

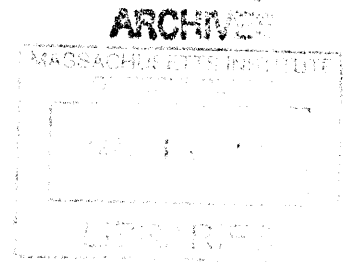
Noise-Induced Cochlear Neuronal Degeneration and Its Role in Hyperacusis- and Tinnitus-Like Behavior

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Abstract

Perceptual abnormalities such as hyperacusis and tinnitus often occur following acoustic overexposure. Although such exposure can also result in permanent threshold elevation, some individuals with noise-induced hyperacusis or tinnitus show clinically normal thresholds. Recent work in animals has shown that noise exposure can cause permanent degeneration of the cochlear nerve despite complete threshold recovery and lack of hair cell damage (Kujawa and Liberman, *J Neurosci* 29:14077–14085, 2009).

Here we ask whether this noise-induced primary neuronal degeneration results in abnormal auditory behavior, indexed by the acoustic startle response and prepulse inhibition (PPI) of startle. Responses to tones and to broadband noise were measured in mice exposed either to a neuropathic exposure causing primary neuronal degeneration, or to a lower intensity, non-neuropathic noise, and in unexposed controls. Mice with cochlear neuronal loss displayed hyper-responsivity to sound, as evidenced by lower startle thresholds and enhanced PPI, while exposed mice without neuronal loss showed control-like responses. Gap PPI tests, often used to assess tinnitus, revealed spectrally restricted, as well as broadband, gap-detection deficits in mice with primary neuronal degeneration, but not in exposed mice without neuropathy. Cross-modal PPI tests and behavioral assays of anxiety revealed no significant differences among groups, suggesting that the changes in startle-based auditory behavior reflect a neuropathy-related alteration specifically of auditory neural pathways.

Despite significantly reduced cochlear nerve response, seen as reduced wave 1 of the auditory brainstem response, later peaks were unchanged or enhanced, suggesting neural hyperactivity in the auditory brainstem that could underlie the abnormal behavior on the startle tests. Taken together, the results suggest a role for cochlear primary neuronal degeneration in central neural excitability and, by extension, in the generation of tinnitus and hyperacusis.

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Acronyms used

ABR – auditory brainstem response
ASR – acoustic startle response
AVCN – antero-ventral cochlear nucleus
BBN – broadband noise
CF – characteristic frequency
CN – cochlear nucleus
CRN – cochlear root neuron
DCN – dorsal cochlear nucleus
DPOAE – distortion product otoacoustic emissions
EPM – elevated plus maze
fMRI – functional magnetic resonance imaging
GABA – γ -aminobutyric acid
GAP-43 – growth associated protein 43
IC – inferior colliculus
IHC – inner hair cell
MOC – medial olivocochlear (efferents)
NBN – narrowband noise
OHC – outer hair cell
PnC – caudal pontine reticular formation
PPI – prepulse inhibition (of acoustic startle)
PTS – permanent threshold shift
PVCN – postero-ventral cochlear nucleus
rms – root-mean-square
SEM – standard error of the mean
SOC – superior olivary complex
TTS – temporary threshold shift
VCN – ventral cochlear nucleus
VGLUT1 – vesicular glutamate transporter 1

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1. Introduction

Acoustic overexposure has been linked to numerous hearing problems beyond elevation of audiometric threshold. Two frequent sequelae are tinnitus, the persistent perception of phantom sound in the absence of stimulation, and hyperacusis, the misperception of moderate-level sounds as intolerably loud (Anari et al., 1999; Schmuziger et al., 2006). It has been suggested that tinnitus and hyperacusis are perceptual indicators of an abnormal increase in the “gain” of the central auditory system resulting from loss of peripheral input (Jastreboff and Hazell, 1993). Indeed, decades of work on acoustic injury in animal models demonstrates a link between cochlear damage and central neural hyperactivity. Increased spontaneous and sound-driven activity have been observed at multiple central stages in the auditory brainstem, midbrain and cortex (Salvi et al., 1990; Kaltenbach et al., 2000; Syka and Rybalko, 2000; Mulders et al., 2011), with concomitant hair cell damage and elevated cochlear thresholds.

Although patients with tinnitus and hyperacusis often report a history of acoustic overexposure, some have clinically normal thresholds (Brandy and Lynn, 1995; Anari et al., 1999; Schmuziger et al., 2006; Coelho et al., 2007), suggesting that these perceptual anomalies can arise through peripheral damage that is undetected by standard audiometry. Recent work in animals has shown that noise exposures causing only transient threshold elevation can nonetheless result in permanent loss of cochlear nerve fibers (Kujawa and Liberman, 2009; Lin et al., 2011b). Cochlear-nerve synapses on hair cells can degenerate after exposure, leaving the surviving cell bodies unresponsive to sound and lacking spontaneous neural activity as if the target hair cell were lost (Liberman and Kiang, 1978). This neuronal loss may be selective for the subgroup of cochlear neurons with high thresholds and low spontaneous discharge rates (Lin et al., 2011a), thus explaining the lack of impact of their loss on cochlear thresholds. Recent human studies have suggested that tinnitus in patients with clinically normal audiograms may be correlated with a peripheral neuropathy, as seen by a reduction in amplitude of the supra-threshold auditory brainstem response (Schaette and McAlpine, 2011; Gu et al., 2012).

Here, using the acoustic startle response (ASR) and prepulse inhibition (PPI) of startle, we examine how noise-induced primary neuronal degeneration changes auditory behavior in mice. The whole-body ASR, elicited by an intense tone- or noise-burst, can be attenuated by the presence of a preceding stimulus called the prepulse. The magnitude of PPI can be a useful measure of stimulus detectability (Hoffman and Ison, 1980). If the prepulse is a silent gap in an otherwise continuous carrier, a decrease in gap-elicited PPI is hypothesized to indicate the presence of a gap-obscuring tinnitus percept (Turner et al., 2006). Here, we show that noise-exposed mice with primary neuronal degeneration exhibit changes in the ASR and PPI compared to both unexposed controls and to noise-exposed mice without neuronal loss. The results suggest that cochlear de-afferentation *per se*, rather than hair cell damage or associated threshold shifts, may be critical to the development of some perceptual anomalies after noise exposure.

2. Background

2.1. Acoustic overexposure reduces cochlear output

The cochlea converts acoustic signals into a neural representation of sound via multiple steps of transduction that depend on the normal functioning of sensory and supporting cells. Stereocilia bundles atop inner hair cells (IHCs) are stimulated by the motion of the cochlear partition, and their deflection allows current to pass into the cell (Hudspeth and Jacobs, 1979). This, in turn, initiates a cascade of ion exchanges that culminates in neurotransmitter release (Fuchs et al., 2003) to the 10-30 cochlear afferent fibers innervating each IHC (Spoendlin and Schrott, 1989; Liberman et al., 1990). These are the Type I afferent fibers, which comprise about 95% of the cochlear nerve (Spoendlin and Schrott, 1989), and each of which makes contact with a single presynaptic “ribbon” at the base of a single IHC (Liberman, 1980). The overall sensitivity of this mechanical-to-neural transduction is maintained by a similar transduction cascade in nearby outer hair cells (OHC). Through their ability to alter somatic stiffness and length, the OHCs amplify cochlear partition movement in a positive feedback loop, providing the mechanical-to-neural transduction process with exquisite sensitivity and tuning that are hallmarks of a healthy and active mammalian cochlea (for review, see Ashmore, 2008).

Overexposure to sound damages cochlear sensory and accessory structures, leading to elevation of cochlear thresholds and reduction in cochlear neural output (Wang et al., 2002b). Noise trauma can irreversibly fuse stereocilia, resulting in decreased current flow and thus reduced spontaneous activity in cochlear nerve fibers, which normally ranges from low (0 spikes/s) to high (~100 spikes/s) firing rates (Liberman, 1978; Liberman and Dodds, 1984a). Damage to, or loss of, OHCs or their stereocilia reduces the “gain” of the cochlea and alters the tuning of individual nerve fibers, leading to more broadly tuned neural responses that require higher stimulus intensities to reach thresholds for a driven response (Liberman and Kiang, 1978; Robertson, 1982; Liberman and Dodds, 1984b; Patuzzi et al., 1989). Synaptic ribbons are often disorganized and detached from the IHC membrane following exposure, suggesting dysfunction of synapses (Kujawa and Liberman, 2009; Lin et al., 2011b). Loss of IHCs from overexposure results in cochlear “dead zones” where any potentially remaining afferent innervation is silenced (Liberman and Kiang, 1978; Robertson, 1982; Liberman and Dodds, 1984b) (Fig. 1, *left*).

With variations in sound exposure duration and intensity, the functional significance of these cellular injuries can vary. Reduced sensitivity of individual cochlear nerve fibers, caused by dysfunction of their target IHCs or neighboring OHCs, results in reduction of the whole-nerve output. This is visible at the gross physiological level in neural responses such as the compound action potential of the cochlear nerve measured at the cochlear base, or the auditory brainstem response (ABR) measured as a far-field potential from the scalp. In cases of severe cochlear damage, these neural measures indicate signs of permanent threshold shift (PTS) such as elevated thresholds and reduced amplitude of supra-threshold responses (Salvi et al., 2000; Wang et al., 2002b). Measures of OHC function, by either the cochlear microphonic response or

otoacoustic emissions, similarly reflect cochlear damage as increased thresholds and reduced supra-threshold amplitudes (Patuzzi et al., 1989; Lonsbury-Martin and Martin, 2003). In contrast, neural and cochlear responses can also reflect a state of temporary dysfunction, or temporary threshold shift (TTS), that may last only a few days. Mild acoustic injury results in transient swelling of cochlear nerve terminals and supporting structures in the cochlea, and disruption of the stereocilia bundle which, in some cases, can self-repair (Wang et al., 2002b; Jia et al., 2009).

2.2. Acoustic overexposure can induce central hyperactivity

Despite the consistent observation of reduced cochlear output, either in spontaneous or sound-evoked activity, the auditory regions of the central nervous system that receive or inherit neural transmission from the periphery often show increased, or hyper-, activity.

Cochlear nerve fibers make first contact within the brain on a variety of cell types in the cochlear nucleus (CN), a large fraction of which are bushy cells that comprise the first stage of the bushy cell neural pathway, which often shows hyperactivity following noise exposure. In the ventral cochlear nucleus (VCN), spherical and globular bushy cells receive direction innervation from cochlear afferents (Liberman, 1991), and respond to sound in a fashion similar to the primary cochlear afferent fibers, hence their “primary-like” or “primary-like-with-notch” response patterns (Rouiller and Ryugo, 1984). Following a PTS-inducing acoustic overexposure, measurement of single-unit responses in the VCN revealed a roughly two-fold increase in spontaneous firing rate in these primary-like units (Vogler et al., 2011). The next steps in the bushy cell pathway are the excitatory ipsi- and contra-lateral projections to the superior olivary complex (SOC), encompassing multiple synaptic stations involved in the neural circuitry for binaural processing of sound (for review, see Oertel, 1999). The SOC has been examined for evidence of post-exposure hyperactivity in animals using *in vivo* imaging of calcium-dependent neural activity, with the result that the signal in SOC can be significantly elevated following both TTS and PTS exposures (Gröschel et al., 2011). These brainstem targets of the CN bushy cells then send largely excitatory input to the inferior colliculus (IC) where ascending auditory inputs from other tracts converge (for review, see Malmierca, 2004).

Included in these other sources of auditory input to the IC are pathways from VCN multipolar cells and the principal cell type of the dorsal cochlear nucleus (DCN). Taking a similarly ventral course through the brainstem but separate from the bushy cell tract are the excitatory projections of CN units with a sound-evoked “chopper” response, corresponding largely to T-multipolar cells in the VCN (Smith and Rhode, 1989) which receive, among other projections, direct input from the cochlear nerve (Liberman, 1991). Single-unit recordings in VCN following PTS-inducing exposures revealed that units with this physiological “chopping” classification showed an enhancement of spontaneous activity, as well as significantly increased rates of sound-evoked firing, compared to choppers from unexposed animals (Cai et al., 2009; Vogler et al., 2011). Taking a more dorsal course to the IC are excitatory projections of the principal cells

of the DCN, which participate in a complex network of excitatory and inhibitory connections of auditory and somatosensory origins (Young et al., 1992; Shore, 2005). Extensive studies on neural activity in DCN of noise-exposed animals have revealed significantly increased spontaneous and sound-evoked firing rates, via single unit recording in the fusiform cell layer (Brozoski et al., 2002) and via multi-unit recording across the DCN surface (Zhang and Kaltenbach, 1998; Kaltenbach and Afman, 2000; Dehmel et al., 2012), although one single-unit study reported no change in spontaneous activity across several physiological classes of cell types in DCN (Ma and Young, 2006). Hyperactivity of DCN has also been inferred through imaging of metabolic neural activity *in vitro* (Imig and Durham, 2005; Middleton et al., 2011).

The IC, receiving input from these ascending auditory pathways, as well as other ascending and descending non-auditory projections (for review, see Malmierca, 2004), is consistently observed to become hyperactive following acoustic overexposure. In contrast to the studies in the CN, those in IC have focused less on specific cell type contributions to noise-induced hyperactivity, yet regularly report signs of increased excitability. Single-unit and multi-channel recordings along the tonotopic axis of the central nucleus of IC reveal significantly increased spontaneous firing rates (Bauer et al., 2008; Longenecker and Galazyuk, 2011), especially among units with best frequencies near the region of most peripheral damage (Ma et al., 2006; Mulders and Robertson, 2009; Mulders et al., 2011) (Fig. 1, *right*), and even increased cross-fiber synchrony in ipsilateral IC where increased spontaneous activity was observed (Bauer et al., 2008). The growth of sound-evoked local field potentials in response to a range of frequencies is often steeper, with high-level response amplitudes significantly enhanced relative to pre-exposure recordings (Salvi et al., 1990; Wang et al., 2002a).

2.3. Relationship between peripheral damage and central hyperactivity

These examples of noise-induced central hyperactivity could, in principle, arise directly from trauma-evoked changes in local neural circuitry during exposure, or indirectly as a response to reduced cochlear neural output. Evidence for indirect effects comes from studies showing that during the first few weeks post-exposure, neural hyperactivity in the IC can be modulated by suppression of cochlear output by medial olivocochlear (MOC) efferent stimulation (Mulders et al., 2010), and can be partially reversed by ablating the cochlea or DCN (Mulders and Robertson, 2009; Manzoor et al., 2012). At longer survivals, central hyperactivity becomes less dependent on input from the periphery, as evidenced by increased spontaneous activity in IC that remains significantly elevated after cochlear ablation 12 weeks post-exposure (Mulders and Robertson, 2011). Although hyperactivity in IC and other central auditory regions may over time become self-sustaining following local plasticity, its dependence on cochlear responses implicates a role for the noise-induced change in peripheral function in its generation.

The majority of these examples of noise-induced central hyperactivity arise with concomitant peripheral damage, as evidenced by reduced counts of remaining IHCs and OHCs (Salvi et al., 1990; Mulders et al., 2011), by significantly elevated cochlear neural thresholds (Wang et al.,

2002a; Cai et al., 2009; Mulders and Robertson, 2009; Vogler et al., 2011), or inferred by elevated brainstem thresholds (Kaltenbach et al., 1998; Brozoski et al., 2002; Ma et al., 2006; Gröschel et al., 2011; Longenecker and Galazyuk, 2011). In particular, the frequency regions with the most noticeable increase in spontaneous activity often correspond with the regions of greatest cochlear threshold elevation (e.g. Mulders et al., 2011) (Fig. 1, *right*). However, in some cases central hyperactivity remains despite threshold recovery (Bauer et al., 2008; Gröschel et al., 2011; Middleton et al., 2011; Dehmel et al., 2012), or despite lack of obvious histopathology in the corresponding frequency locations of the cochlea (Mulders et al., 2011). One possible explanation is that stereocilia damage or subtle hair cell pathology may remain undetected yet impair normal IHC to afferent fiber transmission (e.g. Liberman and Dodds, 1984b) and thus alter the cochlear output to the central nervous system. Alternatively, a reduction in the cochlear nerve response, in the absence of hair cell damage, could potentially elicit these same enhancements in central auditory activity.

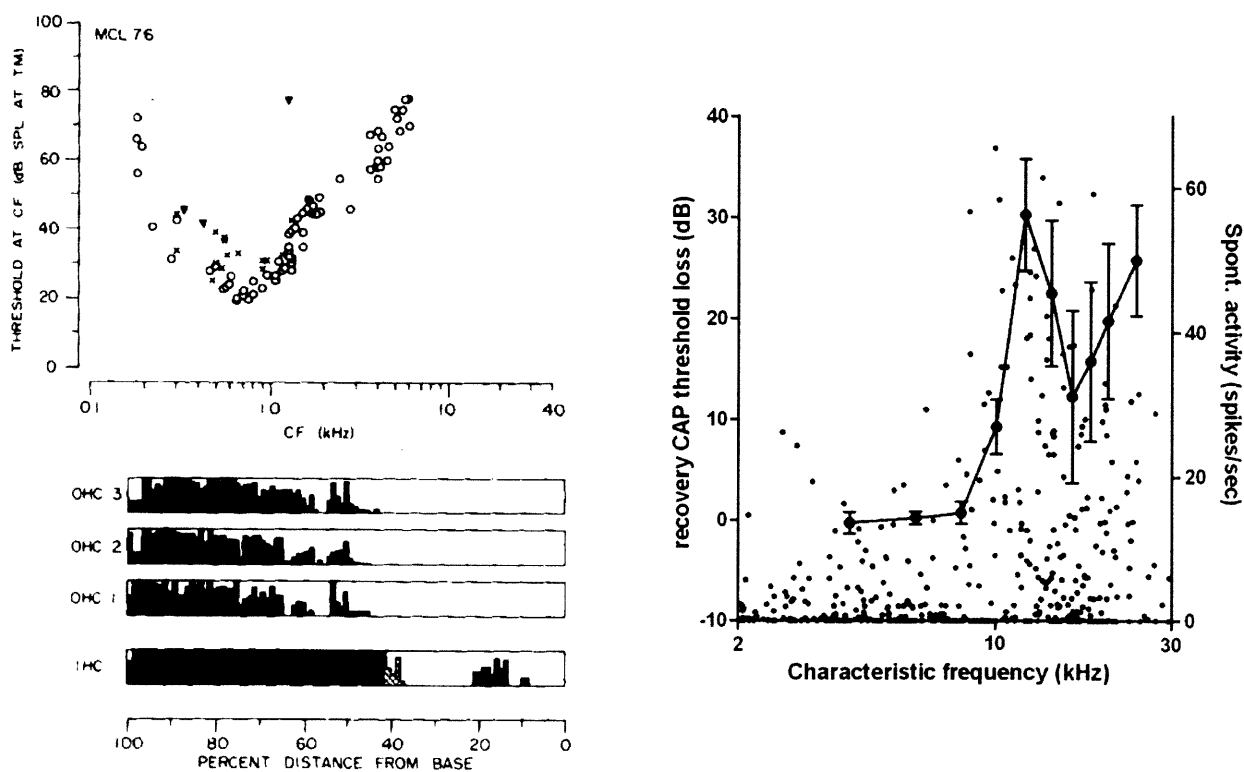


Figure 1: Neural threshold shift and hyperactivity following noise-induced cochlear damage. *Left:* Thresholds of single cochlear nerve fibers are elevated or non-existent (top) in regions of severe outer (OHC) and inner hair cell (IHC) loss (bottom). Shading represents density of remaining hair cells. (From Liberman and Kiang, 1978) *Right:* Spontaneous firing rate recorded in inferior colliculus is elevated in regions corresponding to cochlear threshold shift measured with the compound action potential (CAP). Data from guinea pig. (From Mulders et al., 2011)

2.4. Neuropathic overexposure with intact hair cells

Given that noise-induced PTS is due largely to death of sensory cells or damage to their stereocilia (Liberman and Kiang, 1978; Robertson, 1982; Liberman and Dodds, 1984b), threshold recovery was commonly thought to indicate cochlear recovery. However, recent studies of severe TTS that focused on detailed examination of cochlear nerve fiber synapses and cell bodies over a long post-exposure window revealed evidence for widespread and permanent degeneration of cochlear neurons (Kujawa and Liberman, 2009; Lin et al., 2011b).

Despite recovery of cochlear and brainstem thresholds within 1-2 weeks of exposure, corresponding with hair cell recovery, the observation of loss of pre-synaptic ribbons, loss of post-synaptic cochlear nerve fiber terminals, and loss of cochlear nerve cell bodies over a course of years reflects a noise-induced neuropathy following a TTS exposure (Kujawa and Liberman, 2009; Lin et al., 2011b). Although not detectable in measures of threshold, this neuropathy does have a physiological signature: wave 1 of the ABR, representing the synchronous response of cochlear nerve fibers, is reduced in amplitude for supra-threshold sound levels, both compared to the response from unexposed animals and compared to responses from cochlear frequency regions without synaptic loss (Fig. 2). The degree of ABR amplitude decrement parallels the reduction in synaptic ribbon counts, suggesting that this physiological response is a marker for the amount of cochlear neuropathy. The maintenance of neural thresholds despite widespread neuropathy can be understood if the degeneration is confined to the subset of cochlear nerve fibers with high thresholds (Liberman, 1978; Lin et al., 2011a).

This reduction in sound-evoked cochlear nerve response, in the absence of hair cell damage, could underlie central hyperactivity in cases of no obvious cochlear pathology or threshold shift. More importantly, study of the perceptual consequences of this particular neuropathy could have implications for understanding noise-induced perceptual abnormalities that arise in humans with clinically normal hearing thresholds.

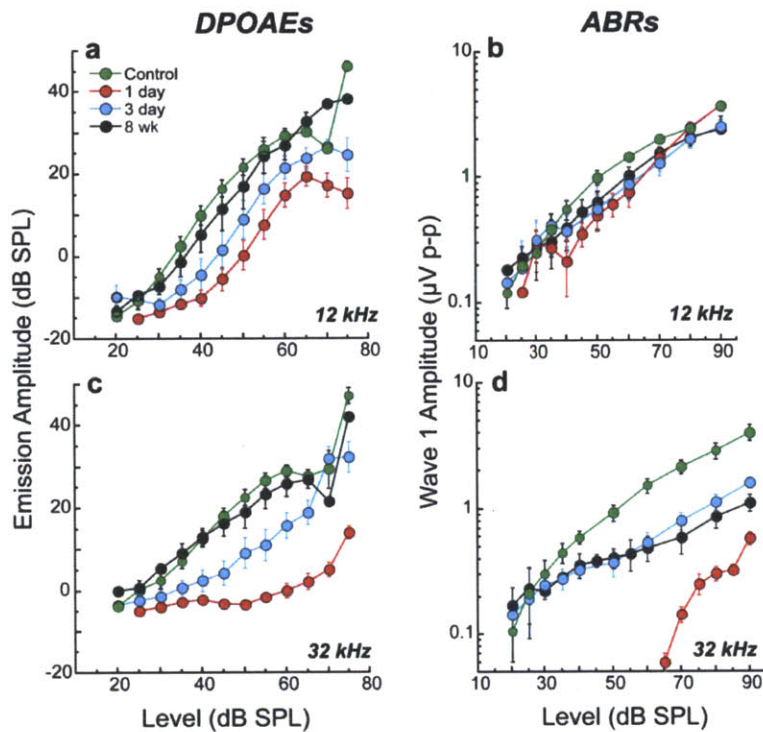


Figure 2: Cochlear function and thresholds recover but supra-threshold neural responses remain impaired following a moderate-level neuropathic exposure. Distortion product otoacoustic emissions (DPOAE) level functions are reduced at one day post-trauma, but recover to unexposed levels (left). Wave 1 of the auditory brainstem response (ABR) is also reduced one day post-trauma, and remains significantly reduced, for cochlear frequency regions with significant neuropathy. (From Kujawa and Liberman, 2009)

2.5. Perceptual sequelae of overexposure: hyperacusis and tinnitus

Hyperacusis, the reduced tolerance for moderate sound levels, and tinnitus, the perception of phantom sounds, are often co-morbid (Anari et al., 1999; Tyler et al., 2008; Kreuzer et al., 2012), and both are seen in patients with a history of noise trauma (Ince et al., 1987; Kreuzer et al., 2012) or can even be triggered by a single episode of acoustic overexposure (Anari et al., 1999; Schmuziger et al., 2006). Given that these anomalies share the attribute of increased sensation of sound, whether the sound is lower-intensity than perceived or is in fact absent, it is natural to speculate that they arise from either sound-evoked or spontaneous hyperactivity somewhere in the auditory neural pathways (Jastreboff and Hazell, 1993).

Tinnitus takes many perceptual forms, described by patients as “ringing”, “buzzing”, “cricket-like”, among other things, and can be perceived continuously or intermittently, and in one ear or bilaterally (Ince et al., 1987; Tyler et al., 2008; Kreuzer et al., 2012). In addition to patient-reported descriptions, the spectral and intensity qualities of the tinnitus percept can be quantified using pitch- and loudness-matching procedures (e.g. Ince et al., 1987). The frequency of tonal tinnitus pitch in patients with elevated thresholds often appears at the “edge” of the normal audiogram (König et al., 2006), and computational modeling of central neural activity based on audiometric losses suggests that this edge-pitch arises due to hyperactivity around these frequency-locations in the brainstem (Schäette and Kempster, 2009). Further evidence for neural hyperactivity in tinnitus comes from observations of increased sound-evoked fMRI signals in IC and auditory cortex, among other regions, of tinnitus patients (for

reviews, see Lanting et al., 2009; Melcher, 2012). Similarly, enhanced sound-evoked activity has been found in midbrain and auditory cortex of patients with abnormal sound level tolerance, considered a mild version of hyperacusis (Gu et al., 2010), suggesting that elevated activity in the neural auditory pathway could underlie abnormal perception of loudness.

Understanding of hyperacusis is challenged in part by a lack of consensus in its definition, arising from various observations of hyperacusis-like symptoms in an array of nominally unrelated conditions and syndromes, including Williams syndrome, facial nerve palsy, migraines, and stress, and hearing impairment (Phillips and Carr, 1998; Katzenell and Segal, 2001; Baguley and McFerran, 2011). Hyperacusis with a negative emotional or fearful association is often termed “misophonia” or “phonophobia”, respectively, specifically indicating the contribution of a non-auditory irregularity in sound perception (Baguley and McFerran, 2011). The term “recruitment”, or “loudness recruitment”, is sometimes used interchangeably with hyperacusis, despite the fact that recruitment is predicated upon elevation of thresholds (see Baguley and McFerran, 2011), while hyperacusis can occur without impaired thresholds (Brandy and Lynn, 1995; Anari et al., 1999). Finally, hyperacusis has also been defined, in some cases, as exceptionally acute hearing at low levels, suggesting better-than-normal sensitivity to threshold-level sounds (see Baguley and McFerran, 2011), which may reflect a different process than reduced discomfort threshold for more intense sounds. The range of hyperacusis definitions are, accordingly, reflected in the varied means of its quantification, such as measurement of situational and emotional contributions with questionnaires, of supra-threshold loudness with the “loudness discomfort level” or “uncomfortable loudness level”, and of loudness across a dynamic range with growth-of-loudness function (Brandy and Lynn, 1995; Anari et al., 1999; Coelho et al., 2007; Gu et al., 2010).

The challenge of objectively measuring tinnitus and hyperacusis in patients, given the diversity of symptoms and potential etiologies, extends to measurement in animal models, and is exacerbated by the assumptions and pitfalls of behavioral techniques. Despite these difficulties, evidence for links between tinnitus- and hyperacusis-like behavior, noise trauma, and increased central neural excitability abounds in animal studies, where increases in spontaneous and sound-evoked activity in the auditory pathway can be confirmed (e.g. Bauer et al., 2008; Yang et al., 2011; Sun et al., 2012).

2.6. Summary and significance

Perceptual abnormalities such as hyperacusis and tinnitus are thought to arise from hyper-excitability of central auditory pathways (Jastreboff and Hazell, 1993), a link that is supported by animal studies where hyper-excitable behavioral and neurophysiological auditory measures share a common origin in acoustic overexposure (e.g. Sun et al., 2012). In humans and animals, evidence for tinnitus or hyperacusis can arise in the presence of noise-induced threshold elevation, signifying clear cochlear damage, as well as in the absence of obvious peripheral pathology (Kaltenbach et al., 2000; Bauer et al., 2008). Given the dependent

relationship between central neural hyperactivity and reduced input from the auditory periphery (e.g. Mulders et al., 2011), the persistence of these perceptual problems with normal thresholds suggests a role for sub-clinical cochlear damage.

Recent evidence for permanent cochlear neuropathy in the absence of hair cell damage in mice and guinea pigs (Kujawa and Liberman, 2009; Lin et al., 2011b) provides a putative explanation for noise-induced central hyperactivity without threshold shift, and could potentially generate a corresponding hyperacusis- or tinnitus-like behavior. Here we examine these possibilities in noise-exposed mice using reflexive measures of auditory behavior, and examination of the peripheral- and central- dominated portions of the ABR. To validate the auditory-specific nature of the behavioral responses to sound, we also examine responses to non-auditory stimuli and signs of anxiety. And, to dissect the role of cochlear neuropathy from other effects of acoustic overexposure, we compare measurements across three groups of mice: neuropathic noise-exposed, non-neuropathic noise-exposed, and unexposed controls. Evidence for a role of cochlear neuropathy in central hyperactivity and hyperacusis- or tinnitus-like behavior would aid in clarifying the primary source of these perceptual problems, and would thus impact diagnosis, treatment, and prevention.

3. Methods

3.1. Animals

CBA/CaJ male mice were used in order to replicate the primary neuronal degeneration phenotype described previously (Kujawa and Liberman, 2009), and because their cochlear thresholds are stable out to 1 year of age (Ohlemiller et al., 2010). Groups of mice were exposed to high-level noise at 16-18 weeks of age, while cage mates served as unexposed age- and sex-matched controls. Additional groups of mice were subject to restraint stress, with unrestrained age- and sex-matched mice serving as their controls. Importantly, variation in reflexive auditory behavior responses with age, sex, and strain (Paylor and Crawley, 1997; Ison et al., 1998; Ison and Allen, 2007) necessitates matching these characteristics across subjects to reduce potential confounds. All procedures were approved by the Institutional Animal Care and Use Committee of the Massachusetts Eye and Ear Infirmary and conform to the guidelines of the Society for Neuroscience.

3.2. Noise exposure

Mice were exposed to octave-band noise (8-16 kHz) for 2 hours at either 100 or 94 dB SPL. Exposures were performed in a small reverberant chamber with an elevated platform in the center where mice were placed, awake and unrestrained, in an acoustically transparent wire cage. The noise waveform was generated digitally using a fifth-order Butterworth filter, amplified (Crown Power Amplifier D75A), and delivered by a compression driver (JBL Model 2446H)

coupled to an exponential horn in the roof of the chamber. Sound levels were verified in the center of the cage with a ¼" Bruel and Kjaer condenser microphone before each exposure and varied by less than 1 dB in the space the cage occupied.

3.3. Physiology: DPOAE and ABR

Auditory brainstem response (ABR) and distortion product otoacoustic emissions (DPOAEs) were measured in the left ears of noise-exposed mice and unexposed controls to assess cochlear function. Most animals were tested at 10-14 days post-exposure, while a subset of mice were also tested at 1 day, 3 days, 6 weeks or 10 weeks post-exposure. For testing, animals were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg) via intraperitoneal injection. Sound was delivered to the ear, and DPOAEs measured from the ear canal, using a custom closed-field acoustic assembly containing two miniature dynamic speakers and an electret condenser microphone (Knowles FG-23329-P07) coupled to a probe tube. The acoustic assembly was calibrated with a ¼" Bruel and Kjaer condenser microphone, and in-ear calibrations were performed before each recording. Custom LabVIEW software controlling National Instruments 16-bit soundcards (6052E) generated all ABR/DPOAE stimuli and recorded all responses.

For DPOAEs, the cubic distortion product $2f_1-f_2$ was measured in response to primaries f_1 and f_2 (frequency ratio $f_2/f_1 = 1.2$, and level ratio $L_1 = L_2 + 10$), where f_2 varied from 8-45.3 kHz in half-octave steps. For each f_2 frequency, L_2 was swept from 10-80 dB SPL in 5 dB steps. Pressure measurements in the ear canal were averaged using spectral and waveform averaging before determining the amplitude of the $2f_1-f_2$ component in dB SPL. Interpolated $2f_1-f_2$ amplitude functions were used to calculate DPOAE threshold using an iso-response criterion of 5 dB SPL.

ABRs were recorded differentially between needle electrodes at the vertex and at the ventral edge of the pinna, with a ground at the base of the tail. Waveforms were measured in response to 4 ms tone pips ($0.5 \text{ ms } \cos^2$ rise-fall) with alternating polarity at a rate of 40/s. Tone pips at 11.3 and 32 kHz were swept in level from 15-80 dB SPL in 5 dB steps. Average waveforms from 512 presentations were amplified 10,000x, bandpass filtered from 0.3-3 kHz, and stored for offline analysis. Threshold was determined by visual analysis of stacked waveforms from highest to lowest SPL, where threshold was the lowest level at which a reproducible peak or trough appeared. Average waveforms at 80 dB SPL from control animals were used to guide visual analysis. Wave 1 amplitude was defined as the difference between a 1 ms average of the pre-stimulus baseline and the wave 1 peak, after additional bandpass filtering using a first-order Butterworth filter from 0.2-10 kHz. For all analyses, measurements of supra-threshold ABR represent the average wave 1 amplitude across presentations of 60, 70, and 80 dB SPL.

3.4. Histology: synaptic ribbon counts

Animals were intracardially perfused with 4% paraformaldehyde while deeply anesthetized, and the inner ears were extracted and post-fixed for 2 hours at room temperature. Ears were decalcified in EDTA, and the cochlear spiral was microdissected into 6 pieces. Cochlear whole mounts were triple-immunostained with primary antibodies overnight at 37°C against the following: 1) CtBP2 to visualize synaptic ribbons (mouse anti-CtBP2 at 1:200, BD Transduction Labs), 2) Na⁺/K⁺-ATPase to visualize primary afferent terminals (goat anti-NKA α 3 at 1:100, Santa Cruz #sc-16052), and Myosin VIIa to visualize inner hair cells (IHCs) (rabbit anti-Myosin VIIa at 1:200, Proteus Biosciences #25-6790). Secondary antibodies were applied for 1 hour at 37°C as follows: biotinylated donkey anti-mouse (1:200) followed by streptavidin conjugated Alexafluor 568 (1:1000); Alexafluor 488-coupled chicken anti-goat (1:1000) followed by Alexafluor 488-coupled goat anti-chicken (1:1000); and Alexafluor 647-coupled donkey anti-rabbit (1:200).

Pieces were imaged at low power and a custom ImageJ plugin was used to create a frequency map. Confocal microscopy (Leica TCS SP2) was used to image the whole mounts at frequency locations of 11.3 and 32 kHz using an oil-immersion 100X objective (1.4 N.A.) at 2X digital zoom and a z-step of 0.25 μ m. Each frequency location was imaged in two adjacent regions, with approximately 10 IHCs per region, for a total of roughly 20 IHCs per location, per ear. Z-stacks of the IHC base were captured, taking care to include all synaptic ribbons, and then analyzed offline using Amira (Visage Imaging). Individual ribbons were isolated within Amira using the *connected components* function and *isosurface* tools, counted, and expressed as synaptic ribbons per number of IHCs in the stack.

3.5. Reflex modification audiometry: ASR and PPI

Acoustic startle response (ASR) and prepulse inhibition (PPI) of ASR were measured in noise-exposed mice and in unexposed, age-matched controls at a variety of post-exposure survivals from 1 day to 10 weeks. These reflexive behavioral measures of sensory processing are based in brainstem neural circuits that generate rapid whole-body responses to sound (Koch and Schnitzler, 1997; Fendt et al., 2001). The ASR and PPI are thought to be involved in protective reaction to, and pre-attentive processing of, relevant environmental auditory stimuli (for review, see Fillion et al., 1998), respectively, and as they are reflexive they require no training (Hoffman and Ison, 1980).

All ASR and PPI tests were conducted in a table-top sound isolation booth (Mac2, IAC) lined with acoustic foam panels to reduce acoustic reflections. Mice were placed in custom, acoustically transparent cages (7 x 5 x 4 cm) with an elliptical floor designed to restrict explorative behavior, while still allowing full expression of the whole-body startle response. Each cage was placed on a cantilevered armature designed to couple vertical cage motion to an accelerometer mounted at the base of the armature. An array of three speakers (Fostex FT17H

Horn Super Tweeter) was mounted around the cage to present startle stimuli via one speaker (above the cage), and the prepulse and background stimuli via two speakers (on either side of the cage). All ASR and PPI stimuli and responses were generated and recorded with custom LabVIEW software running on a 24-bit PXI (National Instruments).

ASR and PPI tests were conducted either in quiet or in the presence of continuous broadband noise (BBN) at 60 dB SPL. Startle stimuli were tone- or noise-bursts, 20 ms in duration with 0.1 ms rise-fall times (Ison and Allen, 2007). For all PPI tests, startle stimuli were BBN bursts at 105 dB SPL. For acoustic PPI tests, the prepulse was 50 ms in duration: tone-burst prepulses had 5 ms rise-fall ramps, whereas gap prepulses had 0.1 ms ramps (Turner et al., 2006). Tone- or noise-burst prepulses and gap prepulses were positioned to terminate immediately (0 ms) before startle onset. For gap PPI tests, the prepulse was a gap in an otherwise continuous carrier at 60 dB SPL. The carrier was either BBN or 0.5-octave bandpass noise centered at frequencies from 5.6-45.3 kHz in 0.5-octave steps. Bandpass filtering of the carrier was performed using cascaded high-and low-pass eighth-order Butterworth filters to achieve a 48 dB/octave roll-off (Turner et al., 2006). For visual PPI tests (Aubert et al., 2006), the prepulse was a 50 ms light burst (~1000 lux) from white LEDs on both sides of the startle cage, with a delay of 100 ms between light offset and startle onset. Light PPI tests were conducted either in quiet, in continuous BBN, or in continuous 0.5-octave bandpass noise centered at 11.3 kHz.

Each ASR and PPI test consisted of 11 blocks of trials, where the first block was excluded from analysis to reduce the effects of short-term habituation (Simons-Weidenmaier et al., 2006). Within each block, each varied parameter was presented once, in randomized order. For ASR and tone- or noise-burst PPI tests, each level was presented once per block, and PPI tests included an additional two startle-only ("baseline") trials in each block. For gap PPI or light PPI, each block consisted of one prepulse trial and two baseline startle trials. For all tests, the interval between trials varied randomly between 15-25 s. Each mouse performed, on average, a total of 15 different ASR and/or PPI tests in randomized order. Mice were tested for up to 3 hours per day, usually 2-3 times per week. Each session began with a two minute acclimatization period in the cage before startle testing began, and all tests were conducted in darkness. The experimenter could observe the mouse during testing via a monitor fed by the output of an infra-red camera.

For each trial, accelerometer output was digitized from 880 ms before, to 120 ms following, startle-stimulus onset. Startle amplitude was calculated as the root-mean-square (rms) of the 100 ms following startle-stimulus onset. In some PPI trials, usually during higher intensity noise-burst PPI trials, the prepulse itself elicited a startle response. Therefore an algorithm was developed to exclude trials from analysis if the response waveform during the prepulse window met two criteria that defined a recognizable startle to the prepulse: 1) one based on rms amplitude of the window relative to the mean rms amplitude of responses in that window across the entire test, and 2) one based on the stereotyped latencies of waveform peaks in the window characteristic of a startle response for our apparatus. Within each test, for a given mouse,

startle amplitudes were averaged across all trials of the same stimulus conditions. PPI was defined as fractional reduction of startle, i.e. 1 minus the ratio of startle amplitude with vs. without prepulse (Ison and Allen, 2007). Thus, a value of 0 means no effect of the prepulse, a value of 1 means complete inhibition of startle, and a negative value indicates prepulse facilitation of the startle response.

3.6. Anxiety: EPM

Exposed neuropathic mice and their non-neuropathic and unexposed controls were assessed for signs of anxiety using the elevated plus maze (EPM) test. In the EPM assay, animals are allowed to freely explore the maze, which includes enclosed and open portions. Animals with increased anxiety or fear spend relatively less time in the open space, and instead restrict their movement to the enclosed areas (e.g. Walf and Frye, 2007). This interpretation of the animal's preferential behavior is supported by observations that administration of anxiogenic, or anxiolytic, compounds can respectively decrease, or increase, the amount of time spent in the open arms (e.g. Pellow et al., 1985). Since repeated exposure to the EPM has been shown to modify the pattern of exploration (Rodgers et al., 1996), naiveté of our mice was ensured by testing each mouse only once, at either 1 day or 5-7 weeks post-exposure. Only data from mice with a full set of whiskers (e.g. not barbered by cagemates) are presented here, given the observation that lack of whiskers prolongs exploration of open arms when tested in dim illumination (Cardenas et al., 2001).

The EPM is comprised of four horizontal beams that join to form a plus-shaped track, with each arm measuring 30 cm in length and 5 cm in width. Two arms are enclosed with 15-cm walls ("closed arms"), while the other two arms have a 1.25-cm lip ("open arms"). The entire track is raised above the ground by 0.9 m. The maze was constructed from polyethylene and acrylic panels for ease of cleaning with Nolvasan disinfectant between mice. Mice were placed individually in the center of the EPM, in dark, and allowed 5 minutes to explore (Walf and Frye, 2007). The animal's movement was captured at a rate of about 6 frames/s with an infrared-capable webcam (Kinamax) attached to the ceiling directly over the maze. Additional infrared LEDs placed next to the camera facilitated video capture and tracking. The movement was tracked as the location of the center of the animal's body over time, using custom software written in C# running on a 24-bit PXI (National Instruments), converted to coordinates relative to the field of view (in cm) and stored for offline analysis. Two measures were calculated from the animal's path: the total number of entries into either of the two open arms, and the total entries into either closed arm, a reduction in which would represent increased anxiety, or reduced locomotor/general activity, respectively (Rodgers and Johnson, 1995).

A subset of mice were restraint-stressed one day before EPM testing using a whole-body restraint technique that has been shown to reliably increase levels of stress hormones and stress-related proteins in mice (Wang and Liberman, 2002; Tahera et al., 2006; Peppi et al., 2011). Each mouse was placed in a 50 ml centrifuge tube (3 by 11 cm) that was perforated with

5mm distributed holes to allow airflow, and remained immobilized in the tube for 2 hours, matching the duration of the noise exposure protocol. The next day, mice were assessed on EPM behavior along with age- and sex-matched controls.

All statistical comparisons, across all tests, were performed using a non-parametric Kruskal-Wallis test (p-value criterion of 0.05) followed by a test of multiple comparisons with Dunn-Sidak correction to reveal significantly different pairs of exposure groups.

4. Results

4.1. Neuropathic vs. non-neuropathic ears

Mice exposed to octave-band noise at 100 dB SPL for 2 hours show a large threshold shift 1 day post exposure, followed by complete threshold recovery within 1-2 weeks (Kujawa and Liberman, 2009). Although cochlear thresholds (and hair cells) recover, there is significant degeneration of cochlear neurons, and a commensurate reduction in the amplitude of supra-threshold cochlear neural potentials, such as wave 1 of the auditory brainstem response (ABR) (Kujawa and Liberman, 2009).

Here, we study the effects of this noise-induced primary neuropathy on auditory behavior, as measured with the acoustic startle response (ASR) and prepulse inhibition (PPI) of startle. To control for extra-auditory effects of high-level noise exposure, we sought to define a “non-neuropathic” exposure, which might cause a similar degree of temporary threshold shift (TTS) (and systemic stress), yet be fully reversible with respect to both hair cell and neuronal function. As shown in Fig. 3A, 2 hour exposures at either 100 or 94 dB SPL both caused a severe TTS (up to 40 dB) in distortion product otoacoustic emissions (DPOAEs), primarily a metric of outer hair cell function (Lonsbury-Martin and Martin, 2003). Similarly, exposure caused temporary shifts of up to 50 dB SPL in ABR thresholds (Fig. 3B). The maximum threshold shifts moved to higher frequencies as exposure level increased, consistent with known level-dependent nonlinearities in cochlear mechanics (Cody and Johnstone, 1981; Robles and Ruggero, 2001). Both exposures also show almost complete DPOAE and ABR threshold recovery when measured as soon as 1 week later.

To assess neuropathy, the amplitude of ABR wave 1 (Fig. 3C), representing the summed activity of cochlear nerve fibers, was measured in response to tone bursts at 11.3 and 32 kHz. Based on prior study of the 100 dB exposure (Kujawa and Liberman, 2009), we expect a large mean amplitude reduction at 32 kHz and very little at 11.3 kHz, as was observed in our 100 dB exposure group (36% vs 7%, respectively) (Fig. 3C, red). Animals exposed at 94 dB (Fig. 3C, blue) showed a smaller reduction in mean wave 1 amplitude at 32 kHz: 15% reduced re controls vs. 36% in the 100 dB group. The later ABR waves, reflecting sound-evoked activity in higher brainstem centers, are not attenuated in the 100 dB group; in fact, wave 5 appears to be

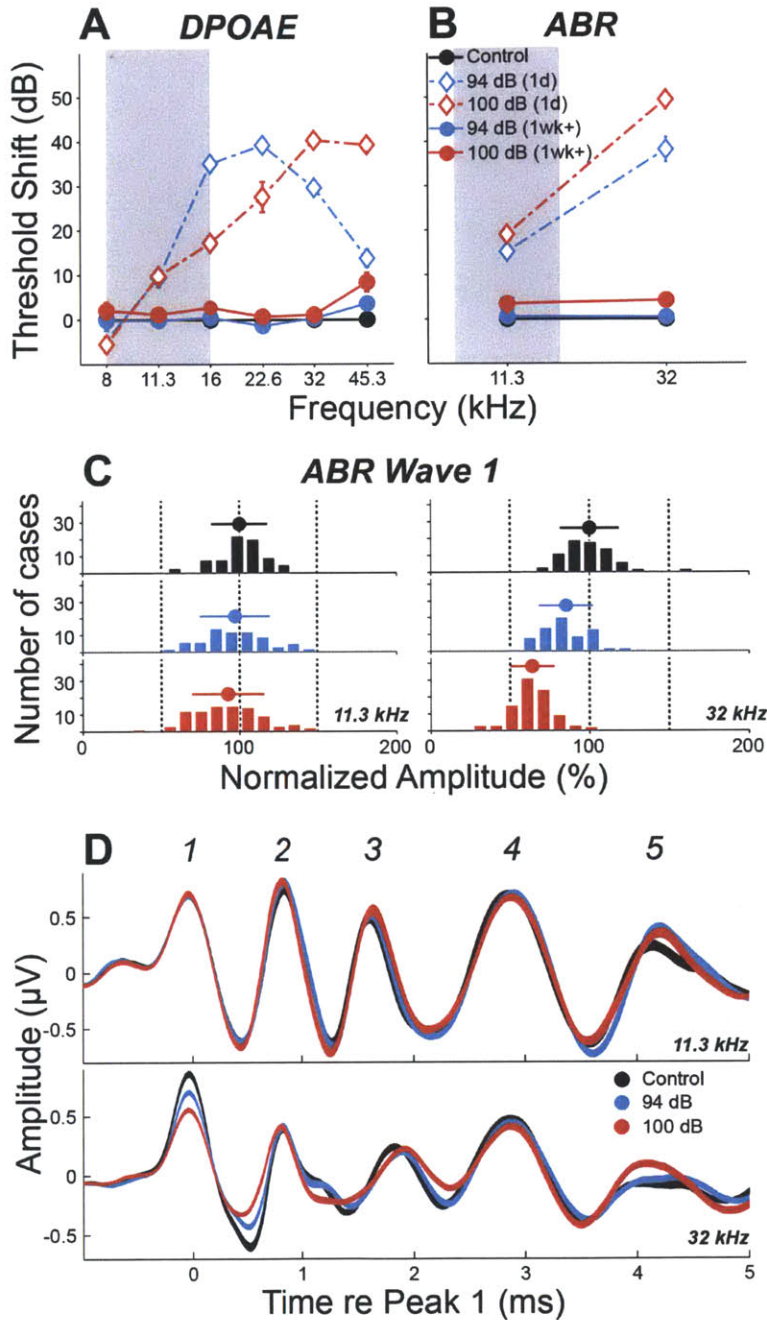


Figure 3: Exposure to octave-band noise causes temporary threshold shifts and permanent reduction in ABR wave 1, especially for the 100 dB exposure (red) and especially at high frequencies. Color key in **B** applies to all panels. **A, B:** Mean threshold shifts (\pm SEMs) by DPOAEs (**A**) or ABRs (**B**) as measured one day (open diamonds) or 1-10 weeks (filled circles) post-exposure. The noise exposure band is indicated by shading. At 1 day post-exposure, $n = 8-9$ mice per group; at 1 week and beyond, $n = 74-93$ samples per group. **C:** Baseline-to-peak amplitudes of ABR wave 1, at 11.3 or 32 kHz, for the same animals as in **A** and **B**, measured 1-10 weeks post exposure. Mean values (\pm 1 SD) are shown above each histogram. **D:** Average ABR waveforms for 11.3 or 32 kHz (at 80 dB SPL), as measured 1-2 weeks post-exposure. Individual waveforms were aligned by peak-I latency before averaging. Data are from a subgroup of the same animals shown in **A** and **B**, $n = 52-65$ samples per group. Waves are labeled 1-5.

enhanced at 32 kHz at 1-2 weeks post-exposure (Fig. 3D) suggesting possible neuronal hyperactivity in central auditory pathways.

To more directly assess the loss of neurons in the inner hair cell (IHC) area (Fig. 4A), we selected for histological analysis three animals from each exposure group with ABR amplitudes near the group mean. Synaptic ribbons, which are easily counted in immunostained cochlear tissue (Fig. 4 B,C,D), provide a reliable proxy for nerve terminal degeneration after acoustic injury (Kujawa and Liberman, 2009). Ribbon counts (Fig. 4E) showed a large and highly significant reduction re controls (45%) at 32 kHz in the 100 dB group ($p = 0.0026$), and a smaller yet significant reduction at 11.3 kHz (15%, $p = 0.0072$). In contrast, the slight reductions in mean ribbon counts in the 94 dB group were not statistically significant at either frequency. Together, the histology and physiology suggest that the 100 dB and 94 dB exposures achieve the intended contrast between neuropathic and non-neuropathic conditions. The neuropathy after the 100 dB exposure is “primary” in the sense that nerve terminals and synapses disappear from surviving hair cells.

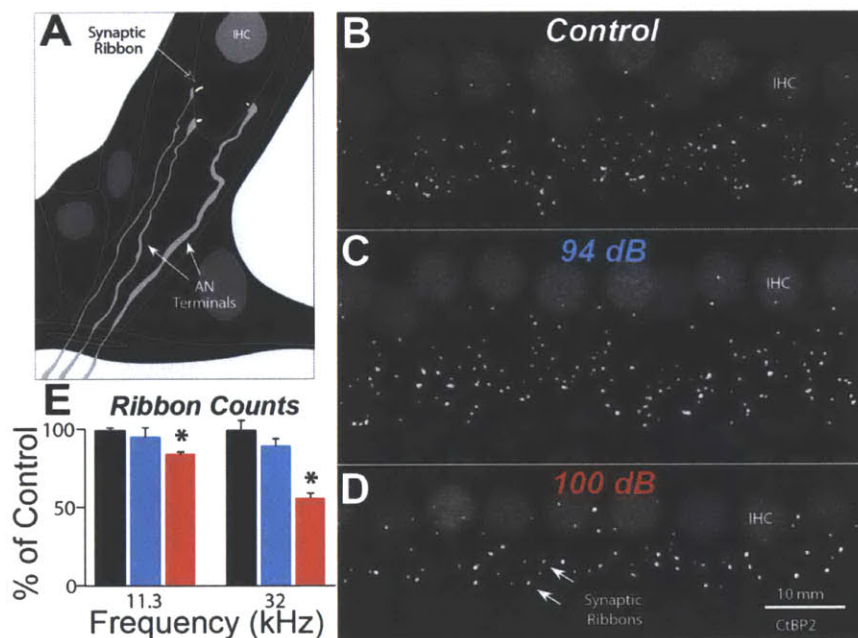


Figure 4: Exposure to octave-band noise at 100 dB causes cochlear neuropathy, while 94 dB does not. **A:** Schematic of the base of an IHC showing 3 of the ~20 cochlear nerve terminals, and their presynaptic ribbons. **B,C,D:** Maximum projections from confocal z-stacks of the IHC area in the 32 kHz region, immunostained for CtBP2 to show synaptic ribbons. The IHC nuclei are also faintly stained. **E:** Mean counts of pre-synaptic ribbons (\pm SEMs) in IHCs, based on confocal z-stacks such as those in **B-D**. Data are extracted from two stacks at each cochlear frequency region in each animal. For each exposure group, 3 animals were chosen with ABR amplitudes near the mean for that group. The 94 and 100 dB groups were studied 11 weeks post-exposure; controls were age-matched. Asterisks indicate significant difference from controls (see text for p values).

4.2. PPI and hearing in noise

Central processing of threshold and supra-threshold stimuli was assessed using acoustic PPI. Prepulses were tone- and broadband noise- (BBN) bursts, measured both in quiet and in a continuous noise background (see insets to Fig. 5B,E).

In a quiet background, the PPI growth functions in control animals (black symbols in Fig. 5A-C) suggest that the prepulse tone bursts at 11.3 (Fig. 5A) or 32 kHz (Fig. 5B) can modulate the startle response at sound pressures as low as 25 dB SPL. The similarity of these PPI “thresholds” to cochlear nerve fiber thresholds (Taberner and Liberman, 2005) and to behavioral thresholds (Radziwon et al., 2009) in the same mouse strain (CBA/CaJ) suggests that PPI can be used to estimate the behavioral audiogram. Addition of background noise should elevate behavioral thresholds for tones, and indeed, PPI thresholds for control mice were elevated by ~20-30 dB by addition of continuous noise at 60 dB SPL (Fig. 5D-F).

Mice with primary neural degeneration (i.e. the 100 dB exposure group) might be expected to show reduced PPI in the presence of background noise, given that the exposure-induced neural loss is selective for the high-threshold cochlear nerve fibers (Lin et al., 2011a) that are normally particularly resistant to masking by continuous background noise (Costalupes et al., 1984). Paradoxically, the neuropathic mice showed significantly greater PPI than controls for 65-75 dB SPL prepulses at both 11.3 and 32 kHz ($p \lll 0.001$; Fig. 5D,E). Even without background noise, PPI for 65-75 dB prepulses was slightly yet significantly enhanced at 32 kHz in the neuropathic group compared to controls ($p = 0.0007$) (Fig. 5B).

PPI enhancement in the neuropathic ears cannot be due to the noise exposure *per se*, because responses of the non-neuropathic group to high-level prepulses did not differ significantly from controls, under any stimulus condition (Fig. 5, blue). Nor is PPI enhancement related to the small residual threshold shift seen in some ears exposed at 100 dB. When the neuropathic (100 dB) group is divided into those with full threshold recovery vs. those with residual shift, both subgroups showed PPI enhancement (Fig. 6A). The PPI enhancement does appear to depend on the degree of cochlear neuropathy. When the 94 dB group is divided into subgroups based on ABR amplitude, the cases with greater reduction in wave 1 show greater PPI (Fig. 6B).

To test whether the hyper-responsivity suggested by the enhanced PPI in neuropathic mice was specific to the auditory pathway, we implemented a cross-modal PPI test using a visual prepulse stimulus preceding the acoustic startle. With appropriate stimulus parameters, a visual stimulus can also suppress startle amplitude (Hoffman and Ison, 1980; Aubert et al., 2006). Using this assay we found no evidence for generalized sensory hyper-responsiveness in the neuropathic mice when compared either to the unexposed controls or to the non-neuropathic exposed mice (Fig. 7A, $p = 0.46$; Fig. 7B, $p = 0.57$; Fig. 7C, $p = 0.13$).

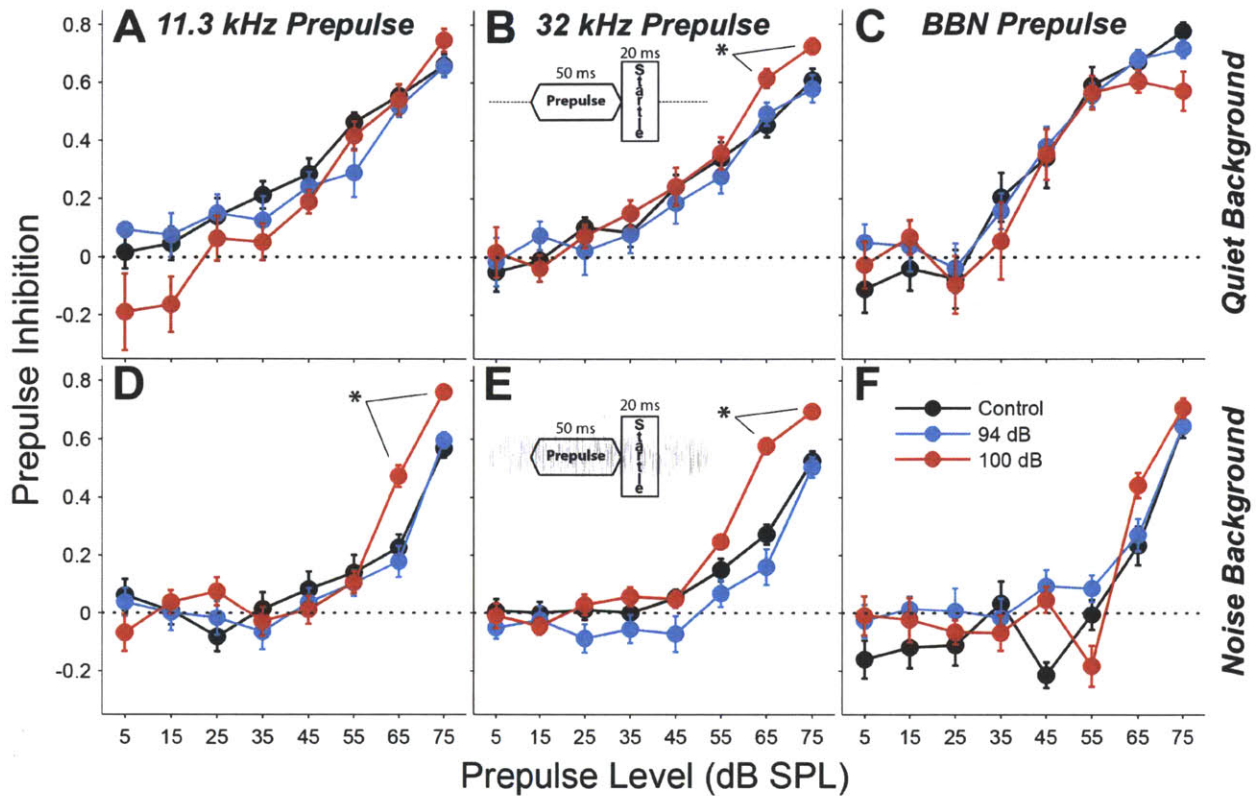


Figure 5: Prepulse inhibition of the acoustic startle is enhanced in mice with noise-induced neuropathy (100 dB exposure). Growth of PPI as a function of level is shown for 11.3 kHz (**A,D**), 32 kHz (**B,E**), or for broadband noise (**C,F**) prepulses. Means (\pm SEMs) are shown (key in panel F applies to all panels). For tone-burst prepulses (**A,B,D,E**), $n = 13-31$ mice per group; for broadband prepulses (**C** and **F**), $n = 9-13$ mice per group. D-F are the same as A-C except that, for the former, PPI is measured in the presence of continuous background noise at 60 dB SPL (compare schematic insets in panels **B** and **E**). Data were obtained at 1-10 weeks post-exposure. Asterisks indicate 100 dB group (red) is significantly different from controls (see text for p values).

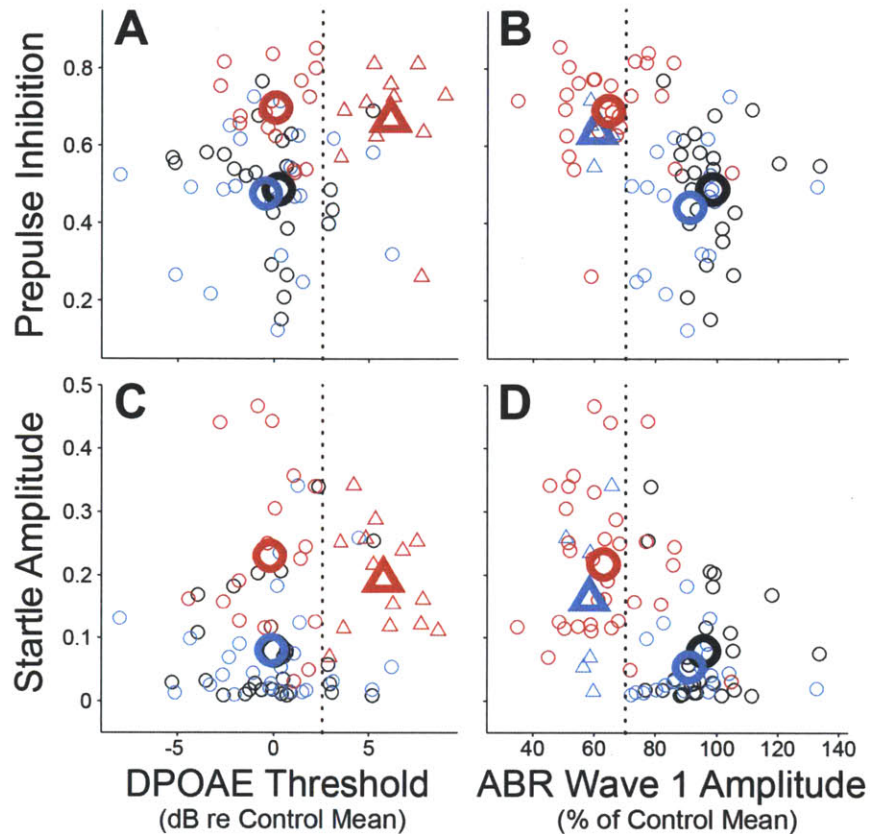


Figure 6: Enhanced prepulse inhibition and acoustic startle response correlate with ABR wave 1 amplitude, not with cochlear thresholds. PPI (A,B) and ASR (C,D) were elicited by 32 kHz stimuli in 60 dB noise, at prepulse level 75 dB or startle level 90 dB, respectively. **A** and **C**: The neuropathic (100 dB) group was divided according to DPOAE threshold: below (red circles) vs. above (red triangles) the group mean (dotted line). Mean PPI (**A**) and ASR (**C**) are shown for each group/subgroup by the bold symbols. **B** and **D**: The non-neuropathic (94 dB) group was split according to ABR wave 1 amplitude: more (blue circles) or less (blue triangle) than 0.5 SD above the 100 dB group mean (dotted line). Mean PPI (**B**) and ASR (**D**) are shown for each group/subgroup by the bold symbols. DPOAE threshold (**A,C**) in each case is averaged across 8-45.3 kHz and expressed re control mean. ABR wave 1 amplitude for 32 kHz tone pips (**B,D**) is calculated as in Fig. 1C. Data are from 1-10 weeks post-exposure (controls are age-matched). Color key from previous figures applies here.

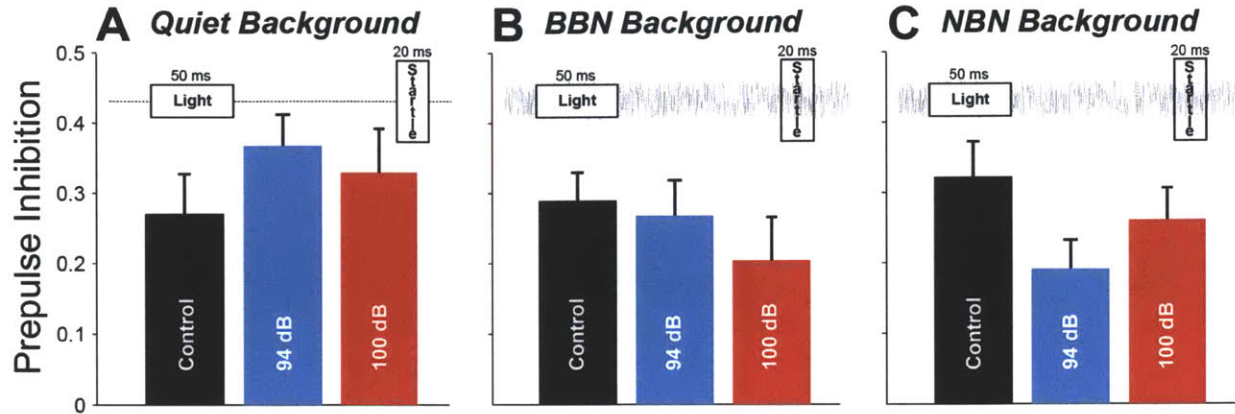


Figure 7: Light-evoked prepulse inhibition of the acoustic startle is not altered for mice with noise-induced neuropathy compared to unexposed or non-neuropathic controls. Mean PPI values (\pm SEMs) in response to a 50 ms light burst are shown for quiet (A) and broadband noise (B) backgrounds, and for a background noise of a half-octave band centered at 11.3 kHz (narrowband noise, NBN) (C) (compare schematic insets across panels). Data were obtained 6-10 weeks post-exposure ($n = 17-21$) samples per group per condition.

4.3. ASR and hyperacusis-like behavior

Because PPI measures are expressed as a fractional alteration in startle magnitude, changes in the startle response itself could complicate the interpretation. Indeed, PPI varies with baseline startle magnitude in both humans and animals (Yee et al., 2005; Csomor et al., 2008). Thus, we measured the thresholds and growth of the ASR, without PPI, in the three groups of mice.

Growth of ASR with level was measured for tonal and BBN startle stimuli, in quiet and in a continuous noise background (see insets to Fig. 8C,F). Startle responses to BBN-bursts at 105 dB SPL (Fig. 8C,F), the startle stimulus used in all the PPI tests, were similar among groups. In particular, ASR to the 105 dB SPL BBN burst in noise is not significantly different between groups ($p = 0.52$), suggesting that the observed PPI enhancement in noise (Fig. 5D,E) does not arise simply because of an attenuated startle response.

At lower startle levels (75-90 dB), the 100 dB exposure group was hyper-responsive in all stimulus conditions: startle thresholds were reduced, and startle amplitudes were significantly increased re control responses ($p < 0.01$, Fig. 8A-F). Like the PPI results, the enhanced ASR is not due to noise exposure, per se, given that the 94 dB group's responses are indistinguishable from unexposed controls (Fig. 8, blue). Furthermore, the ASR hyper-responsiveness does not correlate with incomplete threshold recovery in the 100 dB group (Fig. 6C), yet does correlate with reduced ABR amplitudes in the 94 dB group (Fig. 6D). This suggests that neuropathy, rather than threshold elevation, is key to the development of this type of hyper-responsivity.

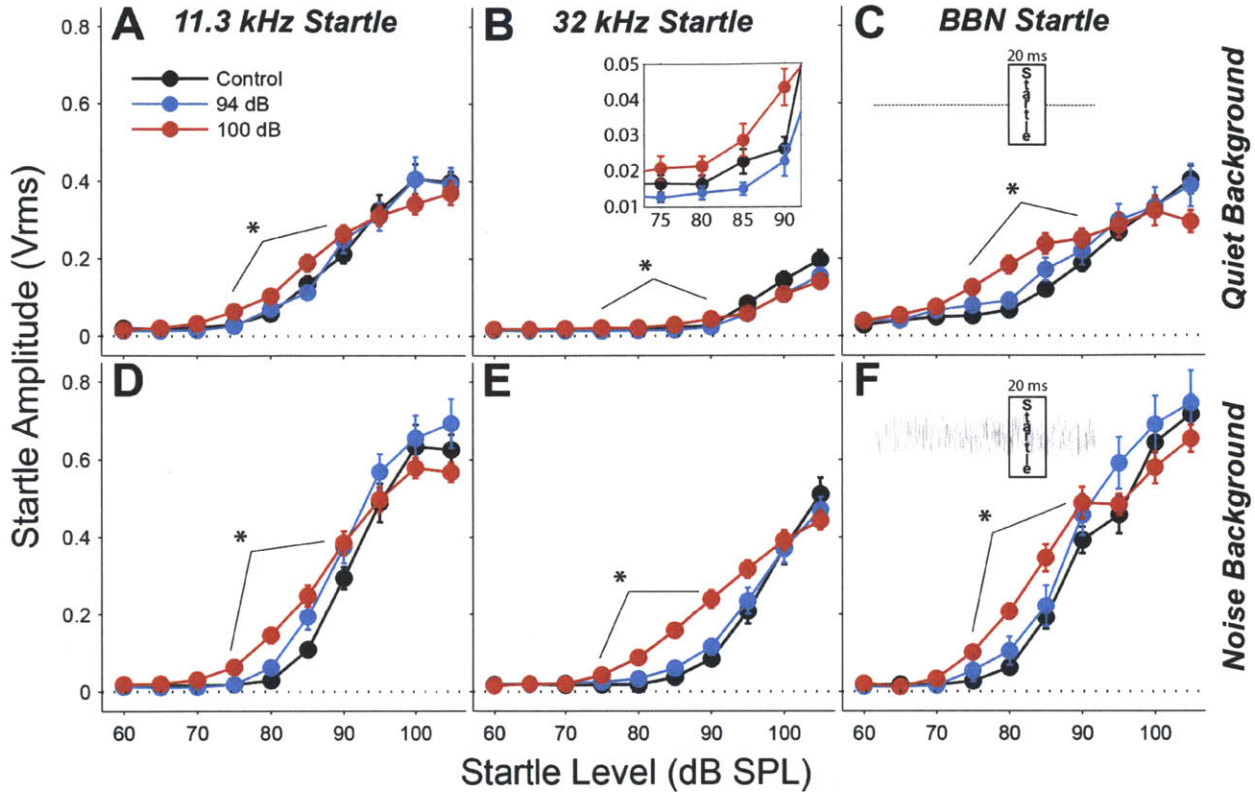


Figure 8: Threshold for the acoustic startle response is reduced in mice with noise-induced neuropathy (100 dB exposure). Growth of startle amplitude with level is shown for startle stimuli at 11.3 kHz (A,D), 32 kHz (B,E), or for broadband noise (C,F) startles. Means (\pm SEMs) are shown (key in panel A applies to all panels). For tone-burst startles (A,B,D,E), $n = 13-37$ mice per group; for broadband prepulses (C and F), $n = 9-17$ mice per group. D-F differ from A-C only by the presence of continuous background noise at 60 dB SPL (compare schematic insets in panels C and F). Inset in B shows the 75-90 dB range of the startle functions in panel B on a different scale. All data were obtained at 1-10 weeks post-exposure. Asterisks indicate 100 dB group (red) is significantly different from controls (see text for p values).

4.4. Gap PPI and tinnitus-like behavior

The gap-prepulse version of the PPI paradigm is now commonly used to probe for the presence of a tinnitus percept in animals (Turner et al., 2006). It is assumed that a gap in an otherwise continuous “carrier” stimulus (see inset to Fig. 9A), will be less detectable if the perceived tinnitus has characteristics of the carrier and “fills” the gap. Gap PPI can be reduced in noise-exposed animals compared to control for narrow-band carriers centered at some frequencies, but not others, (Longenecker and Galazyuk, 2011; Nowotny et al., 2011; Dehmel et al., 2012), suggesting that gap PPI can be used to detect a spectrally restricted tinnitus.

To assess if the hyperacusis-like behavior in our mice was associated with tinnitus-like percepts, we measured gap PPI using broadband or half-octave-band noise carriers centered at multiple frequencies below, within, and above the trauma band (Fig. 9). Mean gap PPI was

significantly reduced in the neuropathic group for gaps embedded in 32 kHz-centered narrowband noise ($p = 0.0048$) and for gaps embedded in BBN ($p = 0.01$) (Fig. 9). Gap PPI was not significantly reduced for the non-neuropathic mice for any carrier. This reduction in gap PPI for the 32 kHz carrier did not correlate with either residual threshold shift or ABR amplitude (Fig. 10).

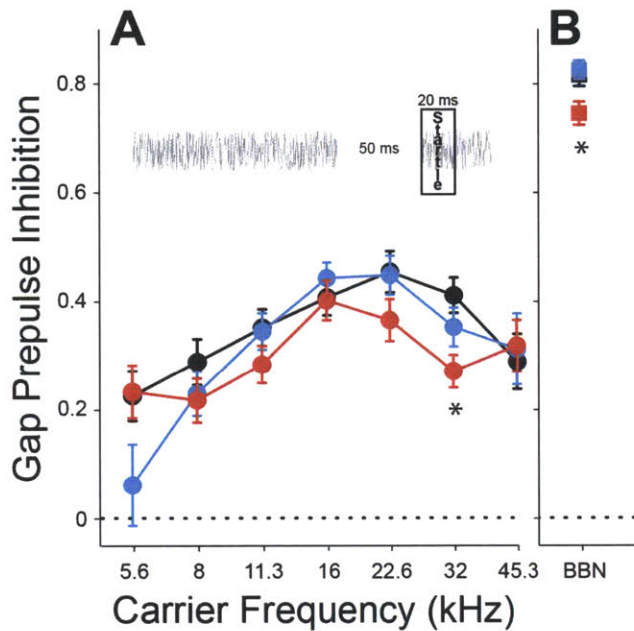


Figure 9: Gap prepulses elicit reduced prepulse inhibition in neuropathic mice for a narrowband-noise gap carrier at 32 kHz, and for a broadband-noise carrier. Mean PPI values (\pm SEMs) are shown for gaps embedded in half-octave carriers centered at 7 log-spaced frequencies (A) and for a BBN carrier (B). Data were obtained from 1-10 weeks post-exposure, $n = 19-40$ samples per group for each carrier condition. Asterisks indicate 100 dB group (red) is significantly different from controls (see text for p values). Color key from previous figures applies here.

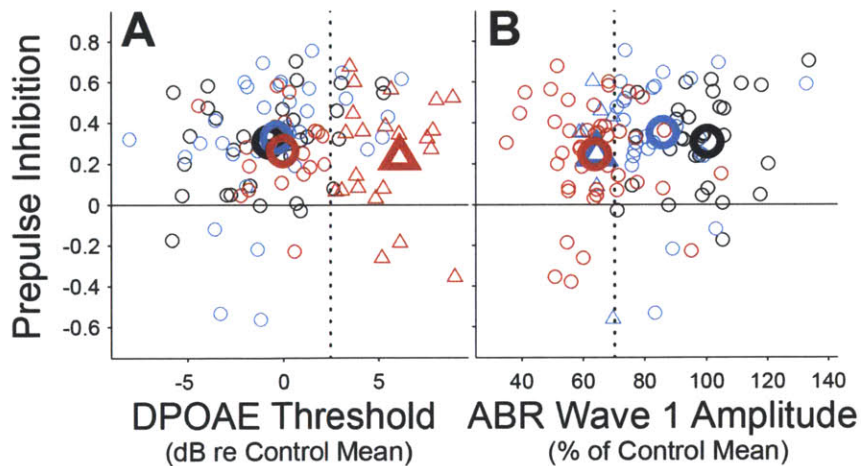


Figure 10: Gap prepulse inhibition does not correlate with either cochlear thresholds or ABR wave 1 amplitude. Gap PPI was elicited by 50 ms gaps in continuous, half-octave narrowband noise centered at 32 kHz and presented at 60 dB SPL. Note that some responses show prepulse facilitation of startle (PPI < 0). Data are from 1-10 weeks post-exposure (controls are age-matched), and are coded and split into groups as in Fig. 6. Color key from previous figures applies here.

4.5. Onset and duration of auditory behaviors

The behavioral data considered thus far were averaged over post-exposure survivals ranging from 1 to 10 weeks. In considering underlying mechanisms, it is informative to examine the onset and offset time courses of the hyper-responsive behavior in the neuropathic exposure group. As shown in Figure 11A, ABR threshold recovery is complete by 1 week post-exposure, by which time ABR wave 1 amplitude has also reached asymptote (Fig. 11B). ABR wave 5 amplitude is initially reduced during the period of TTS, but eventually recovers to, and at some time points exceeds, control responses (Fig. 11C). The enhancements of tone-burst PPI (Fig. 11D) and of ASR (Fig. 11E) are not significant 1 day after exposure ($p = 0.61$ and $p = 0.25$, respectively), when thresholds are still greatly elevated, but appear within 1 week ($p \ll 0.01$) and show no further significant changes, at least out to 10 weeks post-exposure. Gap PPI is consistently reduced for up to 10 weeks post-exposure (Fig. 11F). Overall, the longitudinal data suggest that noise-induced changes in behavior in the neuropathic group appear within days of exposure and are stable for at least 10 weeks.

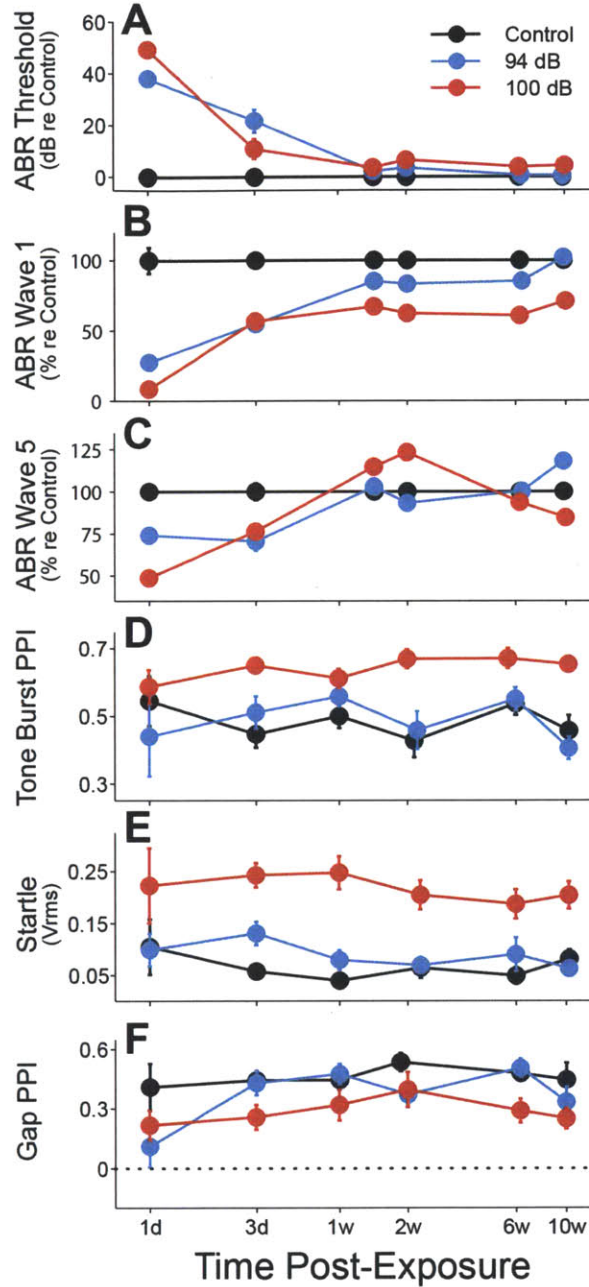


Figure 11: After initial threshold stabilization (~1 week), performance on all startle-based tests was stable with increasing post-exposure time. Threshold (A), wave 1 amplitude (B), and wave 5 amplitude (C) of ABRs at 32 kHz are normalized re control means. Wave 1 amplitudes are calculated as in Fig. 1C. Wave 5 is the rms amplitude of the 80 dB SPL waveform from 4.65-6 ms re stimulus onset. Tone burst PPI for 32 kHz prepulses at 75 dB (D) and tone burst ASR for 32 kHz startle stimuli at 90 dB (E) were measured in 60 dB background noise. Gap PPI (F) is shown for a half-octave band noise carrier centered at 32 kHz. Mean values (\pm SEMs) are shown. Post-exposure times are grouped logarithmically and plotted at the mean time for each group.

4.6. EPM and noise-induced anxiety

To rule out, or rule in, the possibility that the enhancement of ASR and PPI observed in the neuropathic mice reflects increased fear or anxiety, as opposed to a change specifically in auditory processing, a subset of exposed and unexposed mice was assessed for differences in behavioral activity using the elevated plus maze (EPM). In this assay, a decrease in the number of open-arm entries suggests an increase in anxiety or fear (Walf and Frye, 2007).

At one day post-exposure, mean number of open-arm entries was slightly, but not significantly ($p = 0.36$), reduced for neuropathic mice compared to controls and to non-neuropathic mice (Fig. 12A), and the number of closed-arm entries was comparable across groups (Fig. 12E), suggesting no change in general locomotor behavior following exposure (Rodgers and Johnson, 1995). At about 6 weeks post-exposure, open- and closed-arm entries were similar across groups (Fig. 12B,F). Two additional sets of unexposed mice were tested in potentially stressful conditions unrelated to noise exposure, for comparison. In the first cohort, half the mice were subject to restraint stress one day before EPM. Mean number of open- and closed-arm entries was comparable between restraint-stressed mice and their controls (Fig. 12C,G). In the other cohort, half the mice were tested in dark, and the other in light. Light-tested mice made significantly fewer open-arm entries compared to dark-tested mice ($p = 0.029$) (Fig. 12D), consistent with reports of the anxiogenic effects of high illumination in the EPM (Pereira et al., 2005).

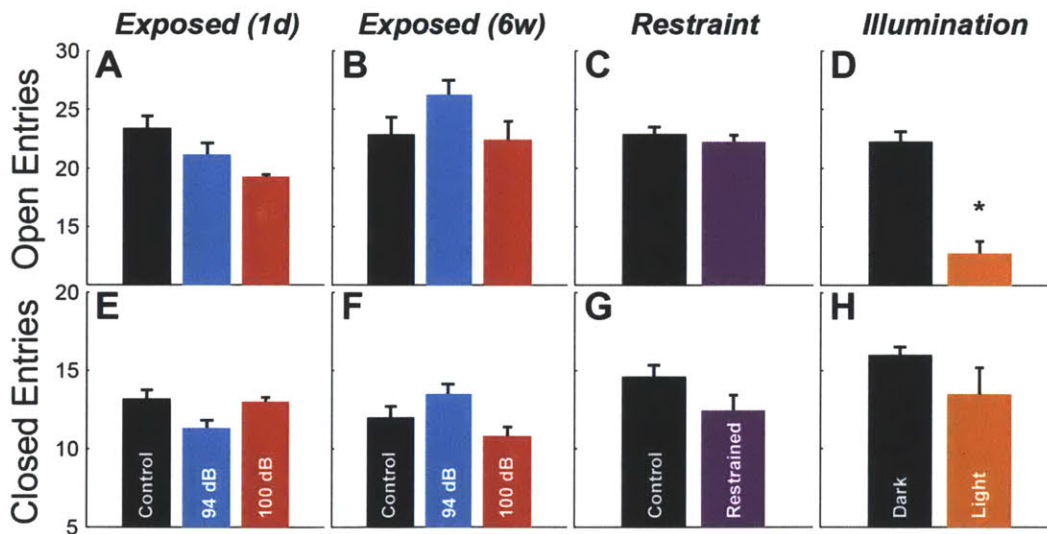


Figure 12: Noise-exposed mice do not exhibit signs of anxiety in the elevated plus maze assay. Mean (\pm SEMs) number of entries into open arms during a 5-minute exploration of the EPM is shown (A-D) along with mean (\pm SEM) number of entries into closed arms during the same session (E-H). Data for noise-exposed mice and their controls were obtained 1 day (A,E) and 6 weeks (B,F) post-exposure ($n = 4-7$ mice per group). Data for restraint-stressed mice and their controls were obtained 1 day after restraint (C,G) ($n = 6-7$ mice per group). Data for light- vs dark tested mice (D,H) were obtained from 4 animals per group. Asterisk indicates performance in light was significantly different from in dark.

5. Discussion

5.1. Cochlear nerve degeneration as an elicitor of central hyperactivity

Noise-exposed mice with cochlear neuropathy, evidenced by concurrent reductions in synaptic ribbon counts (Fig. 4E) and in ABR wave 1 amplitude (Fig. 3C), maintained otherwise normal ABR waveform morphology and amplitudes of later waves, including amplitude enhancement at some post-exposure times (Fig. 3D). Lesioning of different central auditory nuclei, and analysis of their normal response latencies, confirm that the ABR is a summed, synchronous response from the cochlear nerve and its targets in the brainstem and midbrain in cat (Melcher and Kiang, 1996) and in mouse (Henry, 1979). Detailed examination of cellular subpopulations in the cat lesioning study reveals that this response is dominated by the bushy cell pathway, which begins with cochlear nerve input to spherical and globular bushy cells in the VCN, followed by projections to the superior olivary complex (SOC), and its connections to the lateral lemniscus and IC. In the neuropathic group, waves 2-4, generated largely by VCN and SOC (Melcher and Kiang, 1996), showed normal amplitudes despite the wave 1 decrement (Fig. 3D), and wave 5, corresponding with IC activity (Melcher and Kiang, 1996), even showed enhancement. These observations of normalcy and enhancement of the ABR waveform despite noise-induced cochlear nerve degeneration suggest an increase in the “gain” of these cellular populations, perhaps to compensate for the loss of input from the periphery. This increase in central activity could signify a re-equilibration of excitatory and inhibitory influences in the VCN and, specifically, the bushy cell pathway.

5.1.1. Potential mechanisms of central hyperactivity

Several lines of evidence, based on neurophysiological and histological data, suggest that a generalized explanation for the increase in neural excitability following noise-exposure is a shift in the balance of neural excitation and inhibition, subsequent to peripheral de-afferentation. Single-unit frequency-response areas recorded in the brainstem and midbrain typically comprise excitatory and inhibitory regions that shape tuning, yet tuning in DCN and IC units can be broadened after noise trauma (Ma and Young, 2006; Grécová et al., 2009). In principle, this broadened tuning could be inherited from reduced frequency selectivity in the periphery as a result of OHC damage (Liberman and Dodds, 1984b). However, broader tuning of IC units following noise exposure at an early age has been observed in the presence of normal ABR thresholds, suggesting no hair cell damage (Grécová et al., 2009). Furthermore, a significantly smaller number of IC units with inhibitory sidebands was observed in the exposed animals, (Grécová et al., 2009), reinforcing the loss of inhibition as a central mechanism for broadened tuning.

At the level of neural transmission, despite a widespread decrease in cell density in VCN (Gröschel et al., 2010), and even total volume of VCN (Kraus et al., 2011), there is evidence for significant regrowth of synaptic inputs following cochlear de-afferentation. Loss of excitatory,

cochlear nerve connections is visible within one week following de-afferentation. This is evidenced by reduced VGLUT1 labeling in AVCN and PVCN after deafening by intracochlear injection of kanamycin (Zeng et al., 2009), as well as the degenerating appearance of large, excitatory-like terminals in PVCN following acoustic overexposure (Kim et al., 2004). Interestingly, concomitant degeneration of small, presumably glycinergic and GABAergic, inhibitory-like terminals was also observed (Kim et al., 2004). The appearance of GAP-43 labeling in VCN several weeks after noise trauma (Kraus et al., 2011), or within a few days of cochlear ablation (Illing et al., 1997), however, indicates an up-regulation of synaptogenesis, and/or regeneration of input, following de-afferentation. Interestingly, at 10 weeks post-exposure, GAP-43 labeling was strongest in regions of VCN associated with frequencies below the damaged cochlear frequency regions (Kraus et al., 2011). This regional synaptogenesis could still be consistent with a cochlear de-afferentation trigger, given that at long post-exposure survivals, a second wave of synaptic ribbon loss appears more apical to the directly traumatized regions (Kujawa et al., 2011).

Further examination reveals that this neural regrowth in VCN is skewed towards increased excitation, such as greater re-innervation of glutamatergic (excitatory) cell bodies compared to GABAergic (inhibitory) cells (Illing et al., 1997), and greater number of regenerating axosomatic inputs with excitatory profiles (Kim et al., 2004). Consistent with these results is the overall reduction in gene expression of inhibitory neurotransmitters in CN, as well as in IC, following cochlear ablation (Dong et al., 2009). Parallel evidence for reduced GABAergic and glycinergic influence in DCN following noise trauma (Wang et al., 2009; Middleton et al., 2011) suggests that increased excitatory responses likely result from disinhibition, and that disinhibition is common throughout the subcortical auditory pathway following cochlear de-afferentation. These redistributions of excitatory and inhibitory inputs are observed following noise trauma, but also subsequent to administration of ototoxic drugs or mechanical cochlear ablation. From the perspective of the CNS, the common denominator among these methods is the reduction or silencing of cochlear neuronal transmission, thus it is likely that the consequences of any of these techniques could be applicable to the situation of noise-induced primary neuronal loss.

5.1.2. Rapid emergence of central hyperactivity

Enhancement of central activity takes little time to develop: amplitude of local potentials in IC is elevated as soon as 8 hours post-exposure (Salvi et al., 2000), and significant increases in spontaneous firing in DCN is seen within a few days (Kaltenbach et al., 2000). Similarly, we observed greater ASR and PPI within a few days of acoustic overexposure (Fig. 11D,E), indicating that these hyper-responsive behaviors have the potential to arise as a result of this previously described rapid plasticity. Following its induction by peripheral damage, central neural hyperactivity can be reduced by removing the now-aberrant signals from the periphery or suppressing them with efferent activation (Mulders and Robertson, 2009; Mulders et al., 2010), suggesting that the abnormal central responses arise as a consequence of noise-induced changes in the periphery.

For the generation of these enhancements to have root in peripheral changes necessitates that cochlear neuropathy occurs soon after trauma. Indeed, synaptic ribbon loss following the neuropathic exposure is evident within one day (Kujawa and Liberman, 2009) and even immediately following trauma (Liberman, unpublished observations). Similarly, vacuolization of cochlear nerve terminals appears as soon as a few hours post-exposure, signaling excitotoxicity, and foreshadowing neuronal death for some fibers (Robertson, 1983; Wang et al., 2002b; Kujawa and Liberman, 2009). Importantly, this swelling of nerve terminals during the TTS window has been observed even for the lowest exposure level tested (94 dB) (Wang et al., 2002b), suggesting that the non-neuropathic group in the current study likely sustained temporary excitotoxic damage that did not result in neuropathy. Taken together with the fact that both exposed groups here showed significant TTS (Fig. 3A,B), and thus likely sustained nerve terminal swelling, this reinforces the importance of the functional disconnect of cochlear nerve fibers in the generation of persistent central hyperactivity, rather than direct effects of the noise exposure or additional delayed effects of neural degeneration, on the central nervous system. As will be discussed in Section 5.2.2., the functional disconnect of the cochlear nerve, or “neural silencing”, during this TTS window has implications for the behavior of the non-neuropathic mice during the period of reduced cochlear output.

5.2. Cochlear nerve degeneration as an elicitor of hyperacusis

Reflexive measures of auditory behavior revealed signs of hyperacusis-like behavior in the neuropathic mice (Fig. 5,8), occurring within days of noise trauma and persisting for as long as they were studied (up to 10 weeks post-exposure) (Fig. 11). These behavioral indicators of abnormal loudness perception included enhanced PPI for moderate-level tones (Fig. 5B,D,E), a globally reduced threshold for acoustic startle, and an associated increase in startle amplitude for stimuli just above startle threshold (Fig. 8). The PPI trend was specific to auditory stimuli, as light-evoked PPI elicited similar levels of startle inhibition across groups (Fig. 7). To the extent that hyper-responsive ASR and PPI reflect central neural hyperactivity, and specifically hyperactivity of the auditory pathway, these results suggest a role for noise-induced cochlear neuronal degeneration in the development of hyperacusis, especially in cases of patients with normal thresholds.

5.2.1. ASR and PPI as measures of hyperacusis

Current understanding of the neural basis for ASR and PPI implicates substantial contributions from multiple auditory nuclei (Fig. 13), all of which display hyperactivity following noise exposure, suggesting that the ASR and PPI neural circuits themselves may become hyper-responsive.

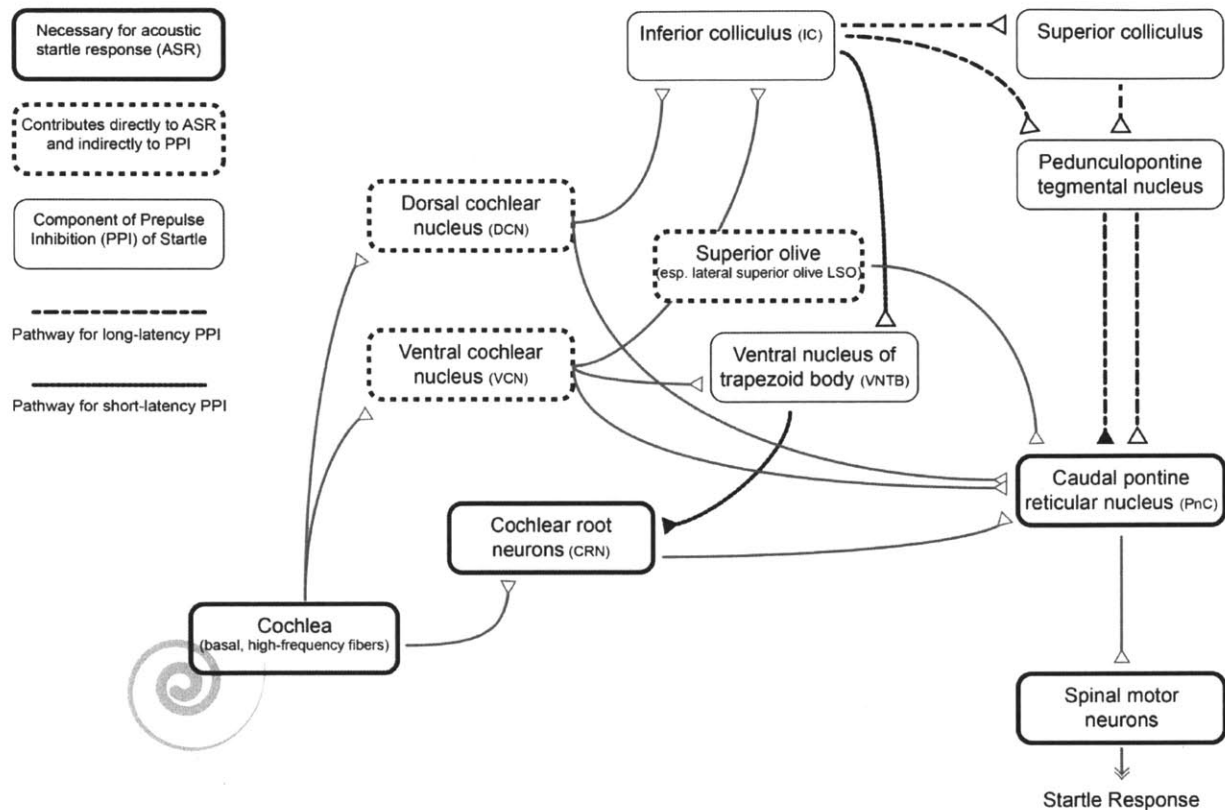


Figure 13: Schematic of subcortical neural circuitry involved in the generation of the acoustic startle response and prepulse inhibition of startle. Based on a combination of tract-tracing, lesion, stimulation and electrophysiological studies, and previously described models for brainstem and midbrain contributions to startle and PPI circuitry (Lingenhöhl and Friauf, 1994; López et al., 1999; Wagner et al., 2000; Fendt et al., 2001; Yeomans et al., 2006; Gómez-Nieto et al., 2008, 2010).

The initial component of ASR circuitry comprises cochlear afferent innervation, from largely basal, high-frequency areas (Osen et al., 1991; Lopez et al., 1993), to cochlear root neurons (CRNs). CRNs reside in the auditory nerve root, just distal to the VCN, and share some characteristics with neighboring globular bushy cells such as appearance, size, axon diameter, and primary-like-with-notch response, although they are much fewer in number (Rouiller and Ryugo, 1984; Merchan et al., 1988; López et al., 1999; Sinex et al., 2001; Gómez-Nieto et al., 2010). CRNs do not send strong projections to higher auditory nuclei, but, critical for ASR, they project to the giant neurons of the caudal pontine reticular formation (PnC), which serves as a sensorimotor interface to the spinal motor neurons that execute the whole-body startle response (Lingenhöhl and Friauf, 1994; Lee et al., 1996; López et al., 1999; Nodal and López, 2003). Retrograde labeling from PnC implicates contributions from VCN, DCN, SOC, and descending projections from IC in the ASR circuit (Lingenhöhl and Friauf, 1994), and indeed ASR amplitude is decreased following lesions of DCN and SOC (Meloni and Davis, 1998; Wagner et al., 2000; although see Schmid and Weber, 2002). Auditory PPI of startle is mediated in large part by IC, which projects indirectly to PnC (Yeomans et al., 2006), and without which auditory, but not

visual, PPI is reduced or eliminated (Leitner and Cohen, 1985; Li et al., 1998). Additionally, a secondary mode for IC involvement in PPI has recently been proposed for prepulses that occur within about 50 ms of the startle stimulus, which involves IC projection to VNTB and inhibitory cholinergic projections from VTNB to CRNs (Gómez-Nieto et al., 2008b, 2010).

Most of these ascending auditory nuclei that contribute to ASR and PPI have been observed to be hyperactive following noise exposure (Kaltenbach et al., 2000; Salvi et al., 2000; Brozoski et al., 2002; Gröschel et al., 2011; Mulders and Robertson, 2011; Vogler et al., 2011), possibly as a result of a de-afferentation-driven change in the balance of excitatory and inhibitory inputs (Kim et al., 2004; Dong et al., 2009). Few studies have characterized CRN response properties in unexposed animals (Sinex et al., 2001; Gómez-Nieto et al., 2010), and nothing is known about their response properties following noise trauma or de-afferentation. However, given that they receive numerous excitatory as well as inhibitory inputs (Osen et al., 1991; Gómez-Nieto et al., 2008a, 2008b), one can speculate that CRNs might be subject to post-exposure redistribution of innervation similar to neighboring cells in the VCN and thus might take on hyper-excitable characteristics. Based on the dual involvement of the VCN, SOC, and IC both in these reflexive behavioral measures, and in the bushy cell pathway that generates the ABR, it is likely that the enhanced ASR and PPI behavior observed in the neuropathic group reflects brainstem and midbrain hyperactivity resulting from noise-induced cochlear neuropathy. This putative relationship is strengthened by the fact that both the hyperacusis-like behavior (Fig. 5,8) and previous observations of neural hyperactivity persist indefinitely, sustained by changes in central mechanisms over time (Kaltenbach et al., 2000; Bauer et al., 2008; Mulders and Robertson, 2011).

Cochlear neuropathy in the absence of hair cell loss may also have a role in the generation of hyperacusis-like behavior in the C57Bl6/J mouse model of progressive sensorineural hearing loss. At 7 months, neuronal counts are reduced throughout the cochlea, whereas hair cells are lost mainly in the basal turn (Hequembourg and Liberman, 2001). As these mice age, PPI and startle amplitude are enhanced for mid- and low-frequency tones (Willott et al., 1994; Carlson and Willott, 1996; Ison and Allen, 2003; Ison et al., 2007). The results from noise-exposed mice with neuropathy presented here suggest that it could be the loss of apical-turn neurons that drives this low frequency hyper-responsivity, rather than solely the central reorganization arising from the basal-turn hair cell loss, as others have suggested (for review, see Willott, 1996).

5.2.2. Relation with hyperacusis in humans

Theoretical discussion of the neurophysiological correlates of hyperacusis relies heavily on the assumption of increased “gain” in the central auditory system (Jastreboff and Hazell, 1993). Although evidence exists, in the form of increased sound-evoked fMRI response in listeners with abnormal sound-level tolerance (Gu et al., 2010), it is rare and still requires further investigation. Interestingly, some indirect evidence arises from the study of loudness perception following temporary conductive hearing loss in humans. In one study, participants were asked to make

categorical loudness judgments before and after two weeks of continuous earplug use, and the stimulus intensity to which listeners ascribed an “uncomfortably loud” rating was lower after this temporary auditory deprivation (Formby et al., 2003). In a later study, measurement of middle ear muscle reflex in similarly input-deprived listeners showed reduced threshold for activation (Munro and Blount, 2009). Taken together, these results suggest that reduced cochlear output led to an increase in the “gain” (Munro and Blount, 2009) of the auditory brainstem regions mediating the stapedial reflex (Lee et al., 2006), with the psychophysical result of enhanced loudness of sounds (Formby et al., 2003), similar to hyperacusis.

The physiological and psychophysical effects of these studies were found to be reversible, and occurred on the order of 1-2 weeks following initiation of deprivation (Formby et al., 2003; Munro and Blount, 2009). Both studies showed that earplug use induced a slight but significant conductive threshold elevation, measured as ~10-20 dB psychophysical sound attenuation, which was sufficient to induce physiological plasticity that presumably underlies the altered perceptual responses. The effect of this transient conductive threshold shift is reminiscent of the results from the non-neuropathic exposed mice in our study, which showed slightly enhanced ASR and PPI during the window of noise-induced TTS (Fig. 11). This comparison reinforces the idea that it is the reduction of cochlear output to the brain that is critical for neurophysiological and perceptual hyper-responsivity following noise trauma, and argues against a primary role of direct effects of the acoustic overexposure on increased central neural excitability.

5.2.3. Alternate explanations for hyper-responsivity

5.2.3.1. Medial olivocochlear efferent hypothesis

In the current study, hyper-responsive startle and PPI behavior following noise exposure appear subsequent to cochlear neuronal degeneration, and are not evident in exposed mice without degeneration, suggesting that the loss of peripheral input is critical to eliciting hyperacusis-like behavior. The neural source of this behavior likely has roots in increased excitability and spontaneous activity in the ascending auditory pathway as a response to the reduced cochlear afferent input. However, it is also possible that the cochlear neuropathy elicits these enhanced auditory behaviors by modifying the medial olivocochlear (MOC) efferent control of cochlear response to sound. Originating in the brainstem, the MOC efferent fibers project to the bases of OHCs and, upon sound- or electrical activation, turn down cochlear gain by hyperpolarizing OHCs and diminishing the positive feedback to basilar membrane motion (for review, see Guinan, 2006). MOC tracts innervate ipsi- and contra-lateral ears through crossed- and uncrossed- projections, respectively, and thus can reduce OHC-based cochlear amplification in either ear or both ears in response to sound (for review, see Guinan, 2006). On the way to the ear, MOC efferent collateral projections also make contact with cells in the cochlear nucleus (e.g. Brown et al., 1988).

The potential role of sound-activated MOC efferent activity in our ASR and PPI paradigms is debatable, especially given the relatively long (~100 ms) time course for MOC efferent-mediated

effects on cochlear responses (see Guinan, 2006) compared to the prepulse-startle onset asynchrony of 50 ms, and the rapidity (<10 ms) of the startle reflex. However, the known “unmasking” effects of MOC efferents by presentation of contralateral continuous noise (Kawase and Liberman, 1993; Kawase et al., 1993) could play a role in our tone-in-noise PPI tests. The driven response of a cochlear nerve fiber to a CF-tone at high levels is reduced in the presence of masking noise due to saturation of the fiber’s firing capacity (Smith, 1979); activation of MOC efferents by a concurrent continuous noise can reverse this firing rate decrement by reducing the nerve fiber’s response to the masker (Kawase et al., 1993). Given the similar nature of this masking paradigm to the tone-in-noise PPI conditions, an intriguing possibility arises that the enhanced PPI in the neuropathic group (Fig. 5D,E) results from increased MOC unmasking. Although the MOC reflex strength in the neuropathic group could be reduced commensurately with the degree of neuropathy, alternatively, the brainstem inputs to the MOC reflex, or the MOC cell bodies themselves, could become hyperactive and result in strengthened MOC unmasking.

The excitatory projections from T-multipolar cells in CN to the MOC cell bodies in VTNB (for review, see Oertel et al., 2011) make them clear candidates for a source of hyperactivity in the efferent response to sound. CN units with “chopper” responses to sound, thought to correspond to T-multipolar cells (Smith and Rhode, 1989), are known to become spontaneously hyperactive and hyper-excitable following noise trauma (Cai et al., 2009; Vogler et al., 2011). This CN cell type is likely to be strongly affected by noise-induced cochlear neuropathy, which is selective for the low-spontaneous rate (SR), high-threshold fibers (Lin et al., 2011a). The multipolar cell population in VCN preferentially receives input from low-SR fibers (Liberman, 1991), and given that T-multipolar make up the majority of this group (Doucet and Ryugo, 1997), they are most likely to become de-afferented and could undergo synaptic changes resulting in hyper-excitability. These cells participate in a positive feedback loop with MOC efferents (for review, see Oertel et al., 2011), as do smaller multipolar cells of the small cell cap in AVCN (Ye et al., 2000) that receive almost exclusively low-SR input (Liberman, 1991), suggesting that a low-SR selective cochlear neuropathy could have implications for MOC-efferent modulation in the cochlea and auditory brainstem. The excitatory nature of this multipolar-MOC feedback loop (Fujino and Oertel, 2001) suggests that a modest amount of hyperactive neural behavior could strengthen itself into significantly elevated neural excitability. Further examination of sound-activated MOC efferent strength in this neuropathic noise exposure model, such as contralateral suppression of DPOAEs in an awake animal (Chambers et al., 2012), could elucidate a potential efferent role in the hyperactive auditory behavior observed here.

5.2.3.2. Stress/anxiety hypothesis

Enhancement of ASR and PPI in the current study is interpreted as signs of hyperacusis-like behavior, resulting from increased neural excitability in the auditory brainstem and midbrain. However, greater ASR and PPI responses are also associated with fear, anxiety, and stress, based on studies of manipulations of the amygdala and hippocampus and their projections to

non-auditory brainstem regions involved in the startle and PPI pathways (Koch and Ebert, 1993; Campeau and Davis, 1995; Du et al., 2011) (for reviews, see Koch, 1999; Bast, 2003), and based on evidence for projections from locus coeruleus to CRNs (Gómez-Nieto et al., 2008a). Recent evidence for a role of acoustic overexposure in generating stress comes from the observation of noise-induced impairment of adult neurogenesis in hippocampus (Kraus et al., 2010), and lack of this neurogenesis nominally leads to increased stress- and anxiety-related behaviors (Revest et al., 2009; Snyder et al., 2011). Taken together with the proposed involvement of the limbic system and auditory-limbic interactions in tinnitus and hyperacusis (Jastreboff and Jastreboff, 2006; Leaver et al., 2011), the possibility must be considered that increased stress or anxiety could have influenced ASR and PPI enhancement in our neuropathic exposure group.

We hypothesized that, if the neuropathic group of mice exhibited enhanced ASR and PPI as a result of increased stress or anxiety, and not in response to changes in the auditory neural pathway, then a similarly hyper-responsive behavior would be observed in another modality. To address this possibility we used a cross-modal PPI test wherein a light prepulse was presented before an acoustic startle. Across different background conditions of no noise, broadband noise, and narrowband noise centered at the trauma band center frequency, light PPI was not significantly different among the exposed and control groups (Fig. 7). This argues for an auditory-specific nature of the hyper-responsivity observed in the neuropathic group, and further suggests, to a first approximation, a lack of difference in baseline stress levels. The similarity of light-PPI responses across groups also argues that the motor component of the startle response is unaffected for neuropathic mice, confirming that the enhancement of ASR and PPI arises as a result of modification to the sensory side of the circuitry.

To more rigorously assess the potential influence of non-auditory factors on auditory behavior in the neuropathic group, we assessed mice from all three groups for signs of anxiety using the elevated plus maze (EPM), namely a reduction in the number of open-arm entries made during a 5-minute exploration period. Open-arm entry counts were similar across control, exposed non-neuropathic, and exposed neuropathic groups at both 1 day and 5-7 weeks post-exposure (Fig. 12A,B), suggesting no difference in anxiety among the three groups. This interpretation is based on the assumption that the EPM should reveal fearful or anxious behavior potentiated by the exogenous stress of noise exposure (Korte and De Boer, 2003), when in fact it is possible that the EPM is more likely to reflect only innate fear of the non-enclosed portion of the maze (Treit et al., 1993), which may or may not be compounded by external sources of anxiety. The significant decrease in open arm exploration under high versus no illumination (Fig. 12D) is consistent with previous reports (e.g. Pereira et al., 2005), but could be interpreted in one of two ways. Our EPM assay could indeed be sensitive to exogenous stressors, and therefore illumination, but not noise and restraint treatments, induces measureable anxiety. However, this result is partially in conflict with previous studies suggesting that immobilization stress reliably leads to measureable anxiety (Padovan and Guimarães, 2000; Korte and De Boer, 2003). Alternatively, our EPM assay could be sensitive only to anxiety driven by fear of the open space,

which could be exacerbated by the addition of visual cues to the whisking-related detection of the lack of enclosure. The EPM results presented here cannot support, or detract from, the possibility that enhanced ASR and PPI could be influenced by increased anxiety. As a pragmatic conclusion, if the distinction between auditory behavior in the neuropathic versus non-neuropathic mice is in fact related to anxiety, it suggests an intriguing role for specifically de-afferentation in its generation, and not acoustic trauma in general.

5.3. Cochlear nerve degeneration as an elicitor of tinnitus

Exposed mice with cochlear neuropathy showed significantly reduced gap-PPI for a narrowband noise carrier situated in a cochlear frequency region of significant de-afferentation (Fig. 9A), as well as for a broadband noise carrier (Fig. 9B). Although this gap variant of the PPI paradigm is commonly used to assess tinnitus in animals, on the assumption that reduced PPI reflects the presence of tinnitus, some conceptual and empirical issues presented below necessitate careful interpretation of these results.

5.3.1. Gap PPI as a measure of tinnitus

Several behavioral methods have been proposed for detecting the presence of tinnitus in animals, following manipulations that often induce tinnitus in humans such as administration of salicylate and acoustic overexposure. These behavioral techniques share the common assumption that a difference in responses between treated and untreated animals on silent intervals, when no external sounds are presented, reflects the interference of a tinnitus percept. Conditioned behavioral responses that are interpreted as evidence for tinnitus include, for example, changes in lever-pressing, water-spout licking, or movement to one side of an apparatus during no-stimulus trials that would suggest that the animal heard an auditory cue (Bauer and Brozoski, 2001; Lobarinas et al., 2004; Heffner and Koay, 2005). In an effort to develop a higher-throughput version of this general task that requires no training, the gap detection variant of PPI was developed, wherein a silent prepulse interval, or gap, in an otherwise continuous carrier is presumed to be less detectible if the tinnitus percept “fills” the gap (Turner et al., 2006).

The premise of the gap PPI assay is valid in the sense that occluding the gap with the addition of a second stimulus, or inserting a carrier-level decrement in place of a full gap, does decrease PPI, suggesting that “filled-gap” interval is less effective in inhibiting the startle response (Ison et al., 1998; Yang et al., 2007). A further potential method for validating the gap PPI test as an indicator of tinnitus is comparison of significant change in behavior on both gap PPI and a trained assay in the same animal. Concomitant changes in two such assays have been observed in animals subject to acoustic trauma (Turner et al., 2006), or following systemic administration of salicylate (Yang et al., 2007), although reliability in detecting a change in auditory behavior across assays does not completely confirm the assay’s reliability in indicating tinnitus.

Assuming that the gap PPI test does reveal presence of a tinnitus percept, further conceptual issues arise regarding determination of the spectral and level characteristics of the perceived sound. In many studies, a strategic combination of broadband, or narrowband or tonal carriers with different center frequencies is used to cover a range of potential tinnitus pitches (Turner et al., 2006; Longenecker and Galazyuk, 2011; Nowotny et al., 2011), yet given the wide spectral variability in human tinnitus percepts (e.g. Ince et al., 1987) it is still possible that certain phantom sounds will not be detected in gap PPI. This potential pitfall could be avoided by using an approach that aims to classify whether the pitch of phantom sounds is above or below a certain frequency (e.g. Yang et al., 2011), although this paradigm would lose the aspect of attempted pitch-matching that is desirable in some experiments. Also problematic is the idea that gap carriers with similar spectral properties as the tinnitus percept could mask tinnitus and reduce the tinnitus percept during the gap via residual inhibition (Roberts et al., 2008), and therefore PPI deficits would be difficult to attribute to the interference of tinnitus.

Since the gap prepulse contains multiple potentially salient cues, nominally the initial noise offset, the silent interval, and the noise onset, it is possible that detection of any or all of these could result in startle inhibition. Accordingly, PPI is consistently observed both for gaps situated at long delays relative to the startle elicitor, where all three of these potential cues are present, and for gaps occurring immediately before the startle elicitor, where the gap prepulse becomes a noise-offset followed by silence (e.g. Ison et al., 2005). Thus, gap PPI deficits could arise if detection of the carrier noise offset or onset is disrupted for reasons unrelated to tinnitus “filling in” the silent interval. The exposed, neuropathic mice in the current study exhibited reduced PPI for gaps embedded in 32 kHz-center frequency narrowband noise, and for broadband noise, when gaps ended immediately before the startle stimulus (Fig. 9A,B). However, the same neuropathic mice showed matched, or even slightly enhanced, gap PPI relative to controls and to non-neuropathic mice when the gap occurred 80 ms earlier (Fig. 14). This shift in performance is not due to an inability of longer latency prepulses to inhibit startle, given that PPI enhancement was also observed in neuropathic mice for long-latency tone burst prepulses in noise (Fig. 15). Although one could interpret the results of the short-latency gap PPI tests (Fig. 9) as evidence for tinnitus in the neuropathic group, the comparison with performance on longer latency gap prepulses suggests additional interpretations and considerations. If gap PPI successfully reveals the presence of tinnitus based on the assumption that it fills in the gap, then animals with tinnitus should show reliable deficits in detection of gaps at either long or short latencies. The lack of consistent PPI decrement in the neuropathic group could, therefore, suggest a lack of tinnitus percept. The fact that a similar model of primary cochlear neuronal loss showed conditioned behavioral evidence for tinnitus following noise exposure (Bauer et al., 2007) suggests the likelihood that our neuropathic group does in fact experience tinnitus, but the variable deficits in the gap PPI test observed here point towards the test’s inconsistency in revealing signs of the percept.

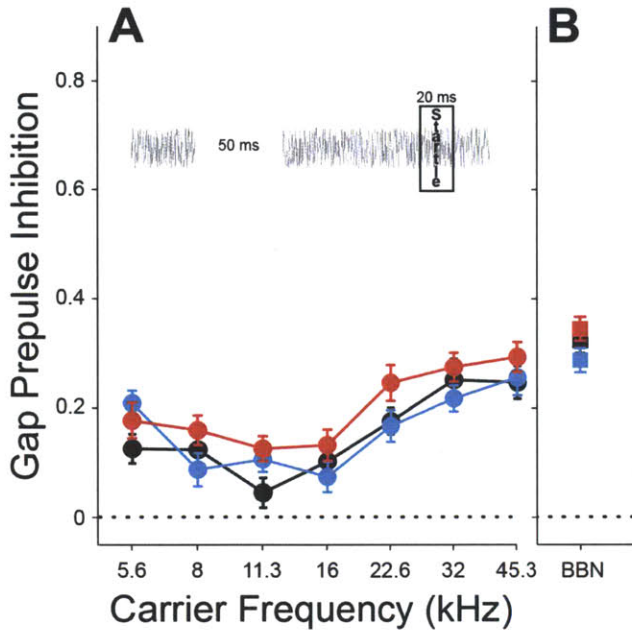


Figure 14: Gap PPI elicited by long-latency prepulses (80 ms from gap offset to startle stimulus onset) is not reduced in neuropathic mice. Mean PPI values (\pm SEMs) are shown for gaps embedded in half-octave carriers centered at 7 log-spaced frequencies (**A**) and for a BBN carrier (**B**). Data were obtained from 1-10 weeks post-exposure, $n = 19-40$ samples per group for each carrier condition. Color key from previous figures applies here.

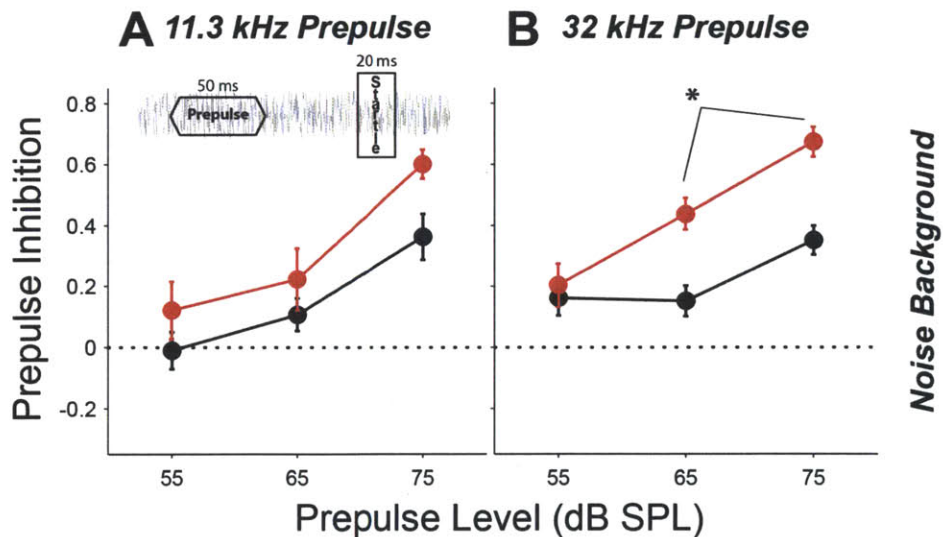


Figure 15: Prepulse inhibition is enhanced in mice with noise-induced neuropathy for long-latency tone-burst prepulses. Growth of PPI as a function of level is shown for 11.3 kHz (**A**) and 32 kHz (**B**) tones in continuous background noise at 60 dB SPL. Means (\pm SEMs) are shown for controls (black) and neuropathic exposed mice (red) ($n = 8-9$ mice per group). Note that these data were collected during the piloting phase before the introduction of the 94 dB non-neuropathic exposure. Asterisk indicates 65-75 dB PPI is significantly different from controls ($p = 0.0001$).

The short-latency gap PPI deficit could, instead, suggest impaired coding of rapid onsets or offsets at the level of the brainstem, given the observation that short-latency, but not long-latency, gaps are unimpaired by lesions of auditory cortex (Bowen et al., 2003). Alternatively, it could signal dysfunction of the putative short-latency PPI pathway, involving projections from IC to VTNB to CRNs (Gómez-Nieto et al., 2008b, 2010), as opposed to the conventional route from IC to the PnC through additional midbrain and brainstem interneurons (Fendt et al., 2001; Yeomans et al., 2006). If this is the case, then the opposite effects of tone-prepulse enhancement, and gap-prepulse reduction, of short-latency PPI in the neuropathic group could indicate differential changes in auditory brainstem or midbrain neurons that encode sound onsets and offsets, respectively. Finally, the observation of occasional prepulse facilitation in the short-delay gap results (Fig. 10) reveals that, for some exposed mice, the gap was detected but appears to be processed in a different way. That a typically inhibitory prepulse becomes excitatory has been taken as evidence for increased excitability of the underlying startle circuit in studies of humans with hyperactive eyeblink reflex (e.g. Schicatano et al., 2000). This latter interpretation is particularly appealing given the concurrent observation of lower startle threshold and greater startle amplitude in the neuropathic group.

Measurement of gap prepulse inhibition of eye-blink startle in humans with tinnitus could begin to address the question of whether reduced gap PPI reliably, if at all, signifies tinnitus with characteristics of the gap carrier. Study in humans is currently underway, with initial results suggesting that the test may not be able to detect tinnitus in a frequency-specific manner (Fournier and Hébert, 2012). Additionally, caution should be taken in considering other psychoacoustical impairments in tinnitus populations. For example, impaired gap detection in humans diagnosed with auditory neuropathy indicates a potential confound in assaying tinnitus that originates from central effects of cochlear neuropathy (Zeng et al., 2005). Interestingly, in the study of gap PPI performance in human listeners, tinnitus participants showed greater startle response magnitude, potentially pointing toward a similar index of hyperacusis in human and animal subjects (Fournier and Hébert, 2012).

5.3.2. Relation with tinnitus in humans

Sound-evoked hyperactivity has been postulated as a neural correlate of tinnitus, based on the theory of increased “gain” in the central auditory system, and based on numerous neuroimaging studies showing increased sound-evoked and tinnitus-modulating activation of auditory cortical regions and IC (for reviews, see Lanting et al., 2009; Melcher, 2012). Recent study of tinnitus- and non-tinnitus subjects with matched, normal-range audiograms similarly demonstrated increased sound-elicited fMRI response in primary auditory cortex of tinnitus subjects (Gu et al., 2010). The observation of decreased ABR wave 1 amplitude, and corresponding enhanced wave 5/1 amplitude ratio, in similar subjects indicates that tinnitus can arise in listeners with normal thresholds and evidence of central neural hyperactivity, potentially as a result of primary cochlear neuronal degeneration (Gu et al., 2010, 2012; Schaette and McAlpine, 2011). Computational modeling suggests that this elevated ABR amplitude ratio, and tinnitus-related

hyperactivity, can arise following primary neuronal degeneration due to homeostatic plasticity of inputs to a DCN-like cell type (Schaeffe and McAlpine, 2011), consistent with the suspected involvement of DCN hyperactivity in animal models of tinnitus (Kaltenbach and Afman, 2000; Brozoski et al., 2002; Middleton et al., 2011). The ABR-based evidence for increased central gain in our neuropathic mice (Fig. 3D) and in humans (Gu et al., 2012) has implications for hyperactivity of subcortical regions participating in the bushy cell pathway, which does not include DCN as an obligatory synaptic station (Melcher and Kiang, 1996) but which could be modulated by DCN activity (e.g. Manzoor et al., 2012). Further investigation into potentially unifying measures of tinnitus-related central hyperactivity, including sound-evoked firing rates and synchrony of cortical responses, and spontaneous activity in primary auditory cortex (Seki and Eggermont, 2003; Yang et al., 2011), could strengthen this putative role for noise-induced cochlear neuropathy in tinnitus.

6. Conclusions

Central neural hyperactivity following acoustic overexposure is a well-documented phenomenon in animal models of noise-induced hearing loss. Its correlation with peripheral damage, with respect to frequency regions of aberrant firing, onset and duration, and short-term dependence on continued peripheral input, suggests that one or more elements of the cochlear injury is critical for eliciting increased central excitability. Here we showed that primary neuronal degeneration appears to result in compensatory gain in the central nervous system, as seen in the later waves of the auditory brainstem response, without the added element of threshold shift that signifies hair cell damage. In addition, we showed that hyper-responsive auditory behavior was correlated with degree of neuropathy, and not degree of threshold shift, suggesting a significant perceptual consequence of noise-induced neuronal degeneration. The onset of hyperacusis-like auditory responses occurred within days of exposure, suggesting that it is the “silencing” aspect of neural degeneration at the peripheral end of the cochlear nerve that is critical for eliciting these physiological and behavioral changes, rather than the delayed effects of neural degeneration at the nerve’s central projections. Taking together the physiology and behavior suggests what others have shown by different techniques, that neurons of the bushy cell pathway are likely to be hyperactive following deprivation of input from the periphery (Fig. 16). We suggest that this occurs due to the immediate functional disconnect of the cochlear nerve, and not as a direct response in the central nervous system to the acoustic trauma. This is supported by the observation that the non-neuropathic exposure, despite significant temporary threshold shift, did not lead to persistent hyper-responsive auditory behavior as the neuropathic exposure did.

Although there was no conclusive demonstration via gap PPI regarding whether the cochlear neuropathy is linked with a tinnitus percept, the evidence for central gain in the ABR waveform is consistent with findings in normal-threshold individuals with tinnitus, and thus this noise-induced neuropathy could serve as an informative animal model for future tinnitus research. The hyperacusis-like behavior resulting from neuropathy in the absence of threshold shift suggests a

specific etiology for clinicians to consider in diagnosing hyperacusic listeners with normal thresholds. Given that this behavior was specific to auditory stimuli, and did not appear concurrently with signs of anxiety, these results could inform a particular distinction of auditory hyper-sensitivity distinct from syndromic forms and from auditory forms confounded by threshold impairment. Overall, the importance of protecting one's hearing, even in moderate noise conditions, remains paramount in preventing subtle, but permanent, changes to cochlear innervation and auditory perception.

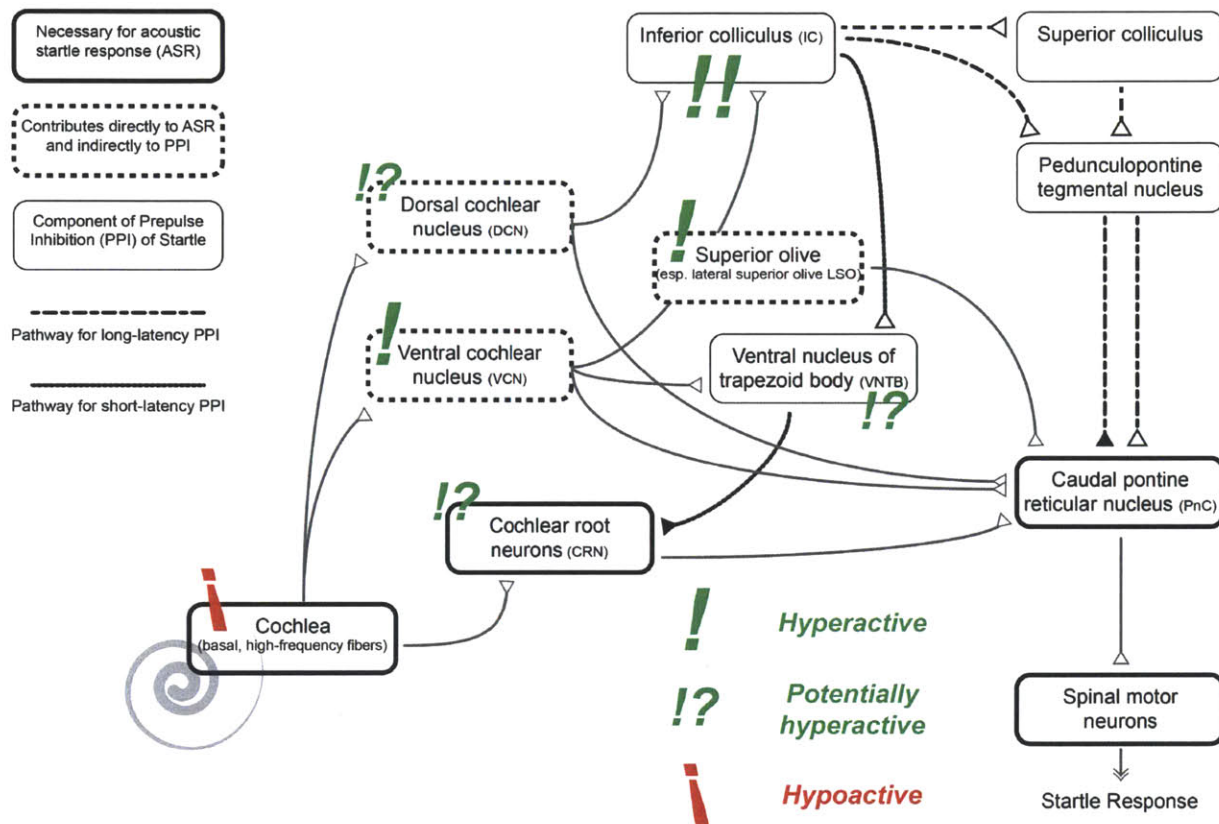


Figure 16: Summary of suggested regions of neural hyperactivity following noise-induced primary neuronal degeneration. Based on ABR wave 1 reduction, cochlear output is reduced, but several higher auditory centers are suspected to be hyper-excitable. Recovery of later ABR wave amplitudes, and enhancement of ASR and PPI, suggests that certain cells of the VCN, SOC, and IC might show increased evoked responses to sound. Less is known about CRNs and VNTB response properties following noise exposure, but given their involvement in ASR and PPI, respectively, it is possible that they would show plasticity in the direction of hyperactivity. Previous studies suggest that DCN becomes hyperactive after noise trauma. Details of schematic sources are as for Figure 13.

7. References

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