The Effects Of Early L-Baclofen Administration On The Development Of Tinnitus Induced By Acoustic Trauma, And GABA$\text{B}_{-R2}$ Expression, In Rats

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Abstract

Tinnitus is an illusory auditory sensation which often manifests as ‘ringing of the ears’. While the pathophysiology of tinnitus is poorly understood, there is currently a strong body of evidence to suggest that an underlying hyperactivity within the central auditory system acts as a pathological mechanism of acoustic trauma-induced tinnitus in affected individuals. As a result, centrally acting drugs that decrease excitation, or increase inhibition, are sometimes prescribed as a treatment. Included among these drugs is baclofen, a γ-aminobutyric acid type B (GABA_B) receptor agonist which increases GABAergic neurotransmission, thus increasing inhibition within the central nervous system. The aim of this study was to investigate the effect of both early L-baclofen administration on the development of acoustic trauma-induced tinnitus, and chronic L-baclofen administration on the psychophysical attributes of established tinnitus, in rats. In addition to this, changes in GABA_B receptor subunit 2 (GABA_B-R2) expression in response to L-baclofen administration were also investigated. The aims of this study were achieved using a unilateral acoustic trauma method of tinnitus induction in rats, followed by assessment of the psychophysical attributes of tinnitus using a conditioned lick suppression model. A foot shock acted as an unconditioned stimulus (UCS) in this model, and speaker off periods as a conditioned stimulus (CS). This resulted in lick suppression of the animals in response to the speaker off periods, with those rats having tinnitus displaying greater lick suppression due to suppressed licking during stimuli that resembled the sensory features of their tinnitus, as well as during the speaker off periods. The acoustic trauma resulted in a significant increase in the threshold of the auditory brainstem response (ABR) of the ipsilateral ear (P ≤ 0.001) which, when re-tested prior to animal sacrifice, was shown to be only temporary in nature. Despite early L-baclofen administration (5 mg/kg, 30 minutes following acoustic trauma), the acoustic trauma had a significant exposure effect for the BBN (P ≤ 0.007), 20 kHz (P ≤ 0.015), and 32 kHz (P


≤ 0.001) tones, indicating the presence of tinnitus in the noise-exposed rats. Chronic L-baclofen administration (3 mg/kg/day for 32 days) resulted in a general drug effect, which affected the animals’ ability to suppress their licking, and was not necessarily an indication of the alleviation of tinnitus in the exposed-baclofen group. Furthermore, there was no significant change in mean GABA_B-R2 density within the cochlear nucleus of L-baclofen-treated animals. These results suggest that L-baclofen administration via subcutaneous injection may have systemic effects which could potentially decrease compliance amongst patients. The general drug effect, found within the current study, made it difficult to assess the outcome of L-baclofen on the psychophysical attributes of tinnitus. Therefore, further studies assessing the effects of different L-baclofen preparations, and other GABA_B agonists, would be of benefit in assessing the potential of L-baclofen as a tinnitus treatment.
Chapter 2: Materials and Methods ................................................. 47

2.1 Animals ..................................................................................................................... 48
2.2 Materials ................................................................................................................... 49
2.3 Sequence of the Project ........................................................................................... 52
2.4 Noise Trauma to Induce Chronic Tinnitus ............................................................ 55
2.5 Hearing Level Assessment ....................................................................................... 57
2.6 Tinnitus Assessment ................................................................................................ 58
   2.6.1 Tinnitus Assessment Chamber ........................................................................... 58
   2.6.2 Tinnitus Assessment Conditions ........................................................................ 59
   2.6.3 Acclimation Phase .............................................................................................. 60
   2.6.4 Pavlovian Conditioned Suppression Training Phase ............................................ 61
   2.6.5 Frequency Discrimination Phase ........................................................................ 62
   2.6.6 A Positive Tinnitus Assessment ......................................................................... 63
2.7 Drug Protocol ........................................................................................................... 65
   2.7.1 Acute Drug Administration Following Noise Trauma ....................................... 65
   2.7.2 Chronic Drug Administration During the Third Testing Period ........................ 66
   2.7.3 Drug Administration Prior to Animal Sacrifice ................................................. 66
2.8 Tissue Removal ........................................................................................................ 67
2.9 Tissue Sectioning ...................................................................................................... 69
2.10 Histology ................................................................................................................ 71
2.11 Immunohistochemistry ............................................................................................ 72
2.12 Quantitative Analysis of GABA<sub>B</sub> Receptors ..................................................... 74
2.13 Statistics .................................................................................................................... 76

Chapter 3: Results .................................................................................. 78

3.1 Auditory Brainstem Evoked Responses ................................................................. 79
   3.1.1 ABRs Immediately Prior to Exposure, and Immediately Following Exposure . 79
   3.1.2 ABRs Immediately Prior to Animal Sacrifice ..................................................... 83
3.2 Tinnitus Assessment and Drug Treatment ............................................................ 84
   3.2.1 First Behavioural Testing Period, Following the Acute Administration of L- Baclofen ......................................................................................................................... 84
   3.2.2 Second Behavioural Testing Period, Following Extended Drug Washout Period .......................................................... ................................................................. 87
   3.2.3 Third Behavioural Testing Period, During Chronic Administration of L- Baclofen ......................................................................................................................... 90
3.3 Histology .......................................................................................................................... 93
3.4 Immunohistochemistry....................................................................................................... 94
3.5 Quantitative Analysis of GABA<sub>B</sub> Receptor ............................................................ 97

Chapter 4: Discussion ................................................................................................. 98
  4.1 Interpretation of the Results, and Comparison to Previous Literature ................. 99
  4.2 Significance of the Study and Clinical Implications .............................................. 107
  4.3 Critical Evaluation of the Experimental Design .................................................... 109
  4.4 Proposed Future Research ..................................................................................... 120
  4.5 Conclusion ............................................................................................................... 123

References ................................................................................................................... 124

Appendix ...................................................................................................................... 159
List of Figures

Figure 1: Anatomy of the ear
Figure 2: Peripheral and central auditory pathways
Figure 3: The CN
Figure 4: The characteristics and proposed generator sites of ABRs
Figure 5: GABA metabolism and inhibitory transmission
Figure 6: The three classes of GABA receptors
Figure 7: Schematic representation of the GABA_B receptor
Figure 8: Chemical structure of GABA and baclofen
Figure 9: Timeline of the behavioural methods
Figure 10: Exposure of an animal to noise trauma
Figure 11: Placement of electrodes during ABR recording
Figure 12: Tinnitus assessment chamber
Figure 13: Acclimation testing phase
Figure 14: Suppression testing phase
Figure 15: Discrimination testing phase
Figure 16: Tissue sectioning plan
Figure 17: Quantitative analysis of GABA_B receptors using ImageJ software
Figure 18: Example of ABR recordings
Figure 19: ABR hearing thresholds at the beginning of the behavioural study
Figure 20: ABR hearing thresholds at the conclusion of the behavioural study
Figure 21: Mean SRs for the first tinnitus testing period, as a function of intensity (dB SPL) and frequency (kHz)
Figure 22: Mean SRs for the second tinnitus testing period, as a function of intensity (dB SPL) and frequency (kHz)
Figure 23: Mean SRs for the third tinnitus testing period, as a function of intensity (dB SPL)
and frequency (kHz)

**Figure 24:** CV staining of a section under 2x magnification

**Figure 25:** Confirmation of GABA$_{B^+R2}$ positive cells in the CN by immunohistochemistry

**Figure 26:** Confirmation of GABA$_{B^+R2}$ staining specificity using negative control sections

**Figure 27:** Mean density of GABA$_{B^+R2}$ staining in the CN of L-baclofen treated animals compared to vehicle treated animals, following a unilateral noise trauma
List of Tables

Table 1: Common causes of tinnitus
Table 2: Pharmacological approaches to the treatment of tinnitus
Table 3: Sequence of methods, in the order they occurred
Table 4: Factors that had a significant effect on the ABR thresholds of the animals
Table 5: Factors that had a significant effect on the SR of the animals during the first
  behavioural testing period
Table 6: Factors that had a significant effect on the SR of the animals during the second
  behavioural testing period
Table 7: Factors that had a significant effect on the SR of the animals during the third
  behavioural testing period
Table 8: Location of GABA_{B} sites in the rat periphery, and the response initiated by receptor
  activation
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1º Ab</td>
<td>Primary antibody</td>
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<tr>
<td>2º Ab</td>
<td>Secondary antibody</td>
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<td>AbDB</td>
<td>Antibody dilution buffer</td>
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<td>ABR</td>
<td>Auditory brainstem response</td>
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<td>AC</td>
<td>Auditory cortex</td>
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<td>AIC</td>
<td>Akaike’s Information Criterion</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>AVCN</td>
<td>Anteroventral cochlear nucleus</td>
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<td>BBB</td>
<td>Blood brain barrier</td>
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<td>BBN</td>
<td>Broadband noise</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CN</td>
<td>Cochlear nucleus</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<td>CS</td>
<td>Conditioned stimulus</td>
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<tr>
<td>CV</td>
<td>Cresyl violet</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-Diaminobenzidine</td>
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<tr>
<td>DCN</td>
<td>Dorsal cochlear nucleus</td>
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<tr>
<td>dH₂O</td>
<td>Distilled water</td>
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<tr>
<td>DPOAE</td>
<td>Distortion product otoacoustic emission</td>
</tr>
<tr>
<td>DPX</td>
<td>Di-n-butyl phthalate in xylene</td>
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<tr>
<td>EPSC</td>
<td>Excitatory postsynaptic current</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<tr>
<td>GABA_A</td>
<td>γ-aminobutyric acid type A</td>
</tr>
<tr>
<td>GABA_B</td>
<td>γ-aminobutyric acid type B</td>
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<tr>
<td>GABA&lt;sub&gt;C&lt;/sub&gt;</td>
<td>γ-aminobutyric acid type C</td>
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<tr>
<td>GABA&lt;sub&gt;B-R2&lt;/sub&gt;</td>
<td>GABA&lt;sub&gt;B&lt;/sub&gt; receptor subunit 2</td>
</tr>
<tr>
<td>GABA-T</td>
<td>GABA-transaminase</td>
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<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
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<tr>
<td>GHSI</td>
<td>Glasgow Status Health Inventory</td>
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<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
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<tr>
<td>HAM-D</td>
<td>Hamilton Depression Scale</td>
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<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>IC</td>
<td>Inferior colliculus</td>
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<tr>
<td>IHC</td>
<td>Inner hair cell</td>
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<td>IPSP</td>
<td>Inhibitory postsynaptic potential</td>
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<tr>
<td>LMM</td>
<td>Linear mixed model</td>
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<tr>
<td>MPI</td>
<td>Multidimensional Pain Inventory</td>
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<tr>
<td>Na&lt;sub&gt;2&lt;/sub&gt;HPO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Disodium hydrogen phosphate</td>
</tr>
<tr>
<td>NaH&lt;sub&gt;2&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Monosodium phosphate</td>
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<td>NaOH</td>
<td>Sodium hydroxide</td>
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<tr>
<td>NDS</td>
<td>Normal donkey serum</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<tr>
<td>OCT</td>
<td>Optimum cutting temperature</td>
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<tr>
<td>OHC</td>
<td>Outer hair cell</td>
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<tr>
<td>PB</td>
<td>Phosphate buffer</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PET</td>
<td>Positron emission tomography</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>PVCN</td>
<td>Posteroventral cochlear nucleus</td>
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<td>QWB</td>
<td>Quality of wellbeing</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>SFR</td>
<td>Spontaneous firing rate</td>
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<tr>
<td>S-HRP</td>
<td>Streptavidin-horseradish peroxidase</td>
</tr>
<tr>
<td>SOAE</td>
<td>Spontaneous otoacoustic emission</td>
</tr>
<tr>
<td>SOC</td>
<td>Superior olivary complex</td>
</tr>
<tr>
<td>SPL</td>
<td>Sound pressure level</td>
</tr>
<tr>
<td>SR</td>
<td>Suppression rate</td>
</tr>
<tr>
<td>SSA</td>
<td>Succinic semialdehyde</td>
</tr>
<tr>
<td>Tap H₂O</td>
<td>Tap water</td>
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<tr>
<td>THI</td>
<td>Tinnitus Handicap Inventory</td>
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<tr>
<td>THQ</td>
<td>Tinnitus Handicap Questionnaire</td>
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<tr>
<td>UCS</td>
<td>Unconditioned stimulus</td>
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<tr>
<td>VAS</td>
<td>Visual Analogue Scale</td>
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<tr>
<td>VCN</td>
<td>Ventral cochlear nucleus</td>
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Chapter 1: Introduction
1.1 The Auditory System

1.1.1 Hearing

The evolution of mammalian hearing has led to the use of frequencies above 1 kHz to improve both detection and localisation of sound sources (Nobili et al., 1998). As a result, various organisms can respond to physical vibrations in the atmosphere within a limited range which is species-dependent and differs with respect to both sound frequency and intensity (Heffner and Heffner, 2007; Raphael and Altschuler, 2003). The process of hearing occurs via the auditory system and, in mammals, this comprises the ears, the vestibulocochlear nerve, and the central auditory nervous system, which can be further divided into the auditory centres within the brain, and their connecting pathways in the brainstem (Musiek and Oxholm, 2000).

1.1.2 Anatomy of the Ear

The human ear is divided into three functional sections: the outer (external) ear; the middle ear; and the inner (internal) ear. These structures are embedded in the temporal bone of the skull, with only the pinna of the external ear protruding from the skull (Forsythe, 2002; Figure 1).
The outer ear consists of two major parts: the pinna and the ear canal (external auditory meatus). The pinna, which is innervated by the great auricular nerve and the auriculotemporal nerve, is an ovoid-shaped structure that serves to receive sound. Situated on a depression of the pinna called the concha, and in the lower part of the pinna, is the entrance to the ear canal (Moller, 2000). The ear canal is an oval shape, having an average diameter of 8 mm and average length of 25 mm in adults (Hammershoi and Moller, 1996). It is protected by cerumen secretions, and serves as an access route which directs acoustic waves from the external environment to the tympanic membrane of the middle ear (Lass and Woodford, 2007).

The middle ear itself is an air-filled cavity approximately 2 cm³ in volume, which is bordered by walls formed from the temporal bone (Dallos, 1973; Yost et al., 1977; Zemlin, 1997). Encompassed within the middle ear is the tympanic membrane and the auditory ossicles: the malleus, incus, and stapes (Moller, 1994). The tympanic membrane is a shallow,
cone shaped, semi-transparent membrane that terminates the ear canal. Its stiffness is not uniform, with the pars flaccida portion of the membrane being more lax than the larger pars tensa portion. This allows the pars flaccida to compensate for changes in air pressure, while the pars tensa simultaneously transmits sound energy between the outer and middle ear (Moller, 2000). Connected to the tympanic membrane are the ossicles, which form a chain linking the tympanic membrane to the membrane covering the oval window of the cochlea. Functionally, the ossicular chain acts as a medium that transfers sound energy from the tympanic membrane of the middle ear to the cochlea of the inner ear. Overall, this process involves the conversion of sound vibrations within the middle ear into fluid vibrations within the inner ear. While usually the large difference between the impedances of fluid and air would result in an inefficient transfer of energy, the structure of the middle ear overcomes this by reducing the mismatch in impedance of the two media (Moller, 1994).

Anatomically, the inner ear consists of three main elements: the semicircular canals, the vestibule, and the cochlea. However, functionally it is comprised of two main elements: the cochlea system, which is responsible for hearing, and the vestibular system, which is responsible for balance (Raphael and Altschuler, 2003). The cochlea is a bony, coiled structure which is divided along its length into three channels of fluid: the scala vestibuli, scala media, and scala tympani (Moller, 1994; Figure 2). The scala vestibuli and scala tympani constitute the bony labyrinth and are connected at the apex of the cochlea via the helicotrema opening. They extend to the base of the cochlea, where the scala vestibuli is terminated by the oval window, and the scala tympani is terminated by the round window. In contrast, the scala media is part of the membranous labyrinth, and is separated from the other two scalae by Reissner’s membrane toward the scala vestibuli, and the basilar membrane toward the scala tympani (Moller, 1994, Moller 2000).
Figure 2: Peripheral and central auditory pathways. Transmission of sound wave energy occurs via the external (outer) and middle ear, before reaching the cochlea system. Frequency dependent processing occurs within the cochlea, which results in the conversion of mechanical energy to neural impulses. Inner and outer hair cells are involved in this process, with the inner hair cells acting as the primary sensory receptor cells in direct contact with afferent nerve fibres, and the outer hair cells acting as an amplifier of auditory action potentials. These action potentials are conveyed from the auditory nerve to the central auditory pathways for further processing. The two central auditory pathways involved are the lemniscal pathway and the extralemniscal pathway. The lemniscal pathway projects to the primary auditory cortex via the ventral thalamus, whereas the extralemniscal pathway projects to the secondary auditory cortex and other associated cortices in the parietal lobe via the thalamus (De Ridder, 2005; Londero et al., 2006).
The basilar membrane supports a number of sensory and non-sensory cells, which collectively make up the organ of Corti (Figure 2). Included in these sensory cells are the hair cells, which are modified epithelial cells with stereocilia. These auditory cells are involved in the conversion of mechanical vibrations within the basilar membrane to neural impulses of auditory nerve fibres (Moller, 1994; Raphael and Altschuler, 2003). The hair cells are arranged along the basilar membrane in rows, with one row of inner hair cells and three rows of outer hair cells. The hair cells are innervated by both afferent and efferent nerve fibres, but to a varying degree. Approximately 95% of the afferent nerve fibres innervate the inner hair cells, with the remaining 5% innervating the outer hair cells (Moller, 1994). As a result, each inner hair cell is innervated by multiple afferent fibres, whereas each afferent fibre innervates multiple outer hair cells (Moller, 1994; Spoendlin, 1970). Functionally, this allows the inner hair cells to act as the primary sensory receptor cells of the cochlea (Moller, 1994). Movement of the basilar membrane, as a result of a pressure wave, excites these cells by the deflection of the stereocilia. Mechanically gated ion channels are mediated via this stereocilia movement, with bending in the direction of the stria vascularis corresponding to depolarization, and subsequent increased discharge of the nerve fibres. Conversely, hyperpolarisation and suppression of discharge of the nerve fibres is caused by bending in the opposite direction, away from the stria vascularis (Flock, 1965; Gulick et al., 1989; Moller, 1994; Raphael and Altschuler, 2003). There are fewer efferent auditory nerve fibres than there are afferent and, as a result, these diverge to innervate multiple hair cells per fibre. Divergence is greater at the level of the outer hair cells, as opposed to the inner hair cells. (Harrison et al., 1974; Desmedt, 1975). Consequently, the outer hair cells are mainly innervated by efferent terminals of the auditory nerve fibres. As such, the primary role of the outer hair cells is to modulate and enhance the initiation of auditory nerve action potentials by the inner cells (Raphael and Altschuler, 2003).
1.1.3 Central Auditory Pathways

The auditory nervous system is comprised of two main auditory pathways: the lemniscal (classical) pathway, and the extralemniscal (non-classical) pathway (Moller, 2003; Figure 2). Anatomically, these two systems diverge at the inferior colliculus (IC). In the lemniscal system, auditory information is processed and interpreted via excitatory and inhibitory input in the ventral thalamus, and its neural projections to the primary auditory cortex (AC) (Caspary et al., 2005). The only input to this system is sound stimuli, with the neurons in the ascending pathways of the lemniscal system consequently being sharply tuned to sound frequencies (Aitkin, 1986; Aitkin et al., 1994).

In contrast to the tonotopic lemniscal system is the extralemniscal system, which has a non-tonotopic organisation. The pathways of this system lead to the secondary AC and association cortices via neural projections from the medial and dorsal thalamic nuclei of the medial geniculate body, thereby bypassing the primary AC (Moller, 2003). Many of these neurons, which fire in bursts, respond to more than one modality of sensory stimulation, and consequently this pathway is less specific to sound. Additionally, the lemniscal pathways provide input to the extralemniscal pathways, which results in a complex interaction between the ascending auditory pathways of these systems (He and Hu, 2002; Hu et al., 1994; LeDoux, 1993).

1.1.4 Cochlear Nucleus

Auditory nerve fibres innervate the cochlear nucleus (CN), which receives and processes auditory input before projecting its output to higher auditory nuclei via parallel pathways. As such, the CN is considered to be the gateway to the central auditory system (Doucet and Ryugo, 1997). It has two main divisions: the dorsal cochlear nucleus (DCN) and the ventral cochlear nucleus (VCN). The VCN is further subdivided into the posteroventral cochlear nucleus (PVCN) and the anteroventral cochlear nucleus (AVCN). These divisions
within the cochlear nucleus are based on cytoarchitectonic features, with each subdivision containing a characteristic array of cell types that differ in their morphology, projection patterns, and physiology (Brawer et al., 1974; Doucet and Ryugo, 1997; Hackney et al., 1990). This diversity in cellular structure and arrangement is particularly prominent in the DCN, which has a distinct layering pattern closely resembling the organisation of the cerebellum, whereas the VCN is not layered (Mugnaini et al., 1980; Oertel and Young, 2004; Ryugo and Parks, 2003). Functionally, the DCN serves to detect spectral features of sound, regulate the VCN, and compensate for head movement, whereas the VCN serves as a relay centre for the conduction of sound from the ear to the binaural brainstem and IC, while also encoding spectral and temporal characteristics of the sound (Forsythe, 2002; Kanold and Young, 2001; Wang et al., 1998).

The afferent auditory nerve fibres that transmit acoustic information to the CN via the 8th nerve bifurcate into an ascending branch that terminates in the AVCN, and a descending branch that innervates the PVCN and the DCN (Lorente de No, 1981; Figure 3). Superficial granule cells in the DCN receive input from the descending branch and project this information via parallel fibres to cartwheel and pyramidal cells (Mugnaini et al., 1980). Cartwheel cells are glycinergic inhibitory interneurons that project into the deep layers of the DCN. These deep layers receive the principal auditory input, and project acoustic information to both giant cells and inhibitory vertical cells (Forsythe, 2002; Golding and Oertel, 1997). The giant cells, along with the pyramidal cells, are the principal output cells. They integrate activity from both auditory nerve fibres and parallel fibres, projecting it to the contralateral IC (Oertel and Young, 2004).
Figure 3: The CN. The CN receives input from auditory nerve fibres via the 8th nerve. The CN act as the initial point of central processing of the auditory input, with the DCN principally involved in the detection of the spectral properties of sound, and the VCN acting as the principal input to the binaural brainstem pathways. The principal locations of various cell types are indicated by the dashed lines and symbols (Forsythe, 2002).

In the VCN, there are four principal neuron types, each with distinct morphology, projections and firing rates. Bushy cells are the relay neurons for the binaural auditory pathway projection to the superior olivary complex (SOC). Spherical bushy cells receiveafferent input from up to four synapses and function to encode information that relates to the timing of acoustic stimuli (Oertal, 1997; Oertal, 1999; Trussell, 1999). In contrast, globular bushy cells receive between 20 and 40 afferent synapses. These cells fire on every stimulus presentation, whereas spherical bushy cells are frequency-specific (Friauf and Ostwald, 1988; Smith and Rhode, 1987; Smith et al., 1991). Multipolar (or stellate) cells, found throughout the VCN, project to the contralateral IC. Their physiological response is referred to as a
“chopper” response, which relates to the regularly spaced firing pattern of action potentials they produce (Smith and Rhode, 1989). Octopus cells project to the IC and the contralateral ventral nucleus of the lateral lemniscus (Adams, 1997; Vater and Feng, 1990). They produce an “onset” response, which relates to their precisely timed firing of action potentials at the beginning of an acoustic stimulus (Forsythe, 2002; Rhode et al., 1983; Rouiller and Ryugo, 1984). Octopus cells are unique in that they respond more reliably to amplitude modulations than other cells of the CN, which is thought to render them useful in analysing and conveying features of speech-like sounds (Adams, 1986; Frisina, 1983).

### 1.1.5 Auditory Brainstem Responses

Auditory brainstem responses (ABRs) are an electroencephalographic response that occurs within the 10 ms proceeding the presentation of a transient sound such as a click or tone stimulus (Moller, 2000; Willott, 2006). In a clinical setting, ABRs are used for diagnostic identification of brainstem pathology, as a measure of brainstem maturity and integrity, and for intraoperative neurophysiological monitoring (Hecox and Galambos, 1974; Mitchell et al., 1996; Moller, 2000; Moore, 1987; Stockard and Rossiter, 1977).

Some of the earliest published evidence of ABRs was contributed by Jewett (1970), who produced characteristic waveforms in response to auditory stimuli in anaesthetised cats. The results indicated that potentials could be detected by widely spaced electrodes at a considerable distance from the source generating those potentials, leading to the conclusion that the technique would allow the study of a sensory system via electrodes outside the system of interest. In current research, ABRs are generally recorded using scalp electrodes. These detect voltages produced as a result of the neural activity throughout the brain. When an ear is exposed to an audible sound stimulus, a chain of neural activity in the ascending auditory pathway is evoked. This begins at the auditory nerve and progresses throughout the brainstem and forebrain. The resultant voltages produced are relatively small compared with other non-
auditory activity. This non-auditory activity produces background noise that masks the presence of the auditory-evoked voltages. However, by repeatedly presenting brief sounds the auditory-evoked voltages, which are consistent in form for each repetition of the sound stimulus, can be superimposed and summated. As the background noise is random and non-consistent, after many repetitions the positive and negative voltages cancel each other out, leaving a set of waves that can be attributed to the auditory-evoked response (Willott, 2006). Muscle and movement artifacts contribute to the background noise and, as a result, the most consistent and clear-cut waveforms are produced when the subject is relaxed, sleeping, or anaesthetised (Markand, 1994).

In human subjects, ABRs are characterised by seven waves (labelled with Roman numerals I-VII) that are identified in the first 10 ms following the stimulus (Figure 4). The first five positive peaks (waves I-V) are considered to be the most clinically significant as they are consistent and found in all normal adult subjects, whereas the final two peaks (waves VI-VII) are variable and not recorded in every ear stimulated (Markand, 1994). Despite small animals having consistency in only four waves, which is likely due to the shorter auditory pathways in these animals compared to those of humans, the interpretation of ABR waves in humans relies mainly on hypotheses formed from a variety of experimental animal studies in cats, mice, and guinea pigs (Buchwald and Huang, 1975; Jewett, 1970; Markand, 1994; Moore, 1987). These studies, along with clinicopathologic correlations in humans, have led to proposed generator sites of the waves. These are: wave I, distal portion of the 8th cranial nerve; wave II, proximal portion of the 8th cranial nerve; wave III, CN; wave IV, SOC; wave V, lateral lemniscus and its nucleus, and IC (Muncie and McCandless, 2011).
Figure 4: The characteristics and proposed generator sites of ABRs. ABRs are far-field evoked potentials. They are characterised in human subjects by seven waves recorded from electrodes in the first 10 ms following a transient sound. Illustrated is an example response of the waveforms that could be produced from a subject using a stimulus that is 60 dB above normal hearing level. Arrows match each wave to the proposed sites of generation in the auditory pathway, which are as follows: wave I, distal portion of the 8th cranial nerve; wave II, proximal portion of the 8th nerve; wave III, CN; wave IV, SOC; wave V, lateral lemniscus and its nucleus, and IC (Mason, 2004).

ABR recordings allow hearing sensitivity to be assessed via the determination of ABR thresholds. While a psychophysical threshold is considered to be the minimal level that
produces a response for at least 50% of the stimulus presentations, this interpretation cannot be applied to ABRs as they are constructed from numerous repetitions that have been averaged. Instead, ABR thresholds are determined by the minimum stimulus intensity that produces an observable ABR response. However, it should be noted that due to the subjective nature of ABR analysis, as well as the variable effects of the recording and sound generation setup for ABRs, ABR thresholds cannot be generalised across laboratories or testing situations (Moller, 2000; Willott, 2006).
1.2  **Tinnitus**

1.2.1  **Tinnitus Introduction**

Tinnitus is an illusory auditory sensation which often manifests as ‘ringing of the ears’ in affected individuals, despite a lack of any external acoustic stimulus (Eggermont and Roberts, 2004). Generally, the sensation is the result of a reversible cause and can dissipate over a period of time, ranging from a few seconds to a few days, upon the removal of the stimulus (Eggermont and Roberts, 2004). However, in approximately 5-15% of the general population, the tinnitus sensation does not subside (Heller, 2003). It can occur at any age, although the prevalence of tinnitus has been found to increase with age, affecting 2.6% of young adults aged less than 30 years old, as opposed to 14.3% of adults aged 60-69 years old (Eggermont and Roberts, 2004; Shargorodsky *et al.*, 2010). For those individuals affected, tinnitus can be a debilitating disorder, and there is growing evidence to support claims that tinnitus can be linked to anxiety, depression, and insomnia (Adams *et al.*, 1999; Fioretti *et al.*, 2013; Halford and Anderson, 1991; Ooms *et al.*, 2012).

1.2.2  **Causes of Tinnitus**

There are a number of factors that have been associated with tinnitus (Table 1). In particular, noise trauma has been shown to be one of the most common causes of tinnitus in humans (Bauer and Brozoski, 2001; Cooper, 1994). Also frequently associated with permanent and temporary tinnitus are drugs, with tinnitus listed as a side effect of more than 300 over-the-counter and prescription medications (DiSogra, 2001; Henry *et al.*, 2005). However, while certain events are known to cause an initial onset of tinnitus, the subsequent mechanisms responsible for sustaining tinnitus remain largely unknown (Henry *et al.*, 2005).
### Table 1: Common causes of tinnitus.

<table>
<thead>
<tr>
<th>Type of Cause</th>
<th>Examples</th>
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</table>
| Otologic      | • Noise-induced hearing loss (e.g. exposure to explosions, brief intense noise, long duration noise) (Bauer and Brozoski, 2001; Cooper, 1994)  
• Meniere’s disease (Schleuning, 1981)  
• Otosclerosis (Schleuning, 1981) |
| Neurologic    | • Head injury (Elgoyhen and Langguth, 2010)  
• Neck injury (Elgoyhen and Langguth, 2010)  
• Multiple sclerosis (Perry et al., 2000) |
| Infectious    | • Meningitis (Schleuning, 1981)  
• Syphilis (Lockwood et al., 2002) |
| Drug Related  | • Prescription and over-the-counter drugs (e.g. salicylates, loop diuretics, aminoglycoside antibiotics, and non-steroidal anti-inflammatory drugs) (DiSogra, 2001; Henry et al., 2005; Lockwood et al., 2002) |
| Other         | • Dental disorders (e.g. temporomandibular-joint dysfunction) (Lockwood et al., 2002) |

Table adapted from Lockwood et al. (2002).

### 1.2.3 Current Tinnitus Treatments

Tinnitus poses a significant challenge to clinicians and patients, as treatment options are severely limited. This is largely due to a lack of understanding surrounding the mechanism of tinnitus, and the inconsistent results which have arisen from those clinical trials which have been conducted on the disorder (Eggermont and Roberts, 2004; Table 2). Currently, there is not a single Food and Drug Administration (FDA)-approved drug on the market specifically targeting tinnitus, resulting in clinicians prescribing a wide variety of drugs on an off-label basis (Elgoyhen and Langguth, 2010). Consequently, treatment effects have been limited, while an unmet need for an effective drug therapy specifically targeting tinnitus remains.
Table 2: Pharmacological approaches to the treatment of tinnitus.

<table>
<thead>
<tr>
<th>Treatment Type and Examples</th>
<th>Proposed Mechanism of Action</th>
<th>Evidence From Clinical Studies</th>
<th>Disadvantages</th>
</tr>
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<tr>
<td><strong>Intratympanic drug treatment</strong></td>
<td>• If the cause is peripheral, tinnitus can be alleviated via the ototoxicity of aminoglycoside antibiotics that reduce activity in the affected ear (Schuknecht, 1957). • Dexamethasone is thought to alleviate tinnitus caused by abnormal excitation of auditory hair cells via sedative and oedema-relieving effects (Cesarani et al., 2002; Sakata and Umeda, 1976) • Lidocaine acts via sodium channel blockade and is thought to reduce activity in the hyper-</td>
<td><strong>Lange et al., 2004</strong> • 57 patients with Meniere’s disease • Single shot or interval gentamicin therapy. • One series of injections included a maximum of three injections during a 15-day period (7 days between subsequent injections), each containing 0.3 mL (12 mg) gentamicin. • 30 patients received one application of gentamicin, 13 patients received two applications, and 5 patients received three applications within a single series of intratympanic injections. 7 patients required a second series of injections, and 2 patients needed three series of injections. • 45.5% of patients reported complete or partial relief of their tinnitus. <strong>Garduno-Anaya et al., 2005</strong> • Prospective, randomized, double-blind study with a two year follow-up. • 22 patients with unilateral Meniere’s disease. • 4 mg/mL dexamethasone (with quantity instilled ranging from 0.5-0.8 mL) or placebo injected intratympanically daily, for five consecutive days.</td>
<td>• Permanent ototoxicity can result from aminoglycoside antibiotics, although a sufficiently long interval between injections can reduce the risk (Lange et al., 2004). • Vestibular symptoms can develop following lidocaine infusion (Sakata et al., 2001). • Due to the local intratympanic application of treatment, success has been limited to tinnitus associated with a peripheral cause (e.g. Meniere’s disease) (Garduno-Anaya et al., 2005; Lange et al., 2004).</td>
</tr>
</tbody>
</table>
| Intravenous lidocaine | functional neuronal pathways that generate tinnitus sensations (Shulman, 1997). | No statistically significant change in THI scores at the 2 year follow-up.  
| | | The mean of the tinnitus and aural subjective improvements in the dexamethasone group (48.1%) was statistically significant when compared to the placebo group (20%).  
| **Lidocaine (Otsuka et al., 2003)** | It has been proposed that lidocaine may work intratympanically, producing either a blockade of sodium channels or vasodilatory effects in the cochlea (Sakata et al. 2001).  
| | The decreased sodium conductance via the sodium channel blockade effect of lidocaine may directly reduce hyperactivity in high discharge neurons, as increased sensitivity is known to be caused by increased | Otsuka et al., 2003  
| | | 103 patients with subjective tinnitus.  
| | | 60 or 100 mg lidocaine administered intravenously over a 24 hour period.  
| | | 89 patients received unilateral treatment and 14 patients received bilateral treatment.  
| | | 70.9% of patients experienced either complete or partial relief of their tinnitus.  
| | | Patients with low- to middle-tone tinnitus, a hearing level of 40 dB or higher, and/or were 60+ years old all showed better response to treatment.  
| **Kalcioglu et al., 2005** | 30 patients with tinnitus.  
| | | 1.5 mg/kg lidocaine administered over a period of 30 minutes.  
| | | 23.3% of patients reported either complete or partial relief of their tinnitus immediately after infusion.  
| | | Subjective relief from tinnitus lasted a maximum of 4 weeks after treatment. | Much of the data on the use of intravenous lidocaine as a tinnitus treatment comes from uncontrolled trials, and it has been suggested that the observed effect may be a placebo effect due to patient expectation (Dobie, 1999; Lockwood et al., 2002).  
| | | Lidocaine is relatively unstable in vivo (Blayney et al., 1985; Simpson and Davies, 1999).  
| | | Effective doses have potentially life-threatening cardiac side effects (Simpson and Davies, 1999).  
| | | Intravenous lidocaine provides only temporary  
| | |}
<table>
<thead>
<tr>
<th>Osmotic regulators and vasodilators</th>
<th>Sodium conduction (Otsuka et al., 2003).</th>
<th>• No significant change in SOAEs or DPOAEs.</th>
<th>Relief, and both continual treatment and the route of administration are impractical (Otsuka et al., 2003).</th>
</tr>
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<tr>
<td>Furosemide (Simpson and Davies, 1999)</td>
<td>Vasodilators increase blood flow, and have been investigated on the basis that this could alleviate tinnitus via increased oxygenation of peripheral and central auditory structures (Simpson and Davies, 1999).</td>
<td>Briner et al., 1993</td>
<td>There is currently insufficient evidence to confirm the efficacy of osmotic regulators and vasodilators for the treatment of tinnitus, with favourable results failing to be replicated in recent studies (Simpson and Davies, 1999; Thirwall and Kundu, 2006).</td>
</tr>
<tr>
<td>Misoprostal (Briner et al., 1993)</td>
<td>Osmotic regulators have been tested as a means of correcting perilymphatic hypertension and ion distribution imbalances in the inner ear, both of which occur in a number of conditions that accompany tinnitus (Simpson and Davies, 1999).</td>
<td>24 patients with severe tinnitus.</td>
<td>- Blinded, placebo-controlled trial.</td>
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<tr>
<td>Glycerol (Filipo et al., 1997)</td>
<td>Intravenous infusions of either 10% glycerol (46 patients) or 18% mannitol (21 patients) were administered 3-6 times, with an interval of 1-3 days between each administration.</td>
<td>Patients were administered either placebo or 200 µg/day misoprostol, increasing every 5 days by 200 µg, until a dose of 800 µg/day was achieved.</td>
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<tr>
<td>Mannitol (Filipo et al., 1997)</td>
<td>There are no placebo responders.</td>
<td>Treatment duration of 1 month.</td>
<td>- Treatment duration of 1 month.</td>
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<td>67 patients with sensorineural hearing loss either with tinnitus and/or aural fullness, or of a sudden origin (but observed 2 months – 5 years on).</td>
<td>After 1 month, placebo patients were crossed over to the active drug for a further month. (All other patients were released from the trial).</td>
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<td>Intravenous infusions of either 10% glycerol (46 patients) or 18% mannitol (21 patients) were administered 3-6 times, with an interval of 1-3 days between each administration.</td>
<td>33% of all patients (3 initially assigned to the active drug, 5 initially assigned to placebo) reported improvement in tinnitus severity, sleep, and concentration.</td>
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<td>- There were no placebo responders.</td>
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<tr>
<td>Benzodiazepines</td>
<td>Davies, 1999.</td>
<td>• Tinnitus was relieved in 13.1% of the glycerol group, and 5.8% of the mannitol group.</td>
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<td><strong>Benzodiazepines</strong></td>
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<tr>
<td>• Alprazolam (Johnson et al., 1993)</td>
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<td>• Benzodiazepines are GABA receptor agonists that increase inhibition in the central nervous system, potentially alleviating tinnitus via a subsequent decrease in hyperexcitability of the central auditory system (Smith et al., 2010).</td>
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<td>• Clonazepam (Gananca et al., 2002)</td>
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| Johnson *et al.*, 1993               |               | • Prospective, placebo-controlled, double-blind study.                                |
|                                       |               | • 40 patients with constant tinnitus enrolled, with 36 completing the study (17 in the alprazolam group and 19 in the placebo group). |
|                                       |               | • 0.5 mg/d alprazolam on days 1-7, 1.0 mg/d on days 8-28, 1.5 mg/d on days 29-84 were the recommended doses. |
|                                       |               | • 76% of the patients in the alprazolam treatment group showed a reduction in the loudness of their tinnitus, based on tinnitus synthesizer and VAS measurements, compared with 5% in the placebo group. |
|                                       |               | • The reduction in loudness of tinnitus within the alprazolam group was statistically significant, when compared to pre-treatment levels, at the end of weeks 4 and 12. |
|                                       |               | • No changes were observed in audiometric data or tinnitus masking levels for either group. |

| Gananca *et al.*, 2002               |               | • Benzodiazepines are associated with a number of adverse side effects e.g. sedation (Johnson *et al.*, 1993). |
|                                       |               | • There is a substantial risk of dependence on benzodiazepines occurring (Dobie, 1999). |
|                                       |               | • There is insufficient evidence of a conclusive benefit of benzodiazepines for the treatment of tinnitus (Elgoyhen *et al.*, 2010). |

| **Prospective, placebo-controlled, double-blind study.** |               | • Prospective, placebo-controlled, double-blind study. |
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| **Benzodiazepines**                  |               | • Benzodiazepines are GABA receptor agonists that increase inhibition in the central nervous system, potentially alleviating tinnitus via a subsequent decrease in hyperexcitability of the central auditory system (Smith *et al.*, 2010). |
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|                                       |               | • There is insufficient evidence of a conclusive benefit of benzodiazepines for the treatment of tinnitus (Elgoyhen *et al.*, 2010). |
### Non-benzodiazepine anti-epileptic drugs

- Valproate (Elgoyhen and Langguth, 2010)
- Carbamazepine (Hulshof and Vermeij, 1985)
- Gabapentin (Bauer and Brozoski, 2006)
- Baclofen (Westerberg et al., 1996)

There are three proposed mechanisms by which anti-epileptic drugs are suggested to alleviate the central auditory hyperactivity believed to be associated with tinnitus. These are:

- Increase GABA-mediated inhibition of the central auditory system.
- Decreased glutamate transmission, thereby reducing the excitation level in the central auditory system.
- Blockade of voltage-dependent sodium channels to halt the depolarisation, and subsequent activation, of cells. (Hoekstra et al., 2001).

#### Hulshof and Vermeij, 1985

- Randomised, double-blind, placebo-controlled trial.
- 48 patients with tinnitus (24 in the carbamazepine treatment arm and 24 in the placebo treatment arm).
- Patients were administered either placebo or 150 mg carbamazepine, three times daily for a duration of 30 days.
- Carbamazepine treatment resulted in a non-significant negative effect in 8% of patients, compared with 13% of patients in the placebo group, measured via a self-assessment scale.

#### Bauer and Brozoski, 2006

- Prospective, single-blind, placebo-controlled trial.
- 39 patients with chronic tinnitus, associated both with and without acoustic trauma.
- Gabapentin was administered in a graduated ascending and descending dose series of 20 week duration, with a peak dose of 2400 mg/d.
- A significant improvement in tinnitus annoyance was found in the acoustic trauma tinnitus patients.
- An improvement of 20% or better in subjective loudness ratings was observed in 4 of the 19 non-trauma patients, and 6 of the 20 acoustic trauma patients.
- The best therapeutic effect was observed in patients whose tinnitus had associated acoustic trauma.

#### A review of the current trials for the use of anti-epileptic drugs as a tinnitus treatment has found that there has been a considerable risk of selection, performance, detection and reporting bias in these studies. Therefore results about the effectiveness of this class of drugs are somewhat inconclusive and further, methodically sound, trials need to be carried out (Hoekstra et al., 2001).

- Anti-epileptic drugs are associated with a number of adverse side-effects e.g. sedation (Simpson and Davies, 1999).
- The initial effectiveness of anti-epileptic drugs often declines over time (Simpson and Davies, 1999).
- Randomised, double-blind, placebo-controlled trial.
- 63 patients with tinnitus (31 in the baclofen treatment arm and 32 in the placebo treatment arm).
- Patients were administered either placebo or baclofen for three weeks (10 mg twice daily during the first week, 20 mg twice daily during the second week, 30 mg twice daily during the third week).
- No statistical advantage of baclofen was found over placebo, with a subjective improvement reported by 9.7% of the baclofen treatment arm versus 3.4% in the placebo arm.
- Marginally statistically significant improvements in the mean THI score of the baclofen patients post-therapy.

There are three proposed mechanisms by which antidepressants are suggested to exert their treatment effect on tinnitus:
- Reduce tinnitus directly by acting on the central auditory system.
- Reduce tinnitus indirectly by treating concomitant

- Randomised, double blind, placebo-controlled trial.
- 120 non-depressed patients with chronic tinnitus (> 6 month duration).
- Patients were administered either placebo or 10 mg/d paroxetine, increasing by 10 mg every 2 weeks (dependent on patient response and tolerance), until maximal dose of 50 mg reached.
- Treatment duration of 100 days.
- No statistical difference between paroxetine and placebo in overall measures of tinnitus loudness, THQ, and QWB.
- Paroxetine treatment group showed significant improvement in QWB question “How aggravated are you by you

- Uncertainty remains about whether the antidepressant efficacy is against the tinnitus itself, or the depression associated with tinnitus (Dobie and Sullivan, 1998).
- Further studies required to determine:
  - Optimal dose of antidepressant drugs for tinnitus treatment.
  - Subset of tinnitus

### Antidepressants

- Tricyclic antidepressants
  - Amitriptyline (Bayar et al., 2001)
  - Imipramine (Baldo et al., 2012)
  - Nortriptyline (Sullivan et al., 1993)

Robinson et al., 2005

<table>
<thead>
<tr>
<th>Antidepressants</th>
<th>There are three proposed mechanisms by which antidepressants are suggested to exert their treatment effect on tinnitus:</th>
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<tr>
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<td>Reduce tinnitus indirectly by treating concomitant</td>
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</table>

Westerberg et al., 1996

<table>
<thead>
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<th>Randomised, double-blind, placebo-controlled trial.</th>
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<td>63 patients with tinnitus (31 in the baclofen treatment arm and 32 in the placebo treatment arm).</td>
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<tr>
<td>Marginally statistically significant improvements in the mean THI score of the baclofen patients post-therapy.</td>
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<tr>
<td>NMDA receptor antagonists</td>
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<tr>
<td>Caroverine (Denk et al., 1998)</td>
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<td>Flupirtine</td>
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</table>

Selectiv serotonin reuptake inhibitors

- Fluoxetine (Baldo et al., 2012)
- Paroxetine (Robinson et al., 2005)

- Reduce tinnitus by having a simultaneous effect on both psychological disturbances and tinnitus itself. (McFerran et al., 2008)

- Post hoc analysis showed that a subgroup reaching maximal 50 mg/d dose of paroxetine showed significant improvement in perceived hearing ability (measured by THQ), QWB questions, and were more likely to have 10 dB drop in tinnitus in either ear.

Sullivan et al., 1993

- Randomised, double blind, placebo-controlled trial.
- 117 patients with chronic tinnitus (> 6 month duration).
- Patients were administered either placebo or 25 mg/day nortriptyline, titrated upwards by 25 mg/week and maintained at 50-150 ng/mL, as determined by blood assessment.
- Treatment duration of 6 weeks.
- Nortriptyline patients showed significant improvement compared to placebo in depression (measured by HAM-D scores), tinnitus related disability (measured by MPI tinnitus interference, internal disability VAS), and tinnitus loudness (measured by tinnitus matching).

Denk et al., 1998

- Placebo-controlled, single blind trial.
- 60 patients with tinnitus (30 patients assigned to the placebo group and 30 patients assigned to the caroverine group).
- Patients were administered either placebo or 160 mg caroverine in a 100 mL saline solution, administered at an

patients who are most likely to benefit from antidepressant treatment. (Baldo et al., 2012)

- NMDA receptor antagonists, even some of those that are selective, have been associated with adverse side effects such as schizophrenia-like symptoms (Lipton, 2006).
| Herbal remedies | (Salembier et al., 2006) Memantine (Figueiredo et al., 2008) | • Memantine (Figueiredo et al., 2008) • Memantine (Figueiredo et al., 2008) Memantine (Figueiredo et al., 2008) receptor antagonists may alleviate tinnitus via their blockade of these receptors, and the resultant reduction in hyperactivity of the central auditory system (Ehrenberger and Felix, 1995; Simpson and Davies, 1999). infusion rate of 2-3 mL/min. (The infusion was stopped in cases of tinnitus reduction or worsening). • 63.3% of the caroverine treatment group showed a statistically significant reduction in tinnitus (which corresponded to a reduction in both the subjective rating and tinnitus matching) following therapy, compared with 0% of patients in the placebo group. Figueiredo et al., 2008 • Prospective, randomized, double-blind crossover study. • 60 patients with tinnitus were enrolled, and 43 patients completed the study. • Patients were administered either placebo or memantine at a dose of 5 mg/d on days 1-7, 10 mg/d on days 8-14, 15 mg/d on days 15-21, and 20 mg/d on days 22-90. Following this was a 30 day washout period, before patients received the opposite treatment, administered via the same protocol as the first treatment. • No significant difference in tinnitus severity (measured by THI scores) was found between memantine and placebo treatment. | Drew and Davies, 2001 • The basis for the use of extracts of *Ginkgo biloba* as a tinnitus treatment is their vaso-regulatory effect which may | • There is no substantial scientific evidence to support *Ginkgo biloba* extracts as an effective tinnitus treatment (Hilton and Stuart, 2010). |
promote increased blood flow, and their changes to neuronal metabolism. It has been proposed that these effects could be particularly beneficial to patients whose tinnitus is a symptom of cerebral insufficiency (DeFeudis, 1991; Hofferberth, 1991; Koltringer, 1989) duration.
- There was no statistically significant difference between *Ginkgo biloba* extract LI 1370 and placebo on tinnitus outcome measures (which included tinnitus loudness, awareness, and impact), as determined by self-assessment scores on questionnaires.

Rejali *et al.* 2004
- Randomised, double-blind, placebo-controlled trial.
- 66 tinnitus patients were enrolled, with 31 patients in the *Ginkgo biloba* arm and 29 patients in the placebo arm completing the trial.
- Patients were administered either placebo or 120 mg sustained release formulation of *Ginkgo biloba* once daily for 12 weeks.
- There were no significant differences in THI scores, GHSI scores, or hearing thresholds between the *Ginkgo biloba* and placebo treatment groups.
- Additionally, a meta-analysis of 6 randomised, double-blind, placebo-controlled studies (including their own) was completed.
- Meta-analysis confirmed no evidence of a *Ginkgo biloba* treatment benefit, with 21.6% of *Ginkgo biloba* patients having gained a treatment benefit versus 18.4% of placebo patients.

- Any perceived benefits of *Ginkgo biloba* extracts are likely to occur in very specific subsets of patients (Hilton and Stuart, 2010).
Abbreviations- DPOAE: distortion product otoacoustic emission; GHSI: Glasgow Status Health Inventory; HAM-D: Hamilton Depression Scale; MPI: Multidimensional Pain Inventory; NMDA: N-methyl-D-aspartate; QWB: quality of wellbeing; SOAE: spontaneous otoacoustic emission; THI: Tinnitus Handicap Inventory; THQ: Tinnitus Handicap Questionnaire; VAS: Visual Analogue Scale.
1.2.4 Pathophysiology of Tinnitus

The underlying pathophysiology of tinnitus is still poorly understood. An important aspect which differentiates some of the proposed tinnitus mechanisms is the site of generation, for which there is evidence that is suggestive of both peripheral and central sites of origin (Henry et al., 2005). Early research in the 1970s by Kiang et al. (1970) was indicative of a cochlear origin, after the discovery of a strong association between hearing loss evoked by cochlear damage, and tinnitus itself. With some degree of hearing loss, and the associated hair cell damage or death, in approximately 85% of tinnitus patients, the idea that the underlying pathology is linked primarily to damage of the sensory apparatus in the cochlea was a reasonable one (Simpson and Davies, 1999). This theory was further reinforced by the fact that patients often perceive their tinnitus sensation from within the ear (Jastreboff, 1990). However, experimental evidence has since revealed that tinnitus is not always reduced following surgical sectioning of the auditory nerve, leading to the argument that while cochlear damage may be an initial trigger of tinnitus, there is likely to be an ultimate involvement of the central nervous system (CNS) in its maintenance (Tyler, 2005). Furthermore, it would be expected that the severity of the tinnitus would correlate to the degree of damage to the ear, if tinnitus was a consequence of hearing loss. It has been demonstrated that this is not the case, as some individuals suffer from severe tinnitus with no evidence of any aural pathology, further reinforcing the limitations of tinnitus mechanisms which focus on the peripheral auditory system (Baldo et al., 2012; Gabr, 2011; Seidman, 2010).

Currently, there is evidence to suggest that damage to the peripheral auditory system at the level of the cochlear receptor may lead to the development of tinnitus via changes in the spontaneous firing rate (SFR) of auditory nerve fibres. This is because one of the most consistent changes to be reported following damage to the cochlea is an increase in neural activity at various levels of the central auditory pathways (Eggermont and Roberts, 2004;
In the auditory system, inhibitory interaction between neurons begins at the level of the DCN. This led Eggermont (1984) to propose that peripheral auditory dysfunction might lead to a reduction in the output of inhibitory interneurons within the DCN, thereby reducing inhibition of the spontaneous activity of the type IV neurons. An abnormally increased output in the SFR of auditory nerve fibres in the affected region results in less inhibition in central auditory structures, ultimately leading to hyperexcitability within the central auditory pathways. Furthermore, once changes in neural activity have been evoked, the CNS has the ability to adapt to these changes via the creation or elimination of synapses, and the masking or unmasking of current synapses. In adults, this neural plasticity generally acts as a corrective mechanism following an altered stimulus environment. However, in some cases the plasticity may be a maladaptive response, resulting in an abnormal sensory perception such as tinnitus (De Ridder and Van de Heyning, 2007; Moller 2007).

The use of imaging techniques further supports the role of the central auditory pathways in maintaining tinnitus sensations in those patients with chronic tinnitus. Positron emission tomography (PET), which detects energy emitted by radio nucleotides, has been used to detect changes in blood flow or glucose metabolism in the brain in response to changes in tinnitus (Baldo et al., 2012). These changes have been induced by a variety of methods including eye and orofacial movements, intravenous lidocaine, and auditory stimulation (Lockwood et al., 1998; Mirz et al., 1999; Osaki et al., 2005). Collectively, studies using PET imaging on patients whose tinnitus can be modulated via these methods have provided evidence of tinnitus-related increases in the neural activity of the central auditory pathways (Eichhammer et al., 2007). As a result, studies have begun to increasingly consider tinnitus as a CNS disorder (Elgoyhen et al., 2012).
1.2.5 Animal Models of Tinnitus

One of the standard ways to test the mechanisms of neurological disorders, and novel drug treatments of these neurological disorders, is by way of animal models. As well as being relatively inexpensive, animal models have a number of advantages over clinical studies. They provide a means of testing novel therapies relatively quickly before they reach human trials, which is crucial for a progression in drug treatment (Darlington et al., 2010). Furthermore, animal models give researchers the ability to have direct control over the method of induction of the disorder of interest, which increases the likelihood that only a single form of the disorder is present and being studied in the animal (Bauer and Brozoski, 2001). This is particularly relevant when studying tinnitus, as quantifying the psychophysical attributes of tinnitus in humans, both reliably and reproducibly, has been proven to be difficult due to the heterogeneous nature of the population and the number of factors that can directly and uncontrollably affect the tinnitus assessment itself (Henry et al., 1999). These include variation in the pitch and loudness of tinnitus between individuals, as well as their fluctuation within individuals (Penner, 1983; Stouffer and Tyler, 1990). The complexity of tinnitus as a psychophysical phenomenon, coupled with a lack of understanding of its mechanism of generation and perpetuation, has created new challenges in the development of a model which will reliably replicate its characteristics in an animal population. A reliable model must soundly address two factors: i) a method of tinnitus induction that represents a leading cause of the tinnitus generation in human patients; and ii) an accurate method for assessing the presence of tinnitus within an animal once it has been subjected to the tinnitus induction (Darlington et al., 2010).

In terms of inducing tinnitus within animals, two methods are commonly used: i) administration of sodium salicylate; and ii) exposure to acoustic trauma. The former has the advantage of being relatively easy to administer via injection, and was demonstrated by Jastreboff et al. (1988) to reliably induce (what appeared to be) tinnitus in rats when
administered at a dose of 350 mg/kg/day via subcutaneous injection. As a result, the sodium salicylate model has continued to be successfully used in a number of studies as a means of inducing tinnitus in animals (Bauer et al., 1999; Ruttiger et al., 2003). However, current research is suggestive of noise exposure being a leading cause of tinnitus within humans, making a model encompassing exposure to acoustic trauma more clinically relevant than the salicylate method, and thereby adding weight to this approach in research (Cooper, 1994; Eggermont, 2005). The acoustic trauma model involves the exposure of anaesthetised animals to a loud noise. Continual refinement has resulted in the determination of a stimulus effective at inducing tinnitus, with most studies now using a narrow band noise with a peak intensity of 105 dB centered at 16 kHz over an exposure time of one hour (or a slight variation of this) as the acoustic trauma (Bauer et al., 1999; Darlington et al., 2010).

The importance of inducing tinnitus via a process similar to that occurring in a significant proportion of the human population is further highlighted by variations of effects in response to the type of induction. Salicylate-induced tinnitus is associated with a shift in the frequency of maximum sensitivity of cortical neurons, which stems from altered peripheral input from the cochlea due to the drug’s rapid entry into the cochlear perilymph (Jastreboff et al., 1986; Stolzberg et al., 2011). However, acoustic trauma has been demonstrated to increase hyperactivity within central auditory centres of the brain, such as the AC and DCN (Eggermont, 2005). The animal-based evidence for neuronal hyperactivity following noise trauma is extensive, and is one of the main driving forces behind the study of centrally acting drugs, such as baclofen, as potential tinnitus treatments (for reviews, see Eggermont and Roberts, 2004; Kaltenbach, 2006; Rauschecker et al., 2010). Early research by Salvi et al. (1990) demonstrated an enhancement of evoked response amplitude-level functions in the IC of chinchillas, following acoustic trauma. The maximum amplitudes of responses were often much greater than those recorded before noise exposure, which led the authors to the conclusion that the enhancement did not appear to originate in the cochlea, but
instead may be the result of reorganisation of neurons within the central auditory pathways. Furthermore, Kaltenbach et al. (2000) demonstrated induction of hyperactivity in the DCN of hamsters following noise trauma. Chang et al. (2002) built on these results by examining the effects of previous acoustic trauma on single neurons in the DCN. This provided evidence of a more specific mechanism of CNS hyperactivity, with the suggestion that intense tone exposure can result in increased bursting neural responses, and a reduction in the regular spontaneous neural activity in the DCN. More recently, Vogler et al. (2011) shifted the focus from the dorsal subdivision of the CN, by providing some of the first evidence of elevated SFRs of neurons in the VCN, in a guinea pig model of cochlear trauma. Collectively, these animal studies highlight the strength of an acoustic-trauma based approach, for the study of therapeutic agents targeted at reducing centrally-occurring neuronal hyperactivity, over peripheral tinnitus induction techniques.

In relation to both practicality and reliability of results, research requires a model that will induce tinnitus in animals for a long period of time, or perhaps even irreversibly in the absence of drug intervention. In this respect, induction by acoustic trauma has again been shown to be favourable, as it has been demonstrated to have a greater permanence than the salicylate model, with animals that have been subjected to a single noise exposure continuing to express tinnitus-like behaviour for the entire duration of studies that, in some cases, have been upwards of one year long (Bauer and Brozoski, 2001). It is also noteworthy that this long duration of tinnitus-like behaviour is achieved and maintained in animals without the need of any drug application. The salicylate model, on the other hand, requires moderate to high doses of sodium salicylate to be effective. From this arises the problem not only of potential drug toxicity in chronic studies, but there is also a possibility of drug interactions that could confound results, hence making this model less reliable in research aimed at testing potential drug therapies for tinnitus (Bauer and Brozoski, 2001; Chen and Jastreboff, 1995; Jastreboff and Sasaki, 1986; Darlington et al., 2010).
Once tinnitus induction has occurred, an accurate way of assessing and confirming its presence must be carried out if an animal model is to be reliable. This has led to the development of behavioural methods that indicate the presence of psychophysical evidence of tinnitus within animals. Problems arise with the fact that standard behavioural approaches require precisely defined stimuli of a short duration, whereas tinnitus sensations are inconsistent and often long in duration (Jastreboff and Sasaki, 1994). The reason the former is required is that it allows a sensory stimulus, which acts as the conditioned stimulus (CS), to be terminated by positive or negative reinforcement, which acts as an unconditioned stimulus (UCS). As a result of this pairing, an animal will learn to associate the CS with the UCS, and will react to the CS in a manner similar to the UCS. However, as tinnitus manifests in an opposite way to the conventional CS by being an unpredictable auditory sensation of a relatively long duration, Jastreboff et al. (1988) developed a behavioural paradigm that reverses the standard paradigm, and employs the use of an offset of a tone (silence) as the CS. In this way, water deprived animals can be trained to associate a mild foot shock (UCS) with the offset of a high frequency tone that resembles tinnitus (CS), by pairing the two together. The fear of the UCS opposes the water-deprived animal’s thirst, resulting in a decrease in drinking activity. An association between the UCS and the CS can be established quickly, causing the animal to stop drinking when the CS is present, even when the UCS is absent. Animals experiencing tinnitus are differentiated from those that are not by the fact that they do not hear silence as the CS, as this is masked by the auditory sensation of their tinnitus, and as such, the auditory sensation of their tinnitus becomes the CS instead of the silence (Darlington et al., 2010; Jastreboff et al., 1988; Jastreboff and Sasaki, 1994).

This lick suppression paradigm, or simple variations of it, is now widely applied in tinnitus research. When utilised after a method of tinnitus induction, in particular the acoustic trauma method, a reliable animal model can be produced to study the mechanisms of tinnitus,
and the efficacy of potential drug therapies (see Darlington et al., 2010 and Jastreboff and Sasaki, 1994 for reviews).
1.3 GABA and GABA Receptors

1.3.1 GABA

\(\gamma\)-aminobutyric acid (GABA) is an inhibitory neurotransmitter that was first identified in the mammalian brain in the 1950s (Bazemore et al., 1957; Krnjevic and Schwartz, 1967). It has been documented in many brain regions including the neocortex, hippocampus, thalamus, basal ganglia, cerebellum, hypothalamus, and brainstem, with multiple cell types or pathways being GABAergic in any one region (Curtis, 1979; Meldrum, 1982). The actions of GABA are extensive, and it plays an important role in trophic effects that include neurite extension, synaptogenesis, survival, and neuronal migration (Barbin et al., 1993; Barker et al., 1997; Mitchell and Redburn, 1996; Obata, 1997). Additionally, GABA has been shown to have excitatory action in the developing brain (Obata et al., 1978).

GABA is synthesized in the brain from glutamic acid via the enzymatic action of glutamic acid decarboxylase (GAD), and the coenzyme pyridoxal phosphate (Figure 5). This synthesis occurs in the presynaptic terminals of inhibitory neurons, where GAD has been shown to exist in high densities. Upon binding to a GABA receptor, GABA mediates inhibition via hyperpolarisation of the post-synaptic membrane, by increasing the membrane conductance of Cl\(^-\) ions (Meldrum, 1982). Inactivation of synaptically-released GABA occurs via its reuptake into nerve endings and glia. GABA is metabolised to succinic semialdehyde (SSA) via the mitochondrial enzyme GABA-transaminase (GABA-T), which also requires pyridoxal phosphate (Meldrum, 1982).
Figure 5: GABA metabolism and inhibitory transmission. In the presynaptic terminal, GABA is formed from glutamate via the enzyme GAD, and is metabolised to SSA via the enzyme GABA-T. Presynaptic GABA is enclosed in vesicles and released into the synaptic cleft in response to a presynaptic influx of Ca\(^{2+}\) ions. The synaptic release of GABA activates GABA\(_A\) and GABA\(_B\) (not pictured) receptors and results in hyperpolarisation of the postsynaptic membrane. Reuptake of GABA occurs across both neuronal and glial membranes (Loscher, 1999).

1.3.2 Classification of GABA Receptors

GABA-mediated post-synaptic inhibition appears to be one of the most widespread forms of inhibition in the brain and, consequently, there is a high expression of GABA receptors throughout the mammalian nervous system (McKernan and Whiting, 1996; Meldrum 1982).

Additionally, GABA itself is a highly-flexible molecule that can exist in multiple low-energy conformation states, allowing it to bind to multiple classes of GABA receptors. Three
major classes of GABA receptors have been identified: γ-aminobutyric acid type A (GABA<sub>A</sub>) receptor; γ-aminobutyric acid type B (GABA<sub>B</sub>) receptor; and γ-aminobutyric acid type C (GABA<sub>C</sub>) receptor (Chebib and Johnston, 1999; Figure 6).

![Image of GABA receptors]

**Figure 6: The three classes of GABA receptors.** GABA<sub>A</sub> and GABA<sub>C</sub> receptors are structurally similar, with both receptor subtypes being ligand-gated ion channels composed of five subunits. Currently, 16 different GABA<sub>A</sub> receptor subunits (α1-6, β1-3, γ1-3, δ, ε, θ, and π) and three different GABA<sub>C</sub> receptor subunits (ρ1-3) have been cloned. In contrast, GABA<sub>B</sub> receptors are seven transmembrane G protein-coupled receptors that are composed of a GABA<sub>B-R1a</sub> or GABA<sub>B-R1b</sub> subunit, and a GABA<sub>B-R2</sub> subunit (Bateson, 2006; Chebib and Johnston, 1999; Enz and Cutting, 1998; Johnston, 1996).

GABA<sub>A</sub> and GABA<sub>C</sub> receptors are ligand-gated ion channels that mediate fast synaptic inhibition. Structurally, these receptors are currently understood to be similar, each being composed of five subunits which functionally form an ion channel. Each subunit is comprised of four transmembrane domains, the second of which forms the wall of the pore.
channel. Additionally, between the third and fourth domain is a large intracellular loop, which is thought to mediate subcellular targeting and membrane clustering (Chebib and Johnston, 1999; Macdonald and Olsen, 1994).

However, despite these structural similarities, GABA_A and GABA_C receptors have been demonstrated to be pharmacologically and biochemically distinct, with each contributing to different physiological mechanisms (Bormann and Feigenspan, 1995; Johnston 1996a). Bicuculline is a selective antagonist of GABA_A receptors, but not of GABA_C receptors. Furthermore, benzodiazepines, steroids, and barbiturates are all known to modulate the GABA_A receptor, whereas little is currently known about chemical modulators of the GABA_C receptor (Johnston 1996; Johnston 1996a; Bormann and Feigenspan, 1995).

In terms of conductance, electrophysiological studies using rat retinal bipolar cells have demonstrated a lower conductance in GABA_C receptors. However, these receptors maintain an open channel for greater time periods than GABA_A receptors, and are less prone to desensitisation (Bormann and Feigenspan, 1995; Johnston, 1996).

In contrast to GABA_A and GABA_C receptors, GABA_B receptors are G protein-coupled receptors (GPCRs) that produce a slow, prolonged inhibitory signal (Chebib and Johnston, 1999). These receptors mediate their effects via the activation of the second messenger systems phospholipase C and adenylate cyclase. Furthermore, the G-coupled proteins of GABA_B receptors regulate the conductance of Ca^{2+} and K^{+} ion channels (Kerr and Ong, 1995).

Structurally, GABA_B receptors are seven transmembrane receptors which have a large extracellular N-terminal ‘Venus fly-trap’ domain, and a cytoplasmic carboxy-terminal tail (Kaupmann et al., 1997; Ong and Kerr, 2000; Figure 7). There is evidence to suggest that agonist activation of the GABA_B receptor results from the closure of the two lobes of the Venus fly-trap structure, which is comprised of leucine-binding protein-like domains. The
resultant change in conformation activates the associated G-protein via its transduction to the transmembrane region (Galvez et al., 1999; Ong and Kerr, 2000).

Figure 7: Schematic representation of the GABA<sub>B</sub> receptor. A GABA<sub>B</sub><sup>-R1a</sup> or GABA<sub>B</sub><sup>-R1b</sup> subunit combines with the GABA<sub>B</sub><sup>-R2</sup> subunit <i>in vivo</i> to form a functional GABA<sub>B</sub> receptor, through an interaction at the level of their intracellular carboxy-terminal tails. GABA<sub>B</sub> receptors are coupled to G-proteins, and their activation, via ligand binding to the ‘Venus fly-trap’ domain, leads to an increase in K<sup>+</sup> and decrease in Ca<sup>2+</sup> membrane conductance, or inhibition of cAMP production via a decrease in AC activity (Bowery and Enna, 2000; Ong and Kerr, 2000; White et al., 1998).

Abbreviations- α, β, γ: G-protein subunits; AC: adenylate cyclase; ATP: adenosine triphosphate; cAMP: cyclic adenosine monophosphate; Kir: inwardly rectifying K<sup>+</sup> channel; R1a: the a isoform of GABA<sub>B</sub><sup>-R1</sup>; R1b: the b isoform of GABA<sub>B</sub><sup>-R1</sup>.

Three GABA<sub>B</sub> receptor subunits have been cloned: GABA<sub>B</sub><sup>-R1a</sup>, GABA<sub>B</sub><sup>-R1b</sup>, and GABA<sub>B</sub><sup>-R2</sup> (Kaupmann et al., 1997; White et al., 1998). Currently, double immunoprecipitation studies have demonstrated that a GABA<sub>B</sub><sup>-R1a</sup> or GABA<sub>B</sub><sup>-R1b</sup> subunit combines with the GABA<sub>B</sub><sup>-R2</sup> subunit <i>in vivo</i> as either a dimer or multimer, making GABA<sub>B</sub> receptors hetero-oligomeric (Chebib and Johnston, 1999; Jones et al., 1998; White et al., 1998). However, there is indication that the GABA<sub>B</sub><sup>-R2</sup> subunit alone is sufficient to cause inhibition of cyclic adenosine monophosphate production, as the combined effect of GABA<sub>B</sub>-
and GABA_{B-R2} in inducing this response is equal to that of GABA_{B-R2} in isolation (Kuner et al., 1998).

In contrast to GABA_A receptors which are located exclusively on postsynaptic membranes, GABA_B receptors are localised both pre- and post-synaptically. Presynaptically, GABA_B autoreceptors provide negative feedback modulation via inhibition of the release of GABA, whereas GABA_B heteroceptors have a role in the release of neurotransmitters via inhibition of Ca^{2+} channel conductance. Postsynaptically, activation of GABA_B receptors has been shown to generate an inhibitory postsynaptic potential via the pertussis toxin-sensitive G protein activation of K^+ channels (Kaupmann et al., 2001).

Unlike GABA_A receptors, GABA_B receptors are insensitive to bicuculline, instead being activated by the GABA analogue baclofen, which remains as the prototypical GABA_B receptor agonist (Bowery et al., 1980). However, siclofen, the sulphinic acid analogue, and 4-amino-3(5-chlorothien-2-yl)-butanoic acid, the thienyl analogue, are also active at GABA_B receptor sites (Carruthers et al., 1995; Ong et al., 1997; Ong and Kerr, 2000). In 1987, Kerr et al. synthesised phaclofen, the phosphonic analogue of baclofen, which was established as a weak partial agonist of the GABA_B receptor. Following from this, it was found that saclofen, which represented a corresponding sulphonic analogue of baclofen, provided a 10-fold increase in antagonist potency when compared to the original phosphonic antagonists (Curtis et al., 1988; Kerr et al., 1988; Ong and Kerr, 2000).
1.4 Baclofen

1.4.1 Baclofen Introduction and Mechanism of Action

Baclofen, (±)-4-amino-3(p-chlorophenyl)-butanoic acid, is a structural analogue of GABA (Figure 8). It acts as a GABA_B receptor agonist, and has been approved by the FDA for the treatment of spasticity and other neurological disorders since 1977, due to its inhibitory effect in the CNS (Lal et al., 2009; Novartis, 1977).

![Chemical structure of GABA and baclofen](Image)

**Figure 8: Chemical structure of GABA and baclofen.** (Taira, 2009).

Baclofen is a racemic drug, comprising equal amounts of L- and D-isomers (Novartis, 1977). Currently, there is evidence that the mechanism of action of baclofen is complicated by the varying actions of the two isomers. It appears that L-baclofen is the more active isomer, with the efficacy of baclofen in treating spasticity being attributed to this isomer (Hill and Bowery, 1981; Olpe et al., 1978; Wuis et al., 1985). Similarly, Fromm and Terrence (1987) reported L-baclofen as being five times more effective than racemic baclofen in relieving trigeminal neuralgia symptoms, in nine out of 15 patients who took part in a double blind
crossover trial. Interestingly, not only has D-baclofen been reported to have less therapeutic potential in treating spasticity and trigeminal neuralgia, but there is also suggestion that D-baclofen may have an antagonistic action on the effects of L-baclofen. This effect has been reported in both the trigeminal nucleus and spinal cord (Fromm et al., 1990; Sawynok and Dickson, 1984; Sawynok and Dickson, 1985; Terrence et al., 1983). Further differences exist in the transport mechanisms of baclofen. Van Bree et al. (1991) determined the blood-brain barrier (BBB) transport profiles of the isomers and racemic mixture. Their results suggest a stereoselective transport of baclofen across the BBB, as it was demonstrated that the BBB clearance and cumulative transported amount were significantly higher for the L-isomer. However, the overall plasma elimination kinetics and cerebro-spinal fluid elimination kinetics were relatively consistent for the isomers and racemic mixture.

In terms of the inhibitory action of baclofen, it remains unclear as to whether this is mediated mainly via an enhancement of inhibition, or a reduction in excitation, as whole cell patch clamp recordings provide evidence of both mechanisms. Ma et al. (2002) demonstrated that the action of baclofen is caused in part by an enhancement of inhibition. Whole-cell patch clamp recordings were made from IC neurons in rat brain slices. Administration of baclofen (10-20 μM) resulted in a reduction in inhibitory postsynaptic potentials (IPSPs), an effect that was completely blocked by phaclofen. Alternatively, a reduction in excitation has been proposed to occur as a result of baclofen’s activation of presynaptic GABA_B receptors that have a modulatory role in glutamatergic excitation. This was demonstrated by Sun et al. (2006), who evoked postsynaptic currents via electrical stimulation of the lemniscal inputs in rat brain slices. A bath application of baclofen (5-10 μM) resulted in a concentration-dependent reduction in the amplitude of excitatory postsynaptic currents (EPSCs) in IC neurons, which was successfully reversed by CGP35348, a GABA_B receptor antagonist. A paired-pulse stimulus paradigm was used to further clarify the involvement of presynaptic GABA_B receptors. The two current pulses allowed the paired-pulse ratio of EPSCs to be
compared before and during baclofen application. The amplitude of the first EPSC showed a much larger reduction than that of the second EPSC when baclofen was applied, suggesting a probable presynaptic mechanism of GABA<sub>B</sub> receptor-mediated modulation of glutamatergic excitation in the IC. These findings by Sun et al. (2006) are consistent with earlier research by Takahashi et al. (1998), who additionally examined the effector pathway of the presynaptic GABA<sub>B</sub> receptor. Baclofen was found to mediate a suppression of presynaptic calcium currents, but had no significant effect on voltage-dependent potassium currents. The relationship found between the baclofen-induced reduction of EPSCs and calcium current was consistent with that found following a reduction in external calcium ion concentration, providing evidence that the presynaptic inhibition mediated by baclofen comes as a result of presynaptic calcium influx suppression.

It has been shown, via the administration of radio-labelled racemic baclofen to dogs, rats, and humans, that baclofen is nearly completely absorbed in the gastrointestinal tract (Faigle and Keberle, 1972; Faigle et al., 1980). This absorption is rapid in humans after oral administration (T<sub>max</sub> ~ 2 h), before being eliminated largely unchanged via renal excretion (Lal et al., 2009). Despite being considered a safe pharmacologic agent, baclofen produces a number of adverse side effects that include sedation, dizziness, and weakness. Additionally, it can produce both visual and auditory hallucinations if cessation of use is abrupt (Lees et al., 1977; Stein et al., 1977).

1.4.2 Current Literature on Studies Investigating Baclofen as a Tinnitus Treatment

Despite positive anecdotal findings from physicians who have prescribed baclofen on an off-label basis as a tinnitus treatment, there is little published data available on the subject from research and clinical trials. The only published clinical trial of baclofen in tinnitus patients is the double-blind placebo-controlled trial by Westerberg et al. (1996). The trial
enrolled 63 subjects, who were then divided into placebo and baclofen treatment groups. Tinnitus was assessed both before the study and at its conclusion, by subjective and objective measures which included the Tinnitus Handicap Inventory (THI), pitch and loudness matching, and the maskability of tinnitus. Patients in the baclofen arm of the trial received a baclofen dose of 10 mg twice daily for the first week, and this was increased by one 10 mg capsule twice daily each week for the following two weeks. Although the results of this trial did not show any clinical or statistical advantage of baclofen treatment over placebo, baclofen should not be discounted as a potentially viable treatment for a number of reasons that are highlighted within the limitations of the study.

Firstly, the lack of statistical significance could in fact be the result of inadequate power, as pointed out by the authors themselves. Before the study, power calculations were performed using expected mean differences in outcomes, and from these it was determined that 45 patients would be needed per group to afford a desirable power. However, not only were these group sizes not achieved initially, but patient numbers also continued to fall throughout the duration of the study, with 11 of the 63 patients withdrawing before its completion. As a result, the final group sizes were 23 for the baclofen treatment arm, and 29 for the placebo treatment arm. This is of substantial consequence, as even small effects that are statistically significant have the potential to provide much needed insight into baclofen’s mechanism of action and effects on patient subsets, which could in turn aid in the development and design of future trials.

Another point of consideration is the variation in pathophysiology among tinnitus patients, which is largely dependent on the underlying cause of the tinnitus. Patients enrolled in the study had a wide array of underlying conditions associated with their tinnitus, such as otosclerosis, presbyacusis, Meniere’s syndrome, and viral upper respiratory illness, and it is unlikely that a single drug would be effective in treating all of these (Moller, 1997). Therefore any significant effect of baclofen in treating a particular subset of tinnitus patients
may have been lost once the baclofen treatment group was diluted with patients outside of that subset. Ideally, stratified analyses of the baseline characteristics should be performed to determine if subpopulations of patients differ in their treatment response. However, as mentioned previously, an inadequate sample size was a limitation to this study, and Westerberg et al. (1996) lacked the statistical power to perform such analyses. Furthermore, patients in the baclofen arm of the study had a significantly higher mean THI score at the beginning of the study, which is indicative of a more severe functional disability. As such, this treatment group may have included a greater number of patients whose tinnitus was refractory to treatment.

Finally, at the time of the trial, the only licensed form of baclofen available was a racemic mixture of both the L- and D-isomers (Szczepaniak et al., 1995). Research has demonstrated that D-baclofen is less effective than L-baclofen at reducing hyperexcitability of neurons within the IC, with some studies indicating that D-baclofen may in fact not only be the less active isomer, but that it additionally antagonises the effect of L-baclofen (Sawynok and Dickson, 1984; Sawynok and Dickson, 1985; Smith et al., 2012; Szczepaniak et al., 1995; Szczepaniak et al., 1996). As a result, the inclusion of D-baclofen within the treatment protocol of the Westerberg et al. (1996) clinical trial may have masked the true potential of L-baclofen, alone, as a tinnitus treatment.

The use of L-baclofen, rather than the racemic mixture, to treat tinnitus in a rat model has been explored more recently in a study by Zheng et al. (2012b). The study used 16 rats that were divided equally into noise-exposed and sham treatment groups. Tinnitus was induced by acoustic trauma in the noise-exposed group, while the sham group was kept under anaesthesia for the same duration but with no noise exposure. The effect of 1, 3, and 5 mg/kg of L-baclofen (s.c.) on the psychophysical attributes of tinnitus was then assessed via a conditioned lick suppression behavioural paradigm, after tinnitus had been confirmed in the noise-exposed rats. The study found that L-baclofen dose-dependently reduced the
behavioural signs of tinnitus in this particular animal model. This was illustrated by the statistically significant attenuation of the shift in the suppression ratio (SR) curve for the 20 kHz stimuli at the doses of 3 mg/kg and 5 mg/kg.

One strength of this study lies in the inclusion of two washout periods: the first between the 3 mg/kg and 5 mg/kg dosing periods, and the second after the 5 mg/kg dosing period. The washout periods gave the study the scope for confirming a real drug effect, as opposed to an effect caused by a reversal of tinnitus as part of its natural progression within the animals. After the first washout period there was a return of the significant separation between the SR curves of the noise-exposed and sham treatment groups, similar to that observed at the beginning of the study before drug treatment. This greater suppression in the noise-exposed group is indicative of the tinnitus returning within that group, resulting in confirmation that the reduction in the psychophysical attributes of tinnitus previously observed was in fact the result of a real drug effect. However, following the second washout period, there remained no significant difference between the SR curves of the noise-exposed and sham treatment groups. Zheng et al. (2012b) gave three possible explanations for this: a natural reversal of the tinnitus may have occurred; the result may have been a long lasting treatment effect caused by what was the highest L-baclofen dose used in the study; or more likely, based on a trend towards separation of the curves, the washout period was not long enough for the complete elimination of the drug. Overall, the results are suggestive of the GABAergic system contributing to the underlying pathophysiology of tinnitus, and provide a more solid grounding for additional tinnitus research to be built upon, in particular that involving GABA<sub>B</sub> receptor agonists such as L-baclofen
1.5 Master’s Project

1.5.1 Rationale for the Present Project

As the precise mechanism of tinnitus continues to elude researchers and clinicians alike, there are very limited treatment options currently available for patients with this debilitating disorder. Previous research on the role of GABAergic transmission, and the potential of baclofen as a drug treatment, clearly highlights this area as one that is both scientifically and clinically significant to neurological research on tinnitus. However, despite this, there remains only one clinical trial on the use of baclofen as a tinnitus treatment, with research employing animal models being similarly limited. Of this small pool of data available, poor study design and inconsistencies in results means that further research is required if the efficacy of this drug as a possible tinnitus treatment is to be fairly and accurately determined. Baclofen itself is already FDA-approved. By testing a drug that is clinically available there is the obvious advantage that if clear evidence for its efficacy exists, the time until its prescription to patients as a tinnitus treatment could be relatively short.

The current project aimed to build on existing research into baclofen as a tinnitus treatment. Zheng et al. (2012b) have previously demonstrated that tinnitus can recur following the cessation of L-baclofen treatment, which suggests a real drug effect as opposed to the tinnitus disappearing by itself over time. This indicates that tinnitus may develop as a result of reduced GABA neurotransmission, which is reversed by the L-baclofen treatment. The next logical step, which is addressed in the present study, was to assess whether an early drug intervention could prevent the reduction in GABA neurotransmission, thus preventing the onset of tinnitus. Furthermore, despite such findings, the changes to GABA\textsubscript{B} receptors in noise-induced tinnitus have never been studied systematically. Therefore by studying the effects of L-baclofen on GABA\textsubscript{B} receptors, the present study provided preliminary results that
can contribute to the elucidation of a possible GABAergic mechanism in the development of tinnitus.

The present project further contributed to the current literature by aiming to address some of the flaws of the previous animal and human studies, while maintaining their points of strength. For example, the present project employed the same acoustic trauma animal model and conditioned lick suppression paradigm that proved effective at inducing and assessing tinnitus by Zheng et al. (2012b), and power calculations were carried out to determine the appropriate sample size of animals to avoid the problem of inadequate power encountered by Westerberg et al. (1996). Furthermore, the use of an animal model provided direct control over the causal condition, decreasing the likelihood that there were population subsets within the treatment groups. Adding to the existing framework gave the current project the capacity to provide further pharmacological evidence of L-baclofen as a viable tinnitus treatment option, and additional evidence towards a GABAergic mechanism.

1.5.2 Objectives of the Project

1) To determine the effect of acute and chronic L-baclofen administration, at different time points, in treating noise-induced tinnitus in rats.

2) To investigate changes in GABA$_{B-R2}$ expression in the CN of rats with noise-induced tinnitus, following treatment with L-baclofen.
Chapter 2: Materials and Methods
2.1 Animals

Thirty two male Wistar rats (300 – 350 g at the beginning of the experiment) were obtained from the Hercus Taieri Resources Unit to be used in this study. The rats were housed in groups of two or three in the animal room in the Department of Pharmacology and Toxicology, which maintains a 12:12 hour light:dark cycle at a temperature of 22 °C. Throughout the study the rats were given free access to food, but were deprived of water during the periods of behavioural testing in compliance with the conditioned lick suppression paradigm. The animals were divided into four groups: noise-exposed + vehicle (n = 8), noise-exposed + L-baclofen (n = 8), non-exposed (sham) + vehicle (n = 8), and non-exposed (sham) + L-baclofen (n = 8). Each rat received treatment with vehicle or L-baclofen 30 minutes after the noise (or sham) exposure using a dose of 5 mg/kg (s.c. injection), and then again every 24 hours for 5 days. The animals were tested for behavioural signs of tinnitus at 2 weeks, and then again at 10 weeks, following noise (or sham) exposure. In addition, beginning at 17.5 weeks after the noise (or sham) exposure, and throughout the third tinnitus testing period, L-baclofen was administered at a dose of 3 mg/kg/day (s.c. injection) for 4.5 weeks. All experiments were conducted in accordance with the regulations of the Otago University Committee on Ethics in the Care and Use of Laboratory Animals.
2.2 Materials

Three mg/kg and 5 mg/kg L-baclofen

One hundred and twenty five mg R(+)-baclofen hydrochloride (Sigma, G013) was dissolved in 5 mL distilled water (dH₂O) to make a weekly stock solution of 25 mg/mL, which was stored at 4 °C. The stock solution was further diluted with saline each day to 2.5 mg/mL, for an every day working solution. For the 3 mg/kg dose the dose volume was 1.2 mL/kg, and for the 5 mg/kg dose the dose volume was 2 mL/kg (see Discussion 4.2 for a full explanation of the reasoning behind changes made to the L-baclofen dose during the study).

Phosphate buffered saline (0.1 M PBS), pH 7.4

Two PBS tablets (Sigma, P4417) were added to 400 mL of dH₂O, before being completely dissolved using a magnetic stirrer. When not in use, PBS was stored at 4 °C.

Paraformaldehyde (8% PFA)

Twenty g PFA (Sigma, 158127) was added to 250 mL of dH₂O, which had been heated to 60 °C on a hot-plate magnetic stirrer in a fume hood. If cloudy, 3 mL of 0.1 N sodium hydroxide (NaOH) (BDH Laboratory Supplies, BDH7222) was added to clear the solution. The solution was then filtered, and left to cool. PFA was made up on the same day of use.

Phosphate buffer (0.2 M PB)

Stock A consisted of 28.39 g disodium hydrogen phosphate (Na₂HPO₄) (BDH Laboratory Supplies, BDH8022), which was dissolved in 1 L of dH₂O, using a magnetic stirrer. Stock B consisted of 24 g of monosodium phosphate (NaH₂PO₄) (BDH Laboratory Supplies, BDH0298), which was dissolved in 1 L of dH₂O, using a magnetic stirrer. To make 0.2 M PB at pH 7.4, 40.5 mL of stock A was mixed with 9.5 mL of stock B. Stock A and
stock B were stored separately at room temperature, and the mixed PB solution made up as required.

**Cresyl violet (0.015% CV) solution**

One hundred and fifty μL of 0.5% CV stock solution was added to 4850 μL of acetic acid, before being double filtered. The laboratory stock solution was stored at room temperature, and the 0.015% CV solution made up each day, as required.

**Gelatin-coated slides**

Five hundred mL of deionised H₂O was heated to 60 °C on a hot-plate magnetic stirrer. Once this had reached temperature, 5 g of gelatin was dissolved into the H₂O, and the resultant solution filtered. Glass slides were placed in slide racks, and these were immersed in the solution at 60 °C for 1 minute. The slides were then removed, the excess solution tapped off, before being left to dry in a warming oven overnight.

**Glycine (0.1 M)**

Zero.seven five g of glycine (Sigma, G6761) was dissolved in 0.1 M PBS using a magnetic stirrer, to make a total volume of 10 mL. Glycine was made up on the day, as required.

**Antibody dilution buffer (AbDB)**

Zero.one g of bovine serum albumin (BSA) (Gibco by Life Technologies, 30063-481) was added to 10 mL of 0.01 M PBS. This was vortexed until dissolved, before 50 μL of Triton X-100 was added, and the solution vortexed again. AbDB was made up on the day, as required.
**Guinea pig (1:500) GABA\textsubscript{B-R2} antiserum**

Twelve µL of guinea pig anti-GABA\textsubscript{B-R2} receptor polyclonal antibody (Millipore, AB5394) was added to 5685 µL PBS, 300 µL normal donkey serum (NDS), and 3 µL Triton X-100, before being vortexed. The solution was made up as required.

**Peroxidase-conjugated donkey anti-guinea pig IgG (1:400)**

Fifteen µL of peroxidase-conjugated donkey anti-guinea pig IgG (Jackson Immuno Research Laboratories, 706-035-148) was added to 5865 µL PBS, and 120 µL NDS, before being vortexed. The solution was made up as required.

**3,3'-diaminobenzidine (DAB) solution**

A DAB peroxidase substrate kit (Vector Laboratories, SK-4100) was used. From this, 4 drops of buffer solution was added to 10 mL of dH\textsubscript{2}O, and the solution vortexed. Next, 8 drops of DAB stock was added, and the solution vortexed again. Finally, 4 drops of hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) was added before the final solution was vortexed. The DAB kit was stored at 4 °C, and the DAB solution made up as required.

**Lysine-coated slides**

Poly-L-lysine solution (Sigma, P8920) was mixed with deionised H\textsubscript{2}O in 1:10 dilution using a magnetic stirrer. Glass slides were placed in slide racks, and these were immersed in the solution for 5 minutes. The slides were then removed, the excess solution tapped off, and left to dry in a warming oven overnight.
2.3 **Sequence of the Project**

Table 3: Sequence of methods, in the order they occurred.

<table>
<thead>
<tr>
<th>Methods</th>
<th>Further Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABR threshold measurements and noise trauma</td>
<td>Every animal was anaesthetised and initial ABR threshold measurements taken. All animals were then exposed to either noise trauma or sham treatment. ABR threshold measurements were taken again, immediately after the noise exposure or sham treatment. Vehicle (saline) or L-baclofen (5 mg/kg) injections were administered 30 minutes post-exposure, and again at the same time every day for the following five days.</td>
</tr>
<tr>
<td>No testing (duration: 2 weeks)</td>
<td>Two weeks of no testing, during which animals had free access to food and water.</td>
</tr>
<tr>
<td>Tinnitus testing period 1 (duration: 5.5 weeks)</td>
<td>Testing period which comprised 6 days of acclimation, 6 days of suppression, and 24 days (8 at each of the three frequencies) of discrimination testing per rat.</td>
</tr>
<tr>
<td>No testing (duration: 2.5 weeks)</td>
<td>Two and a half weeks of no testing, during which animals had free access to food and water.</td>
</tr>
<tr>
<td>Tinnitus testing period 2 (duration: 7.5 weeks)</td>
<td>Testing period which comprised 3 days of acclimation, 3 days of suppression, and 36 days (12 at each of the three frequencies) of discrimination testing per rat.</td>
</tr>
<tr>
<td>Tinnitus testing period 3 (duration: 4.5 weeks)</td>
<td>Testing period which comprised 32 days (10 days broadband noise (BBN), 11 days 20 kHz, 11 days 32 kHz) of discrimination testing per rat. L-baclofen (3 mg/kg) was administered 1 hour before each testing session.</td>
</tr>
<tr>
<td>ABR threshold measurements and tissue removal</td>
<td>Every animal was anaesthetised and final ABR threshold measurements taken. All animals were then given a final L-baclofen injection (3 mg/kg) 1 hour prior to tissue removal via cardiac perfusion.</td>
</tr>
<tr>
<td>Tissue sectioning</td>
<td>The hindbrains of 12 animals (six noise-exposed + vehicle, and six noise-exposed + baclofen) were cut into sections 40 µm thick,</td>
</tr>
</tbody>
</table>
and five sets of serial sections were collected from each animal.

<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histology</td>
<td>Cresyl violet staining was performed on one complete series of sections from each of the 12 hindbrains previously sectioned to help with orientation.</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>GABA&lt;sub&gt;B-R2&lt;/sub&gt; immunoreactivity was measured in the CN of one complete series of sections from each of the 12 hindbrains previously sectioned.</td>
</tr>
<tr>
<td>Quantitative analysis of GABA&lt;sub&gt;B&lt;/sub&gt; receptors</td>
<td>Following immunohistochemical staining, photos of the CN in each section were taken at a 20x magnification level. The mean GABA&lt;sub&gt;B-R2&lt;/sub&gt; density was then determined for the exposed-baclofen and exposed-vehicle groups using ImageJ software.</td>
</tr>
</tbody>
</table>
Figure 9: Timeline of the behavioural methods. ABR threshold measurements were carried out before and after noise (and sham) exposures on all of the animals over a 2 week period. This was followed by 2 weeks of no testing, to allow for the development of tinnitus in the noise-exposed animals. The first tinnitus testing period spanned 5.5 weeks, and was followed by a second period of no testing, which was 2.5 weeks in duration. The second tinnitus testing period spanned 7.5 weeks, and was followed immediately by a third tinnitus testing period which spanned 4.5 weeks. Final ABR threshold measurements and tissue removal were carried out over 1 week. During the behavioural methods there were two periods of vehicle or L-baclofen administration. The first period was 30 minutes post-noise trauma, and again at the same time every day for the following five days. The second period was during the third tinnitus testing period, up until the day of sacrifice.
2.4 Noise Trauma to Induce Chronic Tinnitus

Rats were induced with tinnitus using a unilateral acoustic trauma model (Bauer and Brozoski, 2001). Each animal was anaesthetised with fentanyl citrate (0.2 mg/kg, s.c.), medetomidine hydrochloride (Domitor, Novartis; 0.5 mg/kg, s.c.), and atropine sulphate (50 μg/kg, s.c.), before being placed in a modified stereotaxic head frame inside a sound attenuation chamber. The noise-exposed animals were subjected to a 16 kHz pure tone with an intensity of 115 dB which was generated by a NI 4461 Dynamic Signal Acquisition and Generation system (National Instruments, New Zealand Ltd) for one hour, while the sham animals received the same anaesthetics and were kept under anaesthesia for the same duration as the noise-exposed animals, but without the noise exposure. The noise trauma was delivered unilaterally via a closed field magnetic speaker with a tapered tip (Tucker-Davis Technologies) that was attached to a 3mm cone-shaped speculum that fitted into the external auditory canal of the animal. The unexposed ear was blocked with an earplug to protect it from residual sound exposure (Figure 10).
**Figure 10: Exposure of an animal to noise trauma.** An animal that was to be induced with tinnitus was anaesthetised, before being placed in a sound attenuation chamber. A pure tone was delivered via a speaker fitted to a speculum that was inserted into the external auditory canal, and the unexposed ear blocked by an earplug.

A: sound attenuation chamber; B: speaker; C: speculum; D: ear plug.

Prior to noise exposure the acoustic values were calibrated using a ¼-inch prepolarised free-field microphone (Type 40BE, GRAS Sound & Vibration) that was attached to the speaker via the speculum used to fit into the external auditory canal.
2.5 Hearing Level Assessment

ABR thresholds were used to test hearing levels, and were measured in both ears of all animals before and immediately after the noise trauma or sham treatment, as well as at the conclusion of the behavioural testing, before animal sacrifice. During hearing level assessment, the animals were anaesthetised as previously described (Methods 2.3) before acoustic stimuli were presented using the same set-up that delivered the noise-trauma. The ABRs were recorded using three stainless steel needle electrodes (Chalgren Enterprises, 112-812-48TP): two recording electrodes placed subcutaneously at the vertex and over the bullae; and one reference electrode placed subcutaneously at the occiput (Figure 11).

Figure 11: Placement of electrodes during ABR recording. Subdermal needle electrodes were inserted at the vertex and bullae, with a reference electrode at the occiput. An earplug was placed in the ear that was not being assessed.

Tone bursts of a 5 ms duration (2 ms rise/decay, 1 ms plateau) presented at a rate of 21/s were used to test ABR thresholds, at 32-, 20-, 16-, and 8-kHz, respectively. These tone bursts were presented in a series of decreasing intensity, which began at a level that resulted in observable evoked potentials. Intensities delivered progressed in 20-, 10-, and 5-dB steps, and the hearing threshold was deemed to be the lowest intensity that produced a visually distinct potential.
2.6 Tinnitus Assessment

2.6.1 Tinnitus Assessment Chamber

The presence of tinnitus was first assessed two weeks after the noise trauma using a conditioned lick suppression paradigm. The assessment was carried out in an operant conditioning test chamber (ENV-007, Med Associates Inc., St Albans, VT, USA) within a sound attenuation box (Figure 12).

The operant conditioning test chamber contained a drinking tube that was positioned 5 cm above the floor, on one side of the chamber, behind a v-shaped restrictor plate. The drinking activity of the animals was measured automatically via a lickometer with a photobeam (ENV-251L, Med Associates Inc., St Albans, VT, USA), which was positioned between the restrictor plate, and in front of the drinking tube. This accurately counted each beam break made by the tongue of the animal being tested, allowing the animals’ drinking activity to be quantified by the number of licks during testing.

Mounted on the ceiling of the chamber was a camera used to monitor the behaviour of the animals during testing, and a speaker (ENV-224DM, Med Associates Inc., St Albans, VT, USA), positioned above the drinking tube, which produced BBN (white noise spanning frequencies of 3 kHz to 20 kHz, and with no equalisation), or a pure tone of different frequencies (20 kHz and 32 kHz) and intensities ((BBN = 30 dB, 40 dB, 50 dB, 70 dB; 20 kHz = 70 dB, 80 dB, 90 dB, 100 dB; 32 kHz = 70 dB, 80 dB, 90 dB, 100 dB) via a sound generator (ANL926, Med Associates Inc., St Albans, VT, USA).

The chamber floor consisted of stainless steel rods that were 0.48 cm in diameter and spaced 1.6 cm apart (ENV-005, Med Associates Inc., St Albans, VT, USA), which were used to deliver an electric shock (0.35 mA) that was produced by a constant current shock source (ENV-410B, Med Associates Inc., St Albans, VT, USA) through a scrambler (ENV-412, Med Associates Inc., St Albans, VT, USA).
Figure 12: Tinnitus assessment chamber. Tinnitus assessment of the animals was carried out using a conditioned lick suppression paradigm, which was conducted in an operant conditioning test chamber. A: sound attenuation box; B: camera; C: speaker; D: lickometer; E: drinking tube, F: shock-capable floor.

2.6.2 Tinnitus Assessment Conditions

The animals were water deprived throughout each of the tinnitus assessment periods, having access to water only when inside the testing chamber. Each animal was weighed before the testing period to establish a normal body weight, and then every day prior to testing.
to ensure they maintained a weight of at least 85% of their normal body weight. If an animal fell below 85% of its normal body weight it was given access to water for 30 minutes outside of testing (at least 3 hours after testing to ensure animals did not learn to associate lack of drinking during testing with access to water after testing), or 15 minutes if it was below 90%, but above 85%, of its normal body weight. This resulted in the animals reliably producing approximately 2000 to 6000 licks per testing session.

The conditioned lick suppression paradigm used for the assessment of tinnitus required each animal to be tested every day, for 15 minutes per day, throughout each testing period. During this time animals were subjected to one of three phases: the acclimation phase, the suppression phase, or the discrimination phase.

2.6.3 Acclimation Phase

BBN was played throughout the acclimation phase, and acted as a background noise. In each 15 minute testing session the BBN was interrupted at 10 random intervals, during which a 15 second acoustic stimulus was inserted (Figure 13). Two of these 10 intervals were speaker off periods i.e. silence in the chamber, and the remaining eight intervals were an acoustic presentation of either BBN, or a 20 kHz, or 32 kHz tone, at one of four different intensity levels (BBN = 30 dB, 40 dB, 50 dB, 70 dB; 20 kHz = 70 dB, 80 dB, 90 dB, 100 dB; 32 kHz = 70 dB, 80 dB, 90 dB, 100 dB). Each stimulus was repeated twice during the 15 minute testing session, and occurred in a random order. However, no stimulus presentation could occur during the first and final minute of the testing session, or within one minute of each other. Within a single testing session the type of acoustic stimulus remained constant, but varied randomly between sessions. The purpose of this acclimation phase was to allow the animals to become familiar with the tinnitus-testing chamber and each of the testing stimuli. Each animal was subjected to six sessions of the acclimation phase at the beginning of the first testing period, three sessions of the acclimation phase at the beginning of the second
testing period, and no sessions of the acclimation phase during the third testing period (as this had a discrimination phase that ran directly on from the discrimination phase of the second testing period).

Figure 13: Acclimation testing phase. Fifteen minutes of BBN, interjected randomly with ten 15 second intervals of acoustic stimuli (BBN, 20 kHz, or 32 kHz tones), two of which were speaker off (silent) periods.

2.6.4 Pavlovian Conditioned Suppression Training Phase

Following the acclimation phase, the animals were subjected to conditioned suppression training that involved a mild foot shock (0.35 mA, for 3 seconds) occurring at the end of each speaker off period (Figure 14). The mild foot shock acted as an unconditioned stimulus, while the speaker off period acted as a conditioned stimulus, allowing the rats to learn the association between the shock and speaker off (i.e. silence). Gradually the rats reacted to the foot shock in the speaker off period by exhibiting suppressed licking during this interval. The lick suppression was measured by a suppression ratio (SR) that compared the number of licks in the 15 second interval preceding the stimulus presentation, with the number of licks during the 15 second stimulus presentation. The aim during this phase of testing was for the animals to achieve a conditioned suppression that resulted in an SR of less
than 0.2. Each animal was subjected to six sessions of the suppression phase during the first testing period, three sessions of the suppression phase during the second testing period, and no sessions of the suppression phase during the third testing period (as this testing period had a discrimination phase that ran directly on from the discrimination phase of the second testing period).

\[
SR = \frac{B}{A + B}
\]
Where A is the number of licks during the preceding period, and B is the number of licks during the stimulus presentation period.

Figure 14: Suppression testing phase. Fifteen minutes of BBN, interjected randomly with ten 15 second intervals of acoustic stimuli (BBN, 20 kHz, or 32 kHz tones), two of which were speaker off (silent) periods that were accompanied by a mild foot shock.

### 2.6.5 Frequency Discrimination Phase

The discrimination phase testing sessions presented acoustic stimuli in the same manner as the suppression sessions, however a foot shock occurred only if the animal did not suppress its licking to a level which produced an SR of less than 0.2 for the speaker off period (Figure 15). Each animal was subjected to 24 sessions of the discrimination phase during the
first testing period, 36 sessions of the discrimination phase during the second testing period, and 32 sessions of the discrimination phase during the third testing period.

Figure 15: Discrimination testing phase. Fifteen minutes of BBN, interjected randomly with ten 15 second intervals of acoustic stimuli (BBN, 20 kHz, or 32 kHz tones), two of which were speaker off (silent) periods. Speaker off periods were accompanied by a mild foot shock if licking was not suppressed to a level which resulted in an SR of less than 0.2.

2.6.6 A Positive Tinnitus Assessment

Those animals that did not have tinnitus would hear the speaker off periods as silence, and associate the foot shock with this silence. They would be expected to suppress their licking during the silent periods, but not exhibit lick suppression during the acoustic stimuli presentation period, as the presentation of the stimuli would have no meaning to them. In contrast, if an animal had tinnitus it would not hear the speaker off period as silence, but instead would hear the illusionary auditory sensation of its tinnitus. Therefore throughout the training sessions the tinnitus would become a conditioned stimulus instead of the silence. As a result, in order to avoid the foot shock during the discrimination phase, these animals would not only suppress their licking during the speaker off period, but also during the stimulus presentation period of stimuli with sensory features that resembled their tinnitus. This would
result in a lick suppression that is greater overall compared to those animals that did not have tinnitus, and a positive tinnitus assessment.
2.7 Drug Protocol

2.7.1 Acute Drug Administration Following Noise Trauma

Animals were given a subcutaneous injection of either vehicle (saline) or L-baclofen (5 mg/kg) 30 minutes after the noise trauma while still under anaesthesia, and then again at the same time each day, for the following five days.

The treatment time point of 30 minutes after the noise trauma, aimed at preventing the development of neuronal hyperactivity following noise trauma, and hence the development of tinnitus, was chosen based on a reduction in noise-induced tinnitus being achieved by local application of an N-methyl-D-aspartate receptor agonist (ifenprodil) when administered during the first four days following noise exposure, but not afterwards (Guitton and Dudai, 2007). While this information is based on the local application of a different drug into the cochlea, and therefore is not directly comparable to the current project, it represents the only available time course data on tinnitus treatment. Should a similar effect carry across to the current study, an initial treatment time point of 30 minutes, which falls at the beginning of the 4 day therapeutic window established by Guitton and Dudai (2007), would increase the likelihood that it is detected.

Based on the current literature, 5 mg/kg represents a dose within the range used in humans: using the dose adjustment calculation employed by the US FDA to calculate human equivalent doses, a dose of 5 mg/kg is approximately equal to a human dose of 56.76 mg/day for a 70 kg adult, and is similar to the twice daily 30 mg dose use by Westerberg et al. (1996) to treat tinnitus patients (Regan-Shaw et al., 2007; Smith et al., 2012). Furthermore, a similar study by Zheng et al. (2012b), that assessed the effect of L-baclofen on noise-induced tinnitus, indicated that a 5 mg/kg dose was effective at reducing the behavioural signs of tinnitus in rats.
2.7.2 Chronic Drug Administration During the Third Testing Period

Throughout the third testing period each animal was administered with either vehicle (saline) or L-baclofen (3 mg/kg), via subcutaneous injection, one hour prior to being tested. The dose was altered from the initial 5 mg/kg acute dose due to a sedative effect that was not fully realised until the chronic dosing stage of the study (Discussion 4.2). The sedation caused by a 5 mg/kg dose left the animals unable to perform in the lick suppression paradigm, by reducing their licking activity from the expected 2000-6000 licks per session, to less than 100 licks. A dose of 3 mg/kg was subsequently chosen as it did not produce any observable signs of sedation, and allowed the animals to produce the expected licks per session. Note that 3 mg/kg L-baclofen was also found to be an effective dose in the previous study by Zheng et al. (2012b).

2.7.3 Drug Administration Prior to Animal Sacrifice

Animal sacrifice occurred immediately after the third testing period. As it was not possible to perform cardiac perfusions of all 31 remaining animals in one day, the sacrifice of animals was scheduled over 6 days. To prevent washout of the drug, each animal continued to receive either vehicle or L-baclofen (3 mg/kg) via subcutaneous injection up until the day of sacrifice, and then 1 hour prior to the perfusion on the day of sacrifice.
Each animal was anaesthetised with fentanyl citrate (0.2 mg/kg, s.c.), medetomidine hydrochloride (Domitor, Novartis; 0.5 mg/kg, s.c.), and atropine sulphate (50 µg/kg, s.c.), before final sets of ABRs were measured on the day of sacrifice. Following this, each animal received a final vehicle or baclofen (3 mg/kg) injection. Animal sacrifice, via cardiac perfusion, was scheduled for one hour after this final injection was administered. To perform the cardiac perfusion the animal was attached to the perfusion rack on its back with its limbs spread. The chest was cut half way down. To avoid damage to the animal’s organs the skin was simultaneously lifted as these cuts were made. This exposed the diaphragm, where a hole was cut in the centre, before being extended outwards in both directions. The cut was continued up each side of the chest to allow the heart to be pulled with tweezers to the centre of the chest. Tweezers were then used to grip the right ventricle so that a small incision could be made in the left ventricle, followed by the insertion of a needle through the ventricle wall. Next, the needle was gently moved up into the aorta, and secured in place with a pair of forceps. Additionally, a large hole was cut in the right atrium to ensure a good flow of solution. Once the needle was secure the solutions were pumped through under a pressure of 120 mm Hg. This included an initial 150 mL pre-wash using 0.01 M PBS, before the tissue was fixed using 200 mL of 4% PFA in 0.1 M PB (pH 7.4), followed by 200 mL of 4% PFA in 0.1 M PB containing 0.6 M sucrose (pH 7.4). Once all of the solutions had run the animal was removed from the perfusion rack and flipped over. The neck was then broken, and the head decapitated, before the brain was carefully removed from the skull without any damage to the brain tissue itself. During this removal, the cochlear nerves were cut and only the forebrain was handled with the tweezers to protect the hind-brain and CN tissue. Once removed, each brain was left in a post-fix solution of 4% PFA in 0.1 M PB containing 0.6 M sucrose (pH
at 4 °C for 4 hours. Finally, the tissue was incubated overnight at 4 °C in a post-fix solution of 30% sucrose in 0.1 M PB (pH 7.4).

Each brain was then dissected by a cut made down the midline of the forebrain, to separate the left and right hemispheres from each other, and from the hindbrain. A needle was inserted into the left side of the hindbrain, in an area of tissue not required, so that the sides of the hindbrain could be differentiated from one another. All three of these parts of brain tissue were placed into separate custom-made tinfoil boats. The boats were half-filled with optimum cutting temperature (O.C.T.) compound (BDH Laboratory Supplies), and the front was marked on the outside so that the orientation of the brain tissue inside could be determined during tissue sectioning. Once in the tinfoil boat, the brain tissue was fully covered with O.C.T. compound, before being placed in a beaker of hexane (BDH Laboratory Supplies) which was partially submerged in a canister of liquid nitrogen. When the O.C.T. compound was frozen the boat was removed and the top edges of the tinfoil folded down, before being stored at 20 °C up until the time of tissue sectioning.
2.9 Tissue Sectioning

Due to time constraints, the hindbrains from 12 of the original 32 animals (six noise-exposed + vehicle, and six noise-exposed + L-baclofen) were used for the remainder of the study. To maintain power during statistical analysis, tissue from six rats in two treatment groups was sectioned for analysis, instead of tissue from all four treatment groups. This resulted in groups that were large enough for statistically significant differences to be detected when present. To examine the effect of the L-baclofen administration on GABA$_B$ receptor expression, the two noise-exposed groups were chosen, as a means of eliminating any noise exposure effect when comparing groups.

Each block of hindbrain, previously frozen in O.C.T. compound, was mounted (using more O.C.T. compound) on the cutting stage of a cryostat machine (Leica CM 1850) that was set to a temperature of -16 °C, as this was determined to be the optimal temperature for cutting the tissue. The tissue was cut into 40 $\mu$m thick sections, and the first section taken was selected randomly, with every 5$^{th}$ section onwards being collected. Five sets of 24-well plates were used to collect the sections, so that the final analysis (which used one well plate of sections per brain) included a subset of sections that was random, but separated by a fixed distance (Figure 16). Once collected, sections were kept in the well plates (containing ethylene glycol) at -20 °C until required.
Figure 16: Tissue sectioning plan. A random systematic sampling method was used to ensure every part of the CN had an equal chance of being sampled. The first CN section was selected at random, and from then on sections were collected in five sets of 24-well plates. Therefore, each well plate contained a subset of sections, with each section 5 sections apart from the last. Numbers in yellow circles refer to the well plate, and numbers in white circles refer to the number of the section collected.
2.10 Histology

Cresyl violet (CV) staining was completed on one full series of sections for each of the 12 rats whose brain tissue was to be used for the immunohistochemistry analysis. This was to aid in orientating the sections and identifying the CN. All steps were carried out at room temperature.

The ethylene glycol that the sections were stored in was removed from each well using a pipette. Sections were then washed twice with 0.01M PBS for 5 minutes to remove any residual ethylene glycol still present. This was followed by a 5 minute dH₂O wash to prevent any interactions between the PBS and CV solutions. Next, sections were immersed in 0.015% CV solution for 30 minutes, before being differentiated in dH₂O for 5 minutes, and tap water (tap H₂O) for 5 minutes. All sections were then transferred onto gelatin coated slides and left in dark slide boxes to dry overnight.

Sections were dehydrated in a series of ethanol washes at varying concentrations as follows: 50% ethanol, 1 minute; 70% ethanol, 1 minute; 95% ethanol, 2 minutes; 100% ethanol, 3 minutes. Following the final ethanol wash, a 5 minute xylene wash was completed to remove the residual ethanol, before the slides were cover slipped using di-n-butyl phthalate in xylene (DPX) as a mounting medium.
2.11 Immunohistochemistry

GABA$_{B}$-R2 immunoreactivity was measured on free floating 40 μm sections cut from the 12 rat brains chosen for this phase of the study. The GABA$_{B}$-R2 receptor subunit of the GABA$_{B}$ receptor was studied using an immunohistochemistry protocol adapted from Jamal et al. (2011). GABA$_{B}$ receptors have been demonstrated to exist in major auditory structures of the rat, such as the CN, SOC, and IC, by both in vivo and in vitro pharmacological studies (Bandrowski et al., 2001; Faingold et al., 1989; Isaacson, 1998; Lim et al., 2000; Peruzzi et al., 1997). The GABA$_{B}$ receptor was studied to provide preliminary results that could contribute to the elucidation of a possible GABAergic mechanism in the treatment of tinnitus with L-baclofen. An antibody that specifically recognises the C-terminus of rat GABA$_{B}$-R2 was chosen as this subunit is required for a functional GABA$_{B}$ receptor (Jones et al., 1998; White et al., 1998).

Sections were taken at -20 o C and left at room temperature for approximately 10 minutes, before being transferred to a 24-well plate which contained 0.01 M PBS. Each subsequent step was performed at room temperature, unless otherwise stated. Three 5 minute 0.01 M PBS washes were performed on the sections to remove the ethylene glycol in which they had been previously stored. This was followed by a 15 minute wash in 0.1 M glycine to bind free aldehydes and prevent high background staining. Two more 5 minute 0.01 M PBS washes were then carried out to remove the glycine. Next, heat-induced antigen retrieval was carried out by incubating the sections in citrate (pH 6) at 90 o C for 10 minutes, to break protein cross-links which were introduced during the fixation process, allowing subsequent exposure of antigen sites to antibody (D’Amico et al., 2009). This was followed by a 15 minute cooling period, before two 5 minute washes in 0.01 M PBS. Nonspecific binding of antibodies was blocked by incubating sections in 5% NDS with AbDB for 2 hours, before incubation with a primary antibody (1o Ab) of guinea pig GABA$_{B}$-R2 antiserum. The 1o Ab
was diluted to 1:500, in an AbDB containing 5% NDS, 0.05% Triton X-100 in 0.01 M PBS, and incubated for 72 hours at 4°C.

Three 10 minute PBS washes were carried out, before sections were incubated for 10 minutes in 0.5% H2O2 in PBS to quench endogenous peroxidase. The sections were then incubated with a secondary antibody (2o Ab) of peroxidase-conjugated donkey anti-guinea pig IgG diluted to 1:400 in PBS with 2% NDS for 2 hours. Three 10 minute PBS washes were followed by two 5 minute dH2O washes, before a 20 minute DAB incubation to produce visualisation of the immuno-complex. The resultant brown staining corresponded to positive immunoreactivity to GABAB-R2 (Jamal et al., 2011). Two 5 minute dH2O washes and two 5 minute tap H2O washes were then carried out, before sections were transferred onto poly-L-lysine coated slides with tap H2O, and left to dry overnight in an opaque microscope slide box.

Sections were dehydrated in ascending dilutions of ethanol as follows: 70% for 5 minutes, 95% for 5 minutes, and 100% for 10 minutes. The ethanol was cleared by placing the sections in xylene for 5 minutes, before they were mounted with DPX and left to dry overnight.
2.12 Quantitative Analysis of GABA\textsubscript{B} Receptors

Prior to the quantitative analysis of GABA\textsubscript{B} receptors, sections containing GABA\textsubscript{B-R2} immunoreactive staining were examined under a light microscope (Nikon Elipse Ni-E) at a 10x magnification in order to identify those sections with an intact CN. Following identification of the CN, images were produced and photographs taken at a 20x magnification level using the NIS Elements computer software program, in conjunction with the digital camera attached to the microscope.

To produce the images, the ‘pixel saturation indication’ tool in the NIS Elements program, which highlights under-saturated and over-saturated pixels in contrasting colours, was used to identify an exposure time that would achieve the full dynamic range of the pixels. From this, an optimal exposure time of 2 ms was determined. Additionally, this short exposure time was preferable for the photo-stitch photographs that were taken, as there was less chance of z-plane movement affecting the quality of the final images. The number of fields in the X and Y direction was chosen based on the size of the CN that was to be photographed, and this directly determined the number of images that were stitched together to produce the final photograph of the CN, at the 20x magnification level. Finally, the photo-stitch photograph was captured, and a 100 μm scale bar was burnt onto the photograph before it was saved.

The quantitative analysis itself was carried out using ImageJ software. Each image was converted to an 8-bit file type (Figure 17). Using the known distance from the scale previously burnt onto the photograph, an equivalent scale within the ImageJ program itself was set. Next, the region of interest (CN) was drawn using a freehand tool, before being analysed. ImageJ produced a value for the integrated density of the CN, based on the product of the area drawn, and the mean grey value of this area. Next, the same freehand-drawn region
of interest was transferred to a background area of the photograph where there was no tissue. The integrated density of the background was then calculated, allowing the difference between the two values to be found. The mean densities of the treatment groups (L-baclofen and vehicle) and exposure sides (contralateral and ipsilateral) for the noise-exposed animals were calculated from the values obtained from the ImageJ analysis.

Figure 17: Quantitative analysis of GABA\(_B\) receptors using ImageJ software. The integrated density of the CN was calculated in ImageJ by drawing a region of interest using a freehand tool (1). The background was also calculated, by transferring the freehand-drawn region onto an area of no tissue (2). The mean density was calculated as the difference between these two values.
2.13 Statistics

Before statistical analysis, certain pieces of data were excluded from the data set. If an
animal produced less than 950 licks in any one session (well below the expected 2000 to 6000
licks), the data from that session were excluded as there were not enough licks to provide a
valid SR equation. Furthermore, if an animal’s lick suppression resulted in an SR index that
was greater than 0.2 at the 0 dB SPL stimulus intensity (i.e. they failed to suppress licking in
response to the conditioned stimulus), the data from that session were excluded. The first two
days of data from each phase (acclimation, suppression, discrimination) of each testing period
were also deleted, as there were outliers and increased variation in the animal’s lick
suppression in the transitions between phases. Finally, during the project, one animal became
severely underweight and had to be culled early. Therefore, data from this animal were
included only for the first two tinnitus testing periods.

Following the exclusion of some data, the data sets had missing values, which presents
problems when using a repeated measures analysis of variance (ANOVA) design, as programs
such as SPSS 20 delete subjects with missing data when carrying out this analysis (Gamst et
al., 2008). Sample sizes were calculated to provide sufficient statistical power to detect a
difference if it existed, so excluding these data would have been undesirable in terms of the
power of the project. Furthermore, animals were used to provide the data, and were sacrificed
at the culmination of the project. Therefore, not including the data from animals with missing
values raises ethical concerns. As a result, a linear mixed model (LMM) analysis was chosen,
as it can cope with unequal sample sizes and missing data (Kutner et al., 2005).

All data were tested for the assumption of normality using Kolmogorov-Smirnov and
Shapiro-Wilk goodness of fit tests. In all cases, the null hypothesis that the data came from a
normal distribution was rejected, indicated by a significant result of \( P < 0.05 \). A square root
transformation of the ABR thresholds, and natural log transformations of the mean SR and
GABA\textsubscript{B-R2} densities were made, before the data were re-tested. In all cases, except for the GABA\textsubscript{B-R2} density data, the data showed a more normal distribution but the problem was not resolved (Appendix). However, the sample sizes used to obtain the data for this project were considered large enough that the central limit theorem would protect against the violation of the assumption of normality (Keppel and Wickens, 2004).

LMM is a form of analysis that uses a maximum likelihood estimation procedure to estimate parameters that would result in the maximal probability of the recorded data occurring (Gurka and Edwards, 2011). The data were analysed with between group factors such as exposure, and session, and stimulus intensity as repeated measures. While ANOVAs assume sphericity (i.e. that the data are not correlated), LMM analysis uses covariance matrix structures to model the correlations in the data. This is of significance, as repeated measures data, such as that of the present project, is often correlated, so will violate the sphericity assumption of an ANOVA. The fourteen covariance structures used to model the data in SPSS 20 were: ante-dependence(1); autoregressive(1); autoregressive(1) heterogeneous; autoregressive-moving average(1,1); compound symmetry; compound symmetry correlation metric; compound symmetry heterogeneous; diagonal; factor analytic(1); factor analytic(1) heterogeneous; Huynh-Feldt; Scaled identity; Toeplitz; and Toeplitz heterogeneous. The most appropriate of these covariance matrix structures was chosen based on the goodness-of-fit, as assessed by the Akaike’s Information Criterion (AIC), with the smallest AIC value indicating the optimum covariance structure (Kutner \textit{et al.}, 2005). $P < 0.05$ was considered significant.
Chapter 3: Results
3.1 Auditory Brainstem Evoked Responses

3.1.1 ABRs Immediately Prior to Exposure, and Immediately Following Exposure

ABRs were measured at four different frequencies (32-, 20-, 16-, and 8-kHz respectively) in both ears of all animals immediately before and after the noise exposure or sham treatment. The ABRs were then used to determine the hearing threshold, which was taken as the lowest intensity that produced a visually distinct potential.

Unilateral noise exposure had a significant effect on ABR thresholds, resulting in the elevation of ABR thresholds on the ipsilateral side of the exposed animals at all four frequencies tested (F(1,47.14) = 62.99, P ≤ 0.001; Figures 18, 19a; Table 4). However, there was no apparent elevation of ABR thresholds of the noise-exposed animals on the contralateral side following exposure (Figure 19a). Sham animals showed no elevation in ABR thresholds, on either the ipsilateral or contralateral sides, following the sham treatment (Figure 19b). Additionally, there was a significant exposure x side x time x frequency interaction, showing that the effect of exposure on ABR thresholds varied with respect to these other factors (F(6,328.16) = 3.01, P ≤ 0.007; Figures 19a, 19b; Table 4).
Figure 18: Example of ABR recordings. A comparison of ABR recordings, pre- and post-exposure, that demonstrate the effect of noise-exposure on the ipsilateral side. Recordings are measured in volts, with respect to time in milliseconds. a) Pre-exposure ABR recording in response to a 20 kHz, 50 dB SPL tone. b) Post-exposure ABR recording in response to a 20 kHz, 50 dB SPL tone.
Table 4: Factors that had a significant effect on the ABR thresholds of the animals

<table>
<thead>
<tr>
<th>Factor(s)</th>
<th>Numerator Degrees of Freedom</th>
<th>Denominator Degrees of Freedom</th>
<th>F Value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposure</td>
<td>1</td>
<td>47.14</td>
<td>62.99</td>
<td>P ≤ 0.001</td>
</tr>
<tr>
<td>Exposure x Time</td>
<td>2</td>
<td>143.99</td>
<td>87.91</td>
<td>P ≤ 0.001</td>
</tr>
<tr>
<td>Exposure x Side x Time x Frequency</td>
<td>6</td>
<td>328.16</td>
<td>3.01</td>
<td>P ≤ 0.007</td>
</tr>
</tbody>
</table>
Figure 19a, 19b: ABR hearing thresholds at the beginning of the behavioural study. a) A comparison of ABR hearing thresholds pre- and post-exposure, showing the effect of noise-exposure on ABR thresholds on the ipsilateral side. b) A comparison of ABR hearing thresholds on both the ipsilateral and contralateral sides, pre- and post-sham exposure. Data are represented as means ± SEM.
3.1.2 ABRs Immediately Prior to Animal Sacrifice

ABRs were measured randomly on the ipsilateral side of eight noise-exposed animals, on both the ipsilateral and contralateral sides of a further eight noise-exposed animals, and on both sides of eight sham-exposed animals, immediately prior to animal sacrifice. This was to examine the long-term effects of acoustic trauma on hearing. There was a significant exposure x time interaction which confirmed that exposure had a significant effect on ABR thresholds, and that this varied with respect to the time period (pre-exposure, post-exposure, or before sacrifice) \( \text{F}(2,143.99) = 87.91, \ P \leq 0.001 \); Figures 19a, 19b, 20; Table 4). Elevation of the mean ABR threshold on the ipsilateral side of noise-exposed animals returned to a similar level to that of the contralateral side, and to those of sham-exposed animals, when re-tested prior to sacrifice, indicating that the previous ABR threshold elevation was only temporary in nature.

Figure 20: ABR hearing thresholds at the conclusion of the behavioural study. ABR hearing thresholds on the ipsilateral and contralateral sides of both sham- and noise-exposed animals, immediately prior to animal sacrifice. Data are represented as means ± SEM.
3.2 **Tinnitus Assessment and Drug Treatment**

As was expected in the conditioned suppression task, the SR significantly increased as the stimulus intensity increased, for all of the frequencies tested. Therefore a significant result for intensity will not be discussed further in the results section, but is instead included in the Discussion section. However, where there is a significant interaction between intensity and another factor, it will be included in the results section.

3.2.1 **First Behavioural Testing Period, Following the Acute Administration of L-Baclofen**

The first behavioural testing period spanned 5.5 weeks, and was conducted 2 weeks after the acoustic trauma and initial acute L-baclofen drug treatment. The purpose of this testing period was to assess whether early treatment with L-baclofen could prevent the onset of tinnitus in noise-exposed animals.

When tested under the lick suppression paradigm, exposure had a significant effect on the animals’ lick suppression, causing a downward shift in the SR curve of the exposed groups compared with the sham controls in response to BBN \( (F(1,131.02) = 7.64, P \leq 0.007; \) Figure 21a; Table 5), 20 kHz \( (F(1,25.76) = 6.85, P \leq 0.015; \) Figure 21b; Table 5), and 32 kHz tones \( (F(1,151.01) = 10.65, P \leq 0.001; \) Figure 21c; Table 5), indicating the presence of tinnitus. Additionally, the effect of noise exposure in reducing the SR varied as a function of tone for the BBN \( (F(4,240.98) = 3.40 \leq P \leq 0.010; \) Figure 21a; Table 5) and 20 kHz \( (F(4,578.72) = 3.51, P \leq 0.008; \) Figure 21b; Table 5) tones.

The acute administration of L-baclofen (5mg/kg) resulted in a reduction in lick suppression of the baclofen-treated animals, as indicated by a significant upwards shift in the SR curve when compared with vehicle administration, in response to the BBN \( (F(1,131.02) = \)
8.13, \( P \leq 0.005; \) Figure 21a; Table 5) and 32 kHz tones (\( F(1,151.01) = 3.96, P \leq 0.048; \) Figure 21c; Table 5), but not in response to the 20 kHz tones.

Exposure had a significant effect on the SR, which varied with respect to drug administration and as a function of tone at 20 kHz (\( F(4,578.72) = 2.70, P \leq 0.030; \) Figure 21b; Table 5), but not for BBN or 32 kHz tones. There were no other significant interactions.

**Table 5:** Factors that had a significant effect on the SR of the animals during the first behavioural testing period

<table>
<thead>
<tr>
<th>Factor(s)</th>
<th>Frequency</th>
<th>Numerator Degrees of Freedom</th>
<th>Denominator Degrees of Freedom</th>
<th>F Value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposure</td>
<td>BBN</td>
<td>1</td>
<td>131.02</td>
<td>7.64</td>
<td>( P \leq 0.007 )</td>
</tr>
<tr>
<td>Exposure</td>
<td>20 kHz</td>
<td>1</td>
<td>25.76</td>
<td>6.85</td>
<td>( P \leq 0.015 )</td>
</tr>
<tr>
<td>Exposure</td>
<td>32 kHz</td>
<td>1</td>
<td>151.01</td>
<td>10.65</td>
<td>( P \leq 0.001 )</td>
</tr>
<tr>
<td>Exposure x Tone</td>
<td>BBN</td>
<td>4</td>
<td>240.98</td>
<td>3.40</td>
<td>( P \leq 0.010 )</td>
</tr>
<tr>
<td>Exposure x Tone</td>
<td>20 kHz</td>
<td>4</td>
<td>578.72</td>
<td>3.51</td>
<td>( P \leq 0.008 )</td>
</tr>
<tr>
<td>Drug</td>
<td>BBN</td>
<td>1</td>
<td>131.02</td>
<td>8.13</td>
<td>( P \leq 0.005 )</td>
</tr>
<tr>
<td>Drug</td>
<td>32 kHz</td>
<td>1</td>
<td>151.01</td>
<td>3.96</td>
<td>( P \leq 0.048 )</td>
</tr>
<tr>
<td>Exposure x Drug x Tone</td>
<td>20 kHz</td>
<td>4</td>
<td>578.72</td>
<td>2.70</td>
<td>( P \leq 0.030 )</td>
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</table>
Figure 21a, 21b, 21c: Mean SRs for the first tinnitus testing period, as a function of intensity (dB SPL) and frequency (kHz). The mean SRs are expressed for the noise-exposed and sham control animals, following an acute administration of L-baclofen or vehicle. Data are represented as means ± SEM.
3.2.2 Second Behavioural Testing Period, Following Extended Drug Washout Period

The second behavioural testing period spanned 7.5 weeks, and was conducted 10 weeks after the acoustic trauma and initial acute L-baclofen drug treatment. The purpose of this testing period was to assess whether early treatment with L-baclofen could provide a protective effect against the development and maintenance of tinnitus in noise-exposed animals.

Ten weeks after the noise exposure and acute drug administration, exposure continued to have an effect on the animals’ lick suppression. The SR of exposed animals was significantly lower than sham controls, indicating the presence of tinnitus-like behaviour in these animals. This was significant for the BBN (F(1,25.32) = 6.10, \( P \leq 0.021 \); Figure 22a; Table 6) and 20 kHz tones (F(1,25.75) = 6.22, \( P \leq 0.019 \); Figure 22b; Table 6), but not for the 32 kHz tones. Furthermore, this effect of exposure decreasing the SR varied with respect to tone. The exposure x tone interaction was significant for BBN (F(4,997.69) = 9.70, \( P \leq 0.001 \); Figure 22a; Table 6), 20 kHz (F(4,934.58) = 8.53, \( P \leq 0.001 \); Figure 22b; Table 6), and 32 kHz (F(4,946.68) = 15.77, \( P \leq 0.001 \); Figure 22c; Table 6) tones.

The initial acute administration of L-baclofen resulted in no significant drug effect during this period of the conditioned suppression task. However, there was a significant drug x tone interaction for the BBN tones (F(4,997.69) = 3.16, \( P \leq 0.014 \); Figure 22a; Table 6). No other interactions between factors were significant.
Table 6: Factors that had a significant effect on the SR of the animals during the second behavioural testing period

<table>
<thead>
<tr>
<th>Factor(s)</th>
<th>Frequency</th>
<th>Numerator Degrees of Freedom</th>
<th>Denominator Degrees of Freedom</th>
<th>F Value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposure</td>
<td>BBN</td>
<td>1</td>
<td>25.32</td>
<td>6.10</td>
<td>P ≤ 0.021</td>
</tr>
<tr>
<td>Exposure</td>
<td>20 kHz</td>
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<td>25.75</td>
<td>6.22</td>
<td>P ≤ 0.019</td>
</tr>
<tr>
<td>Exposure x Tone</td>
<td>BBN</td>
<td>4</td>
<td>997.69</td>
<td>9.70</td>
<td>P ≤ 0.001</td>
</tr>
<tr>
<td>Exposure x Tone</td>
<td>20 kHz</td>
<td>4</td>
<td>934.58</td>
<td>8.53</td>
<td>P ≤ 0.001</td>
</tr>
<tr>
<td>Exposure x Tone</td>
<td>32 kHz</td>
<td>4</td>
<td>946.68</td>
<td>15.77</td>
<td>P ≤ 0.001</td>
</tr>
<tr>
<td>Drug x Tone</td>
<td>BBN</td>
<td>4</td>
<td>997.69</td>
<td>3.16</td>
<td>P ≤ 0.014</td>
</tr>
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</table>
Figure 22a, 22b, 22c: Mean SRs for the second tinnitus testing period, as a function of intensity (dB SPL) and frequency (kHz). The mean SRs are expressed for the noise-exposed and sham control animals, following a washout period and no additional drug administration. Data are represented as means ± SEM.
3.2.3 Third Behavioural Testing Period, During Chronic Administration of L-Baclofen

The third behavioural testing period spanned 4.5 weeks, and was conducted 17.5 weeks after the acoustic trauma and initial acute L-baclofen drug treatment. During this treatment period, animals were administered vehicle or L-baclofen (3 mg/kg/day) to assess the effect of L-baclofen treatment on chronic tinnitus.

During this third period of the conditioned suppression task, which followed on immediately from the previous testing period, noise exposure had a significant effect on the animals’ lick suppression at 32 kHz, which resulted in a decrease of the SR in the noise-exposed groups compared with the sham groups for tones of this frequency \( F(1,191.03) = 25.54, P \leq 0.001 \); Figure 23c; Table 7). However, the effect of noise exposure on lick suppression was no longer significant for the BBN or 20 kHz tones. Additionally, the effect of exposure in decreasing the SR varied as a function of tone for the BBN \( F(4,216.72) = 2.88, P \leq 0.024 \); Figure 23a; Table 7) and 32 kHz tones \( F(4,225.22) = 4.34, P \leq 0.002 \); Figure 23c; Table 7) tones.

The administration of 3 mg/kg of L-baclofen throughout this testing period caused a significant drug effect, which resulted in an increase in the SR compared with the vehicle groups. This reduction in lick suppression of the baclofen-treated animals was evident for the BBN \( F(1,172.56) = 28.67, P \leq 0.001 \); Figure 23a; Table 7), 20 kHz \( F(1,20.36) = 10.12, P \leq 0.005 \); Figure 23b; Table 7), and 32 kHz \( F(1,191.03) = 102.77, P \leq 0.001 \); Figure 23c; Table 7) tones. Furthermore, the significant effect of drug treatment varied with respect to tone for the BBN \( F(4,216.73) = 9.00, P \leq 0.001 \); Figure 23a; Table 7), 20 kHz \( F(4,754.13) = 5.00, P \leq 0.001 \); Figure 23b; Table 7), and 32 kHz \( F(4,225.22) = 6.44, P \leq 0.001 \); Figure 23c; Table 7) tones. The effect of this drug x tone interaction on the SR additionally varied with respect to exposure for the BBN \( F(4,216.73) = 3.38, P \leq 0.010 \); Figure 23a; Table 7) and 32 kHz \( F(4,225.22) = 3.08, P \leq 0.017 \); Figure 23c; Table 7) tones.
Table 7: Factors that had a significant effect on the SR of the animals during the third behavioural testing period

<table>
<thead>
<tr>
<th>Factor(s)</th>
<th>Frequency</th>
<th>Numerator Degrees of Freedom</th>
<th>Denominator Degrees of Freedom</th>
<th>F Value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposure 32 kHz</td>
<td>1</td>
<td>191.03</td>
<td>25.54</td>
<td>P ≤ 0.001</td>
<td></td>
</tr>
<tr>
<td>Exposure x Tone BBN</td>
<td>4</td>
<td>216.73</td>
<td>2.88</td>
<td>P ≤ 0.024</td>
<td></td>
</tr>
<tr>
<td>Exposure x Tone 32 kHz</td>
<td>4</td>
<td>225.22</td>
<td>4.34</td>
<td>P ≤ 0.002</td>
<td></td>
</tr>
<tr>
<td>Drug BBN</td>
<td>1</td>
<td>172.56</td>
<td>28.67</td>
<td>P ≤ 0.001</td>
<td></td>
</tr>
<tr>
<td>Drug 20 kHz</td>
<td>1</td>
<td>20.36</td>
<td>10.12</td>
<td>P ≤ 0.005</td>
<td></td>
</tr>
<tr>
<td>Drug 32 kHz</td>
<td>1</td>
<td>191.03</td>
<td>102.77</td>
<td>P ≤ 0.001</td>
<td></td>
</tr>
<tr>
<td>Drug x Tone BBN</td>
<td>4</td>
<td>216.73</td>
<td>8.99</td>
<td>P ≤ 0.001</td>
<td></td>
</tr>
<tr>
<td>Drug x Tone 20 kHz</td>
<td>4</td>
<td>754.13</td>
<td>5.00</td>
<td>P ≤ 0.001</td>
<td></td>
</tr>
<tr>
<td>Drug x Tone 32 kHz</td>
<td>4</td>
<td>225.22</td>
<td>6.44</td>
<td>P ≤ 0.001</td>
<td></td>
</tr>
<tr>
<td>Exposure x Drug x Tone BBN</td>
<td>4</td>
<td>216.73</td>
<td>3.38</td>
<td>P ≤ 0.010</td>
<td></td>
</tr>
<tr>
<td>Exposure x Drug x Tone 32 kHz</td>
<td>4</td>
<td>225.22</td>
<td>3.08</td>
<td>P ≤ 0.017</td>
<td></td>
</tr>
</tbody>
</table>
Figure 23a, 23b, 23c: Mean SRs for the third tinnitus testing period, as a function of intensity (dB SPL) and frequency (kHz). The mean SRs are expressed for the noise-exposed and sham control animals, during chronic administration of L-baclofen or vehicle. Data are represented as means ± SEM.
3.3 **Histology**

CV staining was completed on all sections to identify the CN, and thereby determine the appropriate sections on which to carry out immunohistochemical staining. The purple staining of the Nissl bodies in the neurons and cell nuclei resulted in the CN being readily identifiable by a low magnification (Figure 24).

![Figure 24: CV staining of a section under 2x magnification.](image)

CV staining allowed the CN (indicated by the black arrow) to be easily identified at a low magnification. Scale bar: 100 µm.
3.4 Immunohistochemistry

Immunohistochemistry was carried out to allow for the study of changes in GABA\textsubscript{B-R2} expression in neurons of the CN following induction of tinnitus and either L-baclofen or vehicle treatment. The DAB staining, which was part of the immunohistochemical protocol, resulted in GABA\textsubscript{B-R2} positive cells being stained brown in colour (Figure 25). The immunohistochemistry results demonstrated that the GABA\textsubscript{B-R2} subunit is expressed throughout the CN, as demonstrated in current literature (Jamal et al., 2011). Furthermore, this staining was found to be on the cell membrane of cells, which is consistent with Jamal et al. (2011), who demonstrated punctuate labelling on the somata of cells in the fusiform layer of the CN (Figure 25).
Figure 25: Confirmation of GABA$_{\text{B-R2}}$ positive cells in the CN by immunohistochemistry. Brown staining of GABA$_{\text{B-R2}}$ positive cells in the CN, at 10x magnification. The top inset shows cells in the DCN at 100x magnification, while the bottom inset shows cells in the VCN at 100x magnification. Scale bars: 100 µm (CN), 10 µm (insets).

Negative control sections were also initially used, whereby the same immunohistochemical protocol was performed on sections, but without the addition of the 1$^\text{st}$ Ab. The immunohistochemical result of these negative control sections was no brown staining.
of cells, giving confirmation of the immunopositive staining being specific for the GABA<sub>B-R2</sub> subunit (Figure 26).

Figure 26: Confirmation of GABA<sub>B-R2</sub> staining specificity using negative control sections. The CN of a negative control section, under 10x magnification, showing no brown cell staining. Scale bar: 100 µm.
3.5 Quantitative Analysis of GABA\(_B\) Receptor

The mean density of the GABA\(_B\)-R2 positive staining in the CN of 12 noise-exposed animals was analysed, to assess the effect of L-baclofen treatment on GABA\(_B\) receptor expression. Neither drug treatment nor side of exposure had any significant effect on the mean GABA\(_B\)-R2 density (Figure 27). Furthermore, there were no significant interactions between factors.

![Graph showing mean density of GABA\(_B\)-R2 staining in the CN of L-baclofen treated animals compared to vehicle treated animals, following a unilateral noise trauma. Data are represented as means ± SEM.](image)

Figure 27: Mean density of GABA\(_B\)-R2 staining in the CN of L-baclofen treated animals compared to vehicle treated animals, following a unilateral noise trauma. Data are represented as means ± SEM.
Chapter 4: Discussion
4.1 Interpretation of the Results, and Comparison to Previous Literature

Following a unilateral noise exposure of a 16 kHz pure tone at 115 dB SPL delivered for 1 hour, ABR thresholds were significantly elevated on the ipsilateral side of the exposed animals at all four frequencies tested (8-, 16-, 20-, 32-kHz) (Figures 18, 19a; Table 4). Currently, the peripheral and central mechanisms of this temporary threshold shift are poorly understood, although research is suggestive that peripheral damage caused by noise trauma results in differential effects on central neural activity (Kaltenbach and McCaslin, 1996; Norena and Eggermont, 2003; Qui et al., 2000). Peripherally, Liberman and Dodds (1987) reported cellular swelling and vacuolisation in the OHC and IHC area to be an acute cochlear reaction to noise exposure. This swelling was not present in cochleae which have been fixed several weeks post-noise exposure, bringing the authors to the conclusion that such structural changes are characteristic of acute changes in ABR thresholds, and not permanent threshold shifts. Furthermore, Lataye and Campo (1997) provided evidence of noise exposure-induced damage to stereocilia at the level of the first row of OHCs and the IHCs in rats. A potentially reversible damage to auditory nerve fibre neurites in contact with IHCs has also been reported (Puel, 1995). Centrally, there is evidence to suggest that noise exposure results in a reorganisation of the frequency map in the central neural pathways of the auditory system (Suga et al., 2000). Specifically, Zhang and Kaltenbach (1998) found that tone exposure resulted in effects on both the auditory sensitivity and frequency tuning properties in rats exposed to a 10 kHz tone at 125-130 dB SPL for 4 hours. The elevated response thresholds were reported as being at most tonotopic positions, and were associated with an increase in spontaneous activity in the DCN. Furthermore, Norena and Eggermont (2003) reported increases in the burst-firing activity of neurons in the primary AC in cats which had been
exposed to a 5 or 6 kHz tone at 115-120 dB SPL for 1 hour, which is a similar intensity and
duration to the noise exposure used in the present study.

Increases in the ABR thresholds were greatest at higher frequencies, although
interestingly this was both at, and above, the noise trauma frequency of 16 kHz. In temporary
threshold shifts, there is a widely-cited phenomenon commonly known as the half-octave shift
(McFadden, 1986). Acoustic trauma, when delivered at a constant SPL, has been shown to
produce a maximal desensitisation in neurons with a characteristic frequency that is half an
octave above the exposure frequency, as opposed to the exposure frequency itself. It has been
proposed that this is the direct result of nonlinearities in the basilar membrane (Cody and
Johnstone, 1981). Johnstone et al. (1986) illustrated this by obtaining travelling basilar
membrane vibration wave envelopes, which were a function of distance, at various sound
pressures. With loud sounds there was a basal-ward shift of the maximum vibration by
approximately half an octave.

As expected, there was no significant change in ABR thresholds on the contralateral
side of exposed animals, and neither the ipsilateral nor contralateral sides of the sham
animals, indicating no damage to auditory function in the absence of noise exposure (Figure
19).

There was no significant elevation of the ABR thresholds on the ipsilateral side of the
exposed animals immediately before sacrifice, 22 weeks after the noise trauma (Figure 20).
This result is similar to previous studies which have shown elevated ABR hearing thresholds
of a temporary nature following acoustic trauma in rats (Attias et al., 1990; Zheng et al.,
2012a). The result is indicative of the animals’ long-term hearing levels being unaffected,
with only tinnitus persisting for the duration of the study.

When tested under the lick suppression paradigm two weeks after the acoustic trauma
and acute L-baclofen administration (5 mg/kg), noise exposure had a significant effect on the
animals’ lick suppression at all frequencies (BBN, 20 kHz, and 32 kHz), which was evident
by the downward shift in the SR curve of the exposed groups compared with the sham controls (Figure 21; Table 5). This downward shift of the SR curve of the noise-exposed groups is indicative of tinnitus-like behaviour in these animals. There is currently a large body of evidence to suggest that acoustic trauma-induced tinnitus is linked to a hyperactivity of the central auditory system that is characterised by increased spontaneous activity of neurons (Kaltenbach, 2007). This theory has been strengthened by the wide array of functional measures which have been used to successfully demonstrate hyperactivity in animal models of tinnitus, including c-Fos immunocytochemistry, single- and multi-unit recordings, and metabolic mapping (Eggermont and Kenmochi, 1998; Jastreboff and Sasaki, 1986; Wallhausser-Franke et al., 2003). Furthermore, this has been demonstrated to correlate to human tinnitus patients, with imaging studies of the brains of patients revealing hyperactivation of auditory areas (Andersson et al., 2000; Arnold et al., 1996; Melcher et al., 2000).

The mechanism by which central hyperactivity causes tinnitus in the noise-exposed animals is likely to involve disruption to the inhibition/excitation balance within the central auditory pathways (Kaltenbach, 2007). There is evidence to suggest that the decreased inhibition contributing to hyperactivity is the result of a reduction in glycine release and glycine receptors, and GABAergic neurotransmission (Asako et al., 2005; Bauer et al., 2000; Potashner et al., 2000). Meanwhile increased excitation is likely to be a consequence of increases in glutamate and aspartate release, glutamatergic receptors, acetylcholine receptors, and cholinergic synapses (Jin et al., 2006; Kaltenbach, 2007; Kaltenbach and Zhang, 2007; Muly et al., 2004; Suneja et al., 2000). Collectively, these plastic alterations within the central auditory system are thought to contribute to the development and maintenance of tinnitus (Moller, 2006).

Despite a significant exposure effect for BBN, which indicates the presence of tinnitus-like behaviour in the vehicle and L-baclofen noise-exposed groups, the separation
between the curves of each treatment group was much smaller than those for the 20 kHz and 32 kHz frequencies (Figure 21). This is what we would expect, as the lick suppression in this behavioural paradigm is based upon those animals with tinnitus avoiding the foot shock during the discrimination phase, by suppressing their licking during both the speaker off period, and during the stimulus presentation period of stimuli with sensory features that resemble their tinnitus (see Materials and Methods 2.5.6). Twenty kHz and 32 kHz frequencies have a greater resemblance to the sensory features of tinnitus, and as such the separation between the curves of the exposed and sham treatment groups at these frequencies is greater than that of the BBN. This is indicative of the presence of tinnitus in the exposed animals within the range of 20-32 kHz. While tinnitus in this frequency range is consistent with the current literature using acoustic trauma models in rats, it differs from a number of previous studies which have reported the tinnitus as occurring at a single frequency. Using the same animal model of tinnitus in rats, Zheng et al. (2012b) reported a downward shift of the SR curve of noise-exposed treatment groups as being frequency-specific for 20 kHz. Similarly, Zheng et al. (2012a) reported a frequency-specific downward shift of the SR curve for 32 kHz, which did not occur in response to other stimuli. However, when the perceptual components of tinnitus are characterised in human patients, the tinnitus spectra are often found to occupy a wide frequency range rather than a single frequency, much like the finding in the present study (Norena et al., 2002).

As was expected in the conditioned suppression task, the SR significantly increased as the stimulus intensity increased, for all of the frequencies tested (Figures 21, 22, 23). The animals were conditioned to suppress their licking during speaker off (silence) periods. Higher intensities provided all treatment groups with a greater ability to distinguish the tone stimuli from the periods of speaker off, thereby resulting in decreased lick suppression during these intensities.
The acute administration of L-baclofen (5 mg/kg) immediately following acoustic trauma resulted in a significant drug effect for the BBN and 32 kHz tones during the first tinnitus testing period, which was two weeks after the noise exposure and the L-baclofen treatment. This manifested as a reduction in lick suppression of the L-baclofen-treated animals at these frequencies, and a consequent elevation of the exposed-baclofen SR curve towards the sham treatment groups (Figure 21; Table 5). As baclofen has been shown to have a rapid elimination in human subjects, with a plasma elimination half-life of 3.5 hours, we would expect that the two weeks between the acute drug administration and behavioural testing of the animals was an adequate drug washout period (Faigle et al., 1980). Therefore, this result gives evidence that the acute administration of L-baclofen (5 mg/kg) within 30 minutes of acoustic trauma had a lasting effect on the animals’ perception of tinnitus, as opposed to a direct drug effect on the lick suppression itself. However, the significance of exposure as a factor means this was likely not to have been due to the prevention of tinnitus within the exposed-baclofen treatment group.

As mentioned previously, the current literature is indicative of hyperactivity within the central auditory system as a pathological mechanism of acoustic trauma-induced tinnitus (Eggermont and Roberts, 2004; Salvi et al., 2000). Therefore, it follows that alleviation of this hyperactivity could have a therapeutic effect on the tinnitus. L-baclofen is a GABA_B agonist, and it is proposed that its effects are mediated by an increase in GABAergic inhibition of the ascending central auditory pathways, which occurs via its activation of GABA_B receptors (Bowery et al., 1979). This would thereby decrease the hyperactivity within these pathways, and the associated perception of tinnitus. The fact that there was a partial elevation of the exposed-baclofen SR curve, which was not significant, suggests that either L-baclofen is not effective in the manner used (as an acutely administered preventative agent), or that alternate non-GABAergic pathways are contributing to the hyperactivity within the central auditory system. For example, a net loss of glycinergic inhibition could be an alternate, or
accompanying, plastic change in central auditory structures that results from noise trauma. Wang et al. (2009) demonstrated functional changes in glycine receptors in the DCN of noise-exposed rats, thereby establishing a connection between psychophysical evidence of tinnitus in rats due to acoustic trauma, and glycinergetic neurotransmission. However, the relative weighting of GABAergic pathways versus glycinergetic pathways in contributing to central auditory system hyperactivity remains to be seen, and further research on this is required.

Ten weeks following the acoustic trauma (and acute drug administration), exposure continued to have a significant effect on the animals’ lick suppression with the noise-exposed treatment groups showing greater lick suppression (Figure 22; Table 6), indicating the presence of tinnitus-like behaviour in the acoustic trauma animals. The lack of a significant drug effect during this testing period suggests that any effect on the animals’ lick suppression which was caused by the initial acute administration of L-baclofen, had diminished at this point, and was not sufficient at maintaining the mildly elevated SR curve of the exposed-baclofen group over a 10 week timeframe.

Seventeen and a half weeks following the acoustic trauma, chronic L-baclofen administration (3 mg/kg/day) resulted in noise exposure having a significant effect on the animals’ lick suppression at 32 kHz, but not for BBN or 20 kHz tones, indicating alleviation of tinnitus at this frequency only (Figure 23; Table 7). This result is consistent with the, albeit limited, current literature which has also provided evidence of L-baclofen treatment decreasing the psychophysical evidence of tinnitus in rats. Zheng et al. (2012b) reported no significant differences between the SRs of sham and noise-exposed treatment groups in rats for doses of 3 and 5 mg/kg/day of L-baclofen, when administered daily during testing with the lick suppression model used in the current study.

While the increase in SR of the exposed-baclofen treatment group for BBN and 20 kHz tones indicates the alleviation of the tinnitus-like behaviour, the significant drug effect across all frequencies must be taken into consideration. Theoretically, if the chronic L-
baclofen dosing regimen had resulted in a general drug effect, the sham-vehicle treatment group cannot be used as a control against which to compare the exposed-baclofen group. Therefore, if the L-baclofen is specifically reducing the psychophysical evidence of tinnitus in the exposed-baclofen animals, the SR curve of this group should be similar to that of the sham-baclofen group. At both 20 kHz and 32 kHz there is still separation between the sham-baclofen and exposed-baclofen curves (Figure 23), despite the exposed-baclofen curve shifting above the vehicle treatment curves. This is suggestive that the L-baclofen was in fact having a general drug effect that was inhibiting the animals’ ability to suppress their licking, and is not necessarily an indicator of the alleviation of tinnitus in the exposed-baclofen group.

The general drug effect on the animals’ lick suppression is a new finding in this study, and is one of the main points of difference between earlier research on L-baclofen as a tinnitus treatment. Zheng et al. (2012b) used a repeated measures design which split 16 male Wistar rats into two groups: i) noise-exposed; and ii) sham. As a result, every animal was tested at some point with either vehicle or L-baclofen (1 mg/kg, 3 mg/kg, 5 mg/kg). Consequently, there was no treatment group that had only been administered with vehicle that could act as a comparison to the L-baclofen treatment groups, so it is possible that the same general drug effect which occurred in the present study also occurred in the Zheng et al. (2012b) study, but it was not recognised due to the difference in study design. Therefore, the present results reinforce the necessity of including a control-drug group in pharmacological studies. In addition, the L-baclofen treatment started from 1 mg/kg and gradually increased to 3, and then 5, mg/kg over a few months. Therefore, it is possible that the animals in the previous study may have developed tolerance.

Following sacrifice of the animals, ImageJ analysis of the immunohistochemical staining performed on sections from 12 of the noise-exposed animals showed no significant difference in the mean density of GABA_B^R2 positive staining in the CN (Figure 27). It was expected that the chronic administration of L-baclofen, a GABA_B agonist, would cause a
down-regulation of GABA<sub>B</sub> receptor expression, thereby decreasing the intensity of staining. This is because repeated stimulation of GPCRs has been shown to lead to a decline in response, an important mechanism to avoid overstimulation of their consequent signalling (Ferguson, 2001; Grady <i>et al.</i>, 1997; Kelly <i>et al.</i>, 2008). Despite this, it is likely that some form of desensitisation to L-baclofen occurred during the chronic dosing period, but that this did not manifest as a reduction in GABA<sub>B</sub> receptor protein expression. For example, Lehmann <i>et al.</i> (2003) have demonstrated a complete tolerance to baclofen-induced hypothermia in rats injected daily with 20 µmol/kg baclofen (s.c.) for 14 days, with no corresponding significant changes in receptor binding sites, affinity for GABA, receptor mRNA, or protein levels.

The null result with respect to changes in mean GABA<sub>B</sub>-R<sub>2</sub> density of this project is interesting, and could be an indication that L-baclofen may cause a downregulation of GABA<sub>B</sub> receptors that varies mainly between brain regions, rather than within regions. If this was the case, differences in the mean GABA<sub>B</sub>-R<sub>2</sub> density between L-baclofen and vehicle treated animals was unlikely to come to light in the present study, as only one region of brain tissue, the CN, was analysed. Furthermore, the lack of sham control group as a comparison to the noise-exposed groups means that the results are only suggestive of no significant difference in the mean GABA<sub>B</sub>-R<sub>2</sub> density due to L-baclofen treatment alone, but no conclusion can be drawn with regards to potential changes in the mean GABA<sub>B</sub>-R<sub>2</sub> receptor density of the CN as a pathological result of tinnitus development.
4.2 Significance of the Study and Clinical Implications

Tinnitus is a debilitating condition which is thought to chronically affect 5-10% of the general population, and approximately 10% of the New Zealand population (Davis and Rafaie, 2000; Heller, 2003). While there are many known risk factors for tinnitus, noise exposure is thought to be the most common cause (Bauer and Brozoski, 2001; Cooper, 1994). This adds weight to the need for an effective treatment regimen, as the prevalence of tinnitus is projected to increase in the future due to increases in the availability, affordability, and use of portable listening devices. As a result, the general population is exposed to sounds of higher amplitude, for longer periods of time, and from a younger age (Biassoni et al., 2005; Langguth et al., 2009; Serra et al., 2005). Highlighted by this is the reliability of the acoustic trauma model in producing tinnitus via a mechanism which is most likely to be characteristic of the majority of the patient population.

Currently there is a need for preclinical studies to provide research-based evidence of pharmacological approaches which might be useful in a clinical setting for the treatment of tinnitus. The significance of the present study lies not in testing a novel drug for the treatment of tinnitus, but instead testing a drug which is already clinically available, and for which there is some evidence of its efficacy as a tinnitus treatment (Zheng et al., 2012b). Consequently, favourable results from the present study could be further tested and validated relatively quickly, as baclofen is already licensed and available for prescription. While L-baclofen has not been demonstrated to provide a significant protective effect when administered 30 minutes proceeding noise trauma, it does have a general drug effect on the lick suppression of animals, which requires further research.

Currently, despite a large body of evidence that suggests neuronal hyperactivity within the central auditory system as a contributor to the development and maintenance of tinnitus,
the molecular mechanisms underlying this remain poorly understood. The results of the behavioural component of the present study suggest that alterations in the GABAergic inhibitory system and, in particular, GABA_B receptors, may be important factors which play a role in the development and maintenance of noise-induced tinnitus. The success of the current animal model means that the efficacy of other GABA_B receptor agonists can be reliably tested to gain further understanding of the mechanisms underlying noise-induced tinnitus, while also providing a standard model for the efficacy of potential therapeutic drug therapies to be tested in a comparable manner.

Despite limitations in the immunohistochemical component of the study with regards to the treatment groups analysed, this research adds additional information to the current literature concerned with elucidating any potential GABAergic mechanisms underlying both the development of tinnitus, and the therapeutic effect of GABA agonists in its treatment. Despite no direct clinical significance, research which contributes to identifying underlying cellular mechanisms, and potential drug targets, is vital for the progression of tinnitus treatments.
4.3 Critical Evaluation of the Experimental Design

Variability between the four treatment groups was decreased in a number of ways. Firstly, all 32 animals were male Wistar rats from the same breeding resource unit in Dunedin, and were assigned randomly to the four treatment groups. These animals were caged in the same room, and consequently were exposed to the same light cycle and noise levels outside of testing. Furthermore, all animals had the same access, or restriction, to food and water throughout the duration of the study. The only time this differed was when the weight of an animal fell below 90% of its normal body weight during tinnitus testing. Animals with a body weight between 85% and 90% of normal body weight were given extra water access for 15 minutes, and those that fell below 85% of their normal body weight were given extra water for 30 minutes outside of testing. However, additional water access was not provided within three hours of an animal’s testing. This minimised the risk of the animals associating a lack of drinking during testing with water access after testing. Variability in techniques used for the handling, tinnitus testing, and drug administration of animals was minimised by being carried out by a single person for the duration of the study.

Hearing levels were tested using ABR thresholds, which were measured in both ears of all animals before and immediately after noise trauma or sham treatment, as well as in randomly selected animals at the conclusion of the study. The thresholds themselves were determined by the minimum stimulus intensity that produced an observable ABR response. While this may seem straightforward, the ABR thresholds are subjective in nature and when done manually it cannot be excluded that background noise was mistaken for an observable ABR waveform. The possibility that this had an impact on the overall significance of the results between treatment groups was decreased by having the ABR analysis carried out by a
single person. This resulted in a consistent approach to analysis, with any error in judgement being carried across all four experimental groups.

However, it should be noted that a true ABR threshold that generalises across testing situations, or between laboratories, does not exist (Willott, 2006). Therefore all ABR results of this study must be taken as an indication of an animal’s hearing ability, and possible damage to that hearing, but should not be considered in terms of a hearing level at a specific decibel value. Furthermore, tone stimuli were presented in 5-10 dB steps, so the final threshold obtained was an indication of the level, to the nearest 5-10 dB, below which an animal produced no observable response, but again cannot be considered as an absolute hearing level in decibels. Due to the aforementioned inability to generalise ABR thresholds across testing situations, and the low likelihood that lower intensity steps could reliably be discerned from background noise, reducing the intensity of the steps between the different tone stimuli presented was not deemed to be necessary for this particular study.

ABR thresholds were tested and analysed without blinding of the experimenter to treatment groups, which could have led to unintentional bias in interpretation of the ABR waveforms. Ideally, the treatment group of each animal should not be known by the experimenter; however this was impractical for the current study.

Furthermore, a number of subject- and recording-related factors have been shown to impact ABR measurements. Movement of the subject can produce muscle and movement artifacts that can impact on the ability to correctly interpret the ABR waveforms produced (Markand, 1994). To minimise the effect of this, all animals were anaesthetised during ABR threshold testing to allow for consistent waveforms to be produced. The anaesthesia itself is unlikely to have had an effect on the ABR waveforms, as ABRs are resistant to most sedatives and commonly used anaesthetics (Stockard et al., 1977). Central body temperature of subjects must also be considered. This is because the latency of ABR components, including the inter-peak latency, has been shown to increase with decreasing body
temperature (Markand et al., 1987; Stockard et al., 1978). As rats can become hypothermic when anaesthetised, animals were placed on a hot water heating pad to keep them warm during testing, and to prevent body temperature from artificially prolonging the ABR waveforms (Wixson et al., 1987). However, the temperature of the heating pad itself could not be adjusted, and it was assumed that the heating pad kept the animal’s core body temperature at a suitable level. A more robust method of ensuring that body temperature did not affect the ABR recordings would have been to use a direct current heating pad, in conjunction with a rectal thermal probe to monitor and adjust body temperature during recording.

Before ABR testing, the equipment was calibrated to ensure that the desired stimulus intensities were being achieved. In addition to this, the equipment was re-calibrated on a weekly basis. Changes in the system output from the calibrated values each week were minimal, so it can be assumed that the stimulus intensities produced were fairly consistent throughout the ABR testing periods.

While ABR threshold testing has been used in the current literature to assess hearing thresholds following acoustic trauma, not all studies have re-tested ABR thresholds at the close of tinnitus testing. By doing so in the current study, it was confirmed that elevated hearing thresholds following noise exposure were temporary in nature, and, aside from the tinnitus, the animals’ hearing levels were unaffected. This is of importance as the lick suppression model used relies on the animals having normal hearing so they can distinguish the stimulus presentations, and deafness in the noise-exposed animals would create a source of bias.

Noise exposure in the exposed animals was unilateral. The contralateral ear was blocked with an earplug during the acoustic trauma, and was then assessed via ABR threshold testing. This not only confirmed the success of the acoustic trauma in causing damage to the ipsilateral ear, but also ensured no damage to the contralateral ear, enabling the contralateral
ears of the exposed animals to act as a control for that animal during the lick suppression
tasks. Furthermore, the noise and sham exposures were delivered unilaterally in both left and
right ears of the animals, chosen at random, to reduce lateralisation bias.

Tinnitus testing began two weeks after the acoustic trauma, or sham exposure, in
animals. This was assumed to be an appropriate time course for tinnitus to develop in the
noise-exposed animals based on current literature (Zheng et al., 2012b). The assumption that
two weeks was sufficient for the onset of tinnitus using the current study’s tinnitus-induction
method was then confirmed by the statistical analysis, which showed an exposure effect on
the animals’ lick suppression during the first behavioural testing period. However, Ruttiger et
al. (2013) recently demonstrated that onset of tinnitus in animals exposed to 120 dB SPL at
10 kHz for 1 – 1.5 hours may occur in as few as 30% of those exposed. The four treatment
groups were not altered for the duration of the study, due to the significant exposure effect
which was found in the first period of behavioural testing. While in cases of testing potential
drug treatments, such as this one, it would be desirable to analyse noise-exposed treatment
groups which contain only those animals that have developed tinnitus, it raises ethical concern
to use animals for scientific study, knowing you will be excluding data from a high proportion
of them.

Baclofen is a racemic drug, comprising equal amounts of L- and D-isomers (Novartis,
1977). Research has not only demonstrated L-baclofen to be the more active isomer, but some
studies additionally indicate that D-baclofen may antagonise the effect of L-baclofen
(Sawynok and Dickson, 1984; Sawynok and Dickson, 1985; Smith et al., 2012; Szczepaniak
et al., 1995; Szczepaniak et al., 1996). As a result, the use of a racemic baclofen mixture of
both L- and D-isomers in the Westerberg et al. (1996) clinical trial may have masked the true
potential of L-baclofen, alone, as a tinnitus treatment. Therefore, in the current study the use
of L-baclofen, as opposed to a racemic formulation, was decided upon. This is in line with
research by Zheng et al. (2012b), which demonstrated a reduction in psychophysical evidence
of tinnitus in rats which had been administered L-baclofen at doses of 3 mg/kg/day and 5 mg/kg/day. Furthermore, current research suggests there is a stereoselective transport mechanism for baclofen at the BBB, and a greater cumulative amount of the active isomer is transported into the CNS as a percentage of the intravenously administered dose (Van Bree et al., 1991). However, it should be noted that baclofen prescribed in a clinical setting is generally a racemic formulation which is administered orally. Therefore the results of this study may not translate to the current patient setting, as oral administration of the racemic formulation will affect the bioavailability and clearance of the drug (Van Bree et al., 1991).

Both the drug and vehicle injections were administered subcutaneously to the animals. As a result, the action of the L-baclofen was not localised to the auditory system. Previous functional and autoradiography studies have demonstrated the presence of GABA\(_B\) receptors not only in the CNS, but also at peripheral sites in rats, where they have varying effects (Bowery, 1993; Table 8). The problem with L-baclofen, a GABA\(_B\) receptor agonist, being administered without localisation to the central auditory system in this study is two-fold. Firstly, there is currently a lack of understanding surrounding the mechanism of tinnitus development and maintenance, and the mechanism of action of L-baclofen in treating tinnitus. The present study can be used to formulate hypotheses on these mechanisms, however, no concrete conclusions can be drawn, as the action of the L-baclofen administered cannot be localised to GABA\(_B\) receptors in central auditory system structures, and instead may involve unintended therapeutic effects via the response of receptor activation at sites outside the auditory centres of the brain. Following on from this, the fact that the L-baclofen was not localised to a particular region of interest increases the potential for adverse drug effects to have an impact on the animals, and consequently their performance in the lick suppression behavioural paradigm.
Table 8: Location of GABA\textsubscript{B} sites in the rat periphery, and the response initiated by receptor activation.

<table>
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<tr>
<th>GABA\textsubscript{B} Receptor Location</th>
<th>Effect of Receptor Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atria</td>
<td>Decrease in transmitter release</td>
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<tr>
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<td>Relaxation</td>
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<tr>
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<td>Urinary bladder</td>
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<td>Vas deferens</td>
<td>Decrease in transmitter release</td>
</tr>
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<td></td>
<td>Twitch response</td>
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</table>

Table adapted from Bowery (1993).

Baclofen is associated with a number of adverse effects. In humans, these have been reported to include confusion, dizziness, drowsiness, gastrointestinal upset, and hypotension (Pinto et al., 1972; Westerberg et al., 1996). An L-baclofen dose of 5 mg/kg was initially chosen for the study because it had been shown by Zheng et al. (2012b) to be effective at reducing the psychophysical evidence of tinnitus in rats, with no apparent sign of adverse effects. Additionally, it represents a dose within the range used in humans, giving it clinical relevance. However, it soon became apparent that this dose was too high for the present study, and adjustments had to be made. Initially, the 5 mg/kg dose was administered 30 minutes after noise-exposure (while the animals were still under general anaesthesia), and then again every 24 hours for the following five days. There was no tinnitus testing during this time, and as such the animals’ behaviour and drinking activity was not monitored. As a result, the sedation caused by this dosage was not fully realised until the chronic dosing stage of the study. At this point, when the first three animals were administered with 5 mg/kg they were unable to perform in the lick suppression paradigm, as the sedation reduced their licking activity from the expected 2000-6000 licks per session, to less than 100 licks. Consequently, the dose was adjusted to 3 mg/kg, which produced no observable sedative effects, and
resulted in the level of licks that were obtained during testing in the absence of drug administration.

The sedative effect of a 5 mg/kg dose in this study, but not in previous research by Zheng et al. (2012b), raises the issue of tolerance. The animals in the L-baclofen treatment groups of the current study had only had six L-baclofen injections, administered 17.5 weeks previously, before the chronic dosing segment of the study began. However, the study by Zheng et al. (2012b) employed a design which exposed the animals to a graduated dosing regimen. Each rat was administered L-baclofen at a dose of 1 mg/kg/day for 18 days, followed by 3 mg/kg/day for 18 days, before finally being administered 5 mg/kg/day for 18 days, after an 18 day washout period. It is well documented that repeated stimulation of GPCRs can result in an observable decline in response of the receptors to activation, although relatively few studies with research directed towards identifying mechanisms for GABA<sub>B</sub> receptor-related tolerance have been undertaken (Ferguson, 2001; Grady et al., 1997; Lehmann et al., 2003). Currently, studies have yielded somewhat conflicting results, and it has become apparent that some responses to GABA<sub>B</sub> activation are more prone to tolerance development than others. This is true for hypothermic and analgesic responses, both of which diminish after repeated baclofen dosing, whereas cAMP production does not (Enna et al., 1998; Ghelardini et al., 1998; Gray et al., 1987; Pratt and Bowery, 1993). Lehmann et al. (2003) have demonstrated that despite evidence of complete baclofen-induced tolerance to hypothermia within one week of baclofen administration (4.27 mg/kg/day), there were no accompanying significant changes at the level of GABA<sub>B</sub> receptor binding sites, affinity for GABA, receptor mRNA, or protein levels. This is somewhat similar to the lack of change in GABA<sub>B-R2</sub> density in the CN of L-baclofen treated animals, which occurred in the present study. While the mechanism of tolerance is still not fully understood, it remains an important aspect of baclofen administration in a clinical setting. As mentioned previously, the effects of GABA<sub>B</sub> receptor activation vary in their susceptibility to tolerance. Tolerance could produce
favourable outcomes if the tolerance is in response to adverse side effects of the drug, or unfavourable outcomes if the tolerance occurs in relation to the therapeutic effect of the drug, and would require frequent dose titrations.

No other adverse side effects, besides the sedative effect at the highest dose of 5 mg/kg, were observed. This included no obvious unintended effects on the animals’ behaviour during testing, as well as no apparent stress-related response mechanisms such as excessive porphyrin secretion. However, to completely eliminate the possibility of adverse effects, further examination of the animal tissue after sacrifice would need to be made.

The initial acute administration treatment time point of 30 minutes post-noise exposure was chosen based on research by Guitton and Dudai (2007), which showed a reduction in noise-induced tinnitus being achieved by local application of an NMDA receptor agonist (ifenprodil) when administered during the first four days following noise exposure, but not afterwards. Despite being a different experimental design, and employing the use of a different drug treatment, this study represented the only available time course data on tinnitus treatment. The rationale for the 30 minute time point was that, should a similar effect carry across to the current study, a time point which falls at the beginning of the 4 day therapeutic window established by Guitton and Dudai (2007) would increase the likelihood that it is detected. The resultant lack of a significant drug effect persisting to the second behavioural testing period did not warrant additional time points being tested. Furthermore, it should be noted that this result is not entirely clinically relevant, as it is expected that patients seek drug treatments for tinnitus after it has developed. Administration within 30 minutes of the initial tinnitus-inducing trigger would be difficult, and is therefore most relevant to people who are routinely exposed to known tinnitus triggers on a regular basis. Within such high risk populations, L-baclofen may have potential to be administered as a preventative measure immediately prior to, or following, tinnitus triggers.
It should be noted that, due to time and equipment constraints, not all cardiac perfusions could be performed on the same day. Therefore, some animals had a slightly longer L-baclofen dosing timeframe before sacrifice. As this occurred after the tinnitus testing, it had no effect on the behavioural lick suppression paradigm results. Whether the extra doses of L-baclofen, which were a maximum of five more in rats sacrificed on the last day of cardiac perfusions, had an effect on GABA_B receptor expression is unknown. However, it was deemed more important that the animals continue to be administered L-baclofen up until the point of sacrifice, than for all animals to have an equal number of doses.

The sample size of the study was 32 animals, randomly assigned to four groups. The decision to include eight animals per group was based on power calculations from previous research conducted in the same laboratory (Zheng et al., 2011). This number was calculated as being able to allow a statistical detection of potential differences between the groups, while also being practical in terms of the cost, housing, handling, and testing of the animals. The importance of adequately powered studies is highlighted by the clinical trial of Westerberg et al. (1996), which was likely to have been underpowered. As such, the lack of statistical significance with regards to a baclofen treatment effect in that study may not necessarily mean that one does not exist. In the current study, we can be confident that statistically significant differences were detected when present. This is of importance as even small effects that are statistically significant have the potential to provide further insight into the potential of L-baclofen as a tinnitus treatment, and its mechanism of action, which in turn could aid in the development and design of future research.

CV staining was used to aid in the orientation of the sections, and to identify the CN. From this, it became apparent that due to the fragility of the CN structure in 40 μm thick sections, not all of the CN from all sections was available for the final ImageJ analysis. This was because some parts had become torn, or detached from the rest of the brain structure.
However, the amount of handling required during the sectioning and immunohistochemistry techniques meant that this was, to a degree, unavoidable and expected.

Immunohistochemistry using a GABA<sub>B</sub>-R<sub>2</sub> receptor antibody was carried out on the sectioned tissue. While it is assumed that the commercial antibody had high affinity for, and specific binding to, GABA<sub>B</sub>-R<sub>2</sub> subunits of the GABA<sub>B</sub> receptor, recently there have been questions surrounding the validity of antibodies (Rhodes and Trimmer, 2006). To test for antibody specificity, a number of test runs with a range of conditions were carried out. This involved comparing the differences yielded with varying primary and secondary antibody concentrations (including no-primary controls), incubation times, and incubation temperatures, to ultimately determine a protocol which reduced non-specific labelling. Ideally, the immunohistochemical results should have been further validated by a cross-comparison with other independent techniques, such as autoradiography; however this was not possible due to time constraints.

The CN were photographed and analysed at the 20x objective level of the microscope. The rationale for choosing this setting was to obtain the majority of cells in the focal plane. This level of magnification provided cell body detail, however there was not always good detail of the cell dendrites visible. This places a limitation on the GABA<sub>B</sub> receptor analysis component of the study, as if the L-baclofen administration, noise exposure, or presence of tinnitus, resulted in significant changes at the level of dendritic input to cell bodies, it would not have been detected by the current study.

By using the pixel saturation indicator of the NIS Elements software program, an exposure time of 2 ms was found to be optimal at producing the full dynamic range of pixels. This short exposure time was also well suited to the photo stitch process, as it minimised the risk of movement in the z-plane, thus decreasing the chance of blurring the cell staining of the sections. However, the presence of artifacts in the photographs, such as dust, cannot be ruled out. While slides were wiped with ethanol before analysis to remove most traces of dust and
other artifacts, some traces were unavoidable as they were beneath the cover slip. Where these were obvious, attempts were made to draw around the artificially darkened area during the ImageJ analysis, so it is assumed that the impact on the final \( \text{GABA}_B^{-R_2} \) mean densities was minimal.

When analysing the CN using ImageJ, the area of interest was traced slightly inwards from the edge of the tissue, to exclude the dense non-specific staining of the meninges. As a result, the receptor densities calculated are more likely to directly correlate to the specific \( \text{GABA}_B^{-R_2} \) receptor staining of the tissue. However, it should be noted that the area of interest contained the entire CN. Based on the null results of this analysis, and current knowledge that the CN contains a large number of different cell types of varying functions, it could be argued that it would have been more appropriate to have analysed the DCN and VCN separately, as well as finding their combined density. In the current study, changes in \( \text{GABA}_B \) receptor density within localised regions of the CN may not have been detected if these changes cancelled each other out, and resulted in no overall density difference.
4.4 Proposed Future Research

To further investigate the effects of L-baclofen as a potential treatment for tinnitus, it would be of interest to carry out research of a similar design to the current study, but using an oral L-baclofen dosing regimen instead of administering the drug via subcutaneous injection. This more closely replicates the reality of a clinical setting, in which baclofen is prescribed as an oral medication to patients.

The current study had a treatment time point of 30 minutes post-acoustic trauma, which was used for the acute administration of L-baclofen. To provide a more solid body of evidence for the potential of baclofen as a tinnitus treatment, further research using a greater number of time points is needed. At present, it is unknown whether earlier administration time points would exaggerate a potential protective effect, or if an increased dose could potentially have a greater effect than the time point itself. However, it should be noted that in reality it is unlikely that baclofen would be administered as a treatment immediately following the initial tinnitus trigger (such as an exposure to acoustic trauma), so results relating to the effects of acute doses of L-baclofen on the development of tinnitus could not be widely applied to the tinnitus patient population. Instead, results from such time course research would more likely have to be applied to the development of preventative action for at-risk individuals who are routinely exposed to known tinnitus risk factors, e.g. soldiers in combat conditions (Ylikoski and Ylikoski, 1994).

As previously mentioned, Westerberg et al. (1996) did not find a significant difference between racemic baclofen and placebo treatments in alleviating tinnitus in patients in a clinical trial setting. It would be interesting to carry out research of a similar design to the current study, but with additional D-baclofen and racemic baclofen treatment groups. This would not only provide further insight into the best baclofen preparation for use as a tinnitus treatment (in terms of both efficacy and side effects), but would also provide a more directly comparable result set which could provide evidence for the use of L-baclofen for improved
clinical outcomes, and hence reinitiate interest in baclofen as a potential tinnitus treatment despite the poor clinical trial results.

Based on the evidence of CNS hyperactivity, it would be of interest to carry out further research on the effects of GABA\textsubscript{B} agonists in general, rather than focussing purely on baclofen. Baclofen itself has a severe side effect profile, and can be poorly tolerated among patients. Therefore other drugs which have a similar mechanism of action, but are more widely tolerated in patients, could provide better treatment outcomes as a result of increased patient compliance. In particular, novel pro-drugs, and other structurally similar drugs which have been synthesised to overcome baclofen’s limitations, could be of interest. For example, arbaclofen placarbil, a pro-drug of the L-isomer of baclofen, has been demonstrated to have more desirable absorption, distribution, metabolism, and elimination kinetics, when used as a pharmacological treatment, than L-baclofen (Lal et al., 2009).

To gain a greater understanding of the mechanism by which GABA\textsubscript{B} agonists are exerting their potential therapeutic effects on the development and maintenance of tinnitus, it would be of benefit to carry out research on the effects of local drug application. For example, the use of osmotic minipumps could produce a controlled drug delivery in animals which, when attached to a catheter, can be targeted to a specific area of the brain. However, it should be noted that this is not a realistic and practical long term drug delivery method in patients, so would be research with a focus on tinnitus and treatment mechanisms, as opposed to patient care.

Due to time constraints, ImageJ analysis of immunohistochemical staining was only carried out on the exposed-baclofen and exposed-vehicle groups. This provides insight into the effect of L-baclofen administration on the GABA\textsubscript{B} receptors of exposed animals, but does not provide any information on the potential effect of the exposure itself on GABA\textsubscript{B} receptors, as there is no sham control as a comparison. Therefore, it would be of value to
compare such data to sham-baclofen and sham-vehicle groups, as this would provide further insight into the underlying contribution of the GABAergic system to noise-induced tinnitus.

The immunohistochemistry technique stained GABA\textsubscript{B} \textsuperscript{R2} positive cells, however, from this it was not possible to deduce what type of cell was being stained. Therefore, it would be of interest to carry out additional fluorescent work which uses co-labelling to determine cell type. Furthermore, immunohistochemical analysis is limited by the lack of ability to quantify receptors using the technique. The future use of radioligand binding, to quantify the maximum binding capacity and dissociation constant, would be of benefit in understanding changes to the number and affinity of GABA\textsubscript{B} receptor binding sites in the CN following noise-induced tinnitus and L-baclofen administration.
4.5 Conclusion

An acoustic-trauma tinnitus model in rats, assessed via a conditioned lick suppression paradigm, offers a reliable, replicable, and realistic representation of tinnitus. By employing such a model, the present project did not demonstrate conclusive evidence that L-baclofen administration in noise-exposed rats provides a short-term partial protective effect against the development of noise-induced tinnitus, and alleviation of tinnitus after its onset. This was due to a general drug effect. As a result, a reduction in lick suppression could not be used to confirm an alleviation of tinnitus in the exposed-baclofen treatment group. Therefore additional animal studies are required to further investigate the effects of L-baclofen in treating tinnitus, the most effective method of administration (in terms of both therapeutic and side effects), and appropriate dose ranges.

With an ever-increasing number of novel drugs and drug delivery systems being developed, a sound understanding of underlying cellular processes in both the pathology and treatment of tinnitus is paramount in developing the most effective treatment for this debilitating condition. At a cellular level, the present project showed no significant difference in mean GABA<sub>B-R2</sub> receptor density within the CN of vehicle- and L-baclofen-treated noise-exposed animals. However, the lack of a sham control group as a comparison meant changes in GABA<sub>B-R2</sub> expression as a result of tinnitus development, rather than drug administration, could not be determined from the current results.
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Appendix
Kolmogorov-Smirnov and Shapiro-Wilk tests of normality for data from this thesis. For each data set a histogram and normal quantile-quantile (Q-Q) plot is included, showing the deviation from normality, both before and after transformation. The SR data were square root transformed, and the ABR threshold and GABA<sub>B-R2</sub> data were natural log transformed.

**BBN Data, Tinnitus Testing Period 1:**

Tests of Normality

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<sup>a</sup> Lilliefors Significance Correction

**BBN Data Square Root Transformed, Tinnitus Testing Period 1:**

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<sup>a</sup> Lilliefors Significance Correction
20 kHz Data, Tinnitus Testing Period 1:

Tests of Normality

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<tr>
<td>SR</td>
<td>.136</td>
<td>966</td>
<td>.000</td>
<td>.891</td>
<td>966</td>
</tr>
</tbody>
</table>

a. Lilliefors Significance Correction

20 kHz Data Square Root Transformed, Tinnitus Testing Period 3:

Tests of Normality

<table>
<thead>
<tr>
<th>Statistic</th>
<th>df</th>
<th>Sig.</th>
<th>Statistic</th>
<th>df</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>sqrtSR</td>
<td>.085</td>
<td>966</td>
<td>.000</td>
<td>.956</td>
<td>966</td>
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a. Lilliefors Significance Correction
32 kHz Data, Tinnitus Testing Period 3:

Tests of Normality

<table>
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<tr>
<th>Kolmogorov-Smirnov(^a)</th>
<th>Shapiro-Wilk</th>
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<tbody>
<tr>
<td>Statistic</td>
<td>df</td>
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<tr>
<td>SR</td>
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</table>

\(^a\) Lilliefors Significance Correction

32 kHz Data Square Root Transformed, Tinnitus Testing Period 3:

Tests of Normality

<table>
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<tr>
<th>Kolmogorov-Smirnov(^a)</th>
<th>Shapiro-Wilk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Statistic</td>
<td>df</td>
</tr>
<tr>
<td>sqrtSR</td>
<td>.081</td>
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</tbody>
</table>

\(^a\) Lilliefors Significance Correction
GABA<sub>B</sub>-R<sub>2</sub> Data:

Tests of Normality

<table>
<thead>
<tr>
<th>Kolmogorov-Smirnov&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Shapiro-Wilk</th>
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</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>MeanDensity</td>
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<sup>a</sup> Lilliefors Significance Correction

GABA<sub>B</sub>-R<sub>2</sub> Data Natural Log Transformed:

Tests of Normality

<table>
<thead>
<tr>
<th>Kolmogorov-Smirnov&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Shapiro-Wilk</th>
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</thead>
<tbody>
<tr>
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<td>df</td>
</tr>
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<td>lnMeanDensity</td>
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<sup>*</sup> This is a lower bound of the true significance.
<sup>a</sup> Lilliefors Significance Correction
ABR Threshold Data:

Tests of Normality

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<tr>
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<th>Sig.</th>
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</thead>
<tbody>
<tr>
<td>.377</td>
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<td>.000</td>
</tr>
<tr>
<td>.644</td>
<td>620</td>
<td>.000</td>
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</table>

a. Lilliefors Significance Correction

ABR Threshold Data Natural Log Transformed:

Tests of Normality

<table>
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<th>df</th>
<th>Sig.</th>
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<tbody>
<tr>
<td>.359</td>
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<td>.000</td>
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<tr>
<td>.774</td>
<td>620</td>
<td>.000</td>
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</table>

a. Lilliefors Significance Correction