DIFFERENTIAL PATHOLOGIES RESULTING FROM SOUND EXPOSURE:
TINNITUS VS HEARING LOSS

A dissertation submitted to Kent State University in cooperation with Northeast Ohio Medical University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

by

Ryan James Longenecker

December 2015
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<td>ABR</td>
<td>Auditory brainstem response</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AOE</td>
<td>acoustic over exposure</td>
</tr>
<tr>
<td>ARO</td>
<td>Association for Research in Otolaryngology</td>
</tr>
<tr>
<td>ASR</td>
<td>Acoustic startle reflex</td>
</tr>
<tr>
<td>C.I.</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>COM</td>
<td>center of mass</td>
</tr>
<tr>
<td>COMD</td>
<td>center of mass displacement</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>dB</td>
<td>decibel</td>
</tr>
<tr>
<td>DCN</td>
<td>dorsal cochlear nucleus</td>
</tr>
<tr>
<td>FDA</td>
<td>federal drug administration</td>
</tr>
<tr>
<td>G</td>
<td>gap</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GPIAS</td>
<td>Gap induced prepulse inhibition of the acoustic startle reflex</td>
</tr>
<tr>
<td>HB</td>
<td>high bursting</td>
</tr>
<tr>
<td>HSD</td>
<td>honestly significantly different</td>
</tr>
<tr>
<td>IC</td>
<td>Inferior colliculus</td>
</tr>
<tr>
<td>IP</td>
<td>intraperitoneal (injection)</td>
</tr>
<tr>
<td>ISI</td>
<td>interspike interval</td>
</tr>
<tr>
<td>LB</td>
<td>low bursting</td>
</tr>
<tr>
<td>MGB</td>
<td>medial geniculate body</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>-------------</td>
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<tr>
<td>mGluR</td>
<td>metabotropic glutamate receptor</td>
</tr>
<tr>
<td>NB</td>
<td>no bursting</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NT</td>
<td>no tinnitus</td>
</tr>
<tr>
<td>O.D.</td>
<td>outside diameter</td>
</tr>
<tr>
<td>PnC</td>
<td>nucleus reticularis pontis caudalis</td>
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<tr>
<td>PPI</td>
<td>Prepulse Inhibition</td>
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<tr>
<td>PTS</td>
<td>permanent threshold shift</td>
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<tr>
<td>S</td>
<td>startle</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<td>SPL</td>
<td>sound pressure level</td>
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CHAPTER I
INTRODUCTION

Noise induced hearing loss is one of the most common occupational illnesses in the
United States. Noise exposure initially results in cochlear damage which leads to
maladaptive neuroplastic changes within the central auditory pathway. These changes often
manifest as increased spontaneous firing in auditory neurons and/or elevation of neural
synchrony and bursting activity. This abnormal brain activity has been hypothesized to explain
the development of phantom sound sensation or tinnitus in some individuals. The general goal of
this dissertation is to further elucidate the effect of sound exposure on the auditory system using
behavioral and physiological methods for its assessment. In contrast to previous studies, the
focus was to identify changes in individual mice rather than group averages. This allows for
important clinical comparisons of unique, individual-specific pathologies related noise induced
hearing loss and/or tinnitus.

Tinnitus

Tinnitus, the perception of a sound with no external source, is a complex perceptual
phenomenon affecting the quality of life of 35-50 million adults in the United States (Ahmad &
Seidman 2004). Tinnitus is often persistent, loud, and annoying, causing emotional distress to
those afflicted. Patients with severe tinnitus may have trouble hearing, working and sleeping.
Despite its ubiquity and morbidity, the pathophysiology of tinnitus is inadequately understood,
and there is no universal medically recognized treatment.
Nearly all cases of tinnitus and hearing loss can be explained, at least in part, by exposure to loud, cochlear-damaging sounds. While the underlying causes of hearing loss are continually being elucidated, such progress has been slow in understanding the mechanism(s) responsible for triggering the tinnitus percept. In most clinical cases there is a strong correlation between hearing loss and tinnitus, however, this is not always the case (Weisz et al. 2006). Most commonly patients develop hearing loss in middle and late ages without the presence of tinnitus. Alternatively a person may have tinnitus while demonstrating no audiometric deficits (Job et al. 2007). In order to understand the aforementioned clinical manifestations it is important to unravel the underlying pathologies.

Tinnitus should be considered a symptom and not a disease. The underlying pathologies associated with tinnitus are thought to be manifested centrally as abhorrent neuronal activity (see review Roberts et al. 2010). It is known that exposure to loud sounds can create neuronal hyperactivity in most auditory nuclei and many non-auditory regions of the brain (Eggermont 2005). Although it is uncertain as to whether animals demonstrating neuronal hyperactivity also perceive tinnitus, there have been some indications that clinical patients show similar increases of activity observed by several brain imaging techniques (see review Lanting et al. 2009). Thus, the development of an animal model for tinnitus detection is important to cross validate human studies with research done on animals.

**Gap-induced prepulse inhibition of the startle reflex as a measure of tinnitus**

Several animal models have been developed to accurately assess tinnitus in laboratory animals (Turner 2007; Hayes et al. 2014). Most early studies employed basic mechanisms of conditioning (Bauer et al. 1999; Heffner & Harrington 2002; Guitton et al. 2003; Rüttiger et al. 2003; Lobarinas et al. 2004; Heffner & Koay 2005). These paradigms typically require complex
behavioral manipulations and weeks to months of animal training. Although these models have contributed significantly to the field of tinnitus research, they are time consuming and can be difficult for researchers lacking experience in behavioral techniques.

More recently, a novel method called gap induced prepulse inhibition of the acoustic startle reflex (GPIAS) was proposed (Turner et al. 2006). This method utilizes reduction in the acoustic startle reflex by a preceding silent gap in an otherwise constant acoustic background. Animals with behavioral evidence of tinnitus cannot detect silence and therefore their reduction of the startle reflex is significantly less than in normal animals (Fig. 1A). This method has been used successfully to assess tinnitus induced by salicylate overdose or acoustic trauma in rats (Turner et al. 2006; Yang et al. 2007; Kraus et al. 2010). The most attractive aspects of this paradigm are that it requires minimal animal training and testing sessions can be conducted very quickly, on the order of hours.

Since the original GPIAS model in rats, several additional animal models have been developed (see review Galazyuk and Hébert 2015). Golden hamsters, ferrets, and guinea pigs have all been used as tinnitus animal models. However, perhaps the most used and studied model now is that of the mouse. Mice are the model of choice for genetic manipulations, which makes this an attractive model for potential gene therapies to alleviate tinnitus.

Although the GPIAS methodology is relatively straightforward in terms of the effort it takes to collect data, an in-depth working knowledge of this method can dramatically improve data collection. To this extent, some of the finer details of GPIAS for tinnitus assessment have been recently improved to increase the reliability of data collection (Longenecker & Galazyuk 2012; Grimsley et al. 2015). The more refined resolution of data can ameliorate some of the
Figure 1.1 Two types of stimulus paradigms used for assessing gap detection performance and prepulse inhibition in mice. A Gap detection stimulus paradigm consists of (1) STARTLE trial—a startle stimulus of wide band noise (20 ms duration, 110 dB SPL) embedded in a continuous background narrow band noise centered at 10, 12.5, 16, 20, 25, and 31.5 kHz and presented at 75 dB SPL; (2) GAP STARTLE trial—similar to the STARTLE trial with the addition of a 20 ms gap of silence embedded 100 ms before the startle stimulus. B The prepulse detection stimulus paradigm contains (1) STARTLE trial—a startle stimulus (the same as in A) presented in silence; (2) PREPULSE trial—a startle stimulus was preceded by a prepulse narrow band noise (20 ms duration, 75 dB SPL) centered at six different frequencies (the same as in the gap detection paradigm).
challenging issues with inter-animal variability which can be a function of species, stress level, testing epoch, age, and habituation to the paradigm, etc. Ultimately, these improvements have allowed for high resolution data collection which can permit data analysis on individual animals, in an effort to maximize clinical relatability.

**Assessing hearing damage after sound exposure:**

The prevalence of hearing loss in humans is much higher than tinnitus (Lockwood et al. 2002). However, tinnitus is often found most commonly in patients with some degree of hearing loss. The most common cause of hearing loss is exposure to brief intense noise or to moderate noise over a long period of time (Savastano 2008). In animals, just as in humans, acoustic trauma typically leads to some degree of hearing loss but not always to tinnitus (eg. Kraus et al. 2011; Longenecker & Galazyuk 2011). Unfortunately, tinnitus studies employing animals as subjects often omit the subset of exposed animals which have shown resistance to sound exposure (Middleton et al. 2011; Turner et al. 2006). Tinnitus resistant animals are important to study due to the possibility of identifying mechanisms responsible for tinnitus prevention.

The audiogram of tinnitus patients almost always shows some degree of hearing loss, and in most cases, the tinnitus pitch is prevalent in the hearing loss spectrum (Roberts 2006). For this reason it is important to determine how tinnitus is linked to the hearing loss using an animal model. Since each type of hearing assessment has inherent advantages and disadvantages, to fully test hearing functionality it would be important to use a variety of techniques.

Many tinnitus studies have used the auditory brainstem response (ABR), a noninvasive auditory evoked potential that can be used clinically and in research settings, to access animal hearing thresholds (Turner et al. 2006; Noreña et al. 2003; Bauer et al. 2008). It is known that
following an auditory insult ABRs reliably identify elevated thresholds. This temporary threshold shift is thought to result from swelling of cochlear nerve terminals which is present for days after exposure, yet returns to baseline soon after (Robertson 1983). However, recent work has clearly demonstrated that this observed temporary threshold shift does not account for the trauma-induced damage to ribbon synapses (Kujawa & Liberman 2009). Thus, ABR is capable of detecting temporary threshold shifts post noise-exposure and may not fully represent the complexity of damage to the auditory system (Lin et al. 2011).

Another common approach to assess animal hearing is the behavioral audiogram. Conditioned suppression/avoidance paradigms (Heffner & Masterson 1980) and go/no-go procedures (Prosen et al. 2000, 2003; Klink et al. 2006; Radziwon et al. 2009) produce sensitive and reliable measures of hearing performance in mice. Known as the gold standard, the behavioral audiogram has been constructed within an entire audible frequency range for many species (see review Heffner & Heffner 2007). However, this methodology has demonstrated high variability between labs and requires months to train animals to collect data for a single audiogram. Due to low temporal resolution, this method cannot be used to assess dynamic changes in hearing performance which have often been reported for animals following sound exposure.

As a considerably quicker alternative to the behavioral audiogram, prepulse modulation of the acoustic startle reflex (ASR) has been employed to assess behavioral response thresholds (Fechter et al. 1988). Prepulse inhibition (PPI), a decrease of ASR magnitude when a preceding weaker sound (prepulse) is presented before the startle, has been introduced and successfully tested for over half a century to objectively measure complex neurological systems (Hoffman & Searle 1965; Hoffman & Wible 1970; Graham 1975). Such an approach can be easily adapted for
assessing audiogram-like thresholds if the prepulse is varied in intensity and frequency. This method has successfully identified cochlear damage due to ototoxic drugs (Young & Fechter 1983) and temporary threshold shifts due to pure tone acoustic exposure when a control and experimental group average data were assessed and compared (Walter et al. 2011). Although this method has some advantages over the currently used behavioral paradigms, several important questions need to be explored before this method would be widely accepted for assessing hearing in laboratory animals. First of all it is still unknown whether this method is sensitive enough to assess hearing in individual animals, which would be much more beneficial than group averages. Second, little is known about the extent of PPI threshold reliability from day to day measures. Third, it is unclear whether PPI audiometry can be used to detect permanent threshold shifts caused by either auditory insult or due to age-related hearing loss. These important questions constituted the rationale for the present study.

One additional audiometric assessment that can provide useful information concerning the relative health of the auditory system is the acoustic startle reflex. Just as with ABR thresholds, ASR amplitudes are diminished immediately after sound exposure (Longenecker & Galazyuk 2011; Lobarinas et al. 2013). This can confirm damage to the auditory system after exposure. While this test has not been directly indicated as a useful hearing assessment, preliminary results seem to suggest that it can be a good indicator of hearing damage.

**Tinnitus-associated changes in the central auditory system**

Tinnitus is linked to abnormal changes in neuronal activity at one or more levels along the auditory pathway (Jastreboff 1990; Moller 2003; Rauschecker et al. 2010). Human brain imaging studies have identified altered tinnitus-related activity in auditory (see review Lanting et al. 2009) and non-auditory areas (Lockwood et al. 1998; Husain et al. 2015). Animal models
have helped to identify abnormalities in neuronal activity of auditory as well as non-auditory brain regions that are linked to tinnitus (see review Eggermont 2013). Animals with behavioral evidence of tinnitus typically exhibit neuronal hyperactivity, abnormally high synchrony, burst firing, as well as reorganization of tonotopic maps (Roberts et al. 2010).

The best studied phenomenon related to tinnitus, increased spontaneous activity, has been found acutely after salicylate injection, a drug known to produce a dose-dependent tinnitus percept (see review Stolzberg et al. 2012), or chronically after intense sound exposure throughout the central auditory system. A chronic increase in spontaneous activity has been shown in the dorsal cochlear nucleus (Kaltenbach & McCaslin 1996), the inferior colliculus (Mulders & Robertson 2009), medial geniculate body (Kalappa et al. 2014) and primary auditory cortex (Noreña & Eggermont 2005) following an intense noise exposure. An important addition to the literature came when this increased spontaneous activity was associated to animals showing behavioral evidence of tinnitus (Brozoski et al. 2002, Longenecker & Galazyuk 2011). One potential mechanism hypothesized for this hyperactivity is a shift in the balance between excitatory and inhibitory inputs (Kaltenbach & McCaslin 1996). Following studies confirmed that sound exposure resulted in a reduction of inhibition at multiple levels of the auditory pathway (see review Richardson et al. 2012). It is thought that damage to the cochlea by sound exposure will result in reduced input to the central auditory system (Liberman & Kiang 1978). To compensate for signal strength loss, the central nervous system increases the gain of its neurons, thus creating hyperactivity. The most common finding is that this compensatory mechanism results in the downregulation of GABAergic inhibition in the central auditory system (Brozoski et al. 2007). This finding was recently confirmed on a molecular level in the dorsal cochlear nucleus (Middleton et al. 2011). All of these studies suggest that hyperactivity is
associated with maladaptive changes that are associated with tinnitus; however, it is important not to rule out the underlying hearing loss as a cause for these neuronal changes.

Besides hyperactivity, tinnitus-related changes in the auditory system also include increased neural synchrony and bursting activity. Following sound exposure, abnormal bursting activity occurs in the auditory nerve, dorsal cochlear nucleus, and inferior colliculus, medial geniculate body, and the auditory cortex (Liberman & Kiang 1978; Finlayson & Kaltenbach 2009; Chen & Jastreboff 1995; Bauer et al. 2008; Kalappa et al. 2014; Noreña & Eggermont 2003). This neurophysiological signature of tinnitus could be caused by increased spike regularity, decreased asynchrony of spiking (Dominguez et al. 2006), or increased cross-fiber synchrony (Komiya & Eggermont 2000; Eggermont 1990). There is some evidence that both acoustic trauma and ototoxic drugs can increase synchronous discharge across different levels of the auditory system.

Interestingly, tinnitus was not studied as a function of hearing loss in these experiments. There is a possibility that a number of the observations described in both humans and animals were not indicative of tinnitus but of an underlying hearing loss. Furthermore, most physiology studies have been completed with the use of anesthetics which have been shown to have significant effects on auditory processing (Ter-Mikaelian et al. 2007). Hyperactivity or burst firing could indeed be important markers for tinnitus, but, they could also be a general marker for hearing loss, or be the byproduct of the anesthetics. Any and all of the neural-correlates previously studied cannot be ruled out as causes for tinnitus but should be understood in the framework of hearing loss. Thus, each of these phenomena needs to be studied in more depth. Combining of neurophysiology with a behavioral paradigm which can reliably separate tinnitus
from non-tinnitus animals would be a powerful tool to unraveling the underlying physiological mechanism(s) of tinnitus.

**Summary of specific aims of the dissertation**

The first step in identifying the mechanism(s) responsible for tinnitus development would be to discover a neural correlate that is differentially expressed in tinnitus-positive compared to tinnitus negative animals. Previous research has identified several neural correlates of tinnitus in animals that have tested positive for tinnitus. However it is unknown whether all or some of these correlates are linked to tinnitus or if they are a byproduct of hearing loss, a common outcome of tinnitus induction. Abnormally high spontaneous activity has frequently been linked to tinnitus. However, while some studies demonstrate that hyperactivity positively correlates with behavioral evidence of tinnitus, others show that when all animals develop hyperactivity to sound exposure, not all exposed animals show evidence of tinnitus. My working hypothesis is that certain aspects of hyperactivity are linked to tinnitus while other aspects are linked to hearing loss. To test this hypothesis I will use a tinnitus mouse model that has been developed in our laboratory. Three groups of mice were studied: (1) sound exposed mice that develop behavioral signs of tinnitus, (2) exposed mice that do not develop tinnitus, and (3) unexposed mice, to serve as a control. Tinnitus will be induced by sound exposure via loud narrow-band noise and then assessed using the gap-induced prepulse inhibition of the acoustic startle reflex, a well-established technique in our laboratory. Hearing loss will be evaluated by measurements of: the acoustic brainstem responses (ABR), PPI (prepulse inhibition) audiometry, and acoustic startle responses (ASR). Extracellular recordings will assess spontaneous firing rates and bursting activity in neurons of the inferior colliculus.
If successful, the data from this study will provide an understanding of whether hyperactivity is a unique feature of tinnitus or a more general symptom of hearing deficits following sound trauma. The project will reconfirm or advance knowledge by investigating the following specific aims:

**Specific Aim #1: To develop a mouse model for chronic tinnitus.**

We hypothesized that sound exposure will induce chronic tinnitus in exposed mice. It is hypothesized that after exposure with an intense narrowband noise, some mice will show behavioral evidence of tinnitus while others will not. To test this hypothesis a large group of mice will be exposed to a loud narrowband noise lasting one hour, which is known to induce tinnitus. Tinnitus will be assessed by the prepulse inhibition of the acoustic startle reflex. The presence or absence of chronic tinnitus will be assessed for one year following exposure, a method routinely used in our laboratory.

**Specific Aim #2: To determine the effect of sound exposure on hearing loss in CBA/CaJ mice.**

We hypothesized that sound exposure will induce permanent hearing loss in the majority of exposed mice. Mice will be exposed to a narrowband noise of 116 dB SPL during one hour unilaterally. Hearing will be assessed using input/output functions, auditory brainstem responses, and pre-pulse inhibition of the acoustic startle reflex. The magnitude of the hearing loss will be determined by a comparison of hearing performance in individual exposed mice with the group average of the control mice.
Specific Aim #3: To reveal differences in hyperactivity between animals with and without tinnitus

We hypothesized that sound exposure induced hyperactivity in the auditory system independently of whether an animal exhibits tinnitus. Hyperactivity will be assessed in neurons of the IC using extracellular recording technique. The expectation is that all sound exposed mice will show similar hyperactivity compared to unexposed mice, independently of behavior signs of tinnitus.
CHAPTER II

THE DEVELOPMENT OF TINNITUS AFTER SOUND EXPOSURE IN CBA/CAJ MICE\textsuperscript{1,2,3}

Over the past two decades several animal models for tinnitus have been successfully developed (see review by Turner 2007). The vast majority of these models have employed basic mechanisms of conditioning (Bauer et al. 1999; Heffner & Harrington 2002; Guitton et al. 2003; Ruttiger et al. 2003; Lobarinas et al. 2004; Heffner & Koay 2005). They typically require complex behavioral manipulations and weeks to months of animal training. Although these models have contributed significantly to the field of tinnitus research they are time consuming and can be difficult for researchers lacking experience in behavioral techniques. Recently a novel method was proposed (Turner et al. 2006). It does not require complex behavioral training. Testing can be done quickly in a single one-hour session. This method utilizes reduction in the acoustic startle reflex by a preceding silent gap in an otherwise constant acoustic background.

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\begin{enumerate}
\item Longenecker RJ, Galazyuk AV. 2011. Development of tinnitus in CBA/CaJ mice following sound exposure. JARO 12:647–658
\item Longenecker RJ, Chonko KT, Maricich SM, Galazyuk AV. 2014. Age effects on tinnitus and hearing loss in CBA/CaJ mice following sound exposure. SpringerPlus 3:542
\end{enumerate}
Animals with behavioral evidence of tinnitus cannot detect silence and therefore their reduction of the startle reflex is significantly less than in normal animals. Animal models are critical for efforts aimed toward engineering new therapies to control tinnitus. Mice have an advantage over other tinnitus animal models because they are most easily genetically modified. One mouse strain, the CBA/CaJ, is particularly attractive for tinnitus research. These mice have robust hearing capabilities in comparison to other strains and retain normal hearing through most of their life (Davis et al. 2001; Wu & Marcus 2003). The age-related hearing loss in CBA/CaJ mice is comparable to human hearing models when taking into consideration the differences in life span (Gao et al. 2004). They are also very resilient to noise-induced trauma, which would prevent extreme hearing loss (Davis et al. 2001; Yoshida et al. 2000). Additionally, CBA/CaJ mice have low variability in their responses to noise trauma (Hirose & Liberman 2003), ensuring reliable results during behavioral testing. Perhaps the most important reason for using mice as a tinnitus model is the ability to genetically modify mouse models that show behavioral evidence of tinnitus. The characteristics mentioned above support the rationale to develop the mouse tinnitus model for tinnitus research.

The majority of tinnitus studies on animals have been completed between 28 days and 84 days after sound exposure (Middleton et al. 2011; Chen et al. 2013; Koehler & Shore 2013). While many of the basic elements of tinnitus have been elucidated in these studies, they don’t correlate well to the long term development and stabilization of tinnitus in human patients. Here, we investigate tinnitus in mice up to 360 days post exposure. This model approximates the stereotypical human condition by mimicking an exposure early in life with consequential development of tinnitus at an older age.
Although the method utilizing gap-induced inhibition of the acoustic startle reflex has been successfully used to assess tinnitus in various animals (see review Galazyuk and Hébert 2015), many of the finer details of this methodology, however, have not been refined, but are critical for tinnitus assessment. Thus, this study improved this method by optimizing stimulus parameters and identifying optimal strategies for data analysis.

Methods

Subjects

Twenty-two male CBA/CBJ mice were used. Mice were obtained from Jackson Laboratories and were approximately 12 weeks old with a mean weight of 27.5 g at the beginning of testing. Mice were housed in pairs within a colony room with a 12-h light–dark cycle (8A.M. to 8P.M.) at 25°C. Procedures used in this study were approved by the Institutional Animal Care and Use Committee at the Northeast Ohio Medical University.

Acoustic trauma

Mice were anesthetized with an intraperitoneal injection of a ketamine/xylazine mixture (100/10 mg/kg). An additional injection (50% of the initial dose) was given intramuscularly 30 min after the initial injection. Mice were exposed to a narrow-band noise centered at 16 kHz (4–22 kHz) unilaterally for 1 h. This noise was generated using a waveform generator (Wavetek model 395), amplified (Sherwood RX-4109) to 116 dB SPL, and played through a speaker (Fostex FT17H). The outputs of the loudspeaker were calibrated with a 0.25-in. microphone (Brüel and Kjaer 4135) and found to be ±4 dB between 10 and 60 kHz. A small (2 mm O.D.) plastic tube was used to deliver sound from the speaker to the animal’s right ear. The left external ear canal was obstructed with a cotton plug, a manipulation which typically reduces sound levels by at least 30 dB SPL to a level that does not induce tinnitus (Turner et al. 2006).
**Gap induced inhibition of the acoustic startle reflex:**

The ability of mice to detect a gap of silence preceding the startle stimulus was determined using commercial hardware/software equipment from Kinder Scientific, Inc. Mice were placed in a plastic restrainer situated on a plate with a pressure sensor. Any animal motion was detected by the sensor which measured its amplitude and stored data on the computer hard drive. Kinder Scientific software was used to generate a sequence of stimulus trials including a startle stimulus presented alone (STARTLE) and a startle stimulus paired with a gap (GAP+STARTLE) embedded into continuous background noise; the gap had a 20 ms duration and 1 ms rise/fall time (Fig. 1.1). Background for all these trials was presented as a narrow band (1/3 octave) noise centered at six different frequencies (10, 12.5, 16, 20, 25, and 31.5 kHz). This background noise level was constant (75 dB SPL) throughout the session. The startle stimulus was 20 ms duration white noise presented at 110 dB SPL, with a 1 ms rise/fall time. The gap was 20 ms duration and presented 100 ms before (onset to onset) the startle stimulus (Fig. 1.1A).

Startle amplitude was measured as the peak-to-peak value (expressed in newtons (N)) during the 30-ms time window following startle stimulus onset.

For the gap detection test, parameters of our stimulus paradigm were set to levels which are typical for assuring a robust ∼30% reduction in startle response amplitude caused by a preceding gap of silence in an otherwise continuous background sound (Ison et al. 2002; Turner et al. 2006; Kraus et al. 2010).

The testing session started with an acclimation period lasting 3 min. Immediately afterwards, animals received 10 STARTLE-only trials in order to habituate their startle responses to a steady state level. For each of six background frequencies, we presented five STARTLE only trials and five GAP+STARTLE trials. The STARTLE and GAP+ STARTLE trials were pseudo-randomized. The inter-trial intervals were also pseudo-randomized between 7 and 15 s.
After we completed testing all six background frequencies, the entire session was repeated one more time. Thus, during this testing for each background frequency, the total of 10 GAP+STARTLE trials and 10 STARTLE only trials were presented.

All animals from the experimental group were tested before acoustic trauma, and then at several times points afterward: 1, 3–5, 7 days, weekly for 2 months, and at 84 days, 180 days, and 360 days post-exposure. The control group was tested at the same time points.

**Masking effects of background on startle**

When developing a stimulus session for prepulse inhibition or gap detection tests, it is important to be aware of the masking effects of the background on the startle stimulus (Carlson & Willott 2001). If not addressed appropriately, this suppression may lead to inappropriate conclusions concerning tinnitus identification. It has been shown that a continuous background suppresses an animal's response to startle stimuli. This suppression largely depends on both the background frequency and intensity (Gerrard & Ison 1990). As demonstrated in Fig. 2.1A an individual mouse has shown a “U” shape pattern of frequency dependent suppression of a startle response. We found this suppression in all tested mice without exception. The suppression also increases with increasing background intensity (Fig. 2.1B). We found that the shape of this curve approximates the audiogram of the CBA/CaJ mouse (Radziwon et al. 2009) and several other rodents (Heffner et al. 2001). Such similarity strongly suggests that at the frequencies where mice have the minimal response threshold (12.5–16 kHz) the startle suppression was maximal. To the best of our knowledge, the similarity between these two curves has not been reported before. We hypothesize that this is a universal phenomenon among mammals. This hypothesis requires further validation. Since the startle suppression by the background is very similar for both the trials with or without a gap at a given background frequency, the ratio between the two
Figure 2.1 Startle response amplitude in CBA/CaJ mice depends on both center frequency and intensity of continuous narrow band noise. A) Startle response amplitude as a function of center frequency of continuous narrow band noise presented at 70 dB SPL and recorded three times from the same mouse within a one week period. B) Startle response amplitude as a function of narrow band noise frequency recorded from the same mouse at two noise intensities (50 and 70 dB SPL).
measurements should be frequency independent. However, if this suppression is too strong the startle amplitude will be minimally affected by typical gap suppression (Fig. 2.2, outlined data point). If so, the gap/no gap ratio for this frequency would have a value close to 1, which could lead to an inappropriate conclusion that no gap detection is present and more importantly that this represents the presence of tinnitus at this frequency. To avoid this issue, during data analysis of a gap detection test, it is vital to compare the level of background animal movement when no sound is presented with the level of startle response for each individual frequency when no gap was introduced. In our experiments we determine the mean and standard deviation values for all trials when no sound was introduced. Then, if the mean value of startle (no gap) trials at a given frequency is equal to or less than the mean plus the standard deviation of background movement, the ratio should not be calculated for this frequency (Fig. 2.2, outlined by a circle). If this problem occurs, the startle stimulus intensity should be increased and the testing session should be repeated.

**Choosing an appropriate startle intensity**

As was mentioned previously, ASR studies in the past have employed a range of startle amplitudes. Each species and strain of animals has its own startle stimulus–response function. Such response function curves have been shown for many strains of mice (Bullock et al. 1997). These curves also were obtained in our laboratory from CBA/CaJ mice (Fig. 2.3A). The startle stimuli were presented at the levels ranged from 50 to 130 dB SPL in a random fashion. The interstimulus interval was randomized within a range between 7 and 15 s. Each startle intensity was repeated six times. In average our mice exhibited startle threshold at the level of about 80 dB SPL. At higher startle intensities, the startle stimulus–response function raised steeply until it
Figure 2.2 Non-startle related movements can interact with strongly inhibited startles. If frequency/intensity-dependent suppression of the startle response is not considered, it may lead to an incorrect conclusion regarding tinnitus assessment with the gap detection paradigm. Narrow band noise presented at 70 dB SPL suppresses startle response amplitude at 12.5 kHz center frequency (outlined data point) to a level which is not significantly different from animal movements without any stimulus presented. Horizontal dashed line indicates mean value of animal's movement without any stimulus with the standard deviation represented by a shaded bar.
Figure 2.3 Startle stimulus intensity is critical for gap detection performance. A) Startle stimulus/response function recorded from 7 CBA/CaJ mice. The thick black line represents the average function over 7 mice. The dashed horizontal lines indicate the level of startle response saturation and the level 25% below saturation. B, C) Gap detection performance in the same mice measured at 115 and 105 dB SPL startle stimulus intensities, respectively. Dashed horizontal lines indicate the ratio of 1 that represents poor or no gap detection performance, whereas the ratios less than 1 a better gap detection.
was saturated at the level of about 110 dB SPL. When the mice were tested with the gap detection paradigm at different startle intensities (105 vs. 115 dB SPL) they showed very different gap detection performance. This performance was very poor when startle stimuli were presented at the level of saturation, at 115 dB SPL (Fig. 3B). However, the gap detection performance was greatly improved when startle stimuli were at about 25%–50% below the level of saturation, for instance 105 dB SPL (Fig. 2.3C). This data strongly suggest that avoiding saturated startle response levels is critical when your goal is to alter the startle response with a preceded gap. Thus, before tinnitus assessment using the gap detection paradigm, the startle stimulus/response function should be obtained. Then, using this function the startle stimuli should be set at the levels falling within the rising phase.

**Gap detection test with unilateral conductive hearing loss**

Rationale for this study was to determine whether the sound exposed ear, which has potential hearing loss caused by exposure, contributes to our tinnitus assessment results. A separate group of 8 mice were sound exposed and 84 days post exposure they were assessed for the presence of tinnitus as described above. Based on these test results all mice were separated into two groups: tinnitus positive and tinnitus negative. All animals were then retested with the addition of an ear plug in the exposed ear installed under ketamine/xylazine anesthesia. Before testing, animals recovered from anesthesia in individual cages for two hours. This kind of a plug is known to attenuate sounds by 20–30 dB (Turner et al. 2006), and therefore significantly reduce the contribution of this ear to our test results. Results of these two assessments were compared for every animal from both groups.
Data Analysis

Startle responses showed some variability during the recording sessions: some animals sometimes exhibited an extremely strong startle response or did not startle at all. Therefore, the data in each session were statistically analyzed to remove outliers (Grubbs’ test for outliers). For each background frequency, a total of 10 GAP + STARTLE trials and 10 STARTLE only trials were presented. To calculate the GAP + STARTLE/STARTLE ratio we calculated mean for all startle values. They changed little within one session. Then we divided each of 10 GAP + STARTLE values for a given background frequency by the startle mean value. These 10 ratio values at a given frequency were used to calculate mean and SD values. A one-way analysis of variance (ANOVA) was used to test for differences within a subject. The criterion for the presence of behavioral evidence of tinnitus was a significant reduction in gap detection performance at one or several background frequencies compared to the pre-exposure values.

During our data analysis, we found empirically that the 95% confidence interval is an optimal criterion to demonstrate changes in gap or prepulse detection performance induced by sound exposure.

Results

GAP detection performance in the control mice

We found that all control mice without exception showed a robust suppression of their startle response when a GAP was introduced. The (G+S)/S ratio values computed as an averaged ratio over all six background frequencies varied among mice from approximately 0.3–0.75 (mean±SD=0.617±0.14) yet did not change significantly within each mouse during 3 months of testing. Such ratios also varied from one background frequency to another in individual mice. For a representative mouse in Figure 2.4, this ratio ranged from 0.4 to 0.86 (mean±SD=0.66±0.1)
for six different frequencies. As shown in Figure 2.4, the overall fluctuations of (G+S)/S ratios were not significantly different (F(4,50)=0.14, p=0.966) when measured at different time points, including up to one year after exposure (ratio 0.62 to 0.81 (mean ± SD = 0.68 ± 0.06)).

Consistent with other studies, our control group of mice exhibit robust gap-induced inhibition of the startle response (Turner et al. 2006; Yang et al. 2007; Wang et al. 2009; Kraus et al. 2010), which did not change significantly during 360 days of monitoring.

GAP detection performance in the sound exposed mice

In the experimental mice before the sound exposure, the gap detection performance ((G+S)/S mean±SD= 0.608±0.21) was not significantly different from the control group of mice (F(1,200)=0.76, p=0.384). However, after unilateral sound exposure, the values of (G+S)/S ratios for all exposed mice became much higher (mean±SD=0.94±0.12), indicating that the gap detection was significantly reduced (F(1,200)= 37.19, p G0.0001). A representative mouse in Figure 2.5A before sound exposure showed good gap detection performance with (G+S)/S ratios ranging from 0.25 at 25 kHz to 0.61 at 10 kHz (mean±SD= 0.45±0.12; Fig. 2.5A, top panel).

However, on the first day after exposure, this gap detection performance was significantly decreased at all background frequencies. The averaged (G+S)/S ratio across all six frequencies was increased (mean±SD=0.86±0.09). Until day 21 following sound exposure, this mouse still showed low gap detection performance at 5/6 different frequencies. Beginning at day 28 after exposure, gap detection started to return to the control level at more and more background frequencies. From days 42 to 56, the gap detection deficits were evident only at one or two background frequencies between 16 and 20 kHz. From days 56 to 84, these deficits were shifted to higher frequencies (Fig. 2.5A, bottom panel). At this time period after exposure, the gap
Figure 2.4 Fluctuations of gap-induced suppression of the acoustic startle response over a 360 day period in a control mouse. Open bars represent control means ± SD of \((G + S)/S\) ratios measured at 6 different background frequencies (10, 12.5, 16, 20, 25, and 31.5 kHz). The grey bars represent ratios measured at the same frequencies but at different time points after pre-exposure measurements.
Figure 2.5 Time-dependent changes of gap-induced suppression of the acoustic startle response in mice for a 360 day duration after sound exposure. (A) Changes in gap detection performance in a single sound exposed mouse. Open bars represent mean ± SD of (G + S)/S ratios measured before sound exposure. Grey and black bars represent the ratios which were (black bars) or were not (grey bars) significantly different from the control. (B) The histogram depicts only significant increases in the ratios as a function of background frequency (e.g. indicated by black bars in A) obtained from a sample of 12 sound-exposed mice. 2 out of 12 mice were lost after the 84 day time point so only 10 mice are represented at 180 and 360 day time points. (C) Changes in gap detection performance in a mouse which performance was recovered to the pre exposed level between 180 and 360 days after exposure. (D) Changes in gap detection performance in a mouse which did not show gap detection deficits at 84 days post exposure and continued show no deficits up to 360 days.
detection performance was significantly lower at 20 and 25 kHz. Similarly, the vast majority of the exposed mice (86%, 12/14) developed behavioral signs of tinnitus. Figure 2.5B shows a distribution of significant increases in the ratios as a function of background frequency (e.g., indicated by black bars in Fig. 2.5A) obtained from a population of 12 sound exposed mice. By day 84 after exposure, gap detection deficits were predominantly found between 20 and 31.5 kHz. The range of frequencies with evidence of tinnitus in addition to 20 kHz was similar to that in the mice shown in Figure 2.5A. Four of 12 mice showed gap detection deficits at one additional (higher or lower) frequency, 5/12 mice showed deficits at two additional frequencies, and the three remaining mice exhibited such deficits at three additional frequencies. Our data suggest that the development of behavioral signs of tinnitus after high-level noise exposure in mice is a complex, long lasting, and dynamic process.

When the same mice were continually studied until 360 days post exposure, the story did not change much. We found that the vast majority of mice exhibiting behavioral signs of tinnitus at the 84 day mark (83%, 10/12) continued to demonstrate these signs at 180 and 360 days following sound exposure. A representative mouse in Figure 2.5 presented evidence of tinnitus at the 84 day mark (84 days) between 20 and 25 kHz. At the 180 day time point the range of deficits had shifted to 16 kHz. By 360 days, behavioral signs of tinnitus were evident in this mouse at 16 and 25 kHz. Figure 2.5B demonstrates the distribution of ratios that had significantly increased as a function of background frequency (e.g., indicated by black bars in Fig. 2.5A), which were obtained from a sample of 10 out of 12 sound-exposed mice that showed signs of tinnitus up to 360 days of monitoring. The range of background frequencies where behavioral signs of tinnitus were present at 180 days was similar to those at 84 days. By 360 days following exposure, behavioral signs of tinnitus were predominantly evident within a
narrow frequency range from 16 to 25 kHz. Interestingly, two mice demonstrated an alternative pattern of gap detection deficits as they aged. Although they demonstrated signs of tinnitus at 84 day and 180 day time points, no behavioral deficits representing tinnitus were present when they were tested 360 days after sound exposure (Fig. 2.5C). These mice showed a robust gap detection performance at all frequencies tested which was not significantly different from the performance before sound exposure. It is important to note that 2 mice out of 14 did not develop behavioral signs of tinnitus within an 84 day period after sound exposure, and had no signs of tinnitus at 180 or 360 days after exposure (Fig. 2.5D).

**Potential effects of hearing loss caused by sound exposure on tinnitus assessment**

Sound exposure for tinnitus induction, as well as using gap-induced inhibition of the acoustic startle reflex for tinnitus assessment is becoming a popular technique among scientists using an animal model for tinnitus research. However, potential hearing loss during sound exposure raises some doubts about the gap-induced inhibition of the acoustic startle reflex as a reliable method for tinnitus assessment. To address this issue we conducted a series of experiments on four sound exposed mice with and without behavioral evidence of tinnitus. In agreement with other labs tinnitus animal model, our animals were exposed unilaterally. During exposure one ear was sound protected with a cotton plug, which is known to offer some protection during sound exposure (Turner et al. 2006). Such attenuation provides assurance that the level of sound exposure used for tinnitus induction (116 dB SPL during one hour) was unlikely to cause severe hearing loss in the protected ear. The goal of this study was to determine the feasibility that hearing loss caused by sound exposure in the exposed ear was responsible for the same gap detection deficits that are believed to be an indicator of tinnitus. Our assumption was that the tinnitus percept has a central rather than peripheral origin. If so, then the gap
detection deficits associated with tinnitus should be present in either of the following conditions: When both ears are tested, or when only the protected ear is tested. To test the unexposed ear, the exposed ear was temporarily plugged during behavioral testing. Then, the gap detection performances in these two experimental conditions were analyzed and compared. Each of the four mice tested demonstrated similar gap detection performance between testing conditions (Fig. 2.6). A representative mouse in Figure 2.6A exhibited no significant changes in its gap detection performance after sound exposure when it was tested with two ears unobstructed. When the exposed ear was subsequently plugged, this mouse did not show detection deficits. The remaining three mice showed behavioral evidence of tinnitus. Likewise, their gap detection performance at the two testing conditions was similar. A representative mouse in Figure 2.6B showed robust gap detection deficits at 12.5 and 16 kHz when its two ears were unobstructed. These deficits remained evident at 16 kHz when the testing was performed with the exposed ear plugged.

Discussion

Our results demonstrate that sound exposure triggers permanent changes in the mouse auditory system. The data presented provide evidence that mice can reliably be used in long-term GPIAS studies. ABR thresholds and pre-pulse inhibition ratios recovered to pre-exposure values after 84 days. In contrast, gap detection deficits and ASR magnitudes remained altered when tested through 360 days following acoustic exposure. In addition, an ear-obstruction experiment demonstrates that the behavioral evidence of tinnitus observed in sound exposed animals cannot be easily explained by hearing loss caused by the exposure.
Figure 2.6 Gap detection deficits in sound exposed mice were similar between the following conditions: When both ears are tested, or when only the protected ear was tested. (A) Gap detection performance in a sound exposed mouse showing no gap detection deficits after exposure either when its two ears were unobstructed (top histogram) or when the exposed ear was plugged (bottom histogram). (B) A mouse showing similar frequency-dependent gap detection deficits when it was tested with the same paradigm as the mouse in A. Open bars represent mean ± SD of (G + S)/S ratios measured before sound exposure. Black and grey bars were and were not, respectively, significantly different from pre-sound exposure responses. *p < 0.05, **p < 0.001.
Behavioral evidence of tinnitus remains in animals during 360 days of testing

Our study demonstrated that chronic tinnitus develops within 84 days following exposure to a loud narrow-band noise (Longenecker & Galazyuk 2011). This process is highly dynamic, with gap detection deficits evident over the entire frequency range used for testing (10 – 31.5 kHz) several days after exposure. Following a period of plasticity, the deficits were concentrated to a relatively narrow range at higher frequencies. Results of the present study show that at 180 and 360 days after exposure, gap detection deficits were shifted closer to the noise-exposure center frequency leading to deficits in a narrower range. Similar outcomes have been recently reported for a mixed C57BL/6 X 129 back-crossed mice strain (Turner et al. 2012). It was shown that chronic tinnitus started to emerge around seven weeks after exposure and remained present until the seven month period. This suggests that tinnitus development might be universal among mice, and if lifespan comparisons are made to humans, an important model of chronic tinnitus.

One major inconsistency between these studies was that Turner et al. found diminished prepulse detection beginning at 4 months post exposure in both control and trauma mice. As the authors suggested this reduction can be explained by age related hearing loss, which has been well documented for C57BL/6 mice (Prosen et al. 2003), one of two stains that they crossed in their study. In the present study CBA/CaJ mice showed no sign of presbycusis. PPI values in these mice at 360 days post exposure were not significantly different from those recorded pre exposure. This was expected because CBA/CaJ mice preserve auditory functionality for more than one year of age (Idrizbegovic et al. 2001; Willott et al. 1994). Therefore, this mouse strain provides an opportunity to study age related effects on tinnitus development. For these reasons, CBA/CaJ mice could provide an excellent model for pharmaceutical agents aimed at treating chronic tinnitus.
Our ear plug results argue against hearing loss as the major contributor to frequency-specific gap detection deficits in sound exposed animals. Unilaterally exposed mice showed similar gap detection deficits when either both ears, or more importantly, just the unexposed ear, were tested. Since unexposed ears did not display any sign of ABR threshold increase after exposure, cochlear damage cannot be a reasonable explanation of the gap detection deficits. Although not completely definitive, this new finding further validates the gap detection methodology for tinnitus assessment. Alternatively, GPIAS might detect plastic changes along the auditory neuraxis caused by sound exposure, while not being capable of detecting tinnitus (Kaltenbach et al. 2005; Ma et al. 2006; Noreña & Eggermont 2003).
CHAPTER III

ASSESSMENT OF THE AUDITORY SYSTEM AFTER SOUND EXPOSURE

Quick and accurate assessment of auditory thresholds is a prerequisite for experimental manipulations in the field of auditory research. To date, a variety of protocols have been used to determine audiometric thresholds, yet each has significant limitations which should be taken into account when interpreting data.

Perhaps the most ubiquitous test used to assess hearing performance is the auditory brainstem response (ABR). Rapid assessment of auditory brainstem circuitry makes ABR a good candidate for detecting gross changes in the auditory system. It is known that following an auditory insult ABRs reliably identify elevated thresholds. This temporary threshold shift is thought to result from swelling of cochlear nerve terminals which is present for days after exposure. When measured with ABRs, thresholds return to baseline soon after noise exposure (Robertson 1983). However, recent work has clearly demonstrated that this temporary threshold shift does not account for the trauma-induced damage to ribbon synapses on the inner hair cells (Kujawa & Liberman 2009). Thus, ABR is capable of detecting temporary threshold shifts immediately after noise-exposure but is unable to reliably assess permanent threshold shifts caused by neural degeneration (Lin et al. 2011).

Another common approach to assess animal hearing sensitivity is the behavioral audiogram. Conditioned suppression/avoidance paradigms (Heffner & Masterson 1980) and
go/no-go procedures (Prosen et al. 2000, 2003; Klink et al. 2006; Radziwon et al. 2009) produce sensitive and reliable measures of hearing performance in mice. Known as the gold standard, the behavioral audiogram has been constructed within an entire audible frequency range for many species (see review Heffner & Heffner 2007). However, this methodology has demonstrated high variability between labs and requires experience and training in behavioral science and months to collect data for a single audiogram. More importantly this approach does not allow researchers to assess relatively fast changes in hearing performance which are typical following various hearing insults. Therefore very few studies have used behavioral audiograms to assess hearing deficits after acoustic trauma (Moody et al. 1978; Heffner et al. 2008).

As methodological alternative to such lengthy behavioral training is the acoustic startle reflex (ASR), a reflexive movement in response to an auditory stimulus (Landis & Hunt 1939). The ASR is found in many species and is believed to have evolved as a rapid defense mechanism (Koch 1999). ASR studies in the past have employed a range of startle amplitudes. Each species and strain of animals has its own startle stimulus–response function. Such response function curves have been shown for many strains of mice (Bullock et al. 1997). In our studies with CBA/CaJ mice, we found that these input/output functions were useful for several purposes. First, this assessment is able to assess the relative health of the cochlear output to the lower auditory brainstem. Each animal’s input/output function can be quickly assessed and outliers can be removed from a study. Second, after sound exposure, the input output function will become shallower, representing damage to the auditory system. Finally, though the ASR is fundamentally a reflex movement, both the amplitude and probability of a resulting startle movement can be modulated by a number of external stimuli, and changes in internal state (Hoffman & Wible 1970; Davis et al. 1982; Acocella & Blumenthal 1990). Thus the input/output function can be
used to select appropriate intensity levels for accessory modulatory stimuli which can be used in more advanced ASR studies (Fig. 2.3).

Prepulse inhibition, a decrease of ASR magnitude when a weak preceding sound (prepulse) is presented before the startle, has been used in behavioral paradigms to investigate a wide range of disorders in a range of disciplines including schizophrenia (Swerdlow & Geyer 1993; Grillon et al. 1992; Parwani et al. 2000), alcoholism (Krystal et al. 1997; Stanley-Cary et al. 2002) and psychopharmacology (Phillips et al. 2000; Davis & Menkes 1982). The ASR is also used to develop behavioral tests for neurological disorders such as posttraumatic stress disorder (Weston 2014). It has also been employed to assess behavioral response thresholds to auditory stimuli of different frequencies (Fechter et al. 1988). A prepulse varied in intensity and frequency, will differentially suppress the startle response. This method has successfully identified cochlear damage due to ototoxic drugs (Young & Fechter 1983) and temporary threshold shifts due to pure tone acoustic overexposure (Walter et al. 2011). Although this method has potential advantages over the currently used behavioral paradigms, several important questions need to be explored before this method can be widely applied as a reliable tool for hearing assessment. First of all it is still unknown whether this method is sensitive enough to assess hearing in individual animals, which would be much more beneficial than group averages (Fechter et al. 1988, Walter et al. 2011). Second, the extent of PPI threshold variability from day to day is also largely unknown. Third, little is known whether PPI audiometry can be sensitive to detect permanent auditory threshold shifts caused by such common auditory insults as noise induced hearing loss or aging. These important questions constituted the rationale for the present study.
Methods

Subjects

Twenty-two male CBA/CBJ mice were used. Mice were obtained from Jackson Laboratories and were approximately 12 weeks old with a mean weight of 27.5 g at the beginning of testing. Mice were housed in pairs within a colony room with a 12-h light–dark cycle (8A.M. to 8P.M.) at 25°C. Procedures used in this study were approved by the Institutional Animal Care and Use Committee at the Northeast Ohio Medical University.

Acoustic trauma

Mice were anesthetized with an intramuscular injection of a ketamine/xylazine mixture (100/10 mg/kg). An additional injection (50% of the initial dose) was given 30 min after the initial injection. Mice were unilaterally exposed to a one octave narrow-band noise centered at 12.5 kHz (~8–17 kHz). This noise was generated using a waveform generator (Tektronix AFG 3021B), amplified (QSC RMX 2450) to 116 dB SPL, and played through a speaker (Fostex T925A Horn Tweeter). The outputs of the loudspeaker were calibrated with a 0.25-in. microphone (Brüel and Kjaer 4135) attached to a measuring amplifier (Brüel and Kjaer 2525) and found to be ±4 dB between 4 and 60 kHz. During exposure the speaker was located ~5 cm from the animal’s right ear. During exposure the left external ear canal was obstructed with a cotton plug and a Kwik-Sil silicone elastomer plug (World Precision Instruments), a manipulation which typically attenuates sound by at least 30 dB (Turner et al. 2006, Ropp et al. 2014).

Acoustic Startle Reflex Input/Output functions

All behavioral experiments were conducted via methods previously optimized in our lab (Longenecker & Galazyuk 2012). The startle stimuli were presented at the levels ranged from 50
to 120 dB SPL in a pseudo-random fashion. The inter stimulus interval was randomized within a range between 7 and 15 s. Each startle intensity was repeated twenty times. Thresholds and 75% of the max amplitudes were calculated offline for additional studies.

**Auditory Brainstem Response Testing**

Mice were anesthetized with ketamine/xylazine as during the acoustic trauma. Sterile, stainless-steel recording electrodes (RA4LI Low Impedance Headstage) were placed subdermally, behind each pinna and the reference electrode was placed along the vertex. Tone bursts at 4, 12.5, 16, 20, 25, and 31.5 kHz were presented at increasing sound intensities ranging from 10 to 80 dB SPL in 10 dB steps. Tones were 5 ms duration, 0.5 ms rise/fall time and delivered at the rate of 50/s. ABRs were averaged over 300 repetitions. These waveforms were amplified (TDT RA4PA Medusa Preamp), digitized (TDT RZ6 Multi-I/O Processor), and analyzed offline using custom made software (TDT OpenEx). Thresholds, the smallest sound amplitude that evoked a visible ABR, were determined by visually examining the ABR waveforms in response to every sound frequency presented at different sound levels.

**Reflex modification audiometry: Prepulse detection testing**

All behavioral experiments were conducted via methods previously optimized in our lab (Longenecker & Galazyuk 2012; Longenecker et al. 2014). Testing sessions contained two types of stimuli. First, a startle stimulus (wide-band noise, 100dB SPL, 20ms in duration, 1ms rise/fall) was presented alone; this is referred to in the text as startle only (Fig. 3.1A). The second stimulus type consisted of a congruent startle stimulus preceded by a prepulse (Fig. 3.1A). Prepulse stimuli were 20 ms pure tones with a 1 ms rise/fall time presented at six different frequencies (4, 12.5, 16, 20, 25, and 31.5 kHz) 100 ms before the startle stimulus. These prepulses were played pseudo-randomly in the range of intensities from 10 to 80 dB SPL for a given sound frequency.
Each frequency/intensity combination was presented 39 times. Startle stimuli alone were pseudo-randomly mixed throughout each testing session. Inter-trial intervals were also randomized between 15 and 25 seconds. A complete testing (1872 prepulse trials, and 468 startle alone trials) required 15 hours with a 1 hour break for the animal to rest between hour 7 and 8. Startle-only responses were closely monitored throughout this process to ensure that startle magnitudes were not significantly affected by habituation.

**Reflex modification audiometry: Startle waveform identification and measure**

All waveforms collected during testing sessions were analyzed offline using a recently developed automatic method of startle waveform identification via a template matching paradigm (Grimsley et al. 2015). In this study we used high-speed video recordings to visualize animal startles in order to identify a stereotyped waveform associated with a startle. This allowed us to develop custom software which automatically separates data into either startles or non-startle-related movements. Based on this separation, we have only included trials that resulted in startle responses in our data analysis. We also used a mathematical approach to normalize startle response magnitudes of individual animals to their body mass (Grimsley et al. 2015). Briefly, we use simple mathematical extrapolations to convert startle data (force) into center of mass displacement (height). Startle magnitudes measured in force (Newton (N)) were converted to center of mass displacement (COMD; mm). Force was divided by body mass to yield instantaneous acceleration of the COM. The acceleration vector was then integrated to obtain instantaneous velocity and then integrated again to obtain the instantaneous position of the COM. This mathematical conversion has two benefits: first, the procedure normalizes for mass, allowing legitimate comparisons between animals of different mass and second, it converts the
forces sensed by the piezoelectric startle plate into a more readily understandable unit of height: the center of mass displacement.

**Reflex modification audiometry: Prepulse detection threshold identification**

In each session, the magnitudes of the startles presented alone were compared to the magnitudes of startles preceded by prepulses with various frequency and intensity (Fig. 3.1A). A significant reduction in the magnitude of the startle response by the prepulse compared to the startle presented alone was defined as the prepulse detection threshold (Fig. 3.1B). Identifying this threshold involved several steps. Startle magnitude is known to be quite variable in mice, thus we tested our data for normality using an Anderson-Darling test. The startle magnitude typically was positively skewed, so a square-root transformation was applied, which reduced the skew. For each animal, the transformed startle only data was then bootstrapped to determine 95% confidence intervals for the startle only response magnitudes (Fig. 3.1C). Then for each frequency, a detection function was calculated by fitting a cubic spline from the median transformed startle only magnitude through the median transformed magnitudes for prepulses presented at various intensities. Detection threshold was defined as the sound level at which the fitted detection function crossed the lower confidence interval (Fig. 3.1B).

**Statistical analyses**

Both ABR threshold data and PPI thresholds were evaluated with a repeated measures design because the same animals were used in each condition. Frequency (6) and condition (3) were used as independent variables in the repeated measures analysis of variance (ANOVA). Least significant difference (LSD) post-hoc tests were used to further investigate differences
Figure 3.1 Determining detection thresholds for PPI audiometry. A. Example of stimuli presented, as described in Figure 1. B. Detection function. For each frequency, a cubic spline was fitted to medians of the square-root-transformed response magnitudes for SO and each intensity prepulse. Detection threshold was defined as the sound level where this function intersected the 95% lower confidence interval of a bootstrapped Startle Only response distribution (C). C. Calculations of Startle Only 95% confidence intervals. A Startle Only response magnitude distribution was created by bootstrapping square-root-transformed response magnitudes. The lower 95% confidence interval was used for detection threshold identification (in B).
between specific interactions. A Spearman’s correlation was used to evaluate startle data to identify if habituation was an issue during PPI testing. A paired measures t-test was conducted for the ear plug experiment in a repeated measures design. Values throughout the manuscript are specified as means or medians ± standard error of the mean (SEM). We used an alpha level of .05 for all statistical tests. P values significant at the .05 level are indicated by one asterisk while those significant at the .01 level or lower are indicated by two asterisks.

**Results**

In this study 20 CBA/CaJ mice were divided into three groups. Prepulse detection thresholds were used to construct a curve of each animal’s hearing performance, which we term PPI audiometry. The control (unexposed) group (n=6) was used to test for the consistency of the methodology across different mice and in individual mice across different testing sessions. The other two groups of mice were exposed to a narrowband noise of 116 dB SPL for one hour. The first of these two groups (n=8) was used to determine whether PPI audiometry can detect permanent threshold shifts following noise exposure. The second group of exposed mice (n=6) was aged to assess the effect of combination of noise-induced and age-related hearing loss on PPI audiometry. ABRs and startle input/output functions were also collected in the exposed mice.

**ABR Thresholds Recovery**

ABR thresholds were measured in sound exposed mice (N=14) before, immediately after, and 12 weeks following exposure at six different frequencies (4, 12.5, 16, 20, 25, 31.5 kHz) (Fig.
Figure 3.2 Elevated ABR thresholds recover after 3 months. ABR thresholds from 8 mice exposed to a 116 dB SPL one octave narrow-band-noise centered at 12.5 kHz (grey bar). One day after exposure thresholds, all frequencies were significantly elevated (all frequencies p < 0.001). At 3 months, thresholds were only slightly elevated compared to control levels (p < 0.05 at all frequencies except 16 and 20 kHz, p > 0.05).
3.2). Immediately following sound exposure, all mice showed significant temporary threshold shifts at all frequencies tested. Twelve weeks after exposure, thresholds at most frequencies were recovered and were not statistically different from the values recorded before exposure (Fig. 3.2). However, minute threshold shifts were still evident at 12.5, 25 and 31 kHz.

**Effects of sound exposure on startle magnitude**

The magnitude of the startle response may be greatly decreased after sound exposure (Longenecker & Galazyuk 2011, 2014; Lobarinas et al. 2013). This may confound experimental findings by causing a “floor effect”. To test for this possibility, startle input/output functions were collected before, one day after, and 12 weeks after sound exposure. Since our PPI testing used a 100 dB SPL startle stimulus intensity, we wanted to determine if this startle intensity could evoke a sufficiently strong startle response to ensure that a prepulse could inhibit the response in both control and sound exposed conditions. Although the startle magnitude was significantly lowered after exposure (Fig. 3.3), its magnitude remained more than 2 standard deviations above baseline animal movement (.05 ± .06 mm). Furthermore, our automatic startle waveform identification software provided us with an assurance that only true startle responses were included in our PPI data analysis (Grimsley et al. 2015). This technology is especially effective in separating small startle waveforms from non-stimulus-related movements.

**Consistency of PPI audiometry**

Accuracy and precision are crucial for any methodology that is assessing hearing loss. As such, we tested whether thresholds identified with PPI audiometry were comparable to thresholds determined by behavioral audiograms. Importantly, we also assessed both the variation between mice of the same age as well as the variation over time for individual mice. On
Figure 3.3 Effect of sound exposure on startle response magnitudes. Startle responses across a range of startle intensities were measured before (A; control), 1 day (B), and 3 months after exposure (C). Bold (colored) lines represent mean of all individual mice (thin lines) at each time point. A. The magnitude of the startle response in the control condition to a 100 dB SPL stimulus varied slightly among animals (0.99 ± 0.07 mm). B. One day after exposure the mean startle magnitude under the same conditions was 0.66 ± 0.05 mm. This was significantly smaller than in the control condition (t(91) = 3.77, p < 0.001), suggesting a hearing deficit might be present after exposure. C. 3 months after exposure the mean startle magnitude to a 100 dB stimulus was 0.62 ± 0.05 mm which was not significantly different from one day after exposure (t(91) = 4.48, p < 0.001), suggesting that the startle reflex did not decrease any further.
Figure 3.4 PPI audiometric functions are consistent across time and subjects. PPI audiometric functions from six mice (A-F) over 3 days of testing. Average thresholds were different based on the particular mouse and frequency tested. However, the variation between all conditions was only 5.77 dB (SEM), which suggests low threshold variability.
three consecutive days of testing, thresholds at all frequencies (4, 12.5, 20, 25, 31.5 kHz) were below 40 dB SPL with inter-animal differences of usually less than 10 dB (Fig. 3.4). More importantly, it was possible to obtain audiogram-like thresholds for individual mice. However, this result confirmed a previous prepulse modulation study conducted on humans (Reiter 1981). The variation in PPI audiometry for individual mice over time was minimal. Average threshold variation across frequencies and mice at different days, was only 5.77 dB. Small day to day variations in PPI of individual animals are well described (Willott et al. 2003).

**PPI audiometry can detect threshold shifts caused by noise-induced and age-related hearing loss**

In order to determine whether PPI audiometry is capable of assessing auditory deficits we investigated the effects of sound exposure and aging.

To determine if the PPI audiometry is sensitive to both temporary and permanent threshold shifts we exposed 14 mice to one octave narrowband noise centered at 12.5 kHz and presented at 116 dB SPL for 1 hour. Immediately after exposure PPI thresholds were dramatically increased resulting in a “flatter” cubic spline function (Fig. 3.5B) in comparison to the “steep” monotonic function in the control condition (Fig. 3.5A).

To assess the sensitivity of PPI audiometry to the threshold shifts PPI audiometric functions were collected before, one day, and twelve weeks after exposure. Two representative examples of individualized audiometric functions are shown in Figure 3.6 (Statistical analysis of the population is described in the figure legend). One day after exposure both these mice demonstrated broad temporary threshold shifts. Mouse #3 showed a deficit between 12.5 and 31.5 kHz while Mouse #8 showed a deficit between 12.5 and 25 kHz. These deficits roughly correspond to the non-specific frequency deficits observed with ABRs at the same time points.
Figure 3.5 Audiometric functions change after sound exposure. Representative audiometric functions (tested at 12.5 kHz) from an individual mouse before (A) and after (B) exposure. Threshold was determined by the method shown in Figure 3.1. A. In the control condition the PPI function is both steep and monotonic with the detection threshold located at 9.4 dB SPL. B. In the exposed condition PPI functions tend to become shallower, leading to a higher detection threshold at 34.6 dB SPL.
Figure 3.6 PPI Audiometry can assess noise-induced hearing deficits. Representative PPI audiometric functions for two mice exposed to a 116 dB SPL one octave narrow-band-noise centered at 12.5 kHz (grey bar). Statistics were calculated for the population of 8 mice of the same exposed group. Compared to the control condition, at one day post exposure moderate PPI deficits were observed at 20 kHz ($t(82) = -2.81, p < 0.008$) and 25 kHz ($t(82) = -4.68, p << 0.001$). At 3 months post exposure more substantial deficits were observed at 16 kHz ($t(82) = -5.39, p << 0.001$), 20 kHz ($t(82) = -4.35, p << 0.001$), and 25 kHz ($t(82) = -3.99, p << 0.001$).
Since temporary threshold shifts are typically observed immediately after sound exposure, this is a clear indication that PPI audiometry can assess these changes in the auditory system. Interestingly, both mice exhibited a different, narrower frequency range of deficits at three months following exposure. Similar to previous findings a permanent threshold shift was observed at 0.5 to 1 octave above the frequency range of exposure (Cody & Johnstone 1981) (Fig. 3.6). These data are in direct contrast with ABR data which demonstrated that thresholds at nearly all frequencies returned to the pre-exposed level 12 weeks after exposure (Fig. 3.2).

To investigate whether PPI audiometry is also capable of detecting the aging effect on permanent threshold shifts a second group of exposed mice were aged. These mice were sound exposed at 6 months of age and then aged to 24 months old. At the 20 month epoch, they demonstrated a notched-frequency specific permanent threshold shifts (20 & 25 kHz) (Fig. 3.7). This result was congruent with the deficits seen at the 3 month epoch after sound exposure in the young sound exposed mice (Fig. 3.6). In contrast to the younger group of exposed mice, the aged group demonstrated a minor but significant threshold shifts at 4 and 16 kHz which could likely be attributed to age-related hearing loss, which is known to occur in CBA/CaJ mice at this age (Ohlemiller et al. 2010). At 24 months, however, thresholds were further elevated at most all frequencies. Taken together the results of these experiments suggest that PPI audiometry can assess permanent threshold shifts and could possibly be able to differentiate noise-induced (frequency specific) from age-related (more generalized, from high to low frequencies) hearing loss.
Figure 3.7 PPI Audiometry can assess age-related hearing loss (n=6). In comparison to the control condition, PPI deficits at 20 months of age were observed at most frequencies tested (12.5, p < 0.05, 4, 16, 20, 25 kHz, p << 0.001 (orange asterisks)), with the most pronounced deficits at 20 (t(58) = -11.63, p << 0.001) and 25 kHz (t(58) = -10.51, p << 0.001). Interestingly, PPI thresholds showed further increases at 24 months of age at those frequencies less affected by sound exposure (12.5, 16, 31 kHz, p << 0.001 (blue asterisks)), while 20 and 25 kHz maintained similar thresholds as in the 20 months of age condition (p > 0.05).
Discussion

Prepulse detection for hearing loss

We have shown that prepulse inhibition of the startle reflex can quickly and efficiently monitor auditory thresholds in mice. The acoustic startle input/output functions can suggest that general changes have occurred in the auditory system by an overall decrease in startle amplitude. Furthermore, temporary threshold shifts, resulting from sound exposure, can be detected by PPI audiometry and are similar to those detected by ABRs (Fig. 3.2) and behavioral audiograms (Heffner et al. 2008). However, PPI audiometry was able to detect permanent threshold shifts related to both noise-induced and age-related hearing loss that are not detected by ABRs, making it an attractive method for a quick screening of hearing in laboratory animals.

What does the PPI audiometry measure?

The ASR has been shown to be greatly influenced by preceding stimuli (Hoffman & Searle 1965; Buckland et al. 1969; Willott & Carlson 1995). We have confirmed what was previously found, that prepulses presented at threshold levels can be used to assess an animal’s hearing by modulating the startle motor output (Fig. 3.4) (Fechter et al. 1988). Previous studies have shown that prepulse inhibition of the acoustic startle reflex can be used to detect both ototoxic lesions of the cochlea in rats and guinea pigs (Young & Fechter 1983) as well as temporary threshold shifts due to noise exposure in gerbils (Walter et al. 2012). Furthermore, it has been shown that suprathreshold prepulse modulation of the startle reflex can be diminished in mice with age-related hearing dysfunctions (Willott et al. 1994). Both the noise-induced and age-related deficits observed in the current study corroborate with these previous studies. Our study further revealed that PPI audiometry can assess permanent threshold shifts due to noise-
induced and age-related hearing loss in mice (Fig. 3.6 & 3.7), a popular auditory animal model for auditory research. However, before PPI audiometry can be used routinely for hearing assessment in laboratory animals, it is important to have a clear understanding of what underlying circuitry is being assessed.

The neuronal circuitry underlying the acoustic startle reflex is straightforward and has been studied extensively (Yeomans & Frankland 1996; Koch 1999). The primary startle circuit includes the serial connections between the auditory nerve fibers, cochlear root neurons (in some species), and the nucleus reticularis pontis caudalis (PnC) region (Lee et al. 1996). The motor output is then sent to the interneurons of the spinal cord resulting in a startle. When prepulses are added to this construct, the neural circuitry is far more complex (Swerdlow et al. 2001). The amount of inhibition that a prepulse has on the startle results from complicated neural computations that arise from various brain regions. The primary circuitry for mediating a prepulse’s effect on a startle resides below the level of the colliculi (Davis & Gendelman 1977; Fox 1979). These nuclei send GABAergic projections that terminate at the PnC (Fendt et al. 2001). Although this simple circuitry is directly responsible for generating the direct inhibitory effects on the startle, many higher nuclei can modulate these effects. Prepulse modulatory mechanisms are far more complicated and depend on behaviorally salient factors such as inter-stimulus interval, frequency, stimulus duration, prior auditory experience, and habituation. It has been shown that a large degree of top-down modulation can influence prepulses (Du et al. 2011; Swerdlow et al. 2001; Larrauri & Schmajuk 2006) The amygdala, hippocampus, prefrontal cortex, auditory cortex and many other structures have been flagged as the source of this top-down modulation. To this extent, it seems that PPI audiometry has the potential to assess large
portions of the auditory neuraxis. Therefore it is likely that PPI audiometry could be a useful tool to assess large portions of the auditory system.

**Comparison of PPI audiometry to other methods of hearing assessment**

Many methods exist to assess auditory functionality. Each has a specific purpose accompanied by caveats. The acoustic brainstem response is by far the most popular methodology to assess the auditory system, as it can rapidly assess the connectivity of a subject’s auditory brainstem. These recordings are able to identify gross malfunctions of the auditory system and are also a good tool for assessing the efficacy of sound trauma. More recently, it has been noted that suprathreshold ABR peak amplitudes can provide a better estimate of neuronal efficacy (Kujawa & Liberman 2009). However, ABR thresholds have been shown here (Fig. 3.2), and previously, to be unreliable in detecting long-term neuronal damage caused by sound exposure (Kujawa & Liberman 2009; Singer et al. 2013). In addition, ABRs also express other limitations that are not present with PPI methodologies. First, ABRs in animal models are usually conducted under anesthesia which can alter neuronal activity resulting in unnatural responses (Chambers et al. 2012; Cederholm et al. 2012). Additionally, anesthesia can be lethal for many fragile strains of mice. Secondly, ABRs are only assessing neuronal connectivity of auditory structures below the level of the inferior colliculus (Melcher & Kiang 1996). It is also true that as an animal ages, it becomes increasingly difficult to obtain quality evoked potentials because of increased fat to muscle ratios (Zhou et al. 2006). Lastly, evoked potentials such as ABRs are unable to measure the vast array of neural plastic changes that occur in the auditory system, which are a hallmark of acoustic startle sensorimotor gating experiments (Davis 1984).

The behavioral audiogram collected by classical or operant conditioning methodologies has been the gold standard for threshold detection for decades. These audiograms provide
thresholds that represent the animal’s ability to respond to exceedingly weak stimuli and thus allow insight into an animal’s perception of a sound. Behavioral audiograms for mice have been collected by several different groups over the years (Heffner & Masterson 1980; Prosen et al. 2000, 2003; Klink et al. 2006). However, it is important to note that this method cannot be conducted without some procedural difficulties. First, thresholds differ significantly between labs, sometimes by more than 30 dB SPL (Radziwon et al. 2009). This can be a result of different training programs or criterions. But the greatest disadvantage for behavioral audiograms would be the length of time it takes to obtain data. These studies require experienced behavioral scientists to work with animals for at least an hour per day for weeks to months in order to reach threshold criterion. Following the training process the data collection can also last for months. This presents a major issue for temporally sensitive data collection after experimental manipulations to hearing. This has limited the number of studies that have assessed permanent threshold shifts after auditory insults (Moody et al. 1978). PPI audiometry could be used to obtain permanent threshold shifts, even in experimental designs that test throughout the animal’s lifespan (Fig. 3.7). One important difference between behavioral audiograms and PPI audiometry is the thresholds at various frequencies. In our study, animals in the control condition generally had thresholds that were similar across frequencies. However, this result was also reported in humans (Moody et al. 1978). Although PPI audiometry is undeniably assessing the auditory system in a different way than behavioral audiograms, it could be used as an additional tool to assess auditory functionality.

**Why does monaural exposure lead to binaural PTS?**

In this study we found profound binaural frequency specific PTS’s after unilateral sound exposure. An important question to ask would be: how does a monaural exposure result in
binaural deficits? There are at least three possible explanations. First, it is possible that uncompromised binaural cochlear input is required to obtain normal PPI thresholds observed in control animals. Previously it has been assumed that unilateral exposure would result in damage ipsilateral to the exposure while largely sparing the protected contralateral auditory system. To investigate whether it is possible to obtain normal thresholds monaurally, we conducted PPI audiometry on animals with monaurally obstructed pinna. We found that thresholds in these animals were elevated but were not significantly different from the control (data not shown). It is also true that the behavioral results from a unilateral conductive hearing loss induced by a silicon plug are likely to be different than a unilateral sensorineural hearing deficit. A second option for the deficits could be explained by unintentional binaural exposure. Although this is a possibility, we find it unlikely because even though the exposed ear had ABR threshold shifts between 50 and 70 dBs (Fig. 3.2), the blocked ear did not undergo nearly the same amount of trauma, with thresholds only elevated between 10-30 dB. Third, it is possible that maladaptive plasticity originally triggered in the ipsilateral side following exposure could lead to bilateral neural plasticity throughout the auditory system (Popescu & Polley 2010). It is well established that topographic maps and neuronal activity undergo plastic changes in response to sound exposure (Robertson & Irvine 1989; Eggermont & Komiya 2000; Kaltenbach & Afman 2000; Middleton et al. 2011; Noreña et al. 2010; Wang et al. 2009). This seems like a plausible outcome when taking into account the input/output functions that show a general decrease in startle response (Fig. 3.3). Future studies may identify the mechanism underlying binaural changes in the auditory system triggered by unilateral hearing insult.
Significance of PPI audiometry

Behavioral audiograms are valuable to auditory researchers and clinicians. Interestingly, a methodology similar to the PPI audiograms used here has been successfully used in humans previously (Reiter 1981). In animals, we suggest that PPI can provide a quick and simple solution to assess auditory thresholds. This is especially suited for labs that already employ the ASR for other purposes such as tinnitus, developmental, or psychopharmaceutical studies. It should be noted that PPI tests are performed on multiple animals concurrently with no prerequisite training for the animals or the handlers. In this study we found that we could assess auditory thresholds at 5 different frequencies in just less than two hours, although future work would aim to reduce this length of time even further. This rate of testing would be exceptionally valuable for drug testing which requires large sample sizes. Importantly, this allows for temporally accurate measurements of hearing after induction of trauma. In addition, this methodology is capable of assessing a single mouse’s threshold in a repeated measures design. This allows researchers to treat each as a clinical patient with a unique auditory profile. Future work can compare the results of PPI audiometry with cochlear histology and evoked potential measurements, in addition to traditional behavioral paradigms. PPI audiometry could be used in future studies to track the development of age-related dysfunction over an animal’s entire lifespan.
CHAPTER IV

Effect of sound exposure on neurons of the inferior colliculus in awake mice

It is well established that acoustic over exposure can increase the risk of suffering from hearing loss and/or tinnitus. Tinnitus is most often generated by some degree of sound trauma to the cochlea which leads to reduced input to the central auditory system (see review Lockwood et al. 2002; Galazyuk et al. 2012) Neuroimaging studies have shown that patients with hearing loss and tinnitus have shown abnormal increases in brain activity, which have been implicated as possible neural correlates of either tinnitus or hearing loss (see review Lanting et al. 2009; Roberts et al. 2010). Therefore, it is difficult to parse apart the specific contributions of tinnitus and hearing loss to their specific underlying pathologies.

Animal models of tinnitus have consistently demonstrated both increased spontaneous firing and increased bursting activity at multiple levels of the central auditory system (see review Kaltenbach 2011). Following acoustic over exposure, recordings from anesthetized animals has shown coincidence of hyperactivity and burst firing in the cochlear nucleus (Chang et al. 2002; Finlayson & Kaltenbach 2009; Pilati et al. 2012), inferior colliculus (Ma et al. 2006; Bauer et al. 2008; Coomber et al. 2014), medial geniculate body (Kalappa et al. 2014), and auditory cortex (Noreña & Eggermont 2003). Many of these studies have employed various behavioral techniques to assess tinnitus which was then linked to increases in spontaneous activity and bursting activity. However, it is not well understood if such abnormal brain activity is directly
linked to tinnitus or rather as a general result of sound exposure which may or may not lead to tinnitus. Results from two recent studies on rats and guinea pigs have suggested that increased spontaneous activity is unlikely a neural correlate of tinnitus (Ropp et al. 2014; Coomber et al. 2014).

In the present study, a possible relationship between abnormal brain activity, tinnitus, and hearing loss was tested in individual mice following sound exposure. An improved GPIAS method was used to detect tinnitus whereas PPI audiometry was used to assess for possible hearing loss following sound exposure. Extracellular single unit recordings were used to assess firing activity in inferior colliculus neurons. Both spontaneous and bursting activity in IC neurons were measured and compared with data from control (unexposed) mice. Recordings were conducted in awake animals to exclude a possible effect of anesthesia on the test results. We found that hyperactivity is a typical phenomenon following sound exposure and it is largely independent whether animal exhibits tinnitus. In contrast to previous findings, we found an increase in bursting activity of IC neurons is likely to be linked to severe hearing loss rather than to tinnitus.

Methods

Subjects

Twenty-four CBA/CBJ mice were used. Mice were obtained from Jackson Laboratories and were approximately 12 weeks old with a mean weight of 27.5 g at the beginning of testing. Mice were housed in pairs within a colony room with a 12-h light–dark cycle (8A.M. to 8P.M.) at 25°C. Procedures used in this study were approved by the Institutional Animal Care and Use Committee at the Northeast Ohio Medical University.
Acoustic trauma

Twelve mice were anesthetized with an intraperitoneal injection of a ketamine/xylazine mixture (100/10 mg/kg). An additional injection (50% of the initial dose) was given intramuscularly 30 min after the initial injection. Mice were exposed to a narrow-band noise centered at 16 kHz (4–22 kHz) unilaterally for 1 h. This noise was generated using a waveform generator (Wavetek model 395), amplified (Sherwood RX-4109) to 116 dB SPL, and played through a speaker (Fostex FT17H). The outputs of the loudspeaker were calibrated with a 0.25-in. microphone (Brüel and Kjaer 4135) and found to be ±4 dB between 10 and 60 kHz. The left external ear canal was obstructed with a cotton plug, a manipulation which typically reduces sound levels by at least 30 dB SPL to a level that does not induce tinnitus (Turner et al. 2006).

Behavioral assessment of tinnitus

Twelve mice were sound exposed at 12-16 weeks of age. Mice were tested for tinnitus/hearing loss at 1 day, 1 month, 2 months, and 3 months after exposure. Four control mice and four exposed mice were randomly selected from their respective samples for extracellular recording studies 12 months following exposure.

The ability of mice to detect a gap of silence preceding the startle stimulus was determined using commercial hardware/software equipment from Kinder Scientific, Inc. Mice were placed in a plastic restrainer situated on a plate with a pressure sensor. Any animal motion was detected by the sensor which measured its amplitude and stored data on the computer hard drive. Kinder Scientific software was used to generate a sequence of stimulus trials including a startle stimulus presented alone (STARTLE) and a startle stimulus paired with a gap (GAP+STARTLE) embedded into continuous background noise; the gap had a 20 ms duration.
and 1 ms rise/fall time (Fig. 1.1). Background for all these trials was presented as a narrow band (1/3 octave) noise centered at six different frequencies (10, 12.5, 16, 20, 25, and 31.5 kHz). This background noise level was constant (75 dB SPL) throughout the session. The startle stimulus was 20 ms duration white noise presented at 110 dB SPL, with a 1 ms rise/fall time. The gap was 20 ms duration and presented 100 ms before (onset to onset) the startle stimulus (Fig. 1.1A). Startle amplitude was measured as the peak-to-peak value (expressed in newtons (N)) during the 30-ms time window following startle stimulus onset.

For the gap detection test, parameters of our stimulus paradigm were set to levels which are typical for assuring a robust ~30% reduction in startle response amplitude caused by a preceding gap of silence in an otherwise continuous background sound (Ison et al. 2002; Turner et al. 2006; Kraus et al. 2010).

The testing session started with an acclimation period lasting 3 min. Immediately afterwards, animals received 10 STARTLE-only trials in order to habituate their startle responses to a steady state level. For each of six background frequencies, we presented five STARTLE only trials and five GAP+STARTLE trials. The STARTLE and GAP+ STARTLE trials were pseudo-randomized. The inter-trial intervals were also pseudo-randomized between 7 and 15 s. After we completed testing all six background frequencies, the entire session was repeated one more time. Thus, during this testing for each background frequency, the total of 10 GAP+STARTLE trials and 10 STARTLE only trials were presented.

All animals from the experimental group were tested before acoustic trauma, and then at several times points afterward: 1, 3–5, 7 days, weekly for 2 months, and at 84 days, 180 days, and 360 days post-exposure. The control group was tested at the same time points.
Assessments of hearing loss

Input/output functions of the acoustic startle reflex were created for each animal. All behavioral experiments were conducted via methods previously optimized in our lab (Longenecker & Galazyuk 2012). The startle stimuli were presented at the levels ranged from 50 to 120 dB SPL in a pseudo-random fashion. The inter stimulus interval was randomized within a range between 7 and 15 s. Each startle intensity was repeated twenty times. Thresholds and 75% of the max amplitudes were calculated offline.

ABRs thresholds were collected for each animal. Mice were anesthetized with ketamine/xylazine as during the acoustic trauma. Sterile, stainless-steel recording electrodes (RA4LI Low Impedance Headstage) were placed subdermally, behind each pinna and the reference electrode was placed along the vertex. Tone bursts at 4, 12.5, 16, 20, 25, and 31.5 kHz were presented at increasing sound intensities ranging from 10 to 80 dB SPL in 10 dB steps. Tones were 5 ms duration, 0.5 ms rise/fall time and delivered at the rate of 50/s. ABRs were averaged over 300 repetitions. These waveforms were amplified (TDT RA4PA Medusa Preamp), digitized (TDT RZ6 Multi-I/O Processor), and analyzed offline using custom made software (TDT OpenEx). Thresholds, the smallest sound amplitude that evoked a visible ABR, were determined by visually examining the ABR waveforms in response to every sound frequency presented at different sound levels.

Finally, PPI audiometry was used to assess behavioral hearing thresholds. All behavioral experiments were conducted via methods previously optimized in our lab (Longenecker & Galazyuk 2012; Longenecker et al. 2014). Testing sessions contained two types of stimuli. First, a startle stimulus (wide-band noise, 100dB SPL, 20ms in duration, 1ms rise/fall) was presented
alone; this is referred to in the text as startle only (Fig. 3.1A). The second stimulus type consisted of a congruent startle stimulus preceded by a prepulse (Fig. 3.1A). Prepulse stimuli were 20 ms pure tones with a 1 ms rise/fall time presented at six different frequencies (4, 12.5, 16, 20, 25, and 31.5 kHz) 100 ms before the startle stimulus. These prepulses were played pseudo-randomly in the range of intensities from 10 to 80 dB SPL for a given sound frequency. Each frequency/intensity combination was presented 39 times. Startle stimuli alone were pseudo-randomly mixed throughout each testing session. Inter-trial intervals were also randomized between 15 and 25 seconds. A complete testing (1872 prepulse trials, and 468 startle alone trials) required 15 hours with a 1 hour break for the animal to rest between hour 7 and 8. Startle-only responses were closely monitored throughout this process to ensure that startle magnitudes were not significantly affected by habituation. All startles were analyzed by the automated classifier system briefly described in chapter III (Grimsley et al. 2015).

**Surgery**

Four mice from the control and four mice from the sound exposed groups were used for extracellular recordings. Each mouse was anesthetized using isoflurane inhalation (1.5–2.0%, isoflurane administered by a precision vaporizer) prior to surgery. A midline incision of the skin over the cranium was made. The tissue overlying the skull then was removed and a small metal rod was glued to the skull using glass ionomer cement (3 M ESPE, Germany). Following surgery, animals were allowed to recover for 1–2 days in individual holding cages. Two days after surgery, each mouse was trained to stay inside a small plastic tube, to be used as a holding device during recording sessions. The metal rod on the head of the mouse was secured to a small holder designed to restrain the head of the animal without causing distress, while the ears were unobstructed for free-field acoustic stimulation.
Extracellular electrophysiological recordings in the Inferior Colliculus

Recordings were made from the ipsi- and contra-lateral inferior colliculus (IC) in awake mice inside a single-walled sound attenuating chamber (Industrial Acoustics Company, Inc). Throughout the recording session (2-3h), the animal was offered water periodically and monitored for signs of discomfort. After a recording session, the exposed skull was covered with sterile bone wax and the animal was returned to its holding cage. Experiments were conducted every day for 6 days after which the animal was sacrificed with an IP injection of FatalPlus. No sedative drugs were used during recording sessions. If the animal showed any signs of discomfort, the recording session was terminated and the mouse was returned to its cage.

A small hole (∼50 μm) penetrating the dura was drilled in the skull overlying the IC, through which a recording electrode was inserted into the IC. Extracellular single-unit recordings were made with quartz glass micropipettes (10–20 MΩ impedance, 2–3 μm tip) fabricated using a microelectrode puller (Sutter, P2000) and filled with 0.5 M sodium acetate. The electrode was positioned into the drilled hole by means of a precision (1 μm) digital micromanipulator using a surgical microscope (Leica MZ9.5). The relative position of each electrode was monitored from the readouts of digital micrometers using a common reference point on the skull. Vertical advancement of the electrode was made by a precision piezoelectric microdrive (Model 660, KOPF Instr.) from outside the sound-attenuating chamber. Recorded action potentials were amplified (Dagan 2400A preamplifier), monitored audio-visually on a digital oscilloscope (DL1640, YOKOGAWA), digitized and then stored on a computer hard drive using EPC-9 digital interface and PULSE software from HEKA Elektronik at a bandwidth of 100 kHz.

A search stimulus consisting of a train of 10 frequencies (5-50 kHz) each with a 10 ms duration was presented while the recording electrode was advance in 3 μm steps. Spontaneous
firing of IC neurons was assessed during 38.4 s of recording without any stimulus presented. In order to assess each neuron’s characteristic frequency, sound-evoked activity was recorded in response to 100 ms pure tone pulses which were presented at different sound frequencies and intensities.

IC neurons in control and sound exposed animals often demonstrated spontaneous bursting activity. To be defined as a burst it should satisfy several criteria. (1) Burst should contain 2 or more spikes. (2) Maximum within-burst ISI should be less than 10 ms. (3) ISI between bursts should be greater than 10 ms. These criteria were adapted from Bauer et al. 2008.

To assess bursting, three commonly used criteria were used. 1. Coefficient of variation, a statistic that is derived by normalizing the standard deviation of each unit’s ISI distribution by its mean. Units with high CVs have more irregular ISIs, which suggests that they have more bursting activity. 2. Mode ISI, which represents a unit’s most common bursting frequency. 3. The percentage of bursting activity defined as the number of spikes within bursting events divided by the total number of spikes recorded for a unit over 38.4 sec. Bursting IC neurons were also separated into low and high bursting base % of bursting activity. A low bursting neuron was defined as having 35% or less of its spikes within bursting events whereas a high bursting neuron was defined as having more than 35% of its spikes within bursting events (Fig. 4.4).

Results

Behavioral changes resulting from sound exposure

To test the hypotheses that sound exposure induced hyperactivity in the auditory system will be observed independently of whether an animal exhibits tinnitus; we first needed to assess whether animals have tinnitus. Four mice were randomly selected out of the pool of twelve exposed mice. To test for tinnitus we used the GIPAS methodology (Fig. 1.1). To test for potential hearing loss PPI audiometry was utilized (Fig. 3.1). We also tested ABRs and
input/output functions for each mouse. These assessments were conducted before and three months after sound exposure (1 hour, octave band noise centered at 12.5 kHz, 116 dB SPL), and are summarized in Table 4.1.

**GPIAS assessment of tinnitus**

Tinnitus was defined as a gap detection deficit within a narrow frequency range (usually at or right above the exposure frequency). Using a repeated measures ANOVA we found differences between control and 12 weeks exposed gap functions for mouse #13 [F(5,75) = 666.13, p < .001] and mouse #1 [F(5,61) = p < .001]. An LSD post hoc test showed specific frequency deficits for mouse #13 at 20 kHz [t = 2.78, p < .05] and mouse #1 at 16 kHz [t = 6.79, p < .05], which demarks tinnitus behavior. Mouse 10 and mouse 12 were identified as tinnitus negative as no frequency specific deficits were found (Fig. 4.1).

**PPI assessment of behavioral hearing thresholds**

Hearing thresholds assessed by PPI audiometry revealed different levels of hearing loss between mice (Fig. 4.2). Tinnitus positive mice #1 and 13 demonstrated minor threshold deficits in a narrow frequency range, 25 kHz and 16 kHz respectively. However, mouse 10 showed significant hearing deficits between 12.5 and 25 kHz, suggesting a significant hearing deficit. Finally, Mouse 12 did not demonstrate any threshold deficits.

**ABR hearing thresholds**

ABR thresholds were collected for each mouse before and 12 weeks after sound exposure (see Table 4.1). Mouse #1 and #12 demonstrated minor threshold deficits of 20 dB SPL at 31.5 kHz. Mouse #13 had ABR thresholds completely recovered to the level of pre-exposure. Mouse
<table>
<thead>
<tr>
<th></th>
<th>Mouse 13</th>
<th>Mouse 10</th>
<th>Mouse 1</th>
<th>Mouse 12</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tinnitus (kHz)</strong></td>
<td>Yes (20)</td>
<td>No</td>
<td>Yes (16)</td>
<td>No</td>
</tr>
<tr>
<td><strong>PPI Deficits (kHz)</strong></td>
<td>Yes (25)</td>
<td>Yes (12.5, 16, 20, 25)</td>
<td>Yes (16)</td>
<td>No</td>
</tr>
<tr>
<td><strong>ABR Deficits (kHz)</strong></td>
<td>Recovered</td>
<td>10-20 dB (8, 12, 16, 20, 25, 31.5)</td>
<td>20 dB (31.5)</td>
<td>20 dB (31.5)</td>
</tr>
<tr>
<td><strong>ASR Deficits</strong></td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

**Table 4.1** Summary of behavioral assessments.
Figure 4.1 Tinnitus assessment via GIPAS in four exposed mice. Mice were tested before (control) and three months (exposed 3M) after sound exposure (indicated by a grey vertical bar). Mean gap-induced inhibition and standard errors for each frequency tested are represented as (Gap+Startle)/(Startle). Tinnitus was identified as a significant (*) difference between control and exposed conditions.
Figure 4.2 Auditory thresholds assessed via PPI audiometry. Thresholds from four sound exposed mice recorded before (control) and 3 months after sound exposure (indicated by a grey vertical bar).
Mouse #10 had 10-20 dB deficits ranging from 8 to 31.5 kHz. Similar to PPI audiometric thresholds, ABR thresholds suggest that Mouse #10 had the most significant hearing loss, while the other three mice had either minor or no threshold shifts as a result of sound exposure.

**ASR assessment using input/output functions**

Input/output functions assessing the acoustic startle reflex were collected before and 12 weeks after sound exposure (see Table 1). Startle functions after exposure were significantly reduced for all animals: Mouse 1 [F(10,88) = 718.44, p < 0.001], Mouse 10 [F(10,94) = 805.46, p < 0.001], Mouse 12 [F(9,60) = 520.67, p < 0.001], and Mouse 13 [F(10,76) = 746.76, p < 0.001]. However, the degree of startle reduction differed among animals. Least Honestly Different post hoc tests revealed that mouse # 1 had no significant differences at any specific startle intensity, whereas mouse # 13 and mouse # 10 had moderate deficits ((100 to 115 dB SPL), (105 and 115 dB SPL) respectively) only at high intensity startle values. Interestingly, the mouse # 12 had the greatest depth and range of startle deficits (95 to 120 dB SPL), ranging from low intensities to high intensities.

**Increased spontaneous activity following sound exposure**

To determine if spontaneous firing rates of IC neurons changed in response to sound exposure, single unit recordings were conducted in awake mice. Spontaneous activity was assessed in 118 IC neurons recorded in four control (unexposed) mice and in 383 neurons of the four exposed mice described above. In the exposed mice ipsi- and contra-lateral IC were studied separately because effects of unilateral sound exposure may differ between sides. Comparison of spontaneous firing rates between all neurons in control mice and neurons in ipsi- and contra-lateral ICs in exposed mice revealed a significant elevation of firing rate caused by exposure.
Although the group comparison show significant elevations of firing rate in exposed mice, the pattern of these changes varied dramatically among individual mice. Tukey HSD post hoc tests revealed significant increases in both the ipsi- and contra-lateral IC of mouse # 13. Significant differences were also seen in the ipsilateral IC of mouse # 1, while non-significant increase was observed in the contralateral IC of mouse # 1 and in both IC’s of mouse # 12. In contrast to this pattern of increased activity, mouse # 10 only exhibited contralateral increases, while maintaining spontaneous firing rates very similar to control levels in the ipsilateral IC. Note that all mice were the same age and were sound exposed in an identical manner. Since mouse # 10 and mouse # 12 did not show behavioral evidence of tinnitus, yet all mice had at least some level of increased spontaneous activity, hyperactivity should not be considered as a steadfast neural correlate of tinnitus. These results demonstrate that acoustic trauma caused hyperactivity in all animals, however, only two animals developed behavioral evidence of tinnitus.

**Burst firing properties of IC neurons following sound exposure**

Increases in bursting activity can be the result of either increasing the number of units bursting in a population or increasing the amount to which each individual unit is bursting. In order to test for these possibilities a classification system was developed which separated units into one of three groups: high bursting, low bursting, or non-bursting. The classification of high or low bursting was determined by the “% of spikes of bursting activity” histogram (Fig. 4.4). A Kolmogorov-Smirnov test of normality determined that this distribution was not normally distributed (KS = .176, df = 178, p < .001). However, if the data was separated into low bursting and high bursting units at the 35% mark, the two divisions are normally distributed (Low
Figure 4.3 Changes of spontaneous firing rates in neurons of ipsi- and contralateral ICs in four mice following sound exposure. The mean level of spontaneous activity from neurons of the control group is shown by a black horizontal line (standard error represented by shaded box). Significant differences between each IC and the control are indicated by asterics (*.05, **.001).
Figure 4.4 The distribution of % spiking within bursting events from bursting IC neurons (N=178) from eight mice. Statistical analysis revealed that this skewed distribution could be separated into two normal distributions. Units were classified as low bursting (LB) or high bursting (HB) based on the dashed line demarking 35 percent of spikes within bursting events.
Bursting: KS = .108, df = 130, p = .09; High Bursting: KS = .106, df = 48, p = .20). The percentage of units exhibiting bursting activity was plotted based on no bursting (NB), low bursting (LB), and high bursting (HB) in control mice and in each of ipsi- and contralateral ICs for each of the four exposed mice (Fig. 4.5). In control mice 31% of recorded neurons (n=36) were bursting (24%LB, 7%HB), while 69% of the neurons did not show bursting activity. This distribution is similar to the one recently described in guinea pig (Coomber et al. 2014). Surprisingly this distribution did not change much in three of our four exposed mice (Fig. 4.5). The percentage of bursting neurons in mouse 1, 12, and 13, were very similar to the control. Furthermore, the differences between ipsi- and contralateral IC’s in these mice were surprisingly small. In contrast, mouse 10 demonstrated a unique pattern of bursting. 83% of the neurons (n=54) in the ipsilateral IC in this mouse showed some degree of bursting activity (55%LB, 28%HB). Interestingly, the contralateral IC had only 19% of its neurons displaying bursting; the lowest value among all mice tested. To quantify the changes in bursting activity for individual neurons, we adopted several commonly used temporal measures of neural activity (Ma et al. 2006; Kapalla et al. 2014; Noreña & Eggermont 2003).

Elevation of spontaneous activity in IC neurons after sound exposure can be accomplished via increasing the bursting activity and/or an increasing the rate of regular (non-bursting) spontaneous firing. To assess whether the bursting-related spiking was occurring more frequently in sound exposed animals, we used the coefficient of variation (CV), a statistic that is derived by normalizing the standard deviation of each unit’s ISI distribution by its mean. Units with high CVs have more irregular ISIs, which suggests that they have more bursting activity (Fig. 4.6). The normal physiological range for CVs has been reported as .5 to 1 in cortical
Figure 4.5 Percentage of units with bursting activity. High bursting, low bursting, and units without bursting were classified for the IC of each mouse individually.
Figure 4.6 Example units for each bursting classification. Each unit includes its coefficient of variation which is a measure of spike regularity. A higher CV represents a neuron that is bursting more.
neurons (Christodoulou & Bugmann 2001), however recent studies have shown that after sound exposure values of 1.2 or higher have been shown in the DCN (Pilati et al. 2012). The distribution of CVs across each IC of four exposed mice was viewed with a box plot and tested for significance with a paired t-test was used to evaluate whether sound exposure changed the bursting patterns in IC neurons (Fig. 4.7). A significant CV decreases were observed in contralaterally exposed IC in mouse 13 [t(50) = 2.56, p < .05), mouse 1 [t(40) =2.15, p < .05) and mouse 12 [t(29) =2.92, p < .05). A highly significant CV increase and decrease were only observed in the ipsi- [t(53) = -4.56, p << .001) and contralateral (t(37) =1.44, p > .001) ICs of mouse 10, respectively. This is the only mouse which showed a severe hearing loss (Fig. 4.2).

Another temporal marker of bursting activity is the ISI mode. This can be thought of as the peak in the ISI distribution which results from bursting activity. To determine if the timing between spikes changed as a result of sound exposure, we assessed the mode ISIs of burst firing neurons (Fig. 4.8). The distribution of ISI modes for control mice differed significantly (Wilcoxon signed rank) from the ipsilateral IC of mouse 10 (Z = -2.68, p = .007), and both ipsilateral and contralateral ICs (Z = -2.32, p <.05) (Z = -1.98, p < .05) of mouse 12 which had median modes of respectively. This data suggests that ISIs within bursts of tinnitus positive animals is not changing but does change to some degree in the contralateral IC of the animal with significant threshold shifts.

Finally, we wanted to establish what percentage of all spikes recorded were within bursting events. For each unit the total number of spikes within bursts was divided by the total number of spikes in a given neuron. Interestingly, only mouse 10 demonstrated significant differences after sound exposure compared to control animals (Fig. 4.9). The percentage of spikes within bursts decreased in the contralateral IC (Z = -2.31, p < .05) and increased
Figure 4.7 The distribution of coefficient of variations calculated for ipsi- and contralateral ICs of four exposed mice. Significant differences between control (unexposed) and each exposed IC was determined by a paired t-test (*.05, **.001).
Figure 4.8 The distribution of mode ISIs calculated for ipsi- and contralateral ICs of four exposed mice. Significant differences between control (unexposed) and each exposed IC was determined by a Wilcoxon ranked sum test (*.05).
Figure 4.9 The distribution of % of spikes in bursts for neurons from ipsi- and contralateral ICs of four exposed mice. Significant differences between control (unexposed) and each exposed IC was determined by a Wilcoxon ranked sum test (*0.05, **0.001).
dramatically in the ipsilateral IC of mouse 10 (Z = -3.51, p << .001). This suggests that the percentage of spiking in bursts might be an important indicator of hearing loss but not tinnitus.

**Discussion**

There are three major findings in this study. First, an increase in spontaneous activity following sound exposure was observed in all exposed mice tested regardless of behavioral evidence of tinnitus or hearing loss. Second, the burst firing rate in the majority of exposed animals was not significantly affected by sound exposure. Third, although our sample size was limited, it seems that changes in the IC following sound exposure are most dramatic only in the animal with severe hearing deficits.

**Questioning the link between hyperactivity and tinnitus**

For several decades animal studies have pronounced hyperactivity as a hallmark neural correlate of tinnitus (see review Eggermont 2005; 2013). This study confirmed the results of previous studies that demonstrated that following sound exposure, animals with tinnitus show increased spontaneous activity in the inferior colliculus (e.g. Imig & Durham 2005; Mulders & Robertston 2011). However, in contrast, our study has found that hyperactivity does not always lead to tinnitus development. Half of the exposed mice tested showed clear hyperactivity without a behavioral sign of tinnitus. Recently a similar finding has been reported for rats (Ropp et al. 2014) and guinea pigs (Coomber et al. 2014). Thus this study further advocates that hyperactivity should be considered a generalized neural plastic change related to sound exposure rather than a specific neural correlate of tinnitus.
Bursting activity as a marker of hearing loss but not tinnitus

Many studies have implicated increased bursting activity as one of several possible neural correlates of tinnitus (e.g. Ma et al. 2006; Bauer et al. 2008). Although estimates of bursting are somewhat dependent on distinct definitions of what constitutes a burst, data presented here show that the coefficient of variation decreases in contralateral ICs after sound exposure. This suggests that spontaneous activity becomes more regular, and thus bursts less often occur compared to control animals. This result was evident for all mice tested, including those with behavior signs of tinnitus. This finding was also found in sound exposed rats (Ma et al. 2006), which suggests that this might be a general effect of sound exposure. However, other studies have found that burst firing increases in the IC after sound exposure (Bauer et al. 2008; Coomber et al. 2014). This increase was also evident in the DCN (Finlayson & Kaltenbach 2009; Pilati et al 2012), MGB (Kalappa et al. 2014), and AC (Noreña & Eggermont 2003, 2006). There are several possible explanations as to why this discrepancy occurred. 1. Many studies have started terminal physiological recordings a few days up to a maximum of a couple months after sound exposure. In order to make our study as clinically relevant as possible, mice were sound exposed and tested behaviorally for three months afterwards. Then these mice were allowed to age to middle age before they were used for physiological experiments. The benefit of the CBA/CaJ mouse is that age-related hearing loss is not a significant confound in sound exposure experiments until 20-24 months of age (Ohlemiller et al. 2010). Physiological testing was conducted 12 months after sound exposure, which provided ample time for neural activity (Mulders & Robertson 2011) or behavioral evidence of tinnitus (Longenecker & Galazyuk 2011, 2014) to dynamically change after sound exposure. The progressive nature of tinnitus has also been shown in humans (Gopinath et al. 2010). 2. In this study animals were sound exposed for one hour with a 12.5 kHz narrow-band noise unilaterally at 116 dB SPL. Unfortunately, nearly every hearing loss/tinnitus
study has adapted unique sound exposure parameters, leading to differential exposure effects. Thus direct comparisons between studies are extremely tenuous, especially considering the difficulty of tinnitus assessment and internal confounds arising from various levels of hearing loss resulting from sound exposure. 3. Many of the studies that reported increased bursting activity after sound exposure did not behaviorally assess the presence of tinnitus (Finlayson & Kaltenbach 2009; Pilati et al 2012; Noreña & Eggermont 2003, 2006). Thus it would be impossible to evaluate whether tinnitus was linked to bursting activity in these studies. Furthermore, in the studies that did assess tinnitus behaviorally, the criteria of these assessments varied dramatically (Bauer et al. 2008; Kalappa et al. 2014; Coomber et al. 2014). These factors could explain substantial differences in burst firing activity assessments between studies, thus making connections with tinnitus difficult.

A unique pattern of neuronal activity was observed in the one exposed mouse which demonstrated severe hearing deficits. In agreement with the other three mice the contralateral IC of this mouse also showed increased spontaneous activity, at least contralaterally (Fig 4.3). However the ipsilateral IC did not change its activity compared to the control group. More interestingly, the percentage of neurons that were bursting decreased contralaterally and increased dramatically ipsilaterally (Fig. 4.5). In line with this observation, the CV values and percentage of spikes within bursts was also increased in the ipsilateral IC while decreasing in the contralateral IC (Fig. 4.7). This abnormal activity could be explained by changes in either intrinsic cellular properties of the IC, or result from extrinsic bursting activity arriving to the IC from other auditory centers. Interestingly, these findings are consistent with those measured in cat cochlear nerve fibers after sound exposure (Liberman & Kiang 1978). Following sound exposure, nerve fibers that innervated cochlear regions with mintue damage maintained similar
bimodal distributions of spontaneous firing rates and ISI statistics as untraumatized cats.

However, this pattern changed in certain regions of damaged in traumatized cats, in that the magnitude of change in these firing parameters was directly related to the magnitude of hearing loss. Recordings from single auditory nerve fibers in CF regions that were known to have lost inner hair cells were shown to have lost responsiveness to sound in addition to compressed rates of spontaneous firing and unusual bursting activity. Surprisingly, this pattern of damage was not only seen in the expected CF region corresponding to the acoustic exposure, but also in more basal regions of the cochlea in some animals. PPI audiometric thresholds (Fig. 4.2) used in our study may correlate well to these findings in Liberman & Kiang 1978. In the lone animal with severe widespread deficits, we also found significant increases in bursting activity in the ipsilateral IC with low spontaneous firing rates. More research should be done to conclude whether this was an isolated phenomenon, or if large increases in bursting activity are directly related to the degrees of hearing loss, as only one animal demonstrated this pattern in this study.
The objective of this dissertation was to determine whether hyperactivity developed in the auditory system after sound exposure is linked to tinnitus and/or hearing loss. The specific aims of this dissertation describe how sound exposure can lead to behavioral evidence of tinnitus and/or hearing loss which also result in changes to neuronal firing in the inferior colliculus of mice.

Chapter 2 utilized the gap induced prepulse inhibition of the acoustic startle reflex (GIPAS) to monitor the development of tinnitus in CBA/CAj mice during one year following sound exposure. Immediately after sound exposure, GIPAS testing revealed widespread gap detection deficits across all frequencies, which was likely due to temporary threshold shifts. However, three months after sound exposure these deficits were limited to a narrow frequency band and were consistently detected up to one year after exposure. This suggests the development of chronic tinnitus is a long lasting and highly dynamic process.

Chapter 3 assessed hearing loss in sound exposed mice using several techniques. Acoustic brainstem responses recorded initially after sound exposure reveal large magnitude deficits in all exposed mice. However, at the three month period, thresholds return to control levels in all mice suggesting that ABRs are not a reliable tool for assessing permanent hearing loss. Input/output functions of the acoustic startle reflex show that after sound exposure the magnitude of startle responses decrease in most mice, to varying degrees. Lastly, PPI audiometry
was able to detect specific behavioral threshold deficits for each mouse after sound exposure. These deficits persist past initial threshold shifts and are able to detect frequency specific permanent threshold shifts.

Chapter 4 examined hyperactivity and increased bursting activity in the inferior colliculus after sound exposure in relation to tinnitus and hearing loss. Spontaneous firing rates were increased in all mice after sound exposure regardless of behavioral evidence of tinnitus. However, abnormal increased bursting activity was not found in the animals identified with tinnitus but was exhibited in a mouse with broad-band severe threshold deficits.

Conclusions

CBA/CaJ mice are a good model for both tinnitus development and noise-induced hearing loss studies. Hyperactivity which was evident in all exposed animals does not seem to be well correlated with behavioral evidence of tinnitus but more likely to be a general result of acoustic over exposure. Data from one animal strongly suggest that wide-spread severe threshold deficits are linked to an elevation of bursting activity predominantly ipsilateral to the side of sound exposure. This result is intriguing and should be followed up in further studies. Data obtained in this study provide new insights into underlying neural pathologies following sound exposure and have possible clinical applications for development of effective treatments and diagnostic tools for tinnitus and hearing loss.

Differential pathologies in individual animals following similar acoustic exposure

Perhaps the most significant question that is left unanswered in both the literature and this dissertation is why some animals develop tinnitus after sound exposure, whereas others do not. Tinnitus is known to affect between 35-50 million adults in the United States (Ahmad & Seidman 2004; Shargorodsky et al. 2010), and can be developed coincidentally with hearing loss.
(Lockwood et al. 2002), even after a single exposure to loud sound (Schmuzigert et al. 2006). In most clinical cases there is a strong correlation between hearing loss and tinnitus (Weisz et al. 2006). Alternatively a person may have tinnitus while demonstrating no audiometric deficits (Job et al. 2007; Schmuzigert et al. 2006). Inter-individual differences both in animals and in humans could explain this paradox. The diversity of pathologies seen in this dissertation work could be explained by variations in individual mice in: stress levels, unintentional noise exposure, differential peripheral damage, or unique maladaptive neuroplasticity in the central auditory system.

*Effect of stress*

Many common housing and handling procedures can cause an animal’s stress levels to increase dramatically (see review Balcombe et al. 2004). Stress is known to impair cognitive function (Arnsten, 2009), and thus it can alter the results of behavioral tests (Dawood et al. 2004; Graham et al. 2011; Kaneto 1995). It has been shown that loud unexpected sounds can raise levels of stress-related hormones (Burow et al. 2005). Both restraint and social stress are common in laboratory animals (Stone & Quartermain 1997). Simple handling of an animal, putting it in a new environment, and cage changes can also raise levels of stress in animals (Duke et al. 2001; Seggie & Brown 1974). Animals housed alone show much higher stress levels than animals housed in pairs or groups (Sharp et al. 2003). However, dominance struggles between pairs can cause a great deal of stress to the subordinate animal (Makinson et al. 2015). Any or all of these stressing factors could lead to a potential difference in the effect of sound exposure and/or the neuroplasticity that follows.
Additional unintentional sound exposure

Unintentional sound trauma could be the result of intense sounds in the animal housing facilities. A recent study recorded sounds from an animal care facility found that weekday sound levels could easily reach 70 dB SPL (Liberman et al. 2014). Even if sound levels are not damaging, each animal would get a different auditory experience which could lead to differential plasticity over the length of a longitudinal study. Each mouse is in a slightly different area in reference to each sound source, so it is likely sound levels were not even at all times. Animals can also be unintentionally exposed during transportation to and from a lab or in the lab itself. One study looked at various sounds that animals could be exposed to during an experimental day and found that many are broadband, which include the high frequency range to which rodents have very low thresholds (Sales et al. 1988). Examples of the mouse intense sounds include squeaky doors and animal carts, vacuums, and telephones, which can easily reach intensities over 100 dB SPL. When considering all of these possible noise sources, it is probably that animals will be unequally exposed to sound. This discrepancy might explain why individual mice develop tinnitus, hearing loss, or show the absence of such maladies.

Differential effects of intentional sound exposure

Hearing loss is most commonly a direct result of sound induced damage to the peripheral auditory system. However little is known of how specific structural failures lead to the percept of tinnitus. Data presented in this dissertation suggests that some mice develop tinnitus, sometimes coincidently with hearing loss, while not directly influencing acoustic brainstem response thresholds. This has been replicated in most of the tinnitus literature, but unfortunately the characteristics of sound traumas used across studies varies greatly, making comparisons difficult or inappropriate (see review Galazyuk & Hébert 2015). Additionally, the actual conditions of
exposure for each animal might differ slightly resulting in various degrees of damage. Such factors could include the exact animal placement in relation to the sound source, the time of day the animal was exposed, the exact amount of anesthetic that is absorbed into the blood, as well as all stress-related factors listed above. Current theories of how this peripheral damage leads to tinnitus differs among labs, however there is consensus on certain points. It is known that exposure to loud sounds leads to permanent damage to cochlear nerve fibers, even without direct damage to inner or outer hair cells (Kujawa & Liberman 2009; Lin et al. 2011). This was confirmed in human work that suggested that tinnitus in patients with normal audiograms may be correlated with a peripheral neuropathy, seen as a reduction in amplitude of the first wave of the suprathreshold auditory brainstem response (Gu et al. 2012; Schaette & McAlpine 2011). The resulting decrease of central input leads to maladaptive up regulation of firing in the lower auditory brainstem (see review Roberts et al. 2010). A recent study has suggested that rats behaviorally positive for tinnitus demonstrated the greatest degree of ribbon synapse degeneration at the cochlear nerve terminal (Singer et al. 2013). However, this was only true for intense exposure conditions of around 110-120 dB SPL. An alternative theory suggests that since spontaneous activity is peripherally generated from support cells (Kölliker’s Organ) in the cochlear during the earliest development of the auditory system, that sound exposure could reintroduce this spontaneous activity generator and thus result in tinnitus (Tritsch et al. 2007). However, this has not been tested in sound exposed animals. Although some of these theories provide some explanation of the effect of sound exposure on the peripheral auditory system, the strongest evidence of tinnitus generation is explained by central auditory changes.
Central plasticity following sound exposure

Central neuroplastic changes associated with tinnitus are abundantly studied but remain poorly understood. This dissertation described common neuronal changes related to tinnitus, hyperactivity and bursting activity in the inferior colliculus. However, the conclusions match other recent studies that suggest hyperactivity is not directly associated with tinnitus but is a general effect of sound exposure (Ropp et al. 2014; Coomber et al. 2014). Our new finding was that bursting activity was most prevalent in the animal with the most significant auditory threshold shifts, but less so in tinnitus animals. This suggests that increased bursting activity might be more easily explained by significant cochlear dead zones that result in hearing loss, rather than a tinnitus percept (Liberman & Kiang 1978). In agreement with our findings, one study mentioned above found that the activity-regulated cytoskeletal protein, Arc was downregulated in the amygdala, hippocampus, and auditory cortex in mice with behavioral evidence of tinnitus but upregulated in animals with possible hearing loss (Singer et al. 2013). A similar finding in the cochlear nucleus of rats demonstrated that GAP-43 (a synaptic plasticity associated protein) was upregulated in rats with severe hearing loss but not in rats with tinnitus (Kraus et al. 2011). This suggests that certain neural activity might be downregulated in tinnitus animals but upregulated in animals with greater degrees of hearing loss. Human imaging studies have found neural plastic changes in many brain regions (see review Simonetti & Oiticica 2015). Most studies find that tinnitus patients show increase of the cerebral gray matter in the auditory pathways, and a decrease outside the auditory pathways (i.e. Limbic system, cerebellum, basal ganglia) in comparison to non-tinnitus controls. Future studies should elucidate the common features of neural correlates of tinnitus between humans and animal models.
**Clinical implications and applications**

The studies completed in this dissertation are part of a large goal to ameliorate the percept of tinnitus in a clinical population. Unfortunately at this time, no objective measure of tinnitus exists for humans. Developing such a method would be critical in understanding how tinnitus is initiated, develops, and spreads throughout the human brain. After this process is firmly understood, various clinical interventions with specific drug therapies may be able to selectively relieve suffering from tinnitus.

Many labs have attempted to use gap induced prepulse inhibition of the acoustic startle reflex to assess tinnitus in humans (Fournier & Hébert 2012; Campolo et al. 2013; Mahmoudian et al. 2013; Mehdizade Gilani et al. 2013; Boyen et al. 2015). However, results of these studies have generally concluded that tinnitus cannot be detected by this method. These results could be true for several reasons. First, the stimuli used to assess gap inhibition were different in each study, which makes it difficult to compare the results. Second, these studies did not uniformly assess tinnitus parameters or hear loss spectrums. Not all tinnitus patients have similar tinnitus parameters, thus it would be unlikely that using identical parameters for testing different patients would provide meaningful data. Although it would be a daunting task, each patient’s tinnitus and hearing loss should be fully taken into account in a similar way to our mouse model (Longenecker & Galazyuk 2012). Lastly, and perhaps most importantly, data was averaged among patients. This is a poor way of determining if individual patients display a particular tinnitus, because it is well known that the tinnitus spectrum can differ dramatically between people (König et al. 2006; Chan 2009; Shargorosky 2010). Overall, more work needs to be done to fully understand whether gap detection can indeed detect tinnitus.
Drug therapies for animals with behavioral evidence of tinnitus have been used to various degrees of success. Although the neural correlate(s) for tinnitus have not been definitely identified, many different strategies for tinnitus suppression could exist. One common finding across tinnitus studies has been a noticeable decrease in overall inhibition in animals with behavioral evidence of tinnitus (see review Richardson et al. 2012). A recent study found that GABAergic inhibition was more easily blocked in control mice than in tinnitus mice when recording in dorsal cochlear nucleus neurons (Middleton et al. 2011). A similar result was also found in a study of the auditory cortex, and importantly when a drug enhancing GABAergic inhibition was given to tinnitus rats less rats displayed signs of tinnitus (Yang et al. 2011). Perhaps the most clinically relevant study used the antiepileptic drug Vigabatrin which elevates GABA throughout the central nervous system (Brozoski et al. 2007). When given to rats with tinnitus via drinking water, Vigabatrin reversibly diminished all signs of tinnitus, which replicated an effect in humans. Unfortunately this drug is not a long term solution because of serious side effects. It is thought that if tinnitus is the result of an overactive auditory system, then any drug that lowers this activity might be useful (see review Roberts et al. 2010).

Many other receptors and ion channels could also play a role in tinnitus drug therapy. As mentioned in chapter four, it was shown that high voltage potassium channels decrease after sound exposure (Pilati et al. 2012). Drugs that would modulate the activity of Kv3 channels might be able to prevent abnormal bursting activity. Ih currents, which are present through the auditory system, might also be a potential target for drug therapies to prevent abnormal hyperactivity (Tan et al. 2007). Recent unpublished results from our lab suggest that an mGluR group II drug might suppresses abnormal hyperactivity in the inferior colliculus. When applied during a single unit recording, the neuron almost always decreases its activity for 30 minutes to
an hour prior to recovery (Alghamdi et al. 2015). This drug, LY354740 has already been used in human clinical trials for anxiety (Pilc 2003). When considering these studies together, it is clear to see that in the future tinnitus might be treated in one of many different ways.
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