

The Remarkable Outer Hair Cell

Proceedings of a Symposium in Honor of W. E. Brownell

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Word Count

Introduction 330 Remainder of Manuscript: 4841 (Including Figure Legends)

Number of tables 0 Number of figures 6

Acknowledgements. Funding sources:

²Caruso Dept. of Otolaryngology and NIH/NIDCD Grants R01 DC014450, DC013774, and DC017741 to J.S.O., and NIH/NIDCD Grants F32 DC016211 and R21 DC019209 to J.B.D.

³NIH/NIDCD Grant R01 DC015362

⁴Caruso Dept. of Otolaryngology and NIH/NIDCD Grants R01 DC003687

⁵NIH/NIDCD Grants R01 DC003552 and R01 DC003687

⁷NIH/NIDCD Grant R01 DC012347

⁸NIH/NIDCD Grant R01 DC012347

Abstract

In 1985, Bill Brownell and colleagues published the remarkable observation that cochlear outer hair cells (OHCs) express voltage-driven mechanical motion: electromotility. They proposed OHC electromotility as the mechanism for the elusive “cochlear amplifier” required to explain the sensitivity of mammalian hearing. The finding and hypothesis stimulated an explosion of experiments that have transformed our understanding of cochlear mechanics and physiology, the evolution of hair cell structure and function, and audiology. Here we bring together examples of current research that illustrate the continuing impact of the discovery of OHC electromotility.

Keywords

Electromotility, Prestin, Cochlear Amplifier, Optical Coherence Tomography, Otoacoustic Emissions, Efferent

Introduction

In 1985, Brownell, Bader, Bertrand and de Ribaupierre reported that outer hair cells (OHCs), which are unique to mammalian cochleas, express significant voltage-driven motion, or *electromotility*. They proposed that OHC electromotility could represent the cochlear amplifier originally proposed by Thomas Gold (Gold 1948) to account for the wide dynamic range of hearing. Bill Brownell introduced these findings and ideas to an amazed assembly of hearing researchers attending the 1984 Midwinter Meeting of the Association for Research in Otolaryngology (ARO). The impact on our understanding of hearing was both immediate and long-lasting, galvanizing researchers in areas ranging from inner ear biophysics to clinical testing for hearing impairment and attracting new investigators from outside hearing research. Despite tremendous progress in the last 35 years, many questions remain about the mechanism and function of outer hair cell electromotility – including how it evolved, how it can operate at high frequencies, how it contributes to overall cochlear mechanics, and how it relates to otoacoustic emissions (sounds produced by the cochlea that can be recorded with a microphone in the ear canal).

To celebrate Bill's achievement and highlight how it continues to drive research, the ARO held a symposium at the 44th Midwinter Meeting in 2021: "The Remarkable Outer Hair Cell: Symposium in Honor of Bill Brownell". This report brings together contributions from the speakers and their collaborators. Jonathan Ashmore discusses how OHCs transduce voltage into the mechanical energy of motility. John Oghalai and Elizabeth Olson and colleagues show how visualizing sound-evoked motions of structures inside the cochlea reveals interactions between OHC electromotility and other features to produce amplification. Chris Shera and Alessandro Altoè show that tapering of cochlear structures with frequency, rather than tonotopy of OHC properties, produces tonotopic variation in sound-evoked responses. Carolina Abdala describes new approaches to measuring and analyzing otoacoustic emissions as an accessible indicator of cochlear dysfunction. Ana Belén Elgoyhen shows how the cholinergic receptor proteins that mediate efferent feedback on vertebrate hair cells have undergone selective evolutionary pressure in OHCs.

1. Molecular and cellular mechanisms of outer hair cell electromotility

Jonathan F. Ashmore

Following the discovery that outer hair cells undergo mechanical deformation in response to electrical stimulation, the goal of understanding the molecular and cellular basis of electromotility has been a "Holy Grail" of auditory biophysics. Jonathan Ashmore describes recent developments in this quest to understand the structural basis of outer hair cell function.

It is hard to realize that only a few decades ago the mechanistic role of outer hair cells (OHCs) in cochlear amplification was far from clear although it was appreciated that OHCs were involved in an essential way. The 1985 paper opened up a whole new era of thinking about OHCs: it showed that single cells were electro-motile, lengthening when hyperpolarized and shortening when depolarized (Brownell et al. 1985). The mechanism turned out to be fast enough to be involved in cochlear mechanical tuning and to underpin the requirement of a cochlear amplifier, a mechanism hinted at in earlier hypotheses of how to cancel the viscous damping in the cochlear partition (Gold 1948).

The molecular basis for electromotility is the protein prestin. It was identified in 2000 by the Dallos laboratory from a subtracted hair cell DNA library (Zheng et al. 2000). The surprise is that prestin is not a conventional motor molecule: it is a member of the SLC26 family of membrane transporters, probably forming a tetrameric assembly in the lateral membrane of the OHC. Prestin (SLC26A5) can act as a low efficiency chloride-bicarbonate counter-transporter (Mistrik et al. 2012) and so regulate the intracellular pH of the OHCs. In OHCs its function is largely dominated by an incomplete transport cycle, where the chloride ion is moved from the cytoplasm into the membrane field in the

inward-facing vestibule of the tetramer (Figure 1). This could produce conformational change without moving chloride across the membrane.

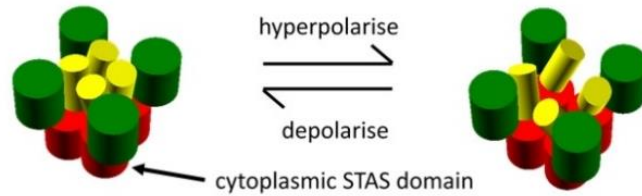


FIG. 1. A scheme for prestin/SLC26A5 function in the OHC membrane. The 8 nm particle seen by electron microscopy is a tetramer, a dimer of dimers, formed from the basic 8-KD (kDa) molecule. Each of the molecule's 14 transmembrane regions is embedded in the membrane and the STAS domain is in the cytoplasm. Hyperpolarization results in anions moving in the cytoplasmic vestibule and producing up to 4% increase in particle diameter (~300 pm) in the plane of the membrane. High molecular crowding then results in the OHC elongating.

The idea that co-transporter mechanisms have been co-opted for electromotility has remained speculative but has received support from recent structural determinations of mammalian prestin/SLC26A5 (Bavi et al. 2021, Butan et al. 2021, Ge et al. 2021). First steps in this direction came from published structures of the bacterial ortholog, dgSLC26, which was found to be an obligate dimer (Geertsma et al. 2015). A closely related member of the same protein family, SLC26A9, is also a dimer with an unusual oligomerization interface in the cytoplasmic portion of the molecule between the STAS domains (Walter et al. 2019). These data suggest that prestin/SLC26A5 has a particular conformation in situ which could be tetrameric, as many lines of evidence have indicated.

A cochlear amplifier based on simple OHC electromotility faces a problem: it is a mechanism driven by transmembrane potential. The filtering by the membrane time constant may limit bandwidth for electromotility although it can be argued that the electrical time constant is substantially reduced towards the cochlear base (Johnson et al. 2011). An idea which now seems plausible is that power can still be delivered to the mechanics if the prestin-based mechanism is modelled as a piezo-electric actuator (Iwasa 2017, Rabbitt 2020). This has been addressed by data recorded from macropatches of the OHC membrane (Santos-Sacchi & Tan 2020). In the case which applies to an OHC mechanically constrained in the intact cochlea, the anion movements through prestin generate sufficient current to cancel the capacitance effects of the membrane. Such a possibility extends the frequencies over which the OHC population can determine the mechanics of the cochlea.

2. Non-invasive studies of cochlear amplification *in vivo*

James B. Dewey and John S. Oghalai

Ex vivo experiments on OHCs present unsolved problems for a variety of technical reasons, including the inaccessibility of the basal cochlea, the limited bandwidth of patch clamp techniques and the relative experimental fragility of the cells. The emerging complementary approach of optical coherence tomography (OCT) overcomes these problems, as discussed in the next two segments by John Oghalai, Elizabeth Olson and their collaborators.

Ever since the discovery that the OHCs change length in response to electrical stimulation (Brownell et al. 1985), experimentalists and modelers have sought to understand how this electromotility might be responsible for amplifying sound-evoked vibrations within the cochlea – a process that is essential for mammalian hearing sensitivity (Dallos et al. 2006; Ashmore 2008; Fettiplace 2020). The dominant view is that OHC electromotility delivers force to the underlying basilar membrane (BM) on a cycle-by-cycle basis, thus enhancing the cochlear traveling wave as it propagates from base to apex (de Boer 1983; Neely and Kim 1983). However, for this scheme to work across the frequency range of mammalian hearing, which can extend beyond 100 kHz (Vater and Kössl 2011), OHC electromotility must be capable of operating at remarkable speeds.

Two key issues have led to ongoing debate regarding the ability of OHC electromotility to subserve high-frequency amplification. The first is whether electromotility can faithfully follow rapid changes in voltage. While the pioneering study of Frank et al. (Frank et al. 1999) demonstrated that electrically stimulated and mechanically constrained OHCs can generate nearly constant force up to at least 50 kHz, recent measurements have characterized electromotility as inherently low-pass in nature (Santos-Sacchi et al. 2019, Santos-Sacchi & Tan 2018). The second issue is the OHC membrane’s electrical impedance, which low-pass filters the transmembrane potential that drives electromotility (Housley & Ashmore 1992, Johnson et al. 2011, Mammano & Ashmore 1996). Given these limitations, it is unclear if meaningful cycle-by-cycle length changes are even elicited by high-frequency sound *in vivo*.

We recently examined the efficacy of high-frequency OHC electromotility *in vivo* by using an optical coherence tomography (OCT)-based approach to noninvasively image through the cochlear bone in live adult mice (Fig. 2a) and measure sound-evoked displacements from within the organ of Corti (Dewey et al. 2021). The relevant methodology has been detailed previously (Dewey et al. 2019, Gao et al. 2014, Lee et al. 2015). Through careful spatial mapping of the vibratory responses we showed that high-frequency sounds cause the top and bottom of the OHC region to move in opposite directions, both tonically and on a cycle-by-cycle basis (Fig. 2b-c). These motions resemble OHC length changes observed *in vitro* (Ashmore 1987, Brownell et al. 1985) and are consistent with the OHC’s electromotile response to tonic and cycle-by-cycle changes in its transmembrane potential. By studying displacement responses at the stimulus frequency and its harmonics, we found that the out-of-phase motions occur at frequencies more than twice the local characteristic frequency. While we saw evidence of low-pass filtering in the displacements, similar to what has been shown in gerbil (Vavakou et al. 2019), motions within the OHC region were still larger than the motion of the underlying BM at all frequencies. These measurements indicate that substantial OHC electromotility is elicited by high-frequency sound *in vivo*, and support the notion that electromotility underlies mammalian cochlear amplification.

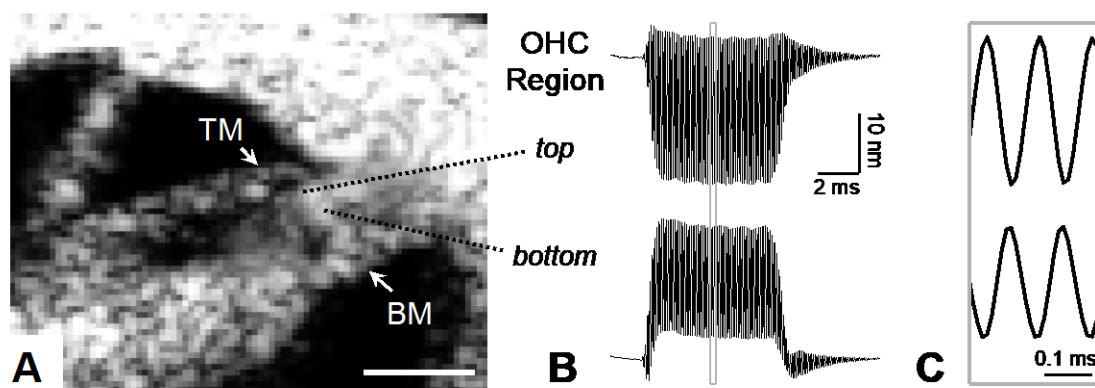


FIG. 2. OCT shows cycle-by-cycle OHC electromotility evoked *in vivo* by high-frequency tone bursts.
a OCT image of the apical turn of the mouse cochlea. TM = tectorial membrane. Scale bar = 100 microns.

b Displacement waveforms of the top and bottom of the OHC region elicited by a 60 dB SPL tone presented at 9 kHz. The asymmetry in the responses reveals a tonic shift in the position of the top and bottom of the OHC region toward one another during the stimulus.
c Highlighted portion of response in **b** on a shorter time scale, showing that the top and bottom of the OHC region move out of phase with each stimulus cycle.

3. Probing the relationship between OHC electromotility and cochlear amplification

Elizabeth S. Olson, C. Elliott Strimbu and Yi Wang

The fundamental signature of the cochlear amplifier is the active, nonlinear tuning of the basilar membrane (BM) at frequencies close to a given location's best frequency (BF). This aspect of the cochlear amplifier was discovered by Rhode (Rhode 1971). Other discoveries supporting the idea that the cochlea was active soon followed: acoustic emissions (Kemp 1978), electrically-induced changes in cochlear mechanics (Hubbard & Mountain 1983), mechanical tuning that was as sharply tuned as auditory nerve fibers (Khanna & Leonard 1982), and nonlinear models of cochlear activity (Kim et al. 1973). It was into this fertile territory that the discovery of outer hair cell (OHC) electromotility arrived (Brownell et al. 1985) and the OHC was adopted as the putative active element driving cochlear amplification.

In mammalian cochleas, the endolymph bathing the mechanosensitive hair bundles has a high positive potential (+80 to 90 mV) which increases the driving force for current through transduction channels and affects the voltage across the prestin-containing basolateral membrane of OHCs. This endocochlear potential (EP) can be reduced or eliminated by intravenous (IV) furosemide. Olson and colleagues used this tool to manipulate EP and observe the impact on cochlear mechanics (Strimbu et al. 2020, Wang et al. 2019). These measurements used an OCT system (Thorlabs), a relatively new technology that allows for imaging and vibrometry within the sensory tissue (Olson & Strimbu 2020, Strimbu et al. 2020). The results show that there is more to cochlear amplification than OHC electromotility.

Figure 3a shows the progression of displacement responses of the basilar membrane (BM) and OHC regions, with time following furosemide injection. Before furosemide injection, the BM region (solid curves) was tuned and nonlinear in the best frequency (BF, ~23 kHz) band (see increasing gain as stimulus level decreased from 80 to 60 dB SPL). In the sub-BF band (below 20 kHz), the BM region responded linearly to changes in level. In contrast, OHC responses (dashed curves) were larger and nonlinear in the sub-BF band. Sub-BF nonlinearity was also present in OHC electrical responses measured close to the sensory tissue. The sub-BF nonlinearity in OHC region motion likely reflects the nonlinearity of OHC electromotile responses, in turn reflecting the nonlinear scaling of the OHC current that drives electromotility (Fallah et al. 2019).

Following furosemide, the BM region motion response was reduced and became approximately linear, such that gain-frequency plots were similar for different input levels at all frequencies, including at and above BF. In contrast, the OHC response was reduced and lost the BF peak but retained nonlinearity at all frequencies.

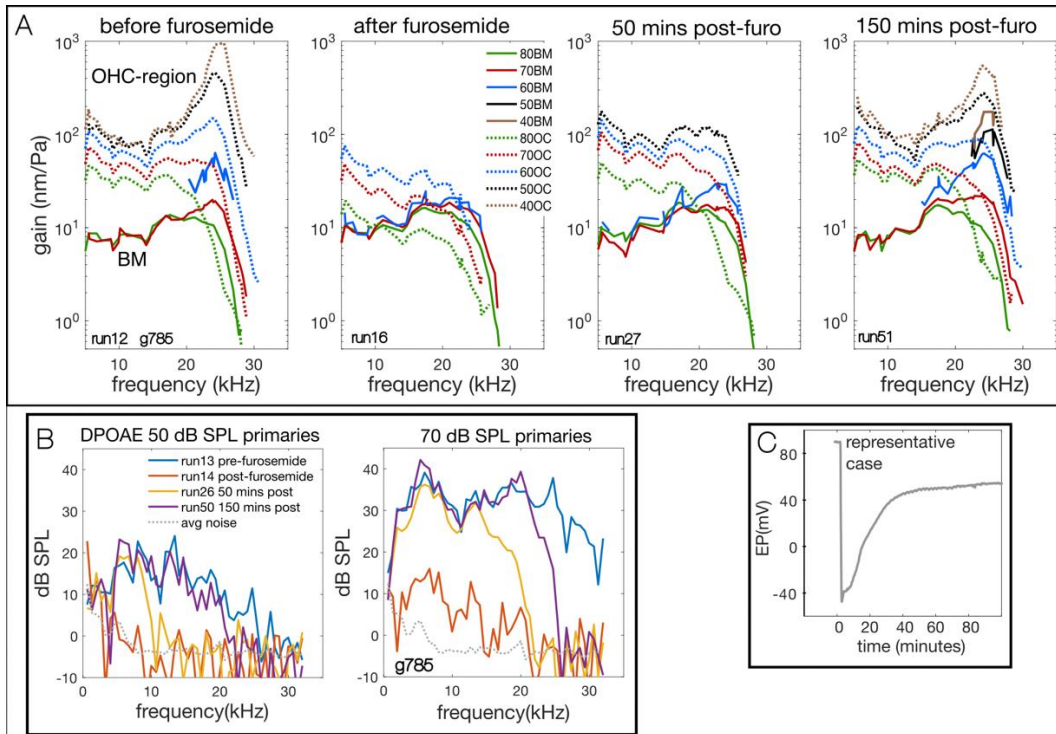


FIG. 3. Furosemide effects on simultaneous sound-evoked displacements at two locations, the BM and OHC regions, distinguish components of the cochlear amplifier.

a Amplitude is shown as gain by normalizing to sound pressure level (SPL). Only amplitude is shown; furosemide-induced changes to phase were minimal. The multi-tone sound stimulus was presented closed-field at the SPLs indicated in the legend (range 40-80 dB SPL). Measurements are shown before IV furosemide injection and several minutes, 50 min and 150 min after.

b DPOAE measured at approximately the same time points as in A, in the same preparation.

c Representative EP measurement following IV furosemide, from a different preparation. The effect of IV furosemide on EP was consistent across preparations (Wang et al. 2019).

Figure 3c shows, in a different, representative animal, the time course of the effect of furosemide IV injection on EP. By 40-50 minutes, EP had substantially, but not completely, recovered and stabilized. Similarly, sub-BF nonlinearity in OHC motion had fully recovered (see 50-minute data in Fig. 3a). However, the tuned, nonlinear BF peak of OHC motion was still diminished; its recovery mainly occurred between 50 and 150 mins, a time window corresponding to stable, sub-normal EP (**Fig. 3c**). OHC electromechanical activity is also reflected in measurements of distortion product oto-acoustic emissions (DPOAEs), further described below. DPOAEs at the BF of the motion measurements (~ 24 kHz) also recovered between 50 and 150 mins (**Fig. 3b**).

Thus, while OHC electromotility and EP recover on similar timescales, other elements of cochlear mechanics that are perturbed by the low EP take longer to recover. One interpretation of these observations is that the recovery of these (still mysterious) elements allows OHC electromotility to successfully engage within the mechanics of the cochlea, to produce the greatly enhanced motion and frequency selectivity that is required for normal hearing. Perturbation studies such as this allow us to probe the constellation of factors that together produce healthy cochlear amplification.

4. Hair Cells or Geometry? Accounting for tonotopic variations in cochlear response properties

Christopher A. Spera and Alessandro Altoè

The discovery of OHC electromotility followed closely after the discovery by Kemp (Kemp 1978) that the inner ear produces sounds, which are called otoacoustic emissions (OAEs). OAEs were quickly recognized as providing strong evidence for cochlear amplification, in addition to being valuable noninvasive indicators of cochlear health. The relation between OAEs, cochlear mechanics, and outer hair cell activity is an area of intense research. Below, Spera and Altoè reinforce that the passive properties of the cochlea determine the substrate for cochlear tuning that the OHCs operate within and analyze how the tapered shape of the cochlea affects spatial variations in cochlear gain, frequency tuning, and response delays.

The physical structure of the cochlea gives it remarkable acoustic properties. When queried with a click, the cochlea responds with a brief “sound rainbow,” a chirp-like echo in which different frequencies return at different times (Kemp 1978). The echoed sounds, known as click-evoked otoacoustic emissions, arise when pressure waves traveling along the cochlear spiral encounter mechanical irregularities in the organ of Corti and scatter back coherently (Zweig & Spera 1995). The sound rainbow reveals important information about how the inner ear processes and analyzes sound. At the broadest level, of course, it shows that the cochlea processes different sounds differently. More particularly, because the cochlea maps frequency onto position, the variation of frequency with time can be reinterpreted as the variation of response delay with cochlear location. That delay, in turn, reveals details of cochlear frequency analysis (Spera et al. 2010). As a well-known example, the rainbow confirms that the sharpness of cochlear tuning varies systematically, being considerably sharper in the cochlear base than in the apex. How do these spatial variations in cochlear signal processing arise?

Biases in our thinking encourage us to seek explanations for the behavior of complex systems in the properties of their components. And what, fundamentally, is the cochlea but an array of hair cells tuned to different frequencies? It is therefore no great leap to hypothesize that the answer lies there, somewhere in the hair cells. Indeed, many characteristics of hair cells are known to vary along the length of the cochlea, including their sizes and orientation within the organ of Corti, the conductances of their mechano-electric transduction channels, and the rate constants of their adaptation kinetics (e.g., (Johnson et al. 2011)). Nevertheless, we set out to test the hypothesis by assuming the contrary (Altoè & Spera 2020a). For purposes of argument, we therefore assume that outer hair cells—aside from whatever contributions they make to determining the local characteristic frequency (CF) (Tobin et al. 2019)—are otherwise functionally identical¹. We implement this assumption in a cochlear model (3D, inviscid fluid) by assuming that the active admittance characterizing the bulk motion of the organ of Corti [adapted from (Altoè & Spera 2020b)] is scaling symmetric. In other words, we assume there are no gradients in cochlear micromechanics other than those contributing to the tonotopic map. If the OHCs in the model do not vary, what does? In a nutshell, we include known macromechanical gradients in cochlear geometry while keeping micromechanical tuning the same at all locations. Thus, we incorporate the cochlear tonotopic map, including its nonexponential form in the apex, and the well-known gradient in basilar-membrane stiffness. And, very importantly, we also incorporate realistic tapering of the cross-sectional area of the cochlear duct.

Remarkably, this simple model accounts for a number of prominent apical-basal differences (Altoè & Spera 2020a).

Figure 4 demonstrates that the model’s responses (shown in orange) nicely reproduce the physiological trends, including apical-basal changes in the shapes of transfer functions and the sharpness of tuning. Thus, global variations

¹ One could say that we take seriously the title of this symposium: “The Remarkable Outer Hair Cell”, with an emphasis on the *The*, as if there were only one. Not literally *one*, of course, as Wheeler and Feynman proposed for the electron, but rather a uniform array with no particular spatial gradient in biophysical response properties other than those arising from—or contributing to—the cochlear map.

in cochlear geometry appear sufficient to account for the data. Evidently, our initial hypothesis was incorrect: spatial gradients in cochlear micromechanics or OHC properties are not needed. Aside from their CF, the outer hair cells, remarkable as they are, might as well all be identical. How does cochlear geometry produce these spatial variations? Although there are multiple factors involved, arguably the most important contribution comes from the tapering of the cochlear duct (see also (Puria & Allen 1991, Sasmal & Grosh 2019, Shera & Zweig 1991). To appreciate the effects of tapering, one needs to know that cochlear gain arises through two mechanisms: The most familiar, of course, is the pumping action (electromotility) of the outer hair cells. Less well known are purely hydrodynamic effects arising from cochlear geometry. Modeling experiments in which a fixed organ of Corti is “transplanted” into ducts of different heights demonstrate that cochlear gain is determined not only by the OHCs, but also by the hydrodynamic environment in which the hair cells find themselves (Shera et al. 2005). Effectively, taller ducts make it easier for the outer hair cells to move the partition, and so it moves more. Thus, high-frequency sounds that peak in the base, where the duct is relatively tall, receive a major boost from the hydrodynamics; low-frequency sounds that peak in the apex, where the duct is shorter, receive less. In this way, tapering creates spatial variations in cochlear gain, and in all things that accompany it, including the sharpness of frequency tuning and the associated response delays. The tapered geometry of the cochlea—a geometry that bears an apt and striking resemblance to a miniature ear horn—bestows upon the cochlea some extraordinary acoustic properties. These properties, manifest in the remarkable sound rainbow, create significant spatial variations in cochlear responses and enhance the signal-processing capabilities of the inner ear.

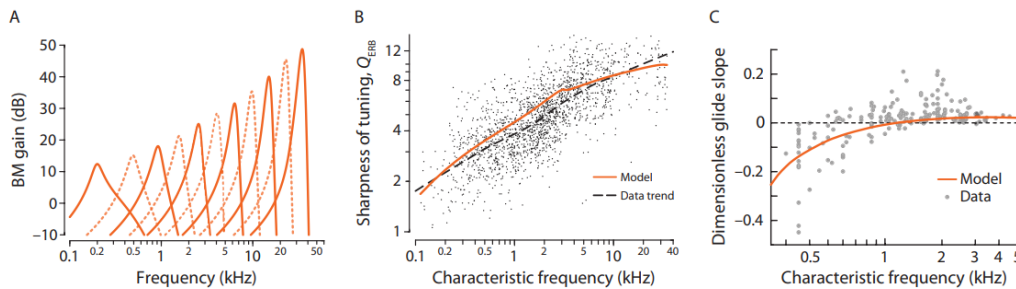


FIG. 4. Model results demonstrate that cochlear geometry can account for observed spatial variations in three measures of cochlear signal processing in cat.
a Basilar-membrane (BM) transfer functions (BM velocity re: pressure at the stapes) computed at 11 locations.
b Variation in the sharpness of mechanical frequency tuning (quantified as Q_{ERB}) compared with values obtained from the cat auditory nerve (Cedolin & Delgutte 2005)
c Variation and sign reversal in the dimensionless glide slope (defined as the time rate of change of the instantaneous frequency of the BM click response, normalized by the square of the local CF) compared with values from the cat auditory nerve (Carney et al. 1999, Shera 2001). Adapted from Fig. 3 of (Altoè & Shera 2020a).

5. Diagnosing hearing loss with Reflection and Distortion Otoacoustic Emissions

Carolina Abdala

The analysis by Altoè and Shera shows how OAEs provide information about fundamental cochlear mechanics. Because OAEs depend on cochlear health and are relatively non-invasive, measurement of OAEs has become a powerful tool for diagnosing human hearing loss. Next, Carolina Abdala describes how various kinds of OAEs reveal complementary information about cochlear function and dysfunction.

It is now recognized that OAEs arise by one (or a combination) of two basic mechanisms within the cochlea, *nonlinear distortion* and *coherent reflection* (Shera & Guinan 1999). Past work has confirmed that distortion- and reflection-type OAEs can be impacted independently by various factors, suggesting that the two OAE classes provide non-redundant information about cochlear function and dysfunction. Although the two types of emissions arise through distinct cochlear mechanisms, they share a common dependence on outer hair cell (OHC) electromotility, which provides the amplification needed to successfully detect and measure OAEs in the ear canal. Hence, both reflection and distortion emissions are linked to and provide a remote gauge of OHC electromotility.

Here we measured both classes of emissions in a group of 20 normal hearing adults and 17 mild-to-moderately hearing-impaired ears, to exploit the unique generation mechanisms of each OAE in characterizing cochlear function. The $2f_1-f_2$ distortion-product OAE (DPOAE) was unmixed to isolate its distortion component while the stimulus-frequency OAE (SFOAE) was recorded as a gauge of cochlear reflection. We presented rapid, swept-tone stimuli to evoke DPOAE and SFOAEs in an interleaved fashion over a 5-octave frequency range (0.5-16 kHz) at 10-12 stimulus levels. Novel OAE metrics characterizing the strength (OAE level re: stimulus at the steepest point on the input/output function) and compressive properties of both OAEs were calculated using advanced fitting and analysis methods and analyzed in a *relational* manner to create a Joint-OAE Profile (Abdala & Kalluri 2017).

In normal-hearing young-adult ears, we saw a characteristic relationship between the generation of nonlinear-distortion and coherent-reflection emissions: (1) SFOAEs have higher levels and greater source strength than DPOAEs and higher levels overall (**Fig. 5a,b**); (2) SFOAE I/O functions show a “compression knee” at higher stimulus levels than DPOAEs (i.e., have an extended linear range) and have steeper compressive growth beyond the knee. In hearing-impaired ears, we find that OAE values fall mostly outside the normative Joint-OAE Profile distribution and can be identified as impaired with high accuracy using joint OAE strength and level measures (92-100%). Furthermore, the distortion and reflection OAEs generated in hearing-impaired ears are not affected in a uniform way. For example, one subject with endolymphatic hydrops showed strongly reduced or non-measurable nonlinear distortion OAEs but present and near-normal SFOAEs, prompting us to speculate about the pathological processes that would disrupt the generation of nonlinear distortion emissions while leaving reflection intact. Since the initial subject was tested, we have seen this same pattern in many more ears and consider it a “signature” Joint-OAE Profile for endolymphatic hydrops. We are currently exploring patterns of OAE disruption for other documented etiologies of hearing loss as well.

In summary, our preliminary work suggests that a Joint-OAE Profile, exploiting two distinct intracochlear generation mechanisms, may better distinguish among hearing losses that have a similar audiogram but different etiologies, underlying pathologies, and perceptual difficulties.

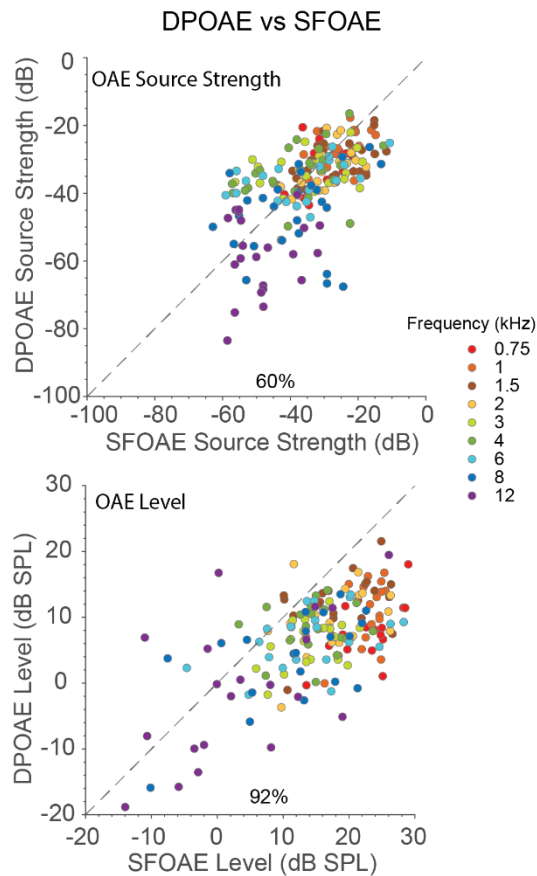


Fig. 5. A normative Joint-OAE Profile of emission source strength and level from 20 normal hearing adults. Each circle represents a paired SFOAE-DPOAE measurement from the same ear. Frequency is denoted by color. The numerical value in the lower right of each panel indicates the percentage of points falling below the diagonal. In these normal-hearing ears, the mechanisms generating SFOAEs are stronger than those generating DPOAEs (see upper panel; slightly more points falling below the diagonal) and SFOAE levels are generally higher than those of DPOAEs at the same fixed stimulus, 65 dB FPL (see lower panel). Some mild-moderate sensory hearing losses disrupt this characteristic relationship between distortion-reflection OAEs. We are currently studying these disruptions to better characterize hearing loss and enhance diagnosis.

6. Efferent control of cochlear amplification: an evolutionary perspective

Ana Belén Elgoyhen

A signature feature of cochlear organization is the separation of afferent output and efferent input, with inner hair cells sending most of the afferent cochlear input to the brain and outer hair cells receiving much of the efferent output of the brain to the cochlea. Olivocochlear efferent synapses on OHCs release acetylcholine onto specialized nicotinic receptors, modulating the hair cell membrane potential and the gain of OHC electromotility. Next, Ana Belén Elgoyhen reviews the evolution of the $\alpha 9 / \alpha 10$ nicotinic receptors that are unique to hair cells and the implications for brain control of OHC electromotility and function.

Upon the transition to land, the hearing organs of tetrapods underwent parallel evolutionary processes, mainly due to the independent emergence of the tympanic middle ear in separate groups of amniotes (Manley 2017). This was followed by other adaptations, like the independent elongation of the auditory sensory epithelia, leading to the extension of the hearing range to higher frequencies and the parallel diversification of hair cell types with differential functions in mammals: phonoreception provided by inner hair cells and sound amplification by outer hair cells (Koppl 2011). In addition, OHCs acquired electromotility (Brownell et al. 1985), a novel mammalian amplification system based on the motor protein prestin (Dallos 2008). Efferent innervation to hair cells is an ancestral feature common to all vertebrate species (Sienknecht et al. 2014). However, only in mammals is the medial olivocochlear efferent system specialized to modulate prestin-driven OHC electromotility. Based on this observation we have suggested that mammalian clade-specific evolutionary processes have shaped the efferent system and we have searched for adaptations on hair cell-specific proteins at the molecular level.

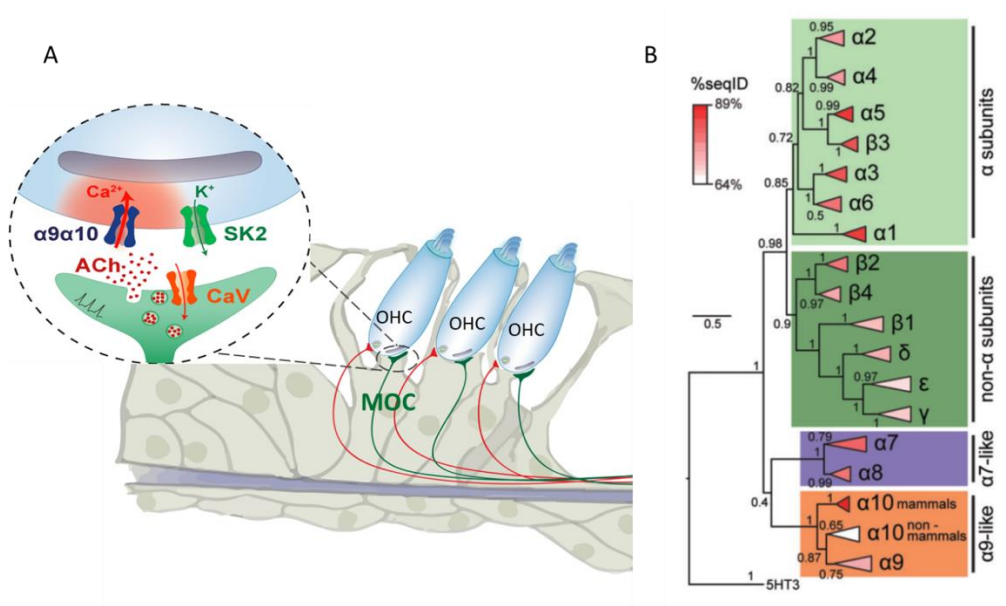


FIG. 6. a Schematics of the medial olivocochlear efferent-outer cell synapse. Acetylcholine released from efferent terminals binds to $\alpha 9\alpha 10$ nAChRs. In mammals these receptors are highly calcium permeable. Intracellular calcium rise activates nearby SK2 potassium channels, leading to hair cell hyperpolarization. *Graphics by Marcelo Moglie.* **b** Phylogenetic relationships between vertebrate nicotinic subunits. The branches corresponding to the same subunits of different species were collapsed up to the node at which one subunit separates from its closest neighbour. Triangles length denotes the divergence on sequence identity from the subunit node. Triangles were coloured according to the average percentage of sequence identity between all pairs of sequences. Numbers in branches indicate the bootstrap value obtained after 1,000 replicates. Note the analysis shows that $\alpha 10$ subunits are unique in presenting a segregated grouping of orthologues with non-mammalian $\alpha 10$ subunits as a sister group to all $\alpha 9$ subunits, and mammalian $\alpha 10$ subunits an outgroup to the $\alpha 9$ /non-mammalian $\alpha 10$ branch. This reflects the overall low % sequence identity of all vertebrate $\alpha 10$ subunits, coupled to high sequence conservation within individual clades, together with the higher rate of non-synonymous substitutions reported for the mammalian clade. *Reproduced from Marcovich et al., 2020.*

Medial olivocochlear efferent innervation to OHCs is cholinergic and is mediated by $\alpha 9\alpha 10$ nicotinic acetylcholine receptors (nAChRs) present at the base of OHCs (Elgoyhen et al. 1994, Elgoyhen & Katz 2012, Elgoyhen et al. 2001, Gomez-Casati et al. 2005). Although the $\alpha 9$ and $\alpha 10$ subunits belong to the acetylcholine pentameric family of ligand-gated ion channels, they are distant members and have a distinct pharmacological profile (Elgoyhen et al. 1994, Elgoyhen et al. 2001, Rothlin et al. 1999, Rothlin et al. 2003, Verbitsky et al. 2000). The atypical features of the $\alpha 9\alpha 10$ receptor prompted the hypothesis that $\alpha 9$ and $\alpha 10$ subunits have undergone a distinct evolutionary history within the family of nAChRs. Using codon-based likelihood models we have shown that mammalian, but not non-mammalian,

α 10 subunits have been under positive selection pressure and acquired a greater than expected number of non-synonymous amino acid substitutions in their coding region (Elgoyhen & Franchini 2011, Franchini & Elgoyhen 2006). Moreover, we have shown that mammalian-specific amino acid substitutions in the α 9 subunit are the basis for the higher relative calcium permeability of mammalian α 9 α 10 receptors (Lipovsek et al. 2014, Marcovich et al. 2020). Overall, the distinct evolutionary history of mammalian α 9 α 10 nAChRs has resulted in differential calcium permeability, current-voltage relationship, channel desensitization profile and choline efficacy across vertebrate species (Lipovsek et al. 2014, Lipovsek et al. 2012, Marcovich et al. 2020). In addition, loss of functional homomeric α 10 receptors (Elgoyhen et al. 2001, Lipovsek et al. 2012, Sgard et al. 2002) and a non-equivalent contribution of subunit interfaces to functional receptor binding sites in mammals has evolved (Boffi et al. 2017). These multiple functional adaptations of the hair cell nicotinic receptor might prove fundamental to faithfully reproduce the high frequency activity of efferent medial olivocochlear fibers (Ballesterero et al. 2011) to fine tune the OHC cochlear amplifier, contributing to the expansion of the mammalian hearing range. It is worth noting that this evolutionary trajectory of α 9 and α 10 subunits is unique within the family of nAChRs, since the entire complement of nAChR subunits is highly conserved across vertebrates and even more so in tetrapods (Dent 2006), suggesting an overall gene family-wide negative selection pressure for the loss of paralogs.

The acquisition of novel functions in vertebrate auditory systems by the selection during evolution of non-synonymous substitutions at the protein level is not restricted to the efferent system. Thus, prestin, together with β V giant spectrin, a major component of the outer hair cells' cortical cytoskeleton which is necessary for electromotility, show signatures of positive selection in the mammalian clade and this may relate to the acquisition of somatic electromotility (Cortese et al. 2017, Franchini & Elgoyhen 2006). In addition, a recent high throughput evolutionary analysis identified signatures of positive selection in 167 inner-ear expressed genes in the mammalian lineage (Pisciottano et al. 2019). Therefore, the evolutionary processes of the auditory system follow the proposal that evolution is a conservative process, since existing structures tend to be modified to accomplish new tasks, rather than the de novo development of functions or structures (Manley 2000). In line with this, our work indicates that evolution-driven modifications in the α 9 α 10 nAChR most likely rendered a medial olivocochlear system highly tuned to serve a differential function in the mammalian cochlea.

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