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Physiological vulnerability of distortion product otoacoustic emissions from the amphibian ear

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The physiological vulnerability of distortion product otoacoustic emissions (DPOAEs) was investigated in the leopard frog, *Rana pipiens pipiens*. For each frog, DPOAEs were recorded from the amphibian and the basilar papillae. Measurements were taken before and after either the arrest of oxygen supply due to cardioectomy, or the destruction of the central nervous system (CNS). DPOAEs in response to high-level stimuli (>75 dB SPL) were rather robust to these insults during the first two hours post surgery. In contrast, DPOAE amplitudes in response to low-level stimuli (<75 dB SPL) decreased significantly. On average, low-level emissions from the amphibian papilla disappeared within 6 min for cardioectomy, and after 13 min for CNS destruction. In the basilar papilla, low-level DPOAEs disappeared more slowly: on average after 34 min following cardioectomy, and after 58 min for CNS destruction. The difference in physiological vulnerability between low- and high-level emissions is similar to that in mammals and a lizard. The difference between the DPOAE decay rate of the frog's amphibian and basilar papillae suggests important differences between the hearing mechanisms of the papillae. © 2003 Acoustical Society of America. [DOI: 10.1121/1.1608957]

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I. INTRODUCTION

Distortion product otoacoustic emissions (DPOAEs) in mammals and lizards are vulnerable to acoustic overstimulation, anoxia, and pharmacological agents (Zurek *et al.*, 1982; Kemp and Brown, 1984; Lonsbury-Martin *et al.*, 1987; Whitehead *et al.*, 1992; Manley *et al.*, 1993; Mills *et al.*, 1993; Rebillard *et al.*, 1993; Frolenkov *et al.*, 1998). While DPOAEs generated by low-level stimuli (below about 60 dB SPL) are particularly vulnerable, high-level DPOAEs (above 60 dB SPL stimulus level) are more robust. This observation has led to the conclusion that low- and high-level DPOAEs are generated by different inner ear mechanisms. In particular, mammalian low-level DPOAEs are assumed to be related to motility of outer hair cells. In contrast, high-level DPOAEs may originate from "passive" nonlinear responses of intracochlear structures.

Somatic outer hair cell motility (Brownell *et al.*, 1985) has been proposed as a mechanism for otoacoustic emission generation. Somatic motility relies on the presence of the protein prestin in the hair cell basolateral membrane (Zheng *et al.*, 2000; Liberman *et al.*, 2003). Prestin-driven somatic motility has been confirmed in mammalian outer hair cells (Zheng *et al.*, 2000) and its effect on otoacoustic emissions shows that it is a necessary and important component of the mammalian cochlear amplifier (Liberman *et al.*, 2003). In

amphibians and other nonmammalian species there is no evidence of somatic motility (He *et al.*, 2003; Manley, 2001). An alternative mechanism for emission generation is the active movements of hair bundles. Although in the frog such bundle movements have only been shown in saccular hair cells at low frequencies (Assad *et al.*, 1989; Assad and Corey, 1992; Benser *et al.*, 1996; Martin and Hudspeth, 1999), it is conceivable that similar motility exists at audio frequencies and in other vertebrate species. Active hair bundle movements have also been shown for hair cells from the turtle basilar papilla (Crawford and Fettiplace, 1985) and the chicken basilar papilla (Hudspeth *et al.*, 2000). Active bundle movements could be a generation mechanism common across vertebrate species, perhaps acting in concert with somatic cell motility in the case of mammals.

Here, we investigated the vulnerability of DPOAEs from the amphibian ear. If DPOAEs in amphibians show similar physiological vulnerability to that in other vertebrates, the hypothesis that similarities exist between DPOAE generation mechanisms in all vertebrates is supported. The common generation mechanism across species may be hair bundle motility, while somatic motility is an additional function that may have become predominant in mammals.

The amphibian inner ear includes the amphibian papilla (AP) and the basilar papilla (BP), both of which are auditory end organs. Based on a comparison between DPOAE frequencies and auditory nerve fiber characteristic frequencies for both papillae, Van Dijk and Manley (2001) concluded that in *Hyla cinerea* the AP generates both spontaneous otoacoustic emissions (SOAEs) and DPOAEs, while the basilar

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papilla generates DPOAEs only. This suggests that important differences between the papillae may exist. We will describe differences between the vulnerability of DPOAEs from the AP and the BP.

II. MATERIALS AND METHODS

Four male and four female leopard frogs, *Rana pipiens pipiens*, were used in these experiments, all obtained from commercial suppliers. DPOAE measurements were performed using a sensitive microphone probe (Etymotic Research, model ER10-C). This probe contains two miniature speakers and a microphone. It is typically used in human subjects with a special foam tip, which fits the human auditory meatus. We used the probe with such a foam tip, but we fit a piece of rubber tubing (length 21 mm, inner diameter 9.5 mm) over the tip, thus extending the probe assembly by about 10 mm. Then, the microphone probe was acoustically coupled to the frog's ear, by placing the open end of the tubing on the skin around the tympanic membrane. The connection between the tubing and the skin was sealed with silicon grease.

The two speakers were used to present two stimulus tones simultaneously, with frequencies f_1 and f_2 , and levels L_1 and L_2 . Stimulus tones were generated using a DA-converter (Tucker Davis Technologies, Gainesville, FL, model RP2).

The microphone signal was analyzed with an FFT network analyzer (Stanford Research, model SR770) set for an analysis range of 780 Hz and frequency resolution of 1.95 Hz. The average amplitude spectrum of 16 successive 50%-overlapping time windows was obtained for each set of stimulus parameters. The peak amplitudes of the distortion products at $2f_1 - f_2$ and $2f_2 - f_1$ were recorded from the average spectrum.

DPOAE input-output functions were measured for two pairs of stimulus tones at $f_1 = 1011$ Hz, $f_2 = 1112.1$ Hz, $f_2/f_1 = 1.1$, and at $f_1 = 2011$ Hz, $f_2 = 2212.1$ Hz, $f_2/f_1 = 1.1$. These stimulus frequencies were chosen to be within the sensitivity ranges of the amphibian papilla (AP) and the basilar papilla (BP), respectively (Ronken, 1990, 1991; Christensen-Dalsgaard and Narins, 1993). The levels of the stimulus tones were equal ($L_1 = L_2$), and were varied from 40 to 90 dB SPL in 5-dB steps. In addition to input-output functions, DP-grams were recorded for frequencies from $f_1 = 211$ to 3011 Hz, in 200-Hz frequency steps, with $L_1 = L_2 = 85$ dB SPL and $f_2/f_1 = 1.1$.

Emission measurements were performed both on live animals and *post mortem*. Initially, animals ($n=8$) were anesthetized with an intramuscular injection of pentobarbital sodium solution (Nembutal, Abbott Laboratories, 50 mg/ml: 1.0–1.3 μ l/g body mass), after which baseline DPOAE measurements were performed. Then, animals were killed by cardioectomy ($n=3$ animals) or by destruction of the central nervous system (CNS, $n=4$). In one animal, both cardioectomy and CNS destruction were applied. In cardioectomy, an incision was made in the animal's abdomen. The heart was located, and removed by cutting its connections with a pair of scissors. CNS destruction was by means of double pithing, a standard technique for frog euthanasia. A sharp needle was

inserted cranially between the skull and atlas, penetrating through the foramen magnum and, by rotation, mechanically destroying the brain. The needle was then withdrawn, and reinserted caudally to destroy the spinal cord. Following cardioectomy or CNS destruction, DPOAE measurements were taken continuously for 2 h. In three frogs, additional DPOAE measurements were performed beyond the 2 h time window. These animals were followed for 45, 51 and 72 h, respectively. Body temperature of the animals was not actively controlled; room temperature was about 20 °C.

The "Principles of Animal Care" (NIH publication 85-23, revised 1985) and USA regulations were followed throughout this study, and protocols were approved by the University of California Animal Research Committee.

III. RESULTS

Prior to euthanasia, DPOAEs were present in all animals investigated. In response to the high-level stimulus, with $L_1 = L_2 = 85$ dB SPL, the distortion product at $2f_1 - f_2$ was typically detectable for stimulus frequencies from $f_1 = 411$ Hz to $f_1 = 2611$ Hz. Within this range, emission levels were highest for $f_1 = 1811$ or 2011 Hz, with peak levels ranging from 20 to 48 dB SPL with an average peak level of 32 dB SPL. The DPOAE at $2f_2 - f_1$ was detectable for $f_1 = 411$ Hz to $f_1 = 2211$ Hz, and peaked at 1611 or 1811 Hz, with peak levels ranging from 22 to 51 dB SPL with an average of 35 dB SPL.

Input-output functions consisted of two segments, separated by a knee point near 75 dB SPL input level (see Fig. 1). We will refer to the corresponding emissions as low-level (<75 dB SPL input level) and high-level (>75 dB SPL input level) DPOAEs, respectively.

Low-level DPOAEs disappeared during the first hour *post mortem*. That is, during this period, the knee point disappeared, and the input-output function essentially became a straight line (see Fig. 1). With CNS destruction ($n=4$ animals), low-level amphibian papilla DPOAEs (i.e., $f_1 = 1011$ Hz) disappeared on average in 13 minutes (range across animals 5–25 min; see Table I). In contrast, in cardioectomized animals ($n=4$, including the animal which underwent both cardioectomy and CNS destruction), low-level DPOAEs in the amphibian papilla always disappeared before the first *post-mortem* recording, i.e., within 6 min. In the basilar papilla, low-level DPOAEs always disappeared slower than those from the amphibian papilla. After CNS destruction the average time to full DPOAE extinction was 58 min on average (range 53–62 min), while after cardioectomy time to extinction was faster: 34 min on average (range 15–52 min). These values are presented in Table I. In summary, the DPOAE decay rate was slower in animals with CNS destruction than in cardioectomized animals. Also, the rate of DPOAE decay was slower in the BP than in the AP.

The high-level DPOAEs either increased or decreased during the first 2 h *post mortem*. Following CNS destruction, amphibian papilla DPOAEs changed on average by +9.3 dB (range -18.4 to +26.9 dB), and basilar papilla DPOAEs changed on average by -1.3 dB (range -5.4 to +3.5 dB). Following cardioectomy, amphibian papilla DPOAEs

FIG. 1. Input-output curves for the DPOAE at $2f_1 - f_2$ for various times *post mortem* (PM). Equal primary levels, $L_1 = L_2$. Ratio $f_2/f_1 = 1.1$. (a) Amphibian papilla, $f_1 = 1011$ Hz, before and after CNS destruction. (b) Amphibian papilla, $f_1 = 1011$ Hz, before and after cardioectomy. (c) Basilar papilla, $f_2 = 2011$ Hz, same subject as panel (b). In live animals, input-output curves consisted of two parts separated by a knee-point near 75 dB SPL input level. The corresponding emissions are referred to as low-level (below 75 dB SPL) and high-level (above 75 dB SPL) DPOAEs. *Post mortem*, the low-level DPOAE disappeared, while high-level DPOAEs are relatively unaffected. Panels (a) and (b) illustrate that the rate of decay of low-level DPOAE was slower after CNS destruction than after cardioectomy. Panels (b) and (c) show that the decay was more rapid in the amphibian papilla than in the basilar papilla. The gray shaded areas indicate system noise (below 70 dB SPL input level) and system distortion (above 70 dB SPL input level).

TABLE I. Decay times of low-level DPOAEs for CNS destruction versus cardioectomy, and amphibian papilla versus basilar papilla. Time to full disappearance is indicated in minutes. The mean across subjects is in bold; the range of values is in brackets.

	CNS destruction ($n = 4$)	Cardioectomy ($n = 4$)
Amphibian papilla ($f_1 = 1011$ Hz)	13 (5–25)	<6
Basilar papilla ($f_1 = 2011$ Hz)	58 (53–62)	34 (15–52)

changed by -9.2 dB (range -17.0 to -0.2 dB), and basilar papilla DPOAEs changed by -3.4 dB (range -9.4 to $+0.8$ dB).

In three frogs, DPOAEs were monitored beyond 2 h *post mortem* ($n = 1$ cardioectomy, $n = 1$ CNS destruction, $n = 1$ cardioectomy and CNS destruction). During this time interval, only high-level DPOAEs were detectable. Initially, DPOAEs would decrease by 20–25 dB to a minimum at 150–400 min *post mortem*. After this initial phase of decrease, DPOAEs reemerged, following a time-course (Fig. 2) which was inconsistent across subjects. Within the same animal, the time-course of the AP- and BP-emissions were also inconsistent. This is related to the observation that after the reemerging of DPOAEs, the DP-grams no longer reflected the frequency ranges of the amphibian and basilar papillae (Fig. 3). In the three frogs, clear acoustic distortion products were still observed at 45, 51, and 72 h after surgery, respectively. At these times the experiment was terminated.

IV. DISCUSSION

The results are qualitatively similar to those in mammals and lizards (Zurek *et al.*, 1982; Kemp and Brown, 1984; Lonsbury-Martin *et al.*, 1987; Whitehead *et al.*, 1992; Manley *et al.*, 1993; Mills *et al.*, 1993; Rebillard *et al.*, 1993; Frolenkov *et al.*, 1998): DPOAEs in response to low-level primary tones (below about 75 dB SPL in frogs) are very sensitive to disruption of inner ear physiology. In contrast, DPOAEs generated by high-level stimuli are more robust.

FIG. 2. Example of the long-term time course of high-level DPOAEs at $2f_1 - f_2$ in one animal. Method of euthanasia: cardioectomy. $L_1 = L_2 = 85$ dB SPL, $f_2/f_1 = 1.1$. Closed symbols: $f_1 = 1011$ Hz, amphibian papilla. Open symbols: $f_1 = 2011$ Hz, basilar papilla. The DPOAE from the amphibian papilla decays to a minimum just after 2 h. Then, it reappears and follows a complex relationship with time. The DPOAE from the basilar papilla follows a similar time-course, but on a different time scale. The initial minimum amplitude for this emission was observed after about 8 h *post mortem*.

FIG. 3. Example of the long-term time course of the high-level DPOAE at $2f_1 - f_2$ following cardioectomy. $L_1 = L_2 = 85$ dB, $f_2/f_1 = 1.1$. The thin vertical line approximately separates the range of characteristic frequencies of auditory neurons from the amphibian papilla (AP) and the basilar papilla (BP) (Ronken, 1991). In the live animal (filled circles) and 2 h *post mortem* (filled diamonds), both the amphibian and basilar papillae emit DPOAEs. After an initial decay of these components (see Fig. 2), DPOAEs reappeared (open symbols) but emission frequencies no longer corresponded to the papillar frequency ranges.

In the experiments described here, no artificial respiration was used. In most frog species, oxygen is absorbed into the animal's body by diffusion through the moist skin, and is distributed through the body via blood circulation. One result of cardioectomy is the arrest of blood circulation. Consequently, the main effect of cardioectomy on hearing is presumably the near elimination of the oxygen supply to the inner ear.

In contrast, with CNS destruction, the heart typically continues to be functional for several hours (our observation), and so oxygen supply to the inner ear is presumably maintained. Rather, CNS destruction disrupts efferent input to the inner ear. In addition, mechanical destruction of the CNS as applied here ("double-pith" procedure), probably also mechanically destroys the endolymphatic and perilymphatic sacs, which are in the brain case. The disturbance of the efferent system will only affect the amphibian papilla, since the basilar papilla in ranid frogs lacks efferent innervation (Frishkopf and Flock, 1974; Robbins *et al.*, 1967).

We showed that low-level DPOAEs decay rapidly following cardioectomy, but slower after CNS destruction. We infer that cardioectomy immediately stops oxygen supply, which in turn results in the rapid decay of DPOAEs. In contrast, the physiological effect of CNS destruction on DPOAEs takes place in several minutes to an hour.

The difference in DPOAE decay time between the amphibian papilla and the basilar papilla, even within the same animal [compare Figs. 1(b) and (c)], is of interest. Apparently, DPOAEs from the AP are more vulnerable to physiological insult than are those from the BP. This suggests that different mechanisms may underlie these emissions. The vulnerability of DPOAEs in the AP correlates with the ability of the AP to generate spontaneous otoacoustic emissions (Van Dijk and Manley, 2001). Possibly, hearing in the AP is supported by an active mechanism *sensu* Gold (1948), i.e., via a feedback mechanism which involves cycle-by-cycle amplification. Such a mechanism could account for the presence of

spontaneous emissions in the AP and is expected to be highly vulnerable to oxygen supply disruption. In contrast, DPOAEs from the basilar papilla, which does not generate spontaneous emissions, could reflect a passive nonlinearity, for example due to the nonlinear stiffness of hair bundles (Howard *et al.*, 1988; Howard and Hudspeth, 1988). Note that "passive" does not imply independence of oxygen supply. In fact, the integrity of the inner ear, and in particular the hair cell tip-links, is essential for generation of such a passive nonlinear response, and is expected to be related in the long term to the inner ear oxygen supply. Rather, "passive" implies the absence of a cycle-by-cycle amplifier, which could generate spontaneous emissions.

A further difference between the AP and the BP relates to their efferent innervation. While in *Xenopus laevis* both the AP and BP receive efferent innervation (Hellmann and Fritsch, 1996), in *Rana catesbeiana* it was found to be present in the AP, but absent in the BP (Frishkopf and Flock, 1974; Robbins *et al.*, 1967). This latter finding can probably be extrapolated to *Rana pipiens*, the congeneric species investigated here. In humans (Veuillet *et al.*, 1991; Collet *et al.*, 1992) and a bird (Manley *et al.*, 1999), stimulation of the efferent system affects otoacoustic emissions. Presumably, the efferent system plays a role in controlling the active mechanism in the inner ear. According to the argument presented above, the presence of efferent innervation in the AP would correlate with the presence of an active mechanism, while in the BP of ranid frogs both the active mechanism and efferent innervation would be absent. Regardless of this possible relationship between efferent innervation and active hearing, the difference in innervation of the AP and BP may contribute to the difference of DPOAE decay rate between the papillae, in particular in the case of CNS destruction.

The robust DPOAEs in response to high-level stimuli reflect the frequency sensitivity ranges of the AP and BP, respectively, at least during the first 2 h *post mortem*. Consequently, an explicit conclusion can be drawn: high-level DPOAEs must originate from structures related to the amphibian and basilar papillae. Our results are therefore consistent with the conclusion of Whitehead *et al.* (1992), who showed that high-level *post mortem* DPOAEs in the rabbit are sensitive to acoustic trauma, and therefore must originate from the hearing epithelium. In guinea pigs, high-level DPOAEs may be present despite hair cell damage (Brown *et al.*, 1989). Apparently, components other than hair cell contribute to these emissions. We speculate that nonlinear (mechanical) properties of auxiliary structures may generate the high-level distortions. Possible candidates in the frog ear are passive nonlinear responses of supporting cells, the tectorial membranes, or the contact membranes, which separate the endolymphatic and perilymphatic fluids in the AP and the BP.

Beyond 2 h *post mortem*, DPOAEs were still present, but emission frequencies were no longer related to the frequency ranges of the AP and the BP. It is unclear how these distortion products are generated, but the changes in frequency range presumably reflect *post mortem* changes in the middle and inner ears. Since these distortions do not seem to be related to "normal" inner ear physiology, they are of no

further interest here. However, their presence shows that the detectability of DPOAEs does *not* imply that the inner ear is functioning normally.

Our main observation is that DPOAE behavior during the first 2 h *post mortem* in the frog is similar to that in the other vertebrates investigated: DPOAEs in response to low-level stimuli are physiologically vulnerable, while DPOAEs for higher stimulus levels are more robust. Nevertheless, our results suggest that important differences exist between the emission characteristics of the two inner ear papillae: the vulnerability of the low-level DPOAEs from the AP is consistent with the presence of an active process, while DPOAEs from the BP need not involve such an active process.

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