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Temporary Reduction of Distortion Product Otoacoustic Emissions (DPOAEs) Immediately Following Auditory Brainstem Response (ABR)

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Abstract

The hearing status of an experimental animal is typically assessed in the laboratory setting by the combined use of auditory brainstem response (ABR) and distortion product otoacoustic emissions (DPOAE), carried out in succession, with the former assay preceding the latter. This study reports a cautionary finding that the use of this accepted regimen yields a reduced DPOAE response. When the DPOAE were performed after ABR testing, transient reduction of the DPOAE amplitudes was observed at all frequencies in both the inbred, C57/B6 and FVB/n, and the outbred, SW mouse strains. DPOAEs were reduced post-ABR in multiple mouse strains suggests that this finding is not strain-specific but a general consequence of the preceding ABR analysis. The reduction in DPOAE was temporary: when re-tested at one hour, DPOAE amplitudes recovered to pre-ABR levels. In contrast to the ABR's impact on DPOAE response, ABR thresholds were not altered or reduced when preceded immediately by DPOAE measurements. The molecular alterations underlying the ABR-induced transient reduction of DPOAE remains to be determined. To investigate the potential role of reactive oxygen species in post-ABR DPOAE reduction, transgenic mice over-expressing SOD1, the cytoplasmic enzyme critical for removal of superoxide radicals were subjected to the same auditory testing regimen. Similar to their wild type littermates, the SOD1 transgenic mice also demonstrated post-ABR DPOAE reduction, and thus do not support a role for superoxide radicals in transient reduction of DPOAE. While toxic noise exposure is known to negatively impact OAE, transient decrease in DPOAE levels following standard ABR assay has not been previously described.

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A practical outcome from this study is a recommendation for reversal of the traditional order for carrying out auditory tests, with the OAE measurements preceding ABR assessment, thus ensuring that the DPOAE response is unaffected.

Keywords

DPOAE; ABR; transient reduction

INTRODUCTION

Evoked otoacoustic emissions (OAEs), are sounds generated within the inner ear in response to stimuli. OAEs are considered to be a byproduct of the cochlear amplification that enhances sensitivity and frequency selectivity. The mammalian outer hair cells (OHC) are considered to play an essential role in the cochlear amplifier. Evoked OAEs are important clinically as they form the basis of a simple non-invasive test for hearing defects in newborns and young children. Distortion product OAE (DPOAE) are evoked using a pair of primary tones f_1 and f_2 (where $f_2 > f_1$). The evoked responses from these stimuli occur at predictable frequencies depending on f_1 and f_2 and are known as distortion products. The most prominent DPOAE is $2f_1 - f_2$ (the “cubic” distortion tone) most commonly used in screening for hearing loss. In the laboratory, DPOAE analysis is used in combination with the auditory brainstem response (ABR) for evaluating hearing in animal subjects.

DPOAEs provide a sensitive measure of the functional integrity of the outer hair cell (OHC). DPOAE analysis is considered particularly relevant in studies investigating acquired hearing loss in which the sensory hair cells are primarily affected. Hearing assessment in noise-exposed groups such as army recruits has identified a general decrease in DPOAE amplitude but without a corresponding permanent increase in the ABR thresholds (Desai et al., 1999; Lapsley Miller et al., 2004; Lapsley Miller et al., 2006). These results suggest a generally greater sensitivity of the DPOAE measurement in detecting impact of the noise damage upon hearing that cannot be detected by the analysis of sound-evoked potentials. DPOAE analysis has also been shown to be more sensitive than ABR-based measurements for revealing subtle auditory dysfunction after exposure to gentamicin, an aminoglycoside, or cisplatin, a chemotherapeutic agent, in separate studies (Shi et al., 1997; Sie et al., 1997). In addition to its relatively greater sensitivity, reduced OAEs have also been shown to have prognostic value. Thus, recent studies have identified reduced OAEs as a risk factor and an indicator of future hearing loss in military personnel exposed to continuous and impact noise (Lapsley Miller et al., 2006; Marshall et al., 2009). Selective OHC toxicity represents the general cytopathology underlying permanent decrease in DPOAE levels that is identified in acquired hearing loss (Hofstetter et al., 1997).

In addition to the permanent changes, transient decreases in DPOAE levels have also been described in response to toxic sound stimuli. Studies in both humans and animals have shown detectable reduction in DPOAEs in response to a sustained and intense noise exposure (Bhagat et al., 2008; Korres et al., 2009; Muller et al., 2008). However, routine ABR testing has not been reported to impact DPOAE. The current study describes a chance discovery of temporary reduction of DPOAE in experimental mice immediately following a routine ABR test. The reduction of the distortion product was not long lasting and established to be a consequence of the order in which the two assays are carried out and not due to artifacts of instrumentation, duration under anesthetic or other irregularities. Reactive oxygen species (ROS) may play a role in the observed phenomena. ROS production is elevated in noise trauma and anti-oxidant treatment has been shown to prevent hair cell damage and hearing loss (Henderson et al., 2006). Potential contribution of ROS was investigated by assessing if the reduction in post-ABR DPOAE could be mitigated in transgenic mice over-expressing superoxide dismutase 1

(SOD1)(Chen et al., 2003; Mele et al., 2006), a cytoplasmic enzyme critical in neutralizing oxygen radicals. SOD1 converts superoxide radicals to molecular oxygen and hydrogen peroxide and thus represents one of the critical regulators of ROS levels within the cells.

Materials and Methods

Experimental Animals

C57BL/6NCrI, FVB/NCrI and CFW mice (henceforth referred to as C57/B6, FVB/n and SW respectively) were purchased from Charles River Laboratories (Wilmington, MA). Transgenic SOD1 mice on C57/B6 background were provided by Dr. Holly Van Rammem (University of Texas Health Center at San Antonio, San Antonio, Texas). These mice were expanded by mating with C57/B6 and the presence or absence of the SOD1 transgene in the progeny was determined using previously described primer sets (Chen et al., 2003). SOD1 expression within the cochleae of transgenics has been shown to be 2.7-fold greater relative to their wild type littermates (Coling et al., 2003); in the transgenic, the SOD1 was widely expressed in the cochlea including outer hair cells (unpublished results from the authors' laboratory). Animals, six to eight weeks of age, were anesthetized with ketamine and xylazine (ketamine 150 mg/kg and xylazine 10 mg/kg) before performing the auditory tests ABR and DPOAE. All experimental procedures involving animals were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and approved by the IACUC at NYU School of Medicine. A minimum of 6 mice, were tested under each experimental condition described below.

Auditory Brainstem Response (ABR) Testing

The ABR thresholds were determined using procedures described previously (Mhatre et al., 2007). Briefly, responses were recorded from silver wire electrodes inserted through the skin at the vertex (active electrode), ipsilateral mastoid (negative) and contralateral mastoid (ground). The scalp-recorded potentials were amplified and sampled at a rate of 25 kHz by an analogue to digital converter using Tucker-Davis software. The broadband (clicks) or frequency specific (tones) stimuli (8, 16, 24 and 32 KHz) were presented at a rate of 33 per second and the responses averaged over 500 trials using free-field ES1 speakers (Tucker-Davis Technologies, Gainesville, FL). The intensity of the input stimulus was initially set at 85 dB SPL and sequentially attenuated in 5 dB steps until a threshold level was reached. The ABR procedure lasted approximately 25 minutes,

Otoacoustic Emission Testing

Distortion Product Otoacoustic Emissions at $2f_1-f_2$ were elicited from test mice using the Real-time Signal Processing System II from Tucker Davis Technologies and procedures described previously (Mhatre et al., 2007). Two level ($L_1 = 65$ dB SPL, $L_2 = 50$ dB SPL) primary signals (f_1 and f_2), with $f_2/f_1 = 1.3$, were generated with test frequencies ranging from 5 to 25 kHz. The primary tones produced by two separate speakers (EC1 close-field speakers, Tucker-Davis Technologies) were introduced into the animal's sealed ear canal through an insert earphone speculum. DPOAE recordings were made with a low-noise microphone ER 10B (Etymotic Research, Elk Grove Village, IL). A peak at $2f_1-f_2$ in the spectrum was accepted as a DPOAE if it was 3 dB above the noise floor.

Statistical Analysis

The data was analyzed using a three way mixed model ANOVA with the mouse strain as a between group variable and the time (pre or post ABR, and one-hour recovery) and frequency (f_2) as the two within group (repeated measures) variables. The significance of specific comparisons was done using the Tukey's HSD Post-hoc test.

RESULTS

I. Impact of ABR upon DPOAEs in C57/B6 and FVB/n Mice

DPOAEs were obtained under three separate conditions from both C57/B6 and FVB/n mice: before, immediately following, and one hour after ABR analysis. Figure 1 shows the DP-grams, for these three conditions, spanning the F2 frequency range of 6 to 24 kHz for C57/B6 and FVB/n. In both C57/B6 (Fig. 1A) and FVB/n (Fig. 1B), the immediate post-ABR DPOAE levels were significantly reduced compared to the pre-ABR DPOAE ($p < 0.0001$). The repeat DPOAE performed one hour following ABR testing showed recovery of the responses in both C57/B6 and FVB/n (Figs. 1C and D, respectively). The Mixed model ANOVA showed that time of measurement (pre, post and after 1 hour recovery) has a significant effect on DPOAE level ($F=23.5$, $df=2$, $p<0.00002$). Post hoc comparisons revealed that the difference between pre and post ($p = 0.00018$) and post and one hour ($p = 0.0013$) were significant but the difference between pre and one hour recovery was not ($p = 0.08$). The pattern seen was independent of the mice strain ($F = 1.9$, $df=1$, $p<0.2$) indicating that recovery was nearly complete at one hour in both C57/B6 and FVB/n.

The ANOVA revealed that frequency was a significant variable ($F = 189.7$, $p<0.000000$, d.f. 15). All two way and three way interactions including frequency as a variable were significant ($p < 0.000000$) indicating that the pattern of damage and recovery was not identical at all frequencies. The interaction between frequency and strain was also significant ($F=11.34$, $p<0.000000$, $df = 15$) indicating that the pattern of level changes differed between the two mouse models. The magnitude of the difference between the pre- and immediately post-ABR DPOAE and between the pre- and one-hour post-ABR DPOAE for individual frequencies in the C57/B6 and FVB/n mice is presented in form of bar graphs, Figure 2A and B, respectively. While both strains display post-ABR reduction of their DPOAE levels across all frequencies, the DPOAE from the C57/B6 mice are affected much more severely at higher frequency primaries than the FVB/n mice. DPOAE levels were also reduced in mouse models at the lower frequencies. These low frequency changes do not recover completely following one-hour recovery period.

To investigate if anesthesia was responsible for the observed DPOAE differences, DPOAE were performed after mice had been under anesthesia for 25 minutes, a time period equivalent to the duration of the ABR test. DP-grams before and after 25 minutes of anesthesia were not significantly different ($p = 0.258$) suggesting that the anesthesia alone does not attenuate DPOAE (results not shown). To test the possibility that performance of DPOAE testing might impact the subsequent ABR responses, ABR testing was performed immediately following DPOAE testing in the C57/B6 mice; broadband and pure tone ABR thresholds did not change when preceded immediately by DPOAE measurements ($p>0.05$; Figure 3).

As with the inbred strains, C57/B6 and FVB/n, ABR-induced DPOAE reduction was also seen in the outbred SW mice, illustrated in Figure 4. Note that the post-ABR reduction of the DPOAE levels is observed at F2 frequencies greater than or equal to 14.2 kHz.

II. DPOAEs in Transgenic Mice Over-expressing SOD1

To assess if elevated ROS levels within the cochlea following ABR analysis are responsible for the temporary attenuation of the OAEs, we tested transgenic mice over-expressing SOD1 and their wild type littermates, before, immediately following, and one hour after ABR analysis for the post-ABR attenuation of the DPOAEs. The comparative DP-grams from SOD1 transgenic immediately following ABR (Figure 5A) and at one hour after ABR (Figure 5B) are similar to those seen for C57/B6. The pre- and immediate post-ABR DP-grams for the SOD1 transgenics and their wild type littermates (not shown) were not statistically different

($p = 0.737$). Hence, SOD1 over-expression within the cochleae of the SOD1 transgenics does not prevent the temporary attenuation of the DPOAE.

DISCUSSION

Reduction in the intensity of DPOAE has been previously described in subjects exposed to toxic sound levels (Marshall et al., 2001). The current study has identified transient reduction in the intensity of DPOAE in response to non-toxic sound stimuli commonly used in the laboratory and clinics to assess hearing acuity: the standard ABR test. The transient but detectable impact of the standard ABR test upon distortion product amplitude has not been previously reported. The temporary DPOAE level shift was demonstrated in two different inbred, C57/B6 and FVB/n, and one outbred, SW, mouse strains, suggesting that post-ABR reduction of DPOAE is a common occurrence. The reduction in DPOAE recovers in one hour.

The DPOAE amplitude reduction induced by the preceding ABR analysis may reflect a direct effect on the mechanosensory hair cells in response to continuous auditory stimulation. Alternatively, central regulation may also be responsible for the reduced activity of the OHC. Medial olivocochlear (MOC) neurons project to outer hair cells (OHC), forming the efferent arm of a reflex that affects sound processing and offers protection from acoustic over stimulation (Liberman, 1989; Liberman et al., 1996). Several studies have shown that activation of the MOC neurons leads to reduction of DPOAE. Thus, the ABR stimuli could activate the MOC neurons that exert suppressive effect upon the OHC and their OAEs. The middle-ear muscle (MEM) reflex can also affect OHC activity. Elicitation of the MEM reflex results in a stapedius muscle contraction, which alters the sound pressure in the ear canal yielding a reduced response by the OHC. Effective activators of the MOC reflex are also activators of the MEM reflex.

The reduction in DPOAE was relatively greater in the higher frequencies of C57/B6 strain compared to FVB/n. It is tempting to speculate that the relatively greater attenuation of DPOAE in the higher frequency range for the C57/B6 mice may be related to their susceptibility to early onset age and noise related hearing loss (AHL) due to the *ahl* gene (Johnson et al., 2000; Johnson et al., 1997). The *ahl* allele corresponds to *Cdh23* which encodes Otocadherin, a type-1 transmembrane protein that is critical for cell adhesion and dependent upon calcium ions for its function (Di Palma et al., 2001). C57/B6 mice contain a single nucleotide polymorphism in exon 7 of *Cdh23* gene, *Cdh23*^{753A}, causing an in-frame skipping of exon 7. The homozygous *Cdh23*^{753A} allele in combination with multiple secondary factors was found to be primary determinant of early-onset AHL in several different inbred strains (Noben-Trauth et al., 2003). Mutations in *Cdh23* cause deafness in humans (DFNB12) and the mouse model *waltzer* (V) (Bork et al., 2001; Di Palma et al., 2001; Wilson et al., 2001). The relatively greater attenuation of the DPOAE in the higher frequency range for the C57/B6 mice raises the possibility that the *Cdh23* encoded variant otocadherin contributes towards the OAEs through a potential role in OHC contractions. Specifically, *Cdh23* has been shown to be a component of the tip-links that interconnect the stereocilia (Siemens et al., 2004; Sollner et al., 2004). Hence, the severe reduction of the DPOAE levels at high frequencies, F2, 20 to 25 kHz, in the C57/B6 relative to FVB/n or SW may reflect the expression of the *Cdh23* allele that is targeted to the structural integrity of the tip links.

The formation and accumulation of reactive oxygen species (ROS) has been identified as an important contributor of acquired forms of hearing loss. Endogenous regulators of ROS provide protection against its damaging effects. Copper/Zinc SOD 1 (SOD1) is widely distributed and is considered to be a key component of the ROS inactivation pathway. Deficiency of SOD1 has been demonstrated to increase the vulnerability of the cochlea to damage associated with normal aging (McFadden et al., 1999) and noise (Ohlemiller et al., 1999), and concomitant

increase in ROS levels. Correspondingly, transgenic mice over-expressing SOD1 were protected against aminoglycoside-induced hearing loss (Sha et al., 2001). To investigate if ROS may be responsible, we tested the susceptibility of transgenic mice overproducing SOD1 to the ABR-induced DPOAE attenuation. The SOD1 transgenic mice also showed a reduction in DPOAE levels similar to their wildtype littermates. These results do not support the role for excess ROS in DPOAE attenuation.

OAE and ABR testing form the common test battery to assess the hearing acuity of the laboratory animal; similarly, they play a critical role in the assessment of hearing in children. A practical outcome of this study is a recommendation for reversal of the assay order for carrying out the auditory tests, with the OAE measurements preceding ABR assessment, thus ensuring that the DPOAE response is unaffected. If, ABR analysis is to precede OAE measurement, then a minimum interval of two hours is recommended between these tests. Alternatively, separate animals from the same strain may be tested for ABRs and DPOAEs, respectively, thus avoiding any possible influence of prior tests.

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Figure 1. DPOAE in C57/B6 and FVB/N mice before, immediately after, and one-hour following ABR

DPOAE were assessed in both C57/B6 and FVB/N mice, spanning the F2 frequency range of 6 to 24 kHz, under three different conditions: pre-ABR; immediate post-ABR and 1-hour-post-ABR. Panel A and B show DPOAE levels pre-ABR, immediately following ABR and the noise floor for C57/B6 and FVB/n across the frequency range, respectively. Relative to the pre-ABR DP-grams, the immediate post-ABR DP-grams from both C57/B6 (A) and FVB/n (B) mice display lower amplitude profile. Panel C and D show DPOAE levels pre-ABR, one hour following ABR, and the noise floor for C57/B6 and FVB/n across the frequency range, respectively. At one hour following ABR, the DP-gram shows recovery of the amplitude to nearly the pre-ABR levels.

Figure 2. Difference in DPOAE response at different time points

The DPOAE data presented in Figure 1 is now presented as difference in the DPOAE amplitude response at different time points in a bar graph. The magnitude of the difference between pre-ABR DPOAE response and the DPOAE response immediately following ABR (black bars) and the magnitude of the difference between pre-ABR DPOAE response and DPOAE response at 1-hour after ABR (grey bars) are shown for C57/B6 (A) and FVB/N (B) mice across the F2 frequencies tested. The difference between DPOAE responses at pre-ABR and immediately following ABR is statistically significant at nearly all of the frequencies (black bar). In general, the post-ABR decrease in the DPOAEs for the C57/B6 mice is much greater in response to primary frequencies of 10 KHz or greater than that observed for the FVB/N mice. In contrast, the difference is not significant when comparing the pre-ABR with 1-hour post-ABR DPOAE responses at nearly all of the frequencies (grey bar) Asterisk denotes statistically significant difference with $p < 0.01$.

Figure 3. ABR thresholds before and immediately following DPOAE test in C57/B6 mice

ABR thresholds were determined for click and pure tone stimuli (8 kHz, 16 kHz, and 24 kHz) in C57/B6 mice before performing DPOAE test (pre-) and immediately following DPOAE test (post-). The ABR thresholds were similar with the differences being statistically insignificant ($p>0.05$).

Figure 4. DPOAE in SW mice before and immediately after ABR

The comparative pre- and post-ABR DP-grams for the outbred SW illustrate the ABR-induced reduction of the DPOAE levels as observed in the C57/B6 and FVB/n strains.

Figure 5. DPOAE in SOD1 transgenic mice before, immediately after, and one-hour following ABR
 In the SOD1 over-expressing mice, reduction in the DPAOE response was seen when performed immediately after ABR testing when compared to the response pre-ABR test (A). At one-hour after ABR, the DPOAE response returned to pre-ABR levels (B). The pre- and post-ABR DP-grams for the SOD1 transgenic mice as seen here were statistically indifferent to the changes seen in their wild type littermates (data not shown) thus suggesting that SOD1 over-expression did not prevent the reduction in DPOAE.