responses, their neurobiological basis and the role of light in other vertebrate species is urgently needed. □

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Auditory illusions and the single hair cell

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LIKE our other senses, the auditory system can produce illusions. Prominent among these are distortion products[1–5]: when listening to two tones, one of frequency f_1 and the second of a higher frequency f_2, an individual may hear not only these primary tones, but also a difference tone of frequency f_2-f_1, a sum tone of frequency f_2+f_1, and combination tones of frequencies such as $2f_1-f_2$ and $2f_2-f_1$. Discovered by Tartini early in the eighteenth century[6,7], these illusory sounds are sufficiently conspicuous that they were employed to carry melodies in classical compositions. Distortion products originate within the cochlea, where they manifest themselves in the basilar membrane's vibration[8]. Here we demonstrate distortion products in individual hair cells of the bullfrog's sacculus, where they emerge from a nonlinearity inherent in the mechanoelectrical transduction process. In addition to offering an explanation for cochlear distortion products, our results suggest that the mechanical properties of hair bundles significantly influence the basilar membrane's motion.

Sound stimulates a hair cell by deflecting its mechanically sensitive hair bundle, which transduces this stimulus into an electrical response[9–11]. Previous work on hair cells of the bullfrog's sacculus indicates that deflection of a hair bundle acts through elastic gating springs to open mechanically sensitive ion channels[10–12]. As a result, a portion of the force (F) necessary to displace a hair bundle through a given distance (X) reflects the work done in pulling a channel's gates ajar. If a channel has three states[12–14], two closed and one open, then

$$F=(\kappa_S+\kappa_G)X+C+[p_1(\Delta\kappa X+Nz_{12})-p_3Nz_{23}] \qquad (1)$$

in which κ_S and κ_G are the stiffnesses of the tilting stereocilia and the combined gating springs respectively, N is the number of channels in a hair bundle, and C embraces several constant terms[10,15]. The probability that a channel is in the first closed state or in the third, open state is respectively p_1 or p_3, and $\Delta\kappa$ is the difference in stiffness between these two states. A channel's sensitivities to bundle deflection, z_{12} and z_{23}, express the change in force when a channel switches from the first to the second closed state, or from the second closed state to the open state. Both of the probabilities for transitions between states are monotonic functions of hair-bundle displacement.

As indicated by equation (1), bundle deflection and the ensuing force are linearly related when the bundle is displaced extensively in the positive direction or negative direction; the bundle's stiffness is nearly constant under these conditions. Over the narrow range of bundle motions in which channels switch from state to state, though, the last term asserts itself: for displacements within about ±40 nm of the resting position, the bundle's stiffness is smaller and varies with displacement. This localized softening of the hair bundle, called gating compliance, has been demonstrated both in anuran[15] and in mammalian hair cells[16].

The final term in equation (1) renders the relation between hair-bundle displacement and force nonlinear. Hypothesizing that this nonlinearity underlies distortion products, we sought to identify these products at the single-cell level by stimulating the bundles of individual hair cells from the bullfrog's sacculus[15–17]. When moved back-and-forth at a single frequency, a hair bundle exerted force against a stimulus fibre at only that frequency and its second harmonic (Fig. 1*a*). When a second frequency was added to the displacement command, however, a more complex response arose (Figs 1*b* and 2*b*). In addition to forces at the two primary frequencies and their second harmonics, the bundle produced forces at the difference and sum frequencies (f_2-f_1 and f_1+f_2) and at combination frequencies ($2f_1-f_2$ and $2f_2-f_1$). These distortion products occurred in all 30 responsive hair cells investigated, for a variety of primary frequencies, and for various ratios of f_2 to f_1. We investigated the distortion products over a broad range of stimulus amplitudes up to ±125 nm. Some of these products, for example the cubic difference product ($2f_1-f_2$), occurred with stimuli as small as ±12 nm. The distortion products accordingly were not dependent on saturating levels of stimulation, but arose from a nonlinearity whose effects were felt at near-threshold levels of stimulation[1]. In their distribution and relative magnitudes, the distortion products resembled those observed upon the basilar membrane[8].

To rule out the possibility that the distortion products observed *in vitro* originated from nonlinearities in the apparatus, we performed four control experiments. Distortion products were detected neither upon deflection of inert objects such as glass fibres, nor during stimulation of hair bundles on damaged cells that lacked gating compliance. When a bundle's transduction channels were irreversibly inactivated by disruption of the gating springs[18,19], all the distortion products promptly vanished (Fig. 1*c*). Iontophoretic application of gentamicin[20], a transduction-channel blocker[21], had a similar but reversible effect on distortion products.

If gating compliance underlies distortion products, it should be possible to predict both the frequencies and the amplitudes of the products from the measured mechanical properties of a hair bundle. After measuring the relation between a bundle's displacement and the consequent restoring force[15] (Fig. 2*a*), we elicited distortion products by subjecting the same hair bundle to sinusoidal stimulation at two frequencies (Fig. 2*b*). We then used equation (1) to calculate the force expected at any instant during stimulation by concurrent displacements at the two frequencies. The theoretical power spectrum of force components (Fig. 2*c*) accorded well with the experimental observations (Fig. 2*b*). A similar quantitative agreement was obtained for each of the three additional hair cells studied in detail, while the results

from all 30 cells were qualitatively consistent. The agreement between the theoretical predictions and experimental results is especially compelling in light of the fact that the former were calculated with no free parameters.

We have demonstrated mechanical distortion products *in vitro* and quantitatively accounted for their origin over the range of hair-bundle displacements associated with gating compliance in hair cells of the bullfrog's sacculus[15]. If gating compliance is to explain distortion products in the cochlea, sound of the intensities that elicit these products must produce hair-bundle motions of comparable size. Distortion products of cochlear origin are readily audible over a roughly 180-fold range of stimulus amplitudes[3,22], at sound-pressure levels from 20 dB to 65 dB. Based upon the basilar membrane's sensitivity to sound[23] and the geometrical relation between basilar-membrane vibration and hair-bundle motion[24], a 20-dB stimulus displaces hair bundles by about ±2 nm, and a 65-dB sound moves bundles by no more than ±25 nm. When two tones are presented together at a frequency ratio typical of psychophysical experiments[1,2,4], hair bundles that respond to both stimuli undergo displacements considerably smaller than the sum of the maximal motions of hair bundles at the characteristic places. We conclude that the stimuli employed to evoke distortion products deflect cochlear hair bundles by no more than a few tens of nanometres, and hence that the bundle motions lie in the range of the gating compliance.

The origin of distortion products from gating compliance has an important implication for studies of auditory frequency tuning frequences, each of ±14 nm. The clamp system measured the force required to effect the commanded motion, which was equal and opposite to the force exerted by the hair bundle. Because the gating of transduction channels in hair cells is not voltage-sensitive[36], we allowed the cell's membrane potential to vary during stimulation. After force records were subjected to Fourier analysis, the results were plotted as one-sided power spectra. The primary frequencies were selected on two grounds: adaptation by saccular hair cells diminishes their responsiveness[37] below ~20 Hz, and the frequency response of the displacement clamp[19] leads to progressively greater underestimation of force at frequencies above 160 Hz. After the force records for *a* and *b* were obtained, but before the data in *c* were acquired, we obliterated the hair bundle's tip links[38], the morphological correlates of the gating springs, by iontophoretic application of a Ca^{2+} chelator[18].

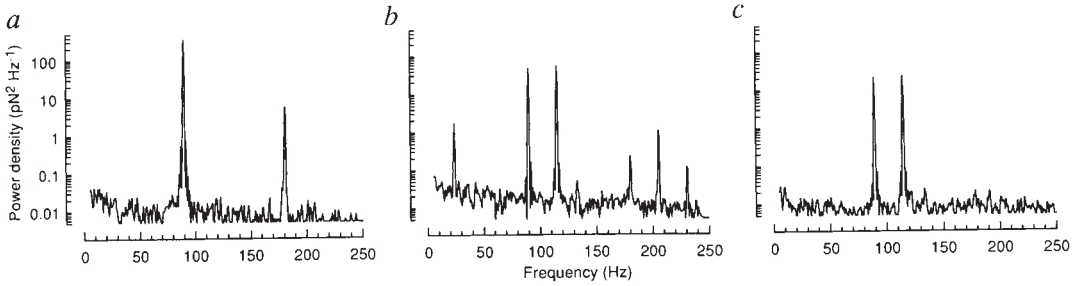

FIG. 1 Distortion products in the force produced by a hair bundle. *a*, When moved back and forth at a frequency of 90 Hz, a hair bundle produced measurable force only at the stimulus frequency and second-harmonic frequency, 180 Hz. *b*, When stimulated with two displacement components of identical amplitude and at frequencies of 90 Hz and 115 Hz, the same bundle exerted forces, not only at the two primary frequencies, but also at the frequencies of several distortion products: 25 Hz ($f_2 - f_1$), 180 Hz ($2f_1$), 205 Hz ($f_1 + f_2$) and 230 Hz ($2f_2$). *c*, Immediately after its transduction apparatus was destroyed, the bundle displayed only the force components corresponding to the primary stimulus frequencies.

METHODS. The saccular macula of the bullfrog's internal ear was dissected and maintained at room temperature in saline solution[30] containing either 100 μM (Fig. 1) or 4 mM Ca^{2+} (Fig. 2). An individual hair bundle was attached near its top to the tip of a horizontal, metal-coated glass fibre about 100 μm in length and 0.8 μm in diameter, whose motion was measured with a calibrated, dual-photodiode displacement monitor[15]. A displacement-clamp system[19] ensured that the fibre's tip underwent the commanded pattern of displacement, either one sinusoidal motion of ±34 nm or the sum of two sinusoidal motions of differ-

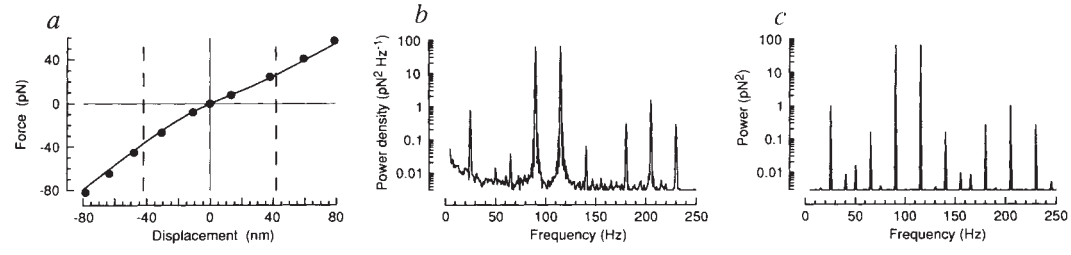

FIG. 2 Comparison of experimental and theoretical distortion products. *a*, The relation between bundle displacement and force was determined by deflecting a bundle with stimulus pulses and measuring the flexion of the stimulus fibre[15]. The data points of this nonlinear relation were fitted to an analytical expression (equation (1); V.S.M. and A.J.H., manuscript in preparation) based on a three-state model[12–14]. The bundle's maximal excursion during the combined stimulation employed for the experiment in *b* is indicated by dashed lines. *b*, Concurrent stimulation of the same hair bundle with ±21-nm sinusoidal stimuli at 90 Hz and 115 Hz elicited a wealth of distortion products: 25 Hz ($f_2 - f_1$), 50 Hz ($2f_2 - 2f_1$), 65 Hz ($2f_1 - f_2$), 140 Hz ($2f_2 - f_1$), 180 Hz ($2f_1$), 205 Hz ($f_1 + f_2$) and 230 Hz ($2f_2$). *c*, Distortion products of similar frequency and magnitude arose in the power spectrum calculated from the bundle's measured stiffness and gating compliance. The primary frequencies and stimulus amplitudes used in the simulation were the same as those employed in *b*, and the scaling of the figures is identical to facilitate their comparison.

METHODS. From the fit in *a*, we inferred that this hair bundle contained $N = 130$ active transduction channels, whose mechanical sensitivities were $z_{12} = 200$ fN and $z_{23} = 100$ fN; the corresponding transition midpoints were $X_{12} = -20$ nm and $X_{23} = 50$ nm. The total stiffness of the bundle's stereociliary pivots was $\kappa_S = 550$ μN m^{-1}, that of the gating springs was $\kappa_G = 300$ μN m^{-1}, and $\Delta\kappa = 450$ μN m^{-1}. The single-sided power spectrum in *b* was obtained experimentally from a force record by the methods described in Fig. 1 legend. Substituting into equation (1) a displacement consisting of two sinusoidal stimuli, each of ±21 nm, we derived an explicit expression for the expected force as a function of time. To predict the power at each of the primary and distortion-product frequences, we evaluated equation (1) and performed Fourier analysis of this oscillatory force.

ing. When a complex sound is analysed by the cochlea, each frequency component evokes a travelling wave whose amplitude peaks at a frequency-dependent position along the basilar membrane. The site of this peak is determined in large part by the stiffness of the basilar membrane[25–27], which is assumed to be dominated by the elasticity of its connective tissue layers[26]. If hair bundles account for distortion tones, however, they too must exert an influence on the basilar membrane's mechanical impedance. The conclusion that hair bundles contribute significantly to the impedance of receptor organs, which is supported by direct measurements in the bullfrog's sacculus[28], is not surprising. For maximal efficiency, the ear should direct as much as possible of the energy in a sound to the mechanically receptive hair bundles. It follows that the bundles should constitute an appreciable part of the mechanical impedance to basilar-membrane motion, and that distortion-product forces produced by hair bundles should reciprocally produce propagating distortion products[29] in basilar-membrane motion. By the same logic, hair bundles may actively enhance the basilar membrane's motion, for they contain myosin (ref. 30, and P. G. Gillespie, M. C. Wagner and A.J.H., manuscript in preparation) and are capable of producing both spontaneous and evoked movements[15,17,31,32]. Hair bundles may by this means contribute to the amplificatory process that underlies the cochlea's great sensitivity[11] and is thought to originate principally from the contractile activity of outer hair cells[33,34].

It seems baffling at first blush that our hearing should be perturbed by a nonlinearity substantial enough to evoke audible distortion products. If these products arise from gating compliance, however, their presence is less obscure: they are an inevitable price paid for a sensory system in which mechanical input is directly coupled to the gating of transduction channels. Natural selection has evidently found the price acceptable, presumably because this mechanism of transduction is both remarkably fast[35] and extraordinarily sensitive[32]. □

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Galanin regulates basal and oestrogen-stimulated lactotroph function

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OESTROGEN, an essential physiological regulator of reproductive function[1], controls lactotroph proliferation and prolactin release[2]. The neuropeptide galanin co-localizes to the lactotroph[3], but its physiological function is largely unknown. Pituitary galanin expression is extremely sensitive to the oestrogen status of the animal. A marked elevation occurs during pregnancy and lactation[4], and exogenous 17β-oestradiol can cause a 4,000-fold increase in messenger RNA levels[5]. Here we report that galanin is secreted by a minority of lactotrophs and is essential for the regulation of basal and vasoactive-intestinal-polypeptide-stimulated prolactin release. Hyperoestrogenization increases the number of galanin-secreting cells and the resulting increase in basal prolactin release is completely abolished by treatment with galanin antiserum. Galanin is a potent lactotroph growth factor and galanin-immunoneutralization completely inhibits the previously reported[6–8] mitogenic effects of oestrogen on the lactotroph. These findings represent direct evidence for paracrine regulation of lactotroph function and demonstrate that the effect of oestrogen on lactotroph proliferation and prolactin release are mediated by locally secreted galanin.

The neuropeptides galanin and vasoactive intestinal polypeptide (VIP) are synthesized and stored in the anterior pituitary[5,9,10] and stimulate the release of prolactin in a number of *in vivo* and *in vitro* systems[11–13]. Galanin is predominantly localized to the lactotroph in female rats[3], but localization of VIP to the lactotroph in the rat[3] has not been confirmed[14,15]. The role galanin and VIP play in lactotroph function was studied using fluorescence-activated cell sorted (FACS) enriched primary lactotrophs and the 235-1 rat clonal lactotroph cell line[16]. Immunoneutralization using antisera raised against galanin[17] (G_{as}) and VIP[18] (V_{as}) was performed on dispersed anterior pituitary cells. Both G_{as} and V_{as} inhibit basal prolactin secretion ($88 \pm 5.1\%$ and $55 \pm 2.7\%$, respectively; Fig. 1*a*). Exogenous galanin and VIP stimulate prolactin secretion to the same extent. G_{as} abolishes VIP-stimulated prolactin release, but V_{as} does not modulate galanin-stimulated prolactin release. Parallel experiments using 235-1 cells demonstrate responsiveness to galanin and G_{as} but not to thyroliberin, VIP or V_{as} (Fig. 1*b*).

Using the galanin cell blot assay, $9 \pm 1\%$ of all lactotrophs are galanin secretors. Galanin secretors are not identified in the lactotroph-depleted population. All 235-1 cells secrete galanin at a low level in the cell blot assay and this was quantified by specific radioimmunoassay[19] (10.3 ± 0.85 fmol per 10^6 cells).

Galanin increases thymidine incorporation into confluent, quiescent 235-1 cells by $315 \pm 25\%$ ($P < 0.001$) and stimulates cell proliferation in log growth phase by $196 \pm 9\%$ ($P < 0.001$). Basal proliferation rate is reduced by $51 \pm 6\%$ ($P < 0.001$) after exposure to galanin antisera (Fig. 2).

Prolactin secretion from FACS-enriched lactotrophs was compared to unseparated dispersed pituitary cells. Inhibition of basal prolactin release by V_{as} is abolished, and the enriched lactotrophs are supersensitive to the addition of VIP (Fig. 3), consistent with VIP release by a cell type other than the lactotroph. Using FACS, lactotrophs were separated into galanin-secreting and non-galanin-secreting subpopulations (see methods, for Fig.

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