Contribution of auditory nerve fibres to compound action potential of the auditory nerve

Short title: Low spontaneous rate fibres: the hidden component

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**ABSTRACT**

Sound-evoked compound action potential (CAP), which captures the synchronous activation of the auditory nerve fibres (ANFs), is commonly used to probe deafness in experimental and clinical settings. All ANFs are believed to contribute to CAP threshold and amplitude: low sound-pressure levels activate the high-spontaneous rate (SR) fibres, and increasing levels gradually recruit medium- and then low-SR fibres. Here, we quantitatively analyze the contribution of the ANFs to CAP 6 days after 30 min-infusion of ouabain into the round window niche. Anatomical examination showed a progressive ablation of ANFs, following increasing concentration of ouabain. CAP amplitude and threshold plotted against ANFs loss revealed 3 ANF pools: i) a highly ouabain-sensitive pool, which does not participate either in the CAP threshold or in the amplitude, ii) a less sensitive pool, which only encoded CAP amplitude, and iii) a resistant pool to ouabain, required for CAP threshold and amplitude. Remarkably, the 3 pools distribution was similar to the SR-based ANF distribution (low-, medium- and high-SR fibers), suggesting that the low-SR fibres loss leaves the CAP unaffected. Single-unit recordings from the auditory nerve confirmed this hypothesis, and further showed that it is due to their delayed and broad first spike latency distribution. In addition to unraveling the neural mechanisms that encode CAP, our computational simulation of an assembly of guinea pig ANFs, generalizes and extends our experimental findings to different species of mammals. Altogether our data demonstrated that substantial auditory nerve fibre loss can co-exist with normal hearing threshold, and even unchanged CAP amplitude.
Introduction

Human hearing covers a large range of frequencies (20 Hz to 20 kHz) and sound pressure levels (0-120 dB sound pressure level; SPL). Sound level encoding is achieved by the auditory transducers of the cochlea, the sensory hair cells, which are innervated by the auditory nerve fibres (ANFs) that convey auditory information to cochlear nuclei. Frequency sensitivity results from low-level amplification and high-level compression of basilar membrane displacements by outer hair cells (OHCs) (Robles and Ruggero 2001). Sound level is encoded in the firing rate of the ANFs, driven by the glutamate release from the inner hair cells (IHCs) (Nouvian et al. 2006). Functional mapping demonstrated that three classes of fibres populate the auditory nerve in the cat: the high-spontaneous rate (SR) (SR > 18 spikes/s), the medium-SR (0.5 < SR < 18 spikes/s) and the low-SR (SR < 0.5 spike/s) fibres (Liberman 1978). While high- and medium-SR fibres are activated in response to lower sound pressure levels but rapidly saturate, low-SR fibres are recruited at higher sound pressure levels and show little or no saturation (Winter et al. 1990). Consequently, the auditory thresholds are primarily coded by high-SR fibres and the sound pressure dynamic range is encoded by the cumulative recruitment of the three ANF populations. Accordingly, loss of ANFs should be readily reflected in the sound-evoked compound action potential (CAP) of the auditory nerve, which presumed to capture the synchronous activation of all three ANF pools.

To investigate the contribution of the ANFs to the CAP responses, we infused the Na+/K+-ATPase inhibitor ouabain into the round window niche in order to selectively destroy the ANFs, as previously shown (Schmiedt et al. 2002). By doing so, we identified i) a pool of fibres that was very sensitive to ouabain and which does not contribute to CAP, ii) an intermediate pool of ANFs, which only encoded CAP amplitude, and iii) a third pool of ANFs with low drug-sensitivity which contributes to the CAP threshold and amplitude. Single unit
recording from the auditory nerve show that the classification based on ouabain-sensitivity (OS) matches the SR-based classification (low-, medium- and high-SR fibres), and that the low-SR fibres are the most vulnerable to ouabain. Simultaneous recording of single unit and CAP further reveals that the high jitter and the long first spike latency (FSL) of the low-SR fibres make them unlikely to contribute to CAP. Finally, the simulation of an assembly of fibres using a computational model of guinea pig cochlea generalizes and extends the contribution of ANFs to CAP to different species of mammals.
**Materials and Methods**

The present study was designed to probe cochlear physiology and morphology 6-days after the ouabain application into the round window niche, as previously described (Schmiedt et al., 2002). Young adult Mongolian gerbils (45–65 g) and guinea pigs (200-450 g) were obtained from Charles River Laboratories (L’Arbresle, France). They were housed in facilities accredited by the French ministry of agriculture and forestry, the “Ministère de l’Agriculture et de la Forêt” (B-34 172 36 - March 11, 2010). All efforts were made to minimize the number and suffering of the animals used.

**Drug preparation**

Artificial perilymph solution (AP) consisted of the following (in mM): 137 NaCl; 5 KCl; 2 CaCl₂; 1 MgCl₂; 1 NaHCO₃; 11 glucose; pH 7.4; osmolarity: 304 ± 4.3 mOsm/kg. Before each experiment, ouabain (Sigma, St. Louis, MO, USA) was prepared in AP to a final concentration of 0, 10, 33, 66, 80 or 100 µM.

**Surgery and round window infusion technique**

Gerbils were anesthetized by an intraperitoneal injection of a mixture of 3 mg/kg Xylazine (Rompun®2%) and 40 mg/kg tiletamine/zolazepam (Zoletil®50). Left cochlea was exposed through a dorsal approach. Once the bulla has been opened, the recording electrode was placed on the bony edge of the round window membrane of the left cochlea leaving enough space in the round window niche for the infusion glass pipette. The infusion glass pipette was filled with AP alone or containing ouabain and introduced into the round window niche (leaving the round window intact) with the aid of a micromanipulator (Warner Instruments). The infusion pipette was connected to a syringe pump (Warner Instruments), which pushed out the solution at a rate of 2 ml/h. After 30 min-infusion, the solutions were
wicked away from the round window niche, and the bulla (including the recording electrode) was closed with dental cement. The round window and the reference electrode placed in the neck were soldered to a plug fixed on the skull. This procedure allowed CAP recordings from the chronically implanted round window electrode 6 days after ouabain infusion without additional surgery.

**Distortion product otoacoustic emissions and compound action potentials**

Distortion product otoacoustic emission (DPOAE) and compound action potential (CAP) were recorded under anaesthesia (3 mg/kg Xylazine and 40 mg/kg tiletamine/zolazepam for gerbils, and 1.6 g/kg urethane for guinea pigs) in a Faraday shielded anechoic sound proof cage. Animals were placed on an anti-vibration table (TMC, Peabody, MA, USA). Heart rate was monitored on an oscilloscope via ECG electrodes. The rectal temperature was measured with a thermistor probe, and maintained at 38.5 °C ± 1°C using a heated underblanket. DPOAEs and CAP of the auditory nerve were recorded 6 days after 30 min-infusion of control AP alone or containing increasing concentrations of ouabain (10 to 100 µM) via the electrode chronically implanted onto the round window membrane.

DPOAEs were first recorded in the external auditory canal using an ER-10C S/N 2525 probe (Etymotic research Inc. Elk Grove Village, IL, USA), consisting of two emitters and one microphone. The two primary tones were generated, and the distortion was processed by the Cubdis system HID 40133DP (Mimosa Acoustics, Champaign, IL, USA). The two tones were presented simultaneously, sweeping f2 from 0.5 kHz to 20 kHz by quarter octave steps, and maintaining the f2/f1 ratio constant at 1.2. The primary intensities of f2 and f1 were set at 60 and 55 dB SPL (ref. 20 µPa), respectively. For each frequency, the cubic distortion product
2f₁-f₂ and the neighbouring noise magnitudes were measured and expressed as a function of f₂.

We then probed the CAP of the auditory nerve. The acoustical stimuli were generated by a NI PXI-4461 signal generator (National Instrument company), consisting of 10 ms tone-bursts with a 1 ms rise and fall time delivered at a rate of 11/s. Sound was delivered by a JBL 075 loudspeaker (James B. Lansing Sound, Los Angeles, CA, USA) in a calibrated free-field condition, positioned at 10 cm of the tested ear (left ear). Cochlear amplification (∗20,000) was achieved via a Grass P511 differential amplifier, averaged 256 times, and filtered with a low-pass filter (cut-off frequency 3.5 kHz) to display the CAP of the auditory nerve. Amplitude-intensity relationships were obtained by varying tone burst level from 0 to 100 dB SPL, in 5 dB steps. The CAP thresholds were 2, 4, 6, 8, 10, 12, 16, 20, 26, and 32 kHz. The amplitude of the potentials was measured between N₁ and P₁, the threshold of the CAP being defined as the dB SPL needed to elicit a measurable response (> 2 µV). After physiological investigations, the cochleae were processed for transmission electron and confocal microscopy.

**Transmission electron microscopy**

Gerbils were decapitated under deep anaesthesia and their cochleae were removed and prepared using our standard protocol for fixation and Epon embedding (Ladrech et al. 2007) before being processed according to the classical surface preparation technique (Spoendlin and Brun 1974). Pieces of the cochlear spiral were selected from four different regions of the cochlear spiral, respectively located around 3.8, 5.19, 6.64 or 8.13 mm from the cochlear apical end and corresponding to the regions coding for 2, 4, 8 and 16 kHz, respectively (Muller 1996). Semi-thin radial sections, including the spiral ganglion, organ of Corti and
stria vascularis, were cut in these four cochlear regions and investigated using photonic microscopy. Ultra-thin sections were cut in the 16 kHz region and analysed under a transmission electron (Hitachi 7100) microscope. Using TEM, the presence of microtubule fascicles highlighted afferent fibers. Conversely, round and clear cytoplasmic vesicles together with some dense-core vesicles identified efferent endings (Satake and Liberman 1996).

Confocal microscopy

For immunostaining, cochleae were perfused with 4% paraformaldehyde in PBS, pH 7.3, and post fixed for 30 minutes in the same fixative at room temperature. The presynaptic IHC ribbons were labelled using a mouse anti-CtBP2 IgG1 antibody (at a dilution of 1:500, BD Biosciences, San Diego, CA). Glutamate receptors were identified using a mouse monoclonal antibody raised against the C-terminus of the GluA2 subunits IgG2a (clone 6C4, 1/200 dilution Millipore, Billerica, MA, USA; (Khimich et al. 2005). The secondary antibodies were an Alexa 488 labelled goat anti-IgG1 mouse antibody together with an Alexa 568 labelled goat anti-IgG2 mouse antibody (1/1000, Invitrogen, Carlsbad, CA, USA). Fluorescent tags were visualized with a Zeiss 5 Live Duo confocal microscope. For quantitative analysis, we selected four different regions located at ~3.8, ~5.19, ~6.64 and ~8.13 mm from the apex, corresponding to 2, 4, 8 and 16 kHz, respectively (Muller 1996). The labelling of IHC nuclei, synaptic ribbons, and AMPA receptor clusters were delimited in each z-plane using threshold procedures. CtBP2/GluA2 receptor co-localization, defining a ribbon-anchored synapse, was determined in each z-plane by intersected ribbon and AMPA receptor labelling delimitation. Because this structure appeared as compact 3D clusters of voxels, we used a hierarchical ascendant classification 3D algorithm (Euclidian distance, nearest neighbour linkage) to detect isolated structures. The centre of mass of IHC nuclei and ribbon synapses was then calculated in 3D coordinates. Each detected ribbon synapse was
assigned to the nearest IHC nucleus by minimizing the distance between the centres of mass.
The number of synapses per IHC corresponded to the sum of detected ribbon synapses
normalized by the number of IHC nuclei. The synaptic ribbon associated with the nuclei of
“incomplete” hair cells at the edge of the image window were excluded from analysis.

Endocochlear potential

Additional animals were used to probe endocochlear potential (EP) 6 days after
control AP alone or containing 100 µM ouabain (3 in each condition). Gerbil cochlea was
exposed by the ventrolateral approach, and the bone over the basal turn of the scala media was
gently shaved and a small fenestrum was made through the thinned bone. A glass
microelectrode (tip diameter 0.1-0.5 µm) was filled with 3M KCl and connected to a direct
current amplifier (WPI, model 773 A, Sarasota, FL, USA). The Ag/AgCl reference wire was
placed in the gerbil’s neck musculature. The recording microelectrode was positioned under
visual control at a point and angle appropriate to be passed through the fenestrum and into the
scala media to record the endocochlear potential.

Extracellular single-fibre recordings

A last set of gerbils was processed for single-units recording. Six days after infusion of
AP alone or containing 33 µM ouabain, animals were placed in a homemade head holder. The
calibrated acoustical stimuli were delivered to the tympanic membrane through magnetic
speakers (type MF1, Tucker-Davis company) coupled to ear bars to produce a sealed
recording environment. The right cochlear nerve was exposed using a posterior fossa
approach. Extracellular action potentials from single auditory nerve fibres were recorded with
glass microelectrodes connected to an Axoclamp 2B amplifier (Molecular Devices), filled
with 3 M NaCl, which had an in vivo resistance between 80 and 110 MΩ. A silver-silver
chloride reference wire was placed in the animal’s neck musculature. The fibre SR was evaluated by averaging the firing (spikes/s) over a 30-s unstimulated period. The fibre characteristic frequency (CF) was determined by threshold-tracking program (10 spikes/s > SR). Driven activity was recorded in response to 10 ms-tone bursts (1 ms rise and fall, 11 bursts/s, 500 or 1000 presentations per level) presented at the CF of the fibre.

Data analysis

Data were analysed with Matlab (MathWorks). Means were expressed ± S.E.M and compared using Mann-Whitney-Wilcoxon test. The goodness of fit was expressed by the coefficient of determination ($r^2$). CAP threshold shift plots against synapse loss were fitted with a piecewise linear model (Vieth 1989): $y = \max(0, ax + b)$, where $a \geq 0$ and $b \leq 0$ are respectively the slope (in dB/%) and the $y$-intercept of the increasing oblique segment (in dB).

CAP amplitude as a function of synapse loss was fitted with a piecewise linear model: $y = \min(0, ax + b)$, where $a \leq 0$ and $b \geq 0$ are respectively the slope and the $y$-intercept of the decreasing oblique segment (in %).

Computational simulation

Simulation of the ANF loss in guinea-pig was achieved using a computational model of sound pressure encoding (Meddis 2006). The input model is an arbitrary sound stimulus. The output is a train of spiking events in one or more parallel ANFs all innervating the same IHC located on the cochlear partition. The model consists of a cascade of six stages recapitulating: stapes velocity, basilar membrane velocity, IHC receptor potential; IHC presynaptic calcium currents, transmitter release events at the ribbon synapse, and firing response including refractory effects.
To upgrade the Meddis’s model, we assessed the number of ANFs per IHC all along the tonotopic axis using confocal imaging in guinea pig cochlea. The counting method was similar as those in gerbils (i.e. CtBP2/GluA2 co-localization, defining a ribbon-anchored synapse). Then, ANFs were divided into three subpopulations (~10, ~15, and ~75% for low-, medium-, and high-SR fibres, respectively) according to (Tsuji and Liberman 1997). Spontaneous firing for low-, medium-, and high-SR fibres were generated by adjusting the time constant of calcium clearance $\tau_{\text{Ca}}$ in the IHC active zone ($\text{SR} = 91.1 \times (\tau_{\text{Ca}})^{2.66}$, with $\tau_{\text{Ca}}$ in ms and SR in spikes/s). The analytic CAP was derived by convolving the sum of peri-stimulus time histograms (PSTHs, $P(t)$) obtained from individual fibres by a damped sine wave ($U(t)$) representing the action potential extracellular waveform (Goldstein and Kiang 1958). This gave $\text{CAP}(t) = K \times \int_{-\infty}^{t} P(t)U(t - \tau)d\tau$, where $K$ was adjusted to simulate a 100 µV CAP ($N_1$ to $P_1$) in response to a 8 kHz tone burst presented at 80 dB SPL and $U(t) = A \times \exp(-kt) \times \sin(2\pi ft)$, with $A = 0.14 \mu \text{V}$, $k = 1440 \text{ Hz}$, and $f = 994 \text{ Hz}$ (Chertoff 2004). The waveform and peak-to-peak amplitude of $U(t)$ were consistent with experimental observations in guinea pig (Prijs 1986). Different ANF subtypes were produced by setting $\tau_{\text{Ca}}$ to 0.1, 0.31, and 0.659 ms for low-, medium-, and high-SR fibres, respectively. Tone bursts from 0 to 100 dB SPL in 1 dB steps were chosen as the input into the computational model. Tone burst characteristics (time rise/fall, duration, inter-stimulation interval, number of presentations) were identical to those used in the present study in both gerbils and guinea pigs in vivo.
Results

Since it has been shown that the infusion of ouabain into the round window niche elicits a massive loss of ANFs 6 days after its application (Schmiedt et al., 2002), we decided to probe cochlear physiology and morphology 6-days after artificial perilymph (AP) containing increasing doses of ouabain.

Ouabain selectivity alters the CAP of the auditory nerve

Because ouabain inhibits the Na/K-ATPase transporter expressed in the fibrocytes of the stria vascularis (Kanoh et al. 2001), we measured the endocochlear potential (EP) in the cochlear basal turn to probe the functional state of the stria vascularis. Survival time of 6 days resulted in normal EP in control AP and in 100 µM-treated cochleae [EP = 75.7 ± 0.7 mV (n=3) versus 74.9 ± 0.6 mV (n=3), respectively]. In addition, consistent with the lack of Na+/K+ ATPase transporter expression in OHCs (Kanoh et al. 2001), 10 to 100 µM of ouabain did not affect DPOAE amplitudes (Fig. 1A).

We then probed the synchronous activation of the ANFs through CAP measurements. While 10 to 66 µM ouabain induced no significant change in CAP thresholds, 80 and 100 µM led to a clear threshold shift up to 40 dB across all frequencies (i.e. mean threshold shift: 22.9 ± 2.1 dB and 40.8 ± 2.7 dB for 80 and 100 µM, respectively, Fig. 1B). CAP amplitude was plotted as a function of sound pressure level (Fig. 1C-F). Infusion of AP alone or containing 10 to 33 µM ouabain did not change CAP amplitude-intensity relationship (Fig. 1C-F). In contrast, 66 µM of ouabain reduced the CAP amplitude without altering auditory threshold (Fig. 1C-F). Increasing the dose of ouabain to 80 and 100 µM led to greater decrease in CAP amplitude and elevation of the threshold (Fig. 1C-F). In addition to their dose-dependency, CAP threshold and amplitude changes were also frequency-dependent. Except for 32 kHz,
ouabain led to larger threshold shifts at high-frequency (50 dB at 16 kHz vs 25 dB at 2 kHz following 100 µM ouabain, respectively). This was also true for the amplitude, with a decrease of 92.1 % versus 79.3 % for 16 and 2 kHz, respectively (Fig. 1C-F).

**Differential sensitivity of ANFs to ouabain**

At the end of the functional assessment, we processed the cochleae for morphological examination. While the organ of Corti appeared normal following a 100 µM ouabain poisoning, we detected a massive loss of afferent neurites and synapses (Fig. 2A-F), when compared with AP control cochleae (Fig. 2G-L). By 3D-confocal imaging, we quantified inner hair cell (IHC) ribbon-anchored synapses (Fig. 3A, B) using juxtaposition of the presynaptic ribbon organelle next to the postsynaptic density (Fig. 3A). Ouabain induced a greater reduction in the number of postsynaptic densities than in the number of presynaptic ribbon (Fig. 3A, B). Plotting the synapse loss against ouabain dose revealed a sigmoidal dose-response curve (Fig. 3C), with an ED₅₀ of 75, 71, 64 and 56 µM for 2, 4, 8 and 16 kHz, respectively. Unexpectedly, 33µM ouabain which did not affect CAP, induced ~20% of synapse loss in the 16 kHz region.

To determine the number of synapses required to elicit a CAP, we plotted the CAP threshold and amplitude against the normalized synapse loss (Fig. 4A,B). Indeed, strong non-linear relationships were found between CAP indexes (threshold and amplitude) and loss of synapses. The critical loss of synapses above which the CAP threshold increased, was 24.2, 38.3, 52.9 and 65.8% at 2, 4, 8 and 16 kHz, respectively (Fig. 4A). The critical loss of synapses above which the CAP amplitude evoked by 80 dB SPL tone-bursts decreased, was 4.5, 5.7, 16.1 and 22.6% at 2, 4, 8 and 16 kHz, respectively (Fig. 4B). By superimposing the non-linear curve fits (Fig. 4C), we were able to distinguish three pools of ANFs: i) a pool with high sensitivity to ouabain (≤ 33 µM), which does not contribute to amplitude and
threshold of the CAP, ii) an intermediate (medium) ouabain-sensitive pool (33-66 µM) which only encoded CAP amplitude and iii) a low ouabain-sensitive pool (≥ 66 µM) which dictated CAP amplitude and threshold.

Greater vulnerability of CAP threshold and amplitude to ouabain in the high-frequency region could be explained by the site of drug application (i.e., cochlear basal turn), leading to a higher ouabain concentration in the basal turn. In this scenario, ouabain would preferentially affect all the ANFs of the basal region. Alternatively, ouabain could preferentially destroy distinct ANFs fractions (because of their drug-sensitivity), irrespective of their location along the tonotopic axis. To discriminate between these two hypotheses, we recorded single-units from the auditory nerve. In control AP cochlea (Fig.5A, B), the distribution of the ouabain sensitive (OS)-ANFs closely mirrors with the tonotopic distribution of ANFs according to their spontaneous rate, i.e., low-, medium- and high-SR fibres, (Fig. 5C). Indeed, the OS-based distribution plots against SR-based distribution shows a linear relationship (Fig. 5D). In this framework, the very sensitive pool to ouabain corresponds to the low-SR ANFs while the intermediate and low ouabain-sensitivity pool corresponds to the medium- and high-SR ANFs, respectively. If this would hold true, ouabain infusion at low concentration (33 µM) should elicit the selective loss of low-SR ANFs along the tonotopic axis, leaving the medium and high-SR ANFs largely unaffected. Accordingly, single-unit recordings demonstrated a massive reduction of low-SR fibres following 33 µM ouabain infusion (Fig. 5E). When we computed the proportion of low-, medium- and high-SR fibres per octave band centred on 2, 4, 8, 16 kHz, the proportion of low-SR fibres over the remaining fibres was reduced, while the proportion of high- and medium-SR fibres was greater than in controls (Fig. 5B and 5F). To verify that the greater proportion of high- and medium-SR fibres was not due to a phenotypic change of the remaining ANFs, we normalized the fibre distribution to the control synaptic count from immunostaining, i.e, taking into account the synaptic loss at 33 µM. Doing so, we
clearly observed that ouabain selectively destroys low-SR fibres, whereas the proportion of medium- and high-SR fibre pools remains unchanged (Fig. 5G). Because low-SR fibres are more abundant in the basal region of the gerbil cochlea (Fig. 5A, B), we plotted the cumulative distribution of CF-fibre from base to apex in control and ouabain-treated cochleae (Fig. 5H). No statistical difference was seen between the control and the ouabain cumulative distributions (Fig. 5H) making unlikely a base to apex gradient of degeneration. In contrast, a clear difference was seen when we compared the distributions from low- to high-SR fibres (Fig. 5I). Altogether, these results demonstrated that ANF pools are differently distributed along the cochlear axis, and that low-SR fibres have a greater vulnerability to ouabain than medium- and high-SR fibres.

**Contribution of ANF pools to the CAP**

In addition to demonstrating a larger ouabain sensitivity of low-SR fibres, our results raise the question as to why low-SR fibres do not contribute to the CAP. This is particularly obvious in the 16 kHz region of the cochlea, where 33 µM induced 20% ANF loss without any effect on CAP amplitude and threshold (Fig. 4C). To investigate the contribution of ANFs to CAP, we simultaneously recorded single unit and CAP of auditory nerve at the characteristic frequency (CF) of the fibre in control gerbils (Fig. 6A-C). When measured from tone-burst onset, the first spike latency (FSL) paralleled with N1-CAP latency (Fig. 7A). Note that N1-CAP latency was always shorter than the FSL (Fig. 7A). This is consistent with the study of Wang (1979) in which the N1 latency of the unitary action potential recorded at the round window is shorter than the spike latency measured at the peak (as done in the present study).

According to the travelling wave delay, both N1-CAP latency and FSL decreased as the probe-frequency increased (Fig. 7A). To overcome the travelling wave delay, we expressed the N1 to FSL interval as a function of CF-fibre, and no correlation was found (Fig. 7B). The
FSL jitter was also independent of the CF-fibre (Fig. 7C). When expressed as a function of the SR-fibre, N1 to FSL interval and FSL jitter significantly decreased as the SR increased (Fig. 6D, E). In response to 80 dB SPL tone-bursts, N1 to FSL interval was respectively 360 µs ± 30 and 665 µs ± 49 for high- and low-SR fibres, with a jitter of 545 µs ± 38 versus 967 µs ± 92. To further examine the involvement of low-, medium, and high-SR fibres in the CAP, we assessed the contribution of unitary action potential at the round window with the convolution model of (Goldstein and Kiang 1958) using PSTH-fibre and action potential extracellular waveform (Fig. 6C). When compared with the high-SR fibres (Fig. 6F, G), the unitary response of low-SR fibres was delayed (465 µs ± 32 versus 848 µs ± 93 for high- and low-SR fibres, respectively) and the amplitude was smaller (35.4 nV ± 1.5 versus 17.4 nV ± 1.6 for high- and low-SR fibres, respectively). As expected, the high degree of synchronization of high-SR fibres supports their major contribution to the CAP recorded at the round window. In contrast, the delayed and the small amplitude of unitary response at the round window, together with the broad FSL distribution of low-SR fibres, makes them unlikely to fire in synchrony and thus they contribute little to CAP amplitude.

**Modelling the ANF contribution to the CAP response**

To further investigate whether the sub-millisecond temporal precision differences on latency and jitter of FSL in low- and high-SR fibres suffice to explain the poor contribution of low-SR fibres to CAP, we used a cochlea computational model. To create a realistic model, we chose to simulate the guinea pig cochlea because its biophysical properties are well documented (Meddis 2006; Sumner et al. 2003; 2002). We therefore simulated a large number of ANFs and the CAP response resulting from their activity, using the computational model of (Meddis 2006), in which we integrated: i) the cochlear place frequency map, ii) the number of ANFs per IHC all along the tonotopic axis, and iii) the proportion of low-, medium and high-SR fibres per IHC.
In this model, we considered 52 IHCs sampled every one-sixth octave along the basilar membrane from 0.14 to 50 kHz. This distribution covered 97.7% of the basilar membrane length from 1% (apex) to 98.7% (base) in 1.9% increments (Tsuji and Liberman 1997). Based on our synapse count in the guinea pig, we found a quadratic relation between the number of synapses ($N$) and the position along the basilar membrane ($x$, in percent from the apex) such that: $N = -0.00291x^2 + 0.339x + 8.28$ where $x = 33.6 + 38.2 \log(f)$ with $f$ the coding frequency in kHz. The IHCs were thus connected by 18 ANFs per IHC in the 2-12 kHz region, 13 ANFs per IHC in the basal region and 9 ANFs per IHC in the apical region of the cochlea, respectively. By adjusting the time constant of calcium clearance $\tau_{Ca}$, we simulated the low-, medium-, and high-SR fibres (i.e. 0.2, 4, 30 spikes/s, respectively). To validate the model, we recorded single units from the guinea pig auditory nerve. Simulated data nicely replicated our experimental single unit recordings from guinea pig auditory nerve (Fig. 8). Note that, whatever the level of stimulation (80 dB SPL or 30 dB above the fibre threshold, Fig. 8C), the FSL jitter decreased as the SR fibre increased.

Based on this result, we simulated an assembly of ANFs (i.e. a neurogram) firing from low- to high-CF and the analytic CAP in response to basilar membrane velocity evoked by increasing sound level (Fig. 9). The ratio of the three ANF pools was determined according to the guinea pig SR-based classification (i.e. ~10, ~15, and ~75 % for low-, medium- and high-SR fibres, respectively) which is homogenous along the tonotopic axis (Tsuji and Liberman 1997). In total, we included 807 ANFs comprising 89 low-, 127 medium-, and 591 high-SR fibres and assumed that each release event elicited one action potential (Rutherford et al. 2012; Siegel 1992), except during the refractory period (Meddis 2006).
Then, we probed different scenarios of fibre loss. Firstly, we simulated a progressive ablation of the ANFs from the basal turn towards the apical turn regardless of SR (Fig. 10A). Analytic CAP evoked by 8 kHz tone bursts showed an abrupt reduction of the amplitude above 20% ANF loss (Fig. 10B), a drastic threshold shift up to 30% fibre loss concomitant with a rapid N1 latency increase (i.e. when the spread of neuronal loss approaches the frequency probe place, Fig. 10C) and no CAP above 50% of fibre loss. Secondly, we probed whether ouabain randomly destroyed the ANFs regardless of their SR (Fig. 10D). Random depletion of the ANFs up to 70% did not significantly affect threshold (shift < 10 dB) while any additional loss of ANFs provoked a sudden threshold elevation with a steep progression (Fig. 10E). Meanwhile, a linear reduction of the CAP with no change in the N1 latency was observed with increasing arbitrary loss of ANFs (Fig. 10E, F). Thirdly, we probed the progressive ablation of ANFs according to their SR and independent of their cochlear location (Fig. 10G). The depletion of the low-SR fibres (i.e. 10% of all the ANFs) did not affect the simulated CAP amplitude or threshold (Fig. 10H). Additional loss of the medium-SR fibre pool (i.e. 25% of all the ANFs) had a minor impact on CAP amplitude (~10% reduction) and left the auditory threshold unaffected (Fig. 10H). However the progressive depletion of high-SR fibres resulted in a drastic reduction of CAP amplitude. The ablation of 70% of ANFs (i.e. 45% high-SR fibres) increased the threshold shift up to 10 dB and a further loss was associated with an even steeper rise in the threshold (Fig. 10H). In the latter scenario, the N1-CAP latency remains constant (Fig. 10I) as observed in our experimental data from gerbil (not shown). Note that the pattern of changes in CAP threshold, amplitude and latency resulting from the progressive ablation from low- to high-SR ANFs goes in line with the CAP behaviour in gerbil (Fig. 4C), and supports the lack of contribution of low-SR fibres to the CAP.
Discussion

It is generally accepted that all the ANFs contribute to the CAP of the auditory nerve across the full dynamic range of sound pressure encoding. Low sound-pressure levels first activate the high-SR fibres. Increasing sound pressure then drives the medium- and low-SR fibres. Selective destruction of ANFs allowed us to determine the contribution of the 3 ANFs pools to the CAP of the auditory nerve, according their sensitivity to ouabain. Unexpectedly, the low-SR fibres, which are the most vulnerable, do not contribute to CAP. To resolve this apparent paradox, we carried out simultaneous recordings of single-unit and CAP. The delayed and small amplitude of unitary response at the round window, together with the broad FSL distribution make the low-SR fibres unlikely to contribute to the CAP amplitude. Computational simulation of an assembly of ANFs and the resulting CAP supports our experimental data and exclude others scenarios of degeneration.

Auditory nerve fibre loss in ouabain-treated cochleae

Ouabain is a well-known cardiac glycoside that specifically binds to Na/K-ATPase (NKA) pump and inhibits its activity with the order of affinity between the α isoforms of α3>α2>>α1 (O'Brien et al. 1994). In the cochlea, the abundant NKA α3 receptor subunit expression both in the afferent terminals (including the bouton-tip shape) and in the membrane of the somata (McLean et al. 2009) as well as the NKA α1 subunit in the somata (Delprat et al. 2007) make the auditory nerve fibers a preferential target for ouabain.

Single unit recording from the auditory nerve shows a selective loss of low-SR fibres regardless of the CF of the fibre. This result contrasts with reports in the rat, in which ANF loss was greatest near the base and decreased toward the apex (Fu et al. 2012). This discrepancy could be due to anatomical differences between species. In gerbil, the internal
auditory meatus is visible through the translucent medial wall of the round-window niche (Chamberlain 1977; Sokolich and Smith 1973). In other words, the auditory nerve is just below the round window niche, where ouabain is applied. Therefore, one can assume that ouabain can directly access the internal meatus through the thin spongy bone of the medial wall of the round window niche and directly bathes the auditory nerve. In rat, the auditory nerve (or the modiolus) is far away from the round window. So, when applied into the round window niche, ouabain probably directly reaches the basal turn scala tympani through the round window membrane, and diffuses toward the apex. Different access to the auditory nerve fibres may also explain the greater ouabain sensitivity of ANFs in gerbil (ED50 in the µM range) than in rat (mM range in rat; (Fu et al. 2012)).

**Differential sensitivity of ANFs to ouabain**

The greater ouabain sensitivity of the low-SR fibres could rely on their differential homeostatic properties. High-SR fibres which have thicker axon diameters and more mitochondria (Liberman 1980; 1982; Merchan-Perez and Liberman 1996) should have a large concentration of Na/K-ATPase pumps to cope with the high firing rate. In this case, relatively low doses of ouabain may not affect the high-SR fibres very much. By contrast, the low-SR fibres which have thinner axons and less mitochondria might not express so many Na/K-ATPase pumps that would confer greater vulnerability to ouabain. By depolarizing the resting membrane potential of axons (Kim et al. 2007), ouabain application may favor the opening of Ca$^{2+}$ channels and toxic effects through Ca$^{2+}$ overload. Prolonged depolarization also leads to Na$^{+}$ overload and to a reversal of the Na/Ca exchanger. So, thinner axons will also suffer from larger increases in Na$^{+}$ and Ca$^{2+}$ concentrations than thicker ones due to the surface-to-volume ratio.
Contribution of the ANFs to the CAP according to their SR

In the basal coil of the gerbil cochlea, up to ~70 % synapse loss did not affect the CAP threshold, in accordance with previous studies showing that synapse loss up to 50% did not alter auditory brain response threshold in sound-exposed mice (Kujawa and Liberman 2009) or guinea pig (Lin et al. 2011). These results can be easily explained if the remaining high-SR fibres, which respond to lowest sound-stimulation remain intact (Furman et al. 2013). Accordingly, single unit recordings from the auditory nerve show that high- and medium-SR fibres are more resistant to ouabain than low-SR fibres. The correlation between synapse loss and the CAP amplitude enabled the discrimination of an additional ANF pool, which does not participate to the CAP and which greatly coincided with the distribution of low-SR fibres.

Given that CAP responses most likely reflect the synchronous activation of the ANFs, we propose that low-SR fibres of ANFs are not synchronized with the stimulus onset. In cat and guinea pig, FSL from low-SR fibres is longer than medium- and high-SR fibres (Rhode and Smith 1985; Versnel et al. 1990). Other studies have demonstrated in mice that low-SR fibres display larger FSL jitter than others (Buran et al. 2010; Oliver et al. 2006). Here, we demonstrated that both mechanisms (delayed and broad FSL distribution) coexist in gerbil low-SR fibres. We further show that low-SR fibres provide a smaller amplitude contribution of unitary response at the round window. As a result, the weakly synchronous firing, the smaller amplitude contribution and delayed latencies do not suffice to build-up far-field potentials, meaning that the low-SR fibres are poorly detectable within the CAP.

Differences in the FSL and its jitter may arise from different degrees of coordination in synaptic release. In IHCs, the vesicular fusion rate shows a strong calcium dependence and, in any given IHC a high variability of the presynaptic calcium signal has been observed (Beutner...
et al. 2001; Frank et al. 2009; Grant et al. 2010; Meyer et al. 2009). So, ANF SR may be
directly governed by the number of calcium channels at the active zone. Additionally, afferent
bouton recordings from rat cochlea *ex vivo* revealed that the vast majority of terminals display
large, monophasic excitatory post-synaptic currents (EPSCs; 70%), whereas a minority have a
preponderance of smaller, multiphasic EPSCs (Grant et al. 2010). Fast and large monophasic
EPSCs probably resulting from a high degree of coordinated vesicular release, should favour
rapid synaptic transfer such as that observed in high-SR fibres. The multiphasic EPSCs
resulting from less coordinated vesicular release could explain the delayed and broad FSL
distribution of the low-SR fibres. This hypothesis is supported by latency measurements of
spike triggered by fast and slow EPSCs (Rutherford et al. 2012). EPSPs with a steep slope
(∼250 mV/ms, probably corresponding to monophasic EPSCs) trigger action potentials with a
short latency (∼300 µs). In contrast, EPSPs with a shallow slope (∼50 mV/ms, likely
corresponding to multiphasic EPSCs) trigger action potentials within millisecond latency
range. Latency differences from *ex vivo* rat cochlea are consistent with the FSL shift (∼400
µs) from high- to low-SR fibres in gerbil. Finally, low-SR fibres could contain a mixture of
monophasic and multiphasic EPSCs (Grant et al. 2010) because their IHC active zone may
have a lower number of docked vesicles or a lower number of Ca^{2+} channels near to the
ribbon (Graydon et al. 2011). Alternatively, the inhibitory effect of dopamine from the lateral
olivocochlear efferent bundle may constitute an additional mechanism that sets the intrinsic
properties of the ANFs (Ruel et al. 2001).

In any case, our physiological data support the hypothesis that low-SR fibres do not
participate to CAP because of their delayed and jittered FSL. This somehow contrasts with the
apparent contribution of low-SR fibres to the CAP in the aged gerbil (Schmiedt et al. 1996)
and in noise exposed guinea pig (Furman et al. 2013). However, the reports showing that low-
SR fibres do contribute to the CAP (i.e., (Furman et al. 2013; Schmiedt et al. 1996)) are studies in which both low- and medium-SR have been grouped into a single pool called "low-SR fibres" in which the spiking discharge rate was below 18 spikes/s, much higher than our cut-off of < 0.5 spike/s for the low-SR pool in the present study.

Comprehensive modelling of ANF contribution towards the CAP response

The question of the ANF contribution to CAP is difficult to directly address because CAP reflects an assembly of fibres spiking in synchrony (Goldstein and Kiang 1958). Up to now, only sequential single unit recordings from different fibres allowed to construct a neurogram, reflecting several fibres responding in synchrony to tone bursts (Kiang et al. 1976; Wang 1979). Interestingly, a computational model can bridge the gap between single unit and CAP of the auditory nerve.

In contrast to gerbil cochlea, which is not so well documented, the accumulation of biophysical and physiological data in guinea pig enables to design a reliable computational model of the cochlea (Meddis 2006; Sumner et al. 2003; 2002). So, we decided to complete the seminal computational model of guinea pig cochlea (Meddis 2006) by adding the different pools of ANFs (800 ANFs distributed along the tonotopic axis according their SR) and the resulting CAP. This computational model allowed us to simulate different scenarios of neuropathy that are difficult to probe in vivo. Doing this, we predict that i) a base to apex gradient of fibre degeneration should lead to a complete disappearance of CAP when the neuronal loss exceeded the frequency probe place (e.g. 50% loss at 8 kHz), ii) a random degeneration should induce a linear reduction of the CAP amplitude (1% fibre loss = 1% CAP decrease), and iii) a low- to high-SR fibre loss should result in a non-linear reduction of CAP, with no effect on CAP amplitude following low-SR fibre deletion. Clearly, the latter scenario
fits with our experimental data in gerbil. In addition to be a good predictive tool, our computational model generalizes and extends our results to another species of mammalian cochlea in which the experimental approach is compromised because ouabain infusion in guinea pig cochlea also damages sensory hair cells (Hamada and Kimura 1999).

**Ecological function of the ANF pool distribution**

One intriguing question that remains is why the distribution of fibres into three pools (high-, medium- and low-SR) across the tonotopic axis, differs between species. One hypothesis would be that the different pools of ANFs are the benefits arising from the ecological functions. Gerbils show heterogeneous distribution of ANFs from base to apex, resulting in two different cochlea-like phenotypes: i.e. majority of high-SR fibres below 3 kHz and a more heterogeneous distribution above 3 kHz (Muller 1996; Ohlemiller and Echteler 1990; Schmiedt 1989). The majority of high-SR fibres in the low frequency range (i.e. below 3 kHz) may reflect the need to detect low-sound level produced by predators (e.g. a hunting owl; see: (Lay 1972; Plassmann et al. 1987)). The more homogeneous distribution above 3 kHz would enable the vocalizations (3 to 45 kHz; see: (Kobayasi and Riquimaroux 2012)) to operate over a large dynamic range supported by high-, medium- and low-SR fibres. Based on the resistance of low-SR fibres to masking noise (Costalupes et al. 1984), the larger amount of low-SR fibres in the vocalization frequency band may provide good inter-individual communication in a noisy environment. If true, one may speculate that the human auditory nerve is populated by a large pool of low-SR fibres in the speech frequency range (~300 – 3,000 Hz). Finally, the greater sensitivity of low-SR fibres to various animal models of trauma as shown in the present study and in the reports of (Kujawa and Liberman 2009; Lin et al. 2011) may explain the discordance between tonal audiogram and poor intelligibility in noisy environments encountered in some patients (Starr et al. 1991; Starr et al. 1996).
Together, these results emphasize the need to perform relevant clinical tests (e.g. speech recognition in noise) to probe the function state of low-SR ANFs in humans.

In conclusion, infusion of ouabain into the round window niche of gerbil cochlea provokes a progressive loss from low- to high-SR fibres in a dose dependant manner. Since low-SR fibre encoding have been shown to resist in noisy environments (Costalupes et al. 1984), it may be interesting to probe signal detection in noise in gerbils, in which low-SR fibres have been selectively destroyed by ouabain. More generally, ouabain may constitute an excellent tool for studying the contribution of each ANF pool in the perception of different auditory scenes.
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References


**Figure Legends**

**Figure 1. Ouabain affects the compound action potential of the auditory nerve.**

(A), Ouabain effects on distortion product otoacoustic emissions (DPOAEs) in the cochlea 6 days after ouabain-poisoning. Note that up to 100 µM, ouabain did not affect DPOAE amplitudes \((n = 7 \text{ per dose})\). *Inset:* Example of DPOAE recording. (B), Ouabain effects on compound action potential (CAP) audiograms. Mean audiograms obtained 6 days after infusion of ouabain into the round window niche \((n = 7 \text{ per dose})\). Note that only 80 and 100 µM ouabain induced threshold elevations \((\text{mean threshold shift: } 22.9 \pm 2.1 \text{ and } 40.8 \pm 2.7 \text{ dB, respectively, } p < 0.001)\). *Inset:* Example of CAP recording. (C-F), CAP amplitude-intensity functions in response to 2, 4, 8 and 16 kHz tone bursts. Horizontal and vertical arrows indicate, respectively, CAP threshold shift and amplitude reduction in percent \((\text{for } 100 \text{ dB sound pressure level (SPL) tone burst})\), for increasing doses of ouabain. All the data are expressed as the mean ± SEM.

**Figure 2. Morphology and ultrastructure.**

Photonic (A, B, G, H) and transmission electron microscopy (C-F, I-L) on radial sections in the 16 kHz region. In ouabain (A) and perilymph (G) infused cochleae, the organ of Corti (OC) and stria vascularis (SV) have a normal appearance. In the spiral ganglion (SG; red shaded) of the ouabain-treated specimens \((n = 2)\), the majority of the neurons are absent \((\text{in A, B})\) and very few nerve fibres (red) can be seen in the spiral lamina \((\text{SL in A; C})\). Note in B, remaining glial cells (arrows) in the spiral ganglion. (C), Magnification of the spiral lamina in the region of the habenula perforata \((\text{hp; square insert in A})\). Two non-myelinated nerve fibres (arrows) are visible. In the perilymph-infused cochleae \((n = 2)\), all the neuron somata (red) are present \((G, H)\) and the spiral lamina \((I, \text{ square insert in G})\) contains densely packed myelinated nerve fibres \((\text{nf})\). In ouabain- \((D)\) and perilymph- \((J)\) infused cochleae, the IHCs have a healthy appearance with typical shape, normal cytoplasmic content, well-positioned nucleus \((n)\) and erect stereocilia (arrows). After ouabain infusion \((D)\), the inner spiral bundle \((\text{isb})\) only contains few nerve fibres. (E), Efferent \((e, \text{ blue})\) and afferent \((a, \text{ red})\) fibres are present underneath the IHC, but no afferent synapses were recognizable onto the hair cell. (F), One axodendritic synapse between an efferent and an afferent. Note the pre-synaptic spicules (arrows) in the efferent and the post-synaptic membrane density in the afferent. (J), In perilymph-infused specimens, numerous nerve fibres were present in the inner spiral bundle \((\text{isb})\) underneath the basal pole of the IHC. (K) Well recognizable afferent dendrites \((a, \text{ red})\)
synapse the basal pole of an IHC. Note presynaptic ribbons (arrowheads) facing afferent
terminals. One efferent fibre (e, blue) contacts an afferent ending. (L) Typical afferent
synapse with a synaptic ribbon (arrowhead) in the IHC and a postsynaptic density (arrows) on
the afferent ending (a, red). Scale bars: A, G = 50 μm; B, C, H, I, D, J = 10 μm; E, F, K = 1
μm; L = 0.5 μm.

Figure 3: Quantification of ouabain-induced ribbon synapse loss.
(A), Confocal microscopy of immunolabelled CtBP2 (green) and GluA2 (red) from the 16
kHz encoding region. Upper panels show the immunolabelled reduction of CtBP2 only (top),
GluA2 only (middle), juxtaposed CtBP2 × GluA2 (bottom) with increasing ouabain dose (0,
66, 100 µM). n indicates IHC nuclei. The lower panel is a 3D magnification and z-projection
of the white square inserts shown above (5×5×5 μm³). (B), Quantitative analysis of CtBP2,
GluA2, and juxtaposed CtBP2 × GluA2 (n = 5 per dose). The reduction of post-synaptic
GluA2 clusters matches the reduction of synapse-anchored ribbons in the ouabain-poisoned
cochlea. Note that 50% of ribbons are still present in 100 µM ouabain infused cochleae. (C),
Tonotopic dependence of synapse loss in response to ouabain. Synapse counts were
performed after electrophysiological investigation (n = 5 per dose). Dashed line represents the
ED₅₀% calculated from sigmoid fit (r² > 0.92). All the data are expressed as a mean ± SEM. *:
p < 0.01, Mann-Whitney-Wilcoxon test.

Figure 4. Mapping of the auditory nerve fibres.
(A, B), Compound action potential (CAP) threshold shift (A) and CAP amplitude (B) evoked
by 80 dB sound pressure level (SPL) tone burst as a function of the loss of synapses at 2, 4, 8
and 16 kHz (n = 5 per dose). Data were fitted by piecewise linear models (r²: coefficient of
determination). Black and red triangles indicate breakpoint x-value as a proxy of ouabain
sensitivity. Vertical and horizontal error bars correspond to means ± SEM. (C) The
superimposition of fitting models shown in (A) and (B) demarcates three pools of auditory
nerve fibres based on their ouabain sensitivity (OS): high-OS (≤ 33 µM), medium-OS (33-66
µM), and low-OS fibres (≥ 66 µM). Vertical dashed lines show the delimitations between the
different pools. Percentages indicate the proportion of fibres per OS-based pool.

Figure 5. Single-fibre recordings after 33 µM ouabain.
(A), Spontaneous rate (SR)-fibre as a function of characteristic frequency (CF)-fibre in
control gerbils (4 ears, 395 ANFs). Horizontal dashed lines demarcate low- (<0.5 spike/s,
green symbols), medium- (0.5-18 spikes/s, blue symbols), and high-SR fibre pools (>18
spikes/s, red symbols). The vertical dashed lines demarcate octave bands centred at 2, 4, 8, 16
kHz. (B), SR-based distribution of fibres per octave band calculated from A. (C), Ouabain
sensitivity (OS) based distribution of fibres per octave band in control animals derived from
CAP recordings (see Fig. 4C). (D), Correlation between OS- and SR-based distributions. The
scatter plot (12 dots) corresponds to the paired data shown in (B) and (C) at 2, 4, 8, and 16
kHz (high-OS with low-SR, medium-OS with medium-SR, and low-OS with high-SR fibres).
A very significant linear relationship \( y = x \) was found (correlation coefficient = 0.92; \( p < 
0.001 \), correlation coefficient test). (E), SR-fibre as a function of CF-fibre in 33 µM ouabain-
poisoning cochleae (5 ears, 424 ANFs). (F), SR-based distribution of fibres per octave band
calculated from E. (G), SR-based distribution of fibres per octave band normalized with our
synapse counting as shown in Fig. 3C. The white rectangles represent the loss of synapses.
(H, I), Cumulative distributions of fibres as a function of CF (H: from base to apex) and SR
(I: from low- and high-SR) in control (black traces) and ouabain-poisoning cochleae (red
traces). Cumulative distributions were computed using logarithmic binning, 20 bins per
decade. In H, no difference base to apex difference was found between the control and
ouabain distributions (two-sample Kolmogorov-Smirnov test, \( p > 0.5 \)) whereas in I, a
significant difference was found originate from the massive deletion of low-SR fibres (\( p <
0.0001 \)). In I, the proportion of low-SR fibres was 14% in control (black) and 2% in ouabain-
poisoning cochleae (red).

**Figure 6. Contribution of single fibres to the compound action potential.**

(A, B), Compound action potential (A) and single-fibre from gerbil auditory nerve (B) were
simultaneously recorded and plotted together for low- (green), medium- (blue), and high-SR
fibres (red). The vertical dashed lines show the CAP N1 latency. Stimulation was a tone burst
presented at the characteristic frequency (CF) of the fibre (1 ms rise/fall, 10 ms duration, 11
bursts/s, 500 presentations, 80 dB SPL, and alternating polarity). B shows the analysis of the
first spike latency (FSL). Dot raster plots (bottom line) were designed as follows: for each
tone burst presentation, small dots indicate spike times, and the time of the first spike evoked
by sound is highlighted by a large dot. The FSL histograms (top line) derived from dot raster
plots were computed using a 100-µs bin width. Note that firing of the low-SR fibre was both
delayed (from N1) and less synchronized than medium- and high-SR fibres. (C, D),
Quantification of data shown in A and B (n=106 ANFs). In C, left panel is N1 to FSL interval
as a function of the SR-fibre and the right panel is the quantification per SR-based pool. In D, left panel is the FSL jitter as a function of the SR-fibre and right panel is the quantification.

(E), Analysis of the unitary response. Top line: peri-stimulus time histograms (PSTHs) derived from dot raster plots shown above (bin width: 20 µs). Inset: Damped sine wave representing the action potential extracellular waveform from (Prijs 1986). Bottom line: simulation of the unit AP (UAP) by convolving PSTH and extracellular waveform as proposed by (Goldstein and Kiang 1958). (F, G), Quantification of data shown in A and C. In F, left panel is CAP to UAP interval (from N1 to N1) as a function of the SR-fibre and the right panel is the quantification per SR-based pool. In G, the right panel is the UAP amplitude (from N1 to P1) as a function of the SR-fibre and the right panel is the quantification. Analysis in C, D, F, G were carried out from the same data (n=106 ANFs). The vertical dashed lines demarcate low-, medium-, and high-SR fibre pools. Oblique lines are fitting models: 

- C: \( y = 0.58 - 0.14 \log (SR) \) 
- D: \( y = 0.82 - 0.19 \log (SR) \) 
- F: \( y = 0.72 - 0.17 \log (SR) \) 
- G: \( y = 22.1 + 7.8 \log (SR) \) with SR in spikes/s. 

***: \( p < 0.001 \), **: \( p < 0.01 \), one-way analysis of variance test and post-hoc multiple comparison procedure.

Figure 7. First-spike latency as a function of characteristic frequency.

(A), First spike latency (FSL) of low- (green symbols, \( n = 30 \)), medium- (blue symbols, \( n = 22 \)), and high-SR fibres (red symbols, \( n = 54 \)) in normal-hearing gerbils (CF-fibre > 2 kHz). FSL was assessed in response to tone burst (80 dB SPL, 1 ms rise/fall, 10 ms duration, 11 bursts/s, 500 presentations, alternating polarity, and probe frequency at the CF-fibre). Black symbols show the N1 latency of the CAP recorded simultaneously for each single-fibre (mean ± SEM, binned values per half-octave band). Note the parallel reduction of latencies from low- to high-CF due to travelling wave delay. (B), N1 to FSL interval as a function of CF-fibre. (C), FSL jitter (i.e. standard deviation) as a function of CF-fibre. N.S.: not significant (\( p > 0.1 \), one-way analysis of variance computed on binned values per octave band).

Figure 8. First-spike latency in experimental and simulated single unit data.

(A, D), Dot raster plots for single-unit in vivo recordings (A) and simulated (D) from auditory nerve of guinea pig. The 3 single-units shown in (A) were recorded from the same animal and had a characteristic frequency close to 8 kHz (range: 7.5 to 8.5 kHz). The CF and SR of the 3 simulated fibres in (D) were adjusted to fit the in vivo fibre properties of (A). For each repetition, the time of the first spike is highlighted by a large dot and the small dots indicate
Figure 9. Simulation of the auditory nerve firing (neurogram).

(A), Computational model adapted from (Meddis 2006). Sound-evoked firing (neurogram, top) and compound action potential (CAP, below) of 807 ANFs distributed along the tonotopic axis from 0.14 to 50 kHz in response to 8 kHz tone burst (1 ms rise/fall, 10 ms duration, 100 presentations per level, 0.5 ms bin width) with increasing level from 20 to 100 dB SPL. Color scale bar indicates firing rate from 0 to 1000 spikes/s. Vertical dashed line indicates the beginning of the stimulation and horizontal solid line shows the probe frequency. Note the spontaneous activity of ANFs outside the probe frequency region (blue speckle) and the post-excitatory inhibition after sound stimulation. (B), Basilar membrane (BM) velocity along the tonotopic axis. BM velocity increases with sound level. Note the reduced frequency selectivity with the increasing intensity. (C), Sound-activated ANFs per IHC along the tonotopic axis. Grey dashed line shows the guinea pig synaptic cochleogram. The criterion for sound-activated ANF was 10 spikes/s above SR.

Figure 10. Simulation of different scenarios of auditory nerve fibre degeneration.

Auditory nerve fibre firing (neurogram) and compound action potential (CAP) response shown as a function of fibre loss in three neurodegeneration scenarios: (A-C) degeneration from base to apex (high- to low-characteristic frequency), independently of spontaneous rate (SR), (D-F) random degeneration, independently of SR and characteristic frequency (CF), (G-I) degeneration from low- to high-SR fibres, independently of the CF. Neurograms and CAPs were simulated in response to 8 kHz tone-bursts (1 ms rise/fall, 10 ms duration, 80 dB SPL,
100 presentations). The horizontal black line in neurograms shows the probe frequency. Fibre loss is indicated as a % at the top of each neurogram in A, D, G. Color scale bar indicates firing rate from 0 to 1000 spikes/s (bin width = 0.5 ms). (B, E, H, C, F, I) Effect of the fibre loss in 1% step increments on CAP threshold shift (left y-axis, black color, B, E, H), CAP amplitude (right y-axis, red color, B, E, H), and the CAP latency (C, F, I). In B and C, vertical dashed lines indicate the probe frequency. In H, vertical dashed lines demarcate the low- (~10%, green), medium- (~15%, blue), and high-SR fibre (~75%, red) pools in guinea pig auditory nerve.