

**IDENTIFYING NEURAL CORRELATES OF TINNITUS: CONSEQUENCES OF
ACOUSTIC TRAUMA ON INFERIOR COLLICULUS ACTIVITY IN UNANESTHETIZED
RATS**

BY

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Doctor of Philosophy.

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ABSTRACT

Tinnitus, or ringing in the ears, is a condition that affects as many as 50 million Americans. It can be caused by numerous factors, and is considered a symptom rather than a disease. Although it is very prevalent in many susceptible populations, the mechanisms of tinnitus are still not well understood. Most commonly defined as the perception of sound when no corresponding external sound is present, tinnitus was originally thought to be generated in the peripheral auditory system. However, it is currently believed that tinnitus is generated and maintained in central auditory structures. Researchers have spent recent decades seeking to identify the neural correlates of tinnitus in the central auditory system, without much success. Before an effective treatment can be developed for individuals who suffer from tinnitus, it is imperative to understand the underlying mechanisms of the condition. This work summarizes our findings in the context of current tinnitus research. We sought to evaluate changes in spontaneous neural activity in the inferior colliculus of awake, freely moving rats following acoustic trauma. We were able to measure levels of spontaneous activity (SA) following acoustic trauma with the use of both single unit electrophysiology and ¹⁴C-2-deoxyglucose assays. By combining these two methods in our animal model of tinnitus, we were not only able to further evaluate SA as a neural correlate of tinnitus, but we were able to address possible problems that tinnitus researchers face, namely the use of anesthesia, varying sound exposure paradigms, and evaluation of both the dominant and non-dominant pathways in the central auditory system.

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My parents, Jim and Sue Kennon

My brother, Josh Kennon

My grandparents, Bill and Helen Kennon

And

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CHAPTER 1: INTRODUCTION

1.1 TINNITUS EPIDEMIOLOGY AND ETIOLOGY

Tinnitus, or ringing in the ears, is often defined as the perception of sound when no corresponding external sound is present. The American Tinnitus Association estimates 50 million Americans experience tinnitus, with 2 million individuals considered to be severely debilitated by their condition (ATA, 2013). Additionally, an estimated 10% of the entire UK population is thought to experience chronic tinnitus (BTA, 2009). It is most common among the senior population, affecting up to 12% of people over 60 years of age (Eggermont and Roberts, 2004), but it is also becoming a concern among younger generations, due to the overuse of ear bud style headphones (Gilles et al., 2013). In addition to these two populations, tinnitus is a serious problem in the military, with approximately 15,000 new cases of tinnitus diagnosed each year, and over 17,000 new cases diagnosed in 2010 alone (Helfer, 2011).

It is reported that men experience tinnitus more often than women (Seidman et al., 2010), but women tend to experience more complex tinnitus than men (Dineen et al., 1997). It has also been reported that Caucasians experience tinnitus at a higher frequency than African-Americans in the United States (Seidman et al., 2010), but there is little data on differences between other ethnic groups. Interestingly, people in the Southern United States are two times more likely to complain of tinnitus than individuals in the Northeast (Seidman et al., 2010).

It is important to note that tinnitus is not considered a disease, but instead it is considered a symptom which people experience due to a number of causal factors. There are two main categories of tinnitus: subjective and objective tinnitus, both of

which can be caused by a variety of factors. Objective tinnitus is defined as the perception of a sound that can be measured by someone other than the patient. Objective tinnitus is often caused by vascular conditions or otoacoustic emissions from the outer hair cells. Conversely, subjective tinnitus can only be perceived by the tinnitus sufferer and the sound cannot be objectively measured in the external environment.

Subjective tinnitus can be induced by many causes. One of the most common causes of subjective tinnitus is acoustic trauma, or sound exposure. Exposure to loud sounds and noises can cause both temporary and chronic tinnitus in humans. Ototoxic drugs are another possible cause of tinnitus. Some drugs, such as salicylates, nonsteroidal anti-inflammatory agents, chemotherapeutic agents such as cisplatin, and quinine can cause tinnitus that is reversible once the drug administration is stopped. However, many aminoglycosides, such as gentamicin, streptomycin, and neomycin can cause irreversible tinnitus (Holmes and Padgham, 2011). Other causes of tinnitus are head and neck trauma, certain diseases, such as Lyme and Meniere's disease, and certain kinds of tumors (ATA, 2013)

Although it is commonly associated with hearing loss, it is possible for tinnitus to present in the absence of any clinically diagnosed hearing loss (Martines et al., 2010). In addition to the wide variety of ways in which tinnitus can be induced in humans, it can also manifest in a variety of ways. For example, patients may experience either bilateral or unilateral tinnitus. They also may perceive the sound in varying intensity, pitch, and loudness. One individual may hear a ringing, while another may experience a buzzing or a roaring sound. Some patients even report their tinnitus to be in the form of distant voices, in the absence of any psychiatric disorder (McKenna, 2011). Due to

the highly variable nature of the condition, tinnitus can be difficult to treat (Seidman et al., 2010).

1.2 CURRENT TINNITUS THERAPIES

Because tinnitus is so prevalent among a wide variety of populations and can severely affect the quality of life for millions of individuals, it is imperative to develop an effective treatment for tinnitus sufferers. There are currently many therapies and treatments that have been tested and approved, although no single therapy or treatment has proven effective for a large percentage of the population of tinnitus sufferers. Table 1.1 outlines a selection of studies that have tested different treatments for tinnitus, as well as the outcome for each study.

A number of pharmacologic agents have been tested in clinical trials for tinnitus patients. Several types of benzodiazepines have been tested in clinical trials for alleviation of tinnitus. Diazepam was found to have no effect on tinnitus loudness (Kay, 1981). However, alprazolam was shown to reduce tinnitus loudness in up to 76% of subjects (Johnson et al., 1993), and clonazepam reduced tinnitus loudness in 32% of subjects (Gananca et al., 2002). The tricyclic antidepressant amitriptyline showed decreases in tinnitus loudness for up to 95% of subjects (Bayar et al., 2001), whereas the SSRI paroxetine showed no improvements over placebo (Robinson et al., 2005). While some of these pharmacologic agents do appear to show improvements in large percentages of subjects in clinical trials, the reduction in tinnitus loudness tends to be moderate. In addition to these drugs, several herbal supplements have been proposed as treatments for tinnitus. Both ginkgo biloba and vitamin B12 have been tested in

Treatment	Outcome	Article
Diazepam	No change	Kay, 1981
Alprazolam	76% of subjects showed reduction in loudness	Johnson et al., 1993
Clonazepam	32% of subjects showed reduction in loudness	Ganancia et al., 2002
Amitriptyline	95% of subjects showed reduction in loudness	Bayar et al., 2001
Paroxetine	No change	Robinson et al, 2005
Sertraline	More effective than placebo	Zoger et al, 2006
Steroids	Unclear	Wei et al., 2013
Gabapentin	Reduction in annoyance	Bauer and Brozoski, 2006
Ginkgo Biloba	No change	Hilton et al., 2013
Vitamin B12	No change	Berkiten et al., 2013
Tinnitus Retraining Therapy	80% of patients show improvement	Jastreboff and Jastreboff, 2000
Vagus Nerve Stimulation	40% of subjects show improvement	De Ridder et al., 2013
Transcranial Magnetic Stimulation	Short term, moderate improvements	Plewnia et al., 2007
Direct cortical stimulation	Possible improvements	Seidman et al., 2008

Table 1.1 Selection of various proposed treatments for tinnitus. This table summarizes various treatments that have been proposed for the alleviation of tinnitus. Select studies have been chosen to represent the outcome of clinical trials for each treatment.

clinical trials, but neither have been shown to have any significant effect on tinnitus (Berkiten et al., 2013; Hilton et al., 2013).

There are also a number of non-pharmacological treatments for tinnitus. One of the most widely used treatments is Tinnitus Retraining Therapy (TRT). TRT combines counselling and tinnitus masking to help patients habituate to their tinnitus, while attempting to decrease the loudness of perception of tinnitus (Jastreboff and Jastreboff, 2000). Another therapy that is occasionally used is transcranial magnetic stimulation (TMS). TMS uses pulses of magnetic fields to redirect neural activity. Some studies have shown improvements in tinnitus patients with the use of TMS, but the changes tend to be short term, on the order of weeks (Plewnia et al., 2007). In addition to these two treatments, vagus nerve stimulation (VNS) is currently being investigated as a new form of therapy for tinnitus. Approved for use in patients with intractable epilepsy and severe depression, VNS stimulates the vagus nerve in short pulses. The stimulation occurs by wrapping an electrode around the left branch of the vagus nerve in the neck, which is accompanied by an implanted stimulation device. Kilgard et al. have paired VNS with tones to redirect cortical plasticity following damage and tinnitus induction. Although the therapy is in the initial stages of development, it appears to be effective in at least 40% of subjects (De Ridder et al., 2013). However, despite the many treatments that have been proposed, there remains no single treatment that is successful in a large percentage of tinnitus sufferers.

1.3 HISTORY OF TINNITUS RESEARCH

Before an optimal treatment can be developed, it is imperative to understand the underlying causes of tinnitus. Little is currently known about the mechanisms of the generation and maintenance of tinnitus, although researchers have studied the condition for decades. It was originally believed that tinnitus was generated and maintained solely in the peripheral auditory system. It was thought that the perception of tinnitus stemmed from abnormal activity in the cochlea, and possibly the auditory nerve. However, initial research into changes in spontaneous activity (SA) in the auditory nerve fibers following the use of noise or ototoxic drugs, which were used to mimic the ways in which humans develop tinnitus, proved inconclusive (Kiang et al., 1976; Dallos and Harris, 1978; Evans et al., 1981; Salvi and Ahroon, 1983).

The tinnitus hypothesis then began to shift away from the peripheral auditory system in the early 1980's, after House and Brackmann published a study examining the state of tinnitus in patients who underwent removal of acoustic tumors with excision of the auditory nerve. In their study, 55% of patients who suffered from tinnitus prior to surgery reported their tinnitus to be either the same as or worse than before having the auditory nerve severed (House and Brackmann, 1981). In a similar study, Gardner reported 52% of patients also experienced their tinnitus with the same or worse intensity following surgery (Gardner, 1984). Tinnitus was relieved in nearly half of these patients, which indicates some influence of peripheral sources. However, these studies indicated the cochlea was not the sole generator for tinnitus perception, as the input from the cochlea was completely abolished and over half of the patients still experienced tinnitus.

Following these studies, researchers began to focus more on central auditory structures, such as the dorsal cochlear nucleus (DCN), ventral cochlear nucleus (VCN), inferior colliculus (IC), and the auditory cortex. Initial studies were performed in animals with tinnitus induced by the use of salicylate. Sodium salicylate is known to cause acute tinnitus in humans, which can be alleviated once the drug is cleared from the system (Mazurek and Szczepek, 2010). This made it an ideal method to create an animal model of acute tinnitus. These studies provided initial evidence for central changes in the auditory system that were related to tinnitus and not just hearing loss. Jastreboff and Sasaki were among the first to find increases in IC SA following the administration of salicylate (Jastreboff and Sasaki, 1986), and Kenmochi and Eggermont found an increase in cortical SA in recording sites with high characteristic frequencies (Eggermont and Kenmochi, 1998). It is important to note that while salicylate has been shown to cause permanent hearing loss at high doses with prolonged exposure (Chen et al., 2013), the changes observed in these studies were due to low doses and short exposure to salicylate, causing temporary hearing loss and tinnitus.

While the salicylate-induced tinnitus studies provided strong evidence for the role of the central auditory system in tinnitus, many humans experience chronic tinnitus, which cannot be properly modeled by using acute doses of salicylate. Therefore, in the late 1990's and early 2000's, researchers developed the first chronic tinnitus animal models. These studies utilized acoustic trauma to permanently damage the peripheral auditory system and mimic conditions in which humans often develop tinnitus (Zhang and Kaltenbach, 1998; Brozoski et al., 2002; Seki and Eggermont, 2003). The majority of these studies found increases in SA in various central auditory structures following

acoustic trauma. These initial chronic tinnitus studies have spurred more work to be done in these auditory areas using animal models of chronic tinnitus.

1.4 CURRENT TINNITUS RESEARCH

It is currently thought that if the peripheral auditory system sustains damage, whether in the form of acoustic trauma, lesions, or ototoxic drugs, the excitatory and frequency specific input from the eighth nerve will be altered. Central auditory structures will lose input from the cochlea and become deafferented. Deafferentation may then cause an imbalance of excitation and inhibition within each structure. Both excitation and inhibition play an important role in each auditory nucleus. For example, fusiform cells, which are important output cells in the DCN, receive inhibitory input from vertical cells (Rhode, 1999). Additionally, the lateral superior olive (LSO) provides a great degree of inhibitory activity to the central nucleus of the IC (Loftus et al., 2004). Alterations along these pathways can cause changes in the balance of inhibition and excitation, significantly affecting any number of nuclei along the central auditory pathway. This may lead to aberrant neural activity, such as hyperactivity, increased neural synchrony, and increased burst firing. These types of aberrant activity may cause the perception of sound, and thus are possible neural correlates of tinnitus (Eggermont and Roberts, 2004).

In the past decade, many researchers have sought to identify the neural correlates of tinnitus. Of particular interest has been the idea of an increase in SA within various central auditory structures following peripheral damage. Tables 1.2 and

1.3 summarize the general methods and results of many of these studies. Although each study aims to evaluate changes in SA following damage to the cochlea, the way in which tinnitus is induced in each study can vary considerably. Some labs continue to use salicylate to temporarily induce acute tinnitus (Salvi et al., 2000; Ma et al., 2006; Parrish, 2008), while others use alternative ototoxic drugs, such as cisplatin, a chemotherapy drug known to cause hearing loss (Kaltenbach et al., 2002; Bauer et al., 2008).

1.5 ACOUSTIC TRAUMA AS A METHOD OF TINNITUS INDUCTION

Although inducing tinnitus with the use of a pharmacologic agent is still widely accepted as a useful tinnitus model, the majority of published studies are currently performed in animals that have received acoustic trauma to induce tinnitus. One reason for the continued use of sound exposure as a tinnitus inducer is the extent to which it can mimic the human condition. A large percentage of tinnitus patients also suffer from some degree of hearing loss due to exposure to loud sounds. However, the degree of hearing loss does not correlate with the severity of tinnitus (Tan et al., 2013). Additionally, patients may present with tinnitus in the absence of any hearing loss or elevated thresholds (Langers et al., 2012). These findings indicate that no single degree of damage is responsible for tinnitus induction in all humans, which may be why there is such variability in sound exposure paradigms used in animal research.

When reviewing tinnitus literature, it becomes apparent that there is no agreement on what sound exposure paradigm is best for tinnitus induction in animals, making it difficult to reach a consensus on the main mechanisms of tinnitus in the

Method of Tinnitus Induction	Animal Model	Area	Post-damage Recovery Time	Type of Anesthesia	Results	Paper
Cisplatin	Chinchilla	IC	2 weeks	Ketamine, Xylazine	↑Mean SA, contralateral	Bauer et al., 2008
Salicylate or quinine	Cat	Cortex, A1	10-90 min, 90-180 min	Ketamine, Pentobarbital	↑ SA low CF, ↓ high CF	Eggermont and Kenmochi, 1998
Salicylate	Guinea pig	IC	2h	Pentobarbital	↑Mean SA	Jastreboff and Sasaki, 1986
Cisplatin	Hamster	DCN	1 mo	Ketamine, Xylazine	↑Mean SA in animals with OHC loss	Kaltenbach et al., 2002
Salicylate	Mouse	IC	2h	Ketamine, Xylazine	Decrease in global SA	Ma et al., 2006
Salicylate	Rat	Cortex	2h, 1-3 days	No anesthesia	↓ after 2h, return to baseline over 3d	Yang et al., 2007

Table 1.2 Summary of studies evaluating changes in SA following tinnitus induction with ototoxic agents. This table summarizes the study design and results from representative studies that evaluated changes in SA following damage to the peripheral auditory system with the use of various ototoxic agents

Method of Tinnitus Induction	Animal Model	Area	Post-damage Recovery Time	Type of Anesthesia	Results	Paper
Unilateral, 1hr, 4 kHz, 85 dB SPL	Chinchilla	IC	2 weeks	Ketamine, Xylazine	↑Mean SA, contralateral	Bauer et al., 2008
Unilateral, 30-60 m, 4 kHz, 80 dB SPL	Chinchilla	DCN	1 week	Ketamine, Xylazine	↑Mean SA, bilateral	Brozoski et al., 2002
Unilateral, 1h, 10 kHz, 124 dB SPL	Guinea pig	IC	2 or 4 weeks	Pentobarbital, Hypnorm	↑Mean SA, bilateral	Dong et al., 2010
Bilateral, 1hr, 16 kHz, 115 dB SPL	Rat	Cortex	11 weeks	Barbiturates - unknown	↑Mean SA	Engineer et al., 2011
Free-field, 4h, 10 kHz, 115 dB SPL	Hamster	DCN	5-6 d	Ketamine, Xylazine	↑Mean SA	Finlayson and Kaltenbach, 2009
Bilateral, 1hr, 6 kHz, 126 dB SPL	Cat	Cortex	7-16 weeks	Ketamine, Pentobarbital	↑Mean SA in tonotopically reorganized areas	Komiya and Eggermont, 2000
Bilateral, 4h, 10 kHz, 105-120 dB SPL	Cat	DCN	28-98 d	No anesthesia, decerebrate	No increase in SA	Ma and Young, 2006
Unilateral, 1hr, 16 kHz, 103 dB SPL	Mouse	IC	13-96 d	Ketamine, Xylazine	No global SA change, bilateral, ↑ SA near CF	Ma et al., 2006
Bilateral, 1hr, 16 kHz, 103 dB SPL	Mouse	IC	13-96 d	Ketamine, Xylazine	No global SA change, bilateral, ↑ SA near CF, ipsilateral	Ma et al., 2006
Unilateral, 1h, 10 kHz, 124 dB SPL	Guinea pig	IC	13-28 d	Pentobarbital, Hypnorm	↑Mean SA	Mulders et al., 2010
Unilateral, 1h, 5 or 6 kHz, 115-120 dB SPL	Cat	Cortex	2h	Ketamine, Pentobarbital	↑Mean SA	Norena and Eggermont, 2003
Bilateral, 2h, 6 kHz, 115 dB SPL	Cat	Cortex	1-16 weeks	Ketamine, Pentobarbital	↑Mean SA in tonotopically reorganized areas	Seki and Eggermont, 2003
Unilateral, 2h, 10 kHz, 124 dB SPL	Guinea pig	VCN	2 weeks	Pentobarbital, Hypnorm	↑Mean SA	Vogler et al., 2011
Unilateral, 2h, 10 kHz, 124 dB SPL	Guinea pig	IC	2 weeks	Pentobarbital, Hypnorm	↑Mean SA in areas corresponding to areas of hearing loss	Vogler et al., 2014

. **Table 1.3** Summary of studies evaluating changes in SA following tinnitus induction with acoustic trauma. This table summarizes the study design and results from representative studies that evaluated changes in SA following damage to the peripheral auditory system with the use of various acoustic trauma paradigms.

central auditory system. Each paradigm differs in frequency of sound exposure stimulus, as well as intensity and duration. Some studies use very mild paradigms, such as broadband noise at 95 dB SPL for 30s (J. F. Willott, 1982), whereas some studies use a very intense paradigm, such as broadband noise at 120 dB SPL for four hours (Shore et al., 2008). It remains to be determined how much of an effect differing acoustic trauma paradigms may have on the development of neural correlates of tinnitus, but it is possible that varying levels of damage may cause different mechanisms of change in the central auditory system. If this is true, then it poses a potential problem for the state of tinnitus research, as noise damage is variable, and hair cell loss varies with sound exposure paradigms.

1.6 ANESTHESIA IN TINNITUS RESEARCH

Another potential problem in current tinnitus research is the use of anesthesia. As outlined in Tables 1.2 and 1.3, almost every published study has used anesthesia during the electrophysiological recordings of SA after tinnitus induction. This is often necessary in order to obtain enough stability for long-term recordings in neurons. However, one study, by Ma and Young, used decerebrate cats in lieu of anesthesia (Ma and Young, 2006). Interestingly, they found no evidence of elevated rates of SA in the DCN in their study. However, their preparation still required the use of anesthesia to perform the surgery, which occurred immediately before their recording sessions. It is possible that residual anesthesia was present in the animal's central nervous system at the time of recording. While the use of anesthesia in these studies is widely accepted, it is important to note that anesthesia has been shown to have significant effects on the central auditory system.

Kuwada et al. examined the effects of anesthesia on rabbit IC neurons, and found changes in various response properties of these neurons, including spontaneous discharge rate, in the presence of anesthesia (Kuwada et al., 1989a). Gaese and Ostwald also analyzed changes in the central auditory system, specifically the rat auditory cortex, in the presence of anesthesia. Similarly, they found changes in frequency tuning and SA in the cortical neurons while using anesthesia (Gaese and Ostwald, 2001). Additionally, Anderson and Young found decreases in the rate of SA of cat DCN neurons with the use of isoflurane anesthesia (Anderson and Young, 2004).

Although the effects of anesthesia on central auditory neuronal response properties have been well documented, it remains the standard protocol for tinnitus research. One may argue that it is acceptable to use anesthesia in these studies, as comparisons made across studies will all be done in anesthetized animals, thereby making the comparisons more appropriate. However, not only have studies shown that the general use of anesthesia can significantly affect the rate of SA in central auditory neurons, but Cheung et al. have shown that the specific type of anesthesia used can have significant effects compared to other types of anesthesia (Cheung et al., 2001). Therefore, even the use of different kinds of anesthesia between studies can affect the results, making cross-study comparisons less appropriate.

1.7 BEHAVIORAL PARADIGMS

Another intriguing and long-standing issue in tinnitus research is determining whether or not animals exposed to loud sounds actually experience tinnitus. It is easy to evaluate tinnitus in humans, as psychophysical evaluations can be made to

determine not only the presence of tinnitus, but the pitch and loudness of the tinnitus as well. The same type of evaluation is much more complicated in animals. Initial behavioral methods developed for evaluating tinnitus required conditioning and training the animals to display a behavior in the presence of tinnitus, as first demonstrated by Jastreboff et al. (Jastreboff et al., 1988). For example, Rüttiger et al. trained rats to actively access a liquid feeder in the presence of white noise. In silence, no reward was provided for accessing the feeder. When their animals experienced salicylate-induced tinnitus, feeder activity was significantly increased in periods of silence, indicating the rats were hearing phantom sounds and provoking them to feed even in silence (Rüttiger et al., 2003).

Another similar behavioral model is employed by Lobarinas et al. In their behavioral paradigm, they utilize schedule-induced polydipsia avoidance conditioning (SIP-AC). This method conditions food-restricted rats to lick water in periods between feedings. When there is a tone present, the rats receive a foot shock, which conditions them to lick water only in periods of silence. Rats that present with tinnitus display decreases in licking during silent periods, again, because they presumably are perceiving sounds that are not present (Lobarinas et al., 2004).

These kinds of conditioning behavioral assays are considered to be fairly reliable in detecting the perception of tinnitus in animals. Some researchers have even developed similar conditioning paradigms to pitch or loudness match tinnitus in their animals, providing results similar to human psychophysical studies (Brozoski et al., 2002). However, these methods require long periods of training for individual animals, which can be cumbersome and require a great amount of expertise in behavioral

research. Also, there are a number of factors that must be controlled for in these studies. Hearing loss, hyperacusis, alterations in memory, and motivational state of the animals can have an effect on the results of the behavioral assays.

Turner et al. introduced a gap detection method for measuring behavioral correlates of tinnitus that bypasses the aforementioned variables (Parrish, 2008). This method requires no additional training, and instead relies on the acoustic startle reflex that is intrinsic in animals. Rats can be placed in an acoustic startle chamber on top of a force plate. A background sound is played in the chamber. During “gap” trials, the background sound is briefly paused, followed by a loud, brief startle stimulus. During “non-gap” trials, the startle stimulus is presented without the gap of silence in the background sound. Normal hearing animals without tinnitus presumably are able to suppress their startle response (measured by the animal’s movement on the force plate) during gap trials, using the gap in background sound as a cue. It is thought that animals experiencing tinnitus do not detect the gap in background sound, and thus do not suppress their startle response. This behavioral assay has been widely used since its introduction, mainly because it provides researchers with the ability to test animals without any prior training or conditioning.

However, the traditional gap detection methodology has recently come into question (Longenecker and Galazyuk, 2012; Lobarinas et al., 2013). There have been reports of a large amount of variability in startle responses in animals that have not even been exposed to noise, which may make the gap detection method more difficult to interpret. It is also possible that hearing loss may affect the acoustic startle reflex. Additionally, although training is not involved in this method, animals may habituate to

the startle stimulus if they are allowed to be tested over numerous trials. Due to these factors, as well as the issues with traditional conditioning behavioral paradigms, there still remains a deficit in proper behavioral testing for tinnitus in animals.

This deficit poses a substantial problem for tinnitus research. If we are not able to properly identify which animals have tinnitus and which do not, then we do not know if we are truly evaluating neural correlates of tinnitus, or if we are simply evaluating neural changes due to damage to the peripheral auditory system. Numerous studies have shown that approximately 50% or fewer animals in a given study develop tinnitus after acoustic trauma. Engineer et al. reported that 62% of their noise exposed rats developed tinnitus (Engineer et al., 2011), while Ruttiger et al. reported a mere 30% of their animals experienced tinnitus after acoustic trauma (Ruttiger et al., 2013). These statistics mimic the human condition, in that hearing loss is not guaranteed to be correlated with the development of tinnitus. However, we must acknowledge that while acoustic trauma is a generally accepted method of inducing tinnitus in animals, until we develop a robust method for behaviorally testing animals for tinnitus, we are evaluating neural changes that correspond with either tinnitus or acoustic trauma.

1.8 IDENTIFYING PUTATIVE TINNITUS GENERATORS

Another intriguing dilemma in identifying the mechanisms of tinnitus is identifying the location of putative tinnitus generators. The auditory system is a vastly complex sensory system (fig. 1.1), which complicates researchers' abilities to conclusively identify one or more nuclei of the central auditory system as playing a primary role in the

generation of tinnitus. Before proposing one or more nuclei as putative tinnitus generators, one must understand the intricate connections of the auditory system.

Beginning at the primary sensory receptor, or the cochlea, sound waves are transduced into neural signals as they travel along the basilar membrane and cause the depolarization of hair cells. Depolarization occurs when stereocilia are deflected and open mechanically-gated ion channels that allow the flow of ions across the membrane. This change in receptor potential then triggers the release of neurotransmitters at the basal end of the hair cell, which then acts on the spiral ganglion cells, causing an action potential to be transmitted by the eighth nerve. Hair cells are arranged in a tonotopic manner along the basilar membrane, with hair cells that respond best to high frequencies positioned at the basal end of the cochlea, and hair cells responding best to low frequencies located near the apex of the cochlea.

From the auditory nerve, information from a given cochlea is processed mainly along the “dominant” pathway to the ipsilateral cochlear nucleus, which is divided into two regions: the dorsal cochlear nucleus (DCN) and the ventral cochlear nucleus (VCN). Both of these regions possess tonotopic organization, which is determined by the projections from the different regions of the cochlea. The cochlear nucleus is a major area of focus in tinnitus research, due to the direct projections from the auditory nerve. However, the cochlear nucleus sends major projections to other auditory nuclei, including contralateral and ipsilateral projections to the superior olivary complex (SOC), and contralateral projections to the inferior colliculus (IC) via the lateral lemniscus (LL).

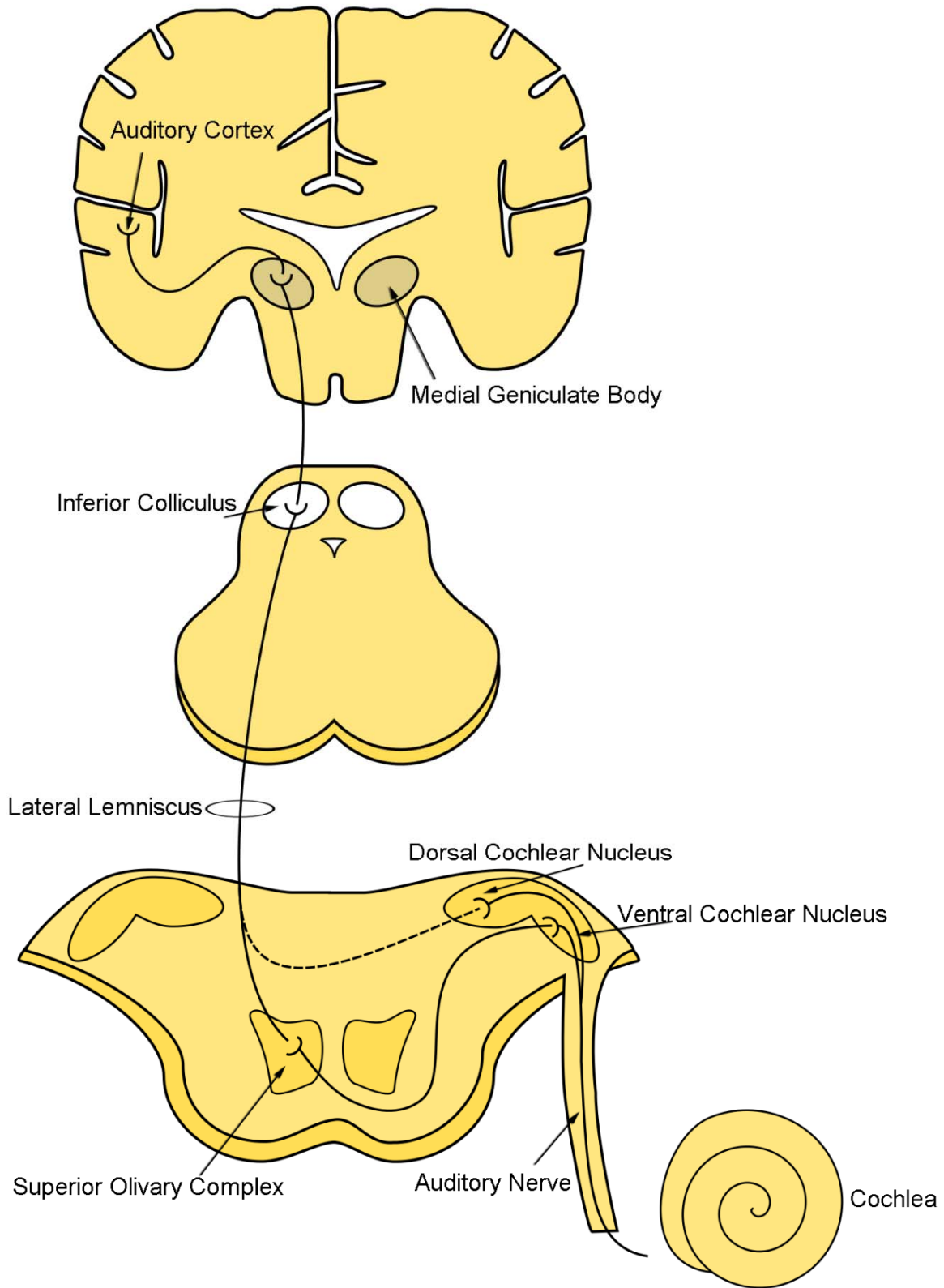


Figure 1.1 Simplified diagram of the auditory system. Major structures of the auditory system are depicted in this simplified diagram. The major dominant pathway connections are represented from one cochlea. The non-dominant pathway connections are not shown.

The IC is another major site of tinnitus research. This is mainly due to the large number of both ascending and descending projections it receives from other auditory nuclei, and because it is an obligatory synaptic relay nucleus en route to the auditory cortex. Additionally, it is easy to identify and access in rodents, making it an ideal candidate for multiple methods of analysis. However, due to the abundant amount of connections made within the IC, it can be difficult to reconcile the anatomical and physiological features of this nucleus. It is known to receive input from the DCN to the central nucleus, which maintains the tonotopic map found in the cochlea and cochlear nucleus. It also features dorsal and external cortices, but the cellular and physiological arrangement of these areas is still not clear. It is important to note that the dominant pathway of the auditory system is considered to include the IC contralateral to a given cochlea, with few projections to the ipsilateral IC. However, there is a small amount of information processed ipsilaterally, as well as between the two colliculi, mainly through commissural projections. Due to the complex nature of IC input and projections, it is thought to be a good representation of changes in activity that may be occurring elsewhere along the auditory pathways.

The IC mainly projects ipsilaterally to the medial geniculate body (MGB), which is part of the thalamus. Although the MGB is not a main focus of tinnitus research, a few studies have been done in this area (Basta et al., 2005; Su et al., 2012). However, the MGB projects to the auditory cortex, which is another area of primary focus in tinnitus research. Functional evaluation of this area can be difficult, due to the highly complex nature of the cortex. Located in the temporal lobes, the auditory cortex is divided into the primary cortex (AI), secondary cortex (AII), and association areas. The borders of

these areas are not always easily defined, and contain multiple tonotopic maps, which can complicate cortical research. Many studies have shown a high degree of tonotopic reorganization following peripheral damage (Komiya and Eggermont, 2000; Seki and Eggermont, 2003; Rajan and Irvine, 2010; Engineer et al., 2011), possibly confounding analysis in tinnitus studies. Additionally, corticofugal pathways have been shown to play an important role in auditory processing, which can have a significant effect on all lower nuclei of the auditory pathway, including the IC and CN (Suga, 2008).

Further complicating the search for putative tinnitus generators is the involvement of non-auditory areas. The limbic system is thought to be significantly affected in tinnitus sufferers, independent of the simple annoyance of tinnitus. (Knipper et al., 2010; Gabr et al., 2011). Areas important for memory are also thought to be significantly affected in tinnitus patients. For example, Laureano et al. found a significant increase in cerebral blood flow to the parahippocampal gyrus in patients with tinnitus (Laureano et al., 2014), and Vanneste et al. showed tinnitus lateralization in humans depends on gamma-band activity of the parahippocampal gyrus (Vanneste et al., 2011). Additionally, Shore et al. have published numerous studies illustrating the involvement of the somatosensory system in tinnitus (Shore et al., 2008; Shore, 2011; Basura et al., 2012; Koehler and Shore, 2013). The ever evolving map of tinnitus-related structures in the brain provides for an arduous undertaking of determining if there is a single primary tinnitus generator, or if alterations in many areas result in a concerted change in activity that is perceived as tinnitus.

1.9 METHODOLOGY

Despite these difficulties, researchers continue to investigate the neural mechanisms of tinnitus using a number of methods. The most widely used method for analyzing changes in the central auditory system following damage is electrophysiology. Studies that utilize electrophysiological recordings employ either single unit or multiunit recording. Single unit recording allows researchers to gather data from a single, discharging neuron, whereas multiunit recording enables researchers to gather data from a cluster of multiple neurons at one time. Not only can this method be used to measure rates of spontaneous activity from single or multiple neurons, but it also can be used to measure various types of response properties in the presence of a stimulus.

Although our study focuses mainly on the rate of spontaneous activity in central auditory structures following peripheral damage, recording changes in response properties following damage can be valuable in evaluating neural correlates of tinnitus. Acoustic trauma or cochlear damage has been shown to cause reorganization of the tonotopic map in various central auditory structures, such as the cortex (Komiya and Eggermont, 2000; Rajan and Irvine, 2010; Engineer et al., 2011), the DCN (Ma and Young, 2006; Middleton et al., 2011), and the IC (Irvine et al., 2003; Mulders et al., 2011). Additionally, changes may occur in other response properties depending on the cell type from which one records. Characterizing and understanding these changes may help reveal more about tinnitus mechanisms.

Another method for analyzing changes in activity in the central auditory system following damage is the ¹⁴C-2-deoxyglucose (2DG) assay. 2DG is a radioactively

labeled glucose molecule in which the 2-hydroxyl group has been replaced by hydrogen. This alteration allows the molecule to be taken up by glucose transporters in metabolically active neurons, but it cannot undergo full glycolysis. Thus, the 2DG molecule accumulates in metabolically active neurons, as glucose is the primary energy substrate of neurons. Because it is radioactively labeled with ^{14}C , an autoradiograph can be created from the tissue, allowing for quantification of changes in metabolic activity across large portions of tissue. This method is not used in tinnitus research as frequently as single unit or multiunit recording, but when used in conjunction with electrophysiology, it can provide a more complete picture of neural consequences following damage to the periphery.

A third method that is becoming more commonly used in tinnitus is manganese enhanced magnetic resonance imaging (MEMRI). MEMRI takes advantage of the fact that Mn^{2+} enters active neurons via voltage gated Ca^{2+} channels and accumulates inside neurons. Cells that are more active will accumulate more Mn^{2+} , making it a good way to visualize and quantify areas of hyperactivity following damage. One important aspect of MEMRI in tinnitus research is the slow clearance time of Mn^{2+} from the region of interest. It can take weeks for the Mn^{2+} to leave the active cells, which allows for uptake outside the MRI machine. This is imperative for tinnitus research, as the MRI machine produces loud sounds during scanning, and the use of Mn^{2+} can be administered in a controlled acoustic environment to allow quantification of SA in the absence of outside stimuli (Cacace et al., 2014). While most studies typically only use electrophysiology to evaluate changes in SA following damage, a combination of all

three of these methods could provide a powerful look into potential mechanisms of tinnitus generation.

1.10 SUMMARY OF WORK

The work that will be presented here aimed to elucidate various aspects of tinnitus mechanisms, specifically changes in SA following acoustic trauma, while addressing some of the aforementioned problems that affect tinnitus research. We utilized single unit recording and 2DG in each of the animals in this study to measure changes in both SA and metabolic activity in the IC, providing a more complete picture of changes that occur in the IC following damage.

To address the issue of the use of anesthesia during recordings, we performed our studies in awake, freely moving animals, as well as animals anesthetized with a ketamine-xylazine mix. Additionally, we sought to determine the consequences of using variable sound exposure paradigms by measuring changes in animals that received either mild or intense acoustic trauma. Finally, in order to gain more insight into potential tinnitus generators, we also measured changes in the non-dominant pathway in the IC ipsilateral to the damaged ear. The data shown here successfully address various aspects of these issues, and potentially reveal more information about possible mechanisms of tinnitus.

CHAPTER 2: EFFECTS OF ANESTHESIA ON SPONTANEOUS ACTIVITY

2.1 INTRODUCTION

Tinnitus is commonly defined as the perception of sound when no corresponding sound is present. It is estimated that as many as 50 million Americans suffer from tinnitus, while 16 million seek out medical treatment (ATA, 2013). Although it is a fairly common condition, the neural mechanisms of tinnitus are not well understood. It is commonly believed that the perception of tinnitus is generated in the central auditory system, as opposed to the cochlea, or the peripheral auditory system. When the peripheral auditory system is damaged, central auditory structures can become deafferented, which may lead to an imbalance of excitatory and inhibitory input in these nuclei. And imbalance of excitation and inhibition could lead to aberrant spontaneous neural activity, which might be perceived as tinnitus. Recent research has focused on identifying neural correlates of tinnitus in various central auditory structures, such as the dorsal cochlear nucleus (DCN) or the inferior colliculus (IC).

Previous studies have shown an increase in spontaneous activity (SA) in the DCN (Zhang and Kaltenbach, 1998), (Brozoski et al., 2002), the auditory cortex (Komiya and Eggermont, 2000), (Noreña and Eggermont, 2003), or the IC (Szczepaniak and Moller, 1995), (Ma et al., 2006), (Bauer et al., 2008), (Dong et al., 2010), (Knipper et al., 2010), (Mulders et al., 2010) following sound damage. However, even though a number of these studies have found an increase in SA following sound damage in tinnitus models, others have shown either no change in spontaneous activity when using electrophysiological recordings (Ma and Young, 2006), or a decrease in metabolic activity in central auditory structures following sound damage when using 2DG (Imig and Durham, 2005).

The seemingly contradictory findings indicate that more work needs to be done to develop a more precise picture of the causes of tinnitus. Comparisons among previous studies are difficult because of several potentially confounding variables. These studies were performed in various species, such as rat, mouse, or chinchilla, and often use a variety of sound damage paradigms, ranging from very mild trauma to severe trauma. Additionally, various recording techniques, such as multiunit or single unit recordings, were used.

Finally, these studies used anesthesia while measuring spontaneous activity, and the perception of tinnitus is evaluated behaviorally in awake animals (Parrish, 2008). Anesthesia has been shown to have an effect on the rate of discharge of neurons, as well as the response characteristics of auditory neurons (Kuwada et al., 1989b; Anderson, 2004), making the results from these studies less reflective of processes in an awake animal. It is possible that the previously observed increases in spontaneous activity of the central auditory structures following sound exposure are not significant in the absence of anesthesia, making the hypothesis that an increase in spontaneous activity causes the perception of tinnitus less likely.

The purpose of this study was to determine if the use of anesthesia has a significant effect on changes in neural activity in the central auditory system following acoustic trauma. We aimed to address this question by not only recording in awake, behaving animals, but also directly comparing our anesthetized results with results from the same model and paradigm under anesthesia. Additionally, we used 14C-2-deoxyglucose, which served as a second method of evaluating changes in spontaneous activity in the same animals, allowing us to gain better insight into overall changes in

activity following damage. Therefore, by using an acoustic trauma model of tinnitus, we were able to analyze changes in SA in the IC with two different methods of measuring changes in activity, either in the presence or absence of anesthesia. We found an overall decrease in activity while using anesthesia, as well as small changes in the pattern of hyperactivity between the anesthetized and unanesthetized animals following acoustic trauma. These differences, although small, may have important implications for future tinnitus research.

2.2 METHODS

Animals

Adult, male Long-Evans rats, weighing between 250-300g upon receipt from the vendor (Charles River Laboratories), were used for all aspects of this study. The Institutional Animal Care and Use Committee of the University of Kansas Medical Center approved the experimental protocol.

Sound Exposure

Rats were anesthetized with a ketamine-xylazine mixture (1ml/mg) and placed in a sound-attenuating booth (Industrial Acoustics Company, Bronx, NY) for the duration of sound exposure. A 16 kHz pure tone was continuously, unilaterally presented to the animals at 118 dB SPL for four hours from a loudspeaker (Radio Shack 40-1310-B) inside a plastic case. The loudspeaker was coupled to the left pinna via ½" flexible plastic tubing. Audalin ear mold compound (All American Mold Lab, Oklahoma City, OK) was used to seal the tube delivering the sound to minimize the occurrence of bilateral damage. The stimulus level outside the tube was measured as 45 dB less than inside the tube when it was sealed to the head. A Macintosh computer with a MaLab

synthesizer, event processor, and software (Kaiser Instruments, Irvine, CA) was used to control noise waveform synthesis.

Surgery

All rats received surgically implanted recording chambers above the right inferior colliculus, contralateral to the damaged ear, prior to electrophysiological recordings. Rats were anesthetized with a ketamine-xylazine mixture and placed in a stereotaxic apparatus with blunt ear bars under aseptic conditions. The scalp was shaved and an incision was made to expose the skull. The craniotomy was made using dental burrs and it was located approximately 2 mm lateral from the midline at lambda. The dura was left intact. A stainless steel tube (4 mm internal diameter) with a removable cap was then positioned over the craniotomy and fixed to the skull using 4 anchoring screws and dental acrylic (Lang Dental Mfg. Co., Wheeling, IL). The skin was then sutured and the rat was allowed to recover with the use of antibiotics (bacitracin) and analgesics (buprenorphine and ketoprofen).

Single Unit Recording

A minimum of one week following sound exposure, single unit extracellular recordings were performed on each rat. A custom-made lightweight microdrive containing a single, tungsten microelectrode was inserted into the surgically implanted recording chamber and secured with a set screw while the rat was anesthetized with either isoflurane or a ketamine-xylazine mixture. Anesthetized rats remained unconscious for the duration of the recording sessions, receiving supplemental doses of ketamine-xylazine. After regaining consciousness, the unanesthetized rats were placed

in a Lucite chamber within a sound-attenuating booth and were allowed to move freely while the microdrive remained attached to a flexible recording cable with a headstage preamplifier. The recording cable was routed through a commutator to a Neuralynx amplifier. The amplifier was computer controlled (Cheetah software, Neuralynx) outside the sound booth to adjust gain and filtering.

Broadband noise bursts were presented to identify neural responses to auditory stimuli. The single microelectrode was incrementally lowered through the depth of the IC, and frequency tuning from clusters of neurons were measured every quarter of a millimeter in depth. When a single unit was encountered, five, five second intervals of spontaneous activity were measured while the rat was not moving or making any self-produced noise, such as tooth grinding. The five intervals were taken consecutively and later averaged. The frequency tuning of the unit was then determined by presenting pure tones at different frequencies and intensities (10 dB SPL-70 dB SPL). In many cases, neurons responded to a restricted range of frequencies and a characteristic frequency (CF) could be determined at the lowest threshold. In other cases, neurons responded unreliably or to a broad range of frequencies, and a CF could not be assigned. In electrode penetrations with frequency tuned units, CFs increased in frequency with increasing electrode depth. The unit's response to noise was determined by playing noise bursts at various intensities.

Finally, the rate of spontaneous activity was again measured in five increments of five second intervals while the rat was silent and not moving. This second measure of the rate of spontaneous activity was used to assess the stability of the cell and its spontaneous activity. Measurements were taken until the electrode reached the end of

the auditory responsive tissue. Multiple electrode penetrations within the IC were made in each rat over a period of 1-2 weeks, during 1-3 recording sessions in each rat.

Lesions

At the conclusion of each single unit recording session, marking lesions were made through the recording electrode at the beginning and the ends of select penetrations. Direct current, electrode tip negative, was applied for 5s at the bottom and top of the penetration.

Data Analysis

Average rates of spontaneous activity were evaluated for all units either within or outside the central nucleus of the IC. All units that were not contained within a tonotopic progression during a given penetration were considered to be outside the central nucleus, regardless of tuning properties. Units within the tonotopic sequence were subdivided into frequency categories based on CF. Units in a tonotopic sequence that did not have a single characteristic frequency or were unresponsive to tones were considered untuned, and were assigned to a frequency category based on their location within the tonotopic sequence. . Figure 2.1 shows an example of a penetration with a tonotopic sequence that contained an untuned unit (Fig. 2.1a), and an example of frequency tuning from a unit with a characteristic frequency (Fig. 2.1c), as well as an untuned unit (Fig. 2.1d). Data are reported with standard error of the mean. Statistical significance was determined using SigmaPlot by a Kruskal-Wallis analysis of variance and post-hoc Wilcoxon pairwise comparison.

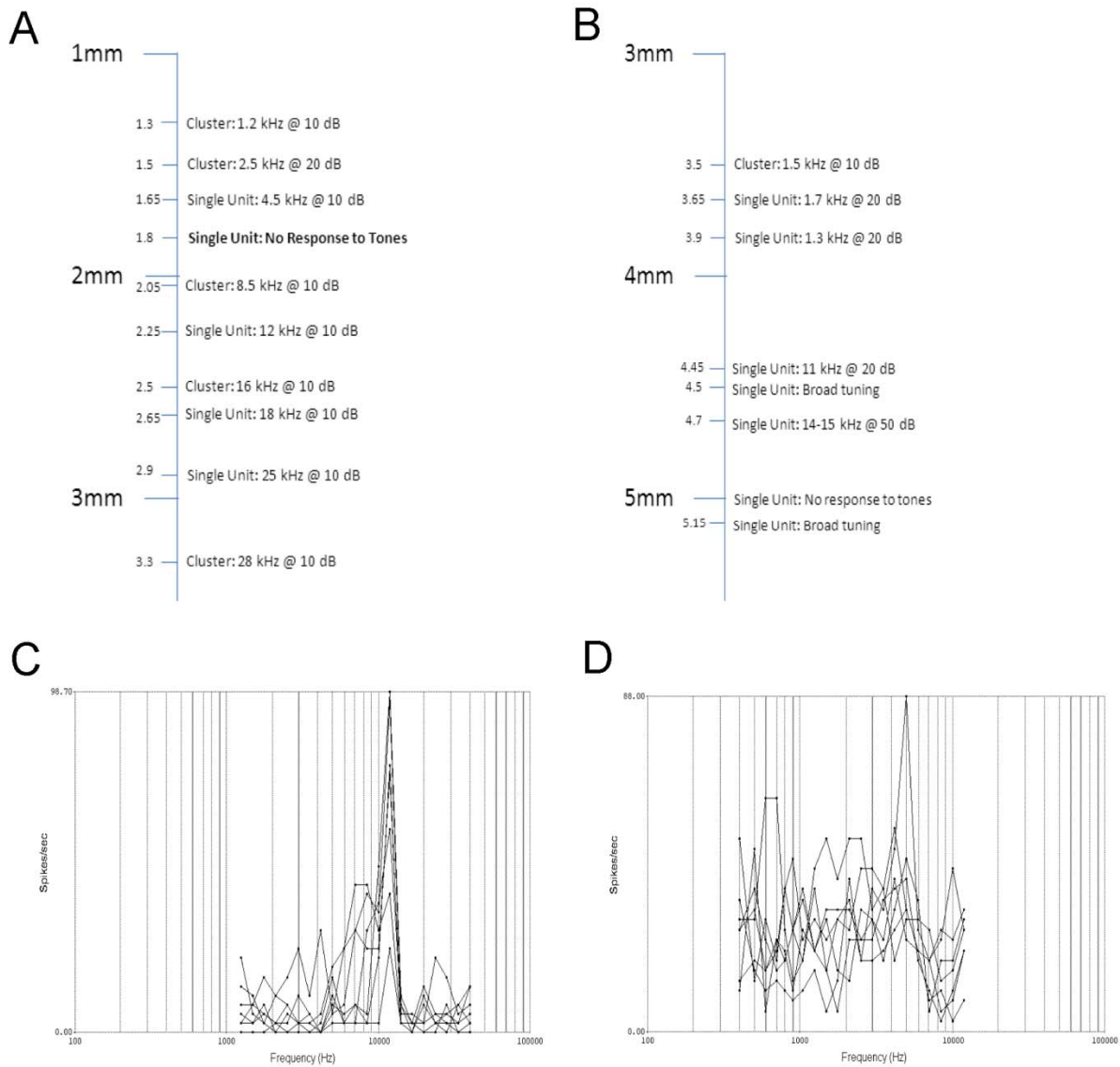


Fig. 2.1 Examples of electrode penetrations. A. Representative frequency tuning from one electrode penetration. Responses to tones were collected from single units and clusters of cells along the depth of the IC. An untuned unit was found at a depth of 1.8mm. This penetration was considered to be within the central nucleus of the IC. B. Representative frequency tuning from an electrode penetration in a different animal. There was no clear tonotopic progression in this penetration, and it was considered to be outside of the central nucleus. C. Frequency response from a single unit with a

characteristic frequency. This unit responded best to approximately 12 kHz at a threshold of 10 dB SPL. D. Frequency response from an untuned unit. This unit responded to a broad range of tones at various intensities.

Auditory Brainstem Response

Following all recording sessions, auditory brainstem response (ABR) thresholds were measured to determine the extent of hearing loss in each animal. Measurements were recorded using the Intelligent Hearing Systems Smart EP program (IHS, Miami, FL). Rats were anesthetized with ketamine-xylazine mixture (1ml/mg) and needle electrodes were placed in the scalp at the base of each pinna and at the vertex of the head. A probe connected to a high frequency transducer was placed in the ear and a series of tone bursts (500 μ s) was presented at a range of frequencies (2, 4, 8, 11.3, 16, 22.6, 32 kHz) and intensities. Threshold was defined as the intensity at which a signal could be reliably observed in three or more repetitions. A high pass filter was used for the 22.6 and 32 kHz frequency sweeps to prevent artificially low thresholds at high frequencies.

¹⁴C-2-Deoxyglucose

In a terminal procedure occurring a minimum of three weeks following acoustic trauma, rats were injected intramuscularly with ¹⁴C-2-deoxyglucose (2DG; 100 μ Ci/kg). Injection occurred in a sound-attenuating chamber, and the rat was placed in a Lucite cage within the sound chamber for 45 minutes. Anesthetized animals received a ketamine-xylazine mixture prior to 2DG injection. Following the 45 minute uptake period, rats were euthanized with a lethal dose of Beuthanasia (5cc/kg) and decapitated. Brains were harvested and flash frozen in heptane cooled to -65° with dry ice. Fresh, frozen brains were sectioned in 40 μ m thick coronal sections on a cryostat. Serial sections were thaw mounted on slides and then apposed to Kodak Biomax MR X-ray film for 1-2 weeks along with ¹⁴C standards. Film was developed and

corresponding sections were stained with thionin for nissl substance, allowing for outlines of each IC to be made on corresponding autoradiographs.

Optical density measurements were made using NIH ImageJ from digital images (QImaging EXi Aqua Camera). Grayscale measurements were calibrated to the 14C concentrations in the standard. 2DG activity measurements were taken in ten sectors on each side of the IC of three representative sections (Fig. 2.2), located approximately 50% through the rostral-caudal axis of the IC. To account for differences in absolute 2DG concentrations among animals, all measurements were normalized to 2DG levels in a portion of the cerebellum and averaged across all three sections. Statistical significance was determined using a one-way ANOVA with a Fisher LSD post-hoc analysis.

2.3 RESULTS

Hearing Thresholds

Figure 2.3 shows results of ABR threshold measurements as a function of frequency, obtained an average of three weeks following acoustic trauma. Compared to unexposed control animals, rats exposed to the 118 dB SPL, 16 kHz pure tone for 4 hours displayed elevated ABR thresholds at every frequency tested (2 kHz, 4 kHz, 8 kHz, 11.3 kHz, 16 kHz, 21.6 kHz, and 32 kHz). The greatest degree of hearing loss was observed at 16 kHz, where thresholds increased by 25 dB SPL. There was also a great degree of threshold shift that occurred at the highest frequency tested, of 21 dB SPL (Fig. 2.3). A one-way ANOVA revealed significant differences in threshold comparing control and sound exposed animals ($P < 0.001$). Fisher-LSD post-hoc tests

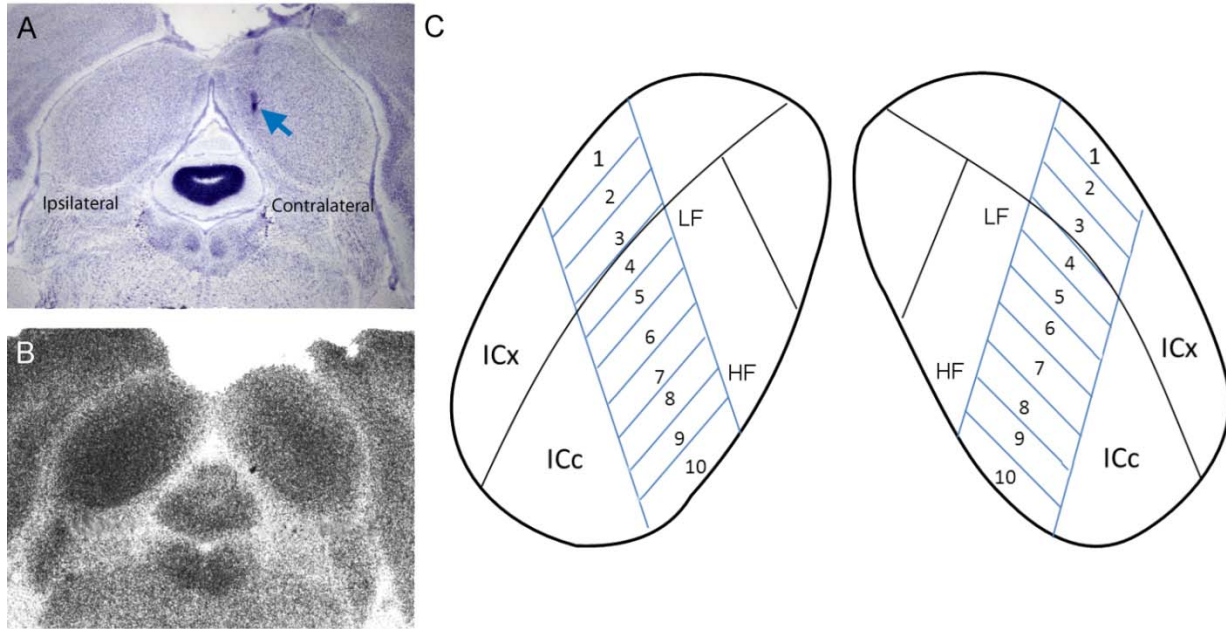


Fig. 2.2 Diagram of ^{14}C -2-deoxyglucose measurements. A. Thionin stained coronal section ($40\ \mu\text{m}$) of representative inferior colliculus. Arrow indicates electrode track. B. Autoradiograph from the same representative section of inferior colliculus. C. Measurements were taken from 10 sectors on each side of the inferior colliculus. The first three sectors correspond to the external nucleus, and sectors 4-10 correspond to the tonotopic arrangement of the central nucleus, from low to high frequencies. ICx=External nucleus of the inferior colliculus; ICc=Central nucleus of the inferior colliculus; LF=Low frequency; HF=High frequency

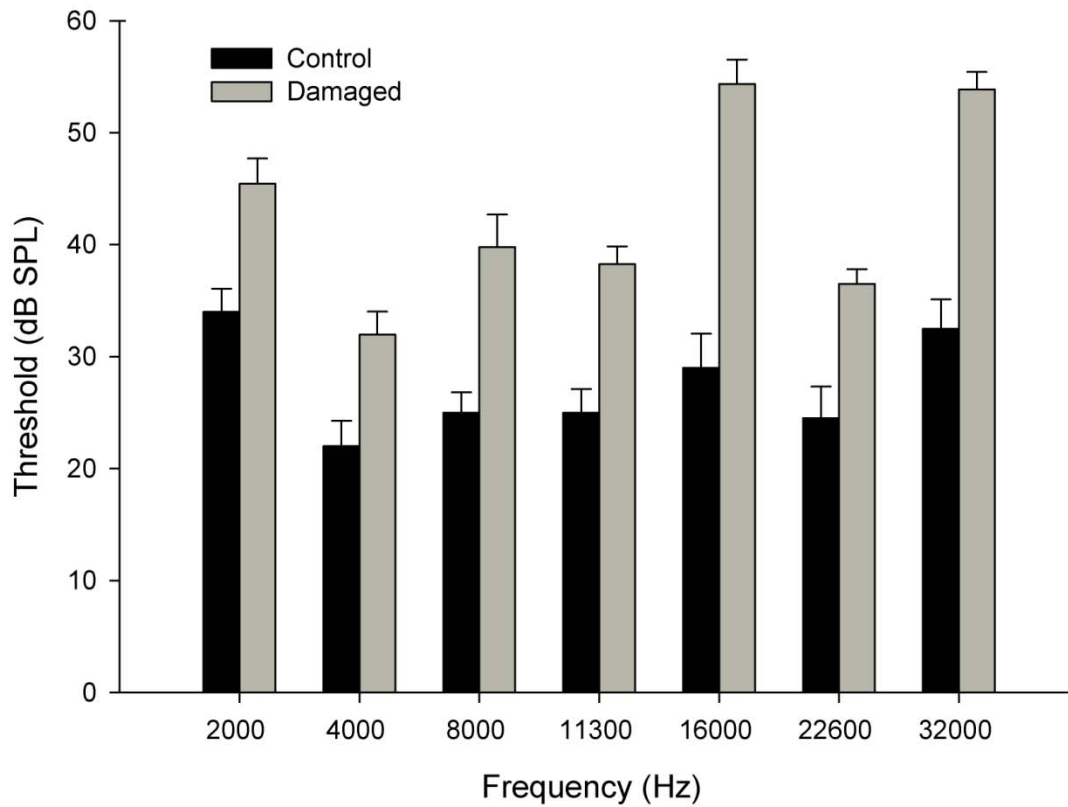


Fig. 2.3 Absolute auditory brainstem response (ABR) thresholds. 7 frequencies were tested (2, 4, 8, 11.3, 16, 22.6, and 32 kHz) at variable intensities until absolute threshold was determined for each animal (n=10 control animals, n=23 damaged animals). All frequencies displayed significant increases in threshold (one-way ANOVA, Fisher LSD post-hoc, $P < 0.001$)

revealed differences at all frequencies tested ($P < 0.001$). These results indicate substantial, permanent hearing loss across all frequencies in sound exposed animals.

Single Unit Recording

The results of our evaluation of spontaneous activity (SA) of single unit recordings are shown in Figure 2.4. Overall, there was no significant difference between control and damaged animals for all units in the unanesthetized or anesthetized groups (Student's t-test, two-tailed, $P = 0.663$, $P = 0.736$). In unanesthetized sound damaged animals, there was no significant difference in the rate of SA for units located within the central nucleus compared to control animals (Student's t-test, two-tailed, $P = 0.741$). This result differs from previously published studies in anesthetized animals that report increases in SA following damage. There was also no increase or decrease in SA for all units located outside the central nucleus (Fig. 2.4a), which includes the external nucleus and the dorsal cortex of the inferior colliculus (Student's t-test, two-tailed, $P = 0.110$). Similarly, for units recorded in anesthetized animals (Fig. 2.4b), there was no significant difference in SA for all units either within or outside the central nucleus (Student's t-test, two-tailed, $P = 0.819$, $P = 0.680$). There was a slight, but insignificant, decrease in SA in units outside the central nucleus in both anesthetized and unanesthetized animals. However, the unanesthetized animals displayed overall elevated levels of SA compared to the anesthetized group, independent of acoustic trauma (Fig. 2.4). There was no difference in SA between the two regions of the inferior colliculus in either group.

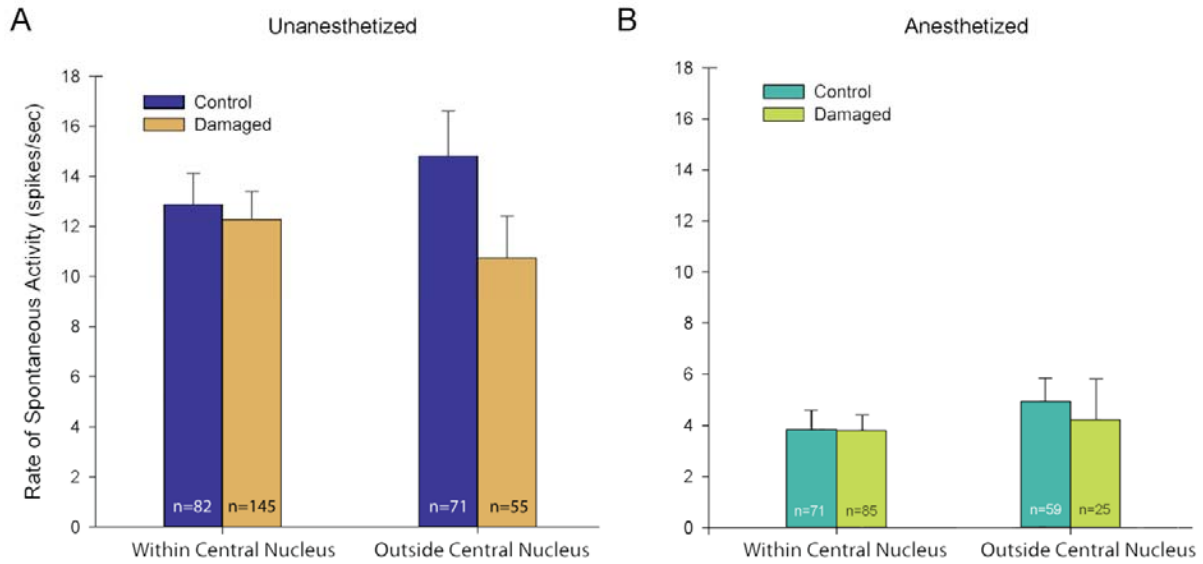


Fig. 2.4 Overall rates of spontaneous activity for units within or outside the central nucleus. A. Awake, freely moving animals displayed no change in rate of SA for all units within or outside the central nucleus. B. Animals anesthetized with a ketamine-xylazine mix displayed no change in the rate of SA for all units within or outside the central nucleus

We evaluated the distribution of rates of SA among all units, which can be seen in Figure 2.5. All groups displayed non-normal distribution, with a large percentage of units exhibiting rates of SA less than 10 spikes/second. Sound exposure did not appear to affect distribution of units for either unanesthetized (Fig. 2.5a,c) or anesthetized animals (Fig. 2.5b,d). However, units in anesthetized animals exhibited a much higher percentage of units with rates of SA below 5 spikes/second.

To address the possibility that small changes may be occurring in the rate of SA in a specific population of cells within the central nucleus of the IC, we evaluated units as a function of CF (Fig. 2.6). Units from unanesthetized animals displayed a marked increase in SA from low to high CFs, particularly in the >8 kHz range (Fig. 2.6a). The rate of SA approximately tripled when comparing the <2 kHz group and the >16 kHz group. Units from anesthetized animals also showed a similar increase in SA across the tonotopic organization, although differences were not as large (Fig. 2.6b). When comparing sound exposed and control animals, units in anesthetized animals (Fig. 2.6b) exhibited a significant increase in SA in one CF group, 2-4 kHz (Kruskal-Wallis, $P<0.001$; Wilcoxon pairwise analysis, $P<0.001$). Units from unanesthetized animals (Fig. 2.6a) displayed a significant increase in SA in the untuned >8 kHz units (Kruskal-Wallis, $P<0.001$; Wilcoxon pairwise analysis, $P=0.047$). These were the only populations of cells to display a significant change in SA following damage. Additionally, there was an increase in the number of untuned units located in the central nucleus following damage, but the increase was not as pronounced in the anesthetized group. Taken together, these results indicate the same overall trend in increasing SA with increasing CF in units recorded with or without anesthesia. However, when

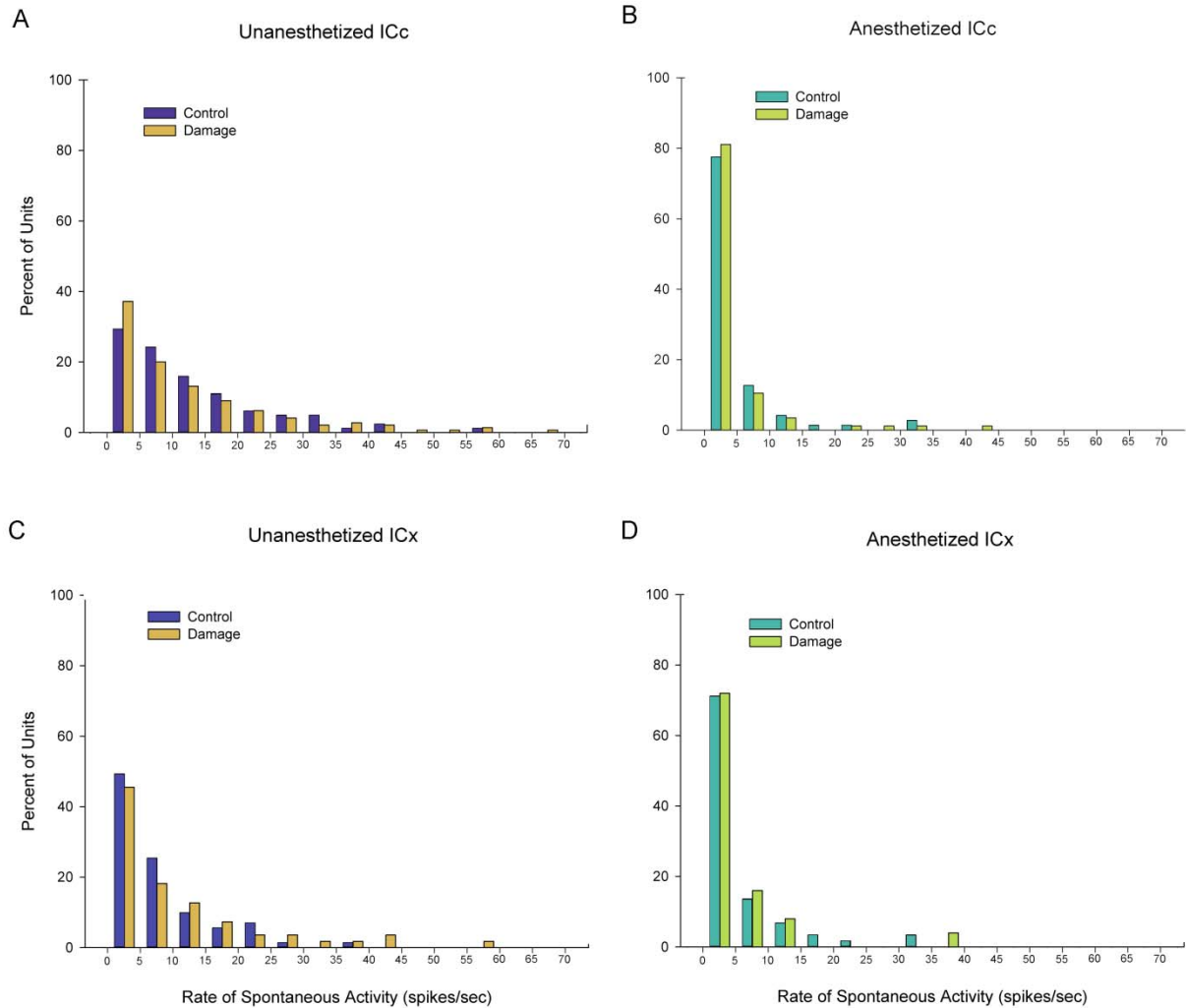


Fig. 2.5 Distribution of rates of spontaneous activity for units within or outside the central nucleus. Percentage of total units within bins of 5 spikes/second are shown for control and damaged animals. A. Unanesthetized animals, ICc B. Anesthetized animals, ICc C. Unanesthetized animals, ICx D. Anesthetized animals, ICx. The anesthetized group displayed a higher percentage of units with rates of SA under 10 spikes/second compared to unanesthetized animals.

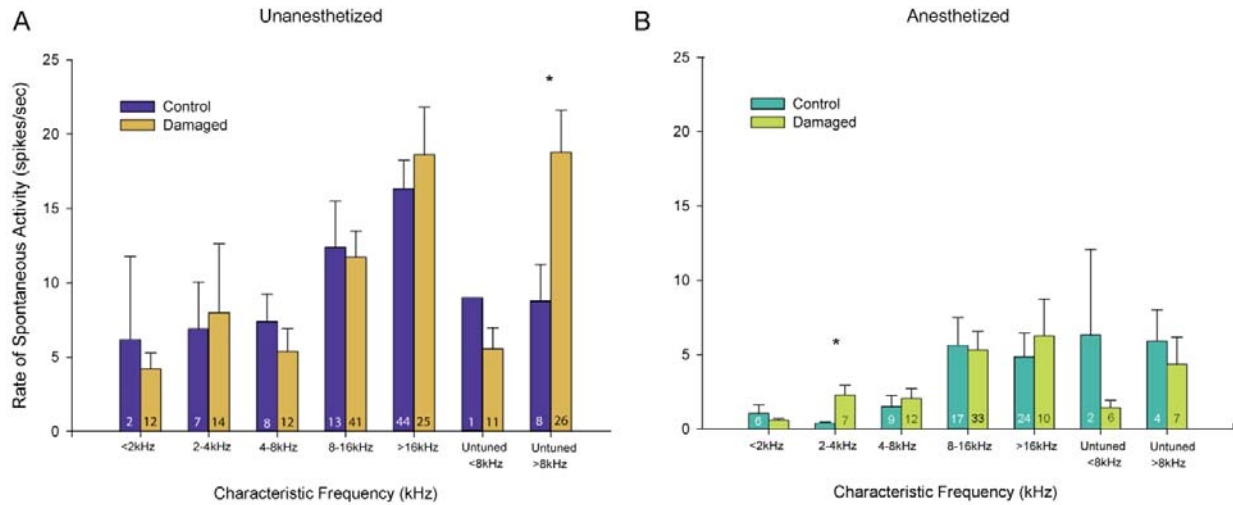


Fig. 2.6 Rates of spontaneous activity as a function of characteristic frequency. Units were categorized as a function of CF, for both the unanesthetized group (panel A) and the anesthetized group (panel B). Unanesthetized animals displayed significant increases in SA in untuned units above 8 kHz. Anesthetized animals displayed significant increases in SA in units with CF between 2-4 kHz (n=8 control). Asterisk indicates $p < 0.05$, Wilcoxon pairwise comparison).

categorized as a function of CF, the two groups exhibited an increase in SA in different populations of cells.

14C-2-deoxyglucose

In addition to single unit recordings, we evaluated IC activity using a metabolic indicator of glucose use. 2DG activity, relative to the cerebellum, was measured as a function of frequency in ten sectors along the depth of the IC. Relative measures are used to decrease variability in dose and metabolism among animals. As seen in Figure 2.2, the first three sectors correspond to the external nucleus of the IC (ICx) and sectors 4-10 correspond to the central nucleus of the IC (ICc), arranged tonotopically from low to high frequencies.

Neither unanesthetized nor anesthetized control animals showed any significant difference in relative 2DG activity between the two sides of the IC across all 10 sectors (Fig. 2.7a,c). However, a gradient increase, followed by a slight decrease in activity was observed from low to high frequencies along the tonotopic map. This gradient was observed in both anesthetized and unanesthetized animals.

There was little difference in these relative measures of 2DG activity when comparing unanesthetized and anesthetized animals (Fig. 2.7a and 2.7c). However, it is important to note that when measuring absolute 2DG activity by averaging raw 2DG values across two sectors from unanesthetized and anesthetized control animals (n=3 unanesthetized, n=3 anesthetized), absolute measures of 2DG activity in unanesthetized animals were approximately 6 times higher than anesthetized 2DG values.

In sound exposed animals, we did observe a difference in 2DG uptake when comparing the ipsilateral IC to the contralateral IC. The unanesthetized animals showed a significant difference in relative 2DG activity between the two sides of the IC, which are either contralateral or ipsilateral to the damaged ear (Fig. 2.7b). The differences occurred at middle and high frequency regions, or sectors 5-10 (one-way ANOVA, $P < 0.001$; Fisher LSD post-hoc, $P < 0.05$). A similar difference was seen in anesthetized animals (Fig. 2.7d), although it was restricted relatively high frequency regions, or sectors 7-10 (one-way ANOVA, $P < 0.001$; Fisher LSD post-hoc, $P < 0.05$). Interestingly, this difference seems to be due to an increase in 2DG activity in the IC ipsilateral to the damaged ear. We expect to see changes in the IC contralateral to the damaged ear, as the majority of the input from the damaged cochlea projects contralaterally. Neither group showed a significant change in relative 2DG activity in the ICx regions, or sectors 1-3.

Not only was there a disparity in the pattern of change between the two groups, but the anesthetized animals displayed slightly lower relative 2DG activity overall (Fig. 2.7). This difference was not as pronounced as the decrease seen in the electrophysiological results, but it was still present at some sectors, indicating an influence of anesthesia on the rate of SA in the IC compared to non-auditory brain regions, such as the cerebellum. Interestingly, the unanesthetized animals show lower relative 2DG activity in the ICx compared to the anesthetized animals in both control and damaged groups.

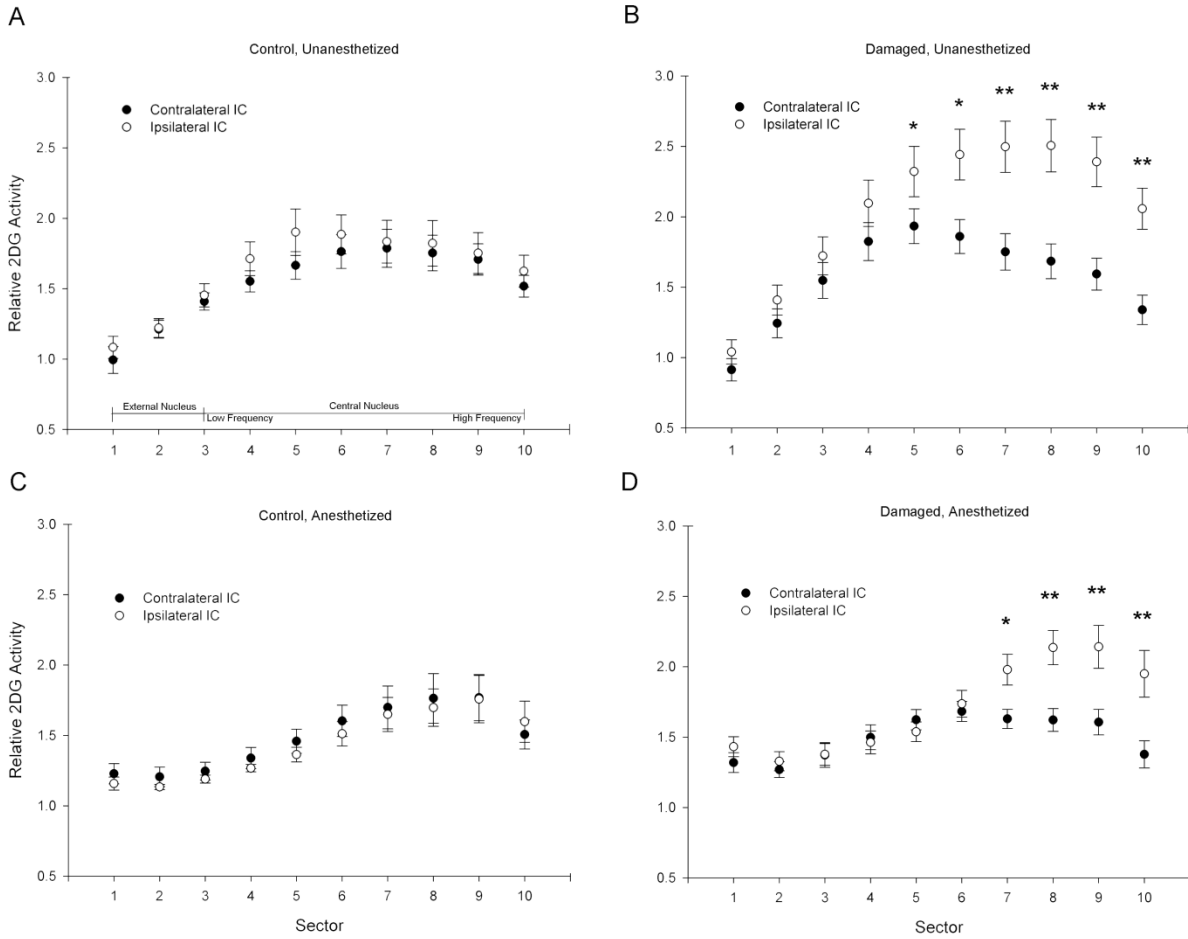


Fig. 2.7 Relative ¹⁴C-2-deoxyglucose measurements. A. Unanesthetized, control animal 2DG measurements B. Unanesthetized, damaged animal measurements C. Anesthetized, control animal measurements D. Anesthetized, damaged animal measurements. *p<0.05, **p<0.001, one-way ANOVA, Fisher – LSD)

2.4 DISCUSSION

Our data show a significant difference in SA between anesthetized and unanesthetized animals. Not only did we observe an overall decrease in SA in the anesthetized group compared to the unanesthetized group, we also observed different patterns of SA across the tonotopic map between the two groups. The anesthetized animals displayed much lower single unit and absolute metabolic activity overall, with or without acoustic trauma. Additionally, we saw an increase in the single unit SA of untuned >8 kHz cells in the unanesthetized group that was not present in the anesthetized group. This discrepancy may have significant implications, as the only variable that was different between the two experiments was the use of anesthesia.

The majority of the previously published studies that measured changes in SA in the central auditory system following acoustic trauma were performed in anesthetized animals (Zhang and Kaltenbach, 1998), (Brozoski et al., 2002), (Ma et al., 2006), (Bauer et al., 2008), (Dong et al., 2010), (Mulders et al., 2010). While almost all of these studies found increases in SA following damage, the use of anesthesia in the studies may have influenced their results. Multiple studies have confirmed the effects of various types of anesthesia on central auditory neurons. Kuwada et al. reported that anesthesia changed response properties, including spontaneous firing rate, of rabbit IC neurons (Kuwada et al., 1989b), and Gaese and Ostwald reported changes in frequency tuning and SA of neurons in the rat auditory cortex (Gaese and Ostwald, 2001). It is difficult to assess the effect of anesthesia in previously published studies, as each study uses different animal models and acoustic trauma paradigms. Here, we compared our anesthetized animals to unanesthetized animals using the same damage paradigm. By

changing only the anesthetized state, we were able to directly compare the effect of anesthesia on our findings.

Based on the previous findings that anesthesia affects central auditory neuron properties, it is not surprising that we observed lower rates of activity with both electrophysiology and 2DG measures of metabolic rate. However, we also observed a difference in the pattern of SA along the tonotopic map of the IC when comparing anesthetized to unanesthetized animals. While the unanesthetized group had one hyperactive population of cells, untuned units >8 kHz, following damage, the anesthetized group showed no difference in SA in any of the CF groupings. Additionally, the unanesthetized group showed elevations in relative 2DG activity across a broader range of the tonotopic map compared to the anesthetized group, which only showed changes at high frequencies. These differences, although seemingly small, may have important implications for future tinnitus research.

It is possible the anesthesia targets a specific type or population of neurons, which could influence any analysis of response properties in the IC. This may explain why our anesthetized group had lower occurrence of untuned units following damage compared to the unanesthetized group. The previously mentioned studies on the effects of anesthesia both used pentobarbital, a GABA receptor agonist (Wan et al., 2003), while our study used ketamine, an NMDA receptor antagonist (Kavalali and Monteggia, 2012), and xylazine, an alpha-2-adrenergic receptor agonist (Albertson et al., 1992). Cheung et al. found isoflurane to have a larger impact on auditory cortical neuron temporal response properties compared to pentobarbital (Cheung et al., 2001). While recording from unanesthetized animals is not always feasible, it is imperative to

consider the mechanism of action of certain types of anesthetic before proceeding with studies that use anesthesia during data collection.

Because our animals were unrestrained and freely moving during our unanesthetized recording sessions, it was difficult to record from the same cell for an extended amount of time. Our method of recording from unrestrained animals does not allow us to assess specific response properties that require longer durations of recording from a single cell. If this problem could be addressed, it would be beneficial to administer anesthesia while recording from a single unit, to see how the presence of the anesthesia directly affects the response properties and SA of that unit. It would also be of interest to administer different classes of anesthetic to see if one might have less of a significant effect than others.

An unexpected finding from our study is an increase in 2DG labeling in the IC ipsilateral to the damaged ear. As the majority of the input from a given ear projects contralaterally to the opposite IC, one might expect to see any damage-induced changes occurring in the contralateral IC. However, we observed an increase in relative 2DG activity in the ipsilateral IC, and no change in the contralateral IC. These findings suggest that evaluation of SA changes with single unit recordings in the ipsilateral IC may prove fruitful.

Another intriguing outcome from our study is the discrepancy between our anesthetized recordings and previously published anesthetized recordings that report overall increases in SA following damage. We did observe a small difference in unanesthetized animals that appeared to be masked by anesthesia, but that does not

account for the differences between results we obtained in our anesthetized animals compared to previously published studies using anesthesia. There are a number of variables that may cause this difference, including the method of damage (i.e. acoustic trauma or ototoxic drugs), or the sound exposure paradigm used in each study. It seems likely that the level of trauma may have a significant effect on the extent of change in SA observed in our animals. Future work needs to be done to determine the significance of sound damage paradigms in tinnitus studies.

CHAPTER 3: EFFECTS OF MILD VERSUS INTENSE ACOUSTIC TRAUMA

3.1 INTRODUCTION

Affecting anywhere between 10-15% of the adult population (Baguley et al., 2013), tinnitus is a pervasive condition that can severely alter the quality of life for many individuals. The American Tinnitus Association estimates that approximately 50 million Americans suffer from tinnitus (ATA, 2013), and of those, 16 million seek medical treatment. The causes of tinnitus are highly variable, ranging from acoustic trauma to exposure to ototoxic drugs, as well as idiopathic causes, and each individual who experiences tinnitus may experience it in a variety of ways.

Interestingly, although tinnitus is commonly associated with hearing loss, the severity of hearing loss does not necessarily correlate with the severity of tinnitus (Tan et al., 2013). An individual with severe hearing loss may not experience tinnitus, whereas an individual with no clinically diagnosed hearing loss may suffer from severe tinnitus. Although there is no correlation between severity of hearing loss and severity of tinnitus, one common animal model for tinnitus is noise exposure, or acoustic trauma. A large number of studies have been published in which animals have been exposed to an intense sound to induce tinnitus. These studies use a wide variety of acoustic trauma paradigms with varying frequencies, intensities, and exposure lengths, which range from a very mild broadband noise at 95 dB for 30s (J. F. Willott, 1982), to a moderate 16 kHz tone at 103 dB for 1hr (Ma et al., 2006), to the intense broadband noise at 120 dB for 4hr (Shore et al., 2008).

Because there is such variability in acoustic trauma paradigms, it is imperative to understand the effects of varying levels of peripheral auditory trauma on central auditory changes in activity. While the majority of these studies report increases in spontaneous

activity (SA) following damage, Ma and Young found no change in SA following damage (Ma and Young, 2006), and Imig and Durham found decreases in metabolic activity following damage (Imig and Durham, 2005). It is possible that the extent of hyperactivity in central auditory structures following cochlear damage may depend on the pattern of damage occurring in the peripheral auditory system, which could be determined by the acoustic trauma paradigm.

To gain more insight into how the paradigm of trauma affects changes in SA in the IC of an animal model of tinnitus, we used two different levels of noise exposure: a 16 kHz, 114 dB SPL pure tone for one hour, which we considered “mild trauma,” and a 16 kHz, 118 dB SPL pure tone for four hours, which we considered “intense trauma.” We performed single unit recording and ¹⁴C-2-deoxyglucose assays on each group in the absence of anesthesia. We found that changing severity of the trauma has significant effects on both hair cell damage as well as changes in SA in the IC.

3.2 METHODS

Animals

Adult, male Long-Evans rats, weighing between 250-300g upon receipt from the vendor (Charles River Laboratories), were used for all aspects of this study. The Institutional Animal Care and Use Committee of the University of Kansas Medical Center approved the experimental protocol.

Sound Exposure

Rats were anesthetized with a ketamine-xylazine mixture (1ml/mg) and placed in a sound-attenuating booth (Industrial Acoustics Company, Bronx, NY) for the duration of

the exposure. The mild trauma group was presented with a 16 kHz pure tone at 114 dB SPL for one hour, and the intense trauma group was presented with a 16 kHz pure tone at 118 dB SPL for four hours from a loudspeaker (Radio Shack 40-1310-B) inside a plastic case. The loudspeaker was coupled to the left pinna via ½" flexible plastic tubing. Audalin ear mold compound (All American Mold Lab, Oklahoma City, OK) was used to seal the tube delivering the sound to minimize the occurrence of bilateral damage. The stimulus level outside the tube was measured as 45 dB less than inside the tube when it was sealed to the head. A Macintosh computer with a MaLab synthesizer, event processor, and software (Kaiser Instruments, Irvine, CA) was used to control noise waveform synthesis.

Surgery

All rats received surgically implanted recording chambers above the right inferior colliculus, contralateral to the damaged ear, prior to electrophysiological recordings. Rats were anesthetized with a ketamine-xylazine mixture and placed in a stereotaxic apparatus with blunt ear bars under aseptic conditions. The scalp was shaved and an incision was made to expose the skull. The craniotomy was made using dental burrs and it was located approximately 2 mm lateral from the midline at lambda. The dura was left intact. A stainless steel tube (4 mm internal diameter) with a removable cap was then positioned over the craniotomy and fixed to the skull using 4 anchoring screws and dental acrylic (Lang Dental Mfg. Co., Wheeling, IL). The skin was then sutured and the rat was allowed to recover with the use of antibiotics (bacitracin) and analgesics (buprenorphine and ketoprofen), administered for 2 days following surgery.

Single Unit Recording

A minimum of one week following sound exposure, single unit extracellular recordings were performed on each rat. A custom-made lightweight microdrive containing a single, tungsten microelectrode was inserted into the surgically implanted recording chamber and secured with a set screw while the rat was anesthetized with isoflurane. After regaining consciousness, the unanesthetized rats were placed in a Lucite chamber within a sound-attenuating booth and were allowed to move freely while the microdrive remained attached to a flexible recording cable with a headstage preamplifier. The recording cable was routed through a commutator to a Neuralynx amplifier. The amplifier was computer controlled (Cheetah software, Neuralynx) outside the sound booth to adjust gain and filtering.

Broadband noise bursts were presented to identify neural responses to auditory stimuli. The single microelectrode was incrementally lowered through the depth of the IC, and frequency tuning from clusters of neurons were measured every quarter of a millimeter in depth. When a single unit was encountered, five, five second intervals of spontaneous activity were measured while the rat was not moving or making any self-produced noise, such as tooth grinding. The five intervals were taken consecutively and later averaged. The frequency tuning of the unit was then determined by presenting pure tones at different frequencies and intensities (10 dB SPL-70 dB SPL). In many cases, neurons responded to a restricted range of frequencies and a characteristic frequency (CF) could be determined at the lowest threshold. In other cases, neurons responded unreliably or to a broad range of frequencies, and a CF could not be assigned. In electrode penetrations with frequency tuned units, CFs increased in

frequency with increasing electrode depth. The unit's response to noise was determined by playing noise bursts at various intensities.

Finally, the rate of spontaneous activity was again measured in five increments of five second intervals while the rat was silent and not moving. This second measure of the rate of spontaneous activity was used to assess the stability of the cell and its spontaneous activity. Measurements were taken until the electrode reached the end of the auditory responsive tissue. Multiple electrode penetrations within the IC were made in each rat over a period of 1-2 weeks, during 1-3 recording sessions in each rat.

Lesions

At the conclusion of each single unit recording session, marking lesions were made through the recording electrode at the beginning and the end of the penetration. Direct current, electrode tip negative, was applied for 5s at the bottom and top of the penetration.

Data Analysis

Average rates of spontaneous activity were evaluated for all units either within or outside the central nucleus of the IC. All units that were not contained within a tonotopic progression during a given penetration were considered to be outside the central nucleus, regardless of tuning properties. Units within the tonotopic sequence were subdivided into frequency categories based on CF. Units in a tonotopic sequence that did not have a single characteristic frequency or were unresponsive to tones were considered untuned, and were assigned to a frequency category based on their location within the tonotopic sequence. Data are reported with standard error of the mean.

Statistical significance was determined using SigmaPlot by a Kruskal-Wallis analysis of variance and post-hoc Wilcoxon pairwise comparison.

Auditory Brainstem Response

Following all recording sessions, auditory brainstem response (ABR) thresholds were measured to determine the extent of hearing loss in each animal. Measurements were recorded using the Intelligent Hearing Systems Smart EP program (IHS, Miami, FL). Rats were anesthetized with ketamine-xylazine mixture (1ml/mg) and needle electrodes were placed in the scalp at the base of each pinna and at the vertex of the head. A probe connected to a high frequency transducer was placed in the ear and a series of tone bursts (500 μ s) was presented at a range of frequencies (2, 4, 8, 11.3, 16, 22.6, 32 kHz) and intensities. Threshold was defined as the intensity at which a signal could be reliably observed in three or more repetitions. A high pass filter was used for the 22.6 and 32 kHz frequency sweeps to prevent artificially low thresholds at high frequencies.

¹⁴C-2-Deoxyglucose

In a terminal procedure occurring a minimum of three weeks following acoustic trauma, rats were injected intramuscularly with ¹⁴C-2-deoxyglucose (2DG; 100 μ Ci/kg). Injection occurred in a sound-attenuating chamber, and the rat was placed in a Lucite cage within the sound chamber for 45 minutes. Following the 45 minute uptake period, rats were euthanized with a lethal dose of Beuthanasia (5cc/kg) and decapitated. Brains were harvested and flash frozen in heptane cooled to -65° with dry ice. Fresh, frozen brains were sectioned in 40 μ m thick coronal sections on a cryostat. Serial sections were thaw mounted on slides and then apposed to Kodak Biomax MR X-ray

film for 1-2 weeks along with ¹⁴C standards. Film was developed and corresponding sections were stained with thionin for nissl substance, allowing for outlines of each IC to be made on corresponding autoradiographs.

Optical density measurements were made using NIH ImageJ from digital images (QImaging EXi Aqua Camera). Grayscale measurements were calibrated to the ¹⁴C concentrations in the standard. 2DG activity measurements were made from ten sectors that crossed the external nucleus and the tonotopic axis of the central nucleus (Fig. 3.6a), from three sections located approximately 50% through the rostral-caudal axis of the left and right IC. To account for differences in absolute 2DG concentrations among animals, all measurements were normalized to 2DG levels in a portion of the cerebellum and averaged across all three sections. Statistical significance was determined using a one-way ANOVA with a Fisher LSD post-hoc analysis.

Cochlear Processing

Harvested cochleae were decalcified with RDO for one hour. Decalcified cochleae were washed using ddH₂O, and myelin stained with 1% osmium tetroxide for one hour, flushing the solution through the cochleae at 15 minute intervals. The osmium tetroxide was removed by washing the cochleae in ddH₂O. Cochleae were dehydrated by washing in a series of PBS, ddH₂O, 50% EtOH, and then 70% EtOH.

Stained and dehydrated cochleae were placed in propylene oxide for 30 minutes and then placed in a 1:1 Araldite:PO mixture. After two hours, cochleae were placed in a 2:1 Araldite:PO mixture and placed on an orbital shaker overnight. The cochleae were then removed from the 2:1 Araldite:PO mixture and placed into de-gased capsules of Araldite. The capsules were placed into a vacuum chamber and allowed to degas for

an additional 2 hours. The capsules were then transferred from the vacuum chamber to an oven set to 60 C and allowed to harden for 2 days, prior to removal.

After being embedded in plastic, the cochleae were cut in sections parallel to the modiolar axis. Sections were made on a microtome in 40 μ m thickness. After placement on glass slides, sections were then counterstained using Toluidine Blue prior to coverslipping with Permount.

Hair Cell Quantification

Hair cell counts were performed by a single person, who had no knowledge of the treatment group to which the cochleae belong. Inner and outer hair cells were counted at 20x using Nomarski illumination on a Nikon Optiphot-2 microscope. The presence of a hair cell was determined by an intact nucleus located in the basal half of the cell. Reconstruction of the basilar membrane was conducted by aligning serial sections using Adobe Photoshop. The coordinates of each basilar membrane point were identified and exported as a Microsoft Excel file. Coordinates of the basilar membrane were expressed as a percent distance along the length of the basilar membrane and synced with the corresponding hair cell count data in Excel. Percent distance along the cochlea was transformed to characteristic frequency using the Greenwood equation (Greenwood, 1996). Differences in hair cell numbers among groups of animals was evaluated using a one-way ANOVA with a Fisher LSD post-hoc test.

3.3 RESULTS

Auditory Brainstem Response Thresholds

ABR thresholds were evaluated as a function of frequency (2 kHz, 4 kHz, 8 kHz, 11.3 kHz, 16 kHz, 21.6 kHz, and 32 kHz) for each acoustic trauma group, which is seen in Figure 3.1. Compared to control animals, rats that were exposed to a mild acoustic trauma displayed permanent, significantly elevated ABR thresholds at 16 kHz (one-way ANOVA, Fisher LSD post-hoc, $P=0.01$). While other frequencies tested also showed increased thresholds, they were not significantly elevated. Conversely, rats that were exposed to an intense acoustic trauma displayed permanent, significantly elevated ABR thresholds at all frequencies tested (one-way ANOVA, Fisher LSD post-hoc, $P<0.05$). These results indicate that the two acoustic trauma paradigms caused significantly different degrees of hearing loss in our animals.

Hair Cell Quantification

The quantification of total hair cells in each group can be seen in Figure 3.2. The mild trauma group displayed relatively few areas of significant outer hair cell (OHC) or inner hair cell (IHC) loss (Fig. 3.2a,b). The greatest degree of OHC loss occurred in the >39 kHz regions, with additional loss occurring at low frequency regions. There were three regions of significant IHC loss (50.2-56.7 kHz, 8.3-10.1 kHz and 0.7-1.6 kHz; one-way ANOVA, Fisher-LSD post hoc, $P<0.05$). Conversely, the intense trauma group displayed IHC and OHC loss in many regions of the cochlea (Fig. 3.2c,d). Significant OHC loss was observed at areas with characteristic frequencies >34.3 kHz, although several areas of mid and low CFs were affected as well. Interestingly, IHC loss was more pronounced at a greater number of locations than OHC and included all CFs > 26.2 kHz and several areas of mid and low CF (one-way ANOVA, Fisher-LSD post hoc, $P<0.05$). The acoustic trauma paradigm not only appears to have an effect on the

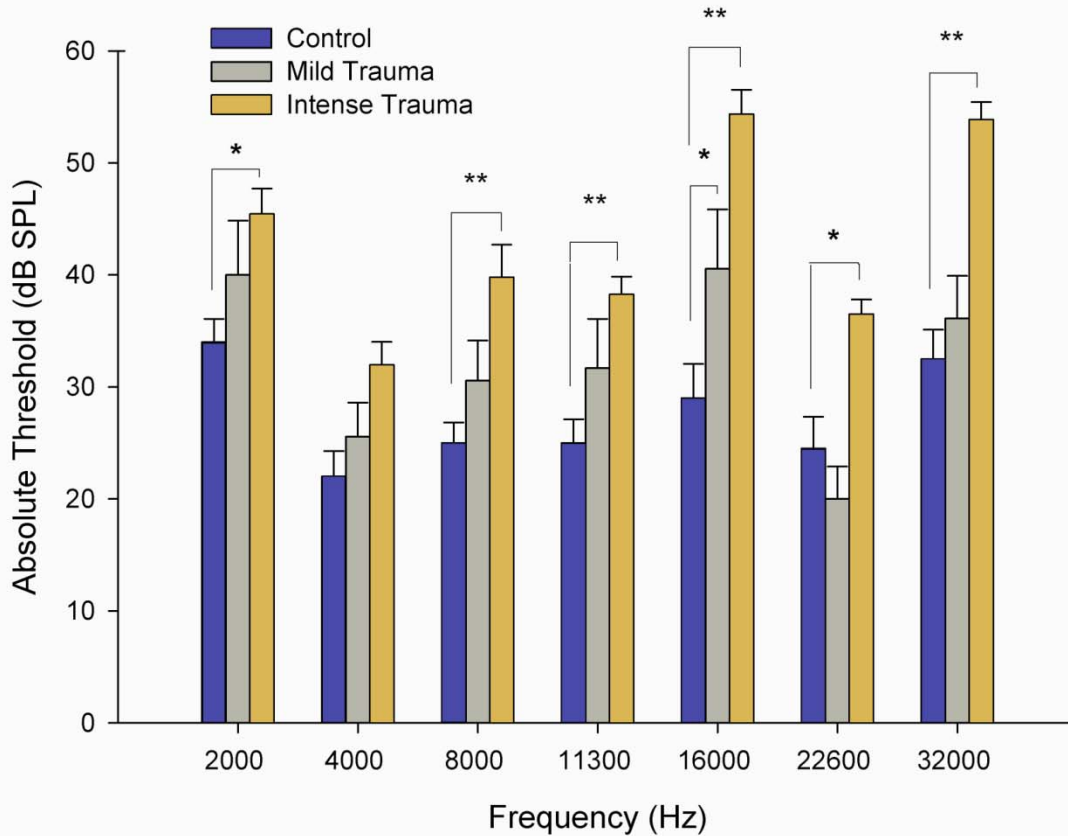


Fig. 3.1 Absolute auditory brainstem response (ABR) thresholds. Auditory brainstem response to 7 frequencies was tested (2, 4, 8, 11.3, 16, 22.6, and 32 kHz) at variable intensities until the absolute threshold was determined for each animal (n=10 control animals, n=9 mild trauma animals, n=23 intense trauma animals). One-way ANOVA, Fisher LSD post-hoc, * $P < 0.05$, ** $P < 0.001$

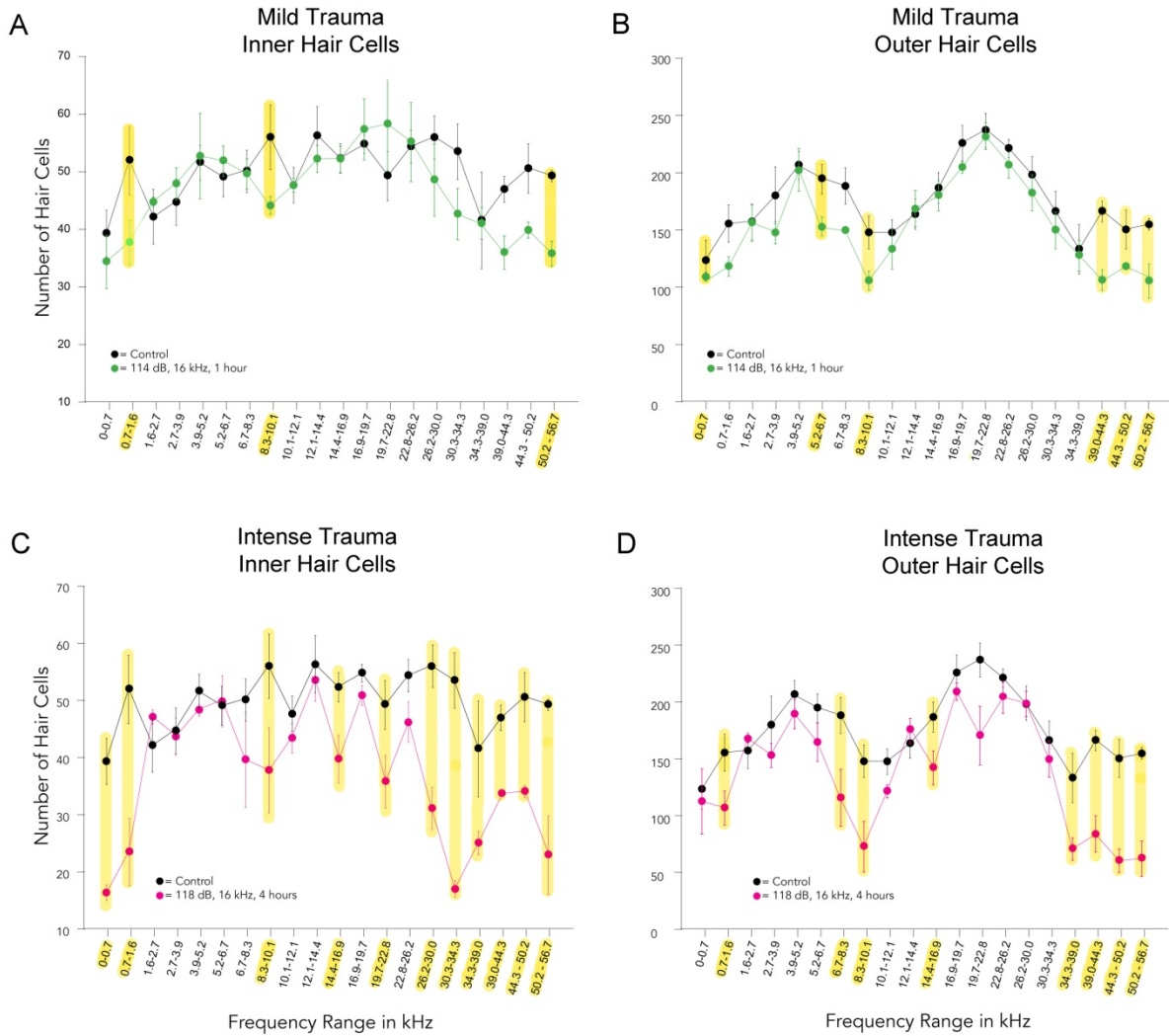


Fig. 3.2 Cochlear hair cell quantification. The total number of hair cells were quantified along the tonotopic axis of the basilar membrane for the mild (A, B) and intense (C, D) trauma groups. Highlighted regions represent areas of significant loss compared to control animals (One-way ANOVA, Fisher-LSD post hoc, $P < 0.05$).

degree of hearing loss in the rats, but it also significantly affects the pattern of hair cell loss in the cochlea.

Single Unit Recording

Figure 3.3 shows the overall rates of SA for all units located either within or outside the central nucleus of the IC for each acoustic trauma paradigm. When all IC units were grouped together according to location either inside the central nucleus or outside the central nucleus (which includes units in the dorsal and external cortices), there was no significant difference in the rate of SA between control and intense trauma animals (one-way ANOVA, Student-Newman-Keul's post-hoc, $P=0.775$, $P=0.207$). However, there was a significant increase in SA for units both inside and outside the central nucleus in the mild trauma group compared to both control (one-way ANOVA, Student-Newman-Keul's post-hoc, $P=0.015$, $P=0.021$) and intense trauma groups (one-way ANOVA, Student-Newman-Keul's post-hoc, $P=0.005$, $P=0.002$). Thus, less severe acoustic trauma is more likely to result in hyperactivity in the IC. Both damage paradigms caused an increase in the percentage of units with a rate of SA below 5 spikes/sec in the central nucleus compared to controls (Fig. 3.4).

The units were then categorized by characteristic frequency to develop a better picture of changes that may occur in specific populations of cells. When categorized as a function of CF, the mild trauma group (Fig. 3.5a) displayed an increase in SA in one population of cells, untuned >8 kHz, but it was not significant (Kruskal-Wallis, $P<0.001$; Wilcoxon pairwise analysis, $P=0.212$). The same population of cells in the intense trauma group (Fig. 3.5b) displayed significantly higher levels of SA compared to control levels (Kruskal-Wallis, $P<0.001$; Wilcoxon pairwise analysis, $P=0.047$). No other groups

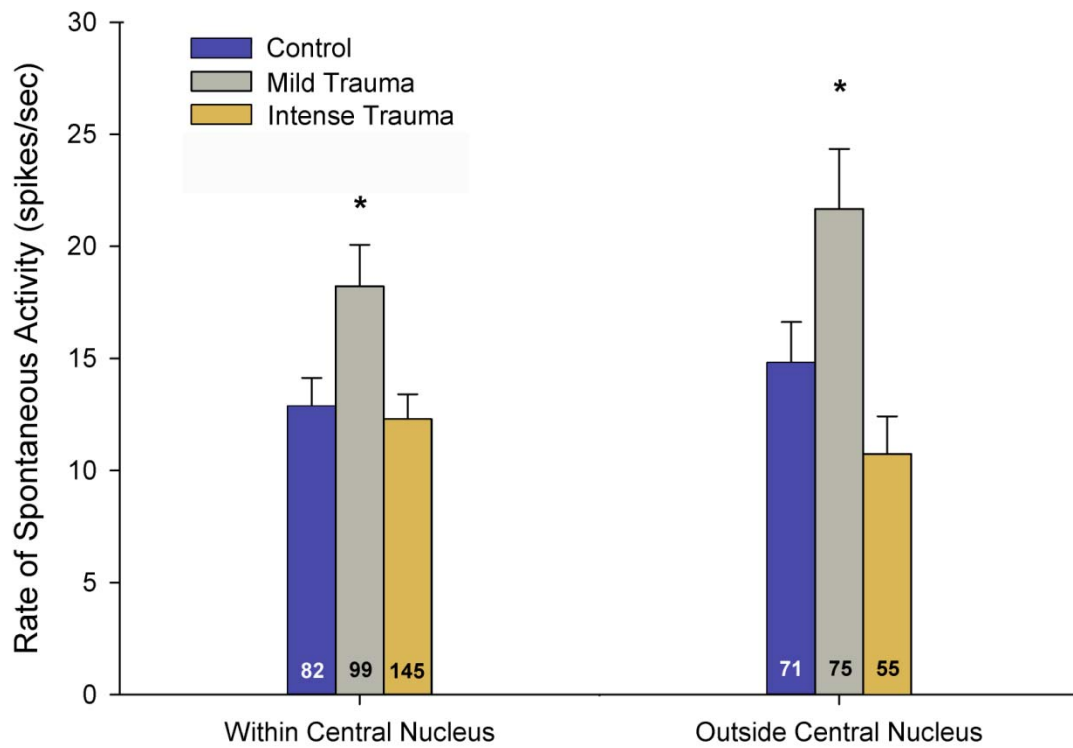


Fig. 3.3 Overall rates of spontaneous activity for units within or outside the central nucleus. Average rate of spontaneous activity was measured for all units either within or outside the central nucleus. One-way ANOVA, Student-Newman-Keul's post-hoc, * $P < 0.05$

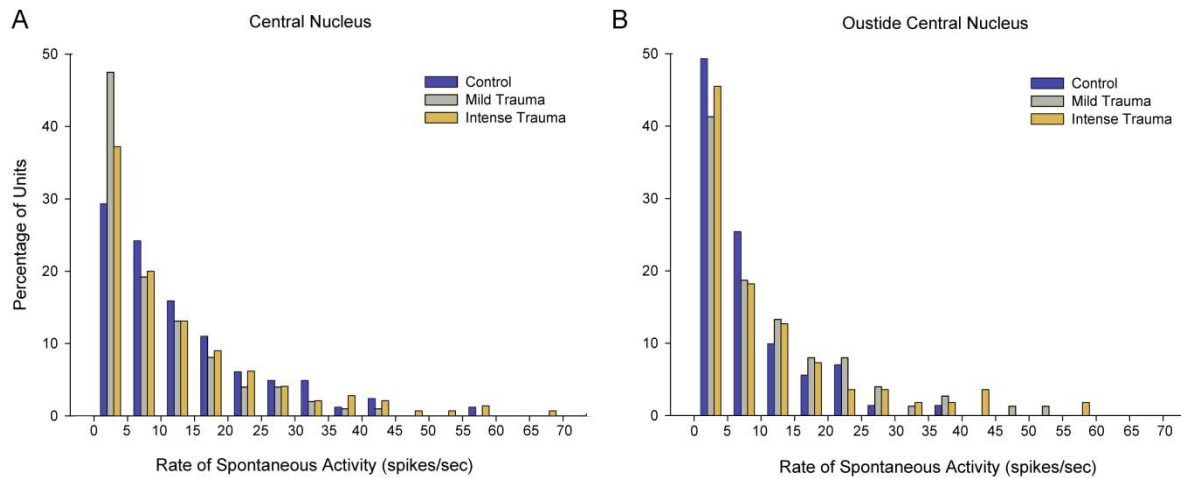


Fig. 3.4 Distribution of rates of spontaneous activity for all units. Percentage of total units displaying rates of spontaneous activity in bins of 5 spikes/sec, either within the central nucleus (A) or outside the central nucleus (B).

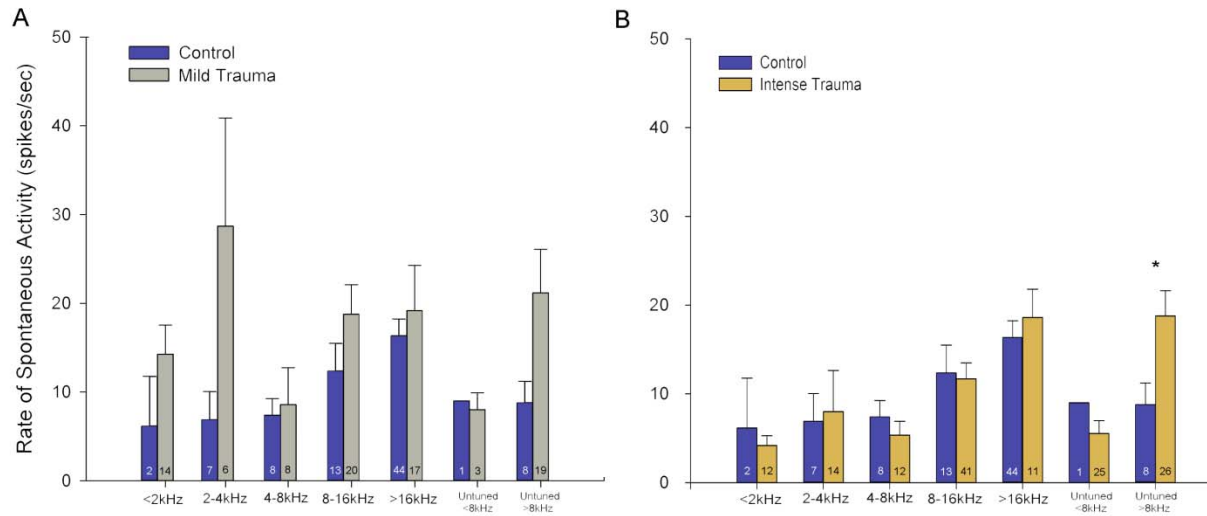


Fig. 3.5 Rates of spontaneous activity as a function of characteristic frequency. Rates of spontaneous activity were measured for units categorized as a function of CF, in both the mild trauma group (panel A) and the intense trauma group (panel B). Kruskal-Wallis, $P < 0.001$; Wilcoxon pairwise analysis, $*P = 0.047$

of units analyzed in the present study showed any significant increase or decrease in SA compared to control levels. Both trauma groups had more than double the number of untuned units compared to controls, indicating a loss of frequency tuning following damage. These results from Figures 3.3 and 3.5 indicate that the severity of trauma does have a significant effect on the changes in SA that are observed in the central auditory system.

14C-2-Deoxyglucose

In addition to electrophysiological measure of SA, each animal also underwent 2DG analysis to obtain metabolic measures of SA. Relative 2DG activity was measured in 10 sectors along the depth of the IC; the first 3 sectors correspond roughly to the external nucleus of the IC, and sectors 4-10 correspond to the tonotopic map of the central nucleus, from low to high frequencies. Both sides of the IC were measured across all 10 sectors. There was no difference in relative 2DG activity between the two sides of the IC of control animals, but there was a gradient increase in activity from low to high frequencies that eventually declines at the highest frequencies.

The mild trauma group (Fig. 3.6c) displayed small changes in the mid to high frequency ranges, or sectors 6-10. There were significant differences in sectors 8 and 9 (one-way ANOVA, $P < 0.001$; Fisher LSD post-hoc, $P = 0.015$, $P = 0.016$). These changes were also observed in the intense trauma group, but to a much greater extent. The intense trauma animals (Fig. 3.6d) displayed a significant change in relative 2DG activity in sectors 5-10 (one-way ANOVA, $P < 0.001$; Fisher LSD post-hoc, $P < 0.05$), which correspond to middle and high frequency areas in the tonotopic map.

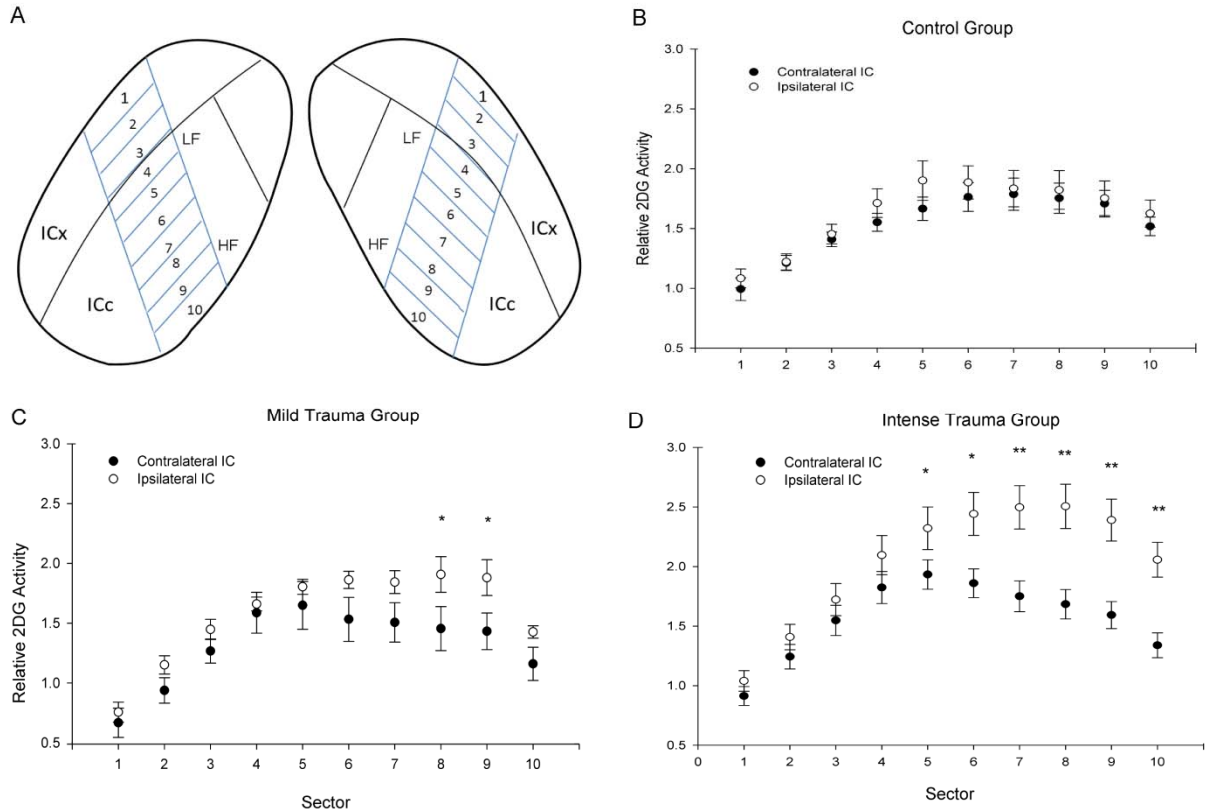


Fig. 3.6 Relative ^{14}C -2-deoxyglucose measurements. A. Diagram of sector measurements on representative drawing of IC B. Control group C. Mild trauma group D. Intense trauma group. Dashed line represents averaged values from contralateral and ipsilateral IC of control group. One-way ANOVA, Fisher LSD post-hoc, * $p < 0.05$, ** $p < 0.001$

Interestingly, the changes occur in the IC ipsilateral to the damaged ear, while the IC contralateral to the damaged ear remains unchanged. Both trauma groups retained the pattern of gradient increase in activity from low to high frequencies that was observed in the control animals. Additionally, neither trauma groups displayed any changes in activity in the external nucleus of the IC. These results differ from the electrophysiological results, where the mild acoustic trauma group showed overall increases in SA in areas within and outside the central nucleus. However, like the electrophysiological findings, these results indicate that the amount of trauma received does have a significant impact on observed changes in SA in the IC following damage.

3.4 DISCUSSION

Our study demonstrates the influence that altered acoustic trauma paradigms can have on changes in SA in central auditory structures in a tinnitus model. By using electrophysiology and a ^{14}C -2-deoxyglucose metabolic assay in animals with either mild or intense trauma, we were able to show differences not only in hearing loss and hair cell loss patterns, but also in the rates of SA in the inferior colliculus of these animals.

The mild trauma group displayed lower ABR thresholds, indicating less hearing loss compared to the intense trauma group. Additionally, the mild trauma group had significantly less hair cell loss of both OHC and IHC. However, the mild trauma group displayed overall higher rates of SA for all the units within or outside the central nucleus of the inferior colliculus when compared to control and intense trauma animals. When analyzed as a function of characteristic frequency, animals in both trauma groups displayed similar patterns of change, with increases in SA in units that are untuned > 8

kHz, but the mild trauma group did not display a significant increase. Similarly, the intense trauma group showed significant changes in relative 2DG activity in the mid to high frequency regions, which was also seen to a very small extent in the mild trauma group. Taken together, these results indicate acoustic trauma paradigms can have a significant effect on rates of SA. Thus, conclusions about tinnitus induced changes in central auditory structure activity are a function of the specific damage paradigm used in each model.

There are many previously published studies that use acoustic trauma as a method to induce tinnitus in animals (Zhang and Kaltenbach, 1998), (Brozoski et al., 2002), (Ma et al., 2006), (Bauer et al., 2008), (Dong et al., 2010), (Mulders et al., 2010), (Komiya and Eggermont, 2000), (Noreña and Eggermont, 2003), (Szczepaniak and Moller, 1995). However, the range in exposure paradigms varies greatly between studies. As we have shown in our study, different acoustic trauma paradigms can cause different patterns of hair cell loss in the cochlea. The animals that received the intense trauma paradigm displayed significant amounts of IHC loss compared to the mild trauma group, which may significantly alter the input from the peripheral auditory system to higher central auditory structures, such as the inferior colliculus. While our animals have not been behaviorally evaluated for the presence of tinnitus, the differences in SA between the two trauma paradigms are still of interest, as an increase SA is widely thought to be a neural correlate of tinnitus (Roberts et al., 2010).

Because tinnitus is a highly variable condition in humans, and can present either in the absence of hearing loss or with very mild hearing loss (Langers et al., 2012), it is imperative to determine how much variability exists in animal models of tinnitus using

different acoustic trauma paradigms. The level of acoustic trauma can cause a wide variety of peripheral changes, such as different patterns of loss of both inner and outer hair cells, loss of synaptic ribbons, and loss of supporting cells, each of which may determine whether or not an animal develops tinnitus. Mulders et al. found that simply changing the duration of noise exposure for one hour causes different degrees of hair cell loss, as well as levels in spontaneous activity in the inferior colliculus of guinea pigs (Mulders et al., 2011). Ruttiger et al. found that equally exposed animals developed tinnitus at a rate of 30%, but that the animals who did display tinnitus behaviors were more likely to have significant loss of synaptic ribbon loss at their inner hair cells (Ruttiger et al., 2013). While our animals were not evaluated for tinnitus behaviors, our results indicate a significant difference in peripheral and central changes based on the acoustic trauma paradigm. It is possible that one damage paradigm is more likely to produce a higher percentage of animals positive for tinnitus behaviors than the other. Testing animals for tinnitus that have been exposed to our two sound exposure paradigms would be of interest.

Interestingly, when changes in activity were evaluated by single unit recording, the mild trauma group displayed significantly elevated levels of SA overall compared to both control and intense trauma groups. However, when analyzed as a function of CF, there were no specific populations of cells that displayed significant increases in SA in the mild trauma group, while the intense trauma group showed significant increases in SA in units that were untuned >8 kHz. These differences may be due to the differences in the pattern of hair cell loss between the two groups, which could lead to divergent changes in peripheral input to the central auditory structures. In the future, it would be

of interest to analyze other differences in peripheral and central changes following the two sound exposure paradigms, such as spiral ganglion cell loss and hyperactivity in the dorsal cochlear nucleus.

CHAPTER 4: ANALYSIS OF CHANGES IN THE NON-DOMINANT PATHWAY

4.1 INTRODUCTION

Tinnitus is a common condition that affects approximately 10-15% of all individuals in the United States and the U.K. (BTA, 2009; ATA, 2013). People may experience tinnitus in a variety of ways, as it is a highly variable symptom that can manifest differently for each individual. It may present unilaterally or bilaterally, and as a broadband noise or specific tones, with a range of presentations in between (Martines et al., 2010). Due to the highly variable nature of tinnitus, it can be difficult to develop an animal model that closely mimics the human experience (Kaltenbach, 2011). Many previously published studies have used either cochlear lesions or acoustic trauma to induce tinnitus in animals. These methods can be applied either unilaterally or bilaterally, depending on the experimental design.

It is thought that damage to the cochlea causes deafferentation of central auditory structures, which may lead to an imbalance of excitatory and inhibitory input. This change may then cause hyperactivity in various central auditory nuclei, leading to the possible perception of tinnitus (Roberts et al., 2010). If the damage is unilateral, one might expect to see changes in spontaneous activity (SA) along the dominant pathways of the auditory system, from the dorsal cochlear nucleus (DCN) ipsilateral to the damaged ear, to the inferior colliculus (IC) contralateral to the damaged ear, and beyond. The majority of published studies have found increases in SA in those structures along the dominant pathway following unilateral damage (Zhang and Kaltenbach, 1998), (Mulders and Robertson, 2011), (Salvi et al., 2000), but few studies have examined the consequences of peripheral damage on central auditory structures along the non-dominant pathway, including the IC ipsilateral to the damaged ear.

Our study aimed to measure any changes in SA that may occur in the IC ipsilateral to the damaged ear following unilateral acoustic trauma by using electrophysiological and metabolic measures of spontaneous activity in awake, freely moving rats. By using the same methods to compare activity in either side of the IC following unilateral damage, we were able to further investigate changes in SA that occur in either the dominant or non-dominant pathways in a tinnitus model.

4.2 METHODS

Animals

Adult, male Long-Evans rats, weighing between 250-300g upon receipt from the vendor (Charles River Laboratories), were used for all aspects of this study. The Institutional Animal Care and Use Committee of the University of Kansas Medical Center approved the experimental protocol.

Sound Exposure

Rats were anesthetized with isoflurane and placed in a sound-attenuating booth (Industrial Acoustics Company, Bronx, NY) for the duration of the exposure. Each animal was presented with a 16 kHz pure tone at 118 dB SPL for four hours from a loudspeaker (Radio Shack 40-1310-B) inside a plastic case. The loudspeaker was coupled to either the left or right pinna via ½” flexible plastic tubing. Audalin ear mold compound (All American Mold Lab, Oklahoma City, OK) was used to seal the tube delivering the sound to minimize the occurrence of bilateral damage. The stimulus level outside the tube was measured as 45 dB less than inside the tube when it was sealed

to the head. A Macintosh computer with a MaLab synthesizer, event processor, and software (Kaiser Instruments, Irvine, CA) was used to control noise waveform synthesis.

Surgery

All rats received surgically implanted recording chambers above the right inferior colliculus, which was either ipsilateral or contralateral to the damaged ear, prior to electrophysiological recordings. Rats were anesthetized with a ketamine-xylazine mixture and placed in a stereotaxic apparatus with blunt ear bars under aseptic conditions. The scalp was shaved and an incision was made to expose the skull. The craniotomy was made using dental burrs and it was located approximately 2 mm lateral from the midline at lambda. The dura was left intact. A stainless steel tube (4 mm internal diameter) with a removable cap was then positioned over the craniotomy and fixed to the skull using 4 anchoring screws and dental acrylic (Lang Dental Mfg. Co., Wheeling, IL). The skin was then sutured and the rat was allowed to recover with the use of antibiotics (bacitracin) and analgesics (buprenorphine and ketoprofen), administered for 2 days following surgery.

Single Unit Recording

A minimum of one week following sound exposure, single unit extracellular recordings were performed on each rat. A custom-made lightweight microdrive containing a single, tungsten microelectrode was inserted into the surgically implanted recording chamber and secured with a set screw while the rat was anesthetized with isoflurane. After regaining consciousness, the unanesthetized rats were placed in a Lucite chamber within a sound-attenuating booth and were allowed to move freely while

the microdrive remained attached to a flexible recording cable with a headstage preamplifier. The recording cable was routed through a commutator to a Neuralynx amplifier. The amplifier was computer controlled (Cheetah software, Neuralynx) outside the sound booth to adjust gain and filtering.

Broadband noise bursts were presented to identify neural responses to auditory stimuli. The single microelectrode was incrementally lowered through the depth of the IC, and frequency tuning from clusters of neurons were measured every quarter of a millimeter in depth. When a single unit was encountered, five, five second intervals of spontaneous activity were measured while the rat was not moving or making any self-produced noise, such as tooth grinding. The five intervals were taken consecutively and later averaged. The frequency tuning of the unit was then determined by presenting pure tones at different frequencies and intensities (10 dB SPL-70 dB SPL). In many cases, neurons responded to a restricted range of frequencies and a characteristic frequency (CF) could be determined at the lowest threshold. In other cases, neurons responded unreliably or to a broad range of frequencies, and a CF could not be assigned. In electrode penetrations with frequency tuned units, CFs increased in frequency with increasing electrode depth. The unit's response to noise was determined by playing noise bursts at various intensities.

Finally, the rate of spontaneous activity was again measured in five increments of five second intervals while the rat was silent and not moving. This second measure of the rate of spontaneous activity was used to assess the stability of the cell and its spontaneous activity. Measurements were taken until the electrode reached the end of

the auditory responsive tissue. Multiple electrode penetrations within the IC were made in each rat over a period of 1-2 weeks, during 1-3 recording sessions in each rat.

Lesions

At the conclusion of each single unit recording session, marking lesions were made through the recording electrode at the beginning and the end of the penetration. Direct current, electrode tip negative, was applied for 5s at the bottom and top of the penetration.

Data Analysis

Average rates of spontaneous activity were evaluated for all units either within or outside the central nucleus of the IC. All units that were not contained within a tonotopic progression during a given penetration were considered to be outside the central nucleus, regardless of tuning properties. Units within the tonotopic sequence were subdivided into frequency categories based on CF. Units in a tonotopic sequence that did not have a single characteristic frequency or were unresponsive to tones were considered untuned, and were assigned to a frequency category based on their location within the tonotopic sequence. Data are reported with standard error of the mean. Statistical significance was determined using SigmaPlot by a Kruskal-Wallis analysis of variance and post-hoc Wilcoxon pairwise comparison.

¹⁴C-2-Deoxyglucose

In a terminal procedure occurring a minimum of three weeks following acoustic trauma, rats were injected intramuscularly with ¹⁴C-2-deoxyglucose (2DG; 100 μ Ci/kg). Injection occurred in a sound-attenuating chamber, and the rat was placed in a Lucite cage within the sound chamber for 45 minutes. Following the 45 minute uptake period,

rats were euthanized with a lethal dose of Beuthanasia (5cc/kg) and decapitated. Brains were harvested and flash frozen in heptane cooled to -65° with dry ice. Fresh, frozen brains were sectioned in 40 µm thick coronal sections on a cryostat. Serial sections were thaw mounted on slides and then apposed to Kodak Biomax MR X-ray film for 1-2 weeks along with 14C standards. Film was developed and corresponding sections were stained with thionin for nissl substance, allowing for outlines of each IC to be made on corresponding autoradiographs.

Optical density measurements were made using NIH ImageJ from digital images (QImaging EXi Aqua Camera). Grayscale measurements were calibrated to the 14C concentrations in the standard. 2DG activity measurements were made from ten sectors that crossed the external nucleus and the tonotopic axis of the central nucleus, from three sections located approximately 50% through the rostral-caudal axis of the left and right IC. To account for differences in absolute 2DG concentrations among animals, all measurements were normalized to 2DG levels in a portion of the cerebellum and averaged across all three sections. Statistical significance was determined using a one-way ANOVA with a Fisher LSD post-hoc analysis.

Cochlear Processing

Harvested cochleae were decalcified with RDO for one hour. Decalcified cochleae were washed using ddH₂O, and myelin stained with 1% osmium tetroxide for one hour, flushing the solution through the cochleae at 15 minute intervals. The osmium tetroxide was removed by washing the cochleae in ddH₂O. Cochleae were dehydrated by washing in a series of PBS, ddH₂O, 50% EtOH, and then 70% EtOH.

Stained and dehydrated cochleae were placed in propylene oxide for 30 minutes and then placed in a 1:1 Araldite:PO mixture. After two hours, cochleae were placed in a 2:1 Araldite:PO mixture and placed on an orbital shaker overnight. The cochleae were then removed from the 2:1 Araldite:PO mixture and placed into de-gased capsules of Araldite. The capsules were placed into a vacuum chamber and allowed to degas for an additional 2 hours. The capsules were then transferred from the vacuum chamber to an oven set to 60 C and allowed to harden for 2 days, prior to removal.

After being embedded in plastic, the cochleae were cut in sections parallel to the modiolar axis. Sections were made on a microtome in 40 μm thickness. After placement on glass slides, sections were then counterstained using Toluidine Blue prior to coverslipping with Permount.

Hair Cell Quantification

Hair cell counts were performed by a single person, who had no knowledge of the treatment group to which the cochleae belong. Inner and outer hair cells were counted at 20x using Nomarski illumination on a Nikon Optiphot-2 microscope. The presence of a hair cell was determined by an intact nucleus located in the basal half of the cell. Reconstruction of the basilar membrane was conducted by aligning serial sections using Adobe Photoshop. The coordinates of each basilar membrane point were identified and exported as a Microsoft Excel file. Coordinates of the basilar membrane were expressed as a percent distance along the length of the basilar membrane and synced with the corresponding hair cell count data in Excel. Percent distance along the cochlea was transformed to characteristic frequency using the Greenwood equation (Greenwood, 1996). Differences in hair cell numbers among

groups of animals was evaluated using a one-way ANOVA with a Fisher LSD post-hoc test.

4.3 RESULTS

Hair Cell Quantification

The focus of our analysis of SA was in the IC receiving input from the cochlea contralateral to the exposed ear. To ensure that our sound exposure did not damage the unexposed ear, we quantified the total number of inner (IHC) and outer (OHC) hair cells in both the exposed and unexposed cochleae, which can be seen in Figure 4.1. The unexposed ear displayed minimal loss of either IHCs or OHCs (Fig. 4.1b, d). However, there was one region of loss in both the IHC (0.7-1.6 kHz, one-way ANOVA, Fisher-LSD post hoc, $P=0.006$) and OHC (6.7-8.3 kHz, $P=0.037$), indicating a very small degree of damage in the unexposed ear. The exposed ear displayed significant loss of both IHC and OHC across the tonotopic arrangement of the basilar membrane, with a high degree of loss in high frequency regions (Fig. 4.1a, c). These results indicate significant effects of unilateral acoustic trauma on the exposed ear with minimal residual effects on the unexposed ear.

¹⁴C-2-deoxyglucose

Each of our animals received ¹⁴C-2-deoxyglucose (2DG) injections to provide a measure of metabolic activity following damage. By measuring relative 2DG activity in 10 sectors along the depth of either side of the IC, we were able to gain more insight into overall changes in spontaneous activity in addition to our electrophysiological results. Figure 4.2 shows the results from the 2DG assay in control and damaged animals. The first three sectors correspond to the external nucleus of the IC, and

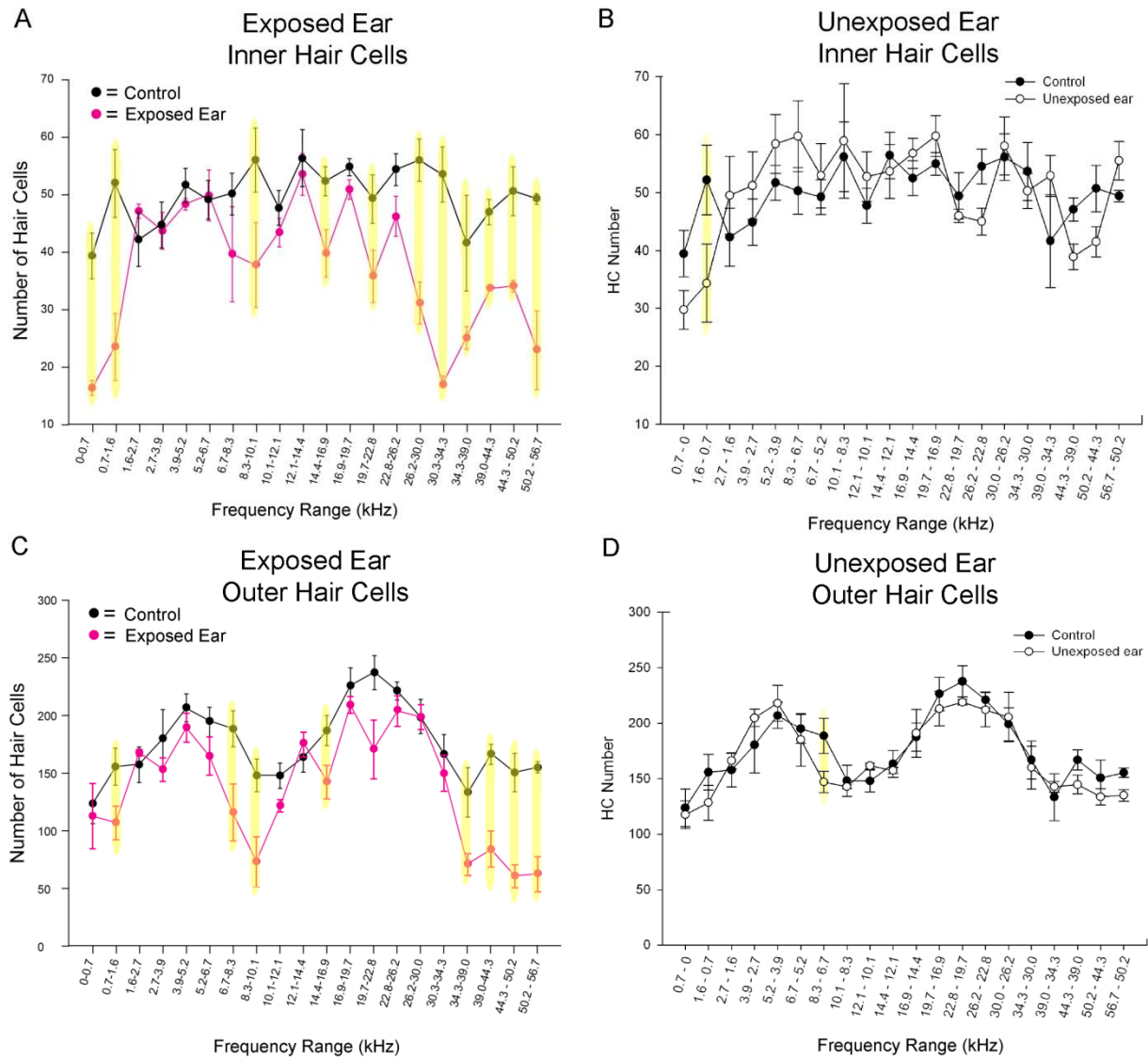


Fig. 4.1 Cochlear hair cell quantification. The total number of hair cells were quantified along the tonotopic axis of the basilar membrane for the exposed ear of unilaterally damaged animals (A, C) and unexposed ear of unilaterally damaged animals (B, D). Highlighted regions represent areas of significant loss compared to control animals (One-way ANOVA, Fisher-LSD post hoc, $P < 0.05$).

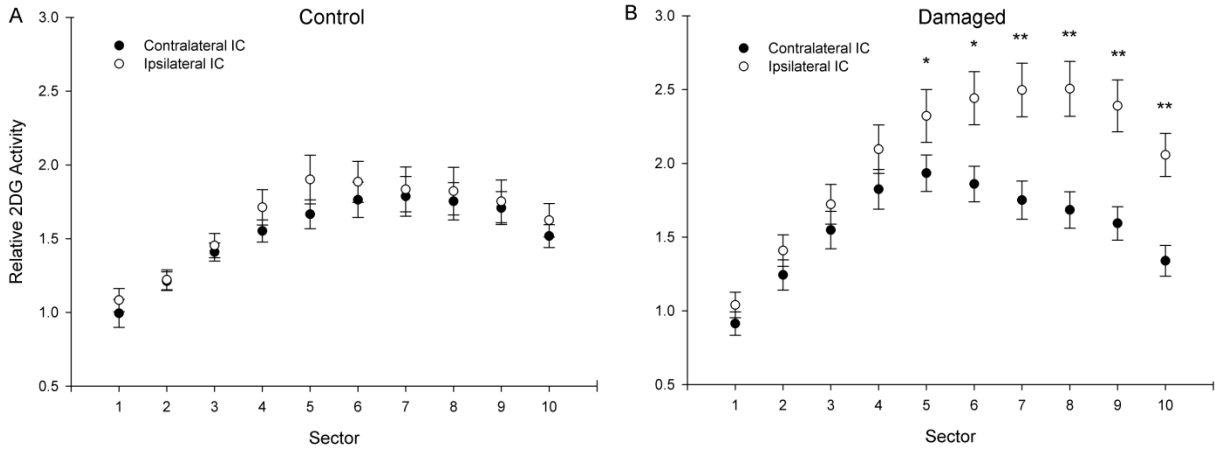


Fig. 4.2 Relative ¹⁴C-2-deoxyglucose activity. Metabolic activity was measured in 10 sectors along the depth of the IC and normalized to an area of the cerebellum. Sectors 1-3 correspond to the external nucleus, sectors 4-10 correspond to the central nucleus, from low to high frequency. A. Control group measurements B. Damaged group measurements. One-way ANOVA, Fisher-LSD post hoc, * $P < 0.05$, ** $P < 0.001$.

sectors 4-10 correspond to the central nucleus, ranging from low to high frequency. Control, unexposed animals did not display any change in relative 2DG activity between the contralateral and ipsilateral IC (Fig 4.2a). However, the damaged animals (Fig. 4.2b) displayed significant difference in relative 2DG activity between the ipsilateral and contralateral IC in sectors 5-10, which correspond to middle to high frequency regions (one-way ANOVA, $P < 0.001$; Fisher LSD post-hoc, $P < 0.05$).

Initially, it was unclear if the differences in the damaged animals were due to decreases in contralateral IC activity, or increases in ipsilateral IC activity. Figure 4.3 shows the results from the contralateral IC (Fig. 4.3a) and ipsilateral IC (Fig. 4.3b) compared to control levels. There was no significant difference in contralateral IC activity compared to controls. However, there was a significant increase in activity in the IC ipsilateral to the damaged ear when compared to the ipsilateral IC of unexposed, control animals. These data indicate an increase in spontaneous metabolic activity in the IC ipsilateral to the damaged ear.

Single Unit Recording

To provide a second measure of activity in the ipsilateral IC, we also performed single unit recording in the IC ipsilateral to the damaged ear in addition to the 2DG assay. Figure 4.4 shows the overall rates of SA for all units within or outside the central nucleus on either side of the IC compared to control rates of SA. There was no change in the rate of SA within the central nucleus in either the contralateral or ipsilateral IC of damaged animals compared to controls (Fig. 4.4). There was also no change in the rate of SA in units outside the central nucleus, which includes the dorsal and external cortices, in either side of the IC of damaged animals. Among units outside the central

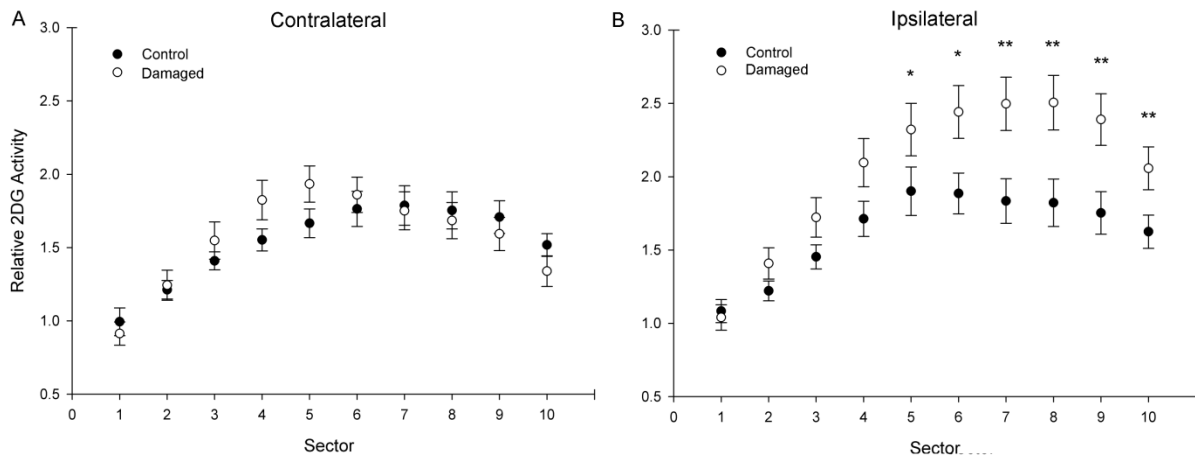


Fig. 4.3 Relative ¹⁴C-2-deoxyglucose measurements in contralateral or ipsilateral IC. Relative 2DG activity was measured in the IC contralateral (A) and ipsilateral (B) to the damaged ear. Measurements are shown compared to control measurements in either side of the IC. One-way ANOVA, Fisher-LSD post hoc, **P*<0.05, ***P*<0.001.

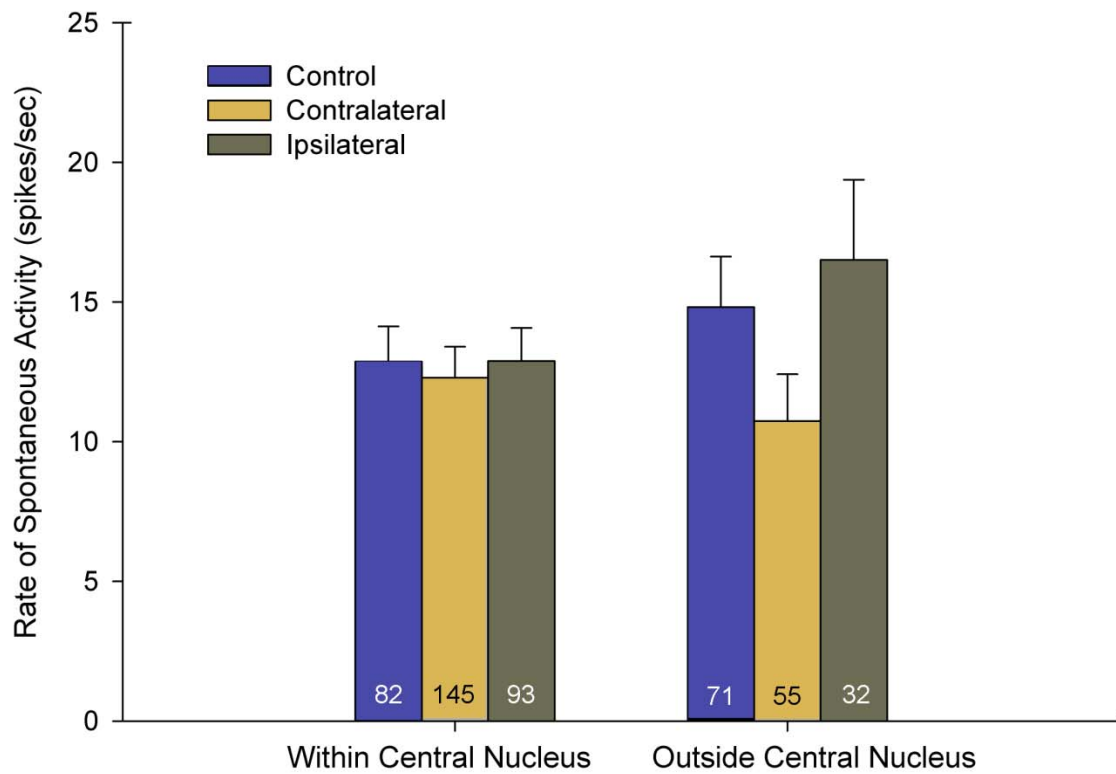


Fig. 4.4 Overall rates of spontaneous activity for all units. Rates of spontaneous activity were measured in all units within or outside the central nucleus for control animals, as well as damaged animals (contralateral and ipsilateral IC). No significant difference was found between any group.

nucleus, there was a small, but statistically insignificant decrease in the rate of SA when comparing the contralateral IC to both control animals and the ipsilateral IC. Nonetheless, there were no significant changes in the overall rates of SA for all units in either IC contralateral or ipsilateral to the damaged ear.

In order to better identify more discrete changes in SA that may be occurring in particular subpopulations of neurons in the IC, we then analyzed the rate of SA in the contralateral and ipsilateral IC as a function of characteristic frequency (CF). These results can be seen in Figure 4.5. Cells were grouped according to their CF by octave up to 16 kHz. Cells that did not respond to a specific CF, as well as cells that did not respond to tones, were categorized as untuned and separated by frequency based on tonotopic location. When analyzing rates of SA in this manner, we found significant increases in one population of units, untuned > 8kHz, in the contralateral IC (Kruskal-Wallis, $P < 0.001$; Wilcoxon pairwise analysis, $P = 0.047$). There were no other significant changes in SA in other populations of cells in the contralateral IC (Fig. 4.5a). There were also no significant changes in SA in any of the cell populations in the IC ipsilateral to the damaged ear (Fig. 4.5b). Interestingly, there were increases in the number of untuned units in the contralateral IC following damage, but this did not occur in the ipsilateral IC.

Taken together, the results from our single unit recordings do not show any change in SA in the IC ipsilateral to the damaged ear. However, they do show changes in SA in the IC contralateral to the damaged ear. Interestingly, these data differ from our 2DG findings.

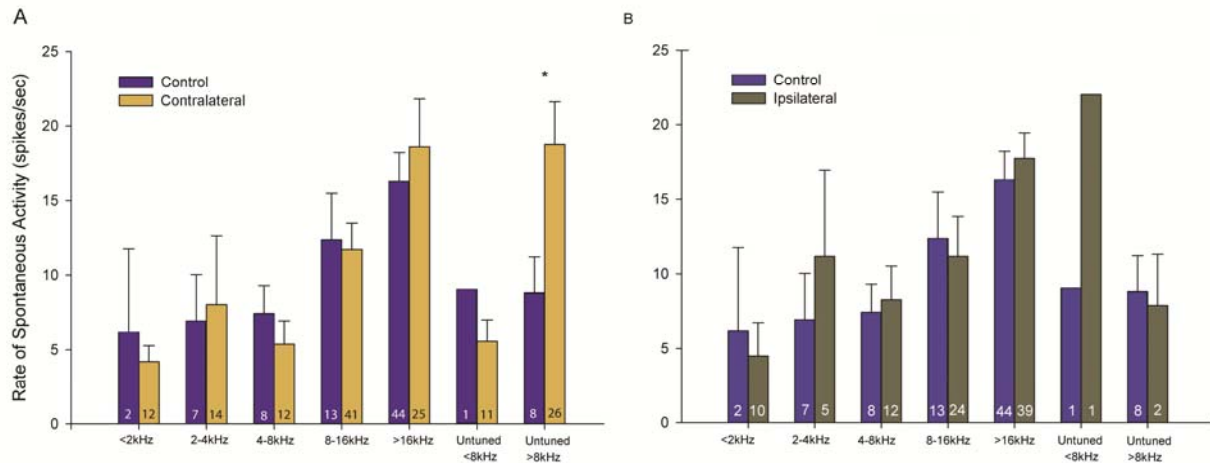


Fig. 4.5 Rates of spontaneous activity as a function of characteristic frequency. Units were categorized as a function of characteristic frequency, arranged from low to high frequencies. Untuned units were separated into < 8kHz or > 8kHz based on tonotopic location. A. Units within the IC contralateral to the damaged ear. B. Units within the IC ipsilateral to the damaged ear. Kruskal-Wallis, Wilcoxon pairwise comparison, * $P=0.047$.

4.4 DISCUSSION

The purpose of this study was to use two methods of measuring spontaneous activity to analyze changes in both sides of the IC of awake, freely moving rats that received unilateral acoustic trauma. Electrophysiologically, we found increases in one population of cells (untuned > 8kHz) in the IC contralateral to the damaged ear, but no change in activity in any cell population in the IC ipsilateral to the damaged ear. We also found no change in overall activity in either side of the IC. These data differ from our ¹⁴C-2-deoxyglucose measurements, in which we found increases in metabolic activity in the middle to high frequency regions of the ipsilateral IC, but no change in the contralateral IC.

It is possible that the changes we observed ipsilaterally are in part due to the small amount of damage occurring in the cochlea contralateral to the sound exposure. Our hair cell quantification indicated two small areas of significant loss in the IHC and OHC of the unexposed ear. Although these areas did not correspond to the damage stimulus, it is possible that a small amount of residual damage may have an effect further up the auditory system in central structures. However, differences in 2DG activity were broad across the tonotopic map of the IC, which does not correspond to the small areas of damage seen in the contralateral cochlea. Additionally, we did not observe an increase in untuned units in the ipsilateral IC, unlike the large increase in untuned units we observed in the contralateral IC. This may indicate a lack of damage in the unexposed ear.

It is also possible that the discrepancies in our electrophysiological and 2DG results reveal an interesting aspect of tinnitus mechanisms. It has been shown that 2DG measures metabolic activity at the synapse, as opposed to the cell body (Nudo

and Masterton, 1986). Auker et al., demonstrated in the visual system that 2DG uptake does not necessarily depend upon the number of action potentials generated (Auker et al., 1983), and Nudo and Masterton showed similar findings in the central auditory system. Because 2DG is measuring activity at the synapse, and not necessarily that of actively discharging neurons, it stands to reason that any changes seen in levels of 2DG activity may be reflective of changes in both net excitatory and inhibitory activity. Our single-unit recording technique measures cell body excitatory activity, which might explain the discrepancy between the two results. It is possible that our 2DG data show an increase in excitatory and inhibitory synaptic activity in the IC ipsilateral to the damaged ear.

It is important to note that the majority of studies that look at the effects of unilateral acoustic trauma on activity in the central auditory system typically analyze changes in either the ipsilateral DCN or the contralateral IC, both of which are considered to be part of the direct pathway from the peripheral auditory system. Such studies typically find increases in SA in the direct pathway following damage. However, there have been a few studies that have measured changes in activity in either the contralateral DCN or the ipsilateral IC following damage. Dong et al. found increases in SA in both contralateral and ipsilateral IC following damage in guinea pigs (Dong et al., 2010). Brozoski et al. found increases in the contralateral DCN of chinchilla following damage (Brozoski et al., 2002). Interestingly, Ma et al. found no global changes in SA in either side of the IC following damage in mice (Ma et al., 2006), and Ma and Young found no change in SA in the DCN of decerebrate cats following bilateral damage (Ma and Young, 2006). The findings of the latter studies correspond well to our present

electrophysiological results. Additionally, our lab previously used 2DG to examine changes in both the direct and indirect pathways following damage, and found subsequent small changes in relative 2DG activity in the indirect pathway (Imig and Durham, 2005).

Because the inferior colliculus is a very complex structure that receives a wide variety of ascending and descending input from other auditory structures (Pollak et al., 2003), we face a challenge in understanding what other structures may have an influence on either side of the IC following damage. Although it is generally accepted that input from a given cochlea projects mainly contralaterally to the opposite IC, we cannot underestimate the influence of the vast array of connections made within the entire structure of the IC. It is also possible that the intrinsic and commissural connections between the two hemispheres of the IC play a role in the changes we observe ipsilaterally. For these reasons, it may be important to analyze changes bilaterally after unilateral damage.

Taken together, our study shows the effects of unilateral acoustic trauma on either side of the IC following damage. By using two methods of measuring spontaneous activity, we were able to show a possible increase in ipsilateral inhibition with an increase in excitation in one population of cells contralaterally. In the future, it may be of interest to continue to measure activity in both sides of the IC following unilateral damage, as well as perform the same studies in bilateral damage models, which may be closer to human experience.

CHAPTER 5: CONCLUSIONS AND DISCUSSION

5.1 PURPOSE OF THIS WORK

The work presented here was designed to analyze changes in SA in the inferior colliculus of rats following acoustic trauma in order to better characterize the central mechanisms of tinnitus. To do this, we used two methods of measuring neural activity, single unit electrophysiology and a ¹⁴C-2-deoxyglucose assay. By combining these two methods in a single animal, we were able to measure SA as well as metabolic activity, which allowed for a more complete picture of changes that may occur in the IC following damage. Additionally, we attempted to address several problems that have hindered current tinnitus research. We sought to address the issues of the use of anesthesia during recording sessions, the variability of acoustic trauma paradigms used to induce tinnitus, and possible changes that may be occurring in the non-dominant pathways of the auditory system that are often overlooked. While some of our results were unexpected, we believe they may reveal interesting aspects of the central mechanisms of tinnitus.

5.2 EFFECTS OF ANESTHESIA ON RATES OF SPONTANEOUS ACTIVITY

As discussed in chapter 2, the majority of tinnitus studies use some form of anesthesia during the electrophysiological recordings. Because anesthesia has been shown to have a significant effect on the rate of SA and response properties of central auditory neurons (Kuwada et al., 1989a; Gaese and Ostwald, 2001; Anderson and Young, 2004), we sought to determine the effects of anesthesia on rates of SA in an acoustic trauma model of tinnitus. To do so, we measured neural activity in the IC of rats exposed to the same acoustic trauma paradigm either in the presence or absence

of anesthesia. As seen in Figure 2.4, the use of a ketamine-xylazine mix during electrophysiological recordings significantly decreased the rate of SA in both control and damaged animals. Additionally, while our unanesthetized animals showed an increase in SA in the units that were untuned > 8kHz, our anesthetized animals did not display an increase in this cell population (Fig. 2.6). Instead, they showed an increase in activity in the cells that had a CF of between 2 and 4 kHz.

Our 2DG assay also showed differences between the anesthetized and unanesthetized groups. Overall absolute 2DG activity was decreased in the anesthetized animals (data not shown), but more intriguingly, the pattern of relative 2DG activity was different between the anesthetized and unanesthetized groups. The unanesthetized animals displayed lower relative 2DG activity in the external nucleus of the IC compared to anesthetized animals. Additionally, the unanesthetized animals displayed significant increases in relative 2DG activity in the IC ipsilateral to the damaged ear across a broader range of the tonotopic map compared to anesthetized animals (Fig. 2.7). These differences, combined with the differences we observed in our single unit recordings, indicate a significant effect of anesthesia on not only the overall rates of SA in the IC, but also the pattern of change following damage.

Understanding the mechanisms of different classes of anesthesia may be important for future studies. Different types of anesthesia target different receptors in the nervous system, which could cause discrepancies between studies. For example, pentobarbital is a GABA receptor agonist (Wan et al., 2003), while ketamine, used in our study, is an NMDA receptor antagonist (Kavalali and Monteggia, 2012), and xylazine is an alpha-2-adrenergic receptor agonist (Albertson et al., 1992). While each

of these anesthetic agents works to inhibit the central nervous system, the different mechanisms of action may have implications for different response properties of central auditory neurons. For example, isoflurane was shown to have a larger impact on auditory cortical neuron temporal response properties than pentobarbital (Cheung et al., 2001). Therefore, if one needs to use anesthesia during recordings, it is important to consider how the specific class of anesthesia will affect the response properties that will be measured.

Because other studies have shown that anesthesia decreases the rate of SA in the central auditory system, we expected to see a decrease in overall rates of SA with the use of anesthesia. However, the observed differences in the pattern of change were intriguing. It is possible that anesthesia targets a specific cell type that would normally display increases in SA following damage, although this remains to be identified. We saw a much smaller increase in the number of untuned units following damage in our anesthetized animals compared to the unanesthetized group. However, both groups of animals received the same amount of sound exposure, which may indicate there is a population of cells that we are not able to record from in the presence of anesthesia.

Another interesting finding was that our anesthetized group did not show overall increases in SA following damage, which conflicts with many previously published studies that do show an increase in SA compared to control animals, even in the presence of anesthesia. Because anesthetic state was the only variable we changed between our two groups, it is possible that another variable, such as the amount of acoustic trauma, is responsible for the differences between our anesthetized recordings

and other previously published results. However, we were still able to successfully show that when anesthesia is the only changed variable, there are significant differences between the two groups.

5.3 EFFECTS OF MILD VERSUS INTENSE ACOUSTIC TRAUMA

In addition to our experiments that analyzed the effects of anesthesia on changes in SA in the IC following damage, we also measured changes in SA with two acoustic trauma paradigms in awake, freely moving rats. We chose to use a mild trauma paradigm, which was a 16 kHz pure tone at 114 dB SPL for one hour, and an intense trauma paradigm, which was a 16 kHz pure tone at 118 dB SPL for four hours. Acoustic trauma paradigms vary greatly among studies, many of which are outlined in Table 1.3. Due to this variability, as well as the findings that the amount of hearing loss in humans does not necessarily correspond to either the severity of tinnitus or the development of tinnitus, we wanted to evaluate the central effects of different acoustic trauma paradigms.

We observed increases in the rate of SA in the cells that were untuned > 8 kHz in both the mild and intense trauma groups, although the mild trauma group failed to reach significance (Fig. 3.5). This is most likely due to the small number of units from which we were able to record. We also saw differences in relative 2DG activity between the ipsilateral and contralateral IC in both the mild and intense trauma groups, but the areas in which differences were observed were very restricted compared to the intense trauma group (Fig. 3.6). Interestingly, although we did not see as many robust changes in activity in the mild trauma group when analyzed as a function of CF, we observed

significant increases in overall rates of SA in this group for all units within or outside the central nucleus, compared to both control and intense trauma animals (Fig. 3.3).

The differences between the two groups indicate the amount of trauma received can be a significant factor in central changes in activity. When analyzing cochlear hair cell loss following the two different trauma paradigms, it is clear that the two paradigms cause different extents of hair cell loss in the cochlea (Fig. 3.2). The intense trauma group displays much greater significant loss of both inner and outer hair cells, and the pattern of hair cell loss has the potential to determine changes that occur at higher levels in the central auditory system via different mechanisms. For example, because the cochlea is tonotopically organized, and the higher structures of the auditory system maintain individual tonotopic maps, loss of hair cells at a specific frequency range could affect the input to specific frequency areas of the tonotopic map in the IC. We observed a large increase in untuned units in the intense trauma group compared to the control group, indicating a loss of frequency tuning in units that correspond to the area of damage. We did not see as great of an increase in untuned units in the mild trauma group, which corresponds with the lesser extent of hair cell loss.

Additionally, it is possible that more intense trauma causes a different cascade of changes in the central auditory system compared to mild trauma. It is intriguing that the mild trauma group displayed overall increases in SA in all units within or outside the central nucleus, while our intense trauma group showed no change. Perhaps different amounts of damage cause different mechanisms or patterns of deafferentation, which could lead to divergent central changes. For example, mild trauma may cause only partial loss of hair cells and supporting cells, and the resulting cellular repair process

may play a role in the deafferentation and reorganization that will eventually occur in central structures. Conversely, severe damage may completely obliterate hair cells and supporting cells to a point that repair mechanisms do not function properly, and the pattern of deafferentation will differ from that seen in the mild trauma group. Additionally, there may be different patterns of change in cochlear structures other than hair cells, such as the stria vascularis. These changes would not be immediately evident in our study, as we only quantified hair cell loss. However, it would be of interest to evaluate changes in supporting cochlear structures as well.

The differences we observed between the two trauma paradigms indicate that it is imperative to fully understand the underlying cellular and mechanistic changes that occur after various levels of acoustic trauma if we continue to use sound exposure as a tinnitus model. If different acoustic trauma paradigms cause different mechanisms of change in the central auditory system, then we must understand what is being modeled in each paradigm, and how those changes relate to human tinnitus mechanisms.

5.4 CHANGES IN THE NON-DOMINANT PATHWAY

Tinnitus is thought to be generated in central auditory structures following damage to the peripheral auditory system. When cochlear input is altered, central auditory structures such as the DCN and IC become deafferented, which can cause an imbalance of excitatory and inhibitory input to these structures. Because a large component of auditory information is processed along the dominant pathway, many studies that use unilateral damage focus on changes in activity only in the dominant pathway, which includes the DCN ipsilateral to the damaged cochlea, and IC

contralateral to the damaged cochlea. However, the auditory system is complex, with many ascending and descending connections between auditory nuclei. Thus, we were interested in analyzing changes in the IC ipsilateral to the damaged ear, which is in the non-dominant pathway.

As shown in Chapter 4, we observed increases in relative 2DG activity in the IC ipsilateral to the damaged ear (Fig. 4.3b). We did not observe changes in the IC contralateral to the damaged ear compared to controls (Fig. 4.3a). These results were unexpected, as the majority of the input from a given cochlea projects contralaterally along the dominant pathway. However, we did not observe changes in SA in the IC ipsilateral to the damaged ear when measuring activity electrophysiologically (Fig. 4.4). We did see an increase in SA in units that were untuned > 8kHz in the IC contralateral to the damaged ear (Fig. 4.5a), but this same population of units remained unchanged in the ipsilateral IC (Fig. 4.5b). These results do not align with our 2DG data.

The conflicting results from our electrophysiological and 2DG data may reveal an interesting aspect of the mechanisms of tinnitus. It has been shown that 2DG measures synaptic activity, rather than actively discharging cell bodies (Nudo and Masterton, 1986), and that 2DG uptake does not depend on action potentials (Auker et al., 1983). Therefore, our 2DG assay has the potential to measure changes in both excitatory and inhibitory synaptic activity, as opposed to only measuring excitatory activity from actively discharging neurons. We may be observing an increase in 2DG activity in the ipsilateral IC in the absence of changes in ipsilateral SA due to increases in inhibitory activity that are not measured by our single unit recording techniques.

An increase in inhibition in the IC ipsilateral to the damaged ear may have several implications for the mechanisms of tinnitus. Because the input to the IC is complex, changes observed in the ipsilateral IC could be caused by input from a number of other locations in the auditory pathway. The increase in inhibition may be due to changes in afferent input along the non-dominant pathway, or it could be due to efferent input from higher structures in the central auditory system. Additionally, there is commissural input from the contralateral IC, which could influence activity in the ipsilateral IC. The ipsilateral IC could be compensating for changes in the contralateral IC, including the increases in SA that we observed in the units that were untuned > 8kHz. Although we are not yet able to fully understand the implications of the observed increases in 2DG activity in the ipsilateral IC, our data indicate that it is important to analyze changes in neural activity in both the dominant and non-dominant pathways following unilateral damage.

5.5 POTENTIAL PROBLEMS

Although our study provides important findings for understanding the mechanisms of tinnitus, there remain some potential problems with our techniques and results. One difficulty in our study is determining whether or not our animals actually experience tinnitus. Acoustic trauma has been widely used and accepted as a model for tinnitus induction in animals. However, some studies have shown as few as 30% of damaged animals develop tinnitus (Ruttiger et al., 2013). We did not use any behavioral assays to determine whether our animals have tinnitus, as we were not able to optimize the gap detection method of detecting tinnitus. Other studies have shown problems with variability and habituation in animals that are tested with this behavioral

method (Longenecker and Galazyuk, 2012; Lobarinas et al., 2013), which we also found in our attempts to use gap detection. Thus, our results may be more applicable to evaluating changes in the central auditory system after acoustic trauma, either in the presence or absence of tinnitus. While we may not be able to definitively show that our acoustic trauma paradigms induce tinnitus, our findings are still important for the study of changes to the central auditory system following hearing loss, which is an even more prevalent public health issue than tinnitus.

Another potential methodology problem that we face in our work is the use of awake, freely moving animals. While we have shown that anesthesia can have a significant effect on the patterns of change in the IC following damage, we must acknowledge that recording from awake, unrestrained animals is not feasible for all electrophysiological studies. Due to the presence of self-produced noise, as well as movement artifacts, we are only able to record SA from units in short intervals while the animal is awake, silent, and not moving. This restriction makes it difficult to gather long periods of SA, as well as more intricate response properties. Therefore, this method is not ideal for all studies.

Additionally, because the animals are allowed to move freely during the 2DG uptake period, it is possible that they are producing noise from grooming and tooth grinding, which may be reflected in 2DG activity. We take many precautions to prevent as much noise as possible, but it is impossible to avoid self-produced noise in unrestrained, awake animals. However, one can assume that the presence of self-produced noise would be reflected in our control measurements. When comparing our

2DG measurements in damaged animals to control animals, we still see a significant difference, indicating minimal effects of self-produced noise on overall 2DG activity.

5.6 FUTURE DIRECTIONS

While the work presented here contributes a significant amount to the current understanding and state of tinnitus research, there remains much work to be done. The first and simplest next step to take in our studies is using a behavioral assay, such as gap detection, to determine how many of our animals are experiencing tinnitus. With the use of an appropriate behavioral method, we could more reliably determine if the changes we have observed are correlated with the presence of tinnitus, or if they are simply the consequences of acoustic trauma.

It would also be of interest to measure molecular level changes in the IC following acoustic trauma. One potential future experiment could be the use of electrochemical detection to measure changes in neurotransmitters along the same penetrations through which we make our single unit recordings. Analyzing changes in excitatory and inhibitory neurotransmitters, such as glutamate and GABA, which occur in correlation with changes we see in SA would present a more complete picture of mechanistic changes that occur in central auditory structures following damage. Additionally, measuring changes in either inhibitory neurotransmitters or receptors in the ipsilateral IC could reveal more information related to the increases in inhibition that we believe are occurring as shown by our 2DG assay.

Another important experiment that could add significant interest to our current studies is to apply the same techniques to evaluate changes in other areas of the

auditory system, specifically the DCN and the cortex. While it would be difficult to measure these changes in the same animal, it would be of great interest to attempt to analyze changes in each of these areas within the same model. It would also be interesting to measure changes in these other structures along the non-dominant pathway to see if there are similar increases in activity that we observe in the IC ipsilateral to the damaged ear. Correlating the changes with detailed hair cell quantification, as well as spiral ganglion cell quantification would potentially provide a more complete picture of changes in tinnitus models from periphery to cortex.

Understanding the mechanisms by which 2DG activity is increased ipsilaterally is imperative for our further understanding of tinnitus mechanisms. One way by which this may be possible is to alter the input to the ipsilateral IC prior to or shortly after acoustic trauma. For example, severing the commissural connections from the contralateral IC either before or shortly after acoustic trauma may eliminate the observed increases in 2DG activity in the ipsilateral IC. An experiment such as this may reveal more about how changes in activity occur during the generation of tinnitus.

A final potential future study that would be of interest is to use MEMRI, which is a non-invasive imaging technique with a high degree of resolution, in addition to electrophysiology and 2DG. A great benefit of using MEMRI in a tinnitus study is the ability to inject Mn^{2+} in an acoustically controlled environment. Uptake of Mn^{2+} can occur in silence and be imaged hours later. This is beneficial because it circumvents the loud sounds the MRI machine makes during imaging. Because Mn^{2+} is transported via voltage-gated Ca^{2+} channels, we would expect the results to be similar to our single unit recording data. However, using MEMRI would give the potential to analyze

changes across the entire central auditory system, instead of a single structure, such as the IC.

5.7 IMPORTANCE OF THIS WORK

Tinnitus is a widespread condition, affecting as many as 50 million Americans (ATA, 2013). It severely affects as many as 2 million individuals in the United States, and can be so bothersome that it can drive people to commit suicide (Roberts et al., 2010). Although tinnitus poses a significant problem for millions of people worldwide, there is still a dearth of understanding of tinnitus mechanisms. Due to this lack of knowledge, no single effective treatment has been found to work for a large percentage of sufferers (Seidman et al., 2010). The work presented here not only addresses some common problems in current tinnitus research, but it also potentially reveals more information about mechanisms of tinnitus.

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