Behavioural and Neural Correlates of Tinnitus

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Thesis Abstract

Tinnitus, often defined as the perception of sound in the absence of an external stimulus, affects millions of people worldwide and, in extreme cases, can be severely debilitating. While certain changes within the auditory system have been linked to tinnitus, the exact underlying causes of the phenomenon have not, as yet, been elucidated. Animal models of tinnitus have considerably furthered understanding of the some of the changes associated with the condition, allowing researchers to examine changes following noise exposure, the most common trigger for tinnitus. This thesis documents the development of an animal model of tinnitus, using the guinea pig to examine neural changes following induction of tinnitus.

In the first study, a novel adaptation of a behavioural test was developed, in order to be able to determine whether guinea pigs were experiencing tinnitus following the administration of sodium salicylate, a common inducer of tinnitus in humans. This test relies on a phenomenon known as prepulse inhibition, whereby a startle response can be reduced in amplitude by placing a gap in a low-level, continuous background noise immediately prior to the startling stimulus. The hypothesis for this test is that if the background sound is adjusted to be similar to an animal's tinnitus (induced artificially following noise exposure or drug administration), the tinnitus percept will fill in the gap and the startle response will not be reduced. The results from this first study indicated that using the Preyer reflex (a flexion of the pinnae in response to a startling stimulus) as this startle measure was more robust in guinea pigs than the commonly-used whole-body startle. Furthermore, transient tinnitus was reliably identified following salicylate administration.

Following the development and validation of this test, a study was conducted to determine whether guinea pigs experienced tinnitus following unilateral noise exposure. Neural changes commonly associated with the condition (increases in spontaneous firing rates and changes in auditory

brainstem responses) were examined, to determine whether there were any differences between animals that did develop tinnitus following noise exposure and those that did not. Two different methods were applied to the behavioural data to determine which animals were experiencing tinnitus. Regardless of the behavioural criteria used, increased spontaneous firing rates were observed in the inferior colliculus of noise-exposed guinea pigs, in comparison to control animals, but there were no differences between tinnitus and no-tinnitus animals. Conversely, significant reductions in the latency of components of the auditory brainstem response were present only in the tinnitus animals.

The final study examined whether the original hypothesis for the behavioural test (that tinnitus is filling in the gap) was valid, or whether there was an alternative explanation for the deficits in behavioural gap detection observed previously, such as changes in the temporal acuity of the auditory system preventing detection of the gap. Recordings were made in the inferior colliculus of noise-exposed animals, separated into tinnitus and no-tinnitus groups according to the behavioural test, as well as unexposed control animals, to determine whether there were changes in the responses of single-units in detecting gaps of varying duration embedded in background noise. While some minor changes were present in no-tinnitus animals, tinnitus animals showed no significant changes in neural gap detection thresholds, demonstrating that changes in temporal acuity cannot account for behavioural gap detection deficits observed following noise exposure. Interestingly, significant shifts in the response types of cells were observed which did appear to relate to tinnitus. The present data indicate that the Preyer reflex gap detection test is appropriate for examining tinnitus in guinea pigs. It also suggests that increases in spontaneous firing rates at the level of the inferior colliculus cannot solely account for tinnitus. Changes in auditory brainstem responses, as well as shifts in response types, do appear to relate to tinnitus and warrant further investigation.

Declaration

I declare that this thesis was composed by myself, that the work contained herein is my own except where explicitly stated otherwise in the text, and that this work has not been submitted for any other degree or professional qualification.

Joel Isaac Berger *Date*

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Publications

Papers

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N.B. The above paper has been modified slightly to be included in both the general methods and the first results chapter

Selected Conference Abstracts

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Berger, J. I., Coomber, B., Shackleton, T. M., Wallace, M. N., and Palmer, A. R. (2012). Examining the behavioural effects of two tinnitus inducers (noise exposure and salicylate) in guinea pigs. *TRI 2012*.

Berger, J. I., Coomber, B., Shackleton, T. M., Wallace, M. N., and Palmer, A. R. (2011). Using the pinna reflex as a behavioural test for tinnitus in guinea pigs. *TRI 2011*.

List of Abbreviations

ABR	Auditory Brainstem Response
AC	Auditory Cortex
AI	Primary Auditory Cortex
AN	Auditory Nerve
AVCN	Anteroventral Cochlear Nucleus
BBN	Broadband Noise
CF	Characteristic Frequency
CN	Cochlear Nucleus
CNIC	Central Nucleus of the Inferior Colliculus
DC	Dorsal Auditory Cortex
DCB	Dorsocaudal Belt
DCN	Dorsal Cochlear Nucleus
DRB	Dorsorostral Belt
fMRI	Functional Magnetic Resonance Imaging
FRA	Frequency-Response Area
GP	Guinea Pig
HL	Hearing Level

IC	Inferior Colliculus
IHC	Inner Hair Cell
IPL	Inter-Peak Latencies
ISI	Inter-Stimulus Interval
LSO	Lateral Superior Olive
MGB	Medial Geniculate Body
MGDT	Minimum Gap Detection Threshold
MSO	Medial Superior Olive
NBN	Narrowband Noise
NT	No Tinnitus
OHC	Outer Hair Cell
PAMR	Post-Auricular Muscle Reflex
PPI	Prepulse Inhibition
PSTH	Post-Stimulus Time Histogram
PVCN	Posteroventral Cochlear Nucleus
RMS	Root Mean Squared
S	Small Field of the Auditory Cortex
SFR	Spontaneous Firing Rate
SL	Sensation Level
SLDT	Sound Level-Dependency Test
SNR	Signal-to-Noise Ratio
SOC	Superior Olivary Complex
SSA	Stimulus-Specific Adaptation
T	Tinnitus
TDT	Tucker Davis Technologies

TRN	Thalamic Reticular Nucleus
VCB	Ventrocaudal Belt
vMGB	Ventral Division of the Medial Geniculate Body
VRB	Ventrorostral Belt
WBS	Whole-Body Startle

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CHAPTER 1

General Introduction

1.1 Introduction to Tinnitus

Tinnitus is defined as the perception of sound in the absence of an external stimulus. It is often perceived as a ringing sound (the word tinnitus actually originates from the Latin *tinnire*, which translates as 'to ring'), but its characteristics can vary depending on the individual. There are two main types of tinnitus: subjective and objective. Objective tinnitus is a rare disorder caused by sounds originating within the body, sometimes through increased perception of vascular processes or contractions of muscles in the ear (Heller and Bergman, 1953). This type of tinnitus may be observed by others, as opposed to subjective tinnitus, which is much more prevalent and can be perceived only by the affected person. This thesis will focus on the subjective form of tinnitus. As such, the term 'tinnitus' will be used to denote subjective tinnitus, unless stated otherwise.

1.1.1 Prevalence of Tinnitus

Tinnitus is not a 'modern' phenomenon. In a letter to a friend, the 18th/19th century composer Ludwig van Beethoven declared that "my ears are buzzing and ringing perpetually, day and night", referring to the disturbance his tinnitus caused him. The naturalist Charles Darwin actually kept daily records of his tinnitus, noting its amplitude and frequency (Shaikh, 2012). Many observations of tinnitus have been made throughout history, with the earliest reference proposed to be the ancient Egyptians, who suggested its cause to be from a 'bewitched' ear (Dietrich, 2004). Despite many cultural references, research into the condition

did not properly begin until the early 1980s (Salvi and Ahroon, 1983). Though most people will experience tinnitus at some point in their lives, perhaps after a rock concert or through operating noisy machinery, recent epidemiological studies suggest that 10-15% of the population suffer from the condition chronically (Heller, 2003). A subgroup of those affected find their tinnitus severely debilitating, sometimes leading to suicide attempts, though it has been shown that the perceived intrusiveness of tinnitus is not intrinsically linked to the actual characteristics of the tinnitus (e.g. Meikle et al., 1984).

1.1.2 Causes of Tinnitus

While the exact underlying mechanisms of subjective tinnitus are currently unknown, the most common trigger for this type of tinnitus is repeated exposure to loud noises (Eggermont and Roberts, 2004). Its onset can also be linked to the intake of ototoxic drugs, such as salicylate or quinine (Ralli et al., 2010). Historically, the origin of tinnitus was believed to reside within the inner ear (Zeng et al., 2011). However, studies have demonstrated that symptoms of tinnitus persists even after the ablation of the cochlea (Zacharek et al., 2002) or severance of the auditory nerve (AN; House and Brackmann, 1981), so it is commonly accepted that tinnitus is the result of aberrant neural activity beyond the level of the AN, possibly triggered by initial changes at the peripheral level. Increased hyperactivity following noise exposure has been observed in the dorsal cochlear nucleus (DCN; Kaltenbach and Afman, 2000), inferior colliculus (IC; Chen and Jastreboff, 1995) and the auditory cortex (AC; Norena and Eggermont, 2003), implicating this phenomenon and these areas as possible contributors to the perception of phantom sounds. Interestingly, it does appear that, at least in the early stages, there is still a peripheral component to the causes of tinnitus, as Mulders and Robertson (2009) found that hyperactivity in the IC of guinea pigs (GPs) could be extinguished by silencing cochlear activity within 6 weeks of unilateral acoustic trauma.

Within certain regions of the auditory system, such as the cortex, different frequencies are represented in spatially distinct areas along a smoothly changing gradient from low to high. This organisation of frequencies is known as tonotopic mapping. Many studies have shown that there is a reorganisation of these maps following noise exposure (for a review

of these, see Eggermont and Roberts, 2004). Some have suggested this tonotopic map reorganisation as a putative mechanism for tinnitus generation (e.g. Muhlnickel et al., 1998). The theory for this mechanism suggests that reduced input to a particular area of the map, which can result from a profound hearing loss, causes an over representation of the frequencies surrounding this area, which along with an increase in spontaneous firing rates (SFRs) and neural synchrony within this area may lead to the perception of tinnitus (particularly in the case of tonal tinnitus). This theory is somewhat disputed (e.g. Langers et al., 2012), although support for this idea comes from studies showing that tinnitus is often represented at frequencies near or at the edge of the hearing loss (Hazell and Jastreboff, 1990). Other studies have seemed to contradict this, showing that tinnitus can occur in the absence of any hearing deficit (Muhlau et al., 2006), but it has been proposed that there may still be a subclinical hearing loss present that is not detected by the audiological assessment (Langguth et al., 2009). Indeed, when the audiological test is altered to include frequencies above 8 kHz, people with tinnitus demonstrate some kind of hearing loss at the higher frequencies (Roberts et al., 2006a).

There is a suggestion that this cortical map reorganization is a necessary prerequisite for the development of chronic tinnitus (Rauschecker et al., 2010), but others propose that this alone may not be sufficient to result in the perception of the phantom sound (Stolzberg et al., 2011). As a consequence of the fact that people with hearing loss do not necessarily develop tinnitus (Lockwood et al., 2002), it is likely that there are further neural changes required for tinnitus generation. Furthermore, there is contradictory evidence to the edge frequency theory (see *Section 1.4.3*), with some patients reporting their tinnitus occurring within the region of hearing loss (Sereda et al., 2011), so the exact mechanisms of how this reorganisation may contribute to the perception of tinnitus are as yet unknown. Ultimately, it appears likely that there will be a number of inter-related factors that lead to the chronic percept of tinnitus, including somatosensory and limbic interactions (Rauschecker et al., 2010), increased SFRs, reorganisation of tonotopic maps and changes in excitatory and inhibitory connections (Scholl and Wehr, 2008) but it may only be necessary for a subset of changes to be present in any one case.

1.1.3 Tinnitus Treatment Considerations

Tinnitus cannot accurately be defined as a homogenous disorder, as patients exhibit a variety of different sensations and aetiologies (Meikle, 2002). This is a consideration that needs to be taken into account when developing standardised methods of clinical diagnoses and also treatment options (Langguth et al., 2011). For example, some patients find that their tinnitus can be masked successfully using various stimuli (Hobson et al., 2012). One type of masking is known as residual inhibition, whereby suppression of the tinnitus continues for some time after the masker is switched off, from a period of seconds to minutes (Roberts, 2007). Others, however, find that their tinnitus gets worse in noise (e.g. Tyler et al., 2008), so using noise maskers is not a viable treatment option for these individuals. Another subset of tinnitus sufferers demonstrate the ability to alter the characteristics of their tinnitus through various somatic manipulations, such as jaw protrusion (Lockwood et al., 1998), neck contractions (Abel and Levine, 2004) and cutaneous stimulation (Cacace et al., 1999), thereby implicating the role of somatosensory interactions in the perception of tinnitus for these patients. A further subset experience tinnitus when gazing in certain directions (known as gaze-evoked tinnitus; Coad et al., 2001), which has been reported to be cured by repetition of gaze movements in a case study of one individual (Sanchez and Pio, 2007). Considering the inter-individual variability outlined here, it is likely that there is not going to be a one-for-all cure, so it has been proposed that creating and defining subtypes of tinnitus is a necessary step in developing treatment options (Landgrebe et al., 2010).

1.2 Overview of the non-pathological auditory system

In order to discuss the various pathological changes in the auditory system that may give rise to tinnitus, it is useful to first describe how the 'normal' auditory system works. Here I will outline the different pathways of the auditory system and briefly illustrate their functional relevance. Fig 1.1 provides a basic schematic of the primary auditory pathway, showing the main ascending connections. There are also many important descending connections, but for the purposes of brevity the ascending system will be mainly focused on here. Where possible, examples will be used specifically for the guinea pig auditory system, as this is the model used in this thesis.

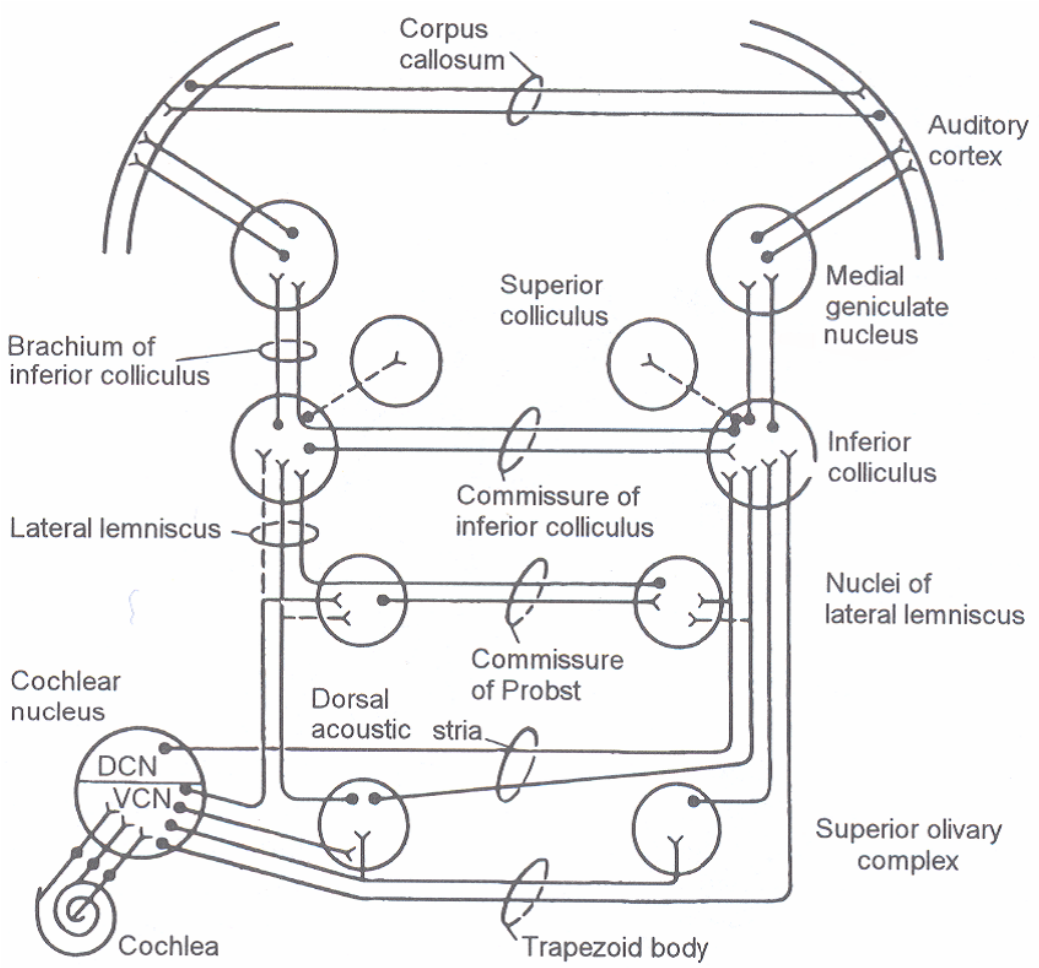

Fig. 1.1: Schematic of the ascending primary auditory pathway. From Ehret (1997).

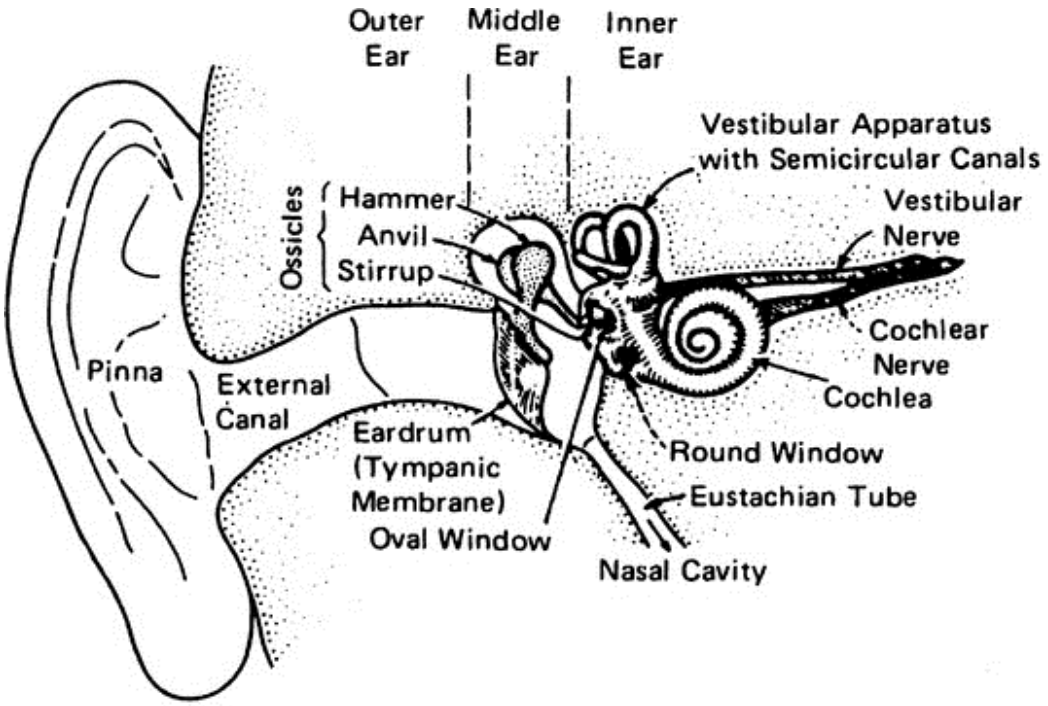

Fig. 1.2: Diagram of the human ear (Flanagan, 1972).

1.2.1 Ear

Fig 1.2 shows the three main peripheral sections of the auditory system - the outer, middle and inner ear. This is where sound waves, rapid pressure fluctuations around atmospheric pressure that convey information in the form of sounds, are first processed. The external auditory meatus (or 'ear canal') is surrounded by folds of cartilage known as the pinna, which is the visible part of the ear. The corrugated shape of this cartilage helps to direct sounds into the external auditory meatus whilst attenuating or amplifying sounds depending on where they are coming from and their frequency, thus creating differences in level and aiding in sound localisation (Hofman et al., 1998). Sound energy is transformed at the external/middle ear border by causing vibration of the tympanic membrane, which in turn produces movement in three small bones (or 'ossicles') located in the middle ear, known as the malleus, incus and stapes. These bones are essential in enabling the sound wave to travel to the inner ear, which has a higher acoustic impedance than air due to the fact that it is filled with fluid. Without this 'impedance matching', much of the acoustic energy would be reflected away from the inner ear. A large part of this impedance matching is due to the fact that the tympanic membrane has a larger area than the

stapes footplate, which means that the force is exerted on a smaller area, thus concentrating the sound pressure. This process is further aided by the lever action of the ossicles, which move in a pivot-like motion as a result of the malleus being longer than the incus.

The middle ear bones move in a lever motion, causing the stapes to drive into the oval window of the cochlea in a piston-like manner. The round window moves in opposite direction to the oval window, in order to compensate for the pressure applied to the incompressible fluid of the inner ear.

The cochlea is vital for transforming energy from acoustic signals into electrical neural impulses. It is a bony-walled, snail-like coiled tube, consisting of three channels - the scala tympani, scala vestibuli and scala media - separated by two thin membranes, known as the basilar and Reissner's membranes (Fig 1.3). The scala tympani and scala vestibuli are filled with a fluid called perilymph, which has a similar ionic composition to cerebrospinal fluid and mainly contains sodium (Na^+) and chloride ions (Cl^-).

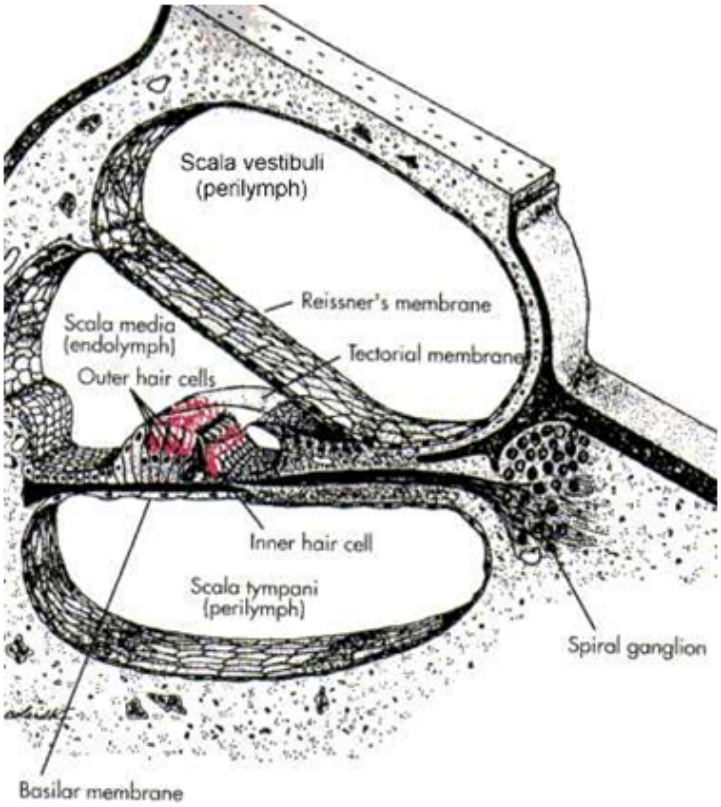

Fig. 1.3: Diagram showing the 3 divisions of the cochlea, along with the dividing membranes. From Nolte (1993).

The scala media is filled with potassium (K^+) rich fluid called endolymph. It receives its potassium ions from the stria vascularis. The high levels of potassium mean that endolymph has much higher positive potential than perilymph (approximately +80 mV as opposed to +5 mV). When the transduction channels open, potassium flows into the hair cells and partially depolarises the negatively charged hair cell. This depolarisation creates a receptor potential, which increases the likelihood that neural activity will be propagated from the auditory hair cells (Konishi et al., 1978).

The movement of the stapes against the oval window causes displacement of the endolymph (the perilymph of the scala vestibuli and scala media is also displaced), which in turn applies pressure on the basilar membrane in a wavelike motion. This travelling wave moves from the base to the apex, with high frequency sounds causing maximal displacement towards the basal end of the basilar membrane and low frequency sounds causing maximal displacement towards the apex. This differential movement of the basilar membrane means that areas are selectively tuned to different frequencies. This is known as tonotopy, a characteristic which is passed on to the auditory hair cells and is preserved throughout the ascending auditory pathway.

The auditory hair cells protrude from the top of the organ of Corti, which is located in the scala media and sits on the basilar membrane, and move against the tectorial membrane (Fig 1.4). There are two types of these cells - inner hair cells (IHCs) and outer hair cells (OHCs).

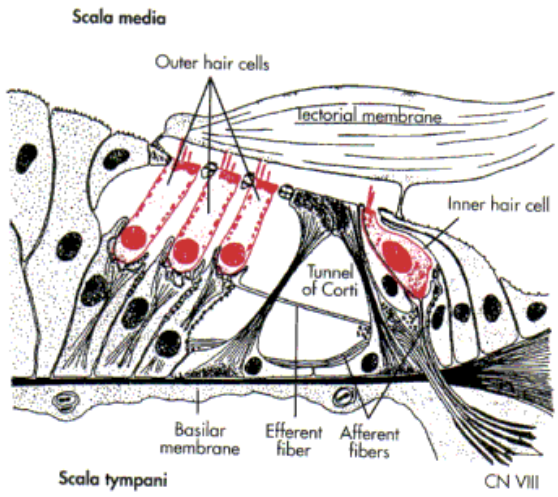

Fig. 1.4: Representation of the organ of Corti resting on the basilar membrane, with hair cells highlighted in red. The AN is labelled here as the eighth cranial nerve (CN VIII). From Nolte (1993).

The majority of afferent activity is caused by activation of the IHCs (Spoendlin, 1967). The tectorial membrane moves laterally above the hair cells, causing motion of the cilia which rest on top of the hair cells. This in turn opens mechanically-gated ion channels on the IHCs, causing an influx of K^+ ions from the scala media. This influx of K^+ ions depolarises the hair cell, which activates the chemical synapse at its base and causes the generation of action potentials along the afferents of the spiral ganglion of the AN. OHCs are embedded in the tectorial membrane and receive most of the efferent input to the cochlea. They are also active, meaning that they can amplify the travelling wave, a useful attribute for detecting quieter sounds. This active process is a result of the OHCs ability to elongate or contract, thereby pushing or pulling on the basilar membrane, and is mediated by a motor protein called prestin (for an overview, see Dallos et al., 2006). When OHCs are largely absent, as seen in patients with severe hearing impairment, hearing sensitivity is dramatically reduced (Chen et al., 2008).

1.2.2 Auditory Nerve

The AN (or eighth cranial nerve) enters the cochlea through an area of the bony cochlear wall known as the modiolus. Consisting of approximately 40000 fibers in humans, this bundle contains two types of neurons with different functionality. The majority (95%) of neurons are myelinated type I fibers, which receive afferent input from the IHCs. Each fiber innervates a single hair cell, whilst each hair cell provides information to a number of nerve fibers. The rest of the AN fibers are unmyelinated type II, which innervate the OHCs (Spoendlin, 1967).

1.2.3 Cochlear Nucleus

The cochlear nucleus (CN) is the first structure of the auditory brainstem (Fig 1.5). It is comprised of three distinct sections - the anteroventral cochlear nucleus (AVCN), the posteroventral cochlear nucleus (PVCN) and dorsal cochlear nucleus (DCN). These areas receive uniform input from the AN and separate this into discrete information which is relayed to higher auditory structures. Cochlear tonotopy is preserved in each region, as AN fibers bifurcate into ascending and descending branches to innervate all three areas. For example, in the DCN, low frequency fibers terminate ventrally and high frequency fibers terminate dorsally, producing

a tonotopic gradient from low-to-high organised from ventral-to-dorsal regions.

The cochlear nucleus contains four distinct primary cell types: bushy (spherical and globular), stellate, octopus and fusiform. These cells are prevalent in different subdivisions, for example, fusiform cells are found only in the DCN and bushy cells in the AVCN. The temporal discharge patterns in response to auditory stimuli differ significantly between cell types, thus aiding in the categorisation of cells when performing electrophysiology (Pfeiffer, 1966). To further aid this categorisation, frequency response areas (which indicate a cell's responsiveness to various frequencies and sound levels) can be obtained and their shapes are different depending on the cell type (Rhode and Smith, 1986; Stabler et al., 1996).

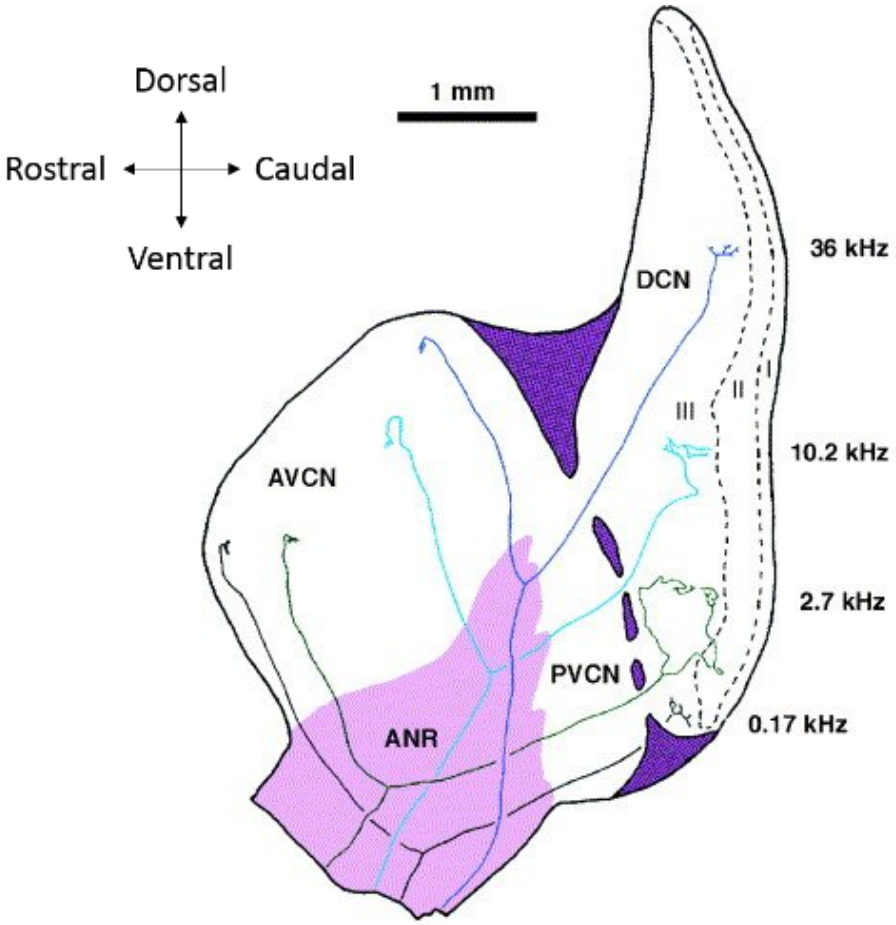

Fig. 1.5: The three sections of the mammalian cochlear nucleus, with approximate tonotopic organisation of the DCN labelled. The AN root and its connections to the various regions are also shown (adapted from Ryugo and Parks, 2003).

1.2.4 Superior Olivary Complex

The superior olivary complex (SOC) primarily receives input from the AVCN and PVCN, via commissural fibers. As it is the first site in the auditory pathway where significant amounts of acoustic information from the left and right ears converge, it plays an important role in sound localisation. The medial superior olive (MSO) is involved in detecting interaural time differences between the two ears (as sounds arrive at each ear at different times depending on the location of the stimulus). This aids in localising sounds on the azimuthal plane and is most sensitive to low frequency stimuli (Wightman and Kistler, 1992). The lateral superior olive (LSO) is mainly involved in detecting differences in sound levels (interaural level differences) and is particularly useful for localising high frequency stimuli (Park et al., 2004). The SOC projects to a midbrain auditory structure, the IC, via an axonal tract known as the lateral lemniscus (Kelly et al., 1998).

1.2.5 Inferior Colliculus

The IC receives most of the ascending projections from the brainstem. It consists of three major sections (Fig 1.6) - the central nucleus (CNIC), the surrounding dorsal cortex and laterally located external cortex. It receives bilateral input from both the MSO and LSO. The site at which most of these afferent inputs terminate is the CNIC, which can be identified histologically, via its laminar organisation (Malmierca et al., 1995), and electrophysiologically by its sharply tuned 'v-shaped' tuning curves (Aitkin et al., 1975). Like many of the auditory structures, CNIC demonstrates clear tonotopic organisation, with low frequencies represented dorsally and high frequencies ventrally (Clopton and Winfield, 1973; Huang and Fex, 1986).

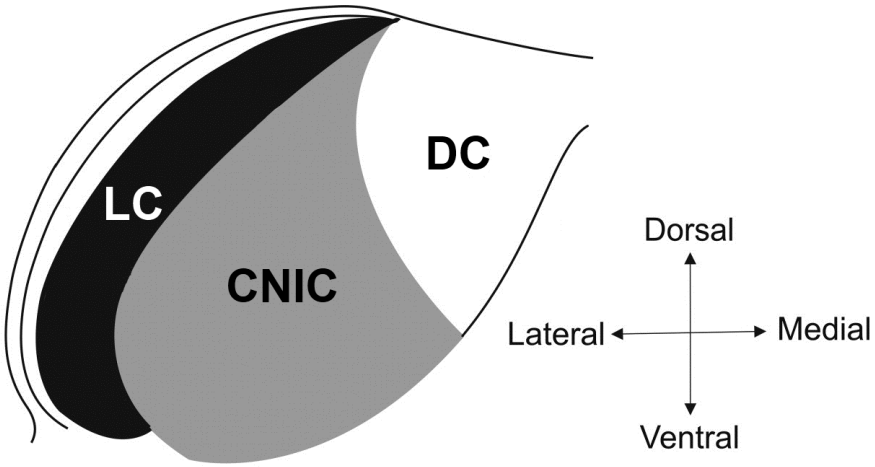

Fig. 1.6: The three divisions of the IC of the rat. Abbreviations - CNIC: Central nucleus of the IC; DC: Dorsal cortex; LC: Lateral cortex. Adapted from Loftus et al. (2008).

The IC is an important site for decoding a variety of auditory information, such as complex temporal structure (Keller and Takahashi, 2000) and sounds that are of particular significance (Casseday and Covey, 1996). IC neurons respond differentially to sounds of a particular duration, frequency or amplitude modulation. This mechanism is vital for detecting gaps in sound and therefore important for understanding a temporally fluctuating stimulus, such as speech (Gordon-Salant and Fitzgibbons, 1993). The IC has also recently been shown to be the first site where a mechanism for detecting infrequent stimuli, known as stimulus-specific adaptation (SSA; Duque et al., 2012; Malmierca et al., 2009), is evident. SSA comes from neurons reducing their responsiveness to a particular repeated stimulus and increasing their firing rate following the detection of a stimulus that is presented infrequently. This aids in identifying biologically relevant sounds, such as the sound of a predator approaching.

1.2.6 Medial Geniculate Body

The medial geniculate body (MGB) receives most of its input from the CNIC (Malmierca et al., 1997), though there are also some direct projections from the DCN (Anderson et al., 2006). It represents the auditory thalamic relay from the IC to AC. Histological and electrophysiological examination of the GP MGB has revealed five subdivisions (Fig 1.7) - the ventral MGB (vMGB), the dorsolateral and suprageniculate (collectively referred to as the dorsal MGB), the medial MGB and the shell MGB (Anderson et al., 2007).

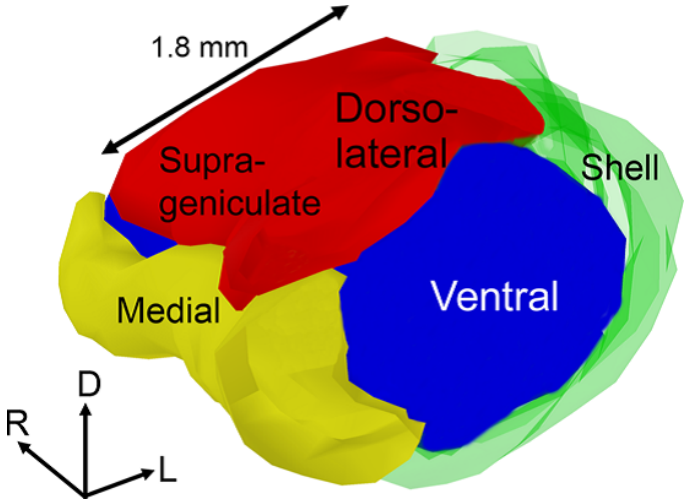

Fig. 1.7: Schematic of the five divisions of the GP MGB. From Anderson et al. (2007).

Neurons in the vMGB mostly have V-shaped tuning curves and are tonotopically organised, whereas the other areas do not show these responses or arrangement. As a result, it is generally believed that the vMGB is the primary thalamic area responsible for relaying intensity, frequency and spatial information to the AC (for a review, see McAlpine, 2009).

1.2.7 Auditory Cortex

Fig 1.8 outlines the distinct regions of the GP auditory cortex (AC), which demonstrates similar organisation to that of other mammals (Redies et al., 1989). AC is comprised of two core tonotopically organised regions: Primary AC (AI) and dorsal cortex (DC). There are two other tonotopic areas: the ventrorostral belt (VRB) and the small field (S) but these appear to be belt areas.

There are also three secondary belt areas that lack a tonotopic arrangement and generally prefer noise to tones - dorsocaudal belt (DCB), dorsorostral belt (DRB) and ventrocaudal belt (VCB). Additionally, a transition zone (labelled 'T' in Fig 1.8) is evident between AI and DC, which does not follow the tonotopic gradient of either region but does respond well to tones (Wallace et al., 2000). These areas show differential responses to stimuli. For example, neurons in DRB respond more strongly to broadband noise (BBN) than pure tones, while all the other belt areas tend to have longer latencies to pure tones than the core areas.

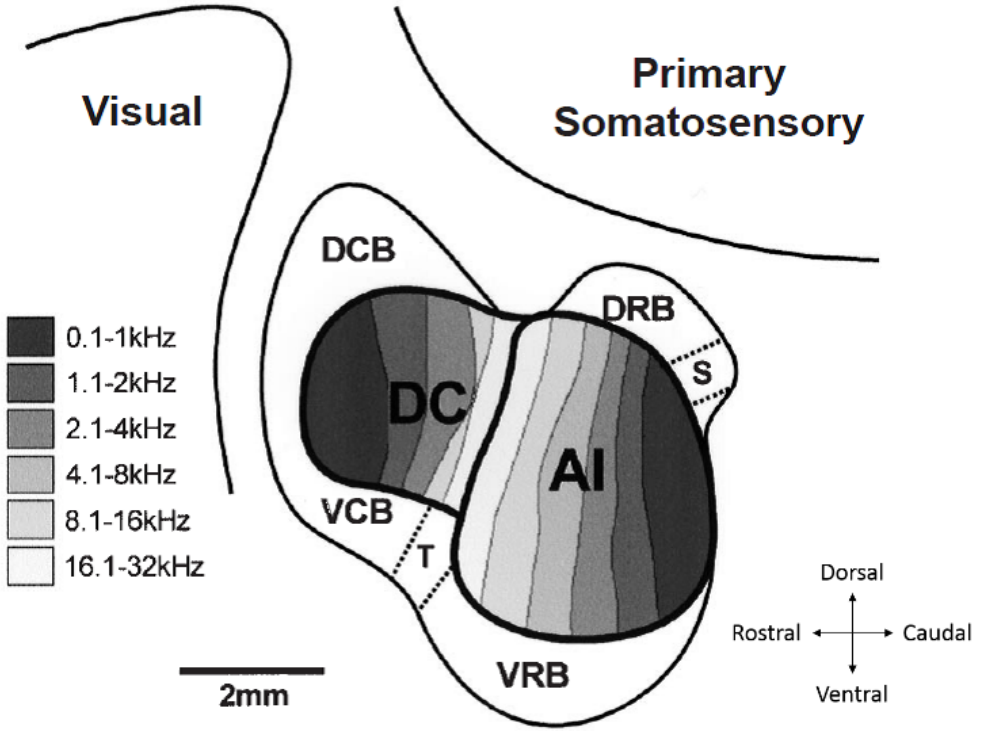

Fig. 1.8: The eight divisions of the GP auditory cortex, with their relative positions to visual and somatosensory areas. Shading indicates the organisation of characteristic frequency along isofrequency bands in dorsal cortex and primary auditory cortex, outlined by the key. See text for abbreviations. From Wallace et al. (2000).

Attempts have also been made to identify tonotopy in the AC of humans, using functional magnetic resonance imaging (fMRI) as a measure (e.g. Langers and van Dijk, 2012), as well as magnetoencephalography (MEG; Lutkenhoner and Steinstrater, 1998). This has proved more difficult than in animals, as the invasive recording techniques used in animal models allow for much greater spatial resolution than any of the human imaging techniques. As a result, there are disparities in the studies examining tonotopic mapping in humans. Some indicate three different tonotopic gradients (Humphries et al., 2010; Langers and van Dijk, 2012), while another recent study found six different tonotopic gradients (Striem-Amit et al., 2011). The main point of agreement seems to be that there are at least 2 areas with different tonotopic gradients, similar to those shown in other mammals, including the GP. It has recently been highlighted that these disparities between studies are likely due to varying degrees of spatial resolutions, as a result of using different imaging techniques (for a review, see either Baumann et al, 2013 or Saenz and Langers, 2013).

1.2.8 Generation of Auditory Brainstem Responses

I will briefly describe the generation of auditory brainstem responses (ABRs), as they are used within this thesis. ABRs are often used in both clinical and experimental settings as a measure of hearing thresholds (Moller, 1999). They reflect the synchronous evoked activity at various levels of the auditory system. In small animals five waves are often defined, while in humans and other mammals seven waves are present (Zhou et al., 2006). ABR waves are measured a variety of ways, such as by their amplitude (as determined by the amplitude change from peak-to-trough), absolute latencies or interpeak latencies (e.g. Vaney et al., 2011). An auditory threshold is usually defined as the lowest sound level which elicits a clear response from a particular wave (wave V in humans; wave IV in small animals). As this thesis is focused on the GP, I shall briefly describe ABRs in small animals.

Fig 1.9 illustrates the typical waveform of a GP ABR. It is generally accepted that fiber tracts within the auditory system generate the major contributions to the ABR waveform (Rudell, 1987). Wave I of the ABR is commonly agreed to be a result of the activity of the AN (Wada and Starr, 1983a; Simha et al., 1988; Melcher and Kiang, 1996; Melcher et al., 1996a; 1996b), while the second wave is thought to be generated by the AVCN and PVCN (Buchwald and Huang, 1975; Simha et al., 1988). Wave III is believed to arise from the SOC and medial nucleus of the trapezoid body, and wave IV from the SOC and lateral lemniscus, i.e., fibers that project to the IC (Buchwald and Huang, 1975; Popelar et al., 2008; Simha et al., 1988; Wada and Starr, 1983b). The fifth wave is then thought to be generated in the IC and lateral lemniscus (Harrison and Palmer, 1984; Melcher and Kiang, 1996; Melcher et al., 1996a; 1996b; Palmer and Harrison, 1984; Popelar et al., 2008). Wave IV (which parallels wave V in humans) is usually the most distinguishable wave in small animals, so it is this wave that is usually used as the measure of detectability of a signal (Boettcher, 2002).

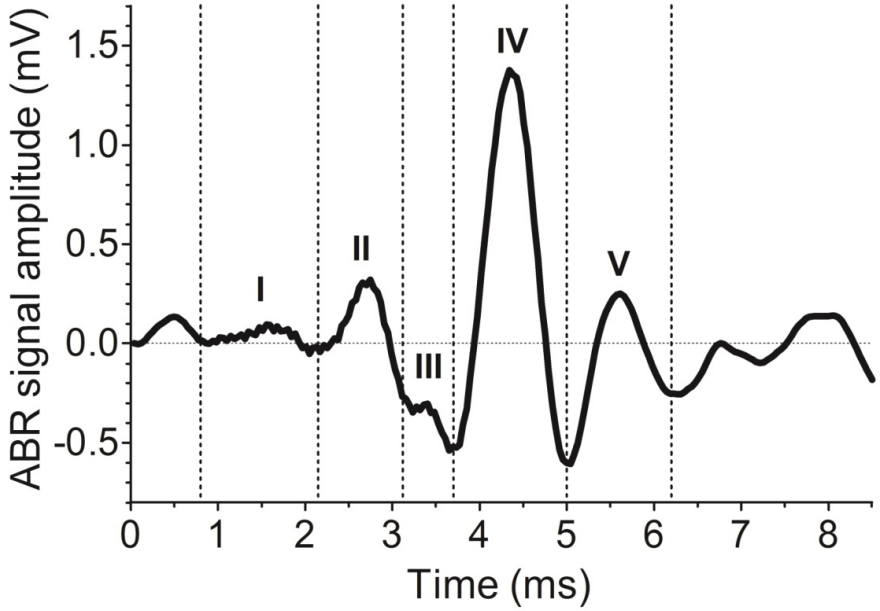

Fig. 1.9: A representative ABR, in response to a 10 kHz tone stimulus presented at 70 dB SPL. Dotted vertical lines indicate approximate time windows when each component wave of the ABR occurs (I-V).

1.2.9 Limbic-Auditory Interactions

As well as receiving and processing auditory input, the auditory pathway has recurrent interactions with the limbic system, the network responsible for emotional responses (Armony and LeDoux, 2010). Many models of tinnitus generation and maintenance implicate involvement of the limbic system, so it is therefore useful to briefly describe the interaction between the two systems. One main limbic region which can be modulated by auditory experience is the amygdala (Sah et al., 2003). The limbic system also has reciprocal connections to the central auditory system, which can affect neuronal activity and plasticity (Marsh et al., 2002; Weinberger, 2007). The main auditory input to the amygdala is sent from MGB and AI (Garrido et al., 2012). These connections terminate in the lateral amygdala, and a reciprocal connection is sent from the basal amygdala to the auditory system, thus creating a feedback loop (Kraus and Canlon, 2012). Such connections are involved in behavioural tests where a conditioned stimulus (e.g. a brief tone) is paired with an unconditioned stimulus (e.g. a foot shock). Briefly, the auditory system processes the conditioned stimulus, whilst the unconditioned stimulus is processed by the somatosensory system, which also projects to the lateral

amygdala. The central nucleus of the amygdala responds by generating an emotional response, such as freezing behaviour. Following conditioning, the tone alone is sufficient to induce the emotional response, due to fear conditioning mediated by limbic-auditory interactions (Weinberger, 2011). These connections also explain emotional reactions to music: patients with grey matter loss in the region of the amygdala show impaired recognition of emotion within musical scenes (Omar et al., 2011), highlighting that limbic-auditory interactions are essential for processing sounds that carry particular meaning.

The amygdala can modulate auditory activity via its connection to the nucleus accumbens (Salimpoor et al., 2013). This area projects to the thalamic reticular nucleus (TRN), which provides inhibitory input to the ascending pathway of the MGB. It has been suggested that this connection may enable habituation to unwanted stimuli by preventing cortical activation (Rauschecker et al., 2010). This can be thought of as an auditory gating mechanism. Reduced activation of this circuit has been proposed to explain the inability to habituate to chronic tinnitus (Rauschecker et al., 2010), an idea which will be discussed later on.

1.3 Inducers of Tinnitus

Different inducers of tinnitus will now be discussed in more detail, in order to provide context to the pathological changes associated with the condition. Tinnitus can be either transient or chronic. Transient tinnitus has a near immediate onset following induction and lasts anywhere between a few seconds to a few days. Chronic tinnitus is defined as lasting more than 6 months and is often more debilitating (Folmer et al., 2004). The most common cause of both transient and chronic tinnitus is known to be noise exposure (Axelsson and Prasher, 2000). This inducer can be subdivided into two different types - occupational and leisure. Occupational noise exposure involves chronic exposure to sounds of a damaging level within a work environment. While sound levels within a work environment are now limited (within the UK, the Control of Noise at Work Regulations 2005 state that the maximum average exposure allowed is 85 dBA without hearing protection and 87 dBA if hearing protection is worn), neural changes associated with tinnitus have been shown to occur in animals following noise exposure to sound levels falling within this limit (Kaltenbach et

al., 2005; Pienkowski and Eggermont, 2012). This suggests that further work is required to determine whether the current standards for noise exposure within a work environment are suitable for protecting hearing and preventing tinnitus.

The other main type of noise exposure - leisure noise - has become more of a concern with the growing popularity of personal music players (PMPs). A recent study of high school students in Canada (Lévesque et al., 2009) found that the mean listening level to PMPs was 82.59 dBA, with some reaching as high as 110.34 dBA. They also found a much higher prevalence of tinnitus in students using these devices at high levels ($>$ 80 dBA) compared to those listening to levels $\leq$ 80 dBA, similar to what others have found (e.g. Meyer-Bisch, 1996). As a result of such research, the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR, 2008) proposed a limit of 85 dBA to all devices sold in the European Union with music playback capability, which came into effect in February 2013, although this limit may be overridden by the user. Leisure noise exposure, however, is not simply limited to PMPs. Rock concerts, night clubs and sporting events have all been linked to increased incidence of tinnitus, most likely due to the high levels of noise exposure that accompany them (Bogoch et al., 2005; Gunderson et al., 1997; Hodgetts and Liu, 2006; Yassi et al., 1993). One method of preventing leisure noise-induced tinnitus is to wear hearing protection. This is, however, rarely used by young adults participating in these activities, perhaps due to the social stigma it carries (Gilles et al., 2012). Ultimately, further public knowledge and changing of social attitudes may be required to prevent hearing damage from leisure noise.

Certain ototoxic drugs have been shown to reliably induce transient tinnitus in both humans and animals. The most common of these is sodium salicylate (for a review, see Stolzberg et al., 2012), the active ingredient in aspirin. In a therapeutic setting, this drug has anti-inflammatory and analgesic effects, as well as aiding in the prevention of certain types of cancer (Paterson and Lawrence, 2001). At high doses, however, it can reliably induce reversible tinnitus in humans (Mongan et al., 1973; Pedersen, 1974) and behavioural evidence of tinnitus in animals (Jastreboff et al., 1988; Lobarinas et al., 2006). In fact, therapeutic dosing of salicylate has historically been performed using the clinical adage, 'Push to tinnitus, then back off slightly', though this is not always recommended, particularly

in patients with a pre-existing hearing loss where such a strategy is not effective (Mongan et al., 1973). Other drugs which have been shown to induce transient tinnitus include cisplatin (Rachel et al., 2002) and quinine (Ralli et al., 2010). As all these drugs induce a type of tinnitus which is reversible, their clinical significance is less relevant than noise exposure, which is often associated with chronic tinnitus.

Finally, tinnitus has been shown to occur with age-related hearing loss, known as presbycusis. There is strong evidence for increased incidence of tinnitus with age (Shargorodsky et al., 2010). Importantly, Schlee et al. (2011) highlight that the level of tinnitus distress is strongly associated with the age of onset: patients who develop tinnitus earlier in life are significantly less affected by the emotional component of the tinnitus than those who have a late onset of tinnitus. The authors argue that this decreased acceptance of tinnitus may be related to the reduction in neuroplasticity seen with advancing age. As this was the first study examining tinnitus-related distress in relation to the age of tinnitus onset, clearly more work is required to determine the neurophysiological mechanisms behind the age of onset as an influencing factor in the distress caused by tinnitus.

1.4 Pathological Changes Following Acoustic Trauma

1.4.1 Damage to the Cochlea and AN

Whilst the middle ear reflex can act to protect against damage from loud sounds, this protection is minimal (Moller, 2011). Ultimately, when sounds are too loud for a prolonged period of time, there is damage to the auditory periphery and compensatory changes within the central auditory system. The IHCs and OHCs are both damaged by acoustic over-exposure, with some completely abolished in profound hearing loss. Excitotoxicity can cause injury to the afferent dendrites of the AN (as a result of increased glutamate release from the IHCs), with this proposed as the main mechanism behind reduced input to the auditory system (Hakuba et al., 2000). In the OHCs, it has been suggested that the motility of the stereocilia is strongly affected with sub-lethal damage (Liberman and Dodds, 1984). This reduction in motility has been measured by observing reduced cochlear

microphonics, assumed to be primarily a measure of the receptor potentials of the OHCs (Santarelli et al., 2006), following prolonged noise exposure in chinchillas (Ferraro et al., 1981). Reduced OHC function has been proposed to be caused by an increased concentration of calcium ions (Ca^{2+}) in OHCs, which may also have excitotoxic effects (Fridberger et al., 1998). The primary effect of degraded IHC and OHC function, or complete loss of a section of hair cells, is reduced sensitivity of hearing (Ulfendahl and Flock, 1998).

1.4.2 Changes in SFRs and Synchronicity

Reduced input to the auditory system may induce central changes as a result of maladaptive compensatory mechanisms (Brozoski et al., 2012). Changes in the SFR of auditory-responsive neurons have been observed at various levels of the auditory system following acoustic trauma. Decreases in the SFR of AN fibers have been observed immediately after acoustic overexposure (Norena and Eggermont, 2003), along with a permanent loss of AN fibers (Furman et al., 2013). Conversely, increases in SFR are well documented for the DCN (Brozoski and Bauer, 2005; Brozoski et al., 2002; Kaltenbach et al., 2004; Zhang et al., 2006), IC (Bauer et al., 2008; Mulders and Robertson, 2009; Mulders et al., 2011) and AC (Engineer et al., 2011; Norena and Eggermont, 2005). While some of these studies did use behavioural tests to identify tinnitus in these animals, an essential prerequisite for linking pathological changes following noise exposure with the condition, none of them examined animals that either only exhibited a significant hearing deficit following noise exposure or simply did not develop tinnitus. This is an important aspect, as there is some ambiguity as to which changes may relate to tinnitus and which may simply be a result of hearing loss.

Based on the evidence that increases in SFR are seen in tinnitus animals as a result of reduced afferent input, Schaette and McAlpine (2011) measured ABRs in tinnitus patients, as well as in a control hearing-matched population. They demonstrated a reduction in the amplitude of Wave I in tinnitus patients compared to controls, indicating some degree of reduced hearing sensitivity (in the absence of a discernible hearing loss as identified by an audiogram). Contrastingly, the amplitudes of wave V were the same as controls in these patients, which equated to an increase in the ratio between Wave I and Wave V in tinnitus subjects. They attributed this to

an increase in central gain, reflecting a compensatory neural mechanism for some degree of loss of input from the periphery, suggesting that this may contribute to the tinnitus percept.

Clearly this model is over-simplistic and does not account for other factors that may affect tinnitus, such as the limbic component or somatosensory input, but it is nevertheless an interesting and compelling theory. It is even more interesting when combined with other theories of tinnitus generation, including Rauschecker's limbic gating mechanism theory (Rauschecker et al., 2010), whereby reduced activation of TRN (through abnormally functioning limbic circuits) may reduce inhibition to the MGB and allow this increased activity to reach cortex, resulting in a phantom perception of sound. The idea of combining these theories to create a more cohesive explanation of tinnitus generation will be discussed further in a later chapter. One cause of the increased excitation outlined here may be a reduction in inhibitory input at certain levels of the auditory system, as the result of an acoustic insult (Gerken, 1996; Roberts et al., 2010). Reduced inhibition (along with increased excitation) has been observed following peripheral deafferentation in the auditory system, at the level of the DCN (Kaltenbach and Godfrey, 2008), IC (Caspary et al., 2008; Dong et al., 2010; Syka, 2002) and AC (Norena et al., 2003; Scholl and Wehr, 2008), similar to what has been documented in other sensory systems following the loss of peripheral input, including somatosensory (Rasmusson and Turnbull, 1983) and visual cortices (Schmid et al., 1995). Furthermore, Middleton et al. (2011) demonstrated hyperactivity in the DCN of mice with behavioural evidence of tinnitus. They attributed this hyperactivity to a decrease in GABAergic inhibition, as an increase in excitation was observed when GABAergic (but not glycinergic) antagonists were applied to the brain slices of tinnitus animals. Combined with an increased strengthening in glutamate transmission observed following noise exposure, this could feasibly result in hyperactivity (Eggermont, 2005). This idea of reduced inhibition is further supported by an age-related down regulation of inhibition which has been reported (Caspary et al., 2008; Frisina, 2010), perhaps helping to explain the increased incidence of tinnitus with age (Shargorodsky et al., 2010).

Changes following noise exposure are not simply restricted to increases in SFR. There is a reasonable body of evidence suggesting that increases in neural synchrony at the level of the cortex may also contribute to

the phantom percept of tinnitus following loss of peripheral input (e.g. Norena and Eggermont, 2003; Seki and Eggermont, 2003). This refers to temporally synchronous activity, the result of multiple neurons firing at the same time, which has been demonstrated to increase around the region of any hearing loss (Eggermont, 2007). The animal literature suggesting neural synchrony as a contributor to tinnitus is supported by human research demonstrating increased oscillatory brain activity in patients with tinnitus (Weisz et al., 2007). This oscillatory activity reflects the synchronous firing of neural populations (Singer, 1999). As all the previous studies examining neural synchrony have been performed in cortical structures, it is unclear whether changes in synchrony observed in the cortex will be evident subcortically. It is possible that increases in neural synchrony as observed in the cortex may reflect a compensatory mechanism for desynchronous activity received from ascending, subcortical pathways (Shulman and Goldstein, 2010).

1.4.3 Reorganisation of the Auditory System

Following loss of peripheral input, reorganisation of the tonotopic/somatotopic maps has been demonstrated in both auditory areas (Harrison et al., 1998) and somatosensory areas (Elbert et al., 1994; Flor et al., 1995; Flor et al., 1998; Merzenich et al., 1984). In the latter, this reorganisation has been attributed as a major cause of phantom limb sensation, whereby pain or feelings of sensitivity are felt in the area where a limb once was following amputation (Ramachandran and Hirstein, 1998), a phenomenon which clearly parallels the perception of tinnitus following peripheral loss. A considerable amount of research into tonotopic reorganisation as a result of noise exposure has been performed on animals, in the CNIC (Irvine et al., 2003; Snyder et al., 2008; Snyder et al., 2000), vMGB (Kamke et al., 2003) and AC (Eggermont and Komiya, 2000; Norena and Eggermont, 2005; Rajan et al., 1993; Robertson and Irvine, 1989). The suggested mechanism behind this reorganisation is that loss of input to a particular area results in a compensatory plastic change within the central auditory system, whereby neurons that once responded to frequencies within the dysfunctional region of the cochlea begin responding to the frequencies represented in surrounding areas (Eggermont and Roberts, 2004; Pienkowski and Eggermont, 2011). This observation of edge-frequency reorganisation is outlined by Fig 1.10.

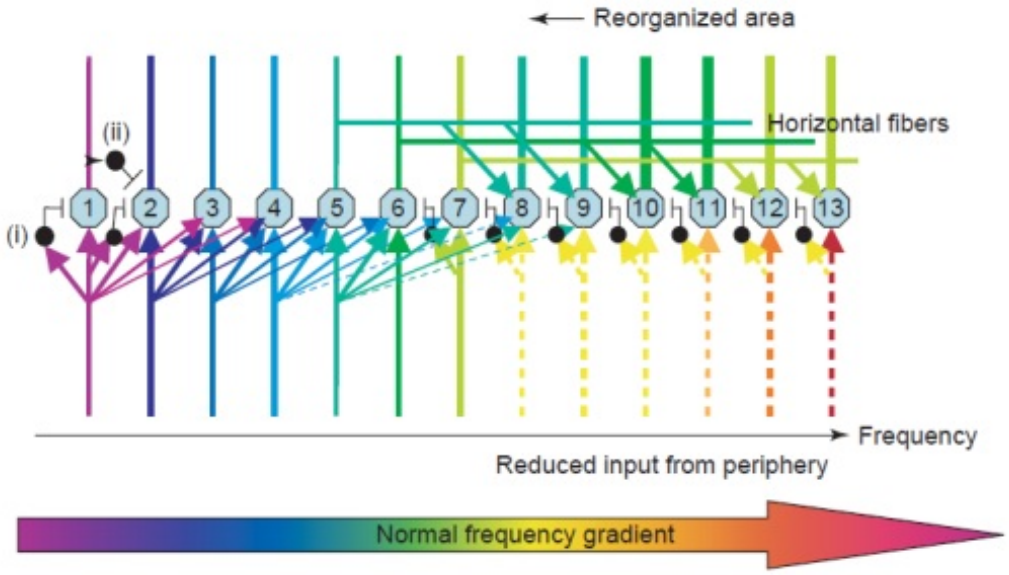

Fig. 1.10: Reorganisation of tonotopic maps. Dotted lines indicate reduced input from the cochlea, which induces homeostatic mechanisms that result in an overrepresentation of the frequencies surrounding the hearing loss. From Eggermont and Roberts (2004).

Based on these findings, if cortical reorganisation was solely responsible for the perception of tinnitus, one would expect the dominant pitch of a person's tinnitus to correspond to the frequency at the edge of the hearing loss, rather than falling within the hearing loss region. While this is sometimes true (Konig et al., 2006; Moore et al., 2010), there are many incidences where the tinnitus pitch often falls within the hearing loss region (Henry and Meikle, 1999; Norena et al., 2002; Roberts et al., 2006b; Sereda et al., 2011) thus questioning the validity of the edge-frequency hypothesis of tinnitus.

Muhlnickel et al. (1998) published one of the earliest studies to indicate that reorganisation of tonotopic maps may relate to tinnitus. Using magnetoencephalography they demonstrated a clear shift in the cortical representation of the tinnitus frequency into a surrounding tonotopic area in tinnitus patients. Since then, there has been much debate as to whether this organisation is actually the cause of the tinnitus percept, or if there are other changes that take place concomitantly that might provide a mechanism for tinnitus (such as increased neural synchrony or hyperactivity). Using fMRI, Langers et al. (2012) did not find any evidence

of tonotopic map reorganisation in tinnitus patients with normal hearing or mild hearing loss. A possible caveat of this study, however, is that the hearing loss may not have been severe enough to cause dramatic cortical reorganisation, or the imaging technique may not have been sensitive enough to detect slight changes associated with the condition. Conversely, Engineer et al. (2011) demonstrated that reversing this reorganisation in rats (through stimulation of the vagus nerve combined with tone-pairing) led to elimination of behavioural evidence of tinnitus. However, they also showed a reduction in other correlates of tinnitus (such as increased hyperactivity), so it is not clear whether a reversal of the reorganisation of the tonotopic map or the reduction of another tinnitus correlate may have caused the cessation of behavioural evidence of tinnitus.

Interestingly, Rajan and Irvine (1996) suggest that a steep hearing loss of ~50 dB per octave is required for tonotopic map reorganisation, based on the fact that they were unable to demonstrate reorganisation in cats with gradually sloping hearing losses, which would explain the lack of cortical reorganisation observed in subjects with normal hearing or a mild hearing loss (Langers et al., 2012). However, more recent studies have indicated that sounds of only a moderate sound pressure level are sufficient to produce tonotopic map reorganisation (Pienkowski and Eggermont, 2009; Pienkowski and Eggermont, 2010a; 2010b), although it is unclear whether these plastic changes caused tinnitus, as no behavioural tests were performed. Ultimately, it appears that tonotopic map reorganisation may be a necessary but not sufficient condition for tinnitus perception (Elgoyhen et al., 2012; Norena and Eggermont, 2003), though clearly further work is required to elucidate precisely how this reorganisation may contribute to the phantom perception of tinnitus.

1.5 Behavioural Models of Tinnitus

It is essential to develop robust animal models of tinnitus to allow research into the condition, as the invasive recording techniques that can be used in an animal model allow researchers to track changes following noise exposure and allow rigorous testing of various drug manipulations (Kaltenbach, 2011). Until the late 1980s, progress in tinnitus research was slow. The lack of validated animal models of tinnitus is thought to have been a major factor in the slow progress (Shaikh, 2012). Jastreboff

et al. (1988) developed the first behavioural test to enable verification of the presence of tinnitus. By pairing electric shocks (an unconditioned stimulus) with the onset of periods of silence (conditioned stimulus), they trained water-deprived rats to lick from a spout only when continuous noise was present. When the unconditioned stimulus was removed and after administering salicylate to an experimental group, the animals were assumed to have tinnitus if they ceased the trained behavioural response quicker than control animals. There have since been slight variations on this method (e.g. Bauer and Brozoski, 2001; Heffner and Harrington, 2002), but for a long time this was the most common behavioural test for tinnitus in animals. Whilst this method does seem effective for detecting the presence of tinnitus, it has some drawbacks. Firstly, it is very time consuming, as it takes weeks to condition the animals to only lick during sound. Also, as it only measures the rate at which the conditioned response extinguishes, it doesn't allow the tracking of tinnitus over time. Finally, it requires attention and short-term memory resources, factors which can be heavily affected by various drugs and so restricts the types of manipulations that can be researched into using this method. Nevertheless, the development of this model formed the basis for research into the subjective condition of tinnitus.

Turner et al. (2006) overcame many of these problems by developing a simple test in rats which relies on a reflex, known as the whole-body startle. When an animal hears a startling sound, they contract their muscles in response. Muscles involved include the leg extensors which are involved in preparation for flight and can be measured by the animal pushing against the floor. Turner et al.'s (2006) test exploited the reduction of this response by the presentation of a gap in continuous noise just before the startle. This paradigm was based on the phenomenon of prepulse inhibition (PPI), whereby the startling stimulus is preceded by a quieter pulse as opposed to a gap, which also causes a diminished startle response (Hoffman and Searle, 1965). Turner et al. (2006) hypothesised that, following noise exposure, animals that were experiencing tinnitus would show deficits in gap detection when the frequency of the background noise was similar to that of their tinnitus, as the tinnitus would effectively 'play over' the gap and therefore cause reduced inhibition of the startle reflex (Fig 1.11) .

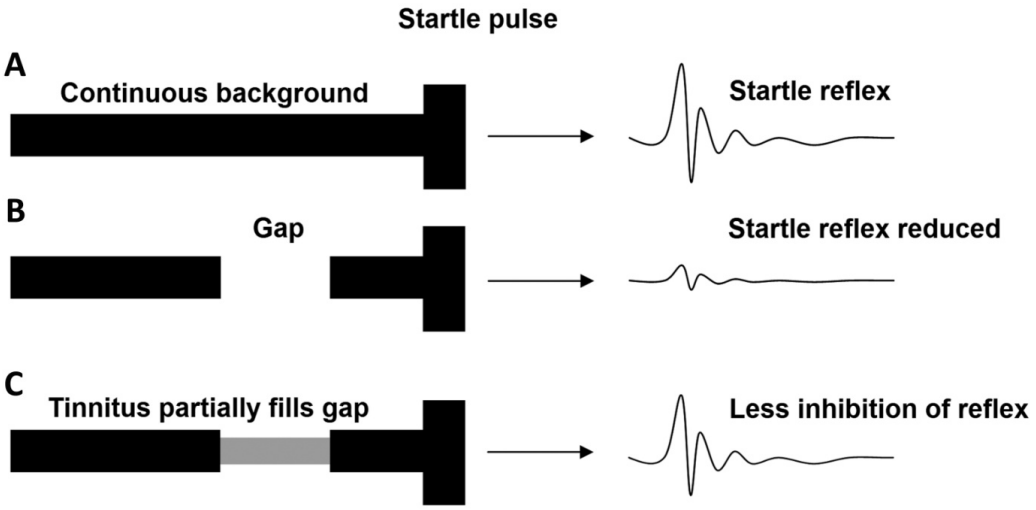

Fig. 1.11: Schematic of the gap detection test, adapted from Turner and Parrish (2008). A startling pulse in continuous background noise elicits a startle reaction (A). When a gap (50 ms) is presented before the startling pulse, this reduces the amplitude of the startle response (B). When an animal is experiencing tinnitus, it will have difficulty detecting the gap, as this will be partially filled in by the tinnitus, and will show less inhibition of the startle response (C).

Using this method, Turner et al. (2006) found that rats exposed to loud noise showed significantly reduced inhibition of the startle reflex only when the continuous noise was centred around 10 kHz, suggesting that the animals might be experiencing tinnitus within this frequency region. They also measured ABRs in the same animals to test hearing thresholds and see whether or not hearing loss could account for this deficit. The ABRs showed that hearing for most animals recovered, thereby ruling out hearing loss as a factor for diminished gap detection and leaving the researchers able to infer that the animals were experiencing tinnitus around the 10 kHz frequency.

Where the ABRs did not recover, a further deficit in gap detection ability was seen at the 16 kHz frequency, thereby implicating hearing loss as the cause of the deficit in this case (see Fig 1.12). The test was further validated by using Bauer and Brozoski's (2001) operant conditioning method of behavioural testing.

There are a number of benefits in using Turner et al.'s (2006) gap detection paradigm as a behavioural test for tinnitus. One key advantage is that it relies on an unconscious and innate reflex, which means that no lengthy or complex training is required. This also means that there are no memory or attentional demands, so drug manipulations can be used without having

to consider the cognitive effects that may affect performance. Furthermore, the factors that can affect this response, as well as the neural circuitry that mediates it, are well researched (Swerdlow et al., 2001). For example, the length of the gap and the delay between gap and startling stimulus can change the amount of startle response reduction (Leitner et al., 1993), as can particular drugs (Davis et al., 1993). Another benefit is that it is possible to repeat this test on many occasions in one animal, as it doesn't require the extinction of a particular behaviour. This enables the observation of the development of tinnitus over time (for instance, Turner et al. demonstrated that tinnitus did not develop until 8-9 weeks post-trauma).

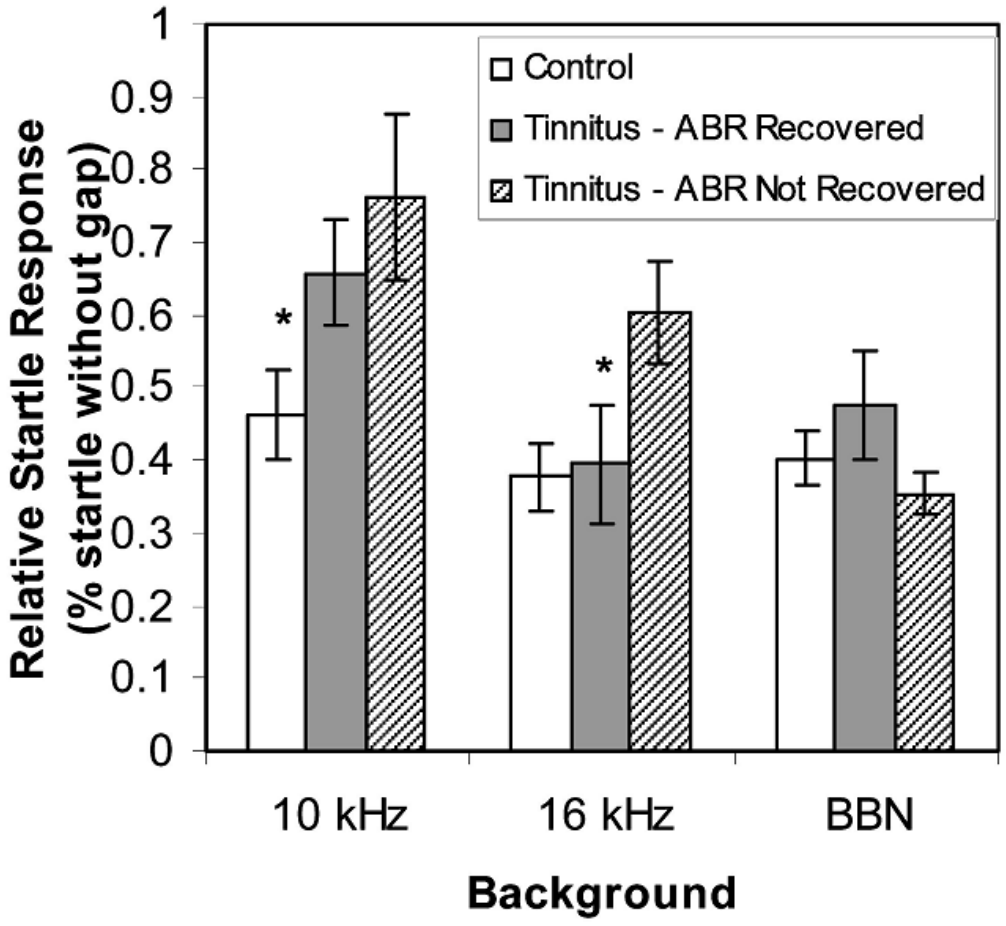

Fig. 1.12: The relationship between gap detection and ABRs following noise exposure. This highlights that gap detection deficits were present at the 10 kHz background frequency regardless of whether ABRs recovered. A further deficit was seen for the 16 kHz frequency in rats where ABRs did not recover. Asterisks indicate a significant difference in gap-induced PPI at that frequency. Adapted from Turner et al. (2006).

Although a number of studies have demonstrated whole-body startle PPI deficits in rats, only two studies have thus far used the method for measuring deficits in GPs (Dehmel et al., 2012a; 2012b). GPs are used in auditory research because they show relatively similar audiograms and tuning curves to humans (albeit their high-frequency hearing is better and their audible range is larger), meaning that they are well-suited to make cross-species inferences from data (Harrison et al., 1981). The problem is that they are not very active animals when required to perform behavioural tasks, possibly due to a supposedly natural resistance to authority or control (Heffner et al., 1971). In fact, it has recently been suggested that aversive and stressful stimuli (e.g. electric foot shocks) are essential for training GPs (Agterberg et al., 2010). Further to this, our early trials suggested that they quickly habituated to the startle and often didn't respond, despite increasing the volume of the startle eliciting stimulus. As a result of this, we developed a similar yet novel test using another startle response, known as the Preyer reflex (Bohmer, 1988).

The Preyer reflex is a flexion of the pinnae in response to a startling stimulus. Often used as a gross measure of hearing in rodents (Jero et al., 2001), the parameters that can modulate this response have been well researched and it has been shown to be affected by PPI (Cassella and Davis, 1986). The neural circuitry mediating this reflex has also been defined, mainly involving the cochlear nucleus, SOC and the IC in the auditory system, as well as areas of the reticular nucleus and facial motor nuclei (Li and Frost, 1996). These anatomical pathways are simple and form a useful basis for observing cellular and behavioural changes following various manipulations (Cassella and Davis, 1986). As the pinna response requires less muscle movement and exertion of energy, it was hypothesised that the GPs will show strong movements over a longer period than they do for the whole-body startle, thus presenting the Preyer reflex as a more reliable behavioural test for tinnitus in this species.

1.6 Aims of this Thesis

This thesis will document a novel development of Turner et al's (2006) gap detection test for use in GPs, using the Preyer reflex as the startle measure, where this behavioural test was used to confirm the presence of tinnitus in GPs. Recordings of SFRs were made in the IC, a midbrain auditory structure, to determine if there are differences between animals with tinnitus and those without following acoustic over-exposure. ABRs were analysed for changes in latency or amplitude in tinnitus and no-tinnitus animals, as changes have recently been demonstrated in tinnitus animals and also humans (Dehmel et al., 2012a; Gu et al., 2012; Schaette and McAlpine, 2011). Finally, neural recordings of gap detection ability were performed to establish whether changes seen in the behavioural test directly related to tinnitus or were merely some form of abnormal temporal processing occurring as a result of noise exposure.

CHAPTER 2

General Methods

2.1 Animals

Experiments were conducted on a total of 51 male and female pigmented GPs from an in-house colony. The animals weighed 300-500g at onset of behavioural testing. GPs were group-housed on a 12: 12 h light: dark cycle, and food and water were freely available. All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986, UK and the approval of the ethical review committee at the University of Nottingham, UK.

2.2 Behavioural Methods

Here I shall describe the methods for behavioural testing common to all the experiments performed within this thesis. Any methodological details which are specific to a particular experiment will be included within the relevant chapter.

2.2.1 Measuring Whole-Body Startle (WBS)

The ability of a GP to detect a gap in background noise - and consequently produce PPI - was initially assessed by quantifying whole-body movement in response to the startling stimulus. GPs were placed in a wire cage (310 mm x 155 mm x 155 mm) on a custom-made startle platform in a sound proof booth; GPs were not restrained and were free to move around the cage. The startle platform was connected to a load-cell (3 kg capacity; Model 1022, Vishay Tedea-Huntleigh, Basingstoke, UK) to measure the downward force applied to the platform following a startling

acoustic stimulus. The output from the load-cell was amplified by a factor of 1000 and recorded in Adobe Audition (Adobe Systems Incorporated, San Jose, CA) via a Tascam US-122 external sound card (44.1 kHz sampling rate, 16-bit resolution; TEAC Professional Division, USA). Synchronisation pulses were recorded simultaneously with the signal from the load-cell; pulses of different size denoted either a 'gap' or 'no gap' presentation. The signal was low-pass filtered at 200 Hz *post-hoc*.

2.2.2 Preyer Reflex

In addition to measuring the WBS response, a motion tracking camera system was used to monitor flexion of the pinna (Fig 2.1). The motion tracking system (Vicon Motion Systems, Oxford, UK) consisted of four infrared cameras. A reflective marker (4 mm diameter) was attached to each pinna using cyanoacrylate adhesive (Fig 2.2), and an additional marker was attached to a central point to determine the orientation of the animal. In initial trials, the central marker was place in middle of the back - in later trials, the centre of the head was used as a central reference.

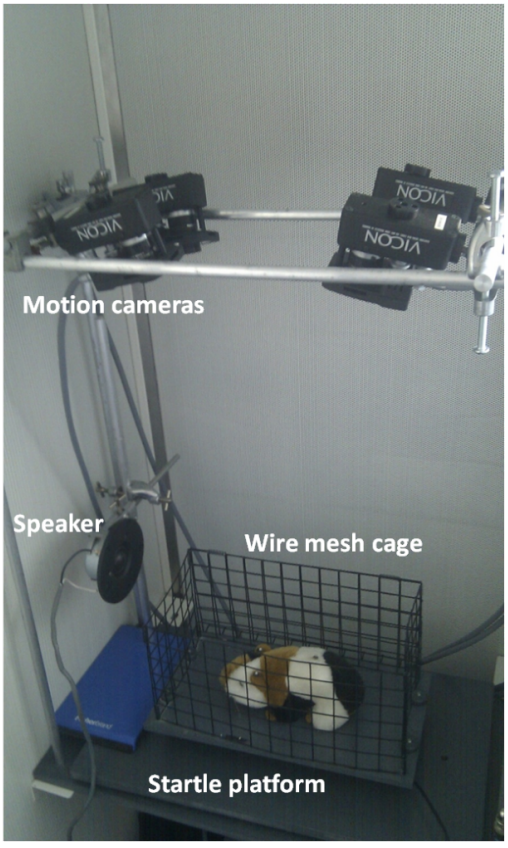

Fig. 2.1: The behavioural test setup, using motion tracking cameras and a startle platform to measure both reflexes.

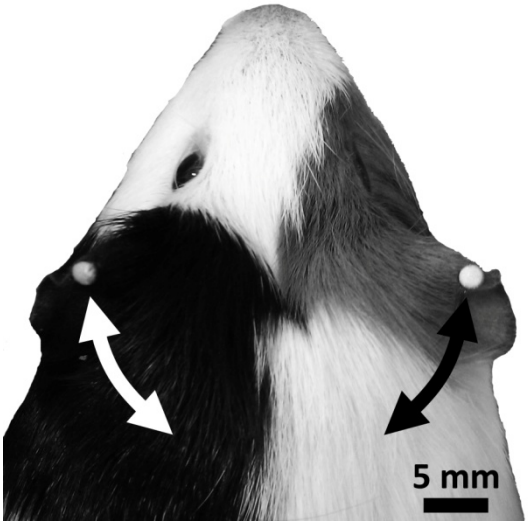

Fig. 2.2: A photograph showing the position of the reflective markers when fixed to the pinnae. Arrows indicate the direction of movement of the pinna in response to a startling auditory stimulus.

The motion tracking system used these markers to triangulate the position of the ears, and subsequently to track pinna movement during the presentation of startling stimuli. In order to track movement effectively, the system requires a minimum of two cameras detecting marker positions. The Vicon system was calibrated at 2-3 week intervals to ensure that the motion tracking cameras were able to correctly establish the positions of the markers; this involved using a static object (a flat calibration object with four reflective markers attached) to determine the lowest vertical level of the platform and a dynamic object (a t-shaped wand with two reflective markers attached) to define the range of movement. Triangulated marker positions were recorded at a sampling rate of 200 Hz using Vicon Workspace software and after each trial raw data (x, y, and z coordinates for each marker over the time-course of a trial) were exported to Matlab® (R2009b, MathWorks, MA, USA) for analysis.

2.2.3 Auditory Stimuli for Behavioural Testing

Auditory stimuli were standard 16-bit digital waveform files (.wav) files using Adobe Audition and presented through a single 25 mm loudspeaker (Peerless DX25, Tymphany, Hong Kong), via the Vicon motion tracking software to enable synchronisation of the onset of recording with presentation of auditory stimuli. Sound pressure level calibration was performed using a ½ inch free-field microphone (Brüel & Kjær Model 4165)

calibrated with a Brüel & Kjær Type 42 Sound Level Calibrator. The speaker was positioned at 18.5 cm above the startle platform and aligned with the front of the cage, on its midline. The position of the GP relative to the speaker did change between, and often within, trials because the animals were not restrained. Consequently, sound levels at the animal were not always constant.

The gap detection method of Turner et al. (2006) requires a continuous background noise; in this thesis, the background noise comprised either narrowband noise (2 kHz bandwidth) centred at 5, 9, 13 or 17 kHz, or broadband noise. Startling stimuli were broadband noise bursts (20 ms; rise/fall time of 1 ms). A single trial consisted of 10 presentations of the startle stimulus preceded by a gap, and 10 presentations without a gap (randomised order of presentation), delivered sequentially for a given background noise condition. The inter-stimulus interval (ISI) was optimised by pilot experiments to 15 or 24 seconds, leading to a single trial taking around 6 minutes and 30 seconds. The background noise was not switched off (apart from during the prepulse gap presentation) during a trial. A gap duration of 50 ms (rise/fall time of 2 ms) and a delay of 100 ms between the gap onset and the startle stimulus onset were selected for all GPs included in the present study. Previous work shows that the size of the WBS response is susceptible to variations in both gap duration and delay between the gap and startling stimulus; the values selected in this study were demonstrated to be optimal for maximising gap detection (Friedman et al., 2004; Leitner et al., 1993). In a single testing session, each background noise condition was presented once, in a randomised order, with ~2 min of silence between each trial. In pilot experiments, the startle stimulus was presented at either 105 or 117 dB SPL, in combination with background noise delivered at 70 dB SPL. In the main experiment the levels were chosen optimally for each GP using a sound level-dependency test (SLDT).

2.2.4 Sound Level-Dependency Test (SLDT)

To avoid saturation of whole-body or Preyer responses (whereby the sound is too loud to be inhibited by a prepulse), or habituation (whereby the sound is too quiet and the startle response is quickly diminished), an SLDT protocol was devised by which to best match the sound level of the background and startle stimuli to achieve optimal gap detection. To do

this, a number of combinations of startle sound levels (95, 100, or 105 dB SPL) and narrowband background noise (4-6 kHz) sound levels (55, 60, or 70 dB SPL) were presented, and PPI quantified. Narrowband background noise was selected for the SLDT, as it was found in earlier experiments that less PPI was elicited when presenting narrowband background noise, compared with BBN, and this was consistent with previous data from others (Turner and Parrish, 2008). The startle/background sound levels for optimal gap detection were selected based on the combination that produced the greatest magnitude of startle response and largest amount of PPI for each individual animal.

Each GP included in all experiments (other than pilot testing) was subjected to the SLDT, prior to commencing baseline testing. The introduction of the SLDT served a dual purpose: Firstly, determining whether GPs exhibited significant PPI enabled us to discard animals that did not, before undertaking time-consuming baseline testing. Secondly, by optimising gap detection at this stage, the probability of retaining robust PPI throughout the duration of behavioural testing was increased. SLDT, as a precursor to extended measurement of PPI, is important as the magnitude of the startle response can significantly decrease after noise exposure. This is more than likely due to a reduced ability to detect the startle-eliciting stimulus (Longenecker and Galazyuk, 2011). However, it should be noted that - owing to the improved reliability of detecting a sustained Preyer reflex (with consistently higher signal-to-noise ratios: see *Section 3.1.2*) over WBS in our pilot experiments - the SLDT focussed on determining optimal sound levels for the Preyer reflex. As a result, the levels for these experiments may have been suboptimal for the simultaneously-measured startle response.

2.2.5 Comparing PPI of WBS and Preyer Reflexes

Gap detection was assessed in 12 GPs prior to any tinnitus induction, using both Preyer and WBS reflexes, and the data obtained for each GP examined for robust and consistent evidence of PPI (*Section 3.2*). These GPs were first subjected to the SLDT, before undergoing testing sessions over a period of two weeks (minimum of three and a maximum of six sessions). Based on data acquired in our initial pilot experiments (see *Section 3.1*), an ISI of either 15 or 24s (necessary to prevent short-term habituation) and a background noise bandwidth of 2 kHz, were used as optimal values for maintaining reliable startle responses and PPI.

2.2.6 Analysis of Behavioural Data

Raw data, encompassing x, y and z coordinates for each of the markers, were exported from the Vicon motion tracking software into comma-separated value (.csv) files. Preyer reflex data were then analysed using custom-written Matlab® software. From these, the absolute positions and Euclidean distance between the markers were calculated. The centre point marker was not used for analysis; this marker was required solely for the purpose of identifying the orientation of left and right pinna markers. Data exported from Vicon were then matched to synchronisation pulses (square waves that marked where each startle pulse occurred and whether there was a gap preceding) included in the sound files. Each individual startle response was plotted to enable manual adjustments for an occasional computer lag (caused by Vicon) between the synchronisation pulse and the sound presentation (up to 1s) - any lag that was present simply meant that all the data were shifted by a fixed amount (lags did not vary throughout a trial). In some trials, marker tracking was disrupted by GP movement occluding the markers from the camera field-of-view. Consequently, the analysis software was designed to remove startle presentations in which occlusion errors (frames during which the markers were not visible) occurred during the 500 ms recording epoch, to avoid distorting the data. The magnitude of the Preyer reflex was calculated for all error-free trials as pinna displacement (change in peak-to-peak distance between right and left pinnae).

The WBS reflex was quantified as the root mean squared (RMS) amplitude of the startle-evoked response (as measured by the load cell) divided by the RMS amplitude immediately prior to presentation of a startle to give an 'amplitude ratio' value (epochs for calculating the RMS values were 150 ms). This accounted for spontaneous movement on the platform and the RMS was used for this reflex as a result of pilot trials highlighting poorer SNR of the WBS (and therefore a less detectable signal). The RMS of the startle evoked response was calculated from 50 ms to 200 ms after the startling stimulus, i.e., the time during which the WBS occurred, in order to avoid any background noise confounding the signal.

Outliers for both Preyer and WBS reflexes greater than two standard deviations from the mean were removed. For each background frequency, the mean displacement (Preyer reflex) and mean amplitude ratio (WBS) for

'gap' and 'no-gap' startle data (10 startle presentations for each condition, per trial) was calculated. A percentage difference between 'gap' and 'no-gap' data was then calculated and PPI was expressed as a percentage decrease in response when a gap was presented, compared with the 'no-gap' condition. Data from all sessions were pooled and the statistical significance of PPI was determined using a Wilcoxon rank-sum test to a 95% confidence rating for each GP at each background frequency. Finally, data from all GPs were pooled according to background noise condition and statistical significance was compared for each reflex, with a two-way ANOVA and Bonferroni *post-hoc* analysis. The variability of PPI for each reflex was assessed with a Coefficient of Variance test.

2.3 Induction of Tinnitus

2.3.1 Sodium Salicylate

The effects of sodium salicylate on PPI of the Preyer reflex and WBS were investigated in a subset of GPs ($n = 4$). Mean baseline PPI was assessed in each GP (minimum three sessions / maximum six sessions over two weeks), prior to administration of sodium salicylate (350 mg kg^{-1}; i.p.) dissolved in saline. Behavioural evidence of tinnitus and neurophysiological effects of salicylate, when administered at this dose, have been demonstrated previously in GPs (Norena et al., 2010). PPI was subsequently measured at 2 h and 5 h post-injection, and again at 72 h to establish whether PPI had recovered to baseline levels. Data from all animals were pooled and the effects of salicylate on the Preyer and WBS responses were assessed statistically for each background noise condition at each time point with a two-way ANOVA and Bonferroni *post-hoc* test. It has previously been demonstrated that the significant decrease in amplitude of the WBS following noise exposure, owing to reductions in hearing thresholds, may render changes in PPI difficult to interpret (Lobarinas et al., 2013). To account for this in our interpretation, raw amplitudes for both reflexes were analysed before and 2 h following sodium salicylate injection.

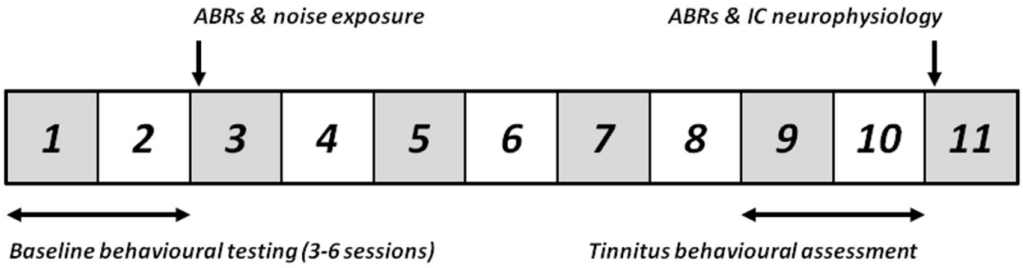

Fig. 2.3: The experimental timeline for noise-exposed GPs. Baseline behavioural testing was performed during the first two weeks prior to noise exposure. ABRs were collected immediately before and after noise exposure. GPs were then retested for evidence of tinnitus at weeks 9 and 10 (7 and 8 weeks post-exposure). ABRs were repeated to assess hearing loss and IC recordings were then performed.

2.3.2 Noise Exposure and ABRs

The timeline for baseline behavioural testing, subsequent noise exposure, behavioural assessment of tinnitus, ABR recording and IC neurophysiology is illustrated in Fig 2.3. Baseline PPI of the Preyer reflex was measured in each GP over a two-week period (minimum of three and a maximum of six testing sessions). GPs that did not exhibit significant PPI in all background noise frequency conditions were excluded from the study - this was usually no more than one in five of the GPs tested. Following baseline data collection, GPs ($n = 24$) were unilaterally exposed to loud noise, in order to trigger the onset of tinnitus pathology - 12 of these animals were also used in *Comparing PPI of WBS and Preyer reflexes* (see *Section 3.2*).

For the noise exposure and ABR measurements GPs were anaesthetised with Ketamine (50 mg kg^{-1}, i.p.) and Xylazine (10 mg kg^{-1}, i.p.), supplemented with further administrations of a mixture of Ketamine and Xylazine, in a ratio of 15:2 (i.m.), throughout the procedure. Core body temperature was monitored throughout and maintained at 38°C $\pm$ 0.5°C using rectal probe linked with a homeothermic heating pad (Harvard Apparatus Ltd., Edenbridge, UK). ABRs were recorded using custom in-house software prior to and immediately after noise exposure to determine hearing thresholds. Once anaesthetised, hypodermic needles were inserted through the skin to act as recording electrodes over the right and left mastoids, and a reference needle inserted at the vertex point. ABR recording electrodes were connected via a Tucker Davis Technologies (TDT) Medusa headstage amplifier (Alachua, FL, USA) to a TDT System 3 interface and checks were

performed to confirm that impedances were low. If impedances were too high, the recording needles were replaced and moved slightly until satisfactory impedances were obtained. Auditory stimuli for ABRs were presented binaurally via 25 mm loud speakers (Peerless DX25). In order to maintain a closed sound system, polyethylene tubes (diameter of 20 mm) were connected to the speakers and placed around each ear to form a seal (Fig 2.4). GPs were placed inside a sound-attenuating chamber and remained there for the duration of the ABR recording and acoustic trauma. The door to the chamber was opened periodically to check for areflexia and administer anaesthetic as required.

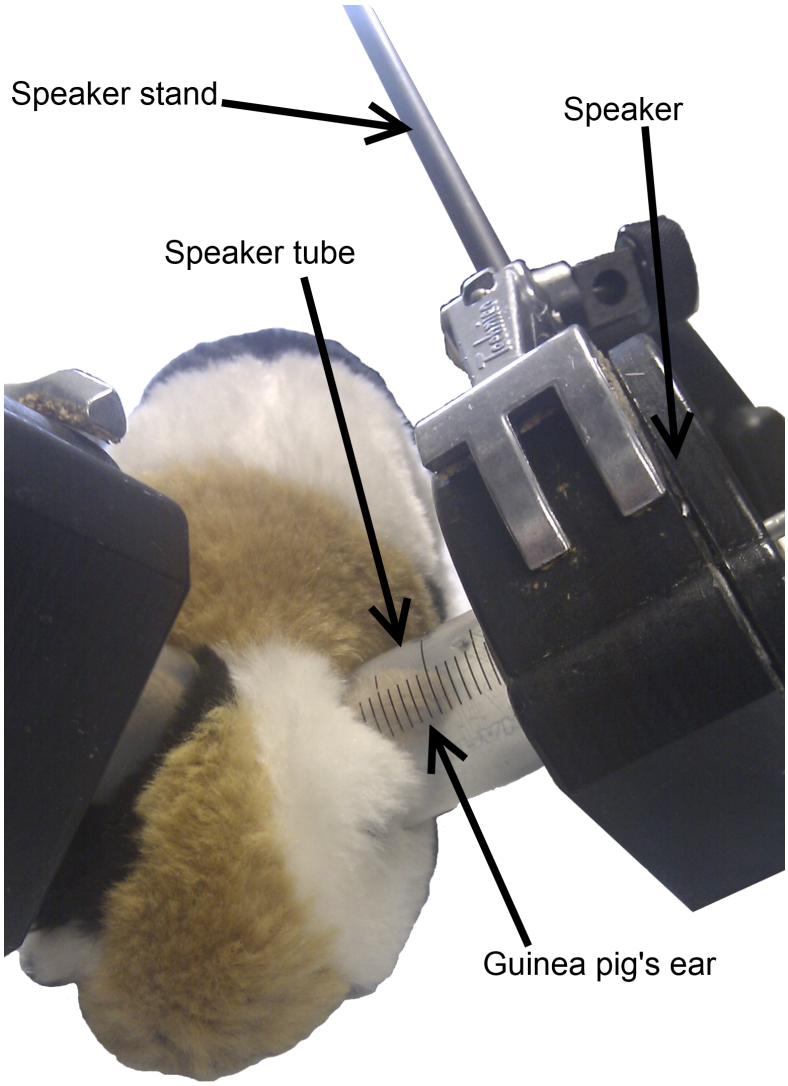

Fig. 2.4: The circumaural speaker arrangement for noise exposure and ABR recording. The seal created by the speaker tubes, combined with cotton wool placed in the tube of the right speaker, allowed for unilateral noise exposure, leaving the right ear unexposed.

2.3.3 Recording ABRs

ABRs were recorded independently for left and right ears in response to ipsilaterally-presented pure tone bursts of 5, 10, and 15 kHz (5 ms duration; rise/fall time of 1 ms), using custom-written software. Tones were presented at progressively decreasing sound levels (5-10 dB SPL steps; starting from 90 dB SPL) until an auditory-evoked response threshold was determined based on the absence of a discernible wave IV from the ABR signal. The signal from the recording electrodes was amplified 25000 times and filtered at 300 Hz - 1 kHz, with a sampling duration of 20 ms. After pre-trauma thresholds had been determined, the right speaker was electrically disconnected and the polyethylene tube was plugged with cotton wool. The right pinna was then folded over, and the plugged tube placed over the ear. This served to reduce the risk of incurring hearing deficits in the right 'control' ear. The success of this protection was later confirmed by the absence of a hearing deficit (as identified using ABRs) in the right ear following noise exposure. The left ear was then exposed to narrow band-passed noise bursts (duration of 500 ms and ISI of 200 ms; centre frequency 10 kHz; bandwidth 1 kHz), presented at 120 dB SPL, for 1 h. These values were selected in order to produce a hearing loss which was somewhat reversible. Control animals were unexposed and only used for neurophysiological recording.

2.4 Classification of Noise-Induced Tinnitus

GPs were initially classified as 'tinnitus' (T) animals if they fitted the following criteria: (1) A complete absence of PPI, i.e., 0%, was required for at least one background noise frequency 7-8 weeks after acoustic trauma. (2) Hearing level (HL) thresholds - as indicated by ABRs recorded at 8 weeks (see below) - had to recover to within 20 dB HL of pre-acoustic trauma thresholds at the ABR frequency closest to the behavioural background frequency at which the PPI deficit was observed. This criterion for the recovery of hearing thresholds was selected as it is conventionally used as the tolerance in human audiograms for "normal" hearing (Houston et al., 1999). GPs that did not exhibit any abolition of PPI, or with PPI abolition accompanied by a permanent hearing loss ($>$ 20 dB HL) after 8 weeks, were assigned to a 'no-tinnitus' (NT) group. This was done to ensure that the T group was not subject to caveats such as

the inability to hear the background carrier due to hearing loss. However, this means that some of the animals in the NT group that showed reduced PPI (along with hearing loss) may have also been experiencing tinnitus. For this reason, when analysing the data, an alternative classification of tinnitus based on significance testing was also applied, in order to minimize any bias caused by the definition of tinnitus used.

The alternative classification was similar to that used by others (e.g. Dehmel et al., 2012a; 2012b; Zhang et al., 2011), whereby a two-way ANOVA with a Bonferroni *post-hoc* test ($p < 0.05$) was applied to the behavioural data to determine whether any significant changes in PPI were evident 7-8 weeks following noise exposure, compared to baseline PPI values. For this alternative criterion, change in PPI was expressed as a ratio. For example, if a GP demonstrated 30% PPI before noise-exposure, this was converted into a ratio using the following formula: (100 - % PPI)/100. In the above example, this would equate to a ratio of 0.7. PPI at the 7-8 week time point was also calculated as a ratio and then expressed as a change in ratio compared to baseline (before/after) for the purposes of displaying the data. Therefore, a value of 1 would indicate no change in PPI from baseline, whereas a value lower than 1 would indicate a reduction in PPI and a value higher than 1 would suggest improvement in PPI. Neural data were then reanalysed under this new behavioural classification.

2.5 Neurophysiological Recording

2.5.1 Surgery

GPs were anaesthetised with urethane (0.5 g kg^{-1} in 20% solution, i.p.; Sigma), Ketamine (40 mg kg^{-1}, i.p.) and Xylazine (8 mg kg^{-1}, i.p.), supplemented with further administrations of a mixture of Ketamine and Xylazine, in a ratio of 15:2 (i.m.), throughout the procedure to maintain a constant state of areflexia. A single bolus injection of atropine sulphate (0.06 mg kg^{-1}, s.c.) was administered to suppress bronchial secretions. ABRs were recorded in noise-exposed GPs (using the method described previously) in order to determine hearing status. GPs were then tracheotomised and respired with 100% oxygen to maintain normal end-tidal CO_2 levels within a range of 28-38 mm of mercury (0.04 - 0.05 atmospheres). Core body temperature was monitored throughout and maintained at 38°C $\pm$ 0.5°C using rectal probe linked with a homeothermic

heating pad (Harvard Apparatus Ltd., Edenbridge, UK). Animals were placed in a stereotaxic frame, with hollow plastic speculae replacing the ear bars, inside a sound-attenuating chamber. The posterior fossa was opened to release the pressure in order to reduce respiratory pulsations of the brain. The bullae were vented on both sides using polyethylene tubes (0.5 mm diameter) to equalize pressure across the tympanic membrane. The tubes were inserted through a small hole in the ethmoid bone and sealed with petroleum jelly (Vaseline ®). Craniotomies were performed over the right and left IC (∼4 mm diameter). Electrodes were positioned above the IC stereotaxically, using coordinates described in the guinea pig atlas of Rapisarda and Bacchelli (1977). This involved determining the point between 11.4 mm back from bregma and the interaural line formed by the speculae that represented ear bar zero. For each side this was point was marked 3 mm away from the sagittal suture.

The dura mater was excised and pairs of electrodes were slowly lowered down at 10° to the vertical plane through the cerebral cortex on each side - such an angle was required to fit both electrodes in at the same time. The exposed cortex was kept moist with intermittent application of 0.9% sodium chloride solution. When necessary, the brain surface was covered in 1.5% agar if stabilisation of recording was required. Search stimuli (noise or tone pips) were presented while lowering the electrodes so that any auditory responses could be identified. When correctly positioned, electrodes encountered auditory driving at a depth of ∼2.8 mm from the cortical surface.

2.5.2 Single-Unit Recording

Extracellular single-units (filtered between 600 Hz and 3 kHz) were recorded simultaneously from right and left IC using two pairs of glass-coated tungsten electrodes (Bullock et al., 1988). These electrodes were made and assembled in-house. For each pair, the tungsten electrodes (∼1-3 MΩ impedance) were attached to a single circuit board using araldite and electroconductive paint, with tips aligned and separated by ∼200 μm, and advanced simultaneously. Electrodes were connected via a TDT Medusa headstage amplifier to a TDT System 3; on-line data collection was facilitated with Brainware (software developed by J. Schnupp, University of Oxford, UK).

2.5.3 Auditory Stimuli & Recording Procedure

Auditory stimuli were delivered diotically through sealed acoustic systems, composed of Etymotic ER-4 earphones (Etymotic Research, Inc., IL, USA), inserted into the hollow speculae. A search stimulus (generated by TDT System 3) was utilised to search for neuronal unit activity in the IC; this comprised a wideband noise (duration 50 ms), gated on and off with cosine-squared ramps lasting 8 ms and a repetition period of 300 ms. Search stimuli were selected to encompass a broad frequency range, thus maximising identification of auditory-evoked neuronal single-unit activity. Once an IC cell had been isolated, a frequency-response area plot (FRA) was generated by presenting pure tone bursts (50 ms; 200 ms repetition rate) over a range of frequencies (50 Hz - ∼25 kHz randomly interleaved at 0.25 octave intervals) and sound levels (attenuations of 0-95 dB in 5 dB steps, from a maximum of ∼100 dB SPL). FRAs were followed by 100 seconds of recording in silence to obtain spontaneous firing rates. Auditory stimuli used in the neural gap detection experiments are described below.

2.5.4 Auditory stimuli for gap detection thresholds

In order to determine the minimum gap detection thresholds (MGDTs) of each single-unit, a gap detection protocol was run following each FRA. Stimuli were delivered using the method described above. These comprised 200 ms of stimulus, followed by a fixed-length gap duration of 1, 2, 4, 8, 10, 20, 50 or 75 ms and another 50 ms of stimulus after the gap. Each gap duration was presented 20 times (with a 700 ms repetition rate) in ascending order, from shortest to longest. Three different stimuli were used - BBN, narrowband noise (NBN) and pure tones. The frequencies used for the narrowband noise matched those used in the behavioural test. The pure tone frequency was selected as the characteristic frequency (CF) of each cell. The sound level used for each animal was the optimal level for the behavioural test, as identified by the SLDT. All gap durations were presented for each type of background.

2.6 Analyses

2.6.1 Analysis of ABRs

In light of previous research linking changes in the relative sizes of ABR peaks with tinnitus (Dehmel et al., 2012; Gu et al., 2012; Schaette and McAlpine, 2011), ABR signals were examined for changes in the component peaks. Pre-noise exposure and 8-week time-point ABRs were compared in response to 5, 10, and 15 kHz tone bursts presented at 70 dB SPL. Since GPs were unilaterally exposed to noise, the ABRs recorded on the right (unexposed) side were able to be used as a within-animal control. This allowed us to gauge the degree of variability between sessions that could occur in a disparate series of ABR recordings, as a result of factors such as depth of anaesthesia or electrode placement. First, ABRs were inspected for shifts in absolute latency and for changes in the inter-peak latencies that might be indicative of pathology at specific stages of the ascending auditory pathway. Previous work demonstrates a clear relationship between ABR latency and sound sensation level (SL): sounds of increasing audibility result in progressively shorter latencies (Prosser and Arslan, 1987). By comparing ABR waveforms collected in response to a suprathreshold stimulus (70 dB SPL), the aim was to avoid increased ABR latencies that might result from a hearing loss. This was not a problem in T animals experiencing only a small hearing loss ($\leq$ 20 dB HL in all cases), where the 70 dB SPL stimulus was equivalent to at least 20 dB SL, but may have contaminated latency analysis in NT animals, as a positive latency shift could be caused by reduced audibility of a 70 dB SPL stimulus. Next, ABRs were inspected for changes in amplitude; the absolute amplitudes of peaks in the ABR signal are not reliable, as variables such as depth of anaesthesia or electrode placement seem to affect signal magnitude. Consequently, relative changes in the peak amplitudes between different component waves of the ABR were assessed, comparing pre- and post-exposure ratios.

2.6.2 Analysis of Spontaneous Firing Rates

The characteristic frequency (CF) of each single-unit was identified in real-time. Characteristic frequencies were defined as the frequency at which the lowest sound level was required to produce an evoked response, as determined by the frequency response area. Cells were selected based on

a short latency and v-shaped tuning curves, indicative of CNIC (Aitkin et al., 1975). It is important to note that these are not the only frequency response types found in CNIC (for a detailed analysis, see Palmer et al., 2013), so some population bias may have been present in the data. However, cells selected were restricted to these types of typical responses in order to ensure that cells were only recorded within CNIC, as no histology was performed to confirm this. Spontaneous firing rates (SFRs) were expressed as spikes-per-second, calculated by dividing the total number of spikes during silence by the stimulus duration in seconds (100). SFRs were first compared between control, T, and NT animals - regardless of hemisphere - and statistically assessed with a Kruskal-Wallis non-parametric test and Dunn's *post-hoc* test. Single-units were then sub-divided according to recording side and their SFRs were compared statistically between groups on the same side, e.g. left-T vs. left-NT vs. left-control, with a Kruskal-Wallis test and Dunn's *post-hoc* test, or between sides within a group, e.g. left T vs. right T, with a Mann Whitney test. A multi-factor repeated-measures statistical test was deemed inappropriate as the data were not normally distributed.

2.6.3 Analysis of Gap Detection Thresholds

Data were analysed in 5 ms time bins. MGDTs of single-units were defined as the minimum gap duration where a significant increase in firing ($>$ 2 standard deviations above the mean firing within the preceding 50 ms, as well as a response of at least 3 spikes within 20 repeats) could be detected following the onset of the post-gap noise. While Matlab® (R2009b, MathWorks, MA, USA) scripts were used to automate analysis, each cell was checked visually (using the Brainware software to visualise responses) to determine the MGDT. Each single-unit was analysed under three categories - BBN minimum gap detection thresholds, the NBN condition that fell within 1 kHz of the CF of each single-unit, and pure tone MGDTs. If a unit did not respond strongly to the first 200 ms of stimulus presentation, it was discarded. Units were also discarded if they exhibited a long ($>$ 20 ms) latency, as these may not have been recorded from CNIC. A small subset of units exhibited offset responses only, i.e. they suppressed their firing during the presentation of auditory stimuli. In these units, MGDTs were determined solely by visual inspection, using a discernible increase in firing around the time of the gap as an indication that the gap had been detected.

MGDTs across animals (but within experimental groups) were expressed as a percentage of responding units for each gap duration. NBN was not useable for any units which did not have CFs within 1 kHz of any of the narrowband frequencies, as the further the CF of a unit is from the frequency of the stimulus, the worse its gap detection is. This is supported by research in humans showing that MGDTs are strongly affected by the width of an auditory filter (Shailer and Moore, 1983), so including units for analysis with stimuli too far from their CF could affect our estimates of MGDT. This phenomenon was evident for single-units in our data and, as a result, the number of units obtained was lower for the NBN condition than the other two conditions. Mean and median MGDTs were compared for control, NT and T animals for each background stimulus condition. A Kruskal-Wallis test and Dunn's *post-hoc* test was performed as a measure of significance within each background noise condition.

CHAPTER 3

Development of a novel behavioural approach to detecting tinnitus in the guinea pig

While the gap detection test of Turner et al. (2006) is widely used in rats and mice, as mentioned previously, only two studies (prior to the publication of these data in Berger et al., 2013) had successfully used the method for measuring deficits in guinea pigs (Dehmel et al., 2012a; 2012b). Here, it is demonstrated that in guinea pigs the Preyer reflex is a more reliable and consistent measure of PPI than WBS. The results reported here were obtained from a number of pilot studies designed to determine the optimal parameters for applying the gap detection method to the Preyer reflex, followed by the main experiment testing the efficacy of the test in detecting tinnitus in guinea pigs.

3.1 Pilot Experiments

3.1.1 Habituation of Startle Response

Pilot experiments were conducted on control animals to examine the effects of randomly varying the ISI between startle stimuli on the magnitude of startle responses, and habituation of PPI. In these pilot experiments two different ISI values were presented in random order within a single trial. The values used were 9 or 15s, 12 or 20s, and 15 or 24s (n = 3 GPs). In the same animals, the effects of varying the bandwidth of the narrow-band background noise (either 1 or 2 kHz) were also assessed.

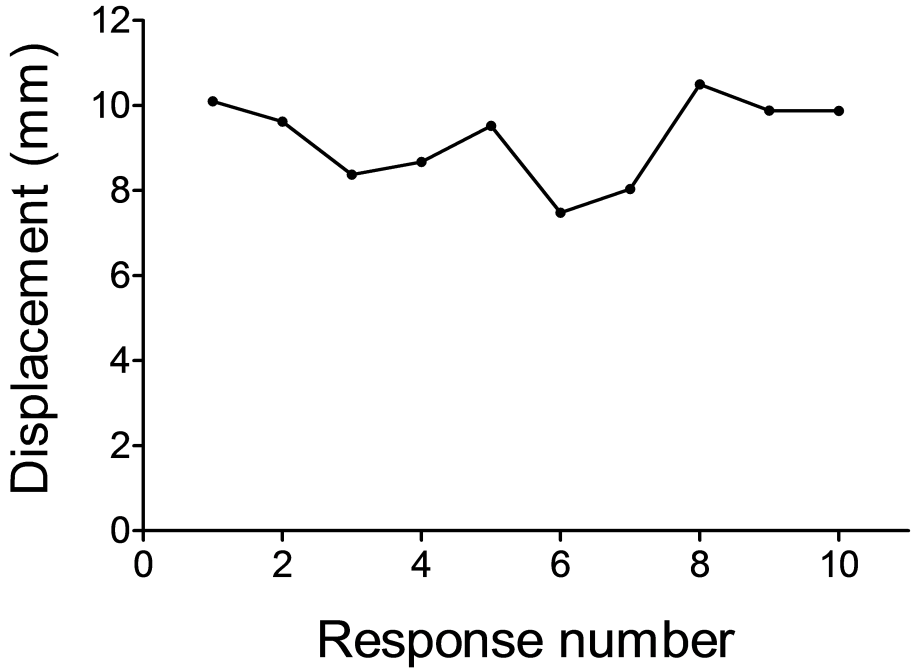

Fig. 3.1: Example from one GP of Preyer reflex amplitudes over the course of one trial, in response to startling stimuli with no gap preceding and using 15-24s ISIs. With these ISIs, no clear short-term habituation was evident and response amplitudes were consistent.

Startle responses were most consistent in amplitude (and habituation was consequently least) with an ISI of 15 or 24s, and a noise bandwidth of 2 kHz. An example of Preyer responses over the course of a trial is shown in Fig 3.1.

It is perhaps not surprising that the longest ISI yielded the least habituation. Moreover, it is plausible that an even longer ISI would result in a further reduction of habituation. However, the duration of a testing session is a limiting factor, as GPs can become stressed if they are solitary for a long time in an unfamiliar environment (Sachser et al., 2007). As a consequence, ISI values randomly varying between either 15 or 24s were selected as the optimal condition and testing was limited to one hour per session. These ISIs were similar to those used in Leitner et al. (1993) and were randomly varied to prevent anticipation of the startling stimulus.

The potential confound of either reflex habituating to startle stimuli, according to the number of trials per week, was further explored in two groups of GPs, over a five-week period: Group One was tested 2-3 times

per week (n = 3), while Group Two was tested once a week (n = 5). For both groups, an ISI of either 15 or 24s was used. No clear changes in the average magnitude of WBS and Preyer reflex startle responses were evident in either group of GPs, indicating that - when tested up to three times per week - animals did not habituate to the startle stimulus on an inter-trial timescale. The only habituation observed was within a single trial where the WBS to the first few stimuli tended to be bigger than the subsequent responses.

3.1.2 Signal-to-Noise Ratios and Variability Between Reflexes

Signal-to-noise ratio (SNR) was compared for each reflex in the long-term (5-week) habituation groups of GPs, described in the previous section (n = 8). This was calculated by taking the mean peak-to-peak signal after startle presentation (for all 'no-gap' startle presentations) from the mean peak-to-peak signal before (500 ms epoch for each). This calculation was performed across all trials for both reflexes. The SNR was calculated as 20 log(signal/noise) and expressed as dB. This process was repeated for each background noise condition, and the difference between Preyer and WBS calculated for all frequencies across all animals. The mean SNR ($\pm$ SEM) of the Preyer reflex (29.2 $\pm$ 6.2 dB) was substantially higher than the SNR for WBS (21.4 $\pm$ 3.8 dB), which equated to $\sim$8 dB improvement in SNR when measuring the Preyer reflex. Further to this, the variability of the startle amplitude for both reflexes was assessed with a coefficient of variance test; Preyer reflex responses exhibited $\sim$50% less variability than WBS.

3.2 Comparing PPI of WBS and Preyer Reflexes

Gap detection and PPI were assessed in a group of GPs (n = 12) by measuring the WBS and Preyer reflexes simultaneously. Representative examples of raw recordings of the responses to a startling sound are illustrated in Figure 3.2. These plots are typical of the recordings obtained for each reflex, and highlight the superior SNR and consistency (i.e., minimal variability) of pinna displacement measurements (Fig 3.2A) versus those acquired from measurements of WBS (Fig 3.2B). Raw signals for all 'gap' stimulus presentations within a trial were pooled and mean RMS and pinna displacement plots derived for WBS and Preyer reflexes respectively;

the same process was applied to 'no gap' presentations. PPI was then quantified for a session for each background noise condition (as described in *Section 2.6.1*).

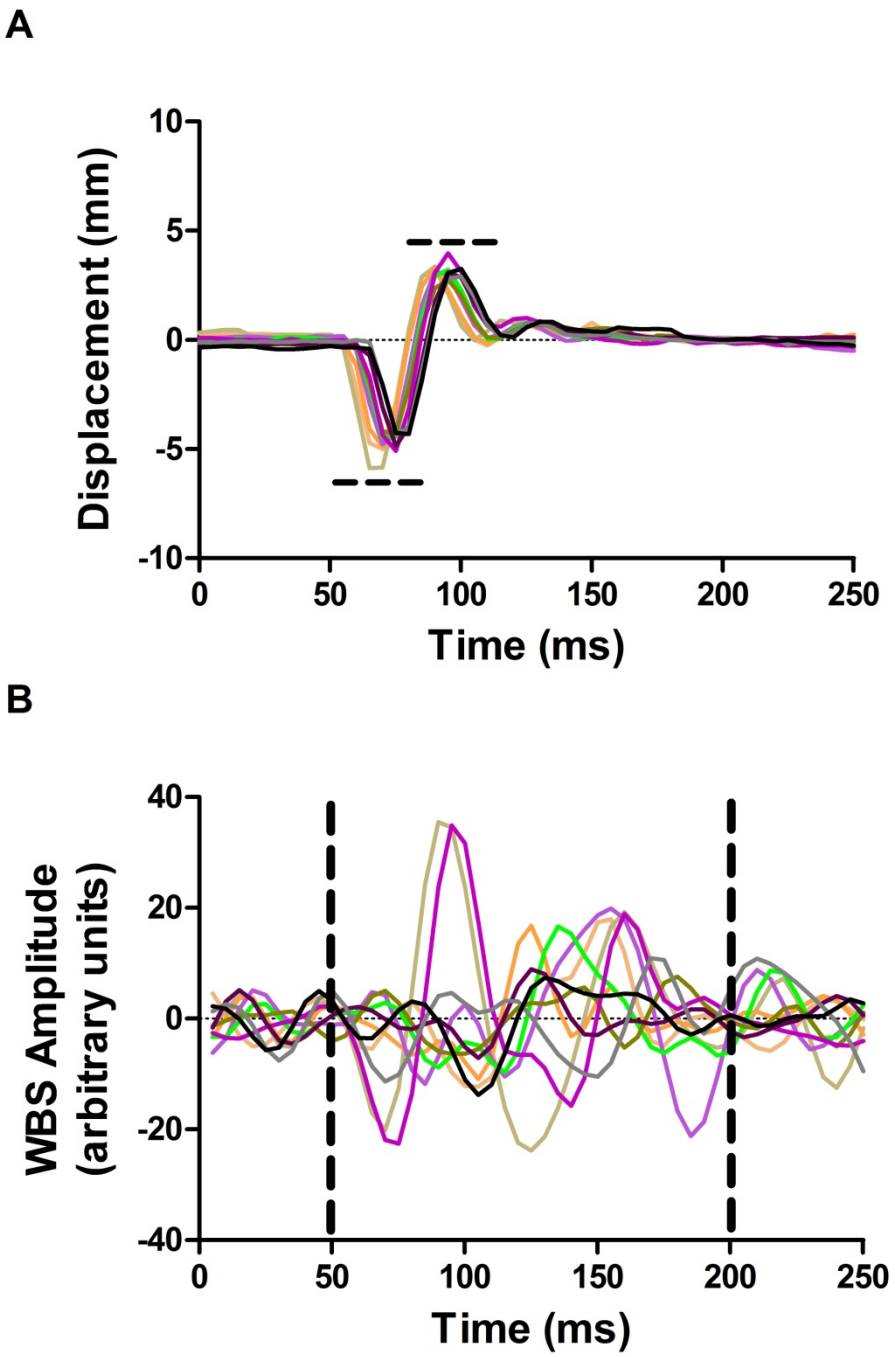

Fig. 3.2: Representative raw traces of 'no gap' startle stimulus presentation (n = 10) overlaid for the Preyer reflex (A) and the WBS (B), taken from a single trial for one guinea pig (BBN background carrier at 70 dB SPL, with a 105 dB SPL startling stimulus). Dotted lines indicate the window of analysis for WBS and peak-to-peak measurement for Preyer reflex.

PPI was assessed in each GP over 3-6 testing sessions. First, the SLDT determined whether a GP was capable of detecting a gap (and the sound levels at which this was best achieved); each animal then underwent testing sessions to obtain significant PPI. In some cases, significant PPI was obtained in three sessions (Wilcoxon rank-sum test; $p < 0.05$), but in other animals additional sessions were required to achieve significance (no more than six sessions - over a period of two weeks - were conducted to avoid the possible confound of habituation).

All 12 GPs exhibited significant PPI of the Preyer reflex at all background noise frequencies ($p < 0.05$). By contrast, significant PPI of the WBS reflex was only apparent at all frequencies in four GPs (see Table 3.1). In some cases, this was due to a complete absence of PPI at a given frequency, and in others to a much higher degree of variability in the WBS response. This was confirmed by a coefficient of variance test; the mean variation (for 'no gap' presentations across all frequencies/animals) of the WBS reflex was 63%, whereas the coefficient of the Preyer reflex was 39%.

GP	WBS (% PPI)					Preyer (% PPI)				
	BBN	4-6 (kHz)	8-10 (kHz)	12-14 (kHz)	16-18 (kHz)	BBN	4-6 (kHz)	8-10 (kHz)	12-14 (kHz)	16-18 (kHz)
1	83	31	31	45	54	34	38	24	30	36
2	79	45	46	68	55	66	22	35	41	45
3	71	33	28	37	35	22	21	25	16	23
4	65	38	59	66	72	24	28	19	26	30
5	73	20	-10	13	20	35	32	13	36	22
6	75	37	43	50	57	20	22	13	13	12
7	57	54	21	50	36	45	23	18	30	31
8	26	-8	-12	-23	-14	24	26	21	21	17
9	54	1	8	8	40	15	24	23	28	25
10	69	-14	46	33	60	45	34	37	36	60
11	51	68	48	51	50	36	48	23	46	35
12	59	4	19	10	5	17	15	23	15	19

Table 3.1: Percentage PPI of the WBS and Preyer reflexes at each background noise frequency (BBN, 4-6 kHz, 8-10 kHz, 12-14 kHz, 16-18 kHz), expressed as % PPI, are shown for all GPs ($n = 12$). Shading indicates significant PPI values, as determined with a Wilcoxon rank-sum test ($p < 0.05$). All GPs demonstrated significant PPI of the Preyer reflex, whereas only four GPs (GP2, GP3, GP6, and GP11) showed significant PPI of the WBS reflex at all frequencies.

When PPI data were pooled across animals and analysed statistically with a two-way ANOVA, significant differences were detected in both background noise centre frequency ($F_{(4,4)} = 4.48$, $p < 0.01$) and reflex type ($F_{(1,4)} = 11.86$, $p < 0.01$) variables, and a significant frequency by reflex interaction was also observed ($F_{(4,55)} = 4.00$, $p < 0.01$). *Post-hoc* analysis indicated significantly higher PPI of the WBS reflex in the BBN condition, compared with the Preyer reflex (t = 4.92, $p < 0.01$), but no significant differences were apparent at other frequencies (Fig 3.3). However, given the limited number of animals demonstrating significant PPI of WBS at all frequencies and the greater range in magnitude of PPI, the WBS measure did not appear to be optimal for determining gap detection abilities across a range of frequencies, and therefore would be less effective for detecting tinnitus perceived as tonal rather than as a broadband noise.

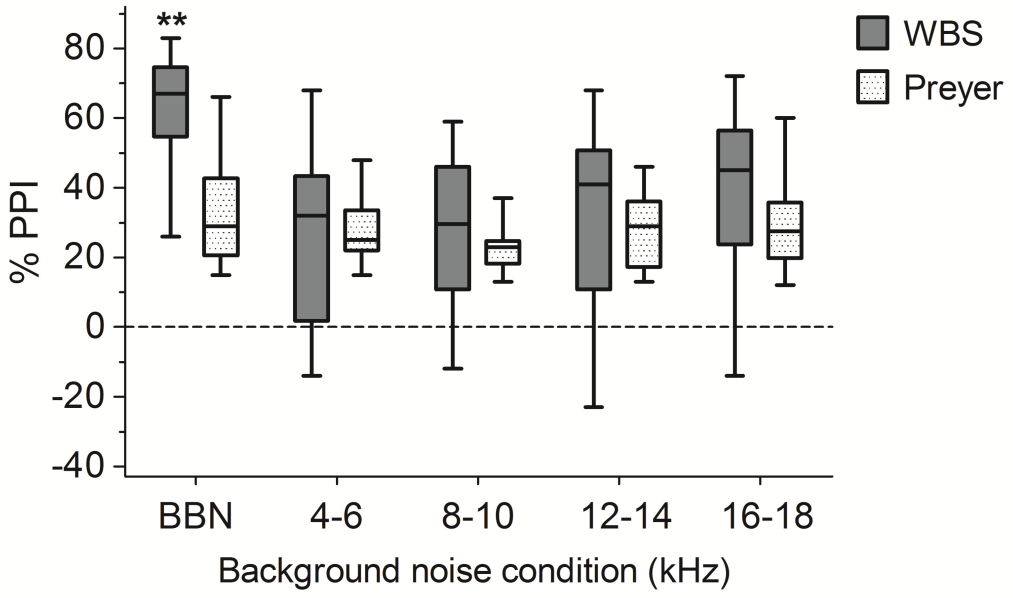

Fig. 3.3: PPI of the WBS and Preyer reflexes. Mean PPI of each reflex at a given background noise frequency is indicated by the solid horizontal line contained within each box; boxes indicate 95% confidence intervals; whiskers indicate the full range of values obtained across all GPs ($n = 12$) for each condition. PPI of WBS was significantly higher than the Preyer reflex in the BBN condition (** $p < 0.01$).

3.3 The Effects of Sodium Salicylate on PPI

In order to compare the efficacy of detecting tinnitus using the Preyer and WBS responses, as measured by reductions in PPI, sodium salicylate was administered to four GPs and PPI of both reflexes was measured at 2 h and 5 h post-injection (Fig 3.4). Across animals, using the Preyer reflex as a measure of PPI (Fig 3.4A), statistical analysis revealed a significant effect in the time-point variable ($F_{(3,12)} = 3.88$, $p < 0.05$), no effect in the background noise centre frequency variable ($F_{(4,12)} = 0.73$, $p = 0.59$), but a significant time by frequency interaction ($F_{(12,45)} = 2.76$, $p < 0.01$). *Post-hoc* analysis indicated that salicylate significantly attenuated PPI of the Preyer reflex in the BBN background noise condition at 2 h (t = 3.98, $p < 0.01$) and in the 8-10 kHz background noise condition at 5 h (t = 3.81, $p < 0.01$). Conversely, no significant reductions in PPI of the WBS (Fig 3.4B) were observed following salicylate administration for either the time-point ($F_{(3,12)} = 0.28$, $p = 0.84$) or background noise centre frequency ($F_{(4,12)} = 1.56$, $p = 0.24$) variables, or a time-frequency interaction ($F_{(12,45)} = 1.33$, $p = 0.24$).

PPI was also measured at 72 h post-salicylate administration to establish whether the effects of salicylate were transient; previous work demonstrated wash-out of salicylate effects occurring within a 72 h period (Mongan et al., 1973). No significant deficits in PPI of the Preyer reflex or WBS were apparent at this time-point in any of the background noise conditions. The deficits in PPI of the Preyer reflex may be indicative of tinnitus and the transient nature is in agreement with the predicted time-course of salicylate-induced tinnitus.

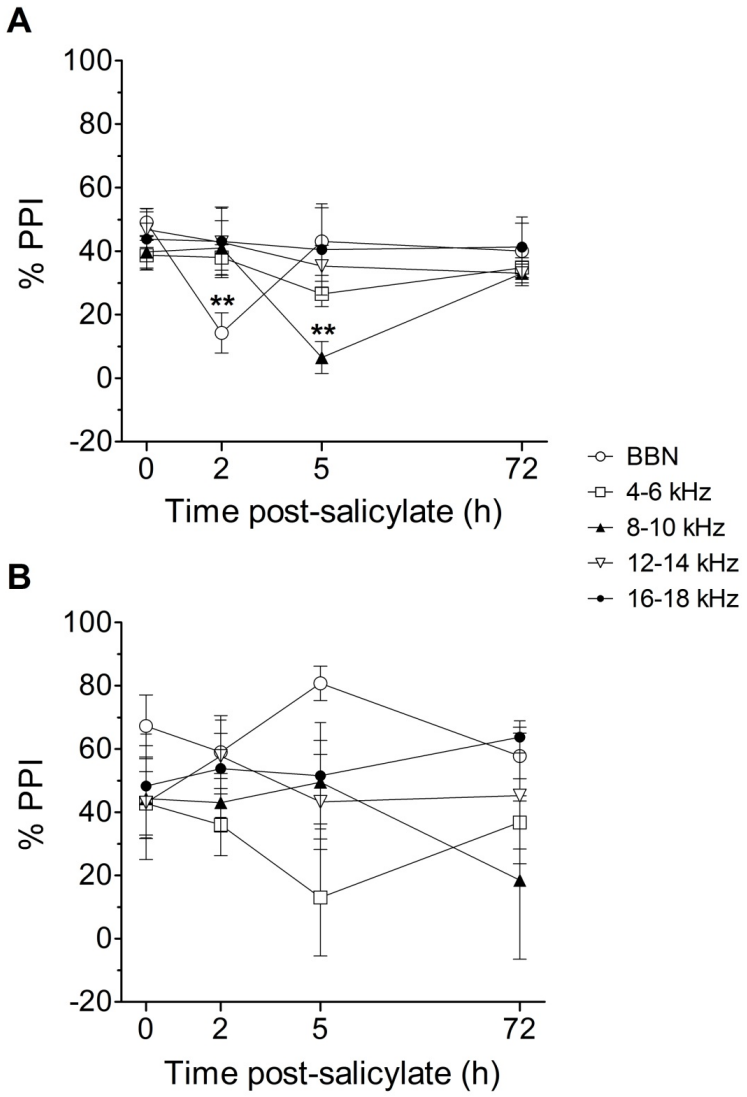

Fig. 3.4: The effects of sodium salicylate on PPI of the Preyer reflex (A) and the WBS (B). Mean ($\pm$ SEM) PPI values for all GPs ($n = 4$) are shown for each background frequency noise condition at 2 h, 5 h, and 72 h post-salicylate administration. For the Preyer reflex, significant reductions in PPI were seen at 2 h in the BBN condition and at 5 h in the 8-10 kHz condition (** $p < 0.01$). For the WBS, no significant changes were observed at any time point.

In order for the Preyer reflex behavioural test to be a useful assay, it is important that differences in gap detection can be detected in individual animals following salicylate administration (as tinnitus characteristics may vary between animals). Therefore, to determine whether significant reductions in gap detection could be identified in individual GPs using the Preyer reflex, the following analysis was applied to the data: every

peak-to-peak Preyer reflex measurement in response to a startle with no gap preceding was compared with every measurement in response to a startle with a gap preceding, for all timepoints (before and after salicylate administration). This was done to provide error values for statistical analysis of PPI, which was required for the timepoints at which only one trial was performed. From this, the mean difference between gap and no-gap Preyer reflex amplitudes was expressed as percentage PPI, to indicate the degree to which a gap was inhibiting the Preyer reflex. Following this, for each frequency, mean PPI at each timepoint following salicylate administration was compared with the baseline PPI values for each animal. Statistical analysis was again performed using a two-way ANOVA with a Bonferroni *post-hoc* test ($p < 0.05$).

The results of this analysis are shown in Fig 3.5. Using this individualised analysis, more variability was evident than when GPs were grouped. At the 2 h timepoint, all four GPs showed a significant reduction in PPI at the BBN background noise condition, two GPs at 4-6 khz (GP1 and GP3), two GPs at 8-10 khz (GP2 and GP4), three GPs at 12-14 khz (GP1, GP2 and GP4), and one GP at 16-18 khz (GP4). At the 5 h timepoint, only two GPs still showed a significant reduction in PPI compared to baseline values at the BBN background noise condition (GP2 and GP4). Two GPs showed a significant reduction in PPI at the 5 h timepoint at 4-6 khz (GP2 and GP4), all four GPs at 8-10 khz, three GPs at 12-14 khz (GP1, GP2 and GP4), and three GPs at 16-18 khz (GP2, GP3 and GP4). With this analysis, significant differences were still evident at the 72 h timepoint for some frequencies. This may be a limitation of the current analysis (there were considerably more data points at baseline compared to the post-salicylate timepoints). Nevertheless, there was still a clear trend towards PPI recovery at all frequencies for all GPs (see Fig 3.5).

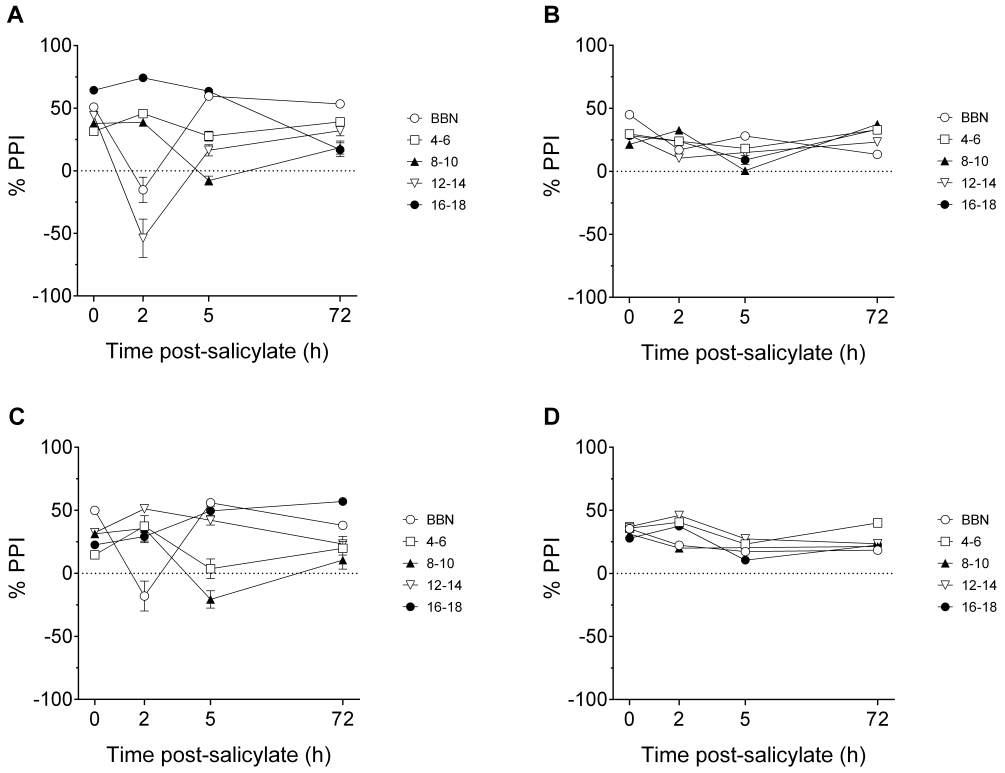

Fig. 3.5: Preyer reflex data for GP1 (A), GP2 (B), GP3 (C) and GP4 (D), using individualised analysis to determine whether any significant changes were present following salicylate administration in individual animals.

3.4 Changes in Reflex Amplitudes Following Salicylate Administration

To determine whether the effects seen here may be purely related to changes in the amplitudes of either reflex following salicylate administration, raw Preyer reflex and WBS amplitudes were analysed before and 2 h after injection (Fig 3.6). As opposed to the decreases observed following noise exposure (Lobarinas et al., 2013), both reflexes exhibited increases in startle amplitudes at all frequencies 2 h following the salicylate injection, indicating that the reductions seen in the PPI of the Preyer reflex were not simply a result of reduced startle amplitudes.

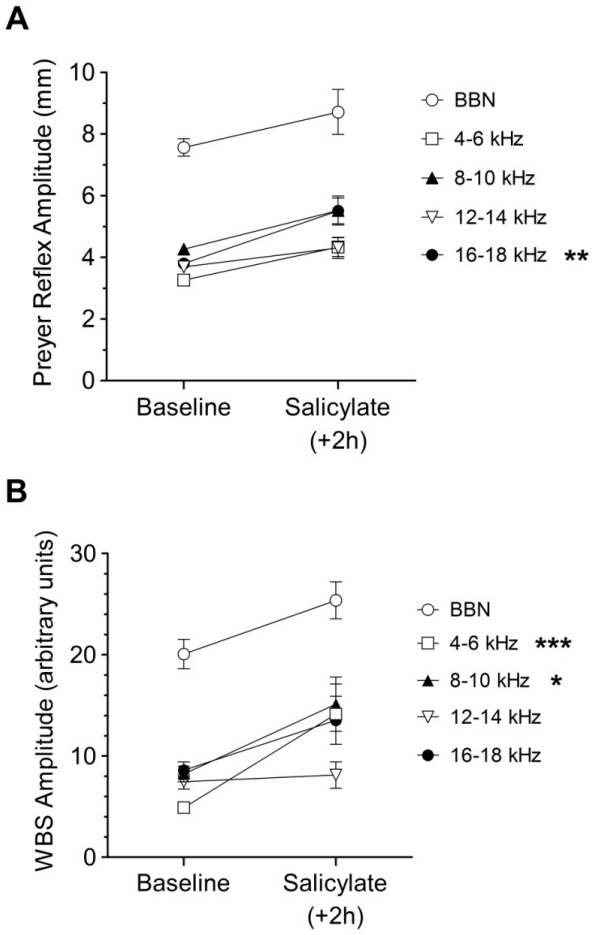

Fig. 3.6: Changes in amplitude of the Preyer (A) and WBS (B) responses. Mean ($\pm$ SEM) values are shown for all guinea pigs ($n = 4$) at each background noise frequency before (baseline) and 2 h after salicylate administration. Significant increases in response amplitude were detected at 16-18 kHz (** $p < 0.01$) for the Preyer reflex, and 4-6 and 8-10 kHz (* $p < 0.05$; *** $p < 0.0001$) for the WBS.

CHAPTER 4

Changes in ABRs and Spontaneous Firing Rates Following Noise Exposure

4.1 Background to Study

Following noise exposure, many studies have attributed changes in SFRs at various levels of the auditory system to tinnitus (Bauer et al., 2008; Brozoski and Bauer, 2005; Brozoski et al., 2002; Kaltenbach et al., 2004; Mulders and Robertson, 2009; Mulders et al., 2011; Zhang et al., 2006). Increases in SFR have commonly been observed in the IC, which are suggested to occur as a result of compensatory mechanisms following loss of peripheral input (Schaette and McAlpine, 2011). While the degree of increase in spontaneous activity in the DCN has previously been correlated with the strength of behavioural evidence for tinnitus (Kaltenbach et al., 2004), no studies to date have examined animals based on categorisation into tinnitus and no-tinnitus groups following noise exposure.

Tinnitus has also been related to changes in ABRs. Schaette and McAlpine (2011) found that tinnitus patients with supposedly normal hearing thresholds, as indicated by their audiogram, had a normal wave V amplitude (the human equivalent to wave IV in GPs), while wave I was significantly reduced. The smaller wave I indicated a degree of reduced hearing sensitivity in tinnitus patients that seemingly did not exhibit hearing loss. It also provided support for a compensatory increase in central gain since wave V was normal. Gu et al. (2012) found similar results, demonstrating that wave III was also increased compared to wave

I in tinnitus patients, which they suggested implicates the ventral cochlear nucleus in tinnitus generation.

In animals, only two studies to date have analysed the amplitude and latency of ABRs following tinnitus induction. Dehmel et al. (2012a) found that animals with behavioural evidence of tinnitus demonstrated an increase in the amplitudes of early ABR waves (I-III), which is inconsistent with the results of Schaette and McAlpine (2011), as well as a significant prolongation of wave I latency, which they attributed to auditory nerve conductivity problems following noise exposure. Furthermore, a recent study by Ruttiger et al. (2013) demonstrated that rats with tinnitus showed a reduction in the average amplitude of all the ABR waves compared to no-tinnitus rats, as well as greater IHC ribbon loss, indicating a greater degree of reduced hearing sensitivity in their tinnitus animals, although this study is not strictly comparable with Schaette and McAlpine (2011) as they induced a significant hearing loss in their tinnitus animals. Nonetheless, the current data available for how changes in ABRs may relate to tinnitus is somewhat contradictory and warrants further investigation.

This current chapter documents neural changes in GPs with behavioural evidence of tinnitus following noise exposure. Initially, using strict criteria (see *Section 2.4*), GPs were categorised into tinnitus and no-tinnitus animals. Prior to this ABRs had been recorded and analysed before noise exposure and after an 8-week recovery period, in order to account for hearing loss. In addition, changes in the amplitudes and latencies of the component waves of the ABR waveforms were assessed, to examine any pathological changes that may relate to tinnitus. Finally, SFRs of neurons in the IC were measured in no-tinnitus and tinnitus GPs, as well as in a control population, in order to test the theory that increased spontaneous activity would be correlated with tinnitus (Mulders and Robertson, 2009).

4.1.1 Alternative Classification of Tinnitus

The initial behavioural criteria for tinnitus were highly conservative, as they attempted to minimise the potentially confounding effect that hearing loss may have on the gap detection test. Therefore, for the purposes of comparison and in order to avoid any bias in interpretation, the data have been reanalysed at the end of this chapter (see *Section 4.4*), using a two-way ANOVA and Bonferroni *post-hoc* test to examine statistically significant

changes in PPI at each background frequency 7-8 weeks following noise exposure, in comparison to baseline PPI values (see *Section 2.4* for a more detailed description of this classification method). This test is similar to that applied by other researchers using the gap detection behavioural test (e.g. Dehmel et al., 2012a; 2012b; Zhang et al., 2011). Under this criterion, any animals exhibiting a significant reduction in PPI at one or more background frequencies were labelled as tinnitus GPs. Animals with no significant deficits at any frequency tested were categorised as no-tinnitus GPs.

4.2 Results

4.2.1 Behavioural Evidence of Tinnitus

Noise-exposed GPs were tested behaviourally for evidence of tinnitus. Under the initial classification, the presence of behavioural tinnitus was determined by two criteria: First, PPI of the Preyer reflex had to be $\leq$ 0% for at least one background noise frequency band. Second, PPI deficits had to occur at a background noise frequency at which there was no more than 20 dB of threshold elevation. The behavioural results for a single GP with tinnitus are shown in Figure 4.1. This GP exhibited a complete absence of PPI in the 16-18 kHz background noise condition (Fig 4.1A), as well as displaying a high frequency (15 kHz) ABR threshold within 10 dB of the baseline hearing threshold (Fig 4.1B).

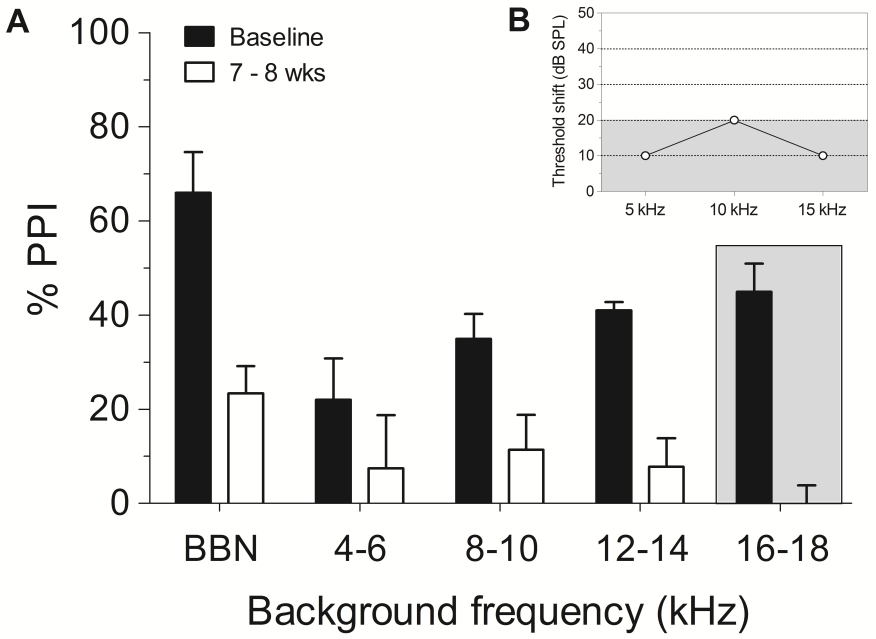

Fig. 4.1: Demonstrating noise-induced tinnitus in a GP. A: % PPI is shown for each background noise frequency (BBN, 4-6 kHz, 8-10 kHz, 12-14 kHz, and 16-18 kHz) under baseline conditions (black bars) and following noise exposure at 7-8 weeks (white bars) in a representative animal. The shaded area indicates that this GP has tinnitus (0% PPI) at 16-18 kHz. B: ABR threshold shift in the same animal at 5 kHz, 10 kHz, and 15 kHz. Shading indicates hearing level threshold recovery to within 20 dB HL. Note: recovery at 15 kHz corresponds with 0% PPI at 16-18 kHz.

Using the original conservative criteria, 8 (of 16) GPs (50%) exhibited behavioural evidence of tinnitus over a range of frequencies at the 7-8 week time-point (Fig 4.2). This figure is similar to that reported elsewhere by others (Dehmel et al., 2012a; 2012b) and the time-point was selected based on the assumption that tinnitus develops within 7-8 weeks of an acoustic insult (Turner et al., 2006; Turner et al., 2012). In some cases, tinnitus behaviour was observed at more than one background frequency. Four animals exhibited 0% PPI and threshold recovery at 4-6 kHz (Fig 4.2B; GP1, GP2, GP13, and GP16), two GPs at 8-10 kHz (Fig 4.2C; GP2 and GP11), and three GPs at 16-18 kHz (Fig 4.2E; GP2, GP5, and GP15). Moreover, two GPs exhibited 0% PPI and threshold recovery (based on a mean threshold taken from the 5 kHz, 10 kHz, and 15 kHz hearing levels) in the BBN condition (Fig 4.2A; GP4 and GP15). Taken together, these findings indicate that behavioural tinnitus was not occurring in a specific frequency band.

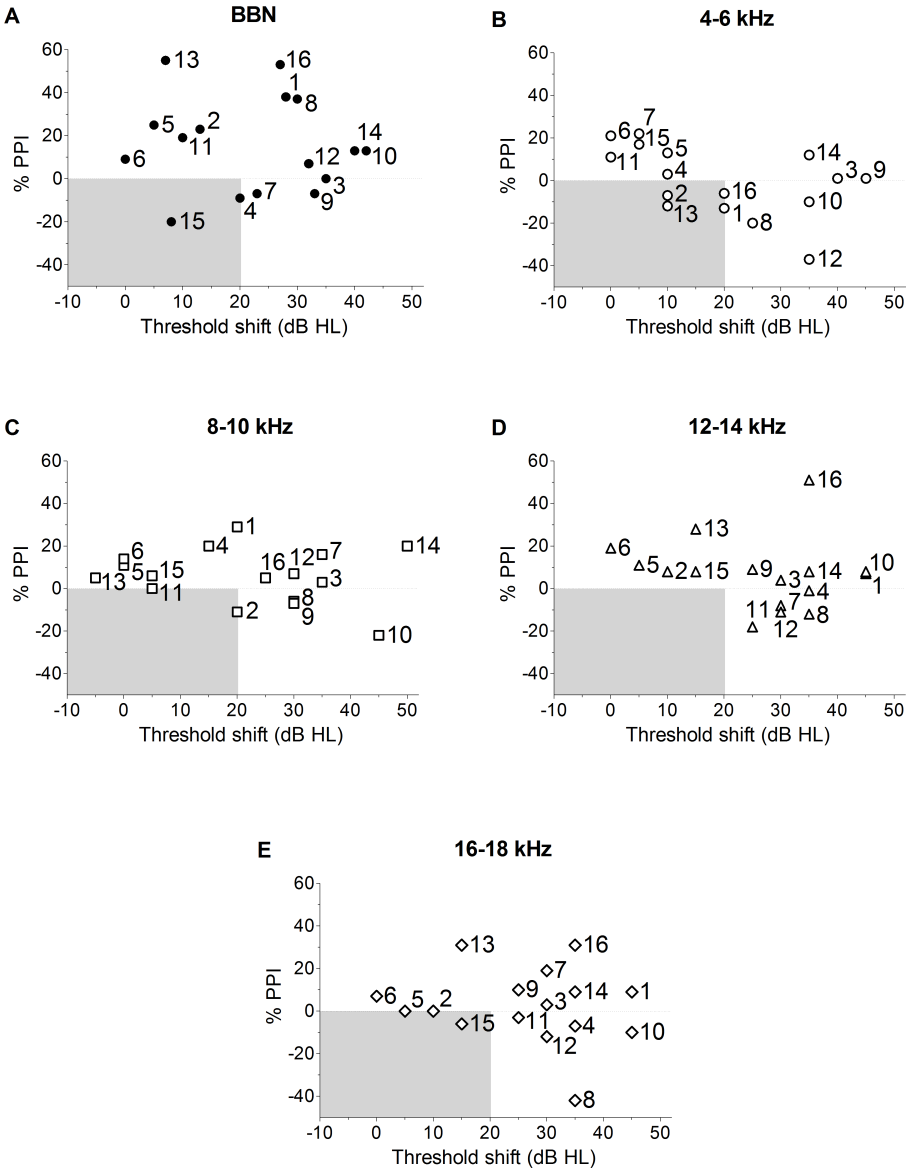

Fig. 4.2: Objective behavioural assessment of tinnitus. The % PPI values for each GP are shown - plotted against hearing threshold shift at the nearest ABR frequency (5 kHz, 10 kHz, or 15 kHz) - at each behavioural background frequency: A: BBN, B: 4-6 kHz, C: 8-10 kHz, D: 12-14 kHz, and E: 16-18 kHz. Numbering denotes GP number. In each plot, the grey shaded area indicates animals that have behavioural evidence of tinnitus with no hearing loss, according to the current criteria (i.e., $\leq$ 0% PPI coinciding with threshold recovery to within 20 dB HL of baseline hearing level). Using these criteria, 7 of 16 animals exhibited tinnitus behaviour.

	BBN		4 to 6		8 to 10		12 to 14		16 to 18	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Baseline	5.7	0.2	3.3	0.2	3.8	0.2	4.0	0.2	4.1	0.2
7-8 Weeks	3.5	0.2	2.3	0.1	2.3	0.1	2.3	0.1	2.4	0.1

Table 4.1: Mean (± SEM) amplitudes of the Preyer reflex before and 7-8 weeks after noise exposure, for each background condition across all GPs in response to 'no gap' startling stimuli, regardless of whether they were classified as experiencing tinnitus or not. Headings indicate background frequency (kHz). Mean values are expressed in mm.

4.2.2 Changes in Reflex Amplitudes

Mean Preyer reflex amplitudes (± SEM) are shown for all animals (both no-tinnitus and tinnitus) before and 7-8 weeks following noise exposure in table 4.1. There were clear reductions in the amplitudes of the Preyer reflex in all animals 8 weeks following noise exposure compared to baseline levels. However, these reductions were observed even when a GP was able to gap detect at a particular frequency following noise exposure. Therefore, it is unlikely that this would have confounded the assumption of the presence of tinnitus.

4.3 Changes in ABRs Following Noise Exposure

4.3.1 ABR Threshold Shifts

A representative example of an ABR recorded in a GP prior to noise exposure is shown in Fig 1.9. This illustrates the series of positive and negative deflections that constitute the five waves of an ABR signal in a GP (as opposed to seven waves seen in a human ABR), in agreement with that shown previously (Dehmel et al., 2012a; Gourevitch et al., 2009; Simha et al., 1988; Wada and Starr, 1983). Component waves of the ABR waveform were categorised based on the time at which they occurred, as indicated by the dotted vertical lines.

The variability in the tinnitus frequency seen in behavioural assessment can be explained by the ABR threshold shifts observed across both groups of animals: Despite the fact that acoustic trauma was induced with a narrow-band stimulus (9.5-10.5 kHz band-passed noise), ABR shifts were evident in response to 5, 10, and 15 kHz pure tones in both NT (Fig 4.3A; $n = 9$) and T (Fig 4.3B; $n = 7$) groups of GPs. ABR shifts across a broad

frequency range are consistent with previous data from animals exposed to a tonal acoustic insult (Chen et al., 2013).

In order to determine whether any shifts in ABR thresholds were statistically significant, a 2-way ANOVA with Dunnett's *post-hoc* comparison was applied to the data. For T GPs, a significant shift was observed in the mean ABR thresholds (compared to baseline hearing thresholds) for all frequencies immediately after noise exposure ($p < 0.0001$). Following recovery, T GPs still exhibited a small, albeit significant shift from baseline levels at 15 kHz ($p < 0.05$), but it is important to note that all these animals still had hearing thresholds within the clinically-relevant 20 dB level at frequencies where behavioural gap detection was abolished. For the NT GPs, there were significant shifts in hearing thresholds at all frequencies, both immediately after noise exposure and 8 weeks later ($p < 0.0001$).

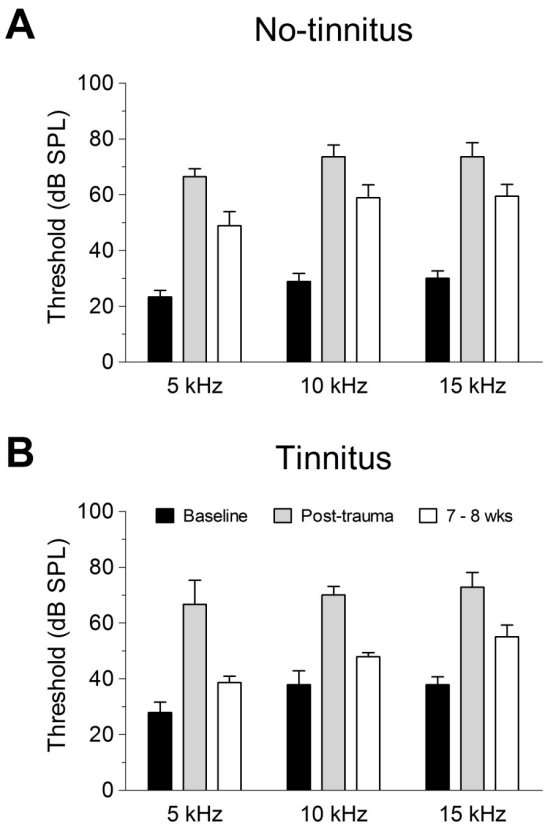

Fig. 4.3: ABR hearing thresholds in N and T animals. In both groups of GPs, threshold shifts were seen across all frequencies. A: Mean thresholds are shown for all NT animals at each ABR frequency (5 kHz, 10 kHz, and 15 kHz) before noise exposure (shown in black), immediately after acoustic trauma (grey), and then at 8 weeks (white). B: Mean thresholds for all T animals.

4.3.2 ABR Latencies in Animals with Tinnitus

ABRs were further scrutinised for changes in latency and the relative amplitude of the constituent peaks. In these data, waves II, IV, and V were confidently and consistently identified. However, wave I and III were not robustly identifiable across all animals, even when using suprathreshold stimuli, and as a result were excluded from further ABR analysis.

The latency of wave IV (the most prominent and consistent wave in the GP ABRs) was first examined: ABRs recorded in T and NT groups, were compared before noise exposure and at 8 weeks, in response to 5, 10, and 15 kHz tones presented at 70 dB SPL. Surprisingly, it was identified that there was a shortening in wave IV latency after trauma in the exposed ear of T animals, in response to the 10 kHz stimulus condition (Fig 4.4). Given the slight reduction in the HL of the ABR stimulus in T GPs, it had in fact been anticipated that latency might increase following noise exposure. When this phenomenon was examined across pooled data from T animals (n = 6 GPs with complete sets of ABR recordings), a significant decrease in the latency of wave IV in the left (exposed) ears of T GPs was evident when compared with within-animal right-side controls (Fig 4.5A; $p < 0.05$). An inspection of the relationship between stimulus sound level and wave IV latency before noise exposure in T GPs confirmed that, as expected, 10 kHz stimuli presented at 50 dB SPL exhibited a longer mean latency than at 70 dB SPL on both left and right sides (Fig 4.5A).

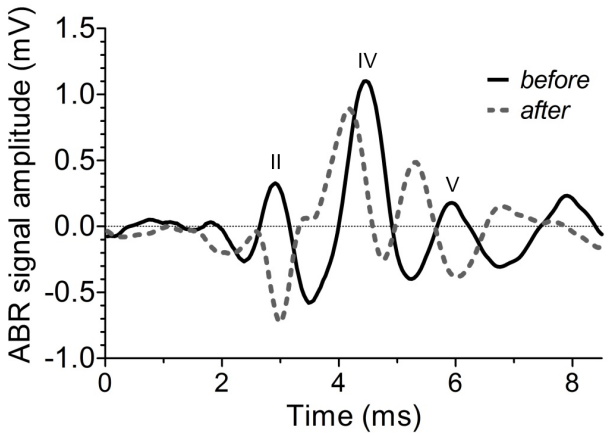

Fig. 4.4: Representative ABRs from a single T GP recorded before noise exposure (solid black line) and 8 weeks later (dashed grey line) to a 10 kHz tone at 70 dB SPL. In this example, waves IV and V are clearly visible and illustrate the decrease in latency seen at 10 kHz in T animals. Roman numerals denote the waves used for latency analysis.

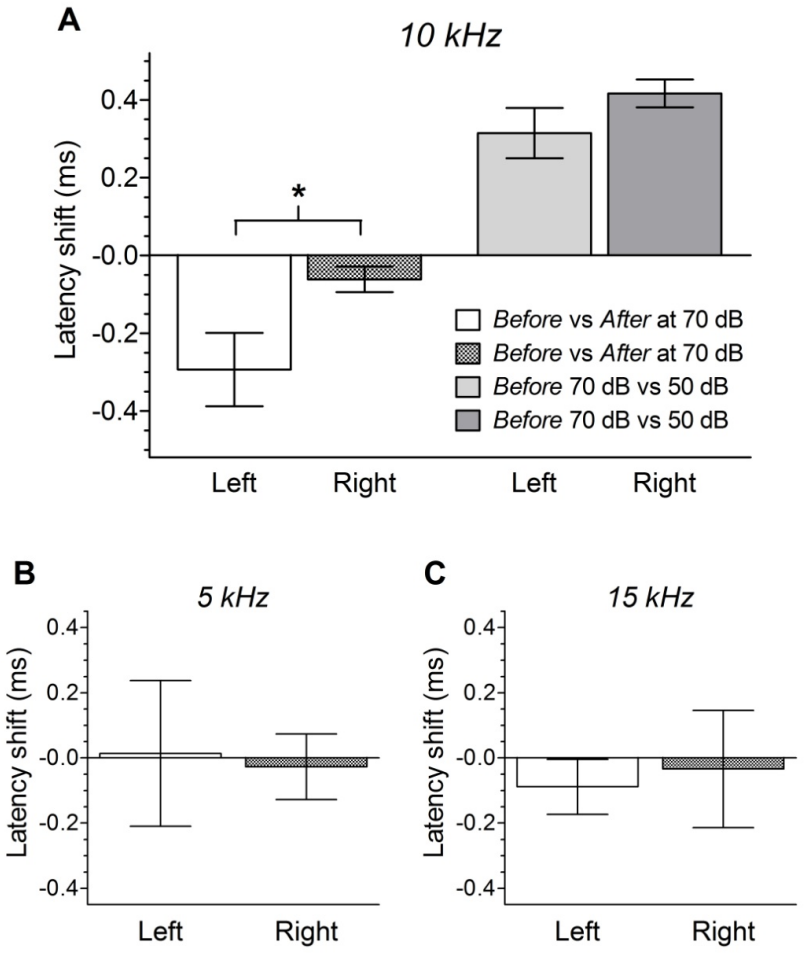

Fig. 4.5: A: Mean ($\pm$ SEM) latency shift (before exposure vs. 8 week time-point) of wave IV in T GPs ($n = 6$) in response to a 10 kHz tone at 70 dB SPL in the left (exposed) ear (shown in white) and right ear (hatched bar). Wave IV latency was significantly shorter in the left ear after noise exposure in T animals compared with right-side controls (* $p < 0.05$). Also shown are latency shifts at decreasing stimuli sound levels (70 dB SPL vs. 50 dB SPL) before noise exposure in T animals. As expected, decreased levels resulted in longer latencies in both left (light grey bar) and right (dark grey bar) ABRs. B: No significant latency shifts were apparent in response to 5 kHz tones at 70 dB SPL. C: Likewise, no significant latency shifts were apparent to 15 kHz tones at 70 dB SPL.

A further comparison of the mean ($\pm$ SEM) latencies of wave IV to 10 kHz stimuli at 70 dB SPL between left- and right-side ABRs (not shown) indicated that, under control conditions, i.e., before noise exposure, the left- (4.37 ± 0.12 ms) and right-side (4.33 ± 0.05 ms) latencies were extremely similar.

By contrast, there were no significant differences in wave IV latencies in response to 5 kHz and 15 kHz stimuli (Fig 4.5B and Fig 4.5C) when pre-exposure and 8-week ABRs were compared. These factors, taken together, indicate that the decrease in wave IV latency in T animals is a robust observation. No significant latency shifts were observed in the NT animals (n = 6; Fig 4.6).

In order to identify whether overall shifts in latency could be attributed to specific waves (and therefore specific components of the ascending auditory system), the inter-peak latencies (IPL) of left- and right-side ABRs were examined, before and after noise exposure. Interestingly, no differences between the IPL were detectable when comparing pre-exposure (II-IV: 1.53 ± 0.01 ms; II-V: 2.81 ± 0.06 ms; IV-V: 1.28 ± 0.06 ms) and 8-week time-point (II-IV: 1.52 ± 0.06 ms; II-V: 2.74 ± 0.04 ms; IV-V: 1.22 ± 0.04 ms) ABRs on the left (exposed) side. This effectively means that the latencies of all measured ABR waves (II-V) in T GPs were reduced after noise exposure, in response to 10 kHz stimuli. Likewise, no differences were apparent in right-side controls, nor was there any variability in IPL between left and right sides before exposure (data not shown).

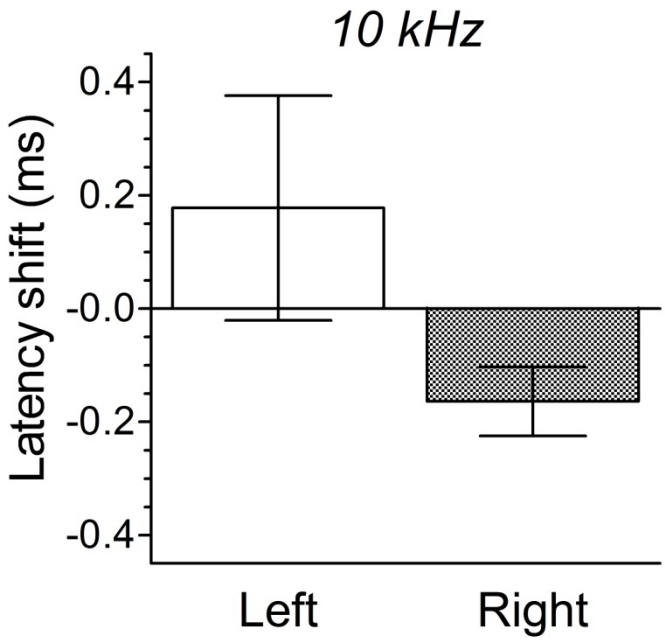

Fig. 4.6: Mean (± SEM) latency shift (before exposure vs. 8 week time-point) of wave IV in NT GPs (n = 6) in response to a 10 kHz tone at 70 dB SPL in the left (exposed) ear (white bar) and right ear (hatched bar). No significant differences were found between the two ears.

4.3.3 ABR Wave Amplitude Ratios

ABR waveforms were also analysed for changes in amplitude. Observationally, in most cases, the amplitude of wave IV was reduced after noise exposure (see Fig 4.4), presumably due to small HL reductions caused by threshold increases; this is in contrast to the effects on latency described in the previous section. ABR recordings from T GPs were subsequently inspected for changes in the relative amplitudes of the constituent waveform peaks at the region of the latency shift, i.e., 10 kHz, in light of previous work conducted in tinnitus patients (Gu et al., 2012; Schaette and McAlpine, 2011). These comparisons were limited by the SNR of the ABR data: waves I and III were unable to be reliably identified, and therefore only the ratios between waves II, IV and V were examined. No significant changes in the amplitude ratios were detected in left- or right-side ABRs. A slight, albeit not statistically significant, increase was observed in the mean ($\pm$ SEM) II/IV (from 0.44 $\pm$ 0.08 to 0.56 $\pm$ 0.04) and V/IV (0.41 $\pm$ 0.07 to 0.78 $\pm$ 0.20) ratios in left-side ABRs. This increase was likely due to the reduction in amplitude of wave IV after noise exposure.

4.3.4 Neuronal Hyperactivity in the IC

Following behavioural testing and ABR recordings, spontaneous neuronal firing was recorded from the left and right IC of each GP, as well as in an additional control group (not exposed to noise) of GPs ($n = 6$). The SFR of each cell - plotted according to CF - is shown for control GPs (Fig 4.7A; $n = 137$ cells), NT GPs (Fig 4.7B; $n = 219$ cells) and T GPs (Fig 4.7C; $n = 187$ cells). These data indicated that, while firing rates appeared to be elevated following acoustic trauma in both groups compared with control GPs, there were no significant trends in relation to CF. This is perhaps not surprising, given the broadband nature of both hearing threshold shifts and behavioural PPI deficits. Statistical analysis of the mean firing rates of all cells recorded in each of the three groups indicated that SFRs were significantly higher in T ($p < 0.0001$) and NT ($p < 0.0001$) groups compared with controls, but that no significant differences were apparent between T and NT groups (Fig 4.8A). Furthermore, the median SFR values, which are arguably more reliable when considering that these cell populations were not normally distributed, clearly showed elevated SFRs in noise traumatised GPs, relative to control GPs (Fig 4.8B).

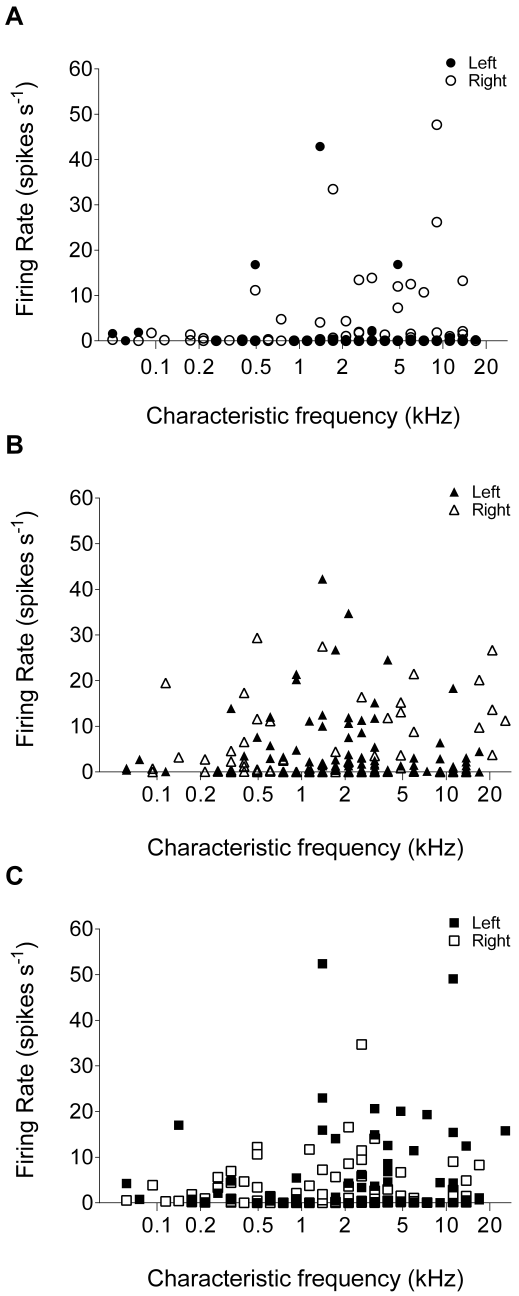

Fig. 4.7: SFRs are shown for each recorded single-unit - plotted logarithmically according to CF - in A: control animals (n = 137 cells), B: NT animals (n = 219 cells) and C: T animals (n = 187 cells). These data have been separated into left and right hemispheres (as denoted on each figure). No trends were apparent between T and NT animals in terms of increases in firing rate at specific frequencies, nor were there any clear hemispheric differences.

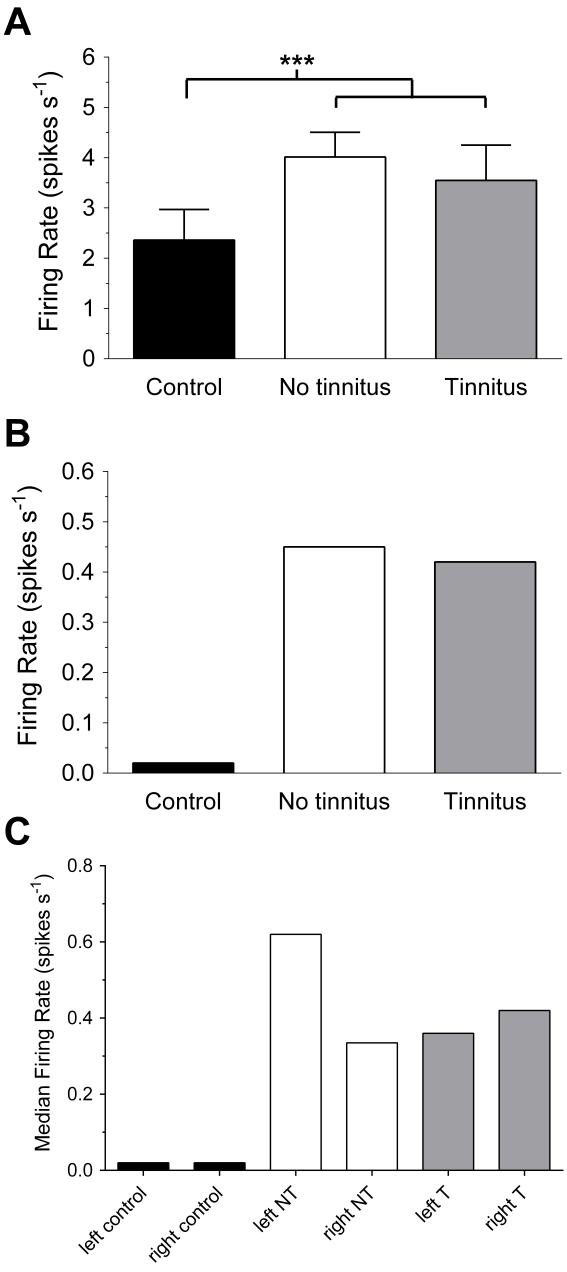

Fig. 4.8: A: Mean firing rate ($\pm$ SEM) for pooled data for all single-units recorded in control (black), NT (white), and T (grey) animals. Firing rate was significantly higher in NT and T groups compared with control GPs (*** $p < 0.0001$). No significant differences were seen between T and NT groups. B: Median firing rates for control (black), NT (white), and T (grey) groups of GPs. Median rates also indicated an increase in firing rate following noise exposure. C: Median firing rates for the three different groups, separated according to hemisphere. While an increase in SFR was clearly evident in NT and T GPs compared with control GPs, there were no significant intra-group hemispheric differences, nor were there any significant differences between NT and T GPs.

In addition to examining changes in firing rate according to CF, the data were also assessed for whether there were any laterality-dependent effects (see Fig 4.7 and Fig 4.8C). This was important, as the noise exposure was unilateral (left-sided), so it is plausible that any effects on SFR may have been restricted to the right IC. However, no 'within-group' differences between neuronal firing rates on left and right sides were observed in control ($p = 0.50$), NT ($p = 0.96$), or T ($p = 0.41$) groups of GPs. When recordings made in the left IC and the right IC were compared independently across the behavioural groups, i.e., left-control vs. left-NT vs. left-T, and then right-control vs. right-NT vs. right-T, it was found that in both cases T and NT groups exhibited significantly higher SFRs than control GPs ($p < 0.0001$). As was the case when, irrespective of CF or side, all single-unit recordings were compared between groups, there were no significant differences between T and NT groups of GPs when subdivided according to side.

4.4 Alternative Classification

The following results show the ABR latency and SFR data under the different behavioural classification (detailed in *Section 2.4*, similar to that used by others, taking a significant reduction in PPI at a particular frequency as an indication of tinnitus presence.

4.4.1 Behavioural Evidence of Tinnitus

Using the alternative criterion, 12 of the 16 noise-exposed GPs exhibited a significant reduction in PPI for at least one frequency, as determined by a two-way ANOVA with a Bonferroni *post-hoc* test ($p < 0.05$). An example of a no-tinnitus GP and a tinnitus GP are presented in Fig 4.9A and Fig 4.9B, whereby change in PPI is expressed as a ratio (see *Section 2.4* for a more detailed explanation). Across animals, there was no clear trend with regard to the frequency of the gap detection deficits, although 16-18 kHz was the most common frequency at which a significant deficit was present, whereas only 1 animal demonstrated significant gap detection deficits at 12-14 kHz (Fig 4.10).

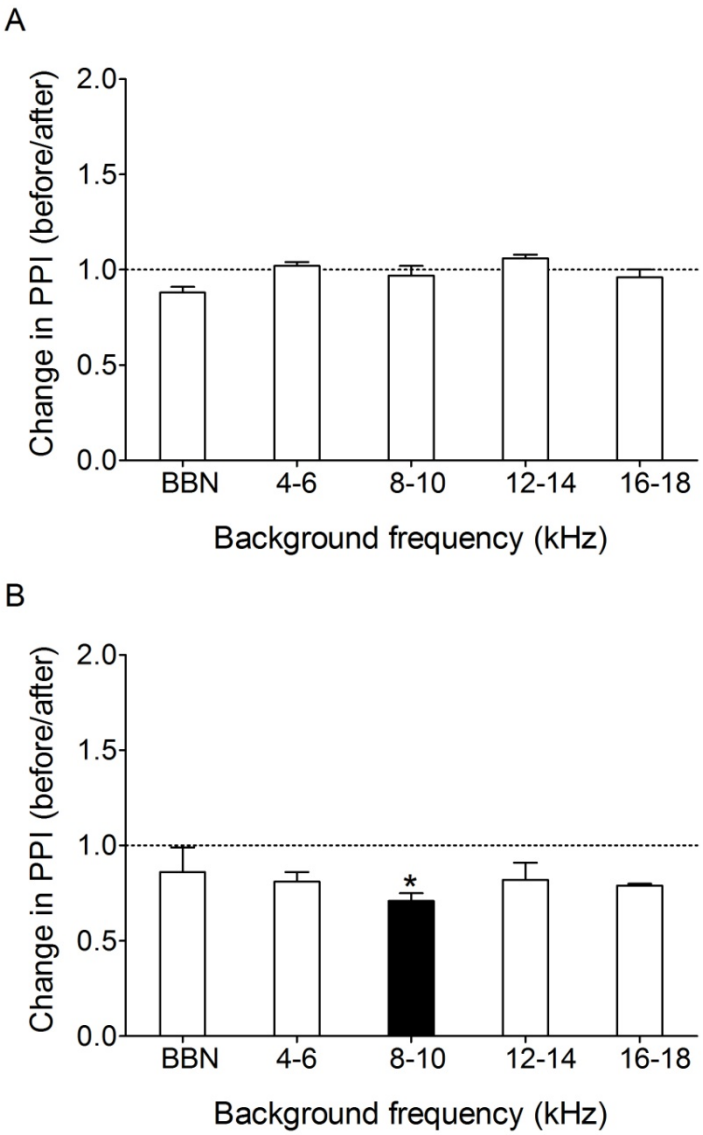

Fig. 4.9: PPI performance 7-8 weeks following noise exposure, normalised to baseline performance for one no-tinnitus GP (A) and one tinnitus GP (B). Performance is expressed as a ratio of the original gap detection ability, whereby a value of 1 equates to the same level of PPI as baseline, a value lower than 1 indicates poorer performance and a value greater than 1 indicates better performance. Dotted line highlights baseline PPI level. White bars suggest no significant change and black bars denote a significant reduction in PPI (* $p < 0.05$).

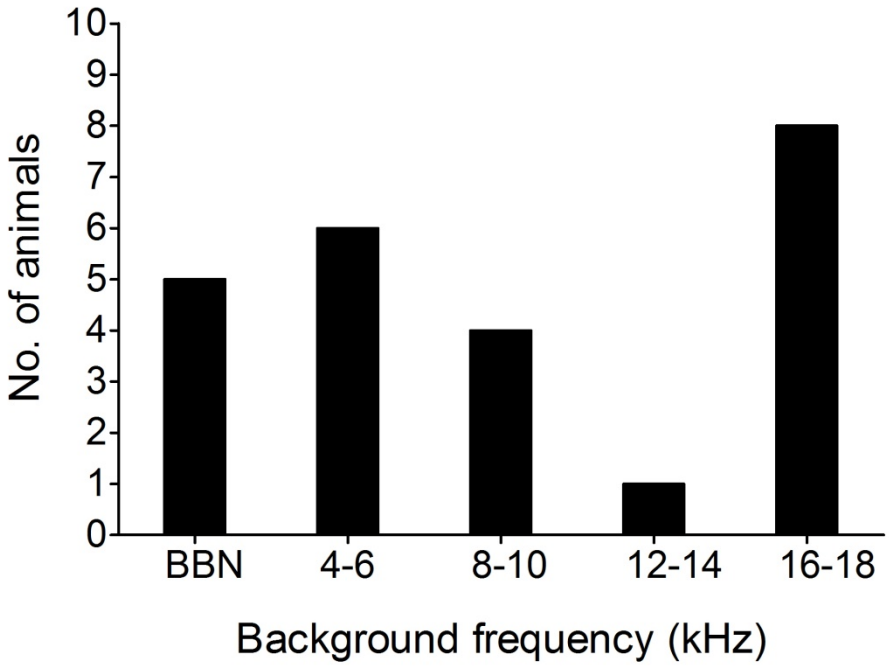

Fig. 4.10: Number of GPs demonstrating significant reductions in PPI following noise exposure, for each background frequency, as determined using a two-way ANOVA with a Bonferroni *post-hoc* test ($p < 0.05$).

4.4.2 Changes in ABR

ABRs were reanalysed for changes in latency, to determine whether the intriguing result examined using the other criteria was still present under the alternative criterion. Given the greater degree of hearing loss for some animals in the new group of T GPs (compared to the other criteria), ABRs were examined only for animals where 70 dB SPL equated to 10 dB SL or greater, in order to reduce the confounding effect of severe hearing loss on latency. A shortening in latency of wave IV was again evident in T animals, solely for the 10 kHz frequency, although there was no longer a significant difference in ABRs between stimulation of the left (exposed) and right (unexposed) ears within T GPs (Fig 4.11).

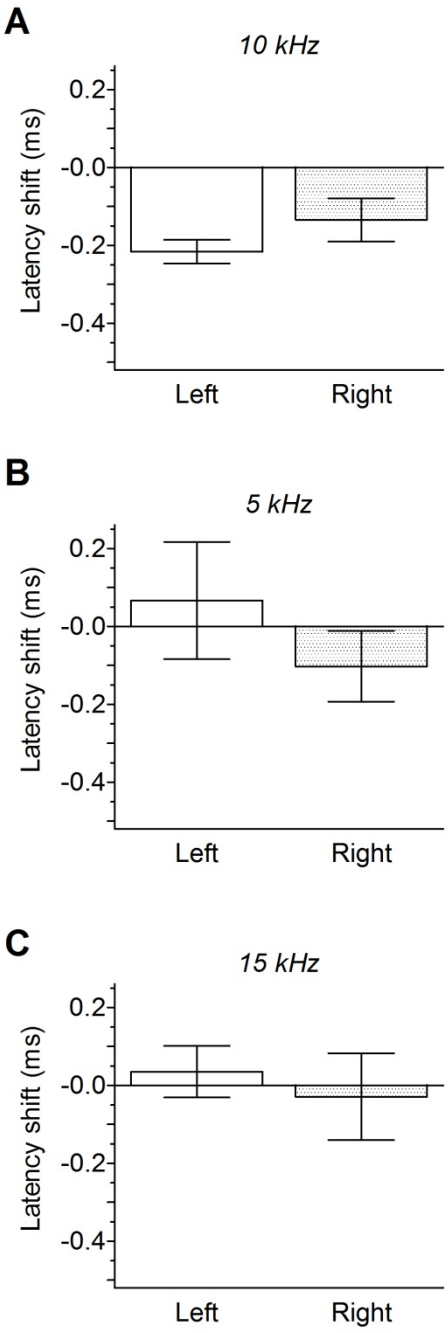

Fig. 4.11: Mean (± SEM) latency shift (before exposure vs. 8 week time-point) of wave IV in T GPs, in response to 10 kHz (A), 5 kHz (B) and 15 kHz (C) tones, using an alternative criterion for tinnitus. A shortening of latency was again apparent in the left (exposed) ear at 10 kHz, though there was no significant difference compared to the right ear in the same animals.

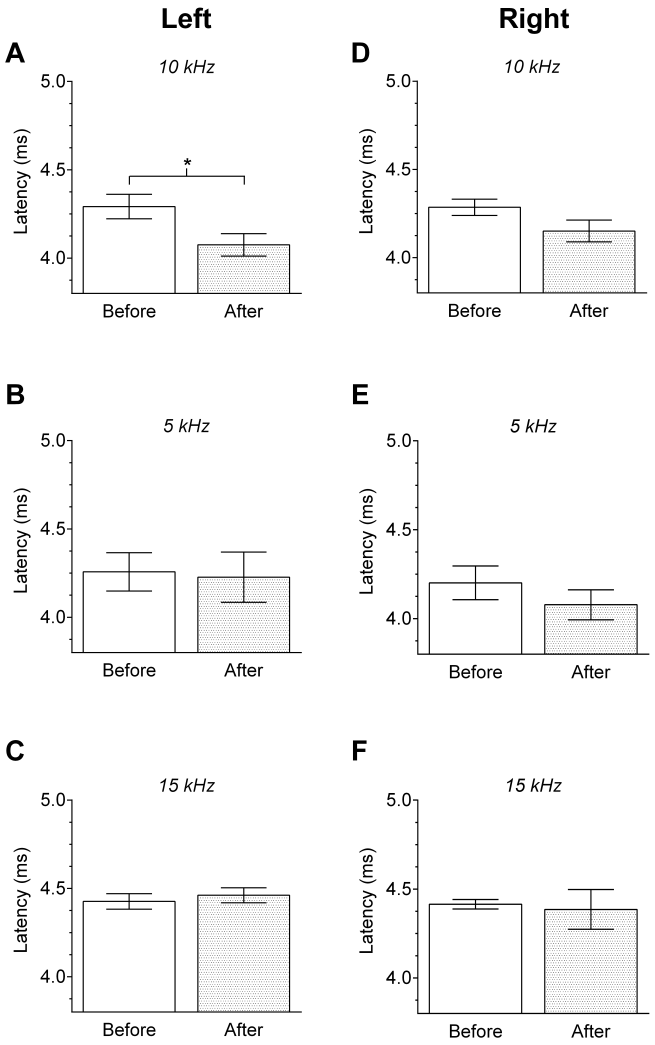

Fig. 4.12: Mean (± SEM) absolute latency of wave IV in the left (exposed) ears of T GPs, in response to 10 kHz (A), 5 kHz (B) and 15 kHz (C) tones, before noise exposure (white bar) compared to 8 weeks following noise exposure (hatched bar), as well as in the right (unexposed) ear in response to 10 kHz (D), 5 kHz (E) and 15 kHz (F) tones, using an alternative tinnitus classification. A significant shortening of latency was only evident for the 10 kHz frequency in the left ear (* $p < 0.05$).

However, when the absolute latency of wave IV for the left (exposed) ear following noise exposure was pooled for all T animals and compared to ABRs recorded prior to noise exposure in the same GPs, the latency of wave IV for 10 kHz proved to be significantly shorter (Fig 4.12A; $p < 0.05$). There were no significant changes in absolute latency for 5 kHz (Fig 4.12B) or 15 kHz (Fig 4.12C), nor were there any significant changes in absolute latency in the right ear (Fig 4.12D, Fig 4.12E and Fig 4.12F).

4.4.3 Changes in SFRs

Spontaneous activity of neurons in the IC was reanalysed under the alternative criterion. The SFR of each cell - again, plotted according to CF and hemisphere - is shown for control GPs (Fig 4.13A; n = 137 cells), NT GPs (Fig 4.13B; n = 101 cells) and T GPs (Fig 4.13C; n = 298 cells). The spread of spontaneous activity suggests there were still no significant trends in relation to CF or hemipshere.

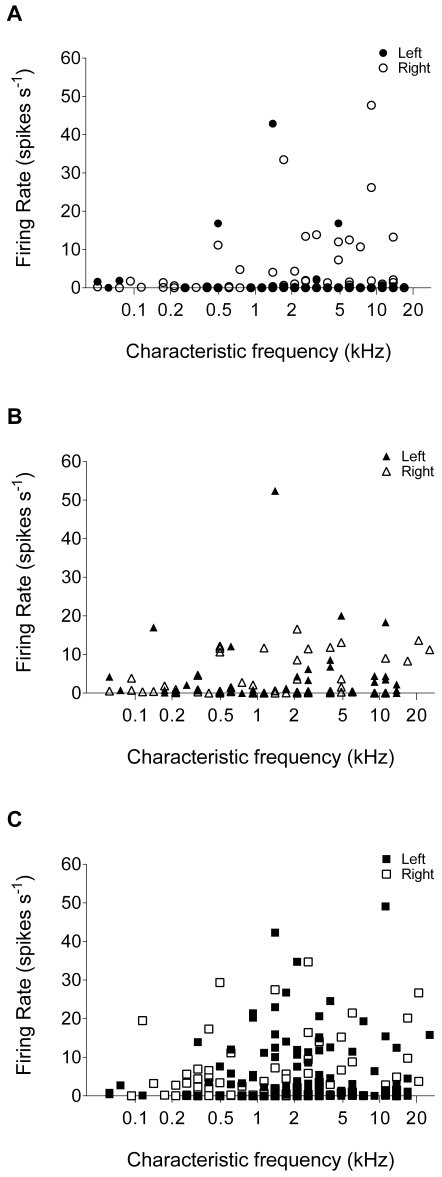

Fig. 4.13: SFRs - plotted logarithmically according to CF and hemisphere - in A: control animals (n = 137 cells), B: NT animals (n = 101 cells) and C: T animals (n = 298 cells), using the alternative criterion for tinnitus classification. Legends on each figure denote hemisphere.

As with the original criteria, statistical analysis indicated that SFRs were significantly higher in T ($p < 0.0001$) and NT ($p < 0.0001$) groups compared with controls, but that no significant differences were apparent between T and NT groups (Fig 4.14A). Again, median SFR values highlight that SFRs were elevated in noise trauma-treated GPs, relative to control GPs (Fig 4.14B). There were no significant hemispheric differences in SFR present within each experimental group. There were still significant increases in SFR for NT and T GPs compared with controls when data were separated according to hemisphere (Fig 4.14C), but not between NT and T GPs.

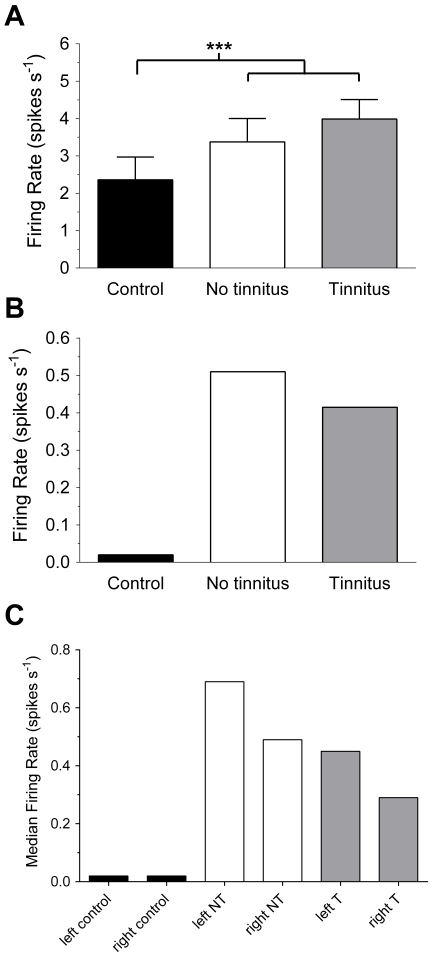

Fig. 4.14: A: Mean firing rate ($\pm$ SEM) for pooled data for all single-units recorded in control (black), NT (white), and T (grey) GPs, using alternative criterion. Firing rate was significantly higher in NT and T groups compared with control GPs (*** $p < 0.0001$). Again, no significant differences were seen between T and NT groups. B: Median firing rates for control (black), NT (white), and T (grey) GPs. C: Median firing rates for the three different groups, separated according to hemisphere.

CHAPTER 5

Neural Gap Detection Thresholds Following Noise Exposure

5.1 Background to Study

The hypothesis of the gap detection test for tinnitus is that tinnitus is perceptually "filling in" the gap, causing a reduction in gap-induced PPI (Turner et al., 2006). While this hypothesis is somewhat supported by research demonstrating gap detection deficits following noise exposure or salicylate (*Chapters 3 and 4*; Chen et al., 2013; Dehmel et al., 2012; Longenecker and Galazyuk, 2011; Turner and Parrish, 2008; Turner et al., 2012), recently it has been suggested that the test may actually reflect a deficit in temporal processing associated with the noise exposure, rather than tinnitus *per se*.

This question initially arose following a study by Fournier and Hebert (2013). Using the eyeblink as a startle measure, they examined the gap detection abilities of human patients experiencing high-frequency tinnitus in comparison to a hearing-matched non-tinnitus control group. In support of the animal literature, the tinnitus patients demonstrated gap detection deficits in comparison to controls. However, these gap detection deficits were not selective to high frequency background carriers (similar to their tinnitus). The deficits were seen at both high and low frequencies, contrary to what would be expected in the presence of a high-frequency tinnitus. The authors concluded that the gap detection deficits seen in tinnitus patients may not necessarily relate to a perceptual "filling in" of the gap, but instead reflect neural temporal processing deficits that somehow relate to

the tinnitus and impair the detection of gaps in noise. A need for evidence to determine whether this is the case has since been proposed by others (e.g. Chen et al., 2013).

While Walton et al. (2008) found no significant differences in gap detection between mice carrying a deafness gene and normal-hearing controls, no study to date has attempted to quantify neural gap detection thresholds following noise exposure. This chapter addresses this question. The minimum gap detection thresholds (MGDTs) of single-units were measured in the IC of control, no-tinnitus and tinnitus GPs. Walton et al (1997) showed that IC gap detection thresholds are also in good agreement with those seen behaviourally. With this in mind, as well as the fact that the IC is an important area for the convergence of ascending auditory information (Casseday et al., 2002), IC is a good candidate for recording neural gap detection thresholds to understand the neural basis of behavioural gap detection. Therefore, recordings from the IC for the three experimental groups were compared to determine whether there were any differences in neural gap detection abilities (which would reflect a temporal processing deficit) following noise exposure, and if any deficits related solely to tinnitus. Ultimately, it was important to determine whether a gap of 50 ms (the duration used in the behavioural test) was detectable from the responses of the majority of neurons in the IC following noise exposure, to establish whether the behavioural gap detection deficits reported in this thesis could be due to temporal processing deficits following noise exposure.

As with the previous chapter, the data were also analysed under the alternative criterion for behavioural evidence of tinnitus, for the purposes of comparison. This follows the analysis using the original criteria (see *Section 5.4*).

5.2 Results

A total of 9 GPs were noise-exposed and tested for behavioural evidence of tinnitus 7-8 weeks following acoustic trauma. The initial criteria used for tinnitus classification are described in *Section 2.4*. A further 6 animals were unexposed and used solely for neurophysiological recordings, in order to serve as controls. Fig 5.1 shows behavioural PPI measures, plotted against ABR threshold shifts. Three out of the nine noise-exposed GPs developed tinnitus based on our original criteria. The other GPs either recovered their hearing and were able to show PPI, or did not recover their hearing. While the number of tinnitus animals is slightly lower than was observed previously using the same criteria (~33% compared to ~44%), it is a similar figure to that found by others (Henderson et al., 2011). Furthermore, this difference in the percentage of animals developing tinnitus is understandable when considering the inter-individual variability of the effects of noise exposure. One of the animals (GP9) showed clear evidence of tinnitus at all four of the narrow background frequencies and was close to showing clear evidence of tinnitus with BBN. However, the other two tinnitus animals were different in that they only showed clear evidence of tinnitus at one narrow background frequency range (4-6 kHz).

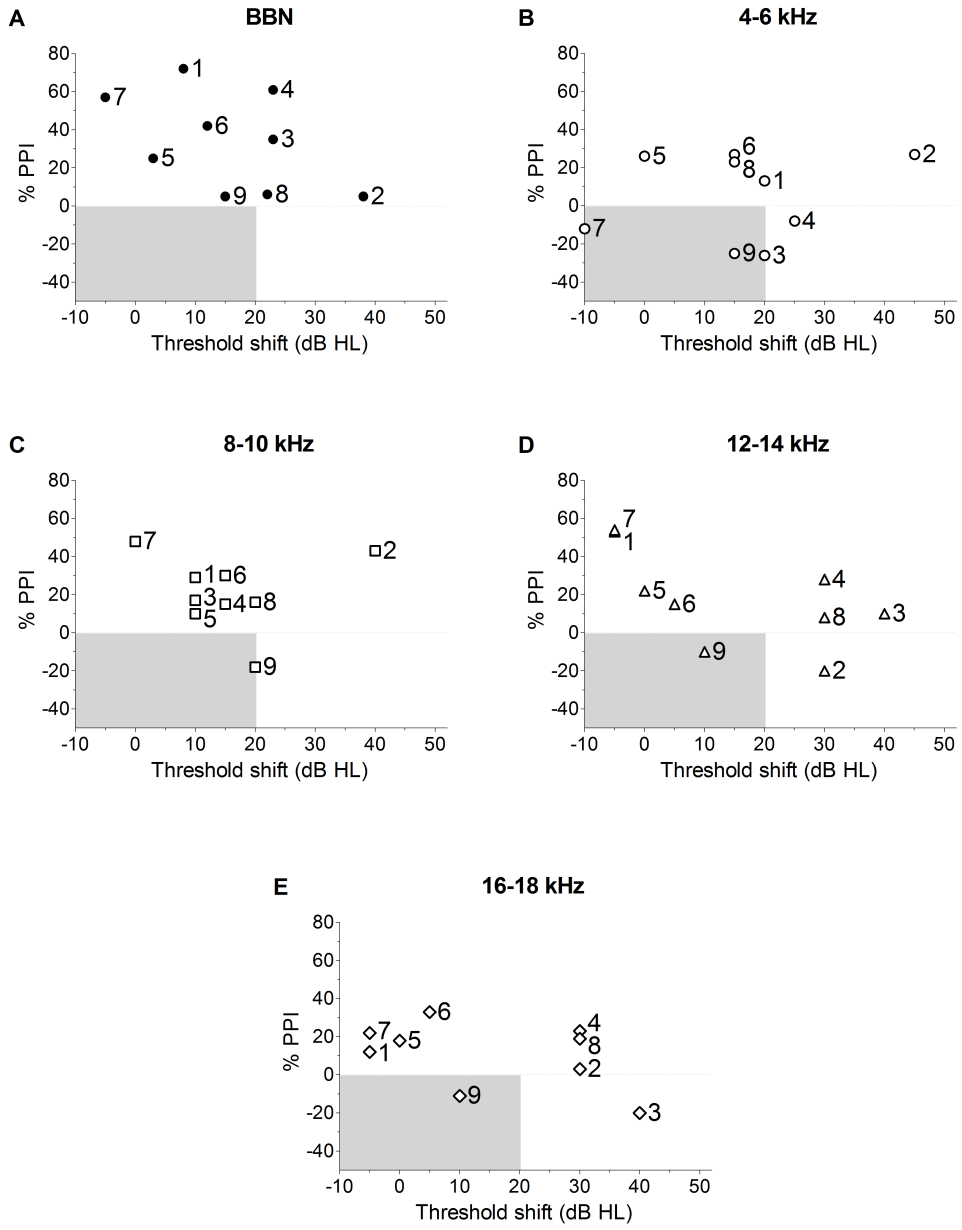

Fig. 5.1: Behavioural assessment of tinnitus. The % PPI values for each GP are shown - plotted against hearing threshold recovery at the nearest ABR frequency (5 kHz, 10 kHz, or 15 kHz) - at each behavioural background frequency: A: BBN, B: 4-6 kHz, C: 8-10 kHz, D: 12-14 kHz, and E: 16-18 kHz. Numbering denotes GP number. In each plot, the grey shaded area indicates tinnitus status, i.e., $\leq$ 0% PPI coinciding with threshold recovery to within 20 dB HL of baseline hearing level. Using these criteria, 3 of 9 animals exhibited tinnitus behaviour.

5.2.1 Neural Gap Detection Thresholds

Following behavioural testing, MGDTs of single-units were examined in the left and right IC of control, NT and T GPs. Fig 5.2 provides an example of a cell responding to stimuli, in order to demonstrate how MGDTs were calculated, which was in a similar manner to that applied by other researchers (e.g. Dehmel et al., 2012a; 2012b; Zhang et al., 2011). MGDTs for each unit were analysed according to the background stimuli used - BBN, NBN (within 1 kHz of the CF of the cell) and pure tones.

Single-units with short latencies (~15 ms or less) were recorded from control GPs ($n = 96$), NT GPs ($n = 94$) and T GPs ($n = 58$). Cells with much longer latencies were discounted, as this may indicate that they were not recorded from CNIC (Syka et al., 2000). The number of cells is somewhat fewer for BBN and NBN than for pure tone, as some cells did not respond to these stimuli or had CFs greater than 1 kHz away from the NBN frequencies.

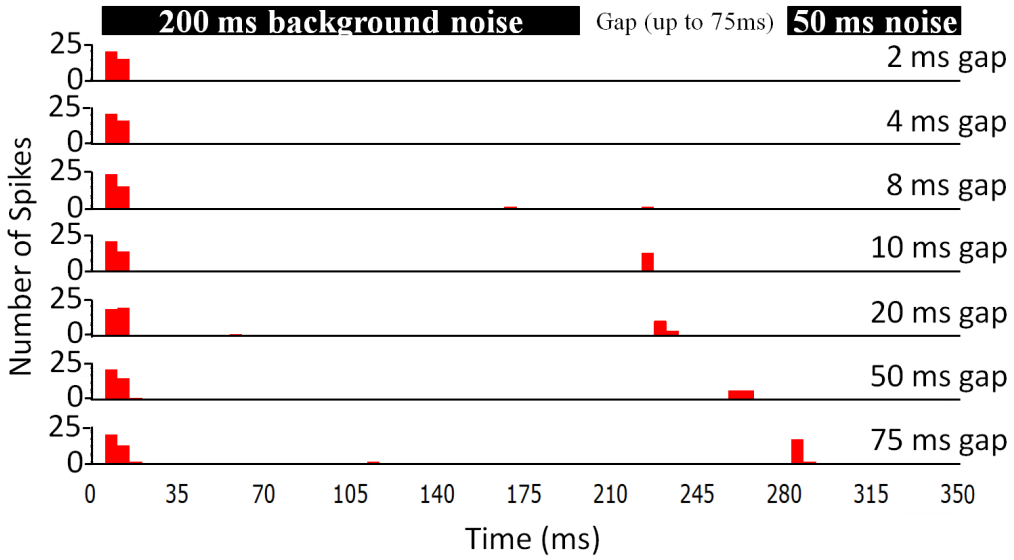

Fig. 5.2: Responses of a single-unit to gaps of different durations, indicating an MGDT of 10 ms. Numbers on the right-hand side signify length of gap. The background stimuli used to elicit responses have been included at the top of the figure. Responses in the 50 ms following a gap were deemed significant if there were a minimum of 3 spikes over 20 repeats which were 2 standard deviations above the firing rate during the preceding 50 ms. All MGDTs were confirmed visually.

5.2.2 Pure Tone MGDTs

The mean and median MGDTs for pure tones, separated according to experimental group, are shown in Fig 5.3 (A and B). MGDTs in response to pure tones are plotted as a function of the percentage of cells responding to gaps of those durations in Fig 5.3C. For pure tones, mean (± SEM) MGDTs were 10.82 (± 1.76; $n = 96$) for controls, 17.28 (± 2.49; $n = 94$) for NT and 11.02 (± 2.40; $n = 58$) for T GPs. Statistical analysis (Kruskal-Wallis with a Dunn's *post-hoc* test) revealed a significant difference between control and NT GPs ($p < 0.05$). In control GPs, 98% of single-units had MGDTs of 50 ms or less. A similar value was observed in T GPs (97%), whilst in NT GPs 89% of cells had MGDTs of 50 ms or less.

To determine whether there were hemispheric differences in gap detection thresholds, pure tone responses for the three different experimental groups were analysed according to the IC that they were recorded from (Fig 5.3D).

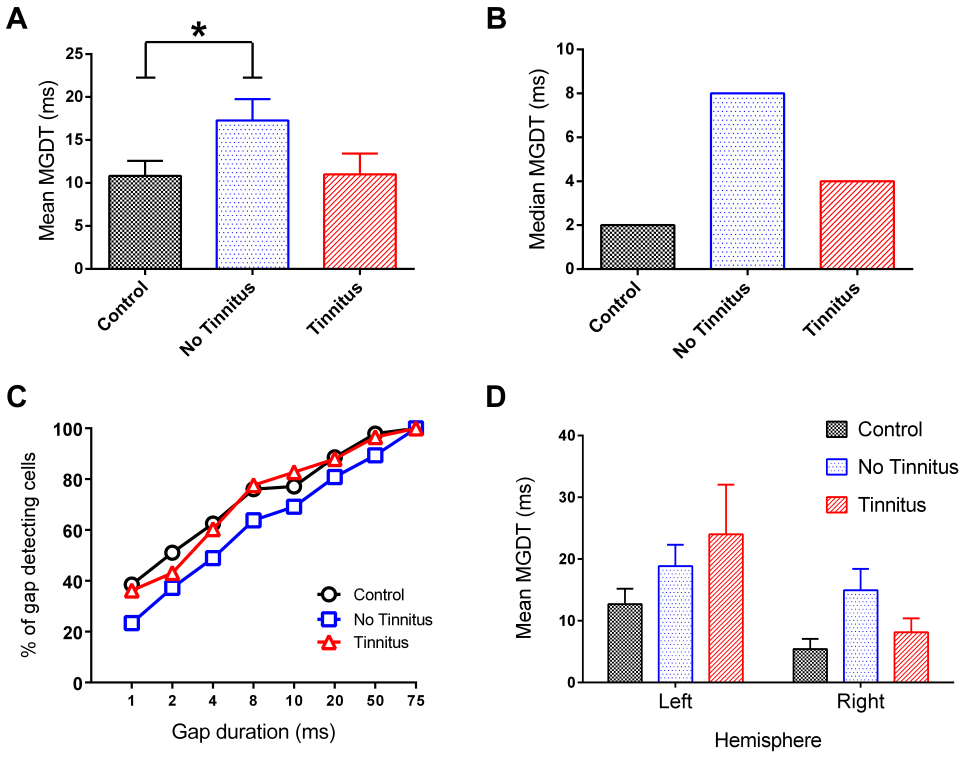

Fig. 5.3: Mean (A) and median (B) MGDTs of single-units for the three different groups in response to pure tones. (C) shows gap detection thresholds as a function of % of responding cells. * $p < 0.05$. (D) shows gap detection thresholds for the three different groups, separated according to hemisphere.

This highlighted that, while noise-exposed GPs generally had longer MGDTs, there were no statistically significant differences in mean MGDTs between any of the three experimental groups when data were separated according to hemisphere. However, there were significant hemispheric differences in the data, in that left IC gap detection thresholds were considerably longer for all three groups than the right IC ($p < 0.05$).

5.2.3 BBN MGDTs

Fig 5.4 shows mean (Fig 5.4A), median (Fig 5.4B) and % of gap detecting cells (Fig 5.4C) in response to BBN. Mean (± SEM) MGDTs in response to BBN were 11.79 (± 2.12; $n = 76$) for controls, 16.04 (± 2.27; $n = 85$) for NT and 21.67 (± 3.46; $n = 55$) for T GPs. There were no statistically significant differences between any of the groups ($p = 0.08$). In control GPs, 96% of single-units had MGDTs of 50 ms or less. A similar value was observed in NT GPs (95%), whilst in T GPs, MGDTs of 50 ms or less were present in 89% of cells.

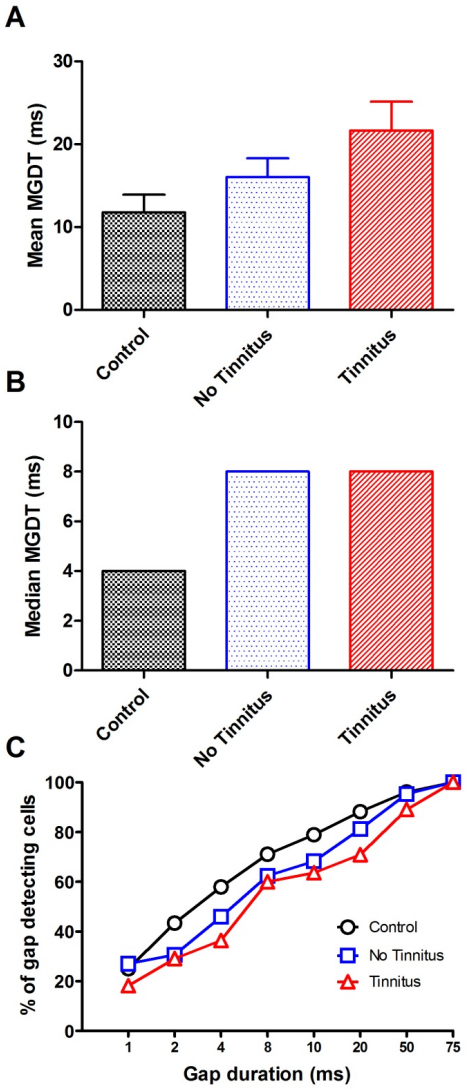

Fig. 5.4: Mean (A) and median (B) MGDTs of single-units for the three different groups in response to BBN. (C) shows gap detection thresholds as a function of % of responding cells.

5.2.4 NBN MGDTs

Fig 5.5 shows mean (Fig 5.5A), median (Fig 5.5B) and % of gap detecting cells (Fig 5.5C) in response to NBN. Cells were analysed in response to NBN, provided their CF fell within 1 kHz of the frequency of the NBN. Mean ($\pm$ SEM) MGDTs were 13.33 ($\pm$ 2.99; $n = 43$) for controls, 19.34 ($\pm$ 3.54; $n = 38$) for no-tinnitus and 20.25 ($\pm$ 6.13; $n = 20$) for tinnitus GPs. In control and NT GPs, 95% of single-units had MGDTs of 50 ms or less. T GPs were slightly less, with 85% of cells exhibiting MGDTs of 50 ms or less. Statistical analysis (Kruskal-Wallis with a Dunn's *post-hoc* test) revealed no significant differences between any of the groups ($p = 0.23$).

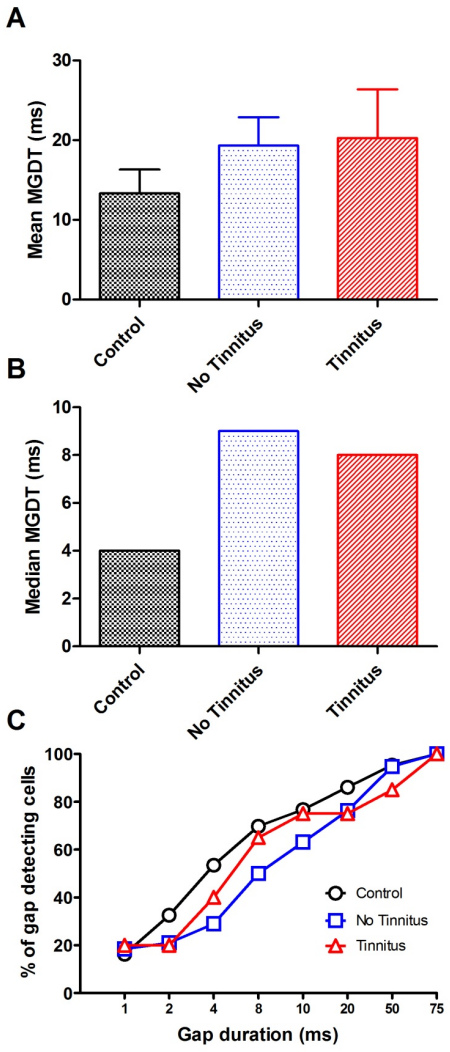

Fig. 5.5: Mean (A) and median (B) MGDTs of single-units for the three different groups in response to the narrowband noise condition that fell within 1 kHz of the CF of each unit. (C) shows gap detection thresholds as a function of % of responding cells.

5.3 Types of Responses

Cells were further analysed according to the types of responses they exhibited. The data were divided into three different classes of units: if a unit responded to the onset of the first 200 ms stimulus but then ceased activity within ~30 ms, it was labelled as an "onset" response; if a unit showed a response that lasted more than 30 ms to the first 200 ms stimulus, it was entitled a "sustained" unit; units which were mostly silent throughout the presentation of both the initial 200 ms stimulus and the second 50 ms stimulus, but responded following the offset of the second stimulus, were categorised as exhibiting offset responses. In determining their MGDT, offset units were classed as having responded to the presentation of a gap if they responded during the silence between the first 200 ms stimulus and the second 50 ms. The first two classes of units were based on the classification used by Astl et al. (1996). Offset responses were classified in a similar manner to Kasai et al. (2012). Examples of the three different unit types are shown in Fig 5.6.

Across the three experimental groups, in response to the pure tone condition, onset cells had a mean (± SEM) MGDT of 16.03 ms (± 2.17; n = 88). The mean MGDT for sustained units was considerably shorter at 7.89 ms (± 1.45; n = 124). There were only a few cells which exhibited offset responses. The mean MGDT of offset units was 30.5 ms (± 10.81; n = 6). These results will be described in more detail in the next section.

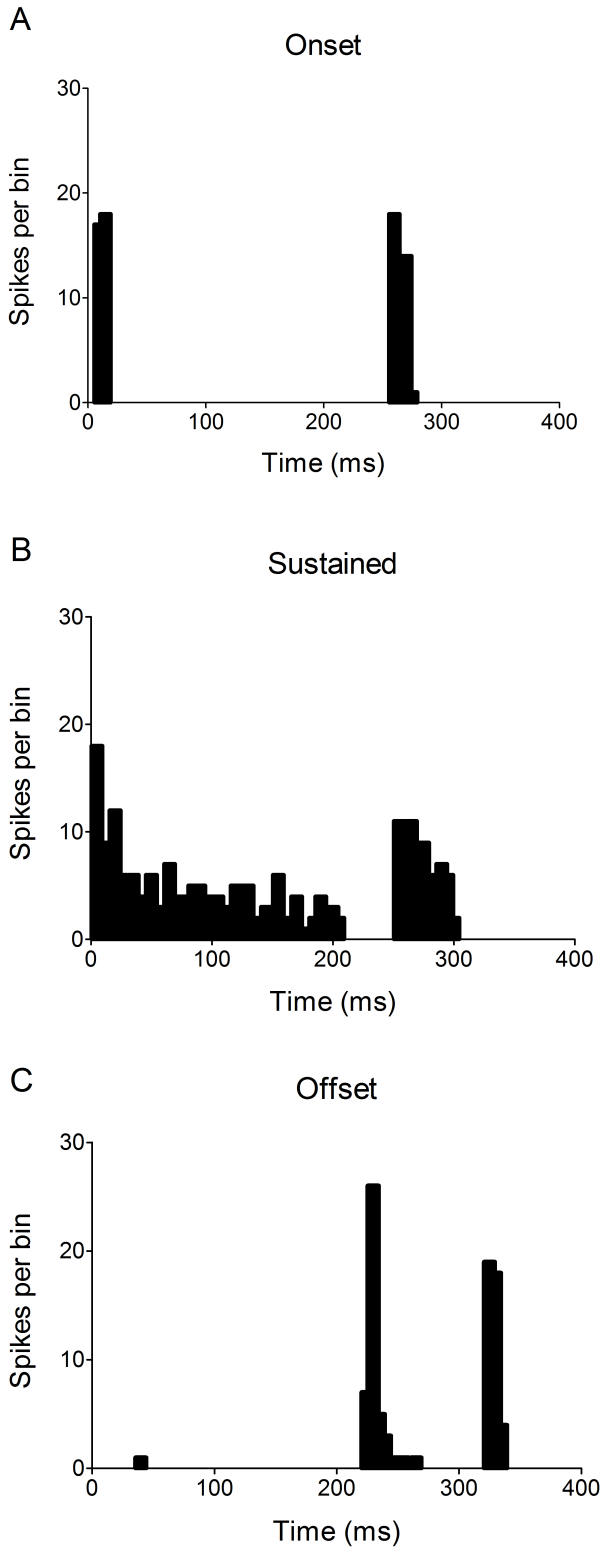

Fig. 5.6: Example responses of three different types of units - onset (a), sustained (b) and offset (c) - in response to the pure tone stimulus condition with a 50 ms gap.

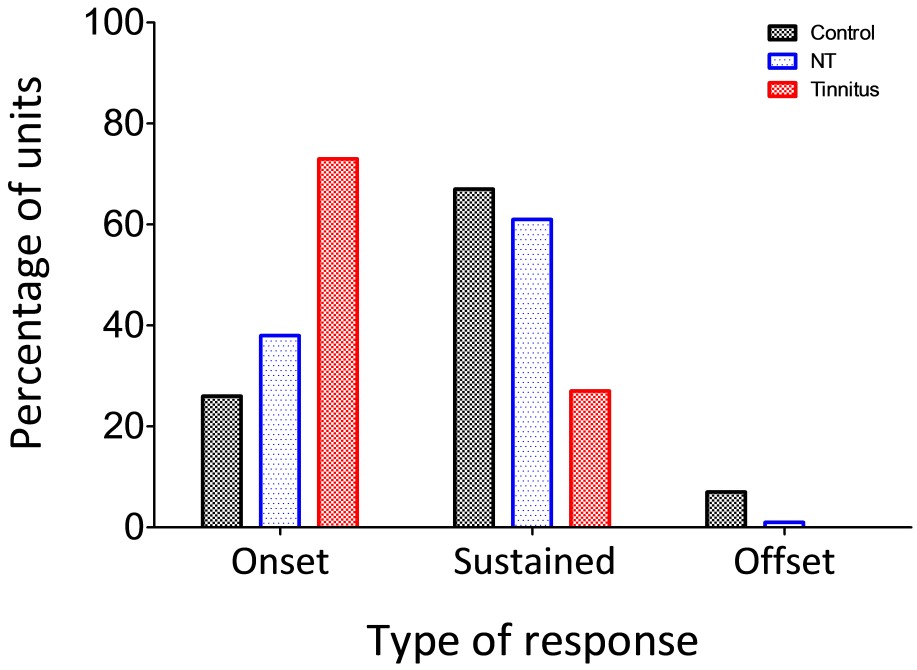

Fig. 5.7: The percentage of unit types for the three different experimental groups. The majority of cells recorded from control animals were classed as sustained units. Contrastingly, onset responses were the most common type for tinnitus GPs.

5.3.1 Changes in Response Type

The three different experimental groups were analysed to determine whether there were any changes in the types of responses expressed by IC neurons. The percentages of unit types for each group are shown in Fig 5.7.

For units recorded from control GPs, 26% exhibited onset responses (n = 23), 67% were sustained (n = 58) and 7% were offset responses (n = 6). For T animals, the majority of units were onset responders (73%; n = 44), while only 27% of units were classified as sustained responses (n = 16) and no units demonstrated offset responses. A chi-squared test was applied to compare the frequency of the different types of units, which revealed that T GPs proportionally had fewer sustained units than controls, but more onset responses (χ^2 (2) = 70.47, $p < 0.0001$). In NT GPs, 38% of units were categorised as onset (n = 36), 61% as sustained (n = 58) and 1% as offset (n = 1). Again, a chi-squared test revealed that there were proportionally more onset units than controls, as well as fewer sustained units (χ^2 (2) =

10.47, $p < 0.01$). Statistical analysis between T and NT GPs highlighted that the proportion of onset units was significantly higher in T GPs than controls, while the reverse was true for sustained units (χ^2 (2) = 31.91, $p < 0.0001$).

Units were also examined for whether their MGDTs (in response to pure tones) varied as a function of response type for each experimental group. The results are displayed in Fig 5.8. Offset units were excluded from analysis due to their low incidence in the sample (6 recorded in total across the three groups). The mean (± SEM) MGDT for onset units in control GPs was 17.43 ms (± 4.19; n = 23), while sustained units in the same animals had a mean MGDT of 6.75 ms (± 1.85; n = 56). Similar figures were observed for NT GPs, with onset units exhibiting a mean MGDT of 17.31 ms (± 3.51; n = 36) and sustained units averaging 10.44 ms (± 2.76; n = 52). The mean MDGT for tinnitus GPs was considerably shorter for both response types, with onset units at 12.71 ms (± 3.06; n = 42) and sustained units averaging 3.56 ms (± 1.34; n = 16).

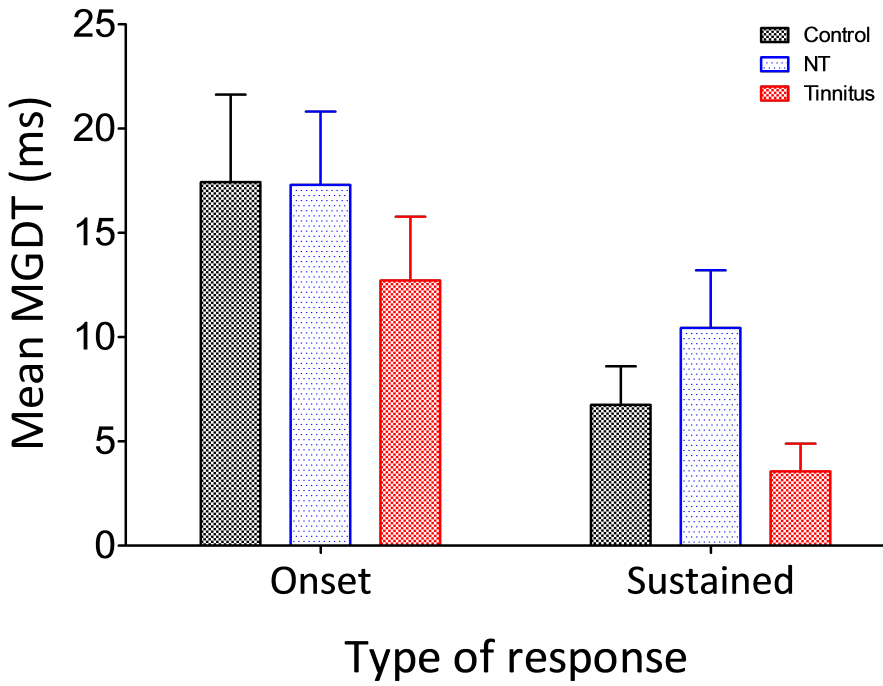

Fig. 5.8: Mean MGDT plotted for the two types of units which occurred most frequently, divided into the three different experimental groups. Error bars represent SEM.

Significance testing was performed using a two-way ANOVA with Bonferroni *post-hoc* test. This indicated that there was a significant overall effect of response type on MGDT, $F_{(1,224)} = 11.17$, $p < 0.01$, consistent with the findings of Walton et al. (2008). The differences in MGDT between the three experimental groups were not significant when analysed according to response type, $F_{(2,224)} = 0.21$, $p = 0.81$.

It is interesting that the mean MGDT was considerably shorter for T GPs than the other two groups when analysed under both response types, and yet slightly longer before cell type categorisation. This can be explained by the fact that there was a significant difference in the unit types expressed in control GPs and T GPs, with control GPs mainly consisting of sustained responses, while T GPs had mostly onset responses. As sustained units had, on average, significantly shorter MGDTs than onset units, this higher incidence in control GPs would mean that they would have shorter MGDTs simply as a result of having more of the types of units that intrinsically had lower MGDTs.

5.4 Alternative Classification of Tinnitus

The data presented in this chapter were reanalysed using the alternative criterion for tinnitus, described in *Chapter 4* under the heading '*Alternative Classification of Tinnitus*'. A total of 7 of the 9 noise-exposed GPs were classified as having behavioural evidence of tinnitus. This number is considerably higher than that found using the original, stricter criteria. However, this equates to ~75% of the animals developing tinnitus, which is consistent with the percentage found in the previous study using the same criterion (see *Chapter 4.4*). Again, across animals, there was no clear trend with regard to the frequency of the gap detection deficits, although only 1 animal demonstrated significant gap detection deficits around the exposure frequency (8-10 kHz background condition; Fig 5.9).

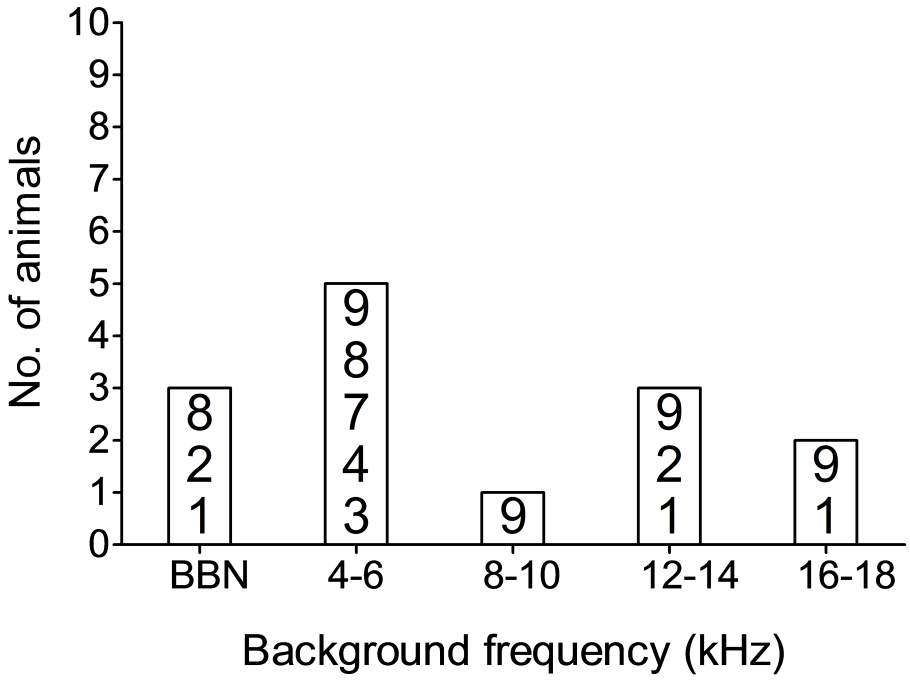

Fig. 5.9: Number of GPs demonstrating significant reductions in PPI following noise exposure, for each background frequency, as determined using a two-way ANOVA with a Bonferroni *post-hoc* test ($p < 0.05$). Numbering denotes GP number.

5.4.1 Pure Tone MGDTs

Fig 5.10 shows mean (Fig 5.10A), median (Fig 5.10B) and % of gap detecting cells (Fig 5.10C) in response to pure tones, analysed under the alternative criterion for tinnitus. Mean (± SEM) MGDTs in response to pure tones were 16.40 (± 3.68; $n = 43$) for NT and 14.29 (± 2.06; $n = 109$) for T GPs. The mean MGDTs for control GPs remained the same for all three background conditions, as these were not affected by the classification. There were no statistically significant differences between any of the groups ($p = 0.13$). In NT GPs, 88% of single-units had MGDTs of 50 ms or less, whilst in T GPs, MGDTs of 50 ms or less were present in 94% of cells.

As with the previous behavioural criteria, data were separated according to the IC that they were recorded from (Fig 5.10D). Again, there were no statistically significant differences in mean MGDTs between any of the three experimental groups when data were separated according to hemisphere. There was still a significant hemispheric difference across the three groups, in that left IC MGDTs were considerably longer than right IC MGDTs ($p < 0.01$).

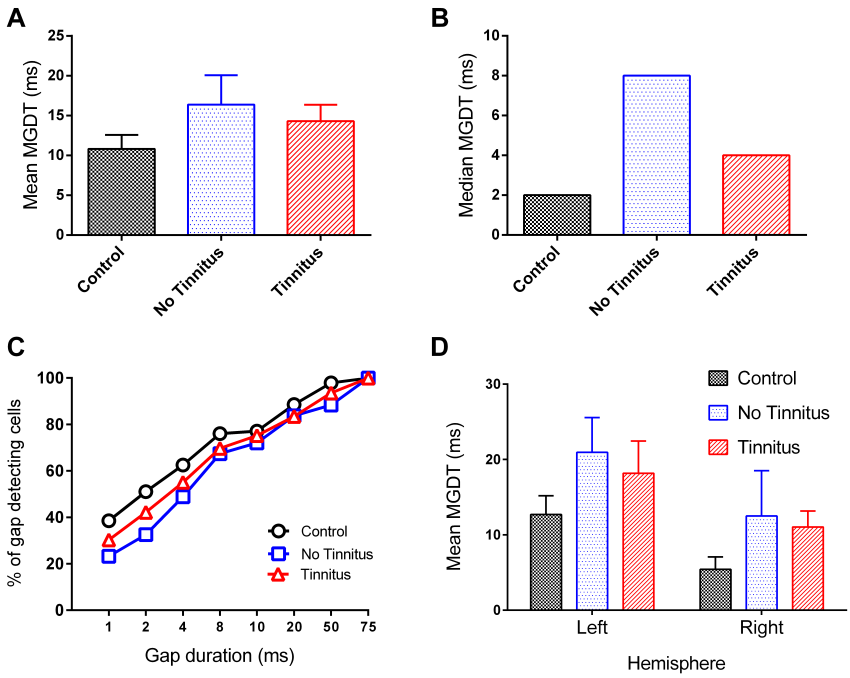

Fig. 5.10: Mean (A) and median (B) MGDTs of single-units for the three different groups in response to pure tones. (C) shows gap detection thresholds as a function of % of responding cells. (D) shows gap detection thresholds for the three different groups, separated according to hemisphere.

5.4.2 BBN MGDTs

Fig 5.11 shows mean (Fig 5.11A), median (Fig 5.11B) and % of gap detecting cells (Fig 5.11C) in response to BBN. Mean (± SEM) MGDTs in response to BBN were 16.76 (± 3.81; $n = 37$) for NT and 18.79 (± 2.27; $n = 103$) for T GPs. No statistically significant differences were evident between any of the groups ($p = 0.07$). In NT GPs, 92% of single-units had MGDTs of 50 ms or less, whilst in T GPs, MGDTs of 50 ms or less were present in 93% of cells.

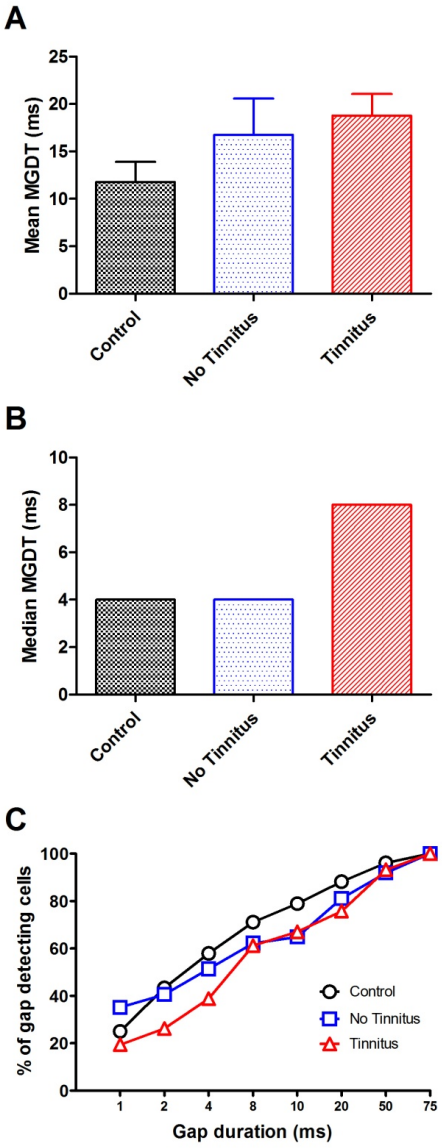

Fig. 5.11: Mean (A) and median (B) MGDTs of single-units for the three different groups in response to BBN. (C) shows gap detection thresholds as a function of % of responding cells.

5.4.3 NBN MGDTs

Fig 5.12 shows mean (Fig 5.12A), median (Fig 5.12B) and % of gap detecting cells (Fig 5.12C) in response to NBN. Cells were analysed in response to NBN, provided their CF fell within 1 kHz of the frequency of the NBN. Mean (± SEM) MGDTs in response to NBN were 20.28 (± 4.95; $n = 25$) for NT and 19.18 (± 4.03; $n = 33$) for T GPs. Statistical analysis revealed that there were no statistically significant differences between any of the groups ($p = 0.18$). In NT GPs, 92% of single-units had MGDTs of 50 ms or less, whilst in T GPs, MGDTs of 50 ms or less were present in 91% of cells.

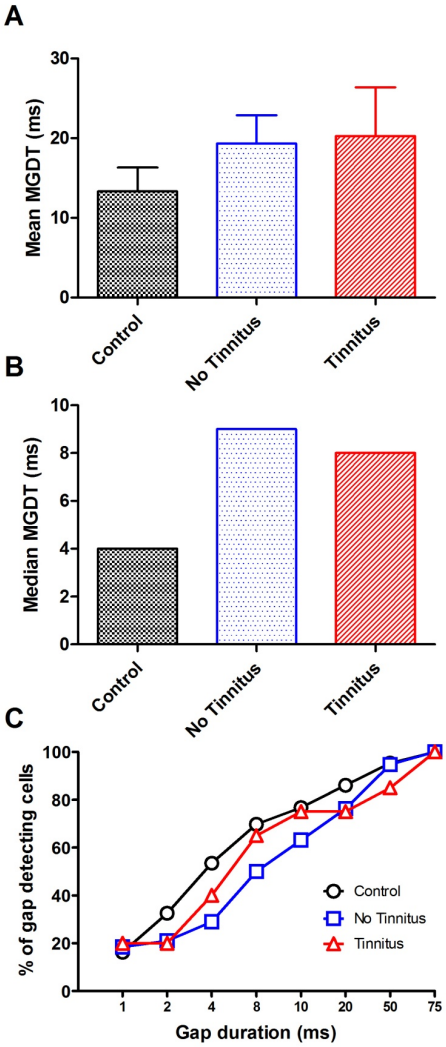

Fig. 5.12: Mean (A) and median (B) MGDTs of single-units for the three different groups in response to the narrowband noise condition that fell within 1 kHz of the CF of each unit. (C) shows gap detection thresholds as a function of % of responding cells.

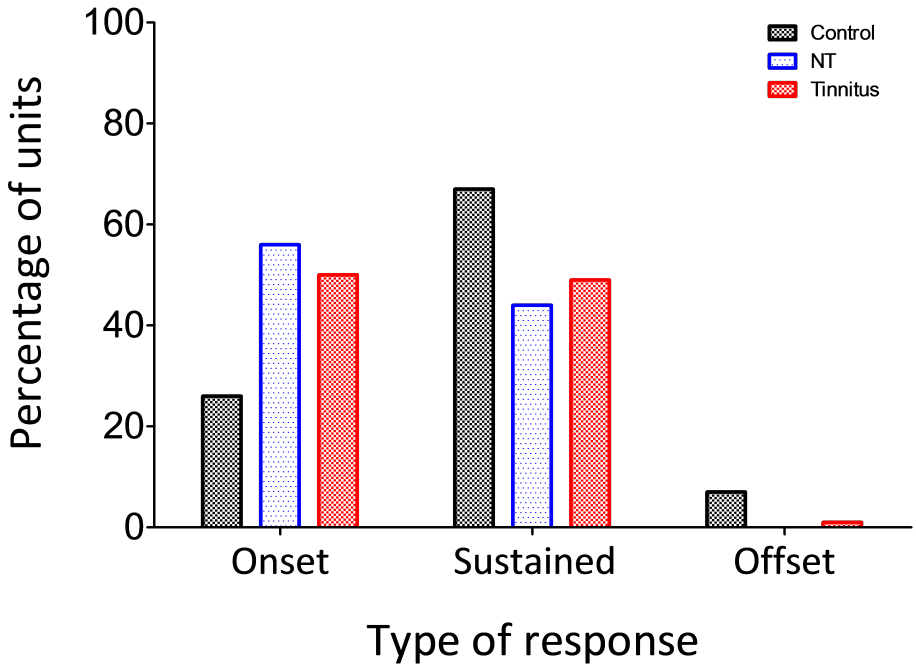

Fig. 5.13: The percentage of unit types for the three different experimental groups. Using the alternative criterion for tinnitus classification, no difference was evident in the type of cells observed between NT animals and T animals.

5.5 Changes in Response Type - Alternative Criterion

Again, using the alternative tinnitus classification criterion, the three experimental groups were analysed for changes in the types of responses expressed by IC neurons. The percentages of unit types for each group are shown in Fig 5.13.

For T animals, using the alternative tinnitus criterion, 50% of units were onset responders ($n = 55$), while 49% of units were classified as sustained responses ($n = 54$) and 1% of units demonstrated offset responses ($n = 1$). Again, despite the lower percentage of onset responses compared to the original criteria, a chi-squared test revealed that there were proportionally more onset units in T animals than controls, as well as fewer sustained units (χ^2 (2) = 35.47, $p < 0.0001$). In NT GPs, 56% of units were categorised as onset ($n = 25$), 44% as sustained ($n = 20$), while no offset units were present. Again, a chi-squared test revealed that there were proportionally

more onset units than in controls, as well as fewer sustained units (χ^2 (2) = 21.69, $p < 0.0001$). Statistical analysis between T and NT GPs was performed, excluding the offset group (as there were no offset cells present in NT GPs to compare to T GPs). The difference in unit types was no longer evident between these two groups, using the alternative tinnitus classification criterion (χ^2 (1) = 1.36, $p = 0.24$).

CHAPTER 6

General Discussion

This thesis documented a number of findings with regard to noise exposure and tinnitus. First, the Preyer reflex gap detection test is a more reliable and robust measure of tinnitus than the WBS in GPs. Second, there is a marked shortening in the latencies of components of the ABR waveform following noise exposure that appears to be linked with tinnitus. Third, increases in the spontaneous firing rate of auditory neurons do not appear to be a strong correlate of tinnitus, but are instead representative of changes following noise exposure (regardless of whether tinnitus is present). Fourth, there is a change in the response patterns of neurons in the IC following noise exposure. Finally, deficits in neural gap detection thresholds following noise exposure do not explain deficits in behavioural gap detection, suggesting that impaired performance on the behavioural task is not a consequence of poor temporal acuity. Here, the impact of these findings will be discussed, along with how they relate to tinnitus.

6.1 Behavioural Measure of Tinnitus Following Salicylate

Objective behavioural measures of tinnitus are essential when creating an animal model of the condition (Kaltenbach, 2011). *Chapter 3* detailed a novel method for quantifying PPI in GPs (published in Berger et al., 2013). This method appears to give robust, consistent responses, and was more reliable than the WBS approach in these GPs. It was also demonstrated that the Preyer reflex method was sensitive for detecting deficits in PPI induced by sodium salicylate, indicative of a tinnitus-like percept in these animals.

The data presented in *Chapter 3* indicate that the Preyer reflex was a more suitable measure than the WBS in detecting tinnitus following salicylate administration. This may be due to the variability of the WBS response in guinea pigs, as highlighted in this study. Since the development of the gap detection model by Turner et al. (2006), the WBS model has been widely used in rats (Wang et al., 2009; Yang et al., 2007), but very few groups have to date successfully induced and measured tinnitus in mice (Turner et al., 2012) or GPs (Dehmel et al., 2012a; 2012b). GPs are notoriously difficult to train without aversive stimuli (Agterberg et al., 2010), and early pilot studies also indicated that they habituated very rapidly to the startle stimulus, or showed a complete lack of WBS responses, which further complicates adaptation of this model for use in GPs. Consequently, it was desirable to find a more-robust method of evaluating PPI in the GP, while still retaining the essential characteristic - measurement of a reflex requiring no training.

The Preyer reflex (described in Bohmer, 1988) appears to offer an elegant solution to these limiting factors. In the present study robust, reproducible startle-evoked responses have been demonstrated that appear far less susceptible to habituation, exhibit a superior SNR overall (meaning that the response was more detectable), and show clear PPI, when compared with the WBS data. Most importantly, baseline PPI of the Preyer reflex was demonstrated at all background noise frequencies in all twelve GPs tested, whereas this was only the case in four of the GPs when evaluating WBS. Moreover, PPI of the Preyer reflex was sensitive to manipulations with sodium salicylate whereas the WBS was not, and thus may present a useful alternative for relating changes in a reflex response to tinnitus.

Salicylate causes transient and reversible tinnitus when administered at high doses in both humans (Mongan et al., 1973) and animals (Ralli et al., 2010). Behaviourally, salicylate has been shown to significantly impair gap detection in rats (Turner and Parrish, 2008), consistent with the results shown in *Chapter 3*. Although the precise mechanisms behind salicylate-induced tinnitus have not - as yet - been elucidated, a number of neural changes have been observed following salicylate administration in animal experiments, including slight decreases in SFRs at the level of the AN, DCN, CNIC and AI, as well as increases in SFRs in the external cortex of the IC and secondary auditory cortex (Basta and Ernst, 2004; Eggermont, 2013; Eggermont and Kenmochi, 1998; Evans and Borerwe,

1982; Lobarinas et al., 2006; Roberts et al., 2010; Wei et al., 2010), although there are conflicting results from different studies (see Stolzberg et al., 2012 for a review). Considering that changes at certain levels of the auditory system may differ from noise exposure (Eggermont, 2006), it is likely that the pathways for tinnitus generation are different dependent on the inducing agent. Nonetheless, salicylate is a useful tool for examining tinnitus. Salicylate treatment reliably induces transient tinnitus; this is proposed (at the central level) to occur as a result of decreased GABAergic transmission and thus a loss of inhibition (Stolzberg et al., 2012).

An important, and potentially confounding, aspect of the gap detection method following tinnitus induction is a reduction in startle amplitudes that has previously been observed after noise exposure (Lobarinas et al., 2013). The potential implication of this finding is that such a decrease may render any gap detection deficits difficult to interpret, as the PPI calculation is a relative measure and thus could be affected by reduced startle amplitudes. In light of the greater degree of variability as well as the poorer SNR of the WBS (compared with Preyer) in these data, it is conceivable that any reduction in amplitudes may often obscure a detectable startle response and render calculations of PPI as meaningless.

In *Chapter 3*, it was documented that reductions in the amplitudes of either Preyer or WBS responses following salicylate administration were not observed. In fact, significant increases were present at various background frequencies. Such increases in amplitude following tinnitus induction have been suggested to relate to hyperacusis (Chen et al., 2013; Sun et al., 2009; Turner and Parrish, 2008), an oversensitivity to sounds that is often present with tinnitus (Dauman and Bouscau-Faure, 2005; Hebert et al., 2004). It is not clear from the data presented in *Chapter 3* whether these increased amplitudes may relate to hyperacusis, as the salicylate injection itself may have caused stress-related augmentation of the startle response in these animals (an idea similar to the fear-potentiated startle; Davis et al., 1993). Interestingly, fear or stress should also cause greater degrees of PPI (Cassella and Davis, 1986; Grillon and Davis, 1997), which is opposite to what has been observed here, further supporting the idea that salicylate-induced tinnitus is causing reduced gap-induced PPI.

It is important to highlight that whilst startle amplitudes did not decrease following salicylate administration, decreased amplitudes are clearly

prevalent following noise exposure (Lobarinas et al., 2013). Consequently, any studies using the gap detection method, regardless of which startle response is measured, should ensure that a clearly detectable response is present following noise exposure. Furthermore, hearing thresholds should have recovered sufficiently in order for an animal to detect and respond to the background stimulus, as this may also confound interpretation of gap detection deficits.

Although the advantages of using the Preyer reflex were clear from the data presented in *Chapter 3* (three times as many GPs demonstrating baseline PPI at every frequency and sensitivity to changes following salicylate administration), an important caveat lies in the design of the SLDT protocol used in these experiments. The SLDT was conducted to determine the optimal sound level combinations (background/startle) for detecting PPI of the Preyer reflex, not the WBS. As a result, the testing conditions were not necessarily optimal for the WBS and hence it is possible that better results might have been obtained for the WBS if the optimisation focussed on this response. For example, the results of Dehmel et al. (2012a; 2012b) suggest that, if optimised, the WBS can be used effectively to measure PPI in GPs. However, from the early pilot experiments that showed inferior SNR and lower levels of detectable WBS, it still seemed likely that the Preyer reflex provided a 'cleaner', more-robust measure in these GPs. The WBS reflex has previously been shown to successfully identify tinnitus in naturally active species, such as rats (Turner et al., 2006) and mice (Turner et al., 2012), but the Preyer reflex may be more suitable in more lethargic animals such as chinchillas or cats (Koka et al., 2011). Furthermore, these animals have large pinnae which would render any Preyer response easier to detect; this may be an important factor in the utility of this approach to measuring gap detection. It would be useful to compare the two methods for measuring PPI in other species.

6.2 Behavioural Measure of Tinnitus Following Noise Exposure

In *Chapter 4*, it was demonstrated that the Preyer reflex gap detection test is a reliable measure of tinnitus following noise exposure, similar to the results of other studies using the WBS as the reflex measure (Chen et al., 2013; Dehmel et al., 2012a; Kraus et al., 2011; Turner et al.,

2006; Turner et al., 2012; Turner and Parrish, 2008; Yang et al., 2007; Zhang et al., 2011). Interestingly, the exposure frequency (10 kHz) was not the most common frequency for gap detection deficits, using either tinnitus classification method. In fact, there was no clear trend with regard to the frequency of the gap detection deficits. This can be explained in part by the ABR shifts observed immediately following noise exposure that occurred across a broad range of frequencies, which would suggest that the exposure frequency would not necessarily predict the frequency of the tinnitus. Another interesting observation is that 4-6 kHz was the most common frequency for gap detection deficits across all noise exposed animals (both *Chapter 4* and *Chapter 5* combined). However, given that deficits were observed at other frequencies in a number of animals, it is debatable as to whether this finding informs us about the precise tinnitus frequency. Other studies suggest that humans often show significant variability when estimating the frequency of their tinnitus (Burns, 1984; Henry et al., 2004; Penner, 1983; Tyler and Conrad-Armes, 1983), with the matched tinnitus pitch fluctuating over time, so the variability observed in these GPs is not surprising. Regardless of any variability between animals, the data presented in *Chapter 3* and *Chapter 4* highlight that the Preyer reflex was suitable for detecting tinnitus in individual animals, without the requirement for animals to be grouped to see differences. This is an important facet of any tinnitus model, as there are clearly interindividual differences in susceptibility of developing tinnitus, the characteristics of tinnitus and the efficacy of different treatments (for an overview of possible genetic differences in interindividual susceptibility, see Sand et al., 2007).

There are limitations to using the reflex response approach that at present remain unresolved. In either form, the method measures - at best - perception of a phantom sound, but fails to assess the emotional components and characteristics that are linked to the level of annoyance produced in the human condition. This, clearly, is a challenging facet to model and quantify in animals, while retaining other features of the current models and not introducing complex behavioural tasks that may interfere with measurement of the phantom sound perception. Despite recent advances in animal tinnitus models, the scope for understanding the pathophysiology of tinnitus remains limited without significant further development. The ideal behavioural test would account for the limbic

components of tinnitus, which clearly have a large impact on how affecting the condition is in humans (Leaver et al., 2012; Rauschecker et al., 2010).

There are concerns that have been raised over the WBS gap detection measure for tinnitus that also need considering for the Preyer reflex test. Reduced startle amplitudes following impaired hearing sensitivity have been shown to affect the measure of prepulse inhibition, which could confound any gap detection deficits. Lobarinas et al. (2013) demonstrated a reduction in startle response amplitude to the level of the noise floor (background movement) in several rats, following unilateral noise exposure. Subsequently, they showed that unilateral conductive hearing loss (induced with earplugs) resulted in rats being classified to a tinnitus group, based on traditional criteria, despite the fact that the earplug testing would likely not cause tinnitus. However, the Preyer reflex, while somewhat reduced in amplitude, is still clearly distinguishable above the baseline noise level, for both 'gap' and 'no-gap' startling stimuli following noise exposure, in animals that are categorised as experiencing tinnitus. Furthermore, by only including animals in the tinnitus group that do not have severe residual hearing loss, as was done in the initial criteria by ensuring that the threshold is no greater than 20 dB HL at the "tinnitus frequency", gap detection deficits caused by reduced audibility of the background carrier are minimised. Also, animals exhibiting gap detection deficits were usually still able to detect gaps at other background frequencies, despite reductions in the amplitude of the Preyer reflex across all background frequency conditions. Therefore, reduced startle amplitudes observed here are unlikely to have confounded measures of gap detection in these animals.

Another major criticism of the gap detection test that has recently been raised is whether it actually measures tinnitus, rather than some form of temporal acuity deficits that may relate to the tinnitus or to the hearing deficit. The initial hypothesis for the test was that gap detection deficits following noise exposure or drug administration reflect a perceptual "filling in" of the gap by the tinnitus (Turner et al., 2006; Turner and Parrish, 2008). With this in mind, it would be expected that gap detection deficits would likely be frequency-specific in tinnitus patients, occurring at or around the frequency of the tinnitus. Fournier and Hebert (2013) recently demonstrated that this was not the case, and that deficits in patients with high frequency tinnitus were also present at much lower frequencies.

As a result, they suggested that gap detection deficits following noise exposure may be caused by reduced temporal acuity of the auditory system preventing detection of the 50 ms gaps.

6.3 Does Tinnitus "Fill in" the Gap?

Chapter 5 detailed the assessment of neural gap detection thresholds in the IC of noise-exposed GPs. This was done to test the validity of the behavioural gap detection test for tinnitus and is the first study to examine neural gap detection thresholds following noise exposure. More specifically, it was of interest to see whether any gap detection deficits observed behaviourally after acoustic trauma may be explained by a reduction in the temporal acuity of the auditory system. While some differences were present, it was clear that 50 ms gaps (the duration used in the behavioural task) were detectable by the majority of single-units in the IC, regardless of whether or not a GP was subjected to acoustic trauma, or subsequently developed tinnitus. Given that IC gap detection thresholds predict behavioural gap detection thresholds in a non-pathological model (Walton et al., 1997), behavioural deficits in gap detection, as seen in the Preyer reflex test, do not appear to be a result of temporal acuity deficits caused by noise exposure.

Interestingly, the only statistically significant difference observed between the three groups was an increase in MGDT for NT GPs compared to controls, for the BBN condition, when using the conservative criteria for tinnitus classification. This suggests that noise exposure does have some effect on gap detection abilities. As some of the GPs in this group sustained a substantial hearing deficit, and given the lack of any significant differences in the other conditions, it is likely that this result is due to a reduction in the audibility of the gap in these animals. However, given that the average MGDT for NT animals in response to BBN (16.04 ms using the original criteria) was far shorter than the duration of the 50 ms gap used behaviourally, this is unlikely to have had a profound effect on behavioural performance. Moreover, many of the GPs in this group still exhibited gap-induced prepulse inhibition at all or most frequencies. Further to this, when the alternative criterion for tinnitus classification was applied, no significant differences were evident between any of the groups for any of the stimulus conditions. With this in mind, it is clear that the slight deficits

in neural gap detection thresholds observed following noise exposure do not explain the gap detection deficits seen behaviourally in T GPs.

It is interesting that there was a significant hemispheric difference in MGDTs across the three experimental groups, in that left IC MGDTs were, on average, considerably longer than right IC MGDTs. This suggests a right hemisphere advantage in temporal processing in these GPs. In humans, some psychophysical studies have previously reported that a left hemisphere advantage was evident in temporal processing (e.g. Brown and Nicholls, 1997; Sulakhe et al., 2003), contrasting with the right side advantage demonstrated in *Chapter 5*. However, other psychophysical studies failed to reproduce this left hemisphere advantage in humans (e.g. Efron et al., 1985; Samelli and Schochat, 2008). Nonetheless, the data presented in *Chapter 5* do conflict with the human psychophysical literature reporting a left hemisphere advantage. It is highly plausible that differences between human studies and the paradigm used here may play a role in any inconsistencies in these findings (e.g. species differences, the use of anaesthetics, or procedural differences, i.e., electrophysiological experiments vs. psychophysical measurements). Interestingly, fMRI studies in humans appear to support the findings of *Chapter 5* and contradict the psychophysical results in humans, showing that increased activation in the right hemisphere was better correlated with performance on a temporal processing task than the left hemisphere (Harrington et al., 2004; Reiterer et al., 2005). Further investigation in animals on hemispheric differences in temporal processing is necessary to address these discrepancies in the literature.

One limitation of the present study is that many of the units did not respond strongly enough to NBN, or their CFs did not fall within 1 kHz of the lower or upper frequency of the noise band, so an MGDT could not be reliably obtained. As a result, the number of units for this condition is substantially lower than the other conditions. In an ideal scenario, these numbers would be equal for all the conditions. Nonetheless, given that the average MGDT for the units that were responsive to NBN was considerably less than the important 50 ms gap duration, it is unlikely that if this sample size was increased the means would substantially change so as to indicate that 50 ms was not detectable by the majority of units. Furthermore, it has been highlighted that psychophysical gap detection thresholds may feasibly be determined by across-channel integration of responsive neurons

(Eggermont, 1999). With this in mind, it can be assumed that the average MGDT to the pure tone gap carrier condition would be a reasonable predictor of behavioural gap detection thresholds. Therefore, the fact that pure tone responses had, on average, the same MGDT for T and control animals suggests that deficits in neural gap detection thresholds are not responsible for behavioural gap detection deficits.

Walton et al. (1998) demonstrated that aged CBA mice with minor sensorineural hearing loss (threshold elevations of 20-30 dB) had poorer gap detection thresholds in the IC than young mice. This is consistent with other studies which have shown that psychophysically-estimated gap detection thresholds are poorer in both aged animals (Barsz et al., 2002; Hamann et al., 2004) and aged humans (Gelfand et al., 1988; Lister and Roberts, 2005; Roberts and Lister, 2004). However, Walton et al. (2008) highlighted that these differences in gap detection thresholds could be solely attributed to age. They examined MGDTs in the IC of middle-aged C57 mice, which are genetically predisposed to develop severe hearing loss (threshold elevations of 40-50 dB) within 6 months, and compared these to young, normal-hearing C57 mice. Their results indicated that there were no significant differences in MGDTs between the middle-aged hearing-impaired mice and normal-hearing young mice. This contrasts somewhat with psychophysical evidence from hearing-impaired humans, showing that gap detection thresholds are significantly longer with a hearing loss when sensation levels are matched to a normal-hearing population, even at a young age (Fitzgibbons and Wightman, 1982).

The present study provides an addition to the considerable body of evidence examining factors that affect gap detection thresholds, showing that while some minor deficits are present following noise exposure, these are generally not significant. It is important to highlight that the estimates of MGDTs found here are considerably longer than those of Walton et al.'s (1998; 2008) studies. However, in their studies, sound levels were matched to the best response of each unit. In order to best model the conditions of the behavioural test, the same sound levels used in the behavioural paradigm were used to determine the sound levels in each neural gap detection experiment. As a result, the levels were not necessarily optimal for each unit, hence the estimates of MGDTs may have been longer. Further to this, the estimates of MGDTs presented in *Chapter 5* are very similar to the psychophysically-estimated thresholds of Fitzgibbons and Wightman

(1982). Regardless of any minor differences, the results here highlight that neural gap detection thresholds following noise exposure cannot explain behavioural gap detection deficits.

The question then remains - what is causing behavioural gap detection deficits following noise exposure? Eggermont (2013) suggested that deficits in gap detection may reflect increased SFRs in subcortical structures. While an animal is under anaesthetic the perception of tinnitus should be abolished (as tinnitus, by definition, requires conscious perception). Thus, if this hypothesis were correct, gap detection would have been expected to be significantly impaired in neurons of the IC, as increased SFRs were indeed present under anaesthetic following noise exposure and this could feasibly result in reduced temporal acuity. However, there was no significant impairment of neural gap detection thresholds observed in tinnitus animals.

Fournier and Hebert (2013) suggested that tinnitus may not be filling in the gap, as the deficits in gap detection they observed in patients with high-frequency tinnitus were not limited to the high-frequency background carrier, but were also present in the low-frequency condition. However, the frequency most similar to patients' tinnitus (16 kHz), as determined using a likeness matching procedure (similar to that used by others; e.g. Moore et al., 2010), was not matched to the frequency of the background carrier (4 kHz for the high-frequency condition; 500 Hz for the low-frequency condition). Therefore, it would have been interesting to determine whether gap detection deficits were even worse at the frequency of the tinnitus than the other frequencies. If this was the case, it would not rule out the hypothesis that tinnitus is perceptually filling in the gap, but would in fact support it. Further studies should be performed using carrier frequencies more similar to the patients' tinnitus, in order to determine whether this hypothesis is correct.

Given the current evidence, Turner et al.'s (2006) original perceptual "filling in" hypothesis still seems a likely candidate for the behavioural gap detection deficits. Nonetheless, further research on humans assessing their performance in detecting gaps similar to their tinnitus would be welcomed.

6.4 Increased SFRs Following Noise Exposure

Many studies have examined hyperactivity throughout the auditory system following noise exposure (e.g. Mulders and Robertson, 2009; Norena et al., 2003). However, few studies objectively assessed animals for noise-induced tinnitus before electrophysiological measurement (Brozoski et al., 2002; Dehmel et al., 2012a; 2012b; Engineer et al., 2011; Kaltenbach et al., 2004; Middleton et al., 2011). At the time of writing, no studies have reported data for noise-exposed NT animals, although Kaltenbach et al. (2004) did demonstrate a correlation between hyperactivity in the DCN and strength of behavioural evidence for tinnitus. In *Chapter 4*, it was reported that spontaneous neuronal firing in the IC was elevated following noise exposure. However, there were no discernible differences between T and NT animals, using either the original criteria or the alternative criterion for tinnitus classification. Neither were there any trends with regard to CF or hemisphere, a result that contrasts with previous reports of a relationship between elevated spontaneous firing and acoustic trauma frequency (Kaltenbach et al., 2004; Mulders and Robertson, 2009; Mulders et al., 2011). Nevertheless, it can be concluded that increases in SFRs appear to relate to the reduced input caused by noise exposure, rather than the tinnitus.

While no previous studies have assessed spontaneous rates in the IC in relation to T and NT (but noise-exposed) animals, Zhang et al. (2004) found that the level of spontaneous activity in the DCN correlated better with the degree of hearing loss than behavioural evidence of tinnitus. The authors concluded that the only reason increased SFRs in DCN relate to tinnitus is because both tinnitus and hyperactivity are caused by the same trigger, i.e. hearing loss. This idea is further supported by the fact tinnitus often occurs immediately following exposure to loud sounds, whereas increases in spontaneous activity are not evident in the DCN until more than 2 days following noise exposure (Kaltenbach and Afman, 2000). This issue is further discussed by Heffner and Koay (2005): it is clearly a contentious point, as many studies still use increased hyperactivity as a neural correlate of tinnitus. Nonetheless, the evidence presented above and in *Chapter 4* suggests that it is likely that increases in SFRs are a direct consequence of damage to the IHCs or OHCs, resulting in reduced input to the auditory system, rather than reflecting the sole underlying cause of tinnitus. This

idea is consistent with the results of studies showing that damage to the IHCs, using the ototoxic drug carboplatin, caused increases in neural activity in the central auditory system (Salvi et al., 2000), as did damage to the OHCs following cisplatin application (Kaltenbach et al., 2002), although these drugs are also known to cause tinnitus (Dille et al., 2010). It is important not to completely rule out increased spontaneous activity in the auditory system as a contributing factor in tinnitus. However, clearly further work is required to determine precisely how this hyperactivity may relate to tinnitus.

The origin of increases in SFRs at the level of the IC following noise exposure was elucidated by Manzoor et al. (2013). They measured SFRs in both the DCN and IC of hamsters following noise exposure, tracking the time-course and the frequency specificity of any changes. The progression of hyperactivity over time was observed in both structures, at similar locations on the tonotopic axis, suggesting that increases in SFRs at the level of the IC were inherited via a passive relay from the DCN. However, SFRs in the IC of noise-exposed animals were considerably lower than those observed in the DCN. This suggests that there is a greater degree of tonic inhibition preserved at the level of the IC following noise exposure. It is uncertain how this relates to tinnitus, as no behavioural tests were performed, although the authors suggest that the relative lessening of increased SFRs at the level of the IC compared to DCN may result in adaptation to the tinnitus signal, leading to lower levels of tinnitus sensation than if levels of IC hyperactivity matched the DCN. Their claim is supported by evidence indicating that tinnitus in humans is often matched to very low sensation levels (Axelsson and Prasher, 2000; Axelsson and Sandh, 1985).

6.5 A Problem of Classification?

One major caveat in the measurements of SFRs in *Chapter 4* is that the classification of the animals as having tinnitus or not relies solely on the behavioural test. The initial criteria used were stricter than many others use, as animals were only considered to have tinnitus if they showed impaired gap detection but minimal hearing loss. This was done to ensure that any gap detection deficits following noise exposure were not simply due to severe hearing deficits preventing detection of the gaps. As a

result, using these criteria, it is not completely certain that all of the no-tinnitus animals did not experience tinnitus, as it was unable to be reliably determined whether tinnitus was present in some of the more hearing-impaired GPs. However, there are a number of factors that support classification of the animals in this manner. First, the percentage of animals that appeared to develop tinnitus following noise exposure is very similar to that found by others using similar levels of noise exposure, with different criteria and operant-conditioning behavioural tests in other species (Henderson et al., 2011; Ruttiger et al., 2013). It is also a similar figure to that reported in humans with a hearing impairment (Lockwood et al., 2002).

Further support for the application of these criteria comes from histological data acquired from GPs used in the SFR experiments (Berger et al., in revision). Histological analysis of the tinnitus and no-tinnitus brains (as identified with the initial conservative criteria), as well as controls, was performed to determine whether there were any changes in the levels of nitric oxide synthase (NOS) expression following noise exposure. Nitric oxide synthase, in its neuronal form (nNOS), is known to modulate synaptic plasticity (see Steinert et al., 2010 for review). High levels of nNOS have also been linked to synaptogenesis in the AVCN following removal of the cochlea (Chen et al., 2004). Using NADPH-diaphorase, which co-localises with NOS (Dawson et al., 1991; Wallace, 1996), significant asymmetries were observed in the VCN of tinnitus GPs compared to controls, but not in the VCN of no-tinnitus GPs (Fig 6.1). As NADPH-diaphorase is not selective to particular isoforms of NOS (of which there are three - neuronal, inducible and endothelial), immunohistochemistry was performed with a monoclonal anti-nNOS primary antibody to confirm that increased expression of NOS was attributable to the neuronal isoform. This is an intriguing result, which suggests that NOS may mediate some of the changes which have been observed in the VCN following noise exposure (e.g. Robertson et al., 2012; Vogler et al., 2011). Further work is required to determine precisely how nNOS may contribute to neural changes associated with tinnitus perception, but the clarity in these nNOS data appears to provide support for the original behavioural criteria.

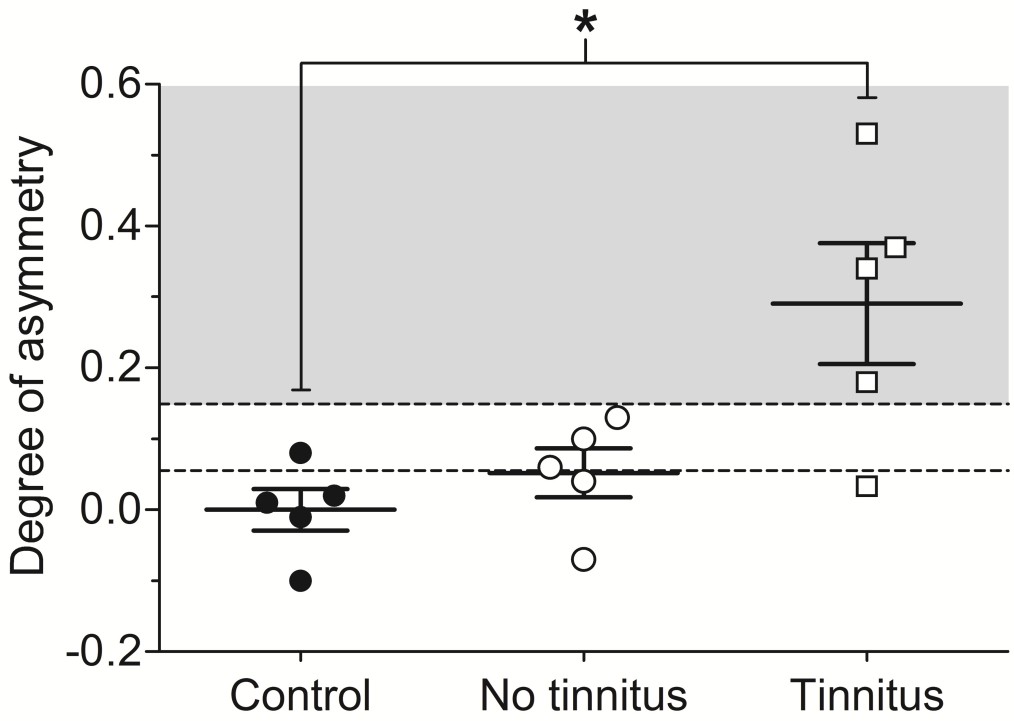

Fig. 6.1: Left-right NOS asymmetry in GPs with tinnitus. The mean ($\pm$ SEM) ratios between left (exposed) VCN and right VCN data are shown - expressed as the degree of asymmetry where values > 0 indicate more NOS-positive cells in the left (exposed) VCN - for control ($n = 5$), NT ($n = 5$), and T ($n = 5$) animals. A significant asymmetry was seen between T and control groups (* $P < 0.05$), but not between NT and control groups. The upper dashed line and grey box indicates the upper 95% confidence limit of the NT group. The lower dashed line indicates the lower 95% confidence limit of the T group. From Berger et al. (in revision).

Also, the frequency-selective changes in ABRs observed were present only in the tinnitus animals. As mentioned previously, any changes in the no-tinnitus animals may have been confounded by significant threshold shifts, but this finding nevertheless further supports the use of these criteria.

Finally, the data presented within this thesis were reanalysed under an alternative criterion, similar to that applied by other researchers. All the results established using the original criteria were replicated using the alternative criterion (including the NOS findings presented above). As a consequence, the lack of differences between tinnitus and no-tinnitus animals in relation to SFRs is an important finding requiring consideration.

6.6 Changes in ABRs Following Noise Exposure

Previous work suggested ABRs as a potential indicator of pathological changes associated with tinnitus. Changes in ABR latency and amplitude were shown in GPs following tinnitus induction (Dehmel et al., 2012a), while a change in the ratio of the amplitudes of the ABR peaks was found in tinnitus patients, supporting the idea of increased central gain in tinnitus (Gu et al., 2012; Schaette and McAlpine, 2011). *Chapter 4* documented a reduction in the latency of ABRs at 10 kHz in T animals, using the original classification criteria. This appeared at least as early as the second wave. The mechanisms behind the reduction at a relatively early stage in the ascending auditory pathway are unclear. However, given the frequency specificity (no latency effects were seen at 5 kHz or 15 kHz), as well as the lack of variability between left and right-side ABRs before noise exposure, this appeared to be robust.

The latency shift was not apparent in NT animals (as classified using the initial criteria). An important caveat, however, is the greater degree of hearing loss in this group and therefore, presumably, a decrease in audibility of the stimuli. This may have increased the latency of the ABRs, which could have obscured any minor reductions in latency that may have taken place. Consequently, it cannot be ruled out that these effects occurred as a non-selective result of noise exposure, and would not therefore be recommended to use these latency shifts as an independent indicator of tinnitus. However, reductions in ABR latency, compared to ABRs measured prior to noise exposure, were observed even when using the alternative criterion for tinnitus classification, which included some animals that had severe hearing loss. Therefore, the observed latency shift shown here is an intriguing result.

This reduction in ABR latency becomes less surprising when viewed in context of human research showing that shorter ABR latencies may be present in hearing impaired listeners, compared to normal hearing listeners (Strelcyk et al., 2009). The explanation for this is that broader filter widths at the level of the cochlea are caused by noise exposure, as a result of decreased frequency selectivity (Dallos and Harris, 1978). This means that each auditory nerve fiber has a broader range of frequencies to which it responds. Based on an idea known as linear systems theory, decreased frequency selectivity at the level of the cochlea would result in a reduction

in cochlear response time (Boer, 1996; Ruggero, 1994). This is further supported by Henry et al. (2011), who demonstrated that noise exposure in chinchillas caused reduced frequency selectivity at the level of the auditory nerve, which was associated with decreases in wave I latency. As a result, it is possible that the reduction in ABR latency observed in *Chapter 4* may simply be a consequence of the noise exposure, rather than tinnitus. This is still unclear though, as significant shifts in ABR latency were only observed in T GPs, around the noise exposure frequency. Furthermore, using the initial, stricter behavioural criteria, the degree of hearing loss was not severe for the T GPs, so any changes in the filter widths as a result of the noise exposure should not have been large enough to significantly affect the latency of the ABR.

Conflicting with the above findings of reduced ABR latencies following noise exposure, Gourevitch et al. (2009) demonstrated that guinea pigs exposed to noise trauma show prolonged Wave III ABR latencies when large temporary threshold shifts were present. Therefore, tinnitus (or its underlying causes) may play a role in the reduction of ABR latencies, as a tinnitus-specific effect on ABR latencies has been shown here. However, more work is required to further elucidate the association between tinnitus and ABR latency. Future studies examining auditory filter widths - which are useful indicators for ABR latencies - in relation to tinnitus would help bridge this gap in the literature.

6.7 Changes in Response Types in the IC

In *Chapter 5*, data was presented on the response types of single units in the IC, for control, noise-exposed and tinnitus GPs. These were broadly categorised into three types based on their post-stimulus time histogram (PSTH) responses to pure tones - onset, sustained and offset. In control GPs, the most common type of unit response was sustained, while approximately 26% of units were onset responders. These figures are consistent with those found by others in the IC of GPs (Astl et al., 1996; Le Beau et al., 1996). Following noise exposure there was a significant increase in the proportion of units exhibiting onset responses, regardless of the behavioural classification of tinnitus applied. In fact, while in control animals the majority of responses were classified as sustained (67%), using the initial classification for tinnitus, units in the IC of T GPs demonstrated

the opposite, resulting in the majority of units (73%) being classed as onset responders. This increase in the proportion of onset responses was still evident using the alternative tinnitus criterion, albeit less dramatic.

Changes in response types of units in the IC have been demonstrated following bicuculline and strychnine (Le Beau et al., 1996). For example, approximately 50% of units demonstrated changes in their PSTH class following application of either drug, most commonly to 'chopper' responses, characterised by a regular discharge pattern of three or more peaks near the stimulus onset. These changes in response type were attributed to the antagonistic effect that these drugs have on GABA and glycine receptors. Furthermore, Wang et al. (1996) examined types of responses in the IC of chinchillas immediately following acute noise exposure, and showed that there was no marked change in the types of responses exhibited by single units. However, the data presented within *Chapter 5* provide the first evidence for long-term changes in response types following noise exposure. It is particularly intriguing that the degree of change in response type was greater for T GPs than NT GPs when using the initial tinnitus classification criteria. This difference between T and NT GPs was no longer evident when using the less strict, alternative criterion. Nevertheless, the change in response types observed following noise exposure was still evident and is a result which warrants further investigation, particularly in determining how this may relate to the manifestation of tinnitus.

There is evidence indicating that the types of responses expressed by IC neurons may represent differing functional roles. For example, Zheng and Escabi (2008) demonstrated that sustained units are effective at encoding the envelope shape of stimuli with low modulation rates, while onset units are most suited to representing repetitive stimuli at high modulation rates. Response properties of units are also determined by inhibitory inputs (Le Beau et al., 1996), mediated by GABA and glycine neurotransmitters. Furthermore, Wallace et al. (2012) found that onset responses are never indicative of laminar cells in the IC, but rather are found to be stellate cells, while sustained units are more likely to be flat laminar cells, highlighting morphological differences between the two response types. As yet, it is unclear how this change in unit type may be associated with tinnitus, although changes in inhibitory circuits, as observed in tinnitus animals (Wang et al., 2011) could feasibly contribute to the changes in the response patterns of units observed above.

Types of units were also analysed in respect to their MGDT. Between experimental groups, when units were divided according to the type of responses they exhibited, no significant differences were found, further highlighting the lack of significant difference in neural gap detection thresholds following noise exposure. Interestingly, sustained units had, on average, significantly shorter MGDTs than onset responders. This is consistent with Walton et al. (2008), who also demonstrated that pure onset units (which mirror the responses that classed as onset here) had slightly longer MGDTs than sustained responses, although their main conclusion from this analysis of response types was that offset (or inhibitory) responses had the longest MGDTs. As the sample size for offset responses was too small, these were not included in the analysis, as they may have skewed the statistical test. However, the results for offset responses were similar to those of Walton et al. (2008), as well as Wilson and Walton (2002), in that the mean MGDT of offset responses was considerably longer than onset or sustained responses (30.5 ms, 10.81 SEM; $n = 6$).

6.8 What Do the Present Results Tell Us About the Causes of Tinnitus?

It is useful to briefly reconsider what the underlying causes of tinnitus may be, in light of some of the data presented within this thesis. It appears that increased SFRs following noise exposure are not sufficient alone to induce a chronic tinnitus percept. Based on Schaette and McAlpine's gain control theory (Schaette and McAlpine, 2011), increased spontaneous rates would result from homeostatic mechanisms as a consequence of reduced input to the auditory system from the periphery following noise exposure. This is supported by the data presented here and consistent with what others have found (Kaltenbach et al., 2004; Mulders and Robertson, 2009; Mulders et al., 2011). However, there must be further mechanisms which induce and sustain the chronic tinnitus percept, as the work presented here and that of Zhang et al. (2004) indicates that increased hyperactivity in the IC and DCN is likely a direct result of noise exposure.

Rauschecker's limbic system gating control mechanism (Rauschecker et al., 2010) is an appealing candidate as a contributing factor. It is evident that, following noise exposure, spontaneous firing rates are increased

throughout the auditory system, up to the level of the inferior colliculus. Then, if there is dysfunction in the limbic gating mechanism, which prevents unwanted sustained activity from reaching the auditory cortex, this could feasibly result in cortical hyperactivity, driven by bottom-up connectivity. It is plausible that this cortical hyperactivity may manifest itself consciously as a tinnitus percept. Patients who suffer badly from tinnitus are often found to also have a major depressive disorder (Dobie, 2003). Additionally, the severity of a patient's tinnitus, as well as tinnitus prevalence, has been shown to be reduced by a reduction in their depressive mood (Hebert et al., 2012). It is therefore possible that depression, or extreme stress, may cause dysfunction in this gating mechanism, via a depletion in serotonin (the neurotransmitter proposed to mediate this gating mechanism; Rauschecker et al., 2010). Clearly the above proposal is an over-simplistic explanation for tinnitus generation. However, further work on animal models of stress, combined with animal models of tinnitus, may help reveal the role that stress plays in tinnitus production.

It is important to remember that tinnitus is not a homogenous disease, but a heterogeneous collection of symptoms. Indeed, with the research field it will likely be necessary to break tinnitus down into different subtypes, as the causes of tinnitus may differ according to the characteristics (Landgrebe et al., 2010). For example, some patients are able to manipulate their tinnitus with jaw movements (Lockwood et al., 1998) or by changing the direction of their gaze (Coad et al., 2001), which implicates a role for the somatosensory system in modulating their tinnitus (Dehmel et al., 2012b). Creating subtypes may prove difficult in animal models of tinnitus, where it is currently impossible to determine the precise characteristics of the tinnitus. It is nevertheless essential for this subdivision to be considered, in order to fully identify the mechanisms behind chronic tinnitus generation and create successful therapeutic interventions.

6.9 Conclusions

- From these data, the Preyer reflex gap detection test for tinnitus appears to be more reliable than the whole body startle measure in GPs.
- Tinnitus is a plausible explanation for gap detection deficits observed following noise exposure, particularly when severe hearing loss is accounted for, as gaps of 50 ms are detected by the majority of IC units following noise exposure, regardless of whether there are behavioural gap detection deficits.
- Hyperactivity alone cannot account for tinnitus perception, as increased SFRs are present following noise exposure even in the absence of behavioural evidence of tinnitus.
- Reductions in ABR latency, changes in levels of nNOS and alterations in the responses of IC neurons do appear to be uniquely related to tinnitus. Further studies should elucidate how these may contribute to the manifestation of the tinnitus percept.

6.10 Suggestions for Further Developments

- Although in this thesis, the Preyer reflex was measured using expensive motion tracking hardware, there is little reason why the reflex could not be measured using simpler hardware, such as accelerometers or low-cost, high frame rate cameras analysed on a frame-by-frame basis. This would provide a cost-effective behavioural test for tinnitus.
- It would be of interest to measure (using electromyography) the post-auricular muscle reflex (PAMR), a vestigial response in humans which produces the pinna reflex in animals (Berzin and Fortinguerra, 1993), to assess the possibility of developing an objective test for tinnitus in humans, based on the gap detection paradigm. The PAMR seems to have a simpler circuitry than the commonly-used eyeblink startle response and appears to be less influenced by attention (Benning et al., 2004; Hackley, 1993; Hackley et al., 1987), so may be a good candidate as a startle measure in a tinnitus test paradigm. This might provide a highly useful tool for clinicians to determine

the characteristics of a patient's tinnitus without solely relying on subjective report. Such a test is not currently available but would be of considerable use (McCombe et al., 2001).

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