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Time Course of Tinnitus Development Following Noise Exposure in Mice

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Gap-induced prepulse inhibition of acoustic startle (GPIAS) has been used in rats and mice to study the problem of tinnitus. The current study demonstrates that similar methods can be used to study the temporal development of tinnitus over time in middle-aged mice. Six-month-old mice on a mixed C57Bl6 × 129 background were anesthetized with isoflurane and exposed to unilateral noise (n = 15), or sham exposure for controls (n = 8), for 1 hr (16-kHz octave band signal, 116-dB SPL). Tinnitus was tested in eight different sound frequency bands before and at postexposure time points of 1, 3–4, 7, 14, 21, and 30 days and monthly thereafter until 7 months postexposure. Noise-exposed mice displayed a number of changes in GPIAS consistent with the presence of hyperacusis and tinnitus. Noise exposure was associated with acute tinnitus measured 1 day later at several frequencies at and above the exposure frequency center. Consistent, chronic tinnitus then emerged in the 24-kHz range. Several time points following noise exposure suggested evidence of hyperacusis, often followed temporally by the development of deficits in GPIAS (reflecting tinnitus). Temporal development of these changes following noise exposure are discussed in the context of the interactions among aging, noise exposure, and the associated neurochemical changes that occur at early stages of auditory processing.

Key words: hearing; cochlear damage; mouse; tinnitus; gap

Tinnitus is the perception of sound in the ears or head when no external sound is present. It has been experienced by 25% of Americans in the last year, and approximately 8% report experiencing tinnitus every day (Shargorodsky et al., 2010). Tinnitus is strongly associated with aging (Podoshin et al., 1997; Rosenhall, 2003), with prevalence rates doubling from the 40s (6.6%) to the 50s (12.5%), before peaking in the 60s (14.3%; Shargorodsky et al., 2010). A recent study by Folmer et al. (2011) showed that veterans were more than twice as likely as age-matched nonveterans to experience tinnitus and that the greatest differences were found in the 50–70-year-old range (Vietnam era), many years beyond their active duty years. This problem is likely to grow in scale as young military personnel age. For example, the U.S. Veterans Administration reports that in 2009 (Department of Veterans Affairs, 2009) tinnitus was the most prevalent new disability claim and the most prevalent overall service-connected disability for those receiving compensation.

The majority of tinnitus sufferers seeking treatment identify no recent, acute trigger, and many report their first experience with chronic tinnitus in middle or late-adulthood, presumably after the impacts of earlier noise trauma and age compound to result in tinnitus (Meikle et al., 2004). In their study to estimate the influence of early noise on tinnitus, Rosenhall and Karlsson (1991) found significant correlations between tinnitus and exposure to earlier occupational noise. Even chronic tinnitus patients who report no onset factors report a noise exposure history indicative of damaging levels of noise (Griest and Bishop, 1998). In a discussion of the medical/legal issues of tinnitus, Coles and colleagues suggest that, although noise-induced tinnitus sometimes seems to appear suddenly, in reality it develops gradually until it, and the related hearing loss, have developed to a point where they can no longer be ignored (Coles et al., 2000). Tinnitus, like noise-induced hearing loss, can arise years after the noise exposure has ceased (Gates et al., 2000).

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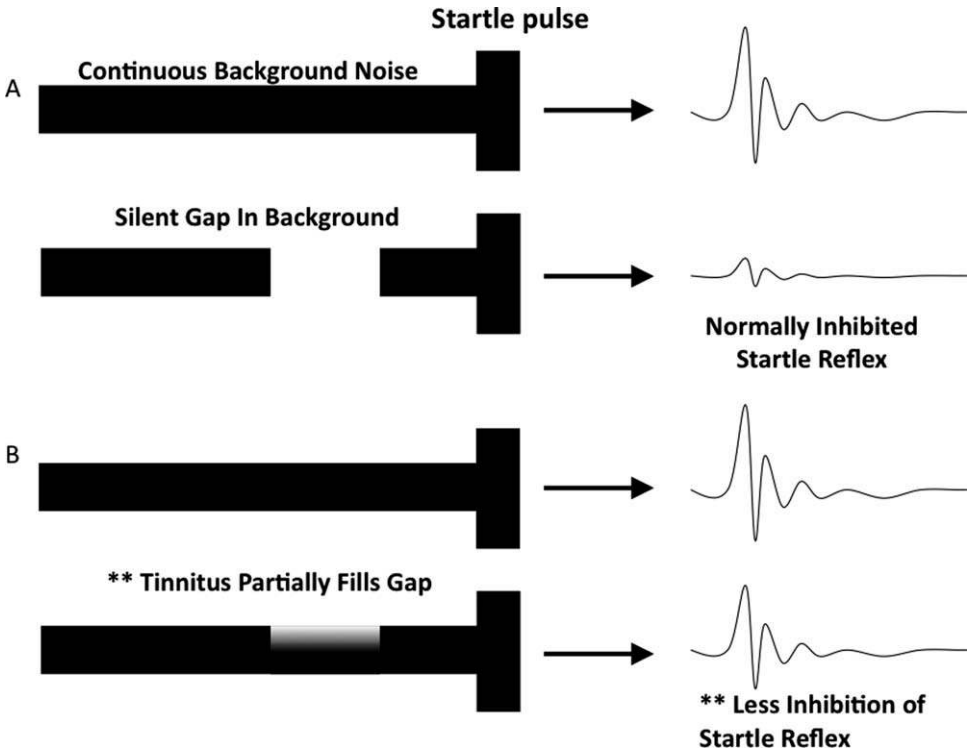

Fig. 1. Summary of the gap prepulse inhibition of acoustic startle (GPIAS) procedure for measuring tinnitus (adapted from Turner, 2007). Briefly, an animal is startled in the presence of a background noise (**A**). In normal animals, a silent gap in the background 50–250 msec before the startle stimulus will reliably inhibit the reflex (**B**). Animals with putative tinnitus with features similar to the background sound exhibit deficits detecting the silent gap, presumably because of their tinnitus. The gap is not as easily detected, so the reflex is not inhibited to the same degree.

Several useful animal models have been developed to study tinnitus. These models generally require training animals to respond distinctively to the absence of an acoustic stimulus. Animals are then given noise or chemical treatments believed to produce tinnitus. Animals with putative tinnitus show a disrupted ability to respond in quiet trials due to their putative tinnitus (Jastreboff et al., 1988; Bauer et al., 1999; Heffner and Harrington, 2002; Guitton et al., 2003; Ruttiger et al., 2003; Lobarinas et al., 2004; Heffner, 2011). Because of the learning, memory, and motivational demands inherent in these behavioral tasks, longitudinal studies are very difficult to conduct. An additional method has recently been developed for tinnitus measurement in animals that does not require training and instead uses reflex modification procedures (Turner et al., 2006; Yang et al., 2007; Turner and Parrish, 2008; Wang et al., 2009; Engineer et al., 2011; Longenecker and Galazyuk, 2011; Middleton et al., 2011). The gap prepulse inhibition of acoustic startle (GPIAS) method is based on the observation that the acoustic startle reflex can be reduced by a preceding signal/stimulus, in this case, a silent gap in an otherwise constant acoustic background (see Fig. 1). Animals exhibit significantly worsened ability to detect silent gaps embedded in a background similar to their putative tinnitus. Because the GPIAS method does not require training and makes use of a reflex, the test is largely resistant to extinction or motivational issues and lends itself well to studies involving repeated testing across the life span of a rat or mouse.

Recent work has shown that tinnitus can be measured in mice with the GPIAS procedures (Longenecker and Galazyuk, 2011; Middleton et al., 2011). The current study extends that work by further developing a mouse model of the temporal development of chronic tinnitus into middle age, when humans begin to experience tinnitus at the highest prevalence rates. Although the current study focused on middle-aged mice, the results are likely applicable to young mice. This study also addresses the delayed effects of noise exposure that occur later in life, including the development of tinnitus. The current work will help bring the full arsenal of modern scientific tools used so effectively with mice to bear on the problem of tinnitus.

MATERIALS AND METHODS

Subjects

Mice were obtained from Johnson and Johnson (Beerse, Belgium) at approximately 3 months of age and held in quarantine for 10–13 weeks before any testing was done. Mice were on a mixed C57Bl6 × 129 background, back-crossed

five times to C57Bl6, leading to a 98% pure C57Bl6. Mice were split into two groups and received either unilateral noise exposure (n = 15, 9 m/6 f) or sham exposure for controls (n = 8, 5 m/3 f). Mice were housed in small groups of three to five within a colony room maintained at 25°C, with a 12/12-hr light/dark schedule and ad libitum access to food and water. All procedures used in the research protocol were approved by the Southern Illinois University School of Medicine Laboratory Animal Care and Use Committee (protocol P190-06-005), conformed to the NIH *Guide for the care and use of laboratory animals*, and followed the Society for Neuroscience's guidelines for the use of animals in neuroscience research.

No comparative data are available on hearing in the mixed C57Bl6 × 129 mice used in the current study, but there is an extensive literature on hearing in the inbred C57Bl 6 (C57) strain. C57 mice first show high-frequency threshold elevations (24 kHz and above) at about 2–3 months of age, and the thresholds continue to rise and step to progressively lower frequencies as the mice age until severe hearing loss is present for most hearing frequencies at 12–16 months (Mikaelian, 1979; Henry and Chole, 1980; Willott, 1986; Hunter and Willott, 1987; Li and Borg, 1991; Willott and Turner, 1999). The result of mixing C57 mice with 129s (which also have age-related hearing loss) results in generally better hearing because of hybrid vigor and much greater heterogeneity in age-related hearing loss, at least in the F1 offspring (Ouagazzal et al., 2006). Back-crossing the C57 and 129 offspring for five generations with C57s would greatly reduce hybrid vigor and produce a phenotype much more similar to that of the C57 parental strain.

Behavioral Testing

Mice were pretested using the GPIAS (Fig. 1) procedure and placed into either noise trauma or control (sham trauma) groups. After noise exposure, mice were then behaviorally tested at days 1, 3–4, and 7–8; then weekly for the first 12 weeks; and then monthly until 7 months postexposure. At pretest and beginning at 1 month, all mice were tested twice weekly, and their mean response for the two tests was used for analysis.

Behavioral testing was conducted using Kinder Scientific's startle reflex hardware and software, customized for this application by the manufacturer (Kinder Scientific, Poway, CA). Background sounds in the startle chamber consisted of 60-dB SPL 1,000-Hz bandpass-filtered noise (48-dB/octave roll off; Krohn-Hite model 3988) centered at 4, 8, 10, 12, 16, 20, 24, and 32 kHz and broadband noise. The 60-dB stimulus is well within the normal hearing range for mice in the 4–32-kHz spectrum (Heffner and Heffner, 2007). Acoustic stimuli were calibrated using a cloth model mouse and a Bruel & Kjaer Pulse System with a 1/2-inch free-field microphone (B&K model 4191). Baseline noise levels in the test chamber (with background test noise turned off) were typically below 20-dB SPL in the 4–40-kHz range. Gap and prepulse inhibition testing used background sounds presented through one speaker (Vifa XT25TG30-04) and startle stimuli presented through a second speaker (Powerline CTS KSN-1005) located in the ceiling of the testing chamber, 15 cm above the animal's head. The sound field was calibrated at a single point in the test chamber to approximate the location of the head of the mouse. Mice would occasionally turn during testing to face the opposite direction. However, the sound source is immediately above the center of the body of the mouse and the sound field should spread equally to the left or the right side of the testing enclosure given the symmetrical properties of the enclosure. The floor of the chamber was attached to a piezo-transducer and provided a measure of startle force applied to the floor. A clear polycarbonate animal holder, with holes cut for sound passage, was suspended above the floor, allowing the animal to turn around freely while minimizing excessive movement. An adjustable-height roof was set to a level that kept animals from rearing up, a behavior that adds variability to the startle response.

Prepulse inhibition (PPI) was periodically measured as a control condition. Prepulse stimuli matched the backgrounds used in gap detection in both spectral and intensity dimensions. PPI testing is essentially the inverse of gap detection testing and serves as a valuable control for hearing loss and temporal deficits that might explain any gap detection deficits. For example, rather than presenting a 60-dB 10-kHz signal in the background continuously and embedding a 50-msec gap before startling the animal, during PPI testing the background is quiet and a 50-msec, 60-dB, 10-kHz prepulse signal would be presented before startling the animal. Deficits in gap processing (GPIAS) accompanied by deficits in PPI would suggest hearing loss or a temporal processing or sensory gating dysfunction as the main source of the gap deficit. The temporal properties of both the PPI testing and GPIAS testing are identical; they both involve a 50-msec event presented 100 msec before a startle stimulus. In PPI the event is the presentation of a stimulus, and in GPIAS the event is the removal of a stimulus. Normal PPI would suggest that the animal could process the rapidly changing stimulus and was not suffering from some form of sensory gating disorder (Braff and Geyer, 1990). However, deficits in GPIAS not accompanied by deficits in PPI might suggest the presence of tinnitus. In addition, improvements in responding during gap trials as well as improvements in responding during PPI trials might suggest the presence of a hyperacusis-like phenomenon, as was recently demonstrated in a salicylate study (Turner and Parrish, 2008).

Each test session, whether GPIAS or PPI, began with a 2-min acclimation period, followed by two trials consisting of an abrupt startle-eliciting noise burst (115-dB SPL, 20-msec duration), which serves to habituate the startle response to a more stable baseline. Data from the two initial trials were not used in the GPIAS analysis. The remainder of the session consisted of startle-only trials pseudorandomly mixed with gap trials. Gap trials were identical to startle-only trials, except for the inserted gap. A variable intertrial interval (5.5-sec average) was used. The frequency of the background used to carry the gap was systematically varied throughout the test session. The targeted frequencies and BBN stimuli used for both gap and PPI were presented in ascending order from 4 kHz to BBN for the first test cycle and in descending order from BBN to 4 kHz for the second test cycle and looped for a total of 290

trials for the gap detection test and 200 trials for the PPI test. Gaps and prepulse stimuli always began 100 msec before the startle stimulus and were 50 msec in duration (0.1-msec rise/fall times). Previous studies suggest that 50-msec gaps beginning 100 msec before a startle stimulus produced stable, asymptotic levels of gap-induced inhibition of the startle reflex in rats (Turner et al., 2006). Startle testing does not cause temporary or permanent threshold elevations in mice (Turner and Willott, 1998).

Noise Exposure

Noise exposure was similar to that used in previous studies with rats (Bauer et al., 1999; Bauer and Brozoski, 2001; Turner et al., 2006). The noise consisted of a peak-calibrated level of 116 dB, centered on a 16-kHz octave band. The noise exposure was presented unilaterally to isoflurane-anesthetized mice for 1 hr. The noise exposure setup allows up to four mice to be noise exposed simultaneously. In previous work with rats, this exposure typically resulted in a 30–50-dB temporary threshold shift across all frequencies, with slightly more elevation at those frequencies around 16 kHz. This exposure, however, leads to little if any permanent hearing loss in rats; it is typical for ABR thresholds to recover to near-normal levels. The results of control studies suggest that unilateral hearing loss alone (simulated by an ear plug in one ear producing a 22-dB threshold shift at 10 kHz) does not significantly affect gap detection (Turner et al., 2006). Additionally, a behavioral control (PPI; see above) was employed in the current study to assess degree of hearing loss at each frequency.

ABR Thresholds

ABR thresholds were obtained for each ear immediately before and after unilateral noise exposure and again at the end of the experiment before mice were euthanized and tissues collected. ABR thresholds are a measure of neural synchrony and are often used to estimate hearing thresholds. Reliable ABR thresholds were available from four controls and six exposed mice. Thresholds were obtained under isoflurane anesthesia for tone pips (2-msec rise/fall, 1-msec plateau) at 8, 10, 12, 16, 20, 24, and 32 kHz. Stimuli were presented from 95- to 5-dB SPL in descending 10-dB steps until a discernible waveform could no longer be detected. Stimuli were presented at a rate of 39/sec, and the averaged response in a 10-msec window was collected for 512 repetitions.

Spiral Ganglion Cell Counts

After the last test animals had been anesthetized, final ABR thresholds were obtained, followed by cardiac perfusion with 4% paraformaldehyde. The cochleas were then removed and shipped in fixative from Illinois to Michigan for spiral ganglion counts. Cochleae were decalcified in 5% EDTA in PBS at room temperature for 24 hr, dehydrated through a graded series of alcohols, and then processed for embedding into JB-4 Plus, a glycol methacrylate plastic. Five-micrometer plastic sections were cut in a paramodiolar plane. The midmodiolar sections were identified; in each such section, there were three cross-sections of Rosenthal's canal. Every such section was picked up and placed on a slide. Slides were rehydrated, dipped in Paragon for 1 min, dehydrated in graded strengths of ethanol, dipped in xylene, and coverslipped with Permount mounting medium. The 12 most midmodiolar sections were selected, and every other section was used for quantitative assessment. Each selected slide was placed in the microscope and digital images were acquired in a Metamorph Image Analysis workstation under brightfield optics. The most basal profile of the Rosenthal's canal was acquired at a low (×2.5–10) magnification. The outline of the profile of the Rosenthal canal was circled to determine the total area using the Metamorph image analysis software. Magnification was then increased (×16–25) for spiral ganglion cell counting.

To be counted, a spiral ganglion neuron had to meet the following criteria: Cells between 12 and 15 μm in diameter with a nucleus between 5 and 9 μm in diameter with eccentricity not greater than 3:1. Every cell within Rosenthals canal meeting these criteria was ''clicked'' and counted. Spiral ganglion neuron density was then calculated by dividing the number of spiral ganglion cells by the area measured. Each of the remaining two more apical profiles was then assessed (as described above) and binned separately, proceeding from base to apex. The next section for the assessment area was then counted, with a total of six sections for each animal assessed.

Data Reduction and Statistical Analysis

Data from gap or prepulse testing yield a single value expressed as a ratio of the mean response in gap trials/mean response in startle-only trials. A response of 1.0 would mean that the startle reflex is the same whether or not it is preceded by a gap or prepulse stimulus (depending on the trial used). The farther the ratio is below 1.0, the better the startle inhibition. Individual and group data are first inspected for outliers and to inspect distributions characteristics. Descriptive statistics were then computed using this ratio value (mean, standard deviations, standard error of the mean, change scores from pretest to various posttest points). Data were then subjected to a mixed model analysis of variance (ANOVA) at certain target background frequencies with treatment (noise trauma vs. control) as a between-subjects variable and time (e.g., pretest, 1 week, 2 weeks) as a within-subjects variable. When appropriate, paired *t*-tests were done to compare pre- vs. postnoise measures. Alpha was set at 0.05 (two-tailed) on all tests, and every effort was made to minimize experiment-wise error rates. All plots and statistical analyses were done in Microsoft Excel, SigmaPlot 11.2 (Systat Software, San Jose, CA), or GraphPad Prism 5.0 for Macintosh (GraphPad Software, San Diego, CA).

RESULTS

Noise exposure resulted in significant behavioral evidence of acute tinnitus measured 1 day after noise exposure, isolated to just those frequencies above the noise trauma (see Fig. 2). This was evidenced by a significant time (pre- vs. postnoise) × background frequency (4–32 kHz) interaction in noise exposed mice, $F(7,112) = 3.15$, $P = 0.004$, which was not present in control mice, $F(7,56) = 0.410$, $P = 0.89$. Followup

paired *t*-tests in noise-exposed mice demonstrated significant worsening of gap responses following noise exposure at 20 kHz ($P = 0.002$) and 24 kHz ($P = 0.003$) and a trend at 32 kHz ($P = 0.088$). Acute ABR threshold elevations were seen immediately after noise exposure, $F(2,14) = 8.987$, $P = 0.009$, across a wide range of frequencies (Fig. 3). Followup Newman Keuls tests showed that thresholds were significantly elevated in the exposed ear relative to both the unexposed ear ($q = 4.229$) and the sham control ears ($q = 5.795$) but were not significantly elevated in the unexposed ear relative to controls. ($q = 1.566$). However, these thresholds shifts were temporary; exit ABR thresholds conducted 7 months after noise exposure at the end of the experiment revealed no significant group differences among sham control, exposed ears, and unexposed ears, $F(2,14)$, $= 0.597$, $P = 0.573$, and no significant Newman Keuls effects were present when comparing individual groups. At 24 kHz, the suspected tinnitus frequency focused on in the behavioral studies, thresholds were nearly identical (difference of 2 dB) in the exposed ear relative to sham control ears.

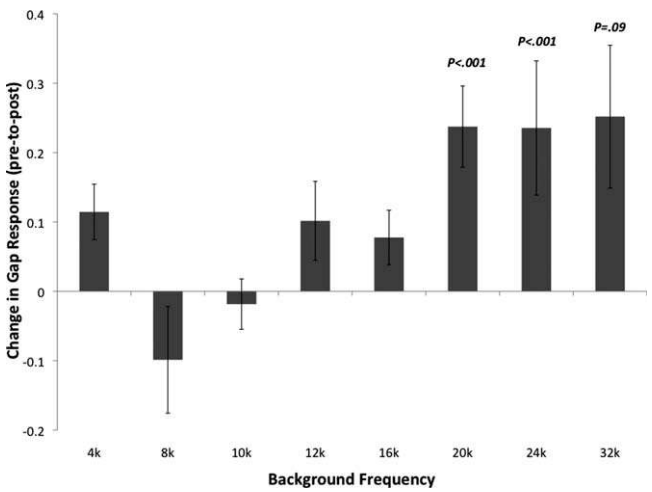

Fig. 2. Gap (GPIAS) change scores from pre- to postnoise (1 day) exposure. Control mice (not plotted) exhibited no statistically significant changes across these frequencies, whereas noise-exposed mice exhibited evidence of worsening gap responses in a range of frequencies above the center of the noise exposure trauma. *P* values obtained using paired *t*-tests. Error bars show standard error of the mean.

Inspection of the longitudinal data suggested that the best evidence of chronic tinnitus should emerge in the 24-kHz range, so additional analyses were done with this frequency. Noise-exposed mice exhibited significant behavioral evidence of both acute and chronic tinnitus at 24 kHz (Fig. 4). A two-way ANOVA for treatment group (noise exposed vs. control) × time (pretest out to 7 months postnoise) resulted in a significant interaction, $F(18,378) = 2.16$, $P = 0.004$, suggesting that the two groups' responses differed significantly over time. Followup one-way ANOVAs for each group showed significant changes across testing times for the noise-exposed group, $F(18,252) = 6.175$, $P < 0.0001$, but not for the control group, $F(18,126) = 1.463$, $P = 0.115$. Followup paired *t*-tests in noise-exposed mice demonstrated significant worsening of gap responses from pretest levels at all time points except for 2 days (16 of 18 time points), when just trends were found (days 7–8, $P = 0.055$, and day 21, $P = 0.083$). The strongest behavioral changes from pretest were found beginning at about weeks 5–7 and extending to the end of testing 7 months after noise exposure. Although noise-exposed mice demonstrated significant worsening of 24-kHz gap responses across nearly all time points after noise exposure, control mice showed significant worsening at only two points, at days 3–4 ($P = 0.044$) and at 7 months ($P = 0.035$). Figure 5 demonstrates the individual variation in controls and noise-exposed mice from pretest to 11 weeks after exposure. This figure demonstrates that, although most of the noise-exposed mice demonstrate tinnitus, not all of them do. In some of these cases it is possible that noise-

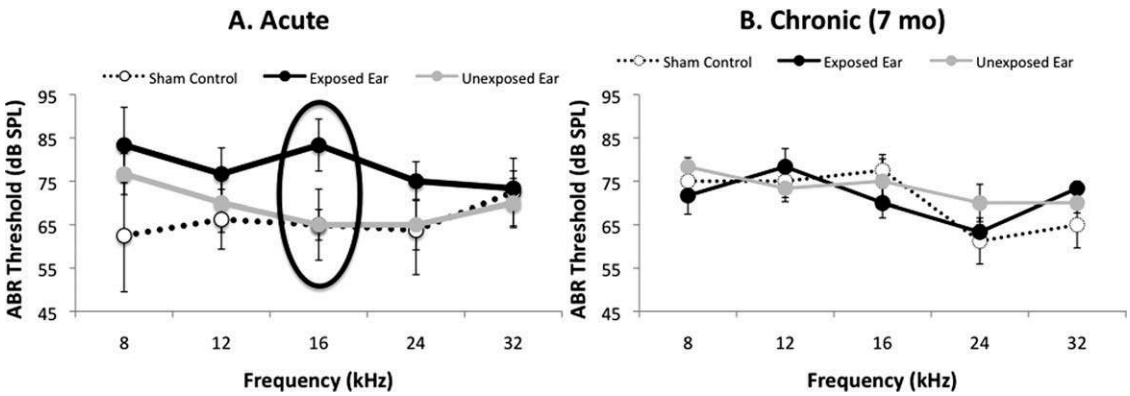

Fig. 3. ABR thresholds immediately following noise exposure (**A**) and 7 months later (**B**), at the end of the experiment. Significant temporary threshold elevations are seen in the exposed ear at the center frequency of the exposure (16 kHz) relative to both the unexposed ear and the sham-exposed control ears. However, these temporary threshold elevations had returned to the level seen in unexposed ears and sham controls by the end of the experiment. Error bars show standard error of the mean.

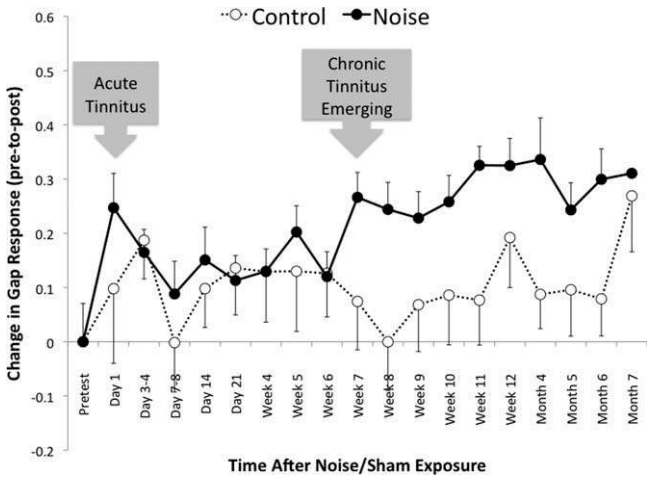

Fig. 4. Longitudinal changes (relative to pretest levels) in gap responses in the 24-kHz background for noise-exposed and sham-exposed control mice. Evidence of acute tinnitus emerged immediately at 1 day postexposure. Gap responding appeared to rebound somewhat from 3 days until about 4 weeks after noise exposure. At that point (5–7 weeks postnoise), strong behavioral evidence of chronic tinnitus emerged. Overall, control mice did not show significant behavioral changes as a function of testing time. Error bars show standard error of the mean.

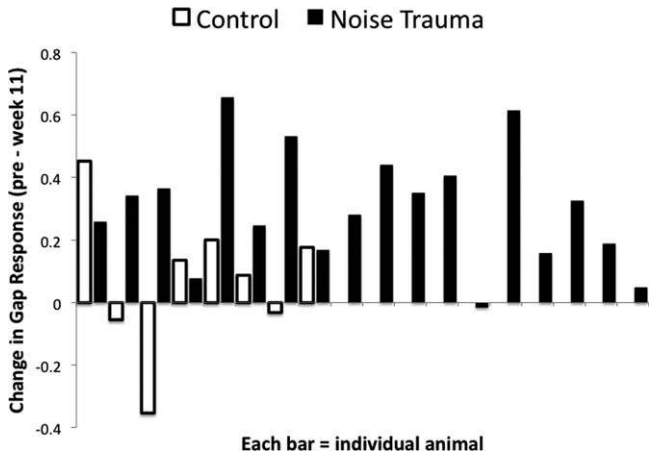

Fig. 5. Pretest to 11-week change scores for individual control and noise-exposed mice. This figure demonstrates that, although over half of the noise-exposed mice appear to demonstrate gap deficits that are consistent with tinnitus, not all of them do. How exactly to define tinnitus is a difficult problem. One approach is to draw a 95% confidence line for control animals and define tinnitus as gap scores worse than that level. In some of these cases, it is possible that noise-exposed animals do have tinnitus but that it is at a frequency other than 24 kHz used in this figure. Also apparent from this figure is that one of the control animals appears to show a deficit consistent with tinnitus. We have seen this in other studies with rats, that approximately 10% of control animals demonstrate deficits in gap responses consistent with tinnitus.

exposed animals do have tinnitus but that it is at a frequency other than 24 kHz.

PPI was also measured at all time points before and after noise exposure using a prepulse probe stimulus matching the frequency and intensity characteristics of the background stimulus used in gap testing. This control helps to determine whether deficits in gap responding are easily explained by hearing loss or temporal processing deficits, which presumably would also be present in PPI testing. Whereas robust gap deficits at 24 kHz were found at virtually every time point after noise exposure, PPI scores were not significantly worse than controls at any of the 18 time points following noise exposure (Fig. 6).

At the conclusion of the study, cochleas were harvested and spiral ganglion cell density was measured in both sham control ears, noise-exposed ears, and unexposed ears. Although no statistically significant differences were found when comparing sham controls to exposed ears ($P = 0.218$), or when comparing exposed ears to unexposed ears ($P = 0.126$), there was a nonsignificant trend toward a modest reduction in spiral ganglion cell density in exposed ears (Fig. 7).

DISCUSSION

Mice exposed to unilateral traumatic noise exhibited behavioral evidence consistent with both acute tinnitus at a range of frequencies at and above the noise-exposure center frequencies, and chronic tinnitus later emerged in middle age, weeks or months later in a narrower window in the 24-kHz range. The deficits in gap responses were not associated with decrements in PPI responses, suggesting that hearing loss and temporal processing deficits could not easily explain the poor gap processing. This evidence is consistent with the hypothesis that tinnitus served partially to fill the silent interval and degrade the signal/noise of the silent cue. These results are consistent with previous studies showing that rats with behavioral evidence of tinnitus from either the lever-pressing approach of Brozoski and Bauer (2006) or the polydypsia approach of Lobarinas and Salvi (Yang et al., 2007) demonstrated a degraded ability to process silent cues using the same GPIAS procedures employed here. Additional recent studies have demonstrated the use of GPIAS methods for measuring tinnitus to address a variety of experimental questions (see, e.g., Wang et al., 2009; Ralli et al., 2010; Zhang et al., 2010). The current study also demonstrates a time of apparent hyperacusis in behavioral responses to prepulse stimuli (Fig. 6) for a period of 2–3 weeks following noise exposure, consistent with a hyperexcitable auditory system.

The dissociation seen in the current study (i.e., gap deficits and normal PPI) suggests that the gap deficits are not easily explained by hearing loss or altered temporal processing, in that such changes would be expected to also alter PPI. In fact, immediately after and for the 2–3 weeks following noise exposure, PPI responses are stronger, indicative of a hyperacusis-like improvement in behavioral responses. However, at approximately 4 months postnoise exposure, both control and trauma mice began showing diminished behavioral responses in the PPI test-

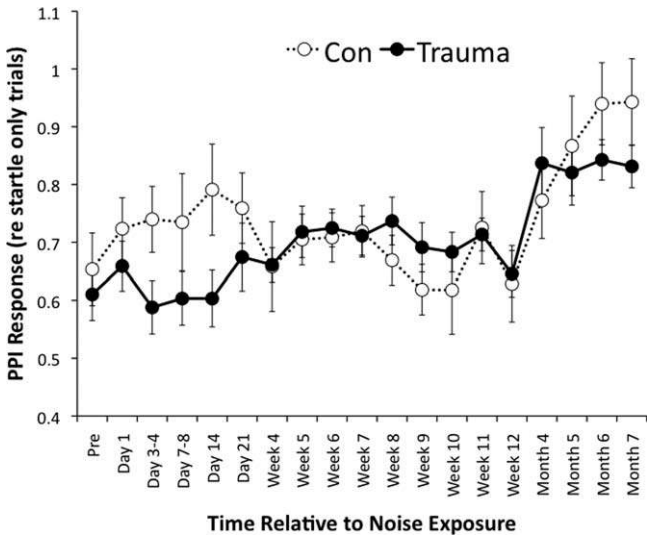

Fig. 6. Prepulse inhibition (PPI) responses for control and noise trauma mice from pretest to 7 months postnoise exposure. Notice the relatively stable PPI responses following noise trauma until about 4 months postnoise exposure, at which point both trauma and control groups appear to become progressively worse as a result of hearing loss. Lack of deficits in PPI for trauma mice from day 1 to week 12 in this figure, coupled with the severe deficits in gap responses after noise exposure shown in Figure 2, is consistent with the presence of tinnitus. Also apparent in this figure is the hyperacusis-like improvement in behavioral responses to prepulse stimuli for the 3–21-day range following noise exposure. Error bars show standard error of the mean.

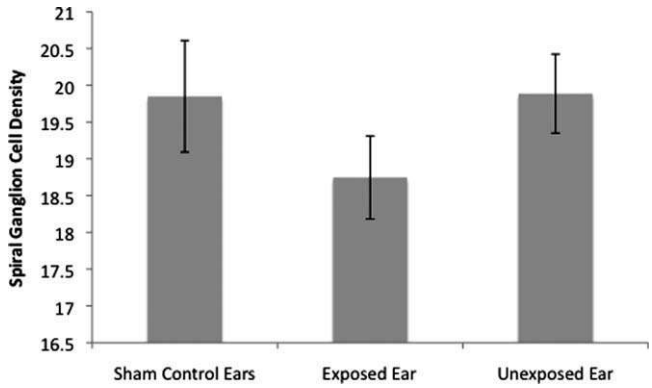

Fig. 7. Mean spiral ganglion cell density for sham control ears, noise-exposed ears, and unexposed ears. Although no statistically significant differences were found when comparing sham controls and exposed ears ($P = 0.218$) or when comparing exposed ears and unexposed ears ($P = 0.126$), there was a trend suggesting a modest reduction in density in exposed ears. Error bars show standard error of the mean.

ing, presumably as a result of hearing loss. For example, noise-exposed mice showed no significant difference in PPI responses from pretest values until 4 months postnoise exposure, after which PPI responses were consistently poor for both control and trauma mice. These results are consistent with advanced, progressive hearing loss in both controls and noise-exposed mice, making the 60-dB, 24-kHz stimulus in PPI testing too difficult to detect reliably. The reduced PPI found after 4 months also suggests that the gap deficits at that point and beyond should be interpreted with caution, because gap deficits then could be due to either tinnitus or hearing loss (or both). It should also be noted that the ABR thresholds suggest that animals could not hear the 60-dB stimuli used in the behavioral tests. However, two factors suggest that this is not the case. First, it is likely that the isoflurane anesthesia used for our ABRs led to worse thresholds than would be expected under different types of anesthesia (e.g., ketamine/xylazine). Second, previous studies have shown that behavioral responses have lower thresholds than do ABR measurements (e.g., Turner and Willott, 1998). This is probably because the ABR threshold is dependent on synchrony of neural firing, whereas behavioral thresholds are not.

There are several benefits to the GPIAS methods for testing tinnitus (for a review of behavioral methods of tinnitus measurement see Turner and Parrish, 2008). The most obvious benefit of the approach is its speed and flexibility and that, because a reflex is used, extinction of a learned response is not a concern. These features combine to allow for the long-term repeated measures so critical in studies of aging (as in the current study), in which aging not only can affect extinction features but might also interact with motivational factors so critical for performance in operant-based methods. Repeated testing, with concerns over extinction and motivation minimized, allows for longitudinal studies to explore developmental changes in features of tinnitus as the processes of aging, hearing loss, and other phenomena change over the life span. The current study tests mice repeatedly for just 7 months after noise exposure, although in previous work with mice we have measured startle and PPI changes longitudinally for well over 1 year (Willott and Turner, 1999). Another benefit of the GPIAS method is that the neural circuitry underlying the startle reflex and its modulation with inhibitory cues have been thoroughly studied (see, e.g., Koch and Schnitzler, 1997; Swerdlow et al., 1999, 2001) because of its relevance for research in fear and sensory gating disorders such as schizophrenia. This literature provides a rich backdrop with which to interpret findings and discover new connections that might have relevance for tinnitus. For example, some limbic system structures such as the hippocampus and amygdala, which appear to play a key role in the neural pathway whereby preceding acoustic stimuli inhibit the startle reflex (Miller et al., 2010), might also play a key role in tinnitus (Rauschecker et al., 2010). A final potential benefit of the GPIAS method is that it might be possible to adapt for use in humans. Eyeblink startle and prepulse inhibition of eyeblink startle are routinely used in research on sensory gating in humans. One of the authors is currently exploring whether it is possible to adapt the basic features of tinnitus measurement used in laboratory animals

and apply it for use in the measurement of human tinnitus. Such an objective measure would be valuable for human tinnitus research, but it would also provide the most direct validation of the animal model.

The current study is in agreement with previous studies demonstrating that mice, like rats, appear to exhibit the behavioral signs of tinnitus following noise exposure (Longenecker and Galazyuk, 2011; Middleton et al., 2011) and further demonstrates the temporal development of such changes. Even though both previous studies with mice used the normal hearing CBA/CaJ strain, and the current study used the C57 × 129 backcross, the findings were remarkably similar. As in the current study, Longnecker and Galazyuk found evidence of acute tinnitus across a wide range of frequencies, and 2–3 months later the tinnitus became more restricted between 20–31 kHz (24 kHz for the current study). Similarly, Middleton et al. showed that mice developed chronic tinnitus at 2–9 weeks after the noise exposure, and it was centered at 24 kHz, the same frequency as that found in the current study.

These findings could open the door for many of the technological advances used so effectively in mice (e.g., genetic knockouts, genetic overexpression) to be applied to the recalcitrant problem of tinnitus. In addition, the short life span of the mouse (approximately 2 years) and the many models of accelerated aging, make them an ideal model for studying the interactions between aging and tinnitus. Their small size and ease of handling make such work cost effective and might make tinnitus a more attractive area of study for some not currently working in the field. However, many details have to be worked out before such work can be streamlined. For example, careful attention should be paid to the strain of mouse used, because it is possible that the strain used can have major implications on the outcome of the studies. For example, workers in the laboratory of one of the authors (J.T.) have noticed similar strain-to-strain differences in susceptibility to noise-induced tinnitus when comparing Long Evans with Fischer brown Norway F1 hybrid rats. Such strain differences, while frustrating, might themselves provide important clues for the likely complex role of genetics in tinnitus.

The underlying pathophysiology responsible for the tinnitus-related behavioral deficits in the current study is not clear. The ABR thresholds indicate a significant threshold shift in exposed ears immediately following noise exposure, but these threshold shifts return to normal over time. However, the spiral ganglion cell density trends observed, in concert with previous research (Kujawa and Liberman, 2009), suggest that there could be more subtle pathophysiological impairments subsequent to noise exposure that are not revealed by ABR thresholds (or possibly even spiral ganglion cell counts) and that might emerge over time following early noise exposure. Tinnitus sufferers often report no recent acute triggers (Meikle et al., 2004) yet have a noise exposure history involving damaging noise exposure levels (Griest and Bishop, 1998). Tinnitus can arise years after the noise exposure has ceased (Gates et al., 2000). These observations of early noise exposure associated with much later development of tinnitus in humans, combined with recent data showing that noise exposure in a mouse model can leave cochlear hair cells intact but can cause a loss of afferent nerve terminals and delayed degeneration of the cochlear nerve (Kujawa and Liberman, 2009), help to provide structure for a theory of tinnitus development that involves more subtle spiral ganglion cell mechanisms that interact with the aging process in ways not yet understood (Weisz et al., 2006). The emerging literature suggests that tinnitus is ultimately a product of plastic changes occurring in central brain structures as a result of reduced afferent input. Studies of cochlear nucleus after varying degrees of spiral ganglion loss demonstrate behavioral and physiological correlates of tinnitus that can be attributed to loss of inhibition (Wang et al., 2011) or compensatory increases in excitation (Shore et al., 2008; Zeng et al., 2009). Consistently with earlier cortical studies (Eggermont, 2008), Engineer et al. (2011) demonstrated that rats with behavioral evidence of tinnitus developed a reorganized primary auditory cortex with an overrepresentation of the tinnitus frequencies. Reversing this plasticity by pairing carefully selected tonal stimuli and vagus nerve stimulation returned the reorganized cortex to normal and reversed the behavioral evidence of tinnitus. By demonstrating that tinnitus can be studied across the life span in mice, the current study offers the possibility that tinnitus will be more quickly understood and treated by combining advanced behavioral, genetic, and neuroscientific tools that have been developed so effectively in the mouse.

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