted in excess after this period, and be responsible for the subsequent decline in improvement observed at Day 16 and Day 32.

The overall aim of this thesis was to investigate the one month time-course of physiological and molecular changes in the rat auditory pathway after NIHL, and correlate any changes with behavioural manifestation of tinnitus, in the hope to identify potential windows of opportunity for therapeutic intervention. This aim has been divided into four specific hypotheses.
The Effect of NIHL on the Physiological Properties of Auditory Neurons

The first specific hypothesis of this study was that NIHL would lead to immediate changes in SA and excitability in the AC, IC and DCN and that the changes would persist over the one month time-course. To test this hypothesis, I performed in vivo electrophysiological recordings in the contralateral AC, contralateral IC and ipsilateral DCN in the young adult rat up to 32 days following NIHL. The stimulus-driven activity (indicator of excitability) and SA were measured and quantified to assess the changes that occurred in each auditory site. The hypothesis was supported by the results of this study, as I have demonstrated that NIHL significantly alters the normal levels of SA and excitability in the auditory pathway of the rat over the one month time-course. My findings support and extend previous investigations that changes in excitability and SA occur after NIHL (see section 1.2.1). Specifically, this study has suggested that small subsets of remaining functional neurons may compensate for the lack of input in the auditory system after NIHL by increasing in excitability. This study also suggests that the lack of significant changes in the medium frequency region of the AC may be due to deafferentation after acoustic trauma. In addition, exposure to the acoustic trauma stimulus used in this study may result in an increase in excitability in the high and low frequency regions of the AC, due to the loss of surround inhibition that “unmasks” the excitatory inputs which were present in the surrounding regions of the AC (Rajan, 1998). This “unmasking” of excitatory inputs may trigger a compensatory inhibitory response in an attempt to reduce increases in excitation. The emergence of NON MON responses could be an indication of this mechanism. Also, the fluctuations observed in stimulus-driven activity and SA, could be another indicator of this attempt to restore normal levels of excitatory/inhibitory processing. The resulting fluctuations may subsequently play a role in either the development or generation of tinnitus, and the
reorganisation of tonotopic maps, and may be a result of changes previously shown in excitatory and inhibitory transmitter systems (refer to table 1.6 in section 1.3.1.3). These changes may serve as part of the underlying mechanisms that lead to acoustic disorders following NIHL.

The Effect of NIHL on Protein Expression

The second specific hypothesis of this study was that NIHL would lead to an increase in excitatory and neuroplasticity marker expression and a decrease in inhibitory marker expression in the AC, IC and DCN over the one month time-course. To test this hypothesis, I utilised western blot techniques to quantify the expression of GABA\(_{A}\alpha 1\), GAD-67, NR2A, Calb1 and GAP-43 in the ipsilateral and contralateral AC, IC and DCN of the young adult rat up to 32 days following NIHL. I have demonstrated that NIHL significantly alters the normal levels of excitatory, inhibitory and neuroplasticity related proteins in the auditory pathway of the rat over the one month time-course. The hypothesis was not completely supported by the results of this investigation. The results of this study suggest that the characteristic decrease of inhibition, that previous researchers have observed after acoustic trauma, may not necessarily be due to significant increases of excitatory neurotransmission-related proteins (refer to section 1.3.1.3); rather it may be that a significant decrease of inhibitory-neurotransmission related proteins leads to an overall increase in excitation. In addition to confirming changes in excitatory and inhibitory-neurotransmission related protein expression, I have shown for the first time that the contralateral AC may play a role in compensation after ipsilateral NIHL. However, the changes observed in the dominant and non-dominant auditory pathways may be a result of the hearing loss itself, or the changes could be a part of a mechanism that serves to restore normal hearing function. Changes in the
ipsilateral and contralateral AC, IC and DCN may reflect a homeostatic mechanism that attempts to re-balance excitatory and inhibitory transmission following NIHL.

The Correlation between Physiological and Molecular Changes after NIHL

In this study, limited correlations could be made between the electrophysiological changes (stimulus-driven activity and SA) and the molecular changes (excitatory, inhibitory and neuroplasticity markers/proteins). However, other protein changes (not investigated in this study) may be responsible for the physiological changes observed after NIHL. This study has demonstrated that NIHL significantly alters the normal levels of excitatory and inhibitory neurotransmission-related proteins and neuroplasticity-related proteins in the auditory pathway of the rat over the one month time-course. I propose that these changes in protein expression may assist other molecular markers to serve as part of the molecular mechanism underlying significant physiological changes that lead to acoustic disorders following NIHL.

The Effects of NIHL on the Development of Tinnitus and Hyperacusis

The third specific hypothesis of this study was that following NIHL, behavioural manifestations of tinnitus would be evident in rats, and the manifestations would vary across animals and across time. To test this hypothesis, I measured the behavioural manifestations of tinnitus in noise-exposed rats using a gap-detection startle reflex behavioural technique up to 32 days following NIHL. This study has identified specific frequency perceptions of tinnitus and hyperacusis following NIHL. In addition, it has highlighted the variations of the perceived frequency of tinnitus across time-points and in individual animals, and yet also highlighted the similarities in the chronic tinnitus spectrum. This study confirms that the rat-model of NIHL I utilised can produce tinnitus and hyperacusis. The data of this investigation supports the initial hypothesis, as I have
shown that NIHL leads to the perception of tinnitus immediately after acoustic trauma and causes a fluctuating chronic tinnitus in the rat. The fluctuations of the perceived tinnitus frequency observed in this study are analogous to that which occur in humans, as many tinnitus patients have reported the perception of fluctuating frequencies over time (Erlandsson et al., 1992, Hallberg and Erlandsson, 1993, Tullberg and Ernberg, 2006). If the chronic tinnitus frequency/frequencies can be somewhat predicted based on the most significant immediate changes in gap-detection performance after NIHL, then those particular frequencies may be focused on immediately; for example, the application of auditory-retraining therapies in the aim of potentially attenuating the development of chronic tinnitus. A small group of animals developed hyperacusis-like responses, which has been reported in other studies (Parrish and Turner, 2008, Turner et al., 2012). This may reflect a compensatory mechanism by the auditory system to increase central gain.

The Effect of ALA Treatment on Hearing Thresholds after NIHL

The fourth and final specific hypothesis of this study was that animals treated with ALA would exhibit less hearing loss than controls (saline treated) after moderate NIHL, as determined by auditory thresholds, and the animals treated with ALA before the acoustic trauma would have a reduced hearing loss compared to animals treated with ALA after the acoustic trauma. To test this hypothesis, I induced a moderate NIHL in young adult rats, treated the animals with ALA or saline before or after the acoustic trauma, and recorded the ABR thresholds over a 64-day recovery period. On the whole, the hypothesis was not supported by the results of this study. This investigation has shown that ALA is not an effective pre or post treatment in a rat model of moderate NIHL. In this study, I have demonstrated that animals pre-treated with ALA experience greater threshold shifts compared to animals pre-treated with saline and that post-treatment of
ALA has no effect on the ABR thresholds after NIHL. Specifically, the initial hearing losses in the Pre-ALA treated animals may suggest that longer durations of treatment and higher doses of ALA may play a role in cochlea toxicity, which leads to hair cell vulnerability. The similarities of the hearing threshold of both Post-ALA and Post-saline groups suggests that either post treatment of ALA is ineffective in treating NIHL after moderate acoustic exposure, or that the acoustic trauma stimulus was not damaging enough to produce increases in free radicals for the ALA to scavenge. As an aside, I have shown that the pre and post administration of ALA significantly reduces weight in ALA-treated animals when compared to their saline counterparts, by increasing energy expenditure.
7.2: SUMMARY OF TIME-COURSE CHANGES

This investigation has identified significant time-dependent changes in a rat model of NIHL. Significant molecular, physiological and behavioural changes were observed over a one month time-course after NIHL (Fig. 7.1). This study suggests that following NIHL, a compensatory mechanism serves to re-balance neural activity. This compensatory mechanism appears to achieve a partially recovered state one week after NIHL. This is a novel finding and indicates that a mechanism around this time-point partially restores normal auditory function. However, a decline in improvement, observed at subsequent time-points, suggests that the mechanism/s involved in this transient recovery may continue and lead to maladaptive changes throughout the auditory system. Alternatively, the decline in improvement could be the result of delayed degenerative changes that are known to occur around the one-week time-point after acoustic trauma and NIHL (Kim et al., 2004a, Lin et al., 2011). These maladaptive changes caused by a prolonged compensatory mechanism, plus the subsequent degenerative changes, may be the underlying mechanism/s of NIHL, and cause or contribute to the generation of auditory disorders such as tinnitus and hyperacusis.
Figure 7.1: Summary of Time-Dependent Changes that occur in the rat (dominant auditory pathway) after NIHL.
Day 0

Immediately after NIHL (Day 0), all animals experienced severe hearing losses and this was accompanied by significant changes in auditory neuron physiology. This finding supports previous investigations that also observed immediate physiological alterations within the auditory pathway after NIHL (Noreña and Eggermont, 2003, Noreña et al., 2003). Significant decreases in stimulus-driven activity and SA were observed in all regions investigated which have also been documented in previous studies after NIHL (Kaltenbach et al., 2000, Tan et al., 2007). Decreases in stimulus-driven activity and SA may serve as an initial neuroprotective mechanism in response to the acoustic insult to prevent overstimulation and excitotoxicity. However, it may also be due to the severe hearing losses the animals experienced, or the decreases in stimulus-driven activity and SA in auditory nerve fibres which has been shown after acoustic trauma (Liberman and Kiang, 1978, Rajan et al., 1993, Norena et al., 2002, Noreña and Eggermont, 2003, Noreña et al., 2003). Though there appeared to be a cessation of neural activity, the remaining neuron populations that were functional in each region increased in excitability, suggesting a mechanism that may serve to maintain a critical level of neural activity. This supports previous work by Gerken and colleagues (1984), who showed that individual neurons, within the IC and CN of cats, became hypersensitive to electric stimulation after hearing loss, and these changes were persistent up to thirty days after NIHL (Gerken et al., 1984).

Though the animals in the current investigation exhibited low levels of overall neural activity, some of the animals exhibited behavioural manifestations of tinnitus, as gap-detection deficits were evident. The most significant deficits were at the low and high frequencies and this shows that the immediate effects of NIHL were not centred on the acoustic trauma spectrum itself (16 kHz). This is in agreement with other studies that
have shown that the range of tinnitus spectrum shifts to other frequencies outside the acoustic trauma frequency range. For example: a 10 kHz tinnitus after a 16 kHz centred noise trauma (Turner et al., 2006), a 24 kHz and 32 kHz tinnitus after a 17 kHz centred noise trauma (Wang et al., 2009), a 10 kHz tinnitus after a 16 kHz centred noise trauma (Middleton et al., 2011), a 20 kHz and 25 kHz tinnitus after a 16 kHz centred noise trauma (Longenecker and Galazyuk, 2011), and a 24 kHz tinnitus after a 16 kHz centred noise trauma (Turner et al., 2012). This suggests that after NIHL (at any centred frequency), the frequency region most affected by the acoustic trauma is not involved in the generation of tinnitus but rather the surrounding frequency regions are. This is supported by the lack of neural hyperexcitability and SA changes observed in the medium frequency region of the AC and the significant fluctuations of neural hyperexcitability and SA in the high and low frequency regions of the AC in the electrophysiological study of this thesis. Tinnitus is thought to be generated by increases of SA and neural hyperexcitability (section 1.2.1); however, the perception of tinnitus could not be attributed to these factors as there was an overall decrease in neuronal excitability and in SA at this time-point. This could suggest that the observed increases in excitability of the remaining functional neurons may have played a role in the generation of the tinnitus in these animals, and may imply that the perception of tinnitus originates from a small subset of aberrant neurons rather than a larger population.

The remaining functional neuronal populations may have experienced significant fluctuations in excitatory and inhibitory proteins; this would have contributed to the change in neuronal excitability. The fact that only a decrease in Calb1 expression was found in the DCN supports the physiological findings of the overall decrease in stimulus-driven activity and in SA. The observation that only Calb1 decreased significantly in the DCN in this study may suggest that it is the first auditory site that
experiences molecular changes after NIHL. There were no significant changes in other excitatory or inhibitory markers at this time-point. This may be due to the low level of neurons/neuronal populations exhibiting increased excitability or it may suggest that other molecular markers (not investigated in this thesis) were involved in changing the physiological state of the neurons. Dong and colleagues (2010) showed that in the ipsilateral CN (lesioned side), the expression of Glycine Receptor Subunit alpha 1 and the Potassium Channel Subfamily K member 15 were decreased immediately after a continuous 10-kHz pure tone trauma at 124 dB SPL for 1 hour. Dong’s findings support the theory of this current study, that other molecular markers may be involved in altering the physiological state of the neurons in the auditory pathway.

Day 4

At Day 4, the animals had moderate to severe hearing losses, with some animals exhibiting a slight recovery of hearing. An overall reduction was observed in the level of excitability, similar to a study by Tan and colleagues (2007), where there were decreases in stimulus-driven activity four days after NIHL (Tan et al., 2007). Though stimulus-driven activity was reduced, the overall SA levels were increased compared to the previous time-point. This may suggest that there is a delayed compensatory mechanism that serves to restore normal levels of stimulus-driven activity and SA. This mechanism was proposed by Schaette and Kempter (2006), in that regions that exhibit decreased levels of stimulus-driven activity after acoustic trauma would increase in SA (Schaette and Kempter, 2006). The excitability of the functional neurons was significantly increased, indicating a hyperexcitable state, which supports the work performed by Gerken and colleagues (1984) and may suggest that the remaining functional neurons increase in excitability to compensate for the overall decrease in neural activity. Though SA was slightly increased compared to the previous time-point, there were no gap-
detection deficits in any of the animals; however, one animal exhibited a hyperacusic-like response. This was a reduced startle response to gap + startle trials after acoustic trauma when compared to the startle response to gap + startle trials before the acoustic trauma, and has been shown in similar studies (Parrish and Turner, 2008, Turner et al., 2012). Hyperacusis is thought to be caused by an increase in central gain (Parrish and Turner, 2008), suggesting that the mechanism involved in increasing central gain may also be involved in increasing the levels of SA and the excitability of the remaining functional neurons. This may support the speculation that tinnitus and hyperacusis share a common underlying pathophysiology (Nelson and Chen, 2004). However, not all animals exhibited hyperacusic-like responses; therefore, the slight recovery of hearing may explain the better performance in gap-detection.

At this time-point, GAD-67 was found to have decreased in the DCN. It is known that GAD-67 is directly involved in the production of GABA. Therefore, a decrease in GAD-67 would ultimately lead to a decrease in inhibitory processes resulting in an increase in excitation. This change in GAD-67 expression could be responsible for the slight increase observed in SA levels. This suggests that the decrease in GAD-67 may serve as, or contribute to, a compensatory mechanism that restores the levels of excitability and SA after NIHL. Previous studies have shown a decrease in the expression of GAD-67 in the IC after acoustic trauma (Abbott et al., 1999, Pouyatos et al., 2004), however, there has been no investigation into the changes in the DCN.

Day 8

The Day 8 group had moderate hearing losses and some animals appeared to have recovered slightly from the NIHL. Fig. 7.2 is a composite graph of the ABR audiograms of all animals used in this thesis. This figure highlights the slight hearing recovery which
is similar across all animals. This finding has been reported in other studies around a similar time-point (Yamashita et al., 2008, Ftoni et al., 2009). The observed recovery of the Day 8 group may suggest a combination of central and peripheral events playing a role in the temporary recovery of hearing function after NIHL. This is in agreement with findings which suggest that peripheral recovery can occur, followed by central degenerative changes (Kim et al., 2004a, Lin et al., 2011) or changes in excitatory transmission (Muly et al., 2004). Though there was a slight hearing recovery, the animals still exhibited decreases in overall excitability, similar to the previous time-point. All regions exhibited an overall increase in SA compared to control and/or Day 0 levels. These results again support Schaette and Kempter’s (2006) proposed compensatory mechanism of the gradual increase of the SA to restore normal levels of stimulus-driven activity and SA after NIHL. As previously mentioned, there was a marked recovery of hearing loss compared to the other time-points. This could suggest that SA initially contributes to the recovery of hearing. Similar to the previous findings in this study, the excitability of the remaining functional neurons were significantly increased suggesting the continuation of a hyperexcitable state, similar to Gerken’s observations (1984). Though there was an increase in SA and excitability in the remaining functional neurons, there were no animals that exhibited tinnitus or hyperacusis–like responses. In addition, there were no significant molecular changes. This suggests that the Day 8 time-point may be a potential therapeutic window, as the hearing had recovered most compared to other time-points (Fig. 7.2) and the animals did not exhibit behavioural manifestations of tinnitus or hyperacusis-like responses. It appears that at this time-point the auditory system has partially recovered after NIHL.
Figure 7.2: ABR audiograms (± SEM) compiled from all animals used in this thesis. All hearing thresholds in graph had a p value <0.02 when compared to pre-lesion values using a student’s t-test.

Day 16

An increase in hearing loss was observed compared to the previous time-point. This has been previously shown in guinea-pigs exposed to severe acoustic trauma, where partial recovery of hearing thresholds was observed (threshold shifts less than 40 dB) one week after NIHL. Subsequently, at the two week time-point, the threshold shifts were increased (threshold shifts greater than 40 dB) (Sendowski et al., 2006). Though, as previously mentioned, partial hearing recovery was observed in Yamashita’s (2008) and Fetoni’s (2009) studies around the 7-10 day recovery period, both of these researchers did not measure hearing loss after that time. This highlights that some previous studies, which have investigated the changes of the auditory pathway after NIHL, were limited by the fact that research was not continued past an acute time-point (i.e. one week). This study has shown that significant changes do occur over one month and that these changes vary at each time-point. Therefore, this highlights the novelty of this current investigation by providing an extended time-course examination of the changes that occur after NIHL. There are many studies that investigate immediate changes and also
long-term changes, with none addressing time-points between 7-14 days. This study has identified that this may be a crucial period and could impact the interpretation of previous findings.

The overall levels of excitability were decreased, however the SA was slightly increased compared to the previous time-point. This further supports the notion that a delayed compensatory mechanism occurs to restore normal levels of stimulus-driven activity by increasing SA. The Day 16 group showed no differences in excitability in the remaining functional neuronal populations; however the threshold responses were much lower than control, suggesting that the neuronal populations had become more sensitive to sound. This could explain why one animal exhibited hyperacusis-like responses and no animals showed behavioural manifestations of tinnitus. This was a similar finding to Turner and colleagues (2012), who showed that exposed mice exhibited hyperacusis-like responses 2-3 weeks after NIHL (Turner et al., 2012). Since there was an increased sensitivity to sound, the remaining functional neurons were not required to become hyperexcitable to compensate for the decrease in neural activity.

Though the remaining functional neurons did not become hyperexcitable, a unique response was identified, the NON MON responses. The presence of NON MON responses may indicate that the excitability of the neurons was being affected by an inhibitory influence, which is supported by the lack of hyperexcitability observed in the remaining functional neurons. This could suggest a mechanism where there is an increase of inhibitory processes to reduce the increased levels of SA, and also in this case, the stimulus-driven activity. However, this cannot be confirmed by the molecular findings, as there was only a significant decrease in $\text{GABA}_A\alpha1$ expression in the AC. In addition, in the western blot study the specific frequency regions were not tested, but rather the proteins in the AC as a whole were analysed. Therefore, it is possible that the
molecular investigation within the AC may not have been sensitive enough to identify changes within the different frequency regions. The presence of NON MON responses could not be linked with the changes in SA or overall excitability.

The emergence of the NON MON responses, changes in inhibitory protein expression, hyperacusis-like responses, and the slight increase in hearing thresholds (compared to Day 8) could suggest that unseen significant changes occur between Day 8 and Day 16, as the Day 8 group appeared to exhibit indicators of partial recovery and the Day 16 group showed a decline in each aspect of the study. The mechanisms that lead to the partial recovery at Day 8 may have resulted in the maladaptive changes observed at Day 16 by overshooting. The decline in recovery after the Day 8 time-point may be due to oxidative stress from the formation of ROS and RNS in the auditory system. It has been shown that free radicals have a delayed formation, reaching peak levels between day seven and ten after NIHL (Yamashita et al., 2004). This increase in free radicals in the periphery may have contributed to the increased loss of hearing, which was observed at Day 16 and Day 32. At a similar time-point, a peak in mononuclear phagocytes has been shown to occur in the cochleae of mice after NIHL (Hirose et al., 2005). Hirose and colleagues proposed that the inflammatory response plays a role in propagating cellular damage in the peripheral nervous system. This delayed inflammatory response may have contributed to the decline after the Day 8 partial recovery period. The degeneration of spiral ganglion cells within the cochlea is also known to be delayed after NIHL (Kujawa and Liberman, 2009). Investigating the central and peripheral changes that occur between Day 8 and Day 16 would be valuable for identifying the exact time-point when the auditory system begins to decline after the partial recovery period.
Day 32

Severe hearing losses were apparent in the animals measured. Others who have used a similar acoustic trauma treatment have shown similar results, in that hearing thresholds remain elevated for months after NIHL (Bauer, 2003, Turner et al., 2006). The overall excitability was slightly increased and the SA decreased (compared to the previous time-point), which further supports the proposed delayed compensatory mechanism serving to restore normal levels of excitability and SA. The animals also demonstrated an increased excitability in the remaining functional neuronal populations and the threshold responses were lower than control. This is a similar finding, compared to the previous time-point, in that the neuronal populations may have become more sensitive to sound. This could suggest a mechanism that serves to increase neural activity by increasing the sensitivity of the remaining functional neurons. This increased sensitivity may have resulted in the slight increase of excitability that was observed in most regions. Also, the decrease in the expression of GABA$_{\alpha}1$ in the AC, may have additionally contributed to the increase of excitability. NON MON responses were evident in most regions, suggesting a slow-acting inhibitory mechanism that serves to restore normal levels of neural activity in response to increased levels of excitation.

At this time-point, animals experienced tinnitus at the high and low frequencies, which were the same frequencies that were shown to be most affected in the Day 0 animals. This supports the previous findings that chronic tinnitus frequencies are established a few weeks after acoustic trauma and are similar to the frequencies that changed most significantly immediately after NIHL (Longenecker and Galazyuk, 2011, Turner et al., 2012).
7.3: Future Directions and Improvements

This current study has identified that the Day 8 time-point may be an important recovery period after NIHL. Understanding the changes that occur specifically between Day 4 and Day 8, and between Day 8 and Day 16, may reveal crucial information about the underlying mechanisms of the auditory system and its response to NIHL. Investigating the changes at and around this time-point may provide a deeper understanding into why the auditory system was ineffectual to maintain the recovery that was observed at Day 8.

Previous investigations into the physiological changes of auditory regions have researched extensively into the changes in the tonotopic map after NIHL and may be highly interrelated with stimulus-driven activity and SA. To date, there has been no investigation into the time-course of changes in tonotopic representation after NIHL. Understanding how SA and the excitability state of the auditory system lead to or are caused by tonotopic reorganisation would be beneficial to the research field.

Though significant fluctuations were observed in the molecular markers after NIHL, there was insufficient evidence to directly link the changes in excitatory/inhibitory/neuroplasticity related proteins/markers to the changes observed in the physiological and behavioural findings in this thesis. Therefore, it is important to investigate additional molecular markers. These could include: other CBPs such as Parvalbumin and Calretinin, other subunits of the NMDA and GABA receptors, other excitatory and inhibitory neurotransmission-related receptors and the appropriate neurotransmitters, and other neuroplasticity markers such as Activity-regulated Cytoskeleton Protein. This would provide a deeper understanding into the relationship between molecular and physiological mechanisms of auditory neurons after NIHL.
Future behavioural studies investigating the manifestations of tinnitus after NIHL would benefit from ensuring that all animals used in the study exhibited a 20-30% reduction in startle when presented with a gap of silence in an otherwise constant background. This confirmation may be crucial to the development of tinnitus in a small subset of animals. As behavioural experiments on animals are usually quite variable, more animals should be used to ensure a homogenous dataset.
7.4: CONCLUSIONS

My thesis has investigated the one month time-course changes of molecular, physiological and behavioural changes in the young adult rat after NIHL. This investigation has presented novel findings in that significant time-dependent changes occur in the auditory pathway after NIHL: the physiological changes over the one month time-course reflect a constant compensatory mechanism between excitability and SA; the molecular changes indicate fluctuations in excitatory and inhibitory neurotransmission-related systems over the one month time-course; and the behavioural manifestations of tinnitus highlight the variability of animals’ perceptions after NIHL. This study has proposed that the Day 8 time-point may be a crucial period after NIHL, whereby the auditory system attains a state of partial recovery. This thesis proposes that the identification of the partial recovery period may impact the interpretation of previous investigations in this field. Understanding the mechanism/s of the subsequent decline in improvement that occurs between Day 8 and 16 and up to Day 32 is of utmost importance for gaining a deeper knowledge of the underlying mechanisms of NIHL. Interpreting these mechanisms will be crucial for understanding the generation and development of acoustic disorders such as tinnitus and hyperacusis, and potentially the development of a therapeutic intervention.
APPENDIX 1

(CHAPTER 3 SUPPLEMENT)
**A1.1: DAY 16 ANIMAL**

**ABR Audiogram**

The ABR audiogram (Fig. A1.1.1) was recorded directly before NIHL and on the day of the experiment (sixteen days after NIHL). The low thresholds across frequencies tested of the pre-NIHL ABR (black line) indicated that this animal had good hearing before the trauma and the post-NIHL ABR audiogram (grey line) indicated that the animal experienced moderate hearing loss sixteen days after the trauma, with slightly less hearing at the lower frequencies.

![ABR Audiogram Graph](image)

*Figure A1.1.1: Individual ABR Audiogram of the ipsilateral (lesioned) ear for the Day 16 animal.*

**Stimulus-driven Response Profiles**

All neural responses were identified in this animal (Table A1.1.1). $\text{MON}^{N/SAT}$, $\text{MON}^{SAT}$ and NON MON responses were observed in the low frequency region of the AC and in the IC. $\text{MON}^{SAT}$ and NON MON responses were observed in the medium frequency...
region of the AC and MON<sub>N/SAT</sub> responses were observed in the DCN. The high frequency region of the AC was not recorded from as the animal died prematurely.

In the medium frequency region of the AC, MON<sup>SAT</sup> and NON MON responses were observed (Fig. A1.1.2 and Table A1.1.1). The NON MON responses were more abundant compared to MON<sup>SAT</sup> responses. The two responses shared the same initial inflection point (threshold response); however, the initial RLF slope (gain) of the NON MON responses was higher than the MON<sup>SAT</sup> responses, which may suggest that the neuronal population exhibiting NON MON responses were more excitable. The RLF of the second slope of the NON MON responses was less than the RLF of the first slope. This indicated that Slope 1 was more excitable than Slope 2. Non-responsive channels were also present, which made up 55% of the total recording channels. These non-responsive channels were distributed throughout the recording channels with no clustering observed.

![Figure A1.1.2: The Inflection Points (threshold responses [bars]) and RLF Slopes (gain function [circles]) of the neural responses present in the medium frequency region of the AC.](image-url)

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In the low frequency region of the AC, \( \text{MON}^{\text{SAT}} \), \( \text{MON}^{\text{N/SAT}} \) and NON MON responses were identified (Fig. A1.1.3 and Table A1.1.1). The NON MON responses were more abundant compared to \( \text{MON}^{\text{SAT}} \) and \( \text{MON}^{\text{N/SAT}} \) responses. The NON MON responses had a relatively low initial inflection point (threshold response) and a low RLF slope (lower gain). The \( \text{MON}^{\text{SAT}} \) responses had a lower inflection point (lower threshold response) and a higher RLF slope (higher gain) than the \( \text{MON}^{\text{N/SAT}} \) responses. This was a common finding when both \( \text{MON}^{\text{SAT}} \) and \( \text{MON}^{\text{N/SAT}} \) responses were identified in the same recording. Non-responsive channels were also present, which made up 22% of the total recording channels. These were found throughout the recording channels.

![Figure A1.1.3: The Inflection Points (threshold responses [bars]) and RLF Slopes (gain function [circles]) of the neural responses present in the low frequency region of the AC.](image)

In the IC, \( \text{MON}^{\text{SAT}} \), \( \text{MON}^{\text{N/SAT}} \) and NON MON responses were observed (Fig. A1.1.4 and Table A1.1.1). The NON MON responses were more abundant compared to \( \text{MON}^{\text{SAT}} \) and \( \text{MON}^{\text{N/SAT}} \) responses. The NON MON responses had a relatively low initial inflection point (lower threshold response) and a low RLF slope (lower gain). The \( \text{MON}^{\text{SAT}} \) responses had a lower inflection point (lower threshold response) than the
MON\textsuperscript{NSAT} responses and a similar RLF slope. Non-responsive channels were also present, which made up 35% of the total recording channels.

**Figure A1.1.4:** The Inflection Points (threshold responses [bars]) and RLF Slopes (gain function [circles]) of the neural responses present in the IC.

In the DCN, only MON\textsuperscript{NSAT} responses were observed (Fig. A1.1.5 and Table A1.1.1). The MON\textsuperscript{NSAT} responses had a similar inflection point and RLF slope to control. Non-responsive channels were also present, which made up 91% of the total recording channels.

**Figure A1.1.5:** The Inflection points (threshold responses [bars]) and RLF Slopes (gain function [circles]) of the neural responses present in the DCN.
### TABLE A1.1.1: STIMULUS-DRIVEN RESPONSE PROFILES AND ASSOCIATED INFLECTION POINTS FOR ALL REGIONS IN THE DAY 16 ANIMAL.

<table>
<thead>
<tr>
<th>Region</th>
<th>Response Type</th>
<th>% of total recording channels</th>
<th>Inflection Point (1) = Slope 1 (2) = Slope 2</th>
<th>RLF Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC (HIGH)</td>
<td>NON MON</td>
<td>42%</td>
<td>(1) 40 ± 0.0 dB SPL (2) 63 ± 0.9 dB SPL</td>
<td>6.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>MON SAT</td>
<td>3%</td>
<td>40 ± 0.0 dB SPL</td>
<td>5.5 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>NR</td>
<td>55%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AC (MEDIUM)</td>
<td>NON MON</td>
<td>55%</td>
<td>(1) 32 ± 2.1 dB SPL (2) 61 ± 2.2 dB SPL</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>MON SAT</td>
<td>9%</td>
<td>42 ± 4.0 dB SPL</td>
<td>4.7 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>MON N/SAT</td>
<td>14%</td>
<td>56 ± 5.3 dB SPL</td>
<td>4.3 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>NR</td>
<td>22%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IC</td>
<td>NON MON</td>
<td>34%</td>
<td>(1) 39 ± 0.1 dB SPL (2) 64 ± 1.5 dB SPL</td>
<td>3.7 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>MON SAT</td>
<td>3%</td>
<td>40 ± 0.0 dB SPL</td>
<td>4.1 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>MON N/SAT</td>
<td>28%</td>
<td>60 ± 2.9 dB SPL</td>
<td>4.2 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>NR</td>
<td>35%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DCN</td>
<td>MON N/SAT</td>
<td>9%</td>
<td>60 ± 0.0 dB SPL</td>
<td>4.1 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>NR</td>
<td>91%</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
A1.2: DAY 32 ANIMAL

ABR Audiogram

The ABR audiogram (Fig. A1.2.1) was recorded directly before NIHL and on the day of the experiment (thirty-two days after NIHL). The low thresholds across frequencies tested of the pre-NIHL ABR audiogram (black line) indicated that the Day 32 animal had good hearing before the trauma; the animal had unusually low thresholds at 1 kHz and 2 kHz. The post-NIHL ABR audiogram (grey line) indicated that the animal experienced moderate hearing loss thirty-two days after the trauma.

![ABR audiogram graph]

**Figure A1.2.1: Individual ABR Audiogram of the ipsilateral (lesioned) ear for the Day 32 animal.**

Stimulus-driven Response Profiles

MON^{NSAT}, MON^{SAT} and NON MON responses were observed in all regions investigated except for the DCN, which only presented MON^{NSAT} responses (Table A1.2.1).
In the AC, MON^SAT, MON^N/SAT and NON MON responses were observed in the high frequency region (Fig. A1.2.2 and Table A1.2.1). Non-responsive channels were also present throughout the recording channels, which made up 9% of the total recording channels. These unresponsive channels had most likely failed. The MON^SAT responses were more abundant compared to NON MON and MON^N/SAT responses. The MON^SAT responses had a lower inflection point (lower threshold response) and a lower RLF slope (lower gain) when compared to the MON^N/SAT responses. The initial inflection point of the NON MON responses was low compared to other regions within the same animal. However, it was high in comparison to the initial inflection points of the NON MON responses in the IC and the medium and low frequency regions of the AC. The RLF slope was low compared to the other NON MON initial RLF slopes. The second inflection point was higher than the first inflection point and was similar to the second inflection points of the other NON MON responses. The second RLF slope was lower than the first RLF slope; however, it was similar to the other NON MON second RLF slopes.

![Figure A1.2.2: The Inflection Points (threshold responses [bars]) and RLF Slopes (gain function [circles]) of the neural responses present in the high frequency region of the AC.](image-url)
In the AC medium frequency region, $\text{MON}^{\text{SAT}}$, $\text{MON}^{\text{N/SAT}}$ and NON MON responses were observed (Fig. A1.2.3 and Table A1.2.1). Non-responsive channels were identified throughout the recording channels, which made up 28% of the total recording channels. The NON MON responses were more abundant compared to $\text{MON}^{\text{SAT}}$ and $\text{MON}^{\text{N/SAT}}$ responses. The $\text{MON}^{\text{SAT}}$ responses had a lower inflection point (lower threshold response) than the $\text{MON}^{\text{N/SAT}}$ responses and a higher RLF slope (higher gain). This was a common finding when both $\text{MON}^{\text{SAT}}$ and $\text{MON}^{\text{N/SAT}}$ responses were identified within the same recording. The initial inflection point of the NON MON responses was low compared to other regions within the same animal. However, it was similar in comparison to the initial inflection points of the NON MON responses in the IC and the low frequency regions of the AC. The RLF slope was low compared to the initial NON MON RLF slopes in the low frequency region of the AC and the IC. The second inflection point was higher than the first inflection point and was similar to the second inflection points of the other NON MON responses. The second RLF slope was lower than the first RLF slope, however was similar to the other NON MON second RLF slopes.

![Figure A1.2.3: The Inflection Points (threshold responses [bars]) and RLF Slopes (gain function [circles]) of the neural responses present in the medium frequency region of the AC.](image-url)
In the low frequency region of the AC, all three response-types were observed (Fig. A1.2.4 and Table A1.2.1). Twenty two percent of channels were not responsive; these channels were identified throughout the recording channels and were not clustered. The MON$^{\text{NSAT}}$ responses were more abundant compared to MON$^{\text{SAT}}$ and NON MON responses. The MON$^{\text{SAT}}$ responses had a lower inflection point (lower threshold response) than the MON$^{\text{NSAT}}$ responses and a higher RLF slope (higher gain). This was a common finding when both MON$^{\text{SAT}}$ and MON$^{\text{NSAT}}$ responses were identified in the same recording. The initial inflection point of the NON MON responses was low compared to other regions within the same animal; however, it was similar in comparison to the initial inflection points of the NON MON responses in the IC and the medium frequency regions of the AC. The RLF slope was higher compared to the initial NON MON RLF slopes in the high and medium frequency region of the AC. The second inflection point was higher than the first inflection point and was similar to the second inflection points of the other NON MON responses. The second RLF slope was lower than the first RLF slope, however was similar to the other NON MON second RLF slopes.

Figure A1.2.4: The Inflection Points (threshold responses [bars]) and RLF Slopes (gain function [circles]) of the neural responses present in the low frequency region of the AC.
In the IC, MON^{SAT}, MON^{NSAT} and NON MON responses were observed (Fig. A1.2.5 and Table A1.2.1). Non-responsive channels were also present, which made up 53% of the total recording channels. The MON^{NSAT} responses were more abundant compared to NON MON and MON^{SAT} responses. The MON^{SAT} responses had a lower inflection point (lower threshold response) than the MON^{NSAT} responses and a higher RLF slope (higher gain). The initial inflection point of the NON MON responses was low compared to other regions within the same animal; however, it was similar in comparison to the initial inflection points of the NON MON responses in the medium and low frequency regions of the AC. The RLF slope was high compared to the initial NON MON RLF slopes in the high and medium frequency region of the AC. The second inflection point was higher than the first inflection point and was similar to the second inflection points of the other NON MON responses. The second RLF slope was lower than the first RLF slope, however was similar to the other NON MON second RLF slopes.

Figure A1.2.5: The Inflection Points (threshold responses [bars]) and RLF Slopes (gain function [circles]) of the neural responses present in the IC.
In the DCN, only $\text{MON}^{\text{N/SAT}}$ responses were observed (Fig. A1.2.6 and Table A1.2.1). Non-responsive channels were also present, which made up 22% of the total recording channels. These unresponsive channels were most likely not working properly. The $\text{MON}^{\text{N/SAT}}$ responses had a similar inflection point (threshold response) and RLF slope when compared to the other $\text{MON}^{\text{N/SAT}}$ responses identified within the same animal.

![Figure A1.2.6: The Inflection Points (threshold responses [bars]) and RLF Slopes (gain function [circles]) of the neural responses present in the DCN.](image-url)
TABLE A1.2.1: STIMULUS-DRIVEN RESPONSE PROFILES AND ASSOCIATED INFLECTION POINTS FOR ALL REGIONS IN THE DAY 32 ANIMAL.

<table>
<thead>
<tr>
<th>Region</th>
<th>Response Type</th>
<th>% of total recording channels</th>
<th>Inflection Point (1) = Slope 1 (2) = Slope 2</th>
<th>RLF Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC (HIGH)</td>
<td>NON MON</td>
<td>13%</td>
<td>(1) 26 ± 2.6 dB SPL (2) 63 ± 1.6 dB SPL</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>MON&lt;sup&gt;NSAT&lt;/sup&gt;</td>
<td>63%</td>
<td>22 ± 0.6 dB SPL</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>MON&lt;sup&gt;N/SAT&lt;/sup&gt;</td>
<td>15%</td>
<td>67 ± 1.5 dB SPL</td>
<td>3.7 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>NR</td>
<td>9%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AC (MEDIUM)</td>
<td>NON MON</td>
<td>53%</td>
<td>(1) 20 ± 0.7 dB SPL (2) 59 ± 1.5 dB SPL</td>
<td>3.5 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>MON&lt;sup&gt;NSAT&lt;/sup&gt;</td>
<td>10%</td>
<td>22 ± 1.8 dB SPL</td>
<td>7.2 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>MON&lt;sup&gt;N/SAT&lt;/sup&gt;</td>
<td>9%</td>
<td>50 ± 1.3 dB SPL</td>
<td>2.9 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>NR</td>
<td>28%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AC (LOW)</td>
<td>NON MON</td>
<td>20%</td>
<td>(1) 21 ± 0.6 dB SPL (2) 69 ± 0.6 dB SPL</td>
<td>6.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>MON&lt;sup&gt;NSAT&lt;/sup&gt;</td>
<td>5%</td>
<td>20 ± 0.0 dB SPL</td>
<td>6.1 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>MON&lt;sup&gt;N/SAT&lt;/sup&gt;</td>
<td>53%</td>
<td>69 ± 1.3 dB SPL</td>
<td>4.7 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>NR</td>
<td>22%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IC</td>
<td>NON MON</td>
<td>16%</td>
<td>(1) 20 ± 0.0 dB SPL (2) 65 ± 5.0 dB SPL</td>
<td>6.1 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>MON&lt;sup&gt;NSAT&lt;/sup&gt;</td>
<td>31%</td>
<td>24 ± 4.0 dB SPL</td>
<td>9.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>MON&lt;sup&gt;N/SAT&lt;/sup&gt;</td>
<td>53%</td>
<td>55 ± 5.5 dB SPL</td>
<td>3.6 ± 0.4</td>
</tr>
<tr>
<td>DCN</td>
<td>MON&lt;sup&gt;N/SAT&lt;/sup&gt;</td>
<td>78%</td>
<td>45 ± 2.7 dB SPL</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>NR</td>
<td>22%</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
APPENDIX 2

(CHAPTER 5 SUPPLEMENT)
SHAM Control Animals

The two-way ANOVA showed that for the first animal (Chapter 5: Fig. 5.6A), there was no main effect of pre/post SIVs, $F(1, 80) = 1.747, p = 0.190$, and there was also no main effect of frequency, $F(3, 80) = 1.938, p = 0.131$. There was no significant interaction between frequency and pre/post SIVs, $F(3, 80) = 0.794, p = 0.501$.

The two-way ANOVA showed that for the second animal (Chapter 5: Fig. 5.6B), there was no main effect of pre/post SIVs, $F(1, 80) = 3.973, p = 0.051$, however there was a main effect of frequency, $F(3, 80) = 10.87, p<0.001$. The Tukey’s test showed that overall the pre/post SIVs at 24 kHz were significantly lower than 4 kHz ($p<0.001$), 10 kHz ($p = 0.007$) and 16 kHz ($p<0.001$). There was a significant interaction between frequency and pre/post SIVs, $F(3, 80) = 3.134, p = 0.031$. The pair-wise comparison analyses determined that the pre SIVs at 4 kHz were higher than 10 kHz ($p = 0.017$), 16 kHz ($p = 0.012$) and 24 kHz ($p<0.001$), suggesting that before the second test the animal had better gap detection at 10 kHz, 16 kHz and 24 kHz compared to 4 kHz. In addition, the post SIVs at 24 kHz were lower than 4 kHz ($p<0.001$), 10 kHz ($p = 0.001$) and 16 kHz ($p<0.001$), suggesting that after the second test the animal had better gap detection at 24 kHz compared to 4 kHz, 10 kHz and 16 kHz.

The two-way ANOVA showed that for the third animal (Chapter 5: Fig. 5.6C), there was no main effect of pre/post SIVs, $F(1, 80) = 0.917, p = 0.341$, however there was a main effect of frequency, $F(3, 80) = 5.985, p = 0.001$. The Tukey’s test showed that overall the pre/post SIVs at 24 kHz were significantly higher than 4 kHz ($p = 0.010$) and 16 kHz ($p = 0.001$). There was a significant interaction between frequency and pre/post SIVs, $F(3, 80) = 6.886, p<0.001$. The pair-wise comparison analyses determined that the pre SIVs at 10 kHz were lower than 24 kHz ($p = 0.019$), suggesting that before the second
test the animal had better gap detection at 10 kHz compared to 24 kHz. In addition, the post SIVs at 4 kHz were lower than 10 kHz \( (p = 0.001) \) and 24 kHz \( (p = 0.002) \) and 16 kHz was lower than 10 kHz \( (p<0.001) \) and 24 kHz \( (p<0.001) \). This suggests that after the second test the animal had better gap detection at 4 kHz and 16 kHz when compared to 10 kHz and 24 kHz.

The two-way ANOVA showed that for the fourth animal (Chapter 5: Fig. 5.6D), there was a main effect of pre/post SIVs, \( F (1, 80) = 4.668, p = 0.034 \), and the pair-wise comparison analysis determined that there was a significant decrease in SIV at 10 kHz \( (p = 0.01) \) after NIHL. There was no main effect of frequency, \( F (3, 80) = 1.306, p = 0.279 \) and no interaction between frequency and pre/post SIVs, \( F (3, 80) = 1.343, p = 0.267 \).

The two-way ANOVA showed that for the fifth animal (Chapter 5: Fig. 5.6E), there was a main effect of pre/post SIVs, \( F (1, 80) = 7.198, p = 0.009 \), and the pair-wise comparison analysis showed that there was a significant decrease in SIV at 24 kHz \( (p = 0.011) \) after NIHL. There was no main effect of frequency, \( F (3, 80) = 0.593, p = 0.621 \). There was an interaction between frequency and pre/post SIVs, \( F (3, 80) = 2.764, p = 0.048 \). The pair-wise comparison analysis showed that the pre SIVs at 10 kHz were lower than 24 kHz \( (p = 0.009) \), suggesting that after the second test the animal had better gap detection at 10 kHz compared to 24 kHz.

Day 0 Animals

The two-way ANOVA showed that for the first animal (Chapter 5: Fig. 5.7A), there was a main effect of pre/post SIVs, \( F (1, 80) = 59.997, p<0.001 \), and the pair-wise comparison analyses determined that there was a significant increase of SIV at 4 kHz \( (p<0.001) \), 10 kHz \( (p = 0.012) \) and 24 kHz \( (p = 0.002) \) after NIHL. There was also a main effect of frequency, \( F (3, 80) = 10.624, p<0.001 \), and the Tukey’s test showed that
the pre/post SIVs at 4 kHz were significantly higher than the pre/post SIVs at 10 kHz (p<0.001), 16 kHz (p<0.001) and 24 kHz (p = 0.001). There was also an interaction between frequency and pre/post SIVs, F (3, 80) = 9.594, p<0.001. The pair-wise comparison analyses showed that the post SIVs at 4 kHz were higher than 10 kHz (p<0.001), 16 kHz (p<0.001) and 24 kHz (p<0.001), suggesting that after NIHL the animal had poor gap detection at 4 kHz compared to 10 kHz, 16 kHz and 32 kHz.

The two-way ANOVA showed that for the second animal (Chapter 5: Fig. 5.7B), there was a main effect of pre/post SIVs, F (1, 80) = 8.326, p = 0.005, and the pair-wise comparison analyses determined that there was a significant increase of SIV at 16 kHz (p = 0.002) and 24 kHz (p = 0.033) after NIHL. However, there was no main effect of frequency, F (3, 80) = 2.537, p = 0.063. There was an interaction between frequency and pre/post SIVs, F (3, 80) = 3.773, p = 0.014. The pair-wise comparison analyses showed that the pre SIVs at 16 kHz were lower than 4 kHz (p = 0.046) and 10 kHz (p = 0.044), suggesting that before NIHL the animal had better gap detection at 16 kHz compared to 4 kHz and 10 kHz. In addition, the post SIVs at 4 kHz were lower than 10 kHz (p = 0.003) and 16 kHz (p = 0.015), suggesting that after NIHL the animal had better gap detection at 4 kHz compared to 10 kHz and 16 kHz.

The two-way ANOVA showed that for the third animal (Chapter 5: Fig. 5.7C), there was no main effect of pre/post SIVs, F (1, 80) = 0.600, p = 0.441, and there was no main effect of frequency, F (3, 80) = 0.655, p = 0.582. There was an interaction between frequency and pre/post SIVs, F (3, 80) = 3.618, p = 0.017. The pair-wise comparison analyses determined that the post SIVs at 10 kHz were lower than 4 kHz (p = 0.04) and 16 kHz (p = 0.021), suggesting that after NIHL the animal had better gap detection at 10 kHz when compared to 4 kHz and 16 kHz.
The two-way ANOVA showed that for the fourth animal (Chapter 5: Fig. 5.7D), there was a main effect of pre/post SIVs, $F (1, 80) = 20.769$, $p<0.001$, and the pair-wise comparison determined that there was a significant increase in SIV at 10 kHz ($p<0.001$) and 24 kHz ($p = 0.005$) after NIHL. There was also a main effect of frequency, $F (3, 80) = 6.048$, $p = 0.001$. The Tukey’s test determined that the pre/post SIVs at 16 kHz were significantly lower than the pre/post SIVs at 10 kHz ($p = 0.001$) and 24 kHz ($p = 0.018$). There was a significant interaction between frequency and pre/post SIVs, $F (3, 80) = 4.655$, $p = 0.005$. The pair-wise comparison test showed that the post SIVs at 10 kHz were higher than 4 kHz ($p<0.001$) and 16 kHz ($p<0.001$). Also, 24 kHz was higher than 4 kHz ($p = 0.016$) and 16 kHz ($p = 0.003$), suggesting that after NIHL the animal had poor gap detection at 10 kHz and 24 kHz compared to 4 kHz and 16 kHz.

The two-way ANOVA showed that for the fifth animal (Chapter 5: Fig. 5.7E), there was no main effect of pre/post SIVs, $F (1, 80) = 0.738$, $p = 0.393$, and there was no main effect of frequency, $F (3, 80) = 1.784$, $p = 0.158$. There was a significant interaction between frequency and pre/post SIVs, $F (3, 80) = 4.807$, $p = 0.004$. The pair-wise comparison analyses showed that the pre SIVs at 24 kHz were lower than 10 kHz ($p = 0.008$) and 16 kHz ($p = 0.003$), suggesting that before NIHL the animal had better gap detection at 24 kHz compared to 10 kHz and 16 kHz. In addition, the post SIVs at 16 kHz were lower than 10 kHz ($p = 0.013$) and 24 kHz ($p = 0.028$), suggesting that after NIHL the animal had better gap detection at 16 kHz compared to 10 kHz and 24 kHz.

Day 4 Animals

The two-way ANOVA showed that for the first animal (Chapter 5: Fig. 5.8A), there was a main effect of pre/post SIVs, $F (1, 80) = 5.238$, $p = 0.025$, and the pair-wise comparison analysis determined that there was a significant decrease of SIV at 4 kHz ($p$
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= 0.022) four days after NIHL. There was no main effect of frequency, F (3, 80) = 0.434, p = 0.729. There was no significant interaction between frequency and pre/post SIVs, F (3, 80) = 1.632, p = 0.189.

The two-way ANOVA showed that for the second animal (Chapter 5: Fig. 5.8B), there was a main effect of pre/post SIVs, F (1, 80) = 23.841, p<0.001, and the pair-wise comparison analyses determined that there was a significant decrease in SIV at 4 kHz (p = 0.008) and 10 kHz (p<0.001) four days after NIHL. There was also a main effect of frequency, F (3, 80) = 9.281, p<0.001. The Tukey’s test showed that the pre/post SIVs at 4 kHz was significantly higher than the pre/post SIVs at 16 kHz (p = 0.003) and 24 kHz (p<0.001). In addition, the pre/post SIVs 10 kHz were significantly higher than 24 kHz (p = 0.041). There was no significant interaction between frequency and pre/post SIVs, F (3, 80) = 1.206, p = 0.314.

The two-way ANOVA showed that for the third animal (Chapter 5: Fig. 5.8C), there was no main effect of pre/post SIVs, F (1, 80) = 0.030, p = 0.864, and there was no main effect of frequency, F (3, 80) = 1.168, p = 0.328. There was no significant interaction between frequency and pre/post SIVs, F (3, 80) = 0.659, p = 0.580.

The two-way ANOVA showed that for the fourth animal (Chapter 5: Fig. 5.8D), there was no main effect of pre/post SIVs, F (1, 80) = 2.510, p = 0.118, and there was a main effect of frequency, F (3, 80) = 4.264, p = 0.008. The Tukey’s test determined that the pre/post SIVs at 10 kHz were significantly higher than the pre/post SIVs at 24 kHz (p = 0.004). There was no significant interaction between frequency and pre/post SIVs, F (3, 80) = 2.591, p = 0.059.

The two-way ANOVA showed that for the fifth animal (Chapter 5: Fig. 5.8E), there was a main effect of pre/post SIVs, F (1, 80) = 5.666, p = 0.020. The pair-wise comparison
analyses showed that there was a significant decrease in SIV at 24 kHz (p = 0.044) and a significant increase in SIV at 4 kHz (p<0.001) and 10 kHz (p = 0.004) four days after NIHL. There was no main effect of frequency, F (3, 80) = 1.908, p = 0.136. There was a significant interaction between frequency and pre/post SIVs, F (3, 80) = 8.190, p<0.001. The other pair-wise comparison analyses determined that pre SIVs at 24 kHz were higher than 4 kHz (p = 0.015) and 10 kHz (p = 0.018), suggesting that before NIHL the animal had poor gap detection at 24 kHz compared to 4 kHz and 10 kHz. In addition, the post SIVs at 4 kHz were higher than 16 kHz (p<0.001) and 24 kHz (p<0.001) and 10 kHz was higher than 16 kHz (p = 0.007) and 24 kHz (p = 0.011), suggesting that after NIHL the animal had better gap detection at 16 kHz and 24 kHz compared 4 kHz and 10 kHz.

Day 8 Animals

The two-way ANOVA showed that for the first animal (Chapter 5: Fig. 5.9A), there was no main effect of pre/post SIVs, F (1, 80) = 1.657, p = 0.202, and there was no main effect of frequency, F (3, 80) = 1.107, p = 0.352. There was no significant interaction between frequency and pre/post SIVs, F (3, 80) = 1.526, p = 0.215.

The two-way ANOVA showed that for the second animal (Chapter 5: Fig. 5.9B), there was no main effect of pre/post SIVs, F (1, 80) = 2.534, p = 0.116, and there was no main effect of frequency, F (3, 80) = 0.649, p = 0.586. There was a significant interaction between frequency and pre/post SIVs, F (3, 80) = 5.004, p = 0.003. The pair-wise comparison analysis showed that the pre SIVs at 4 kHz were lower than 24 kHz (p = 0.03), suggesting that before NIHL the animal had better gap detection at 4 kHz compared to 24 kHz. In addition, post SIVs at 24 kHz were lower than 4 kHz (p = 0.04)
and 10 kHz (p = 0.002) suggesting that after NIHL the animal had better gap detection at 24 kHz compared to 4 kHz and 10 kHz.

The two-way ANOVA showed that for the third animal (Chapter 5: Fig. 5.9C), there was no main effect of pre/post SIVs, F (1, 80) = 0.577, p = 0.450, and there was no main effect of frequency, F (3, 80) = 2.150, p = 0.101. There was a significant interaction between frequency and pre/post SIVs, F (3, 80) = 5.455, p = 0.002. The pair-wise comparison showed that the pre SIVs at 24 kHz were lower than 4 kHz (p = 0.037) and 16 kHz (p = 0.005), suggesting that before NIHL the animal had better gap detection at 24 kHz when compared to 4 kHz and 16 kHz. In addition, the post SIVs at 4 kHz were lower than 10 kHz (p = 0.002) and 24 kHz (p = 0.048), suggesting that after NIHL the animal had better gap detection at 4 kHz compared to 10 and 24 kHz. Also 10 kHz was lower than 16 kHz (p = 0.005), suggesting that after NIHL the animal had better gap detection at 10 kHz compared to 16 kHz.

The two-way ANOVA showed that for the fourth animal (Chapter 5: Fig. 5.9D), there was no main effect of pre/post SIVs, F (1, 80) = 2.562, p = 0.114, and there was no main effect of frequency, F (3, 80) = 0.754, p = 0.524. There was no significant interaction between frequency and pre/post SIVs, F (3, 80) = 2.008, p = 0.120.

The two-way ANOVA showed that for the fifth animal (Chapter 5: Fig. 5.9E), there was no main effect of pre/post SIVs, F (1, 80) = 0.002, p = 0.965, and there was no main effect of frequency, F (3, 80) = 0.829, p = 0.482, nor was there a significant interaction between frequency and pre/post SIVs, F (3, 80) = 0.205, p = 0.893.
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The two-way ANOVA showed that for the first animal (Chapter 5: Fig. 5.10A), there was no main effect of pre/post SIVs, $F(1, 80) = 2.638, p = 0.109$, and there was a main effect of frequency, $F(3, 80) = 3.289, p = 0.025$. The Tukey’s test demonstrated that the pre/post SIVs at 4 kHz were significantly higher the pre/post SIVs at 24 kHz ($p = 0.025$). There was no significant interaction between frequency and pre/post SIVs, $F(3, 80) = 1.714, p = 0.172$.

The two-way ANOVA showed that for the second animal (Chapter 5: Fig. 5.10B), there was a main effect of pre/post SIVs, $F(1, 80) = 9.615, p = 0.003$, and the pair-wise comparison analyses determined that there was a significant reduction in SIV at 16 kHz ($p<0.001$) and 24 kHz ($p = 0.008$). These significant decreases could not be attributed to high pre-NIHL SIVs, as pre values were normal. This suggests better gap detection sixteen days after acoustic exposure. There was a main effect of frequency, $F(3, 80) = 3.793, p = 0.014$. The Tukey’s test showed that the pre/post SIVs at 24 kHz was significantly lower than the pre/post SIVs at 4 kHz ($p = 0.043$) and 10 kHz ($p = 0.036$).

There was also a significant interaction between frequency and pre/post SIVs, $F(3, 80) = 3.824, p = 0.013$. The pair-wise comparison analyses showed that the post SIVs at 4 kHz were higher than 16 kHz ($p = 0.001$) and 24 kHz ($p = 0.001$) and 10 kHz was higher than 16 kHz ($p = 0.002$) and 24 kHz ($p = 0.002$). This suggests that after NIHL, the animal had poor gap detection at 4 kHz and 10 kHz compared to 16 kHz and 24 kHz.

The two-way ANOVA showed that for the third animal (Chapter 5: Fig. 5.10C), there was no main effect of pre/post SIVs, $F(1, 80) = 0.851, p = 0.359$, and there was no main effect of frequency, $F(3, 80) = 0.689, p = 0.561$. There was no significant interaction between frequency and pre/post SIVs, $F(3, 80) = 1.651, p = 0.185$. 299
The two-way ANOVA showed that for the fourth animal (Chapter 5: Fig. 5.10D), there was no main effect of pre/post SIVs, $F(1, 80) = 3.250, p = 0.076$. However, there was a main effect of frequency, $F(3, 80) = 2.747, p = 0.049$. The Tukey’s test showed that the pre/post SIVs at 24 kHz was significantly higher than the pre/post SIVs at 4 kHz ($p = 0.037$). There was also a significant interaction between frequency and pre/post SIVs, $F(3, 80) = 3.251, p = 0.027$. The pair-wise comparison test determined that the post SIVs at 4 kHz were lower than 16 kHz ($p = 0.004$) and 24 kHz ($p = 0.002$), suggesting that after NIHL the animal had better gap detection at 4 kHz compared to 16 kHz and 24 kHz. Also 10 kHz was lower than 24 kHz ($p = 0.031$), suggesting that after NIHL the animal had better gap detection at 10 kHz compared to 24 kHz.

The two-way ANOVA showed that for the fifth animal (Chapter 5: Fig. 5.10E), there was no main effect of pre/post SIVs, $F(1, 80) = 0.738, p = 0.393$, and there was a main effect of frequency, $F(3, 80) = 4.317, p = 0.007$. The Tukey’s test determined that the pre/post SIVs at 24 kHz were significantly higher than the pre/post SIVs at 4 kHz ($p = 0.005$). There was no significant interaction between frequency and pre/post SIVs, $F(3, 80) = 1.284, p = 0.286$.

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The two-way ANOVA showed that for the first animal (Chapter 5: Fig. 5.11A), there was a main effect of pre/post SIVs, $F(1, 80) = 8.010, p = 0.006$, and the pair-wise comparison analyses showed that there was a significant increase of SIV at 4 kHz ($p = 0.023$) and 24 kHz ($p = 0.017$). There was also a main effect of frequency, $F(3, 80) = 3.268, p = 0.026$. The Tukey’s test determined that the pre/post SIVs at 4 kHz were significantly higher than the pre/post SIVs at 10 kHz ($p = 0.027$). There was no
significant interaction between frequency and pre/post SIVs, F (3, 80) = 2.259, p = 0.089.

The two-way ANOVA showed that for the second animal (Chapter 5: Fig. 5.11B), there was a main effect of pre/post SIVs, F (1, 81) = 13.200, p = 0.001, and the pair-wise comparison analysis determined that there was a significant increase of SIV at 24 kHz (p<0.001). There was also a main effect of frequency, F (3, 81) = 2.982, p = 0.037. The Tukey’s test determined that the pre/post SIVs at 24 kHz were significantly higher than the pre/post SIVs at 16 kHz (p = 0.015). There was a significant interaction between frequency and pre/post SIVs, F (3, 81) = 5.141, p = 0.003. The other pair-wise comparison analyses showed that the post SIVs at 24 kHz were higher than 4 kHz (p = 0.001), 10 kHz (p = 0.003) and 16 kHz (p<0.001), suggesting that after NIHL the animal had poor gap detection at 24 kHz compared to 4 kHz, 10 kHz and 16 kHz.

The two-way ANOVA showed that for the third animal (Chapter 5: Fig. 5.11C), there was a main effect of pre/post SIVs, F (1, 81) = 4.397, p = 0.039, and the pair-wise comparison analyses determined that there was a significant increase of SIV at 4 kHz (p = 0.037) and 24 kHz (p<0.001). There was also a main effect of frequency, F (3, 81) = 5.942, p = 0.001. The Tukey’s test determined that the pre/post SIVs at 24 kHz were significantly higher than the pre/post SIVs at 10 kHz (p = 0.018) and 16 kHz (p<0.001). There was a significant interaction between frequency and pre/post SIVs, F (3, 81) = 5.313, p = 0.002. The pair-wise comparison analyses showed that the post SIVs at 4 kHz were higher than 10 kHz (p = 0.028) and 16 kHz (p = 0.002), suggesting that after NIHL the animal had poor gap detection at 4 kHz when compared to 10 kHz and 16 kHz. Also 24 kHz was higher than 4 kHz (p = 0.045), 10 kHz (p<0.001) and 16 kHz (p<0.001), suggesting that after NIHL the animal had poor gap detection at 24 kHz when compared to 4 kHz, 10 kHz and 16 kHz.
The two-way ANOVA showed that for the fourth animal (Chapter 5: Fig. 5.11D), there was no main effect of pre/post SIVs, $F(1, 81) = 0.014$, $p = 0.906$, and there was a main effect of frequency, $F(3, 81) = 7.448$, $p < 0.001$. The Tukey’s test determined that the pre/post SIVs at 4 kHz were significantly higher than the pre/post SIVs at 10 kHz ($p = 0.001$), 16 kHz ($p = 0.001$) and 24 kHz ($p = 0.024$). There was a significant interaction between frequency and pre/post SIVs, $F(3, 81) = 7.329$, $p < 0.001$. The pair-wise comparison analyses showed that the post SIVs at 4 kHz were higher than 10 kHz ($p < 0.001$), 16 kHz ($p < 0.001$) and 24 kHz ($p = 0.001$), suggesting that after NIHL the animal had poor gap detection at 4 kHz compared to 10 kHz, 16 kHz and 24 kHz. Also, 24 kHz was higher than 10 kHz ($p = 0.016$) and 16 kHz ($p = 0.046$), suggesting that after NIHL the animal had poor gap detection at 24 kHz compared to 10 kHz and 16 kHz.

The two-way ANOVA showed that for the fifth animal (Chapter 5: Fig. 5.11E), there was no main effect of pre/post SIVs, $F(1, 81) = 0.050$, $p = 0.824$, and there was no main effect of frequency, $F(3, 81) = 1.723$, $p = 0.170$. There was an interaction between frequency and pre/post SIVs, $F(3, 81) = 3.963$, $p = 0.011$. The pair-wise comparison analyses showed that the post SIVs at 4 kHz were lower than 10 kHz ($p = 0.002$), 16 kHz ($p = 0.034$) and 24 kHz ($p = 0.003$), suggesting that after NIHL the animal had better gap detection at 4 kHz compared to 10 kHz, 16 kHz and 24 kHz. This highlights the variability between animals; there was no trend between frequencies across animals within the same group.
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