Resting neural activity patterns in auditory brain areas following conductive hearing loss

by

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Abstract

**Introduction:** A common cause of conductive hearing loss in young children is otitis media, which when chronic or recurrent can effect speech and language development. However, little is known about the effects of such conductive loss on resting activity levels in the auditory system.

**Hypothesis:** Conductive hearing loss will change spontaneous activity levels at the inner hair cell synapse, and lead to auditory deprivation of central auditory pathways.

**Experiments:** In a mouse model, a 50-60dB conductive loss was produced by blocking the ear canals. Resting neural activity patterns were quantified in cochlear nucleus and inferior colliculus using c-fos immuno-labelling. Conductive hearing loss subjects were compared to normal hearing controls and subjects with bilateral cochlear ablation.

**Results:** Subjects with conductive hearing loss showed a trend in reduction in c-fos labelled cells in cochlear nucleus and a more significant reduction of labelled cells in the central nucleus of inferior colliculus compared to normal controls.

**Conclusions:** There is a reduction in resting neural activity in auditory brainstem and midbrain as a result of conductive hearing loss. Such changes may influence the developing auditory brain during early postnatal years when the system is highly plastic.
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<tr>
<td>ABR</td>
<td>auditory brainstem response</td>
</tr>
<tr>
<td>AMPA</td>
<td>2-amino-3-(5-methyl-3-oxo-1, 2-oxazol-4-yl) propanoic acid</td>
</tr>
<tr>
<td>AOE</td>
<td>acute otitis media</td>
</tr>
<tr>
<td>AVCN</td>
<td>anterior ventral cochlear nucleus</td>
</tr>
<tr>
<td>CHL</td>
<td>conductive hearing loss</td>
</tr>
<tr>
<td>CIC</td>
<td>central nucleus of inferior colliculus</td>
</tr>
<tr>
<td>CN</td>
<td>cochlear nucleus</td>
</tr>
<tr>
<td>COM</td>
<td>chronic otitis media</td>
</tr>
<tr>
<td>dB</td>
<td>decibel</td>
</tr>
<tr>
<td>DCIC</td>
<td>dorsal cortex of inferior colliculus</td>
</tr>
<tr>
<td>2-DG</td>
<td>2-deoxyglucose</td>
</tr>
<tr>
<td>ECIC</td>
<td>external cortex of inferior colliculus</td>
</tr>
<tr>
<td>GluR</td>
<td>glutamate receptor</td>
</tr>
<tr>
<td>IC</td>
<td>inferior colliculus</td>
</tr>
<tr>
<td>IEG</td>
<td>immediate early gene</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>kHz</td>
<td>kilo Hertz</td>
</tr>
<tr>
<td>NeuN</td>
<td>neuronal nuclei</td>
</tr>
<tr>
<td>OME</td>
<td>otitis media with effusion</td>
</tr>
<tr>
<td>PVCN</td>
<td>posterior ventral cochlear nucleus</td>
</tr>
<tr>
<td>SPL</td>
<td>sound pressure level</td>
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1 CHAPTER: INTRODUCTION

This chapter contains a brief introduction to the thesis, including explanation of rationale for the study, and providing a road map for contents of the thesis.

1.1 Research Objectives

Middle ear infections such as otitis media with effusion are common in young children. Health Canada has reported that in 2008/2009, 50% of Canadian children had at least one ear infection before their third birthday, and 13% of these children had reoccurring ear infections i.e. Chronic Otitis Media with Effusion (1). Chronic ear infections causing conductive hearing loss especially in children at a critical time in their development have been shown to affect speech and language development, behaviour, cognition, and academic achievement. However, relatively little is known about the resting activity state of the auditory system during conductive hearing loss. Past studies on the effects of conductive hearing loss (2-4) have explored changes to the auditory system using auditory brainstem evoked response (ABR’s), electrode recording from the round window and 2-deoxyglucose labelling techniques. Results from these authors are varied. On balance, the data from these studies do indicate that there are changes to central auditory areas. The objectives of this thesis are to add clarity, and to specifically investigate what changes occur in resting neural activity patterns in the central auditory pathway during a conductive loss. Results from this study will give more information on changes seen in the
ascending pathway during a sound attenuation and will help to determine how severely the auditory pathway is affected in patients that suffer from chronic episodes of otitis media.

1.2 Thesis Outline

This chapter presents the motivation for undertaking the present study. An outline of the thesis and research objectives is given.

Chapter 2 presents relevant background information. There is a review the anatomy of the ear and the physiology of hearing, followed by a description of conductive hearing loss. Then there is a review of the role of spontaneous neural activity in hearing and a discussion on some of the ways in which changes in hearing can lead to plasticity in the auditory pathway.

Chapter 3, details the experimental methods. Included is a description of the experimental model, experimental design and procedure. The method used for analysing the results is also described here.

Chapter 4 presents the results of the experiments.

Chapter 5 contains a discussion of the results and other various studies related to this work. Included is a discussion on the clinical significance of this study.

Chapter 6 contains some conclusions drawn from this study with some comments of future research directions.

Chapter 7 outlines the protocol used in this thesis, and extra graphs displaying results from this work as an appendix.
2 CHAPTER: BACKGROUND

In the following sections of this chapter, reviews of background areas of knowledge related to the experiments of this thesis are presented.

2.1 Anatomy and Physiology of the Mammalian ear

The ear consists of three parts: outer ear, middle ear and inner ear. The outer ear consists of the pinna, concha and the auditory meatus (Figure 2-1). These three components act to capture acoustic signals and direct them to the ear canal, where the acoustic signal travels to the tympanic membrane. Once acoustic waves enter the middle ear, they are converted into mechanical vibrations by the middle ear ossicles: the malleus, incus and stapes (Figure 2-1). These bones connect the tympanic membrane to the oval window of the cochlea of the inner ear.

The inner ear consists of a spiral shaped boney structure called the cochlea. A cross section through the cochlea reveals three chambers: the scala vestibuli, the scala tympani and the scala media. Figure 2-2 shows the placement of these three chambers relative to the Organ of Corti, where the apical surface of the hair cells are bathed in a positively charged, (high in potassium ion concentration) solution called endolymph. During sound stimulation, the mechanical vibrations induced via the middle ear ossicles reach the cochlea and transmit vibrations which activate motion of the basilar membrane. The basilar membrane is a structure that runs the length of the cochlea and to which is attached the sensory organ of Corti. A special feature of the basilar membrane is the way it vibrates in response to acoustic signals.
Figure 2-1 The human ear showing the outer, middle and inner ear. Adapted from Neuroscience Second Edition, D. Purves et al. 2001, Pg 280.

Figure 2-2 Cross-section through the cochlea. Image adapted from Neuroscience Second Edition, D. Purves et al. 2001. Pg 284.
At the base of the cochlea, the basilar membrane is narrow and stiff allowing it to vibrate strongly to high frequency components of sound signals. At the apex, the basilar membrane is wider and more flexible allowing it to respond best to low frequency sounds. This physical feature (stiffness gradient) gives rise to the cochlear place mapping of frequency (cochleotopic or tonotopic organization) within the inner ear. This cochleotopic organization is maintained in neural arrays from the cochlea, and is a mainline feature of auditory pathways through brainstem, midbrain, and cortex. Figure 2-3 shows part of the organ of Corti, where vibrations in the basilar membrane cause the stereocilia on the outer hair cells to bend against the tectorial membrane, and fluid movements of endolymph cause deflection of stereocilia on the inner hair cells.

In the cochlea there is one row of inner hair cells, and three rows of outer hair cells. The outer hair cells have structure and function consistent with their role as a biological amplifier within the cochlea. (i.e. they act like acoustical pre-amplifiers). The outer hair cells boost up mechanical vibrations of sound stimuli, which are detected by the inner hair cells. Outer hair cell function is further described in section 2.1.2. Thus the inner hair cell is the true sensory receptor of hearing. At each inner hair cell synapse there is a transfer of information to cochlear afferent nerve fibres projecting into the brain. This information transfer from haircell to neuron is through neurotransmitter release (glutamate) at the chemical inner hair cell synapse.

Deflection of stereocillia on the inner hair cell (Figure 2-4) results in opening and closing of membrane ion channels associated with ‘tip-links’, which allows the active flow of potassium ions from the endolymph, where there is high potential (+80mV), into the hair cell, where there is low potential (-50mV). Resulting depolarization opens voltage-gated calcium channels, allowing calcium into the cell, which induces the release of vesicles containing neurotransmitter (glutamate) at the hair cell synapse. The released neurotransmitter diffuses from the presynaptic
terminal to the post-synaptic terminal of the afferent nerve fibre (spiral ganglion cell), depolarizing the peripheral dendrite and triggering an action potential. This signal will travel from these first order neurons to the cochlear nucleus of the brainstem, and then through the ascending auditory pathway to the auditory cortex (refer to Ascending Auditory Pathway section 2.1.3).

Figure 2-3  Movement of basilar membrane creates a shearing force that bends the stereocilia of the hair cells. Image adapted from Nature Reviews in Neuroscience.
Figure 2-4 Mechanoelectrical transduction mediated by hair cells. Image adapted from Neuroscience-second edition, D. Purves et al. 2001, Pg 280

2.1.1 The Inner Hair Cell

A closer look at the synaptic region of the inner hair cell reveals a unique structure that acts like a reservoir for vesicles containing neurotransmitter: the ribbon synapse (Figure 2-5). This type of synapse consists of a presynaptic electron-dense core, which is surrounded by vesicles attached through filamentous tethers (5). Synaptic ribbons are morphologically diverse, with mammalian hair cells containing ribbons ≤ 200nm in width, and harbouring ~100-200 tethered synaptic vesicles (6). Their role in sensory systems has been extensively researched to show that they play an important part in maintaining a graded synaptic transmission that improves temporal acuity as well as encoding sounds of extremely differing intensities (7). Synaptic ribbons are able to meet this demand by storing and increasing the pool of release-ready vesicles, as well as transporting and/or timing delivery of these vesicles to the plasma membrane (8). Electrode recordings from the oval window of the cochlea have shown that even in the absence of sound stimulation, there
is a baseline of spontaneous neural activity generated at the inner hair cell synapse. It can be speculated that due to the requirement of graded synaptic transmission, the tip-links (ion channels) in the stereocillia and the ribbon synapse are ‘leaky’, and contribute to the spontaneous release of neurotransmitter and baseline afferent neural activity. Research has indicated that there are differences in structure and function of ribbon synapses in hair cells along the length of the cochlea. Work by Fuchs et al have shown that ribbons in the chick’s cochlea can vary in size and vesicle numbers along the cochleotopic axis (9). More recent research has also suggested that calcium entry through voltage gated calcium channels occurs close to ribbons in hair cells, and can vary in number among ribbons in a single cochlear hair cell (6, 10, 11). This could account for differences in spontaneous rates and depolarization thresholds of individual afferent neurons contacting an inner hair cell (8).

Figure 2-5 The role of the ribbon synapse in neurotransmission from the inner hair cells to afferent nerve fibres.
Once the neurotransmitter diffuses across the synaptic cleft, it activates an AMPA-like receptor in the post-synaptic terminal. Using immunogold labelling, Otterson et al were able to show glutamate receptor subtypes (GluR) 2, 3 and 4 in the postsynaptic afferent fibre (12). Glowatzi & Fuchs (2002) also showed that the excitatory postsynaptic currents from rat inner hair cells were mediated by AMPA receptors (13). Therefore, since glutamate is thus far known to be the primary neurotransmitter used at the hair cell synapse, and there is a continuous baseline release at this synapse, how is glutamate saturation and excitotoxicity avoided? Research by Furness et al and Rebillard et al have shown that excess glutamate is taken up by glutamate transporters into supporting cell transporters (14, 15), which are abundant on the pillar inner hair cell side where high spontaneous rate fibres are located (16).

2.1.2 The Outer Hair Cell

As previously mentioned, the function of the outer hair cell system is as an acoustic pre-amplifier by boosting mechanical vibrations of sound stimuli detected by inner hair cells. It is able to do this by contracting or elongating following changes in intracellular potential. This movement in outer hair cells is known as electromotility, and is thought to be modulated by the cochlear efferent system neurotransmitter, acetylcholine. These efferent terminations on the outer hair cell are mostly efferent axons originating at the superior olivary complex of the brainstem. This arrangement would indicate there may be some spontaneous activity generated by these outer hair cells, although levels of activity would perhaps be much lower than those seen with inner hair cells.

Otoacoustical emissions produced by outer hair cells can be detected by placing a microphone at the eardrum and recording the response after presenting a sound stimulus. These
emissions can also occur spontaneously, indicating that the outer hair cell process is capable of producing an acoustic signal. We know that inner hair cells can change shape in response to small electrical currents, therefore, it is likely that outer hair cells amplify acoustic input, and also improve on the (passive) frequency selectivity of the basilar membrane. It is known that stimulation of the crossed olivocochlear bundle, causes broadening of eighth nerve tuning curves which likely reflects the inhibitory effects of cochlear efferents on the bio-mechanical function of the outer hair cells. These outer hair cell mechanisms modify the stimulus that is detected by inner hair cells which are the true receptors of the auditory system. The work of the present thesis concerns neural activity in ascending pathways, and thus is primarily concerned with inner hair cell function.

2.1.3 Ascending Auditory Pathways

The nervous system has been extensively studied for decades and early work describes well defined pathways within the brain. A pioneer in anatomically defining the sensory pathways was Ramon y Cajal. Some of the first observations made by Cajal included the one directional movement of nerve impulses through the brain and spinal cord (17), as well as illustrations on morphologically distinct cell types in the nervous system (18).

As described above, once sound waves have travelled through the periphery (ear), and are converted into electrical signals by the inner hair cells, nerve impulses will initially travel through the cochlear afferent nerve fibres. Axons from these cochlear afferents come together in the modiolus to form the cochlear nerve. The majority of cochlear afferent nerve fibres (95%) terminate on the inner hair cell (19), whereas the other 5% forms outer spiral fibres that can project to as many as 20 outer hair cells (20). The axons of these nerve fibres are fine and
unmyelinated, so that any electrode recordings of these nerve fibres are able to reflect the activity of individual hair cells (21). Another important feature of these afferent cochlear neurons is that they have very high rates of spontaneous activity (discharge in the absence of stimulation). Liberman was able to identify three spontaneous rate groups: low (<0.5 spikes/sec), medium (0.5-18 spikes/sec) and high (>18 spikes/sec). The most sensitive afferent neurons (low thresholds of response) have the highest spontaneous rates and attach to the inner hair cell closest to the modiolus (22-24). It is this spontaneous activity together with small amounts of driven activity at rest (sounds made during movement in the cage or individual animals breathing or vocals) is of interest in this thesis. The present study is exploring the ‘resting levels’ of activity in neurones central to these highly spontaneously active cochlear afferent neurons.

In the ascending auditory pathway, there are six major areas that contain cell bodies of neurons that make up the central pathway. These are the cochlear nucleus, the superior olivary complex, the nuclei of lateral lemniscus, the inferior colliculus, the medial geniculate body and the auditory cortex (Figure 2-6). Within the cochlea, the sensory epithelium has a tonotopic arrangement such that hair cells responding to high frequencies are found at the base, and hair cells responding to low frequencies are found at the apex. This tonotopic organization continues through the ascending auditory pathway, and can be seen in all six major areas that make up the central pathway. The afferent nerve fibres from the inner hair cells all project to three subdivisions of the cochlear nucleus: the dorsal nucleus (DCN), and two ventral areas, the anterior (AVCN) and posterior ventral cochlear nucleus (PVCN). Since the 1930’s, work carried out by Lorente de Nó reported that the cochlear nucleus contained “no less than 40 or 50 types of neurons”. He used a Golgi staining technique where neurons are labelled with a silver stain that allows them to be traced (25). This was later confirmed in the 1960’s, where the different cell
types were then identified (26, 27). In the dorsal cochlear nucleus, cell populations include fusiform (pyramidal) cells, giant cells, a variety of small cells and granule cells. In the ventral cochlear nucleus, cell populations include spherical bushy cells, globular bushy cells, octopus cells, cochlear root neurons (not found in all species), multipolar cells, small cells and granule cells.

Axons from the ventral cochlear nucleus project to the superior olivary complex on both the ipsilateral and contralateral sides, whereas axons from the dorsal cochlear nucleus mainly project to the contralateral central nucleus of the inferior colliculus (midbrain) (Figure 2-6). The superior olivary complex plays a role in sound localization. It is the first site in the auditory pathway to receive binaural input (28). There are three main nuclei that make up the superior olivary complex: lateral superior olivary nucleus, the medial superior olivary nucleus and the medial nucleus of the trapezoid body. Information coming to the superior olivary complex is mainly from the AVCN, although neurons from the PVCN and the DCN also project to the ipsilateral and contralateral lateral superior olivary nucleus, respectively (28). The neurons in the cochlear nucleus have the important job of being the first relay neurons in the pathway, and it is these neurons that are targeted in this thesis as they receive direct information from the inner hair cell synapse.
Figure 2-6 Schematic representation of the ascending auditory pathway in the mammal. Input from one ear is depicted. Adapted from The Biology of Hearing and Deafness, Harrison RV, 1988. DCN - dorsal cochlear nucleus; AVCN - anteroventral cochlear nucleus; PVCN posteroventral cochlear nucleus; MTB - medial nucleus of the trapezoid body; MSO – medial nuclues of the superior olive; LSO – lateral nucleus of the superior olive; C of P – commissure of Probst; C of IC – commissure of inferior colliculus; PN – pericentral nucleus; CN – central nucleus; EN – external nucleus; VN – ventral nucleus; MN – medial nucleus; DN – dorsal nucleus; CC – corpus callosum

Following the brainstem auditory nuclei, the next major area in the ascending pathway is the nucleus of the lateral lemniscus. Nerve fibres extend from the lateral superior olivary nucleus ipsilaterally and contralaterally, whereas fibres from the medial superior olivary nucleus will
only project ipsilaterally (29). From the lateral lemniscus, the majority of the fibres terminate in the central nucleus of the inferior colliculus (28).

The inferior colliculus has been described as the principal midbrain nucleus of the auditory pathway, and receives ascending input, via many relays, from periphery neurons as well as descending input from the cortex. Originally, Ramón y Cajal distinguished three regions within the IC, which have later been labelled as dorsal cortex, central nucleus and the external nucleus/cortex (Figure 2-6). The central nucleus has neurons that respond exclusively to auditory stimulation (30) and is essential for normal hearing (31). The external cortex has neurons that respond to multisensory stimulations and has little auditory input (32) whereas the dorsal cortex neurons receive input from the cerebral cortex (33) and the auditory cortex, although its role in hearing is not well known.

Neuroanatomical methods using retrograde and anterograde axonal transport of tracers have shown that ascending projections from the DCN of the cochlear nucleus (larger cells) and superior olivary complex converge in the central nucleus of the IC (34-37). The bimodal neurons in the central IC can therefore be described as 2nd, 3rd or 4th order neurons depending from where in the pathway they receive input. Integration at the IC is integral in the binaural system for sound localization. Experiments carried out in the barn owl, have shown that convergence of binaural inputs in the midbrain necessitates a topographical representation of auditory space (38). The neurons in this auditory space map respond to sound from a specific region of space and have both preferred elevation and horizontal (azimuth) location. Neurons in the IC are also able to process sounds with complex temporal patterns i.e. speech, and therefore is the first stage in the pathway that can analyse sounds of significance (39). It is because of this important role, that the IC was also chosen in this study to measure resting activity levels. As mentioned above, the
neurons found in the central nucleus of the inferior colliculus mainly respond to auditory stimulation, and it is this area of the IC that is of particular interest. Any changes in resting activity levels in this area will indicate if there are any deficits in neuronal activation of these bimodal neurons that are responsible for processing sounds, and may help to better understand some of the clinical finding regarding effects to speech and language development in children with otitis media. From the IC, neuronal fibres will either project to the medial geniculate or to the contralateral IC (40).

The medial geniculate body is divided into three main regions: the ventral, dorsal and medial divisions (Figure 2-6). The ventral division largely receives ascending auditory information from the central IC, whereas the dorsal division receives few connections from the collicular region. Instead, its projections are largely to non-primary auditory cortex (41), and like the ventral division; it receives descending cortico-geniculate input (42). The medial division receives projections from the IC as well as thalamic regions (43). Not all cell responses are auditory related, with some neurons responding to somatosensory and vestibular stimulation (41). Axonal projections from the ventral and medial divisions of the medial geniculate body terminate in the primary auditory cortex (Figure 2-6 and Figure 2-7).

The auditory cortex can be found within the sylvian fissure in humans, and is subdivided into the primary auditory cortex (A1) and the surrounding secondary auditory cortex (peripheral, or belt region) (Figure 2-7). The primary auditory cortex is arranged into six definable layers. It receives its main input from the ventral division of the medial geniculate body, with projections mainly terminating in layer IV (28). As described before, an interesting feature of the auditory pathway is the tonotopic organization of frequency tuning in neurons that extends from the cochlea through the major areas in the central auditory pathway. Tonotopic
organization in the cochlea is shown by frequency selectivity of inner hair cells, which continues in a point-to-point format from the cochlea nucleus to higher order nuclei. At the inferior colliculus, tonotopic organization is shown in the form of iso-frequency sheets (44, 45), where orthogonal to the axis of tonotopicity, there appears a system of binaural coding relating to sound organization.

Figure 2-7 The human auditory cortex. The primary auditory cortex is shown in blue and the surrounding areas of auditory cortex are shown in pink. Image adapted from Neuroscience – second edition, edited by D. Purves et al. 2001; Pg 294.

This tonotopic organization continues to the cortex, where in the primary auditory cortex, tonotopicity is in the form of iso-frequency strips (Figure 2-7), and binaural representation is again orthogonal to the tonotopic axis (46, 47). Sensory processing that occurs in other areas of the auditory cortex are not well understood, but are thought to be used for higher-order processing of natural speech sounds (48). Past animal studies have shown that ablating the auditory cortex causes loss in the ability to discriminate between two complex sounds (49). Therefore, we know that the correct development of the central auditory pathway is essential and critical for the normal processing of everyday sounds from the environment.
Neurons in the auditory cortex receive their information from the medial geniculate body, where cell responses can be multisensory related i.e. auditory, somatosensory and vestibular. Cortical neurons here are thought to represent biologically significant acoustic events and recognition would be based on a population of cells rather than the electrophysiological study of a single cortical neuron (28). Therefore, resting levels of activity in the cortex are not explored in this thesis. It is very unlikely that any spontaneous activity occurring at the inner hair cell synapse would influence resting levels of activity at the cortical level.

2.2 Conductive Hearing Loss

Conductive hearing loss occurs when acoustic signals are attenuated as they pass from the external environment to the inner ear. This means that the threshold for hearing a sound has increased, making it difficult for subjects to hear and process acoustic signals from the environment. In most cases, a conductive hearing loss involves a simple blockage of sound conduction. In some cases there is physical change or damage to the middle ear structures. For example in otosclerosis, mostly found in older patients, where an abnormal bone growth in the middle ear makes it difficult for sound vibrations to reach the inner ear. A very common cause of conductive hearing loss is otitis media, found mainly in young children, where a middle ear infection has caused fluid build up in the middle ear immobilising the middle ear bones, which again makes it difficult for sound vibrations to reach the inner ear (Figure 2-8). Otitis media is discussed below in more detail. The present study was in large part conceived because of the concerns that chronic or recurring otitis media may have some effect on neural activity patterns in the auditory pathways.
In all cases of pure conductive hearing loss, inner hair cells have not been damaged or affected, and can function normally. Therefore, if the blockage is removed without affecting the middle ear bones, sound processing can return to normal. In patients suffering from otosclerosis, surgery can be an option, and in patients with otitis media, antibiotics can clear the middle ear, allowing the patient to resume normal hearing. In this study, we are interested in the effects of conductive hearing loss on the auditory pathway, and will refer to otitis media as the clinical disorder we are interested in.

In animal studies carried out on the effects of conductive loss to the auditory system, results have been varied. Some investigators have shown that during a conductive loss, there are structural changes that occur in the size of neurons (50, 51), whereas others have not seen this (3, 52). It would seem that the controversy surrounding the effects of conductive loss on the auditory pathway will depend on a number of factors such as the critical time in development that the attenuation has occurred, and the method in which conductive loss was induced. In the present study, conductive hearing loss was induced by blocking the external auditory canal. Therefore, the middle ear and the inner ear are still able to function. In this case, air conduction has been blocked; however, bone conduction to the inner ear via vibrations in the skull can still occur. We know that there are many factors that can contribute to “hearing changes” seen with conductive hearing loss, e.g. the type of blockage and where it occurs in the pathway from the external ear to the internal ear, bone conduction factors and occlusion effects. An example of this is seen in clinical research done by El-Sayed, where patients with chronic otitis media had clinically significant bone conduction threshold elevations (53). Clearly, conductive hearing loss cannot be viewed too simplistically. However, basically, a conductive block contributes to an overall reduction in acoustic signals activating the cochlea. In the present study, we should emphasize
that our study of neural activity levels has not been designed to model otitis media (or any specific etiology) but rather to give a general understanding of effects on neural activity in the auditory pathway after a conductive block of acoustic stimuli. However, we also recognize that otitis media, because it is a very common cause of conductive hearing loss in young infants, is a particularly important type of hearing loss to understand more fully.

### 2.2.1 Otitis Media

There are many different types of otitis media that are classified by the presence of either an inflammation or an infection of the mucosal lining of the middle ear cleft: Acute Otitis Media (AOE), Mucoid Otitis Media or Otitis Media with Effusion (OME), Serous Otitis Media (SOM) and Chronic Otitis Media (COM). Of these, chronic otitis media can be the most detrimental to development of the auditory pathway due to the continuing effects of conductive loss.

As mentioned before, conductive hearing loss from otitis media occurs when infections such as the cold and flu cause swelling and fluid accumulation in the space behind the eardrum (Figure 2-8). The rhinoviruses (nose viruses) infect the eustachian tube that runs from the back of the nose to the middle ear. This tube is used for pressure equalization as well as a more functional use for lateral draining of fluids from tissues on either side of the skull. This tube in a newborn is horizontal, which deters its function as a drainage system. As the child grows, the eustachian tube shifts to a 45 degree angle, thus allowing for better drainage. Therefore, in very young children suffering from colds, it is common for this tube to fill up, and without effective drainage, this fluid accumulation in the middle ear traps bacteria and viruses and increases
pressure on the eardrum. The increased pressure causes the eardrum to bulge, which leads to symptoms of ear pain, pressure, and decreased hearing (Figure 2-9).

Figure 2-8  Image showing a middle ear infection (Otitis media). Image adapted from website: functionalmedicinesolutions.com

In young children who are unable to communicate clearly, it can be difficult to initially diagnose an ear infection, and if left untreated, otitis media can lead to permanent hearing loss (54). It should be noted that children born with poor eustachian tube function e.g. children with Down syndrome, will experience more frequent and severe cases of otitis media.
Past studies carried out in children, those suffering from repeated bilateral episodes of otitis media compared to normal controls showed deficits in higher-order auditory processing (55). These studies showed that a reduction in a child’s hearing at a time that is critical for speech and language development can later lead to hearing sensitivity, longer wave V latencies and difficulties in recognising speech in background noise (56)(57). In other work carried out in 2007 by Xu et al., it was shown that by inducing a conductive loss, neurons in the auditory cortex displayed a faster rate of synaptic depression and smaller asymptotic amplitude, as well as less spike frequency adaptation when compared to normal. These results were similar to neurons that were affected by sensorineural hearing loss (58). These results supported data found in children that have suffered from otitis media, in that conductive hearing loss alters temporal properties of auditory cortex synapses and contributes to higher-auditory processing deficits.
2.3 Spontaneous/Resting Activity

Spontaneous or resting activity is described as neural activation in the absence of stimulation. It is commonly seen throughout the brain and has been described as essential for neuronal survival and for mapping neural circuits in the brain (59). There are essentially two sources that can contribute to spontaneous activity. The first is action potentials fired in the afferent nerve fibre triggered by stochastic release of neurotransmitter from the hair cell (60-62). The second is auditory nerve activity triggered by very sensitive cochlear afferents that respond to basic physiological activity i.e. heart beat (63). Work carried out by McKay et al. has identified the necessity of spontaneous activity during development to maintain synaptic strength in the cochlear nucleus (endbulb of Held) (64). Rates of spontaneous activity have been shown to be highly non-random in nature and it seems likely that this endogenous activity may provide information on how neural pathways should be wired through the course of development (23, 65, 66). For this reason, monitoring levels of this endogenous activity during a conductive loss may give an insight on how sound attenuation can affect development in the young plastic brain. In this thesis, references to resting levels of activity are mentioned. Resting levels of activity include spontaneous activity together with small amounts of driven activity caused by movement of the animal in the cage and sounds produced by the animal during its confinement in a sound proof room.

In the developing auditory system, before hearing onset, Bergles et al. have shown that spontaneous activity originates in the Kölliker’s organ, where spontaneous release of ATP causes inner hair cells to depolarise, releasing glutamate that triggers bursts of spontaneous neural activity in afferent nerve fibres (67). This activity during development slows down after hearing onset; however, there are still baseline levels of neural activity generated at the inner hair cell
that continue even after hearing onset. It is thought that this baseline of spontaneous neural activity is used to maintain already formed neural pathways, and any change to spontaneous rates in afferent fibres and first order neurons, can relate back to changes occurring at the hair cell synapse. We know from Liberman’s work that neurons can have differing rates of spontaneous activity although the reason for this is unclear (22). Anatomical studies on the inner radial fibres in a cat cochlea have revealed that 60% of these fibres have a higher density of mitochondria, which could contribute to higher spontaneous rates in these fibres (68). In other studies carried out by Liberman et al. and Tucci et al., results have shown that following the onset of sensorineural deafness spontaneous rates of neural activity decline (52, 66, 69). We also know from more recent work investigating inner hair cells, that ribbons found at the hair cell synapse could also play a role in spontaneous discharge during rest. Since changes in baseline neural activity can relate back to changes at the inner hair cell synapse, and it is known that conductive hearing loss does not damage hair cells, it is assumed that conductive loss would have no affect on baseline spontaneous rates. Studies carried out on the effects of conductive hearing loss have shown mixed results. Work done by Tucci et al in 1987 on chicks has shown that conductive hearing loss does not affect spontaneous neural activity (52). However later work in 1999, 2001 and 2002 showed a decrease in neuronal activity in the central auditory system after a conductive loss in gerbils (2, 70, 71). Results seem to be affected by the method used to induce the conductive hearing loss and the species of animal used. Experimental methods that have been used in the past to detect levels of neural activity include detecting 2-deoxyglucose (2-DG) uptake and recording spontaneous neural activity from the oval window.
2.4 Plasticity in the auditory system

The term ‘plasticity’ is a very broad description of changes that occur in the brain in response to experience. Plastic changes can occur over long periods of time (long-term plasticity), weeks or months, or over shorter periods of time (short-term plasticity), a few hours or days. These changes can be seen on a large systems scale, e.g. shifts in iso-frequency bands in response to a cochlear lesion (72) or at a much smaller sub-cellular level e.g. synaptic plasticity seen in studies on learning and memory (73, 74).

One important aspect of brain plasticity is that it is most often age related. Thus the auditory system is very plastic during an early post-natal period, and at this time activity patterns at the cochlear level can have significant influence on more central pathway development. The questions posed in the present thesis were motivated in part because the effects of conductive hearing loss in infants may have an important (negative) influence on auditory brain development.

In the auditory system, much research on synaptic plasticity has been done at a large axosomatic terminal, called the end bulb of Held. This synaptic terminal is found in the AVCN and is significant in its size and function. This ending also shows an impressive degree of plasticity in its shape and synaptic structure. Studies done in the cat have observed the effect of sensorineural hearing loss caused by tetrodotoxin application, and found changes to the end bulb synapse in its shape i.e. more branching and small terminal swellings. Observations have also shown changes in patterns of nerve fibre contact with second order neurons (75). Even more drastic changes to the end bulb of Held were seen in 6 month and 6.5 year old deaf white cats, where there was an attenuation of tertiary branching and a loss of the fine interconnecting
meshwork around the endbulbs (76). Further testing in these congenitally deaf cats showed a restoration in morphology of these endbulbs and their synapses by the introduction of a cochlear implant at three-months (77, 78). These results have confirmed the need for early intervention in sensorineural deaf subjects and emphasize how important the critical period of early development is in the auditory system. A question then arising from the work on sensorineural deafness is how severe is the effect on auditory development during conductive hearing loss, where sound stimulation is attenuated instead of absent?

Some studies that have looked at plastic changes during a conductive loss have described more subtle changes such as the redistribution of AMPA and glycine receptor subunits in the CN in response to monaural earplugging (79). These results are of particular interest to this study, as they could explain what is happening during glutamate transport at the inner hair cell synapse in conductive loss. Changes seen in resting activity levels may be influenced by this change in distribution of AMPA and glycine receptor subunits. Other studies have shown that monaural sound deprivation caused a distortion in tonotopic maps and weakened the deprived ear’s representation while strengthening the open ear’s representation. It also disrupted binaural integration of interaural level differences, showing a form of competitive plasticity (80). Could these results then partly explain some of the results seen in clinical research regarding otitis media patients having difficulties in recognising speech in background noise?

From these studies and many more, it is clear that the synaptic plasticity induced by changes in the periphery is significant and its susceptibility to these changes is greater during a critical period in development.
2.5 Hypothesis

I hypothesize that conductive hearing loss will alter levels of driven and spontaneous neural activity and lead to significant auditory deprivation in the cochlea nucleus and inferior colliculus of the brain.
3 CHAPTER: EXPERIMENTAL METHODS

In the following chapter, the experimental model, experimental design and procedure are described. The method used for analysing the results is also presented here.

3.1 The Mouse Model

The animal chosen for this study was the wildtype mouse; strain: CBA/J (Jackson Laboratories-JAX® Mice and Services, Bar Harbor, Maine, USA). This animal model was chosen for the following reasons: first, the mouse model has been used extensively in auditory research to study changes from behaviour to genetics. Second, mice are altricious animals, meaning that they are born deaf, with no physiological response from the cochlea until they are eight days old. Past studies have indicated that mice behavioural thresholds do not mature until 16 to 18 days of age (81), and that the critical period for neuronal growth is before 45 days of age (82). Using this information, young male adult mice (6 weeks old) were chosen, whose neurons have already matured and whose auditory pathway is well established. Lastly, the immuno-labelling method used for analysis in this study has been extensively used in the mouse (83-85).

3.2 Experimental Design and Procedure

There were three main experimental groups: Control (normal hearing); n=8, Conductive loss (CHL); n=8 and the Deafened Control (cochlea ablation); n=4. All experimental groups were bilaterally affected, so raw data collected from each ear was viewed independently.
For the control group, mice were exposed to normal environmental sounds. ABR (auditory brainstem response) measurements were carried out to check hearing thresholds (a detailed description of ABR methodology is given in section 3.2.1), and then the mice were isolated from environmental sounds by being left in a sound proof booth for 12 hrs. Mice were freely able to move about in the cage. Twelve hours isolation was chosen as mice were left overnight in a sound proof booth and under normal circadian rhythm, would likely be sleeping during most of this time. This minimised the activity of the mice in their cages before they were sacrificed. Any movement or noise created by the mice themselves during this time could affect levels of neural activity detected. After 12 hrs of isolation, the mice were anesthetised intraperitoneally using a combination of xylazine (10mg/kg) and Ketamine (150mg/kg). Mice were then sacrificed and their brain tissue prepared for staining (a detailed explanation of tissue preparation is given in section 3.2.2).

The experimental timeline for the control group is shown below:

For the Conductive loss group, mice were again exposed to normal environmental sounds. Initial ABR measurements were carried out to estimate hearing thresholds, after which a bilateral conductive hearing loss was induced by blocking both ears with dental cement (Integrity - TempGrip, DENTSPLY Caulk, Milford, USA). Fig 3-1 is a schematic of how dental cement
was placed in the external canal (this figure shows the human ear; in our study of course the dental cement was placed in a similar position in the mouse ear canal). Dental cement was chosen because its properties allowed easy manipulation and application into the ear canal (injected) before it hardened. It also formed a good bond with the skin lining the ear canal, which ensured that the mouse could not easily remove the block using its paws. This method of blocking the external ear canal provided a non-invasive approach in inducing a conductive hearing loss. Another material that was originally chosen to block the external ear canal was silicone earplugs; however these were easily removed and eaten by the mice.

![Diagram of the ear with dental cement blocking](image)

**Figure 3-1 Schematic of the ear showing the area in the external ear canal that was blocked using dental cement.**

Once the ear canal was blocked, a second set of ABR measurements were carried out to check any changes in hearing thresholds. Mice were observed for three days in this condition in a normal environment. Mice were freely allowed to move around in their cage. Behaviourally, these mice did not move around as much as the control mice, and may have found the blocked ear canals heavy in weight. After 3 days, the mice were being left in a sound proof booth for 12
hrs to ensure that only resting levels of activity would be detected during immunocytochemistry. After 12 hrs of isolation, the mice were anesthetised intraperitoneally using a combination of xylazine (10mg/kg) and Ketamine (150mg/kg). Mice were then sacrificed and their brain tissue prepared for staining (a detailed explanation of tissue preparation is given in section 3.2.2).

The experimental timeline for the conductive hearing loss group is shown below:

For the Deafened control group, mice were again exposed to normal environmental sounds. Initial ABR measurements were carried out to estimate hearing thresholds, after which mice were bilaterally deafened under anaesthetic. A needle was inserted from the external ear (in both ears), through the tympanic membrane and middle ear to cochlea in the inner ear. Using the tip of the needle, a hole was made in the cochlea and sterile water was injected inside to cause ablation. Injury occurred in part by osmotic damage to the hair cells. A second set of ABR measurements were then carried out to ensure bilateral deafferentation. Mice were then left for 5 days to ensure any initial neural excitotoxicity had passed before mice are again left for 12 hrs in a sound proof booth. During this time, they were freely able to move about in the cage. After 12 hrs of isolation, the mice were anesthetised intraperitoneally using a combination of xylazine (10mg/kg) and Ketamine (150mg/kg). Mice were then sacrificed and their brain tissue prepared for staining (a detailed explanation of tissue preparation is given in section 3.2.2).
The experimental timeline for the deafened control group is shown below:

![Timeline Diagram]

### 3.2.1 ABR Measurements

To verify normal hearing thresholds in each animal, pure tones auditory brainstem responses (ABRs) were measured from 2 kHz to 32 kHz. Mice were anesthetised using a combination of xylazine (10mg/kg) and Ketamine (150mg/kg). Electrodes were then placed in a vertex-to-mastoid configuration, and using the Intelligent Hearing Systems Smart-EP system, tonal ABR measurements are taken by placing a probe in one ear to produce tonal stimuli from intensities ranging 10dB to 90dB. Figure 3-2 is an example of mean ABRs (averaged over 512 sweeps) from a single subject during the presentation of 32 kHz tonal stimuli at 10-90 dB SPL. In control and deafened mice, the probe is place in the external ear canal. In conductive hearing loss mice the probe is placed up against the blocked ear.
Figure 3-2 Auditory Brainstem responses to 32 kHz tonal stimuli. Each ABR waveform reflects the average response from 512 sweeps presentations. Responses are presented in descending order from 90 to 10 dB SPL. Waveforms are clear and reproducible down to 20dB SPL (*).

### 3.2.2 Tissue Preparation

Prior to sacrificing the mice, anaesthetic, a combination of xylazine (10mg/kg) and Ketamine (150mg/kg) was administered intraperitoneally. The toe-pincho reflex was used to ensure mice were deeply anaesthetized before carrying out intracardiac perfusion, where the chest cavity was opened to expose the heart. By inserting a needle into the left ventricle and making an incision in the right atrium, the body was first flushed with room temperature saline, followed by cold 4% paraformaldehyde to fix the tissue. Successful perfusion was observed when contracting muscle movement could be seen in the limbs and the tail. Mice brains were
carefully removed from the skull intact, and placed in cold 4% paraformaldehyde at 4°C over night. The next day, 60µm free-floating coronal slices were cut from the base of the brainstem to the end of the inferior colliculus using the Vibratome 1000plus sectioning system (The Vibratome Company, Evergreen, St. Louis, USA). Samples are then prepared for immunolabelling. A detailed protocol of the method used for immunolabelling of c-fos and NeuN is given in the appendix section 1.

3.2.3 C-fos Labelling

Active neurons in brain slices were fluorescently labelled for the proto-oncogene c-fos. The protocol used to label these cells is detailed in the appendix section 1. In 1989, Bishop M and Varmus H discovered the cellular origin of retroviral oncogenes. C-fos belongs to this group of genes responsive to trans-synaptic stimulation and their activity has been recorded in many studies over the years (85-88). Once the Fos protein is produced in the cytoplasm it migrates to the nucleus where it forms heterodimeric transcription complexes with other proteins and eventually regulates the transcription of other genes (87). During the staining process, the anti-fos polyclonal primary antibody (Calbiochem, cat# PC 38; 1:10000) first binds to the Fos protein at the nucleus. This is followed by the fluorescent-conjugated secondary antibody (Alexa Fluor 568 goat anti-rabbit/mouse, Invitrogen; 1:1000) binding to the primary antibody-Fos complex. Figure 3-3 shows these c-fos active neurons as seen under an epiflorescent microscope.

Since c-fos activation can be induced by a large range of stimuli, it has been used in numerous studies (22) as a functional tracer to map neuronal pathways with cellular resolution (89). In the auditory system, c-fos has also been used to show frequency bands in response to pure tones in several brainstem nuclei (83, 85, 90). The use of immediate early gene for mapping
neural pathways follows previous studies that used electrophysiological recordings or metabolic markers such as 2-deoxyglucose (2-DG) to map brain activity. Immunocytochemistry makes it possible to study large regions of the brain in a single experiment, while also viewing cells at a higher spatial resolution (Figure 3-3). C-fos was chosen as the protein complex to label as it is activated in cells in response to auditory stimulation. Neuron activation will depend on threshold levels for that particular neuron, so some neurons may display higher levels of c-fos i.e. high intensity and other neurons will display lower levels of c-fos i.e. low intensity. In all experimental subjects, changes to cell activity will be independent of neuron threshold levels. Any changes seen in neural activation patterns will be equally affected in all subjects.

Figure 3-3 Image of c-fos fluorescently labelled active neurons as seen under a microscope. Images viewed at 1X and 20X resolutions.

Major limitations in using immunocytochemistry (ICC) to map neuronal patterns are cross-reactivity of the primary antibody of interest with proteins of close homology, in this case
other members of the Fos family (e.g. Fra-1, Fra-2), and species specificity of primary antibodies. The use of more specific antibodies recently available has reduced the problem of cross-reactivity. To overcome the problem of species specificity, we are using antibodies specific to the mouse.

### 3.2.4 NeuN Labelling

NeuN (neuronal nuclei) is a neuron-specific nuclear protein found in most neuronal cell types and is identified using the monoclonal anti-body, anti-NeuN. Anti-NeuN originally called mAb A60, was discovered when a battery of monoclonal antibodies (mAbs) against brain cell nuclei was generated by repeated immunizations. The NeuN protein appears at developmental time points corresponding to neuron withdrawal from the cell cycle or with initiation of terminal differentiation of the neuron (91). Since its discovery, NeuN has been used in many studies as a diagnostic neuronal marker and as a counter-stain (92).

In this study, the NeuN protein is recognized using a mouse monoclonal anti-NeuN primary antibody (Chemicon; 1:1000) and a fluorescent-conjugated secondary antibody: Alexa 568 goat anti-mouse (Invitrogen; 1:200). Figure 3-4 shows a NeuN stained tissue slice at low and high magnification. Both the cell nuclei and the cytoplasm are stained.
Figure 3-4 NeuN stained tissue as seen under the microscope. Images viewed at 1X and 20X resolutions.

3.2.5 Method of Quantification

Fluorescently labelled cells were viewed using the Zeiss epiflorescent microscope, and were quantified using Volocity 5 software, which enabled an objective measure of active neurons in a sample. Areas of interest were first identified on a stained section using stereotaxic coordinates based on the mouse brain (93). These areas were then viewed at 20X resolution to quantify labelled neurons. Work carried out by Rieux et al. compared different methods used to quantify c-fos labelling, and concluded that computer-assisted image analysis e.g. optical density image analysis provides an accurate and rapid method to determine the relative amount of Fos protein in a sample (94). In this study, the Fos protein was identified using a fluorescent marker and was objectively quantified based on intensity rather than optical density, and on the size of the nucleus. Figure 3-5 shows how intensity and size parameters were used during analysis. Due
to the thickness of sections, different focal planes are used to view cells deeper in the section. Following NeuN staining, due to large numbers of cells present, an estimated number of cells were counted by taking an average of the number of cells in 5 random areas in the sample and extrapolating that figure to fit the area of the sample.

Figure 3-5 Screen print showing method of analysis when quantifying labelled neurons.
3.2.6 Scanning Electron Microscopy

Scanning electron microscopy (SEM) was used to take topographical images of inner and outer hair cells in conductive hearing loss subjects and control subjects. This was done to ensure that following conductive loss induction, inner and outer hair cell integrity remained intact. The protocol used to prepare cochlea samples for SEM is shown in the appendix section 2.

The SEM works by focusing a beam of electrons on the sample surface. X-ray, backscatter electrons and secondary electrons are ejected from the sample surface and are collected by detectors to display an image of the topography of the sample. For this study, hair cells in the cochlea were prepared using a critical point dryer to remove water molecules from the sample and then splutter coated in gold to ensure good conductance. Images of inner and outer hair cells from conductive hearing loss subjects were collected and compared to control subjects.
4 CHAPTER: EXPERIMENTAL RESULTS

In this section we present the changes to hearing thresholds resulting from experimental conductive hearing loss, and cochlear ablation, and the resulting changes in resting neural activity levels in the auditory areas of interest (cochlear nucleus and inferior colliculus). Also presented are some SEM (control) studies of the cochlea.

4.1 ABR Results

Mice with ear canal blockage to simulate conductive loss displayed an elevation in hearing thresholds of 50-60dB SPL (Error! Reference source not found.) across all frequencies (2 kHz to 32 kHz). Mice with bilateral cochlear ablation showed no discernable ABR’s even over 100dB SPL, indicating complete cochlear destruction and deafferentation.

Figure 4-1 ABR threshold elevations (50-60dB) in mouse model of conductive hearing loss (CHL). Deaf subjects had no measureable ABR’s even at intensities over 100dB.
4.2 SEM Results

Scanning Electron Microscopy was used to view the morphology of hair cells after conductive loss. Not all experimental animals were examined, and not all cochlear areas were examined systematically. However, from the samples examined we observe no obvious changes to the morphology of the hair cells and stereocilia. Typical appearance of haircells in conductive loss ears are as illustrated in Figure 4-2.

![Inner Hair cells](image1) ![Outer Hair cells](image2)

Figure 4-2 Inner and outer hair cells show normal morphology after induced conductive loss.
4.3 RESULTS: Immunocytochemistry

4.3.1 Cochlear Nucleus

Results from the cochlear nucleus showed a trend in the reduction of c-fos labelled cells from the control group to the conductive hearing loss group to the deafened group. These results were calculated using Kruskal-Wallis One Way Analysis of Variance and gave a p value of 0.007 (Figure 4-3). The reduced number of c-fos active neurons in deaf subjects indicates that cochlear deafferentation results in almost no resting activity, whereas the reduced neural activity in conductive hearing loss subjects (where there is no hair cell damage), indicates suppressed activity at rest. Results on the differences in c-fos labelling between the dorsal and ventral cochlear nucleus are shown in the appendix section 3, figure 0-2. Results in pair-wise comparisons between the control and conductive hearing loss (CHL) group gave a p value of 0.195. Pair-wise comparisons between the control and deafened group gave a p value of 0.051, and between the CHL group and the deafened group gave a p value of 0.004 (Figure 4-3). These results have a power of 40% and show that although there was no statistical significance in the reduced number of c-fos labelled cells, with an increased number of subjects, significance may become more apparent.
Figure 4-3 Results at the cochlear nucleus indicate a trend of reduction in resting activity levels after induced conductive loss. Overall analysis using ANOVA (p=0.007). Pair-wise (t-test) comparisons of *Control vs CHL (p=0.195), CHL vs Deaf (p=0.004) and Control vs Deaf (p=0.051).

4.3.2 Inferior Colliculus

In the inferior colliculus, active cells are quantified from all regions of the IC (dorsal, central and external). Literature has identified the central nucleus of the IC as the primary area for auditory activity, and although results show a general decline in all areas of the IC, the central nucleus of the IC showed the most significant reduction in c-fos active neurons (Figure 4-4). Statistical significance between all three experimental groups was calculated with One Way Analysis of Variance, resulting in F=13.005 and p=<0.001. A pair-wise comparison of control vs CHL reveals a p value of 0.058. This result shows borderline significance, however with more
subjects, the difference in labelled neurons between control and conductive hearing loss subjects may become more significant.

Figure 4-4 Results at central nucleus of IC show a significant reduction in c-fos labelled neurons. Overall analysis using ANOVA (F (2, 15) =13.005, P=<0.001) during a conductive hearing loss. Pair-wise (t-test) comparisons of **Control vs CHL (p=0.058), ***CHL vs Deaf (p=0.006) and *Control vs Deaf (p<0.001).

An overall result in the whole IC is shown in the appendix section 3. Data collected in the dorsal cortex shows no difference in c-fos labelled neurons between the control and conductive hearing loss subjects (Figure 4-5). These results confirm that there is very little ascending auditory activity in the dorsal cortex of the inferior colliculus. Results on the external cortex (Figure 4-6) indicates a slight trend in reduced activity, although a pair-wise comparison between the control and conductive hearing loss subjects gave a p value of 0.096. Therefore, there results indicate
that there may some ascending activity to the external cortex of the inferior colliculus but no significant reduction in c-fos labelled neuron activity. Neural activity in both the dorsal and external cortex are also affected my somatosensory stimulation and have descending pathway connections from cortico geniculate areas.

![Graph](image)

**Figure 4-5** The resting level of activity between CHL and Control are very similar, whereas deaf mice show a large decrease in activity. F (2, 15) = 5.648, P = 0.015. Pair-wise (t-test) comparisons of **Control vs CHL (p=0.524), ***CHL vs Deaf (p=0.026) and *Control vs Deaf (p=0.017)**
Figure 4-6 There is some decrease in activity between CHL and control subjects. Deaf subjects show a larger decrease in activity. ANOVA analysis $F(2, 15) = 5.938$, $P=0.013$. Pair-wise (t-test) comparisons of **Control vs CHL ($p=0.096$), ***CHL vs Deaf ($p=0.110$) and *Control vs Deaf ($p=0.011$).

4.3.3 Percentage of c-fos active neurons in the brainstem and midbrain

NeuN detection was used as a counter stain to measure approximate numbers of neurons in the CN and IC of the brain and compares it to active neurons in these areas at rest. The percent of c-fos active neurons in the cochlear nucleus and the central nucleus of inferior colliculus is
shown in Figure 4-7. The c-fos labelled cell counts show relatively low numbers of neurons active in the resting (non sound stimulated) state. In the cochlear nucleus for normal controls 0.17% of cells were (c-fos) active. In conductive hearing loss this falls to 0.12%. After cochlear ablation only 0.01% of cells were c-fos active. In central inferior colliculus, 2.21% of cells were active in the resting state. In conductive loss 1.87% of neurons were active and only 0.34% after bilateral cochlear ablation.

![C-fos active cells chart]

**Figure 4-7 Percentage of c-fos active cells in the cochlear nucleus and central nucleus of IC.**

Immunocytochemistry using NeuN and c-fos labelling makes it possible to visually identify differences in neural activity patterns between the different experimental groups. Figure 4-8 compares images of the left inferior colliculus between a NeuN stained control, c-fos stained controls, c-fos stained CHL subjects and c-fos stained deaf subjects. NeuN control image shows
neurons taken from one slice of the IC. C-fos images are obtained by converging data from 14 slices of the IC.

Figure 4-8 Images of the left IC comparing the difference in resting neural activity between control, CHL and deaf subjects. Images are converted from fluorescence to monochrome format.
5 CHAPTER: DISCUSSION

In the following chapter, a discussion of the results of this thesis work and other various studies related to this work is given. Also included is some commentary and speculation on the clinical significance of this study.

5.1 Discussion of experimental protocol & results

To gain a better understanding of the effects of conductive hearing loss on the auditory pathway, immunocytochemistry was employed to detect changes in resting neural activity patterns. Results obtained from c-fos immuno-labelling showed a decreased neural activity between conductive loss subjects and control subjects in two major auditory areas i.e. cochlear nucleus and central nucleus of the inferior colliculus. These areas of the central auditory pathway consist of second and third order neurons. It is supposed that these central changes in resting neural activity in these areas reflect an alteration of driven and spontaneous neurotransmitter release at the level of the inner haircell synapse.

In the past, central effects of conductive hearing loss have been studied in various ways, but results were inconsistent and sometimes contradictory. Anatomical studies in the mouse have indicated that sound deprivation during postnatal development resulted in smaller nuclei in the cochlear nucleus and medial nucleus of the trapezoid body (50). However, a similar study carried out in rhesus monkeys showed no discernible change in neuronal size or any changes to ABR latencies (3). It seems that the age at which a conductive loss was induced i.e. before hearing onset in altricious animals, would result in a reduction in the size of nuclei. In precocious
animals, with mature cochleae, the effects of conductive loss were less obvious, and results showed no effect on neuronal size. In other functional studies looking at the effects of conductive loss on the auditory pathway, results in chicks and gerbils showed no change in neuronal activation patterns (52), or changes in neuronal metabolic activity (2-deoxyglucose uptake) (95). Other studies however, revealed a decrease in spontaneous activity recorded at the round window (neural ensemble activity; round window noise) (71) and a reduction in neuronal 2-deoxyglucose uptake (2). Here, it seems results were dependent on the method of inducing a conductive loss, and the type of species used. In another study, a unilateral conductive loss resulted in increased neuronal activity in the auditory cortex contralateral to the manipulated ear (95, 96). This result could be explained by the anaesthetic used (pentobarbital and ketamine), which has been identified to cause enhanced stimulus evoked increases in relative 2-DG uptake (97). These results could also be explained by the induction of unilateral conductive hearing loss. In the auditory system, both ears behave in a homeostatic fashion, where damage to one ear causes changes in the other (undamaged) ear, which compensates for the loss in hearing from the damaged ear. Therefore, changes seen in metabolic activity could be resulting from the fully functional ear, where competitive plasticity (between the pathways from each ear) would ensure that the ‘good’ ear compensates for the sound attenuated ear. Due to this reason, bilateral conductive loss was induced in the mice for this thesis.

The method used in this study to detect changes in neural activity patterns after a conductive loss differs from the previous studies that mainly used 2-deoxyglucose uptake to measure changes in neuronal metabolic activity. A disadvantage in using 2-DG uptake for this purpose is that it is only able to show regions of activity in the brain, and may be influenced by external manipulations. The advantage of using c-fos as a marker for neural activity at rest,
allows detection in large regions of the brain, while providing a spatial resolution high enough to see individual cells. In past studies using c-fos detection, investigators chose to use a secondary detection system involving the avidin-biotin-complex and diaminobenzidine (83, 90, 98). This protocol worked well, however in this study, a fluorescent-conjugated secondary antibody is chosen because fewer steps were needed to stain samples, and detection using the software available is compatible with this technique. Most limitations pertaining to immunocytochemistry are overcome by using a conventional animal model (mouse), where species specific antibodies are available for this model, and using antibodies specific to the antigen c-fos to avoid cross-reactivity. The protocol used for staining can found in the appendix.

Results from auditory brainstem responses indicate a 50-60dB SPL rise in hearing threshold in conductive loss subjects compared with controls (Figure 4-1). These results together with scanning electron microscopy images of the hair cells clearly indicates that sound attenuation leading to higher thresholds does not induce physical changes in morphology, indicating no hair cell damage (Figure 4-2). In deaf subjects, cochlea ablation results in no measurable ABR’s. These results ensure that by ablating the cochlea, hair cells are destroyed, and that by inducing a pure conductive loss, sound is attenuated with no hair cell destruction. By blocking the ears bilaterally using dental cement, bone conduction could occur. In clinical setting, sound attenuated by otitis media also causes some small amount of bone conduction. Results from this thesis are only showing changes in neural activity during a conductive block. The level of bone conduction was not a concern as we were not emulating otitis media but were just observing effects from conductive hearing loss. To avoid bone conduction, middle ear bone removal may have been a better way to induce a conductive block.
As mentioned earlier, mice in each experimental group are bilaterally affected indicating another difference between this study and previous studies. In doing so, it eliminates the need to tease out and identify ipsilateral and contralateral pathways, as experimental results are seen on both sides. In the central auditory pathway, the regulation of excitatory and inhibitory activity may be influenced by a unilateral conductive hearing loss (99). This is seen in a study carried out in guinea pigs, where the regulation of D-aspartate release and uptake is used to measure transmitter release from glutamatergic presynaptic endings and glutamate inactivation. They found that unilateral attenuation eventually induced changes consistent with abnormally weakened excitatory glutamatergic transmission in the ipsilateral cochlea nucleus (100). Using c-fos immunocytochemistry, such changes during a unilateral conductive loss will be difficult to distinguish especially in areas further up the pathway, where neurons can project to and from multiple areas. Therefore, by inducing bilateral manipulation any changes in the pathway are uniform and equally affected in both hemispheres.

The two auditory pathway areas of interest in this study are the cochlear nucleus and the inferior colliculus. As mentioned previously, the cochlear nucleus contains the first relay neurons to receive information directly from inner haircells. Therefore, active cells in the cochlear nucleus will directly reflect excitatory activity at the inner haircell synapse. The other major area of interest is the inferior colliculus, more specifically the central nucleus of the inferior colliculus, where bilateral integration is integral in the binaural system for sound localization. This area is further up the auditory pathway, and is almost exclusively auditory related. Bimodal neurons found here are described as second, third and fourth order neurons depending from where in the pathway they receive input (34, 36). Therefore, data collected from these neurons will give an impression on the pattern of activity that occurs in the pathway, even if it is several
relays away from the source (inner haircell synapse). Other cortical areas further up the pathway are not targeted in this study, as neurons here start to integrate information from multisensory areas, and resting neural activity patterns will not reflect activity at the periphery.

The results from this study corroborate with some of the later studies carried out by Tucci et al. on the gerbil (2, 70), where reductions in neural activity were detected in models of conductive loss. The changes in activity seen at the cochlear nucleus (Figure 4-3) between the conductive loss subjects and the control subjects is not significant (p=0.195), however, data does indicate a trend in reduction. In the central nucleus of the inferior colliculus (Figure 4-4), changes in resting neural activity between conductive loss subjects and controls is slightly more significant (p=0.058). This result found higher up the auditory pathway is interesting and may indicate that perhaps a longer period of conductive loss or a larger population of subjects would help signify the reduction in activity levels seen in the cochlear nucleus. These results also show that the effects of sound attenuation can be seen from the brainstem to the midbrain and does not dissipate completely as it travels from periphery through the pathway. In deaf subjects, the reduction in activity is severe, indicating that by destroying hair cells, neuronal activation in the ascending pathway almost stops. The very low levels of activity seen in the cochlear nucleus and the central nucleus of the inferior colliculus are probably related to other sensorineural activity and any descending pathway activity. Work done by Winer et al. on the midbrain has shown that the central nucleus of the IC has light projections from the primary auditory cortex and projections from the ectoslyvian gyrus, where neurons are associated by tonotopic auditory areas and retinotopic visual areas (33). Another study done by Schofield et al. has shown that there are connections from the auditory cortex to the cochlea nucleus that then projects to the inferior
These studies identify other pathways connections that can explain the low levels of activity seen in the deafened mice.

The results in resting levels of activity under these experimental groups are put into perspective when compared with the number of neurons available in these auditory areas (Figure 4-7). This data clearly shows that there is a low percentage of cells are active in the resting state, yet changes in these levels can still be seen during a conductive hearing loss, which may have an effect in pathway development.

Interestingly, when comparing the reduction in activity between conductive loss subjects with controls, and deaf subjects with controls, as expected, there is a significant decline in active neurons in deaf mice, where hair cells are damaged. However, in conductive hearing loss subjects, there is no hair cell damage (detected using SEM (Figure 4-2)), yet there is still a reduction in the number of active neurons. A major question, therefore, resulting from this study is what is happening at the inner hair cell synapse to cause this decrease in activity during a conductive loss? From research and literature, we know that at the inner haircell synapse, ribbon synapses and the distribution of Ca+ channels can contribute to neurotransmitter (glutamate) release (6-11). Therefore, it seems that during a conductive hearing loss, sound attenuation contributes to a suppression of glutamate release as a ‘feedback’ mechanism. Work done by Lee et al. on the hair cells in the chicken described one potential mechanism for regulating calcium channels at the hair cell synapse; calcium-dependent inactivation (102). This L-type calcium channel is modulated by calmodulin-like calcium-binding proteins, and is thought to control opening of calcium channels to maintain the set-point for spontaneous glutamate release. A question then is how is conductive loss able to influence this ‘feedback’ mechanism? It is prudent to point out that in such a homeostatic system, any changes in excitatory activity at the
hair cell would induce changes to any inhibitory activity in the system, and this type of feedback mechanism could be a result of trying to maintain a balance in the system. Further research is needed to fully answer this and other questions that have arisen from this work.

5.2 Clinical significance of results

The findings from this study have begun to answer some of the questions surrounding conductive hearing loss and elucidate its effect on the auditory pathway. Many more questions still need to be answered to gain a better understanding of this disorder.

There have been mixed reviews on the effects of otitis media in hearing development. Some studies have found deficits in higher-order auditory processing in children (8-9 years old) after their initial diagnosis (7-39 months old) ((55, 56). In other studies and reviews, it is suggested that in typically developing children, otitis media may not be a substantial risk factor for later speech and language development or academic achievement (103). Most research in this area has concluded that although effects of early otitis media may resolve by school age (7+years), there seems to be a link between otitis media patients and speech perception in noise (104). It seems that the number of times a patient gets an ear infection, and the length of time the patient has suffered from otitis media will determine the severity of its effect in the auditory pathway. Some complications that can arise from severe chronic otitis media include mastoiditis, cholesteatome and permanent damage to the middle ear bones.

Results from this study show that conductive hearing loss can affect resting neural activity patterns throughout the auditory pathway, which clinically could relate to altered central auditory pathway development in infants suffering from chronic episodes of otitis media. These clinical
implications suggest that children suffering from conductive hearing loss for extended periods of time should be monitored regularly and earlier intervention is necessary.
6 CHAPTER: CONCLUSIONS & FURTHER DIRECTIONS

This Chapter contains some conclusions drawn from this study with some comments of future research directions.

We can conclude that conductive hearing loss does not only reduce the level of stimulus driven activity in the cochlea, but also appears to down-regulate transmitter release at the inner haircell synapse without causing physical damage to these hair cells. This translates to lower resting activity levels in central auditory areas. These findings focus our concern on sensory deprivation in infants with conductive hearing loss, especially during early post-natal years when developmental plasticity of the auditory brain is very high. Perhaps altered neural wiring at this stage could contribute to deficits in higher-order auditory processing as reported in outcome studies on children with chronic, re-occurring otitis media (56, 57).

Further work to understand the mechanisms involved at the inner haircell synapse (i.e. lowering glutamate release) during a conductive loss can help to answer some of the questions that have arisen during this investigation. In this study, acute experiments with mice enduring 3 days of conductive loss was carried out; however could longer periods of conductive loss have a more profound effect in the auditory pathway, and do the lower levels of resting activity persist after resolution of the conductive loss? Other ABR studies involving data from this thesis involved removing the conductive loss (dental cement) after the three days to determine any changes in brain stem response. Fig 0-4 in section 4 of the appendix shows these results, where thresholds are lowered after 15 days mainly in the high frequency region of the cochlea. This
indicates that there seems to be some damage to the tympanic membrane while inducing the conductive loss causing higher thresholds in low frequency regions. More work refining the method of conductive loss, and increasing the pool of data will yield more information from this study. Another factor to consider is that the ‘side-effects’ of chronic conductive loss can vary, and attention needs to be given in the method of inducing a conductive loss in animal studies such that conditions resulting from it closely represent those found in the clinical setting. Further work looking at the critical time period (how long), when the effects of a conductive loss become irreversible also needs to be considered.

Further questions regarding the level of activity in the brainstem include what changes (if any) are seen in stimulus driven activity once resting levels of activity are reduced? Such an experiment would involve stimulating normal and experimental groups to the same sound intensity, and then measure levels of neuronal activity in regions of the brain. Any differences in neuronal quantification should be compared to data from this study to see if changes in stimulated activity reflect data from resting levels of activity.

A limitation in this study is that c-fos detection of central auditory neurons is an indirect measure of activity levels at the inner hair cell synapse. A more direct approach of measuring activity at the synapse or in first order afferent neurons is warranted. This can be done by either the electrophysiological study of function or by immunolabelling studies of proteins involved in vesicular release at the inner hair cell synapse. Further work in this area will help answer some of the questions mentioned above, as well as tease out some of the complexities involving feedback mechanisms and other cortical responses to external stimuli. Such work will involve studies on descending pathways (from the cortex down) and how they can influence information received and travelling through ascending pathways.


5. Fuchs P. Why do hair cells have ribbons? focus on "synaptic ribbon enables temporal precision of hair cell afferent synapse by increasing the number of readily releasable vesicles: A modeling study". J Neurophysiol. 2008 Oct;100(4):1695-6.


APPENDIX 1

1. c-FOS/NeuN Immuno-labelling Protocol

1. Intracardiac perfusion of animal with saline to wash vascular system followed by freshly made 4% Paraformaldehyde in 0.1M phosphate buffer (PB) at pH 7.4. Remove the whole brain from the skull.

2. Cut the brain using vibratome 60 microns thick. Collect sections in 0.1 M phosphate buffer and store in 0.02% sodium azide storing sol. at 4C.

3. Rinse the sections 3X5 mins with PBS 0.1M on shaker.

4. Incubate in primary antibody:
   - c-fos staining: Rabbit anti-Fos polyclonal primary antibody (e.g., Calbiochem, cat. no.PC 38) for 48hrs at 4°C on shaker. Optimal dilution 1:10,000 (4µl in 40ml of blocking sol.)
   - NeuN staining: Mouse monoclonal anti-NeuN primary antibody (Chemicon) for 48hrs at 4°C on shaker. Optimal dilution 1:1000.

5. Rinse the sections 3X5 mins with PBS 0.1M on shaker.

   - C-fos staining: Alexa Fluor 568 goat anti-rabbit/mouse IgG (H + L) (e.g., Invitrogen, cat. no. A11011) for 2hrs at room temp. Optimal dilution ratio 1:1,000 in sol. containing 0.1M PBS with 0.3%Triton-X. (20µl in 20ml). Carry out in a dark room. DO NOT EXPOSE SAMPLES TO LIGHT.
- NeuN staining: Alexa 568 goat anti-mouse (Invitrogen) for 2hrs at room temp.
  Optimal dilution ratio 1:200 in sol. containing 0.1M PBS with 0.3% Triton-X.
  Carry out in a dark room. DO NOT EXPOSE SAMPLES TO LIGHT.

7. Rinse the sections in 3X5mins with 0.1M PBS on shaker.

8. Transfer sections to a slide using a brush.

9. Add antifade mounting medium (PermaFluor) and place cover slips. Allow to dry for 2 days.

10. **PAUSE POINT** Mounted brain sections at 4°C may be stored in the dark for up to 2 months before imaging with microscope.

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2. **Scanning Electron Microscopy sample preparation**

**Note:** Solution preparations are given at the end of the protocol.

**Note:** Samples are kept rotating on the rotorack for all rinses/incubations.

**Note:** Cochlea once removed from the animal must always be kept in solution and should not be exposed to air, as air bubbles can form in the cochlea affecting the results.

**DAY 1**

1. Transcardial perfusion with saline, followed by 2.5% gluteraldehyde in sodium cacodylate buffer (pH 7.4; fixative).
2. Remove bulla, punch hole in bulla and remove outer bone to expose cochlea. Remove inner ear bones from the oval window entrance. Using a 30G needle and 1-mL syringe, gently flush cochlea with fixative 2 or 3 times. Incubate in fixative for 2 hours.
3. 1 x 15 min cacodylate buffer rinse.
4. Place samples in a new solution of cacodylate buffer overnight at 4°C.
**DAY 2**

5. 1 x 15 min cacodylate buffer rinse.
6. Prepare 2% buffered osmium tetroxide (**in PB**). Using a 30G needle and 1-mL syringe, flush cochlea 2-3 times with OsO₄. Incubate in OsO₄ for 1.5 hours.
7. 2 brief rinses with cold saline.
8. Dehydrate in 1 x 15 min rinses of cold 35%, 50% then 70% alcohol on rotorack (samples can remain in 70% alcohol in fridge for a few days).

*NOTE: SAMPLE SHOULD NOT BE EXPOSED TO AIR ONCE DEHYDRATION STEPS BEGIN. Remove most of the previous fluid, leaving some inside to cover the sample. Fill vial with the next solution and repeat (remove most alcohol, fill).
9. In 70% alcohol, open up the bony structure around the cochlea to reveal hair cells. For mice use forceps. For chinchillas use a drill to thin the outer bone. Use forceps to open the bony structure (can leave in 70% in fridge for a few days).

**DAY 3**

10. Switch on the CPD machine for approx. 10mins before cooling the CPD chamber. This heats the valves to avoid them freezing during the cooling process.
11. 1 x 15 min rinses in each of 90% and 95% alcohol. 3 x 15 min rinses in 100% alcohol. Rinses are done as above (see *NOTE*). While washes are being carried out, proceed to cool the CPD chamber.

**TO COOL CPD CHAMBER** - Make sure chamber bolts are all closed securely. Make sure fill, vent drain and vent rate are closed. Open the gas (on the tank), then open vent and vent rate a little bit. Open fill (pressure should rise), close fill. Once the pressure drops to 500psi, open fill again (pressure should increase again). Keep repeating this process (opening and closing fill) until the temperature in the chamber is at or below 10ºC. Once the temp has reached 10ºC, close all valves. Open drain to reduce the pressure to zero. The chamber can now be opened to place the samples inside.
12. Place SEM cage into absolute alcohol (ensure alcohol fully covers the cage), and transfer cochlear turns from the glass vial into the SEM cage using a pipette with the tip cut off (wider mouth). *NOTE – SAMPLE SHOULD NOT BE EXPOSED TO AIR.
13. Place closed SEM cage quickly into critical point dryer (CPD). Close bolts securely.
14. **FOR CO₂ WASH** – CPD should be cooled with the temp close to 10ºC. Open fill and ensure sample is covered in CO₂ solution. Pressure should go up to 800 psi. Open vent and vent rate a little bit. The vent rate controls the head of gas above the CO₂ solution in the chamber. Adjust fill and vent rate to maintain a meniscus 2/3 of the way up in the window on the chamber.
15. Rinse for one minute and then close fill, vent and vent rate. Pressure should stay at 800 psi.
16. Leave CPD to allow the sample to bath in solution for 10 min. Start again (from step #14) and carry out rinse 3 times in total.
17. After last wash, close vent, vent rate and fill. Switch to heat → red light comes on. Psi goes up to 1000 - 1500 and temperature reaches 42°C. Red light will switch off automatically and the white light will switch on.

18. Before removing the samples, the pressure needs to be reduced by opening vent and vent rate a little. Pressure needs to go down very slowly (100 – 200 psi/min). Once pressure reaches zero, close vent and vent rate. Open drain to ensure all pressure has gone. Close drain. The chamber can now be open to remove the samples.

19. Remove specimens and mount on to carbon stickers ready to view under SEM.

*For very small specimens (neonates), go to OTOTO procedure found in the lab manual section 5. After step #8, rehydrate sample and carry out OTOTO for 15 mins each. In between carry out water wash 5 times.

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**Solution Preparation**

1) **Saline**

900 mg of sodium chloride in 100 ml distilled water

2) **Preparation of 100ml sodium cacodylate buffer (pH 7.4)**

   Preparation of stock solution:
   
   **Stock A: 0.2M Sodium Cacodylate:**
   
   4.28 g sodium cacodylate in 100 ml of distilled water. Store at 4 degrees C

   **Stock B: 0.2M HCl**

   1.7 ml Hydrochloric Acid in 100 ml distilled water. Store at 4 degrees C

   **Preparation of Buffer:**

   Add 25 ml of Stock A + 1.4 ml of Stock B made up to 100 ml with distilled water.

   Store at 4°C.

3) **Preparation of 2.5% Gluteraldehyde in Sodium Cacodylate buffer (pH 7.4)**

   To prepare 100 ml, add 5 ml of 50% gluteraldehyde + 95 ml sodium cacodylate buffer.

   Store at 4°C.

4) **Preparation of 1% buffered Osmium Tetroxide**

   Add 2 ml of 4% Osmium Tetroxide to 6 ml of 0.1M Phosphate buffer.

   (Refer c-Fos Methodology for 0.1M phosphate buffer preparation)

   (Everything inside fume hood!)
Preparation of 0.1M phosphate buffer (PB):

Solution A [0.2M Sodium phosphate dibasic]
- 2.84 g sodium phosphate dibasic Na2HPO4 (anhydrous)
- make up to 100 ml with distilled water

Solution B [0.2M Sodium phosphate monobasic]
- 2.76 g of Sodium Phosphate monobasic Na2HPO4.H2O
- made up to 100 ml with distilled water

Prepare 0.2M phosphate buffer (pH 7.4)
- 40.5 ml of solution A + 9.5 ml of solution B
  (OR)
- 81 ml of solution A + 19 ml of solution B

Prepare 0.1M phosphate buffer (pH 7.4)
- mix equal amounts of 0.2M phosphate buffer with distilled water.

5) Preparation of 1% aqueous Osmium Tetroxide
   Add 2 ml of 4% osmium tetroxide to 6 ml of distilled water.
   (Inside fume hood!)

6) Preparation of 1% aqueous Thiocarbohydrazide (TCH)
   0.3 g of TCH dissolved in 30 ml of distilled water by stirring in a magnetic stirrer
   for about an hour.
   Then the above supernatant is taken.
   TCH should be prepared fresh on the day of use.
3. Results: Immunocytochemistry in the IC and CN

There is a decrease \( F (2, 17) = 10.461, P = 0.001 \) in resting level activity in the whole IC.

0-1 There is a decrease \( F (2, 17) = 10.461, P = 0.001 \) in resting level activity in the whole IC.
Resting level of activity in the cochlea nucleus. Graph compares results between the dorsal and ventral cochlea nucleus. DCN ANOVA (p=0.004). VCN ANOVA (p=0.228)
4. ABR Results: after removal of conductive block

Change in hearing thresholds over time after removing dental cement

0-3 ABR thresholds start to drop after removing conductive block. High frequency regions recover faster