Persistent Hair Cell Malfunction Contributes to Hidden Hearing Loss

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20 ABSTRACT

21 Noise exposures that result in fully reversible changes in cochlear neural
22 threshold can cause a reduced neural output at supra-threshold sound intensity.
23 This so-called “hidden hearing loss” has been shown to be associated with
24 selective degeneration of high threshold afferent nerve fiber-inner hair cell (IHC)
25 synapses. However, the electrophysiological function of the IHCs themselves in
26 hidden hearing loss has not been directly investigated. We have made round
27 window (RW) measurements of cochlear action potentials (CAP) and summing
28 potentials (SP) after two levels of a 10kHz acoustic trauma. The more intense
29 acoustic trauma lead to notch-like permanent threshold changes and both CAP
30 and SP showed reductions in supra-threshold amplitudes at frequencies with
31 altered thresholds as well as from fully recovered regions. However, the
32 interpretation of the results in normal threshold regions was complicated by the
33 likelihood of reduced contributions from adjacent regions with elevated
34 thresholds. The milder trauma showed full recovery of all neural thresholds, but
35 there was a persistent depression of the amplitudes of both CAP and SP in
36 response to supra-threshold sounds. The effect on SP amplitude in particular
37 shows that occult damage to hair cell transduction mechanisms can contribute
38 to hidden hearing loss. Such damage could potentially affect the supra-threshold
39 output properties of surviving primary afferent neurons.

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42 INTRODUCTION

43 The traditional view of reversible acoustic trauma has been that it affects
44 primarily the functioning of the outer hair cells (OHCs), whose role is to amplify
45 cochlear mechanical responses to sound and so determine the absolute
46 sensitivity of the neural output from the inner hair cells (IHCs) (Ashmore, 2002;
47 Patuzzi et al., 1988; Yates et al., 1992). Full recovery of neural thresholds after
48 acoustic trauma (temporary threshold shift, or TTS), signifies a full recovery of
49 OHC sensitivity and until recently it was presumed that in such cases overall
50 cochlear function also recovered. However, a number of recent studies have
51 elegantly shown that despite the presence of normal neural thresholds after loud
52 sound exposures, cochlear neural responses to supra-threshold acoustic stimuli
53 can remain depressed (Furman et al., 2013; Kujawa et al., 2015; Liberman, 2015;
54 Lin et al., 2011). This reduced neural output, that has been referred to as “hidden
55 hearing loss” is associated with neuropathic changes at the IHC synapse; in
56 particular, with a selective loss of synapses between IHCs and the high threshold,
57 low spontaneous rate population of primary afferent neurons (Furman et al.,
58 2013; Liberman, 2016).

59 Most previous studies of hidden hearing loss have used the Wave I amplitude of
60 the auditory brainstem response (ABR) to assess cochlear neural output and
61 therefore lack an independent measure of hair cell function. One group has used

62 otoacoustic emissions (DPOAEs) in mice and guinea pigs (Kujawa et al., 2009; Lin
63 et al., 2011), to monitored full recovery of OHC function, but no specific
64 electrophysiological measures of either OHC or IHC output were employed.

65 Therefore, we have made detailed measurements of both hair cell and neural
66 electrophysiological responses after loud sound exposures of varying severity.
67 We show that changes in the supra-threshold magnitude of the summing
68 potential (SP) also occur after full recovery of neural thresholds, suggesting that
69 hidden hearing loss may reflect not only specific synaptic neuropathy, but also
70 lasting changes in IHC electrophysiological function.

71 72 METHODS

73 Eighteen pigmented guinea pigs of either sex, weighing between 282 and 558g
74 at the time of acoustic trauma, were used. The experimental protocols
75 conformed to the Code of Practice of the National Health and Medical Research
76 Council of Australia, and were approved by the Animal Ethics Committee of The
77 University of Western Australia. Details of all anaesthetic and surgical
78 procedures have been presented in previous publications from this laboratory
79 (Mulders et al., 2009; Mulders et al., 2013; Mulders et al., 2011).

82 *Acoustic trauma*

83 For initial acoustic trauma, animals were anaesthetized by intraperitoneal
84 injection of Diazepam (5mg/kg), followed 20 minutes later by an intramuscular
85 injection of Hypnorm (0.315mg/ml fentanyl citrate and 10mg/ml fluanisone; 1
86 ml/kg). Animals were allowed to breathe unassisted and the left ear was exposed
87 to either 1hr (n=6) or 0.5hr (n=6) of a pure tone acoustic trauma (10kHz, 124dB
88 SPL) using a calibrated closed sound delivery system as described previously
89 (Mulders et al., 2011). The right ear was blocked with plasticine during the
90 exposure. A silver wire electrode was placed on the round window (RW) with a
91 reference wire adjacent to the tympanic bulla and an indifferent in the neck
92 muscles, and cochlear neural thresholds (CAP thresholds) for tone bursts ranging
93 from 4 to 24kHz were assessed immediately before and after exposure
94 (Johnstone et al., 1979). Animals were then allowed to recover for 2 weeks. A
95 third group of animals (n=6) served as sham controls and received identical
96 treatment without loud sound exposure.

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98 *Post-recovery electrophysiology*

99 After the recovery period of 2 weeks, all animals were re-anaesthetized by an
100 intraperitoneal injection of pentobarbitone sodium (30mg/kg) and a 0.15ml
101 intramuscular injection of Hypnorm. The maintenance anaesthetic regime
102 consisted of full Hypnorm doses every hour and half doses of pentobarbitone

every 2 hours. Animals were placed on a heating blanket in a sound proof room and artificially ventilated on carbogen (95% O₂ and 5% CO₂). CAP thresholds were again measured as described above and then detailed input-output (I/O) functions were recorded at 4, 8, 14, and 20kHz at 5dB intensity increments. At the end of each experiment, the 4kHz I/O function was repeated in order to control for any general deterioration of the recording conditions. No changes were seen. To record both CAP and summing potential (SP) waveforms, low and high frequency cut-offs on the recording amplifier (DAM 80, X1000 gain) were 1Hz and 3kHz, respectively. Averaged waveforms (32 presentations) were recorded using a 40kHz sampling rate (AD Instruments Powerlab 4ST and Scope software) and amplitudes were analyzed off-line. For 4kHz tones, waveforms at higher intensities were significantly contaminated by cochlear microphonic (CM) despite the low pass filtering employed at the recording stage. A four point smoothing was therefore carried out offline in order to yield a clean CAP waveform for peak-peak measurements.

Figure 1 near here

Figure 1A,B shows typical examples of the RW waveforms recorded in response to a 20kHz tone burst 25 dB and 45dB above CAP threshold (1ms rise-fall time). CAP amplitudes were measured as the N1-P1 peak-to-peak amplitude. As described in detail previously (Brown et al., 2010; McMahon et al., 2008; Sellick et al., 2003) the summing potential (SP) can be observed as the d.c. shift in RW potential occurring both at the onset and offset of the tone and there are

arguments for and against using either as the SP measure. The onset SP could be under-estimated because of the start of the negative-going N1 wave of the CAP, whereas the slower slope of the offset SP is probably the result of contamination by changes in asynchronous neural firing (Sellick et al., 2003). Figure 1C shows that in the present study, there was no difference in the SP magnitude estimated in these two ways in normal animals. Furthermore, we found that changing the tone burst rise-time from 1ms to 0.5ms (which would allow more time for the onset SP to reach its maximum before the CAP response began) caused a negligible change in the measured SP amplitude. For these reasons and because of its steeper rise, the onset SP was used throughout this study for statistical analysis, but in addition the results of offset SP measurements are also shown. SP I/O functions were measured at 14 and 20kHz only, because unlike the remotely generated CAP, which can be recorded in an unbiased manner using a RW electrode (Brown et al., 2010), the SP is generated locally, mainly by the inner hair cells (McMahon et al., 2008; Sellick et al., 2003; Zheng et al., 1997). SP waveforms recorded from the RW in response to low frequencies become complex and difficult to interpret.

Statistical analyses

To compare CAP audiograms, CAP I/O functions and onset SP I/O functions between groups, two-way ANOVA with Sidak's multiple comparisons post hoc tests were used. All statistical analyses were performed in GraphPad Prism.

RESULTS

CAP thresholds

Figure 2A,B show the average CAP thresholds from the three groups of animals. Figure 2C shows the same data as shown in Figure 2A, expressed as changes in CAP threshold immediately after acoustic trauma using a 10kHz tone for either 1 or 0.5hr. Both acoustic trauma groups showed the typical pattern of immediate CAP threshold loss described previously (Mulders et al., 2009; Mulders et al., 2011). Thresholds at 4kHz were unaffected by the 10kHz exposure, while thresholds at higher frequencies showed increasing loss of sensitivity which was maximal between 12 and 24kHz. The threshold changes were significantly less for the 0.5hr exposure group compared to the 1hr exposure, for most frequencies between 8 and 20kHz. Figure 2D shows the difference between initial CAP thresholds measured pre-exposure and those measured from the same animals 2 weeks later. The average CAP thresholds for the 1hr exposure group showed a persistent CAP threshold loss, with a notch-like peak at 12kHz, near complete recovery between 14 and 16kHz and a rising threshold loss between 18 and 24kHz. This pattern of hearing loss has been previously described (Mulders et

al., 2009; Mulders et al., 2011; Robertson et al., 2013; Wang et al., 2002). The notch-like residual threshold change is consistent with an approximate 1/2 octave shift of damage above the exposure frequency that arises from nonlinear cochlear mechanics (Cody et al., 1981). The high frequency loss is not well understood and may be related to differential metabolic sensitivity of the extreme base of the cochlea (Sha et al., 2001). In contrast to the results for the 1hr exposure, the 0.5hr exposure group showed no significant loss of threshold compared to the shams, indicating a full recovery of CAP sensitivity in the 0.5hr exposure group.

CAP I/O functions

Figure 3 shows the CAP I/O functions after 2 weeks recovery for all three groups. For CAP responses to 8kHz tones, there was a significant reduction ($p < 0.05$) in amplitudes between shams and both exposure groups for the two highest intensities of tone burst stimulation (Fig. 3C). The reduction in CAP amplitudes at supra-threshold tone levels was most apparent for tone stimuli at 14 and 20kHz (Fig. 3A,B). At these frequencies, there was a significant reduction ($p < 0.05$) in amplitudes in both exposure groups compared to shams, for all tone intensities at ~60dB and above (1hr exposure) and from ~70dB for the 0.5hr exposure.

The results obtained at 8, 14 and 20kHz provide evidence of hidden hearing loss and confirm the findings of others. In particular, the reduced CAP amplitudes in

response to supra-threshold tones in the 0.5h exposure group are especially convincing, since in this group there was a complete recovery of all CAP thresholds (Fig. 2B,D). The results for the 1hr exposure group are less simple to interpret, because the reduced supra-threshold CAP amplitudes could be partly a result of a reduced contribution from adjacent regions whose thresholds may be elevated.

At all frequencies above 4kHz that were tested, it is important to stress that there were significant threshold changes immediately after the acoustic trauma, suggesting that the persistent effects, seen on supra-threshold CAP amplitudes after 2 weeks recovery, were a consequence of the initial trauma at that region. However, a surprising result was obtained for the CAP response to 4kHz tones. At this frequency, CAP thresholds were not affected by the initial acoustic trauma at either exposure duration (Figure 2A,C) and consistent with this, there were no significant differences between the CAP amplitudes at any intensity when the sham and 0.5h exposure groups were compared. In contrast, however, in the 1hr exposure group, although the recovered CAP thresholds at 4kHz were not different from the pre-exposure thresholds, supra-threshold CAP amplitudes were significantly reduced ($p < 0.05$) at 70, 75 and 90dB (Fig. 3D).

SP I/O functions

Figure 4 shows the final recovery SP I/O functions for the three experimental groups using tone bursts at 14 (Fig. 4C,D) and 20kHz (Fig. 4A,B). Figures 4A,C

207 show the results using the onset SP and figure 4B,D show the results using offset
208 SP measure. The offset SP data are more limited at lower SPLs than for the onset
209 SP because of the difficulty in defining a discrete SP step for the more sluggish
210 offset waveform. However, at moderate to high SPLs, there is excellent
211 agreement between the onset and offset SP data in all groups as also shown in
212 Figure 1C. In the 1hr exposure group there was a large reduction in SP amplitude
213 compared to the shams at all intensities $>\sim 65$ dB SPL and in the case of 20kHz a
214 rightward shift was also apparent that was consistent with the average loss of
215 neural sensitivity at this frequency. It is notable however, that the pattern of
216 CAP threshold change in the 1hr exposure group was highly variable and
217 therefore Figure 5 shows an example from this group of the 20kHz SP I/O function
218 in one animal with a notch-like loss of CAP sensitivity peaking at 12 kHz but
219 recovered thresholds at 20kHz that were not different from normal (Fig. 5B,C).
220 Comparison with the average SP I/O function from the sham group (Fig. 5A)
221 shows that even in this individual case, the SP supra-threshold amplitudes are
222 markedly reduced, although as for the CAP amplitudes in this exposure group, it
223 cannot be ruled out that this is the result of a reduced contribution to the
224 response from other regions whose thresholds are elevated.

225 As for the CAP I/O results, the co-existence of normal CAP thresholds with
226 reduced supra-threshold SP amplitudes, is reinforced by the data from the 0.5hr
227 exposure group in which CAP thresholds at all frequencies returned to normal 2
228 weeks after the initial acoustic trauma. Figure 4 shows that even in this group,

229 supra-threshold SP amplitudes measured at either 14 or 20 kHz $>\sim 80$ dB SPL were
230 significantly reduced.

231 Figure 6 shows the relationship between the 14 and 20 kHz SP and CAP
232 amplitudes for the three groups of animals. If the effects of acoustic trauma on
233 the CAP were solely due to a reduced IHC receptor potential (either because of
234 damage to the OHC amplifier, or to the IHC transduction mechanism itself) then
235 the trauma values should lie on the same curve as the sham group). This is not
236 the case for the 30 minute exposure group at both 14 and 20 kHz. This is
237 consistent with the reduced CAP output at higher sound pressures not being fully
238 explained by the SP change alone, and a likely synaptic neuropathy is present
239 that is selective for high threshold nerve fibres.

240 The result of this SP/CAP comparison is less easy to understand for the 1hr
241 exposure group as the range of CAP and SP amplitudes after this trauma is
242 markedly reduced and the results are also influenced by the significant threshold
243 loss at these frequencies. The data appear to lie on the same curve as the sham
244 group with only a minor deviation at the higher end of the curve. However this
245 does not necessarily mean that there is no neuropathy present after this more
246 severe trauma. It is more likely that the residual CAP amplitude is almost
247 exclusively generated by the recruitment of lower threshold nerve fibres which
248 are known to have a limited dynamic range (Furman et al., 2013; Winter et al.,
249 1990).

250

251 DISCUSSION

252 The present results using the RW CAP response confirm previous studies which
253 have shown that the overall cochlear neural output for supra-threshold stimuli,
254 is depressed some weeks after acoustic trauma and that this can occur despite a
255 full recovery of neural thresholds. The novel finding in this study is that in
256 addition to depressed CAP amplitudes, the SP recorded from the RW in response
257 to high frequency tones, is similarly depressed. There is compelling evidence
258 that SP is dominated by the receptor current generated by the IHCs (McMahon
259 et al., 2008; Sellick et al., 2003; Zheng et al., 1997). Intracellular recordings of
260 the receptor potential transfer function from hair cells (Russell et al., 1986) show
261 that the operating point of IHCs is asymmetric and they therefore generate a
262 large d.c component in response to sinusoidal input. The operating point in OHCs
263 is, in contrast, close to the middle of the transfer curve and they therefore do
264 not contribute a major component to the externally recorded SP (Russell et al.,
265 1986). There is thought to be an additional small, slower negative-going
266 contribution to the onset of the RW response before the start of the N1, that
267 emanates from the post-synaptic dendritic potential (DP) (Dolan et al., 1989;
268 Sellick et al., 2003) and this would presumably be reduced when post-synaptic
269 neural elements are lost. However, such a post-synaptic contamination of the
270 onset SP cannot explain the present results since a reduction in the DP should
271 lead to an increase in the positive-going SP, rather than the observed decrease.

272 Furthermore, the fact that the results from onset and offset SP measurements
273 were identical, strengthens the argument that such contamination of the onset
274 response is unimportant in our measurements. On balance, the present results
275 strongly suggest that hidden hearing loss need not be a pure neuropathy,
276 involving only the IHC-afferent synapse. For the particular forms of acoustic
277 trauma used in this study, hair cell malfunction can also be involved.

278 The precise relative contributions of neuropathy and hair cell malfunction to the
279 reduced neural output cannot be readily ascertained from the present data.
280 Figure 6 shows clearly that the SP changes cannot fully account for the observed
281 CAP changes and therefore strongly suggests that neuropathy is present
282 involving the high threshold, low spontaneous rate fibres as shown previously by
283 others (Furman et al., 2013; Kujawa et al., 2009; Lin et al., 2011). However the
284 20 kHz data (Fig. 6B) show an apparent greater contribution of neuropathy than
285 the 14 kHz data (Fig. 6A) for the 30 min AT. This is not readily explained as the
286 immediate threshold loss at both frequencies is the same (Fig. 2A) and at both
287 frequencies thresholds completely recover (Fig. 2B). One possibility is that there
288 is larger protective effect at 14 kHz from olivocochlear efferent feedback
289 (Maison et al., 2013)

290 The nature of the proposed hair cell malfunction is yet to be determined. One
291 possibility is that IHC function is normal in all respects, but the reduced SP
292 amplitude reflects a reduced supra-threshold contribution by the OHCs to the
293 organ of Corti vibration that provides the mechanical drive to IHCs. This

explanation seems unlikely for several reasons. First, normal CAP thresholds imply normal levels of OHC amplification, and it has been shown that there is a direct correlation between the magnitude of OHC currents (measured as cochlear microphonic) and CAP threshold (Patuzzi et al., 1989a). Furthermore, it is known that the OHC contribution to cochlear vibration amplitudes becomes less at higher sound intensities because of the saturation of the cochlear amplifier effect (Johnstone et al., 1986; Yates et al., 1990; Yates et al., 1992). In addition, although their acoustic trauma regimes were not identical to those used in the present study, Liberman and co-workers (Kujawa et al., 2009; Lin et al., 2011) have reported that I/O functions of the DPOAE (reflecting the electromechanical amplifier function of the OHCs) can fully recover after loud sound exposures that result in hidden hearing loss as detected by supra-threshold neural response amplitude changes.

An alternative possibility is that there is damage to or loss of IHCs which are responsible for the generation of the SP. The SP recordings at 14 and 20kHz, although localized to the basal turn, are graded responses and hence patchy loss of, or damage to, some but not all of the IHC population could be responsible (see for example (Mulders et al., 2011)). This might result in a reduced SP amplitude at higher stimulus levels, but provided there are enough normally-functioning IHCs, the SP and CAP responses near threshold could still be normal. The 14kHz SP data after recovery from the milder acoustic trauma (Fig. 4B)

provide a clear example of the fact that SP amplitudes can be indistinguishable from normal over a significant range above threshold and the reduction in amplitude only appears at higher intensities. This phenomenon is strikingly similar to the CAP outcome for which the current explanation is the presence of a higher threshold population of afferents that are more prone to degeneration after loud sound (Furman et al., 2013). However, unlike for the cochlear neural output, there is no evidence for a specific population of high threshold IHCs that are more prone to damage. A more likely possibility therefore is that the reduced supra-threshold SP amplitudes despite normal CAP sensitivity, reflect a reduced supra-threshold output of individual IHCs, even when their transmitter release (and hence excitation of their intact afferent neurons) at threshold sound levels is normal. This reduced supra-threshold SP could be a consequence of loss of a proportion of the IHC transduction channels or associated structure such as stereocilia tip links, as a consequence of acoustic trauma (see for example, (Patuzzi et al., 1989b)).

A final issue is the puzzle of the 4kHz CAP supra-threshold responses which were found to be depressed in the 1hr exposure group despite there being no initial effect on the CAP thresholds immediately after the exposure or after 2 weeks recovery, unlike all the other frequencies investigated. This result could have two explanations, one trivial and the other significant. First, because of the low frequency tails of tuning curves of auditory nerve fibres, higher level CAP responses to 4kHz tones could receive a remote contribution from more basal

regions of the cochlea and these remote contributions could be reduced as a consequence of threshold loss and/or neuropathy at higher frequencies, particularly in the 1hr exposure group. Such a mechanism might also contribute to the changes seen at 8kHz. Another possibility is that damage resulting from the acoustic trauma can spread, over time, to more remote apical cochlear regions unaffected by the initial exposure. If this result is confirmed, the nature of such spreading damage will require further investigation.

In summary, the results of the present study show that hidden hearing loss may involve defects in the supra-threshold behavior of IHCs. This finding could have important implications for the consequences of hidden hearing loss, because the supra-threshold behavior of all surviving nerve fibers receiving input from the IHCs could potentially be affected by such IHC pathology. It would therefore be instructive to measure I/O functions of individual surviving afferent neurons in such cases. Finally, the results in the present study are reminiscent of findings in human patients with auditory neuropathy, in which electrocochleography has identified a subset of patients in whom there is evidence of a possible pre-synaptic contribution to this pathology (McMahon et al., 2008).

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360 FIGURE LEGENDS

361 Figure 1.

362 A,B: Examples of normal RW recording in response to 20kHz tone bursts 25 dB
363 (A) or 45dB (B) above CAP threshold (average of 32 stimulus presentations).
364 CAP amplitude defined as N1-P1 peak to peak amplitude. SPon denotes onset
365 summing potential. SPoff denotes offset summing potential. C. Comparison
366 of SP magnitudes estimated from the d.c. change at tone onset and offset in
367 sham animals.

368 Figure 2.

369 A,B: CAP threshold audiograms in dB SPL, C,D: Changes in cochlear compound
370 action potential (CAP) thresholds. A,C: showing audiograms immediately after
371 the 30 min and 1 hr acoustic trauma (AT) as well as the audiogram of the sham
372 animals for comparison. B,D: Results after two weeks recovery in sham, and 30
373 min or 1 hr AT animals. Mean $\pm$ SEM for each group. N=6 for all. Symbols in C
374 depict statistical significant difference between 30 min AT and 1 hr AT groups.
375 Symbols in D depict statistical difference between 1 hr AT group with sham as
376 well as with 30 min AT group. * $p < 0.05$, ** $p < 0.01$, # $p < 0.001$.

377 Figure 3.

378 CAP I/O functions showing CAP N1-P1 amplitude plotted against sound intensity
379 at 20 kHz (A), 14 kHz (B), 8 kHz (C) and 4 kHz (D) in sham animals and animals
380 exposed to a 30 min or 1 hr AT. Mean $\pm$ SEM for each group. N=6 for all except

for the 1 hour AT group in A (n=5). Symbols depict statistically significant differences. * significant difference between sham and 1 hr AT only; ^ significant difference between sham and 1 hr AT only as well as between 30 min AT and 1 hr AT; # significant differences between all 3 groups; & significant difference between sham and 1 hr AT only as well as between sham and 30 min AT.

Figure 4

SP I/O functions showing SP amplitude at 14 (C,D) and 20 kHz (A,B) plotted against sound intensity. A and C show onset SP and B and D show offset response. Mean $\pm$ SEM for each group. N=6 for all except for the 1 hour AT group in A (n=5). Statistical analysis was performed on the onset SP. Symbols depict statistical significant differences. * significant difference between sham and 1 hr AT only; ^ significant difference between sham and 1 hr AT only as well as between 30 min AT and 1 hr AT; # significant differences between all 3 groups.

Figure 5.

A, Example of SP I/O function for one animal (solid line) in the 1hr acoustic trauma group which had normal CAP thresholds at 20kHz after recovery. Average SP I/O function for sham group is shown for comparison (dotted line). B. CAP threshold audiogram in dB SPL of the animal shown in A before AT and

402 after recovery. C. CAP threshold changes (recovery versus pre-exposure) for
403 single acoustic trauma animal in A, showing narrow threshold notch at 12kHz
404 and normal thresholds at other frequencies.

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406 Figure 6.

407 Plots of average CAP versus average SP amplitudes for sham, 30 min at and 1 hr
408 AT groups at 14 kHz (A) and 20 kHz (B).

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