Towards a diagnostic test for hidden hearing loss

CHRISTOPHER J. PLACK[*], GARRETH PRENDERGAST, KAROLINA KLUK, AGNÈS LÉGER, HANNAH GUEST, AND KEVIN J. MUNRO

The University of Manchester, Manchester Academic Health Science Centre, Manchester, England

Cochlear synaptopathy (or "hidden hearing loss"), due to noise exposure or ageing, has been demonstrated in animal models using histological techniques. However, diagnosis of the condition in individual humans is problematic because of: (i) test reliability, and (ii) lack of a gold standard validation measure. Wave I of the transient-evoked auditory brainstem response (ABR) is a non-invasive electrophysiological measure of auditory nerve function, and has been validated in the animal models. However, in humans Wave I amplitude shows high variability both between and within individuals. The frequency-following response (FFR), a sustained evoked potential reflecting synchronous neural activity in the rostral brainstem, is potentially more robust than ABR wave I. However, the FFR is a measure of central activity, and may be dependent on individual differences in central processing. Psychophysical measures are also affected by inter-subject variability in central processing. Differential measures, in which the measure is compared, *within an individual*, between conditions that are affected differently by cochlear synaptopathy, may help to reduce inter-subject variability due to unrelated factors. There is also the issue of how the metric will be validated. Comparisons with animal models, computational modeling, auditory nerve imaging, and human temporal bone histology are all potential options for validation, but there are technical and practical hurdles, and difficulties in interpretation. Despite the obstacles, a diagnostic test for hidden hearing loss is a worthwhile goal, with important implications for clinical practice and health surveillance.

INTRODUCTION

Hearing ability is usually assessed using pure tone audiometry (Johnson, 1970), which measures the smallest detectable level of pure tones at a range of frequencies. The resulting audiogram is sensitive to dysfunction of the outer hair cells and, to a lesser extent, inner hair cells (IHCs) in the cochlea. However, it is becoming increasingly clear that the audiogram is not sensitive to some types of peripheral auditory dysfunction. In particular, results from rodent models suggest that noise exposure and/or aging, can cause permanent loss of synapses between the IHCs and auditory nerve fibers, *without permanently affecting sensitivity to quiet sounds* (Kujawa and Liberman, 2009; Sergeyenko *et al.*, 2013). The disconnected nerve

---

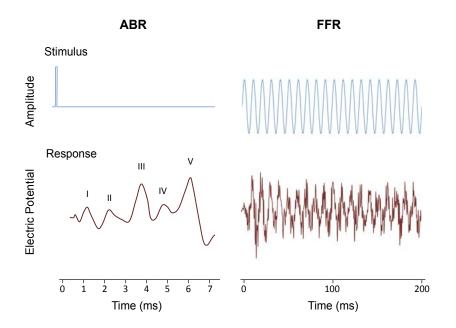*Corresponding author: chris.plack@manchester.ac.uk

fibers subsequently degenerate. This disorder has been variously termed "cochlear neuropathy", "cochlear synaptopathy", and popularly "hidden hearing loss" (Schaette and McAlpine, 2011), because the loss is not thought to be detectable using pure-tone audiometry. The loss seems to affect selectively the low spontaneous rate (SR) fibers that have high thresholds and are thought to be responsible for coding sound intensity at moderate-to-high levels (Furman *et al.*, 2013). This may explain why the loss does not affect sensitivity to quiet sounds.

Several research groups are currently trying to determine the extent to which hidden hearing loss is a contributor to hearing difficulties experienced by humans. There is evidence that listeners with a history of noise exposure but with normal audiograms have deficits in speech perception and temporal processing (Alvord, 1983; Kumar *et al.*, 2012). Similarly, the aging process may affect speech perception in noise even when there are no significant increases in audiometric threshold (Dubno *et al.*, 1984; Rajan and Cainer, 2008). An open question concerns the extent to which these deficits are a consequence of cochlear synaptopathy, or other types of dysfunction, for example, IHC dysfunction, or central neural dysfunction.

A major obstacle to the academic investigation of hidden hearing loss, and to the eventual incorporation of the research findings into clinical practice, is the absence of a reliable and validated diagnostic test for the disorder. In the animal models, selective immunostaining and confocal microscopy can be used to determine directly the loss of synapses. However, such invasive procedures are not possible in humans, at least pre-mortem. In this article we will consider non-invasive measures of hidden hearing loss, their potential as a diagnostic test, and the challenges faced in developing them to this stage. Table 1 provides a summary of the techniques that will be discussed.

Diagnostic technique	Hypothesized effect of synaptopathy	Pros	Cons
ABR	Reduction in wave I amplitude at high levels	Relatively direct measure of auditory nerve function	Highly variable in humans
FFR	Reduction in synchrony to amplitude modulation	Robust response; objective	Affected by variability in central processes
Behavioral	Increase in discrimination thresholds at high levels	Easy to measure	Affected by central processes; hypothesized effects are small

Table 1. A summary of potential diagnostic techniques for hidden hearing loss.

Fig. 1. An illustration of typical stimuli and recorded waveforms for two electrophysiological measures of auditory neural coding; the auditory brainstem response (ABR) and the frequency following response (FFR).

MEASURES OF HIDDEN HEARING LOSS IN HUMANS

The auditory brainstem response

The click-evoked electrophysiological auditory brainstem response (ABR, see Fig. 1) is a prime candidate for a measure of hidden hearing loss in humans. The ABR can be recorded in humans using electrodes placed on the scalp; typically an electrode is attached to a mastoid and to another location such as the contralateral mastoid, forehead, or vertex. The differential response to the two electrodes determines the recorded ABR. Wave I of the ABR reflects auditory nerve function, and in the rodent models has been shown to be sensitive to the effects of noise exposure (Kujawa and Liberman, 2009) and aging (Sergeyenko *et al.*, 2013). In these models, the amplitude of Wave I is reduced at moderate-to-high levels but not at low levels, consistent with a selective loss of low-SR fibers. Furthermore, Wave I amplitude correlates strongly with the proportion of intact synapses (Kujawa and Liberman, 2009; Sergeyenko *et al.*, 2013), which provides validation for the measure in rodents.

In humans the evidence is less compelling, but both aging (Konrad-Martin *et al.*, 2012) and, recently, noise exposure (Stamper and Johnson, 2015) have been shown to be associated with a reduction in ABR Wave I amplitude for high-level clicks, in the absence of, or controlling for, an increase in audiometric threshold. In addition, Wave I amplitude for high-level clicks is reduced in listeners with tinnitus even when the audiogram is normal (Schaette and McAlpine, 2011). It is suggested by

91

Schaette and McAlpine that loss of auditory nerve fibers may induce tinnitus due to a compensatory increase in central neural gain. However, there are some problems associated with the use of ABR Wave I as a diagnostic test of hidden hearing loss. First, unlike the rodent models in which the ABR can be measured accurately using sub-cutaneous electrodes, in humans ABR Wave I has a relatively low amplitude and shows high variability both between individuals and within individuals on repeated tests (Beattie, 1988; Lauter and Loomis, 1988). This variability may be the result of a number of factors unrelated to cochlear synaptopathy, including sex, head size, variations in tissue resistance, and variations in electrode placement (Schwartz and Berry, 1985). The use of intra-canal electrodes, including tympanic membrane electrodes, can increase the amplitude of Wave I, but may increase the variability (Stamper and Johnson, 2015). Hence, at present, while Wave I may be useful for demonstrating *group* differences in synaptopathy, between those noise exposed and those not for example, it is probably not useful for determining if an *individual* has hidden hearing loss.
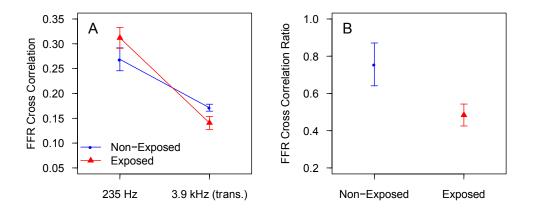
Another issue is that the amplitude of Wave I in response to a broadband click is strongly influenced by activity in basal regions of the cochlea (Don and Eggermont, 1978). Even if the audiogram is normal over the clinical range, up to 4 kHz or 8 kHz say, hair cell loss in higher characteristic frequency regions may affect the amplitude of the response. Hence to identify synaptopathy, the results may have to be controlled for high-frequency audiometric thresholds, or, alternatively, the high-frequency region may be masked using high-pass noise during recording of the ABR to prevent the basal region contributing to the response (Don and Eggermont, 1978).

The frequency-following response

The frequency-following response (FFR) is a sustained auditory evoked potential, thought to reflect neural activity in the brainstem synchronized (phase locked) to the waveform of the stimulus (Krishnan, 2006; see Fig. 1). The FFR is particularly sensitive to amplitude modulation at modulation rates of a few hundred hertz, although it also reflects phase locking to temporal fine structure for frequencies up to about 1 kHz. Over recent years the FFR has become popular as a measure of auditory temporal coding. The FFR can be recorded using similar electrode montages to the ABR, and for lower frequencies at least, is a more robust measure than ABR Wave I, with most participants showing a clear response above the noise floor. Importantly, FFR amplitude can be measured objectively using a discrete Fourier transform of the response at the component frequency, whereas ABR Wave I measurement sometimes requires a subjective intervention to analyze the waveform and determine the peak location.

There is evidence that the amplitude of the FFR to both stimulus envelope and temporal fine structure decreases with increasing age even when controlling for absolute threshold (Clinard and Tremblay, 2013; Marmel *et al.*, 2013; Bones and Plack, 2015). The FFR is also predictive of behavioral performance on tasks such as frequency discrimination (Marmel *et al.*, 2013) and modulation discrimination (Bharadwaj *et al.*, 2015) for listeners with normal audiometric thresholds. There is

also preliminary evidence that the FFR is reduced in noise-exposed ears for listeners with normal absolute thresholds (Plack *et al.*, 2014, see Fig. 2). These results suggest that the FFR may be sensitive to synaptopathy.

Fig. 2. Results from the conference presentation of Barker *et al.* (2014) reported by Plack *et al.* (2014). A: FFR synchrony to a 235-Hz pure tone and to a 235-Hz tone transposed to 3.9 kHz (i.e., a 3.9 kHz pure-tone carrier amplitude modulated at 235 Hz), for groups of listeners with (red triangles) and without (blue circles) a history of recreational noise exposure. For each stimulus, the dependent variable was the coefficient of correlation between the FFR and a 235-Hz pure tone. B: The ratios of the coefficients between the two frequencies (3.9 kHz : 235 Hz). Error bars are standard errors.

However, unlike ABR Wave I, the FFR is produced largely by generators in the brainstem, the largest component from the region of the inferior colliculus (Krishnan, 2006). Hence differences in central auditory processing may well contribute to individual differences in FFR amplitude. For example, it is known that musicians and tone language speakers have stronger FFRs for certain types of stimuli (Krishnan *et al.*, 2005; Wong *et al.*, 2007), likely due to experience-related plasticity. Aging affects central neural function (Konrad-Martin *et al.*, 2012), so an FFR deficit due to age could be a consequence of a combination of peripheral and central factors. Like the ABR, the FFR is also limited by between- and within-subject variability due to factors such as tissue resistance and electrode placement.

Behavioral measures

Behavioral measures, such as psychophysical thresholds, require a subjective response from the listener. Hence, they don't have the "objectivity" of electrophysiological measures, and may potentially depend on processing at all stages from the auditory periphery to the motor commands sent to the finger that presses the response key. As is the case for the FFR technique, there is the concern that performance may be influenced by central factors unrelated to synaptopathy. As well as purely auditory factors, these may include higher-level functions such as

memory and attention. However, behavioral techniques have been shown to provide reliable measures of some aspects of peripheral function, in particular frequency selectivity and cochlear compression (Oxenham and Plack, 1997).

Reduction in the numbers of low-SR fibers might be expected to affect discrimination tasks at high sound levels. However, as pointed out by Oxenham and Heinz (personal communications) if considered in terms of signal detection theory, a 50% fiber loss (similar to that in the animal studies) would reduce the discrimination index, d-prime, by a factor of $\sqrt{2}$ only. This would result in a barely measurable increase in threshold, about 1 dB in the case of the intensity difference limen (Buus and Florentine, 1991), for example. Considering the between-subject variability in performance expected due to central factors, it is not clear that psychophysical measures have the necessary sensitivity to diagnose synaptopathy, *unless* almost all the synapses with low-SR fibers are lost in a given region of the cochlea.

There are little available data directly relating synaptopathy to behavioral performance. Tinnitus patients with normal hearing, who exhibit a reduction in ABR Wave I amplitude consistent with synaptopathy (Schaette and McAlpine, 2011), have elevated intensity discrimination thresholds (Epp *et al.*, 2012). Noise exposure and aging have been related to deficits in temporal processing tasks and speech discrimination in noise (Alvord, 1983; Dubno *et al.*, 1984; Kumar *et al.*, 2012).

MANAGING VARIABILITY

A common problem for measures of hidden hearing loss in humans is that of variability. *Within*-subject variability may be minimized for the electrophysiological techniques by using careful procedures, and ensuring electrode placements and impedances are tightly controlled. For psychophysical tests, practice and the use of a procedure that is easy to learn can ensure that performance is at asymptote (King *et al.*, 2013). An approach for minimizing both within- and between-subject variability is to use a differential measure, in which two measures are compared for each individual: one measure that is assumed to be affected by synaptopathy and one that isn't. Ideally, both measures should be affected equally by other sources of variability so that effectively this variability can be cancelled out or at least minimized. Such an approach may be effective for both electrophysiological and behavioral measures, and help to reduce or eliminate confounds due to central factors for the FFR and for the behavioral measures. There are two clear options for differential measures of synaptopathy; comparisons across frequency and comparisons across level.

Comparisons across frequency

One differential approach is to compare measures between a low-frequency region and a high-frequency region. It is generally reported that noise exposure causes most damage in higher frequency regions (around 4 kHz), hence the low-frequency measure can be used as a within-subject comparison. A preliminary study used this technique by comparing the FFR to a 235-Hz pure tone with that to a 235-Hz

modulator imposed on a 4-kHz carrier (Plack *et al.*, 2014, see Fig. 2). The participants were audiogram matched. The noise-exposed group had no reduction in FFR amplitude to the low-frequency tone, but showed a reduction in the amplitude of the FFR to the envelope of the high-frequency stimulus. Furthermore, the difference between the groups was greater when the ratio of high-frequency to low-frequency responses was used as the measure.

For the ABR, filtered or masked clicks can be used to probe different frequency regions, and hence allow a cross-frequency comparison. For behavioral measures it is relatively simple for narrowband stimuli to compare performance in different frequency regions. Whenever narrowband stimuli are used, it may be advisable to include a broadband masking noise to ensure that high-SR fibers do not contribute to the response due to spread of excitation. However, a problem with using across-frequency comparisons is that it is not yet clear that synaptopathy only affects high-frequency regions.

Comparisons across level

An alternative is to rely on the finding that synaptopathy is selective for low-SR fibers, which have high thresholds and code intensity information at high levels, above the saturation level of the high-SR fibers. Hence evoked-response amplitude, and behavioral performance, should be selectivity impaired at high levels. By comparing the measure across different levels, it may be possible to isolate the effects of synaptopathy from other sources of variability. In the study of Schaette and McAlpine (2011) it was observed that the reduction in ABR Wave I was greater for the 100 dB pe SPL click than for the 90 dB pe SPL click. Bharadwaj *et al.* (2015) have taken a similar approach for their FFR measures, by measuring the FFR to a modulator imposed on a high-level carrier. They reasoned that the FFR for a low modulation depth would be determined primarily by the response of low-SR fibers, whereas the FFR for a high modulation depth would depend in part on the response of high-SR fibers, since the dips in the modulation would fall within their level range. Bharadwaj *et al.* (2015) showed that the slope of the function relating FFR strength to modulation depth correlated more strongly with behavioral modulation detection performance than did FFR strength in isolation.

THE PROBLEM OF VALIDATION

In the rodent models, validation of electrophysiological or psychophysical measures is possible because researchers can count synapses and nerve fibers post-mortem using histological techniques. While human temporal bones are available to researchers, and have been used to provide estimates of auditory nerve fiber loss due to aging (Makary *et al.*, 2011), it is not trivial to validate a test performed on a living human using a post-mortem measure! The problem essentially is that we currently lack a "gold-standard" measure of synaptopathy that can be used with a living human to validate the diagnostic test. We are hence confronted by the serious problem of being unable to confirm that our diagnostic test is measuring what we

want it to. There are, however, a number of potential approaches to validation that may be productive.

Validation with animal models

One approach to validation is to assume that between-species differences are insignificant with regard to the diagnosis of synaptopathy, and validate the measure using animal models. For the ABR, for example, there is good evidence from comparisons with synapse counts that Wave I is a reliable measure of synaptopathy in animals with normal sensitivity to quiet sounds (Kujawa and Liberman, 2009; Sergeyenko *et al.*, 2013). The FFR could be validated in a similar way, and it should be possible to validate simple behavioral measures, such as psychophysical discrimination thresholds, in animals suited to behavioral tasks such as the chinchilla. These measures can then be compared with post-mortem synapse counts taken shortly after threshold measurement.

Computational modeling

There are now a number of computational models of the peripheral auditory system (e.g. Zilany *et al.*, 2009), based on animal and human data, that could be adapted to make predictions of the expected effects of synaptopathy on evoked potentials and behavioral performance. These results could help validate diagnostic tests based on these measures, to determine whether the pattern of results is consistent with the expected effects of synaptopathy. However, there are still too many uncertainties in these models to rely on them entirely, and these models of course cannot determine the actual synaptic loss for an individual. The utility of these models may lie in their use in conjunction with the animal data.

Auditory nerve imaging

Imaging techniques, in particular magnetic resonance imaging (MRI), have the potential to provide a direct measure of nerve fiber loss. At present it is not possible to image the auditory nerve non-invasively in humans with the resolution required to detect a proportional reduction in nerve fibers. However, it is conceivable that techniques such as diffusion tensor MRI may be refined to the point at which we can provide a direct estimate of the loss of fibers due to synaptopathy. Although such a measure may not itself be cost-effective or practical for routine use in the clinic as a diagnostic test, it could be used to validate a simpler clinical test, for example, by imaging a relatively small number of individuals with normal audiograms, with and without suspected hidden hearing loss.

Human temporal bone histology

Direct nerve fiber and synapse counts are certainly possible in humans post-mortem using donated temporal bones. The problem then is how to use this information to validate a test, without having to repeatedly perform that test on the individual until they die to account for changes in performance over time. Terminally ill patients may be one option if consent can be obtained, although these individuals are

predominantly elderly and may have a number of hearing-related complications, including hair cell loss. Another option is to test young participants in the military, or other occupations with higher than average mortality, who have agreed to donate their temporal bones.

CONCLUSIONS

The discovery of cochlear synaptopathy, or hidden hearing loss, has potentially major implications for audiological practice, health surveillance, and noise exposure regulations. Investigations of the disorder in humans are hampered by the lack of a reliable diagnostic test. The amplitude of Wave I of the ABR is the most direct non-invasive measure of auditory nerve function in humans, but is limited by variability. The FFR and behavioral measures are less direct, and influenced by central factors, but may prove more reliable. Variability may be reduced by the use of differential measures, that compare performance across frequency or level for example, to isolate the effects of synaptopathy form other sources of variance. There is also the problem of test validation. It may be necessary to rely on animal data relating comparable electrophysiological and behavioral measures with direct histological measures, although it is conceivable that technological innovations in neuroimaging may allow a direct estimate of auditory nerve fiber loss in humans, permitting validation of a more clinically useable test.

ACKNOWLEDGMENTS

REFERENCES

Alvord, L.S. (**1983**). "Cochlear dysfunction in "normal-hearing" patients with history of noise exposure," Ear. Hearing, **4**, 247-250.

Barker, D., Hopkins, K., Baker, R., and Plack, C.J. (**2014**). "Detecting the early effects of noise exposure," in *Midwinter Meeting of Association for Research in Otolaryngology* (San Diego).

Beattie, R.C. (**1988**). "Interaction of click polarity, stimulus level, and repetition rate on the auditory brainstem response," Scand. Audiol., **17**, 99-109.

Bharadwaj, H.M., Masud, S., Mehraei, G., Verhulst, S., and Shinn-Cunningham, B.G. (**2015**). "Individual differences reveal correlates of hidden hearing deficits," J. Neurosci., **35**, 2161-2172.

Bones, O. and Plack, C. J. (**2015**). "Losing the music: aging affects the perception and subcortical neural representation of musical harmony," J. Neurosci., **35**, 4071-4080.

Buus, S. and Florentine, M. (**1991**). "Psychometric functions for level discrimination," J. Acoust. Soc. Am., **90**, 1371-1380.

Clinard, C.G. and Tremblay, K.L. (**2013**). "Aging degrades the neural encoding of simple and complex sounds in the human brainstem," J. Am. Acad. Audiol., **24**, 590-599.

Don, M. and Eggermont, J.J. (**1978**). "Analysis of the click-evoked brainstem potentials in man unsing high-pass noise masking," J. Acoust. Soc. Am., **63**, 1084-1092.

Dubno, J.R., Dirks, D.D., and Morgan, D.E. (**1984**). "Effects of age and mild hearing loss on speech recognition in noise," J. Acoust. Soc. Am., **76**, 87-96.

Epp, B., Hots, J., Verhey, J.L., and Schaette, R. (**2012**). "Increased intensity discrimination thresholds in tinnitus subjects with a normal audiogram," J. Acoust. Soc. Am., **132**, EL196-201.

Furman, A.C., Kujawa, S.G., and Liberman, M.C. (**2013**). "Noise-induced cochlear neuropathy is selective for fibers with low spontaneous rates," J. Neurophysiol., **110**, 577-586.

Johnson, E.W. (**1970**). "Tuning forks to audiometers and back again," Laryngoscope, **80**, 49-68.

King, A., Hopkins, K., and Plack, C.J. (**2013**). "Differences in short-term training for interaural phase difference discrimination between two different forced-choice paradigms," J. Acoust. Soc. Am., **134**, 2635-2638.

Konrad-Martin, D., Dille, M.F., McMillan, G., Griest, S., McDermott, D., Fausti, S. A., and Austin, D.F. (**2012**). "Age-related changes in the auditory brainstem response," J. Am. Acad. Audiol., **23**, 18-35.

Krishnan, A., Xu, Y., Gandour, J., and Cariani, P. (**2005**). "Encoding of pitch in the human brainstem is sensitive to language experience," Cogn. Brain Res., **25**, 161-168.

Krishnan, A. (**2006**). "Frequency-following response," in *Auditory evoked potentials: basic principles and clinical application*. Eds. R.F. Burkhard, M. Don, and J. Eggermont (Lipincott Williams and Wilkins, Philadelphia), pp. 313-333.

Kujawa, S.G. and Liberman, M.C. (**2009**). "Adding insult to injury: cochlear nerve degeneration after "temporary" noise-induced hearing loss," J. Neurosci., **29**, 14077-14085.

Kumar, U.A., Ameenudin, S., and Sangamanatha, A.V. (**2012**). "Temporal and speech processing skills in normal hearing individuals exposed to occupational noise," Noise Health, **14**, 100-105.

Lauter, J.L. and Loomis, R.L. (**1988**). "Individual differences in auditory electric responses: comparisons of between-subject and within-subject variability. II. Amplitude of brainstem Vertex-positive peaks," Scand. Audiol., **17**, 87-92.

Makary, C.A., Shin, J., Kujawa, S.G., Liberman, M.C., and Merchant, S.N. (**2011**). "Age-related primary cochlear neuronal degeneration in human temporal bones," J. Assoc. Res. Otolaryngol., **12**, 711-717.

Marmel, F., Linley, D., Carlyon, R.P., Gockel, H.E., Hopkins, K., and Plack, C.J. (**2013**). "Subcortical neural synchrony and absolute thresholds predict frequency discrimination independently," J. Assoc. Res. Otolaryngol., **14**, 757-766.

Oxenham, A.J. and Plack, C.J. (**1997**). "A behavioral measure of basilar-membrane nonlinearity in listeners with normal and impaired hearing," J. Acoust. Soc. Am., **101**, 3666-3675.

Plack, C.J., Barker, D., and Prendergast, G. (**2014**). "Perceptual consequences of "hidden" hearing loss," Trends Hear., **18**.

Rajan, R. and Cainer, K.E. (**2008**). "Ageing without hearing loss or cognitive impairment causes a decrease in speech intelligibility only in informational maskers," Neuroscience, **154**, 784-795.

Schaette, R. and McAlpine, D. (**2011**). "Tinnitus with a normal audiogram: physiological evidence for hidden hearing loss and computational model," J. Neurosci., **31**, 13452-13457.

Schwartz, D.M. and Berry, G.A. (**1985**). "Normative aspects of the ABR," in *The auditory brainstem response*. Ed. J.T. Jacobson (Taylor and Francis, London), pp. 65-97.

Sergeyenko, Y., Lall, K., Liberman, M.C., and Kujawa, S.G. (**2013**). "Age-related cochlear synaptopathy: an early-onset contributor to auditory functional decline," J. Neurosci., **33**, 13686-13694.

Stamper, G.C. and Johnson, T.A. (**2015**). "Auditory function in normal-hearing, noise-exposed human ears," Ear. Hearing, **36**, 172-184.

Wong, P.C.M., Skoe, E., Russo, N.M., Dees, T., and Kraus, N. (**2007**). "Musical experience shapes human brainstem encoding of linguistic pitch patterns," Nat. Neurosci., **10**, 420-422.

Zilany, M.S., Bruce, I.C., Nelson, P.C., and Carney, L.H. (**2009**). "A phenomeno-logical model of the synapse between the inner hair cell and auditory nerve: long-term adaptation with power-law dynamics," J. Acoust. Soc. Am., **126**, 2390-2412.