Chapter 3
General methods

3. General Methods

3.1 Animal preparation

105 adult pigmented guinea pigs (*Cavia porcellus*) with normal hearing thresholds (Patuzzi et al., 1989b), external ears free of debris, and body weights from 200 – 470 g were used[1]. Guinea pigs were obtained from the Biological Sciences Animal Unit at the University of Western Australia, and most were fasted overnight prior to experimentation. All anaesthetic and surgical protocols were approved by the Animal Ethics Committee of the University of Western Australia (Approval No. 02/100/184).

3.1.1 Anaesthetic regime

Following premedication with a subcutaneous injection of 0.1 mL atropine sulphate (0.6 mg/mL; Apex Laboratories, NSW), guinea pigs were anaesthetised with 30 mg/kg body weight Nembutal (60 mg/mL pentobarbitone sodium; Merial Australia, NSW) and 0.15 mL Hypnorm (0.315 mg/ml fentanyl citrate and 10mg/mL fluanisone; Janssen Pharmaceutica, Belgium). Anaesthesia was subsequently maintained by 15 mg/kg Nembutal given every two hours, and 0.15 mL Hypnorm given every hour. Incision sites were anaesthetized locally with lignocaine hydrochloride (20 mg/mL; Troy Laboratories, NSW).

Once surgical anaesthesia was obtained (indicated by the absence of a pedal-withdrawal reflex), animals were paralysed with 0.1 mL pancuronium bromide (2 mg/mL; AstraZeneca, NSW, Australia) to eliminate middle-ear muscle activity and extraneous myogenic potentials. As this also eliminated foot-withdrawal, adequate depth of anaesthesia was determined by monitoring of the electrocardiogram (ECG): animals were given further doses of either Hypnorm or Nembutal if the heart rate increased to a period of less than 175 ms.

Due to supply difficulties, a different anaesthetic regime was used for eight animals (GP#102 to GP#109), which were anaesthetized with 1.5 g/kg urethane (Sigma-Aldrich Chemie, Germany), with hourly doses of 0.15 mL Hypnorm. These animals were also paralysed with pancuronium, and their ECG was monitored as above.

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[1] Some collaborative experiments required differing animal identification schemes. Guinea pigs that were part of this project were numbered (in order): 1-24, 31-34, 52-115, PS91-PS92, PS99, and 116-125.

3.1.2 Surgery

All experiments were conducted in electrically-shielded sound-proof rooms. Following surgical anaesthesia, the trachea was cannulated and the animals were artificially ventilated with Carbogen (95% O_2, 5% CO_2) using a Bioscience 8000-1 ventilator (6 cubic cm tidal volume, 51 cycles/minute; Bioscience, Sheerness, Kent, UK). Both tragi were removed and the head was mounted between hollow ear cones, with head position stabilised by a bite-bar and nasal clamp. The ear cones were placed at an angle that allowed a clear and unobstructed view of the tympanic membrane through the cone.

Body temperature was maintained at 38° C by a heating pad controlled by rectal thermostat. Air temperature in the sound-proof room was approximately 22° C for GP#01 to GP#57. To prevent cooling of the exposed cochlea (Brown et al., 1983), a 12 V DC light globe was placed near the head of the guinea pigs numbered GP#58 to GP#81. A room heater was used to maintain sound-proof room air temperature between 30° C and 38° C for all subsequent animals.

Following removal of the right pinna, a small hole was made in the bulla using a scalpel, allowing a dorsolateral approach to the round window and first-turn of the cochlea. In the event that the right cochlea was damaged or deafened, surgery and experimentation were carried out in the left cochlea.

3.1.3 Cochleostomy

For experiments involving cochlear perfusions or DC current injections, a hole was drilled in the first-turn, approximately 1 mm from the round window, using either a hand-held twist drill (0.11 mm diameter; Titex Plus, Germany), or the bevelled end of a 27-gauge needle (Terumo Medical Corp., MD, USA) which created a hole of similar diameter. For perfusion experiments, a small exit hole was made in the otic capsule at the apex of the cochlea using a sharp wire hook. A drop of Heparin Injection BP (heparin sodium – porcine mucous; David Bull Laboratories, VIC, Australia) was applied to this apical opening to prevent its blockage by blood clots[2].

3.1.4 Electrode placement

Electrical signals were recorded using Teflon-coated silver wire (0.008” bare wire diameter; A-M Systems, Inc., WA, USA). Prior to use, the Teflon coating was removed

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[2] Although intracellular heparin is a potent blocker of IP3 receptors (Nilsson et al., 1988; Sugasawa et al., 1996), the perilymph flow in the opened cochlea is in the base-to-apex direction (Salt et al., 1991), ensuring diffusion of heparin to the basal regions of the cochlea would be limited.

from the final 1 mm of the wire, and the exposed end was chlorided in a saline solution by applying a 20 μA current for two to five minutes.

The active electrode was placed on (or occasionally through) the round window with the aid of a micromanipulator (Narishige Co. Ltd., Tokyo, Japan). Recordings were made with reference to an indifferent electrode placed in the temporalis muscle overlying the temporal bone. An earth electrode, wrapped in saline-soaked tissue, was placed in the neck. The exposed and chlorided portions of the indifferent and earth electrodes were 1 – 2 cm long. In some of the later perfusion experiments, the active electrode was placed within the perfusion pipette. In these cases, the Teflon coating was removed from the final 1 cm of the wire, and the exposed end was etched in a potassium cyanide solution to produce a sharpened tip that could be placed close to the pulled end of the pipette.

For the experiments investigating the application of force to the cochlear wall conducted with Dr. Peter Sellick (see Chapter 5), electrical signals were recorded from etched wires placed in scala tympani and scala media through first-turn cochleostomies. Small beads of silicone (RTV-734, Dow Corning, MI, USA; RTV-102, GE Plastics, VIC, Australia) were placed along the shank of the electrode to prevent the leakage of cochlear fluids.

ECG signals were recorded from two 26-gauge needles (Terumo Medical Corp., MD, USA) inserted between the digits of the left fore-paw and hind-paw.

3.2 Recording system

Experiments presented here were conducted using two experimental setups within the Auditory Laboratory of the University of Western Australia. Most experimentation was carried out in SPR2, while those involving measurement of otoacoustic emissions were carried out in SPR4. The recording systems for both setups are described below.

For experiments conducted in SPR2, electrical signals from the round window were amplified using a custom-built pre-amplifier (40 dB gain), and low-pass filtered at 5 kHz using a Stanford SR650 programmable filter (Stanford Research Systems, Inc., CA, USA) which provided an additional 10 dB gain. Use of the low-pass-filter at the 5 kHz setting introduced a delay of around 0.14 ms to CAP latencies. The amplified and filtered signals were then passed to the line input of the soundcard (Creative Ensoniq AudioPCI CT4810, Creative Labs, NSW, Australia) and recorded using the Cricket data acquisition software described in Chapter 4. The soundcard line-input had a flat frequency response (less than ± 2 dB variation) in the range of 6 Hz to 19 kHz.

For experiments conducted in SPR4, electrical signals from the cochlea were amplified and filtered using a DAM 50 differential amplifier (0.1 Hz - 10 kHz pass-band, 40 dB gain; World Precision Instruments Inc., FL, USA), before being passed via a custom-built relay to the line input of the soundcard (CardDeluxe, Digital Audio Labs, MN, USA) and recorded using the Cricket data acquisition software described in Chapter 10. For measurement of otoacoustic emissions, ear-canal sound pressure was monitored with a Brüel & Kjær 4134 ½-inch condenser microphone, amplified by a Brüel & Kjær 2609 measurement amplifier with an A-weighted filter, and analysed using the Cricket software. The A-weighted spectra were then mathematically converted to dB SPL.

3.3 Data Acquisition Software

Stimulus-generation and data acquisition were controlled by The Cricket, custom-written software that enabled automated and rapidly-interleaved measurements of i) the CAP thresholds at multiple frequencies, ii) the CAP waveforms elicited by supra-threshold stimuli, iii) low-frequency (207 Hz) CM waveforms for online Boltzmann analysis, iv) the spectrum of the neural noise (both spontaneous and driven), and v) distortion-product otoacoustic emissions. Details of the software are presented in Chapter 4.

3.4 Acoustic stimuli

The Cricket used custom-generated sound stimuli for eliciting CAPs, the CM, and DPOAEs. These stimuli will be discussed in detail in Chapter 4.

3.4.1 Stimulus delivery methods differed between setups

For experiments conducted in SPR2, the stimuli used for eliciting CAPs were delivered using a reverse-driven Brüel & Kjær 4134 ½-inch condenser microphone (Brüel & Kjær, Denmark) coupled to the external ear through the hollow ear-cone. Low-frequency acoustic stimulation for CM analysis was delivered using a Beyerdynamic DT48 headphone (Beyerdynamic, Heilbronn, Germany) for animals GP#GOB01 to GP#GOB23, and a low-frequency driver (of unspecified origin) for subsequent animals. The low-frequency driver was used in preference to the Beyer headphone because it was lighter and less cumbersome to place, and was able to produce a more intense 207 Hz pure tone for the same electrical drive. Both the Beyer headphone and the low-frequency driver were coupled to the ear canal using a 3 cm length of polyethylene tubing.

For experiments conducted in SPR4, both the CAP and CM stimuli were delivered using a Beyerdynamic DT48 phone coupled to the cones in the ear canal using a 3 cm length of polyethylene tubing. High-level distortion-product otoacoustic emissions were evoked by primary tones delivered using a pair of Beyerdynamic DT48 phones that were also coupled to the ear canal by 3 cm polyethylene tubes.

3.4.2 Calibration

Low-frequency sound-pressure levels were calibrated at 207 Hz using a Brüel & Kjær 4192 ½-inch condenser microphone with a probe-tube cone to monitor the ear-drum sound-pressure produced by the low-frequency driver used in these experiments. The electrical output of the B&K microphone in this configuration was calibrated against the output produced when the microphone was coupled to a Brüel & Kjær 4230 Sound Level Calibrator, which produced a 1 kHz tone at 94 dB SPL. An unattenuated signal from the soundcard produced 115 dB SPL at the ear drum with the low-frequency driver.

A biological reference was used for high-frequency sound-pressure levels. The tracked thresholds of the ten guinea pigs that had the best overall hearing levels were averaged at each frequency and were used to derive a correction factor to convert the tracked attenuation levels (specific to the SPR2 setup) into a biological reference that could be used to compare the thresholds of individual guinea pigs to those of the rest of the population used in this study, and allowed quicker assessment of the state of an animal during an experiment. This reference scale was referred to as “guinea pig hearing level” (dB GPHL), akin to the “hearing level” (dB HL) used in human audiometry. The GPHL correction factors are presented in Chapter 4.

3.5 Experimental manipulations

The surgical aspects of the experimental manipulations are described in Section 3.1 above. The techniques used to perturb cochlear function are described in the Methods sections of the relevant chapters.

3.6 Issues relating to the Boltzmann analysis of CM waveforms

Boltzmann analysis of the CM was discussed in Section 1.2.2 of Chapter One, and will be discussed further in Chapter Four. Results obtained using the Boltzmann analysis technique are presented in Chapters Five, Six, Seven, and Eight. As mentioned earlier, the transfer curve relating the OHC receptor current to the assumed angular displacement of the hair bundle can be estimated from the Lissajous figure obtained by plotting the CM potential against the instantaneous phase-shifted sound pressure level in the ear canal (Nieder and Nieder, 1971; Avan and Legouix, 1988; Patuzzi and Rajan, 1990).

The low-frequency CM recorded in the first turn of the guinea pig cochlea has been shown to be proportional to stapes velocity (Dallos, 1973), which phase-leads the ear canal sound pressure by 90º. As the stapes velocity increases at 6 dB/octave relative to sound pressure, the ability of an instantaneous ear canal sound pressure to displace the basilar membrane also increases by 6 dB/octave. However, at any given (fixed) frequency, the angular displacement of the hair bundle can be assumed to be proportional to the instantaneous phase-shifted sound pressure level in the ear canal.

3.6.1 Use of a 1st-order Boltzmann function

The opening probability of the MET channels has been described by a first-order Boltzmann function (Holton and Hudspeth, 1986; Patuzzi, 1995; Sirjani et al., 2004), a second-order Boltzmann function (for example, Kros et al., 1992), and other, more empirical, functions, such as polynomials (Chertoff et al., 2000). The use of a *first-order* Boltzmann function derived initially from noise-analysis studies of the MET channels from the bullfrog sacculus: Holton and Hudspeth (1986) found the relationship between the angular displacement of the hair bundle and the current through the transduction channels to be consistent with a two-conductance-state model, following a sigmoidal first-order Boltzmann activation function. The first-order Boltzmann activation function describes a population of independent and identical channels that have two conductance states – an open (conducting) state, and a closed (non-conducting) state. The stochastic flickering of the population of channels may be biased by displacement of the hair bundle.

In the two-state model (1st-order Boltzmann function), the channels may redistribute between the open and closed states with given rates of transition, as follows:

$$closed \underset{kco}{\overset{koc}{\leftrightarrows}} open \qquad \textbf{[1]}$$

where k_{co} and k_{oc} are the rate constants for opening and closing transitions, respectively.

In the three-state model (2nd-order Boltzmann function), the channels may redistribute between the open and closed states with given rates of transition, as follows:

$$closed1 \underset{kc1o}{\overset{koc1}{\rightleftarrows}} closed2 \underset{kc2o}{\overset{koc2}{\rightleftarrows}} open \qquad \textbf{[2]}$$

where k_{co} and k_{oc} are the rate constants for opening and closing transitions, respectively.

Kros et al. (1992) found the transfer function of cultured neonatal mouse OHCs to be well described by a 2nd-order Boltzmann activation function. However, the transfer curves obtained in that study (shown in Figure 3.1) were grossly asymmetric, and bear little resemblance to the derived transfer curves obtained from CM Lissajous figures (shown in Figure 3.2), or from microelectrode recordings from OHCs *in vivo*.

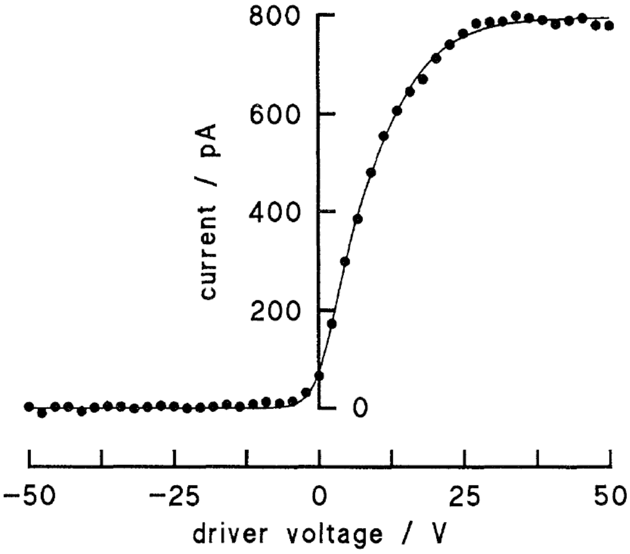
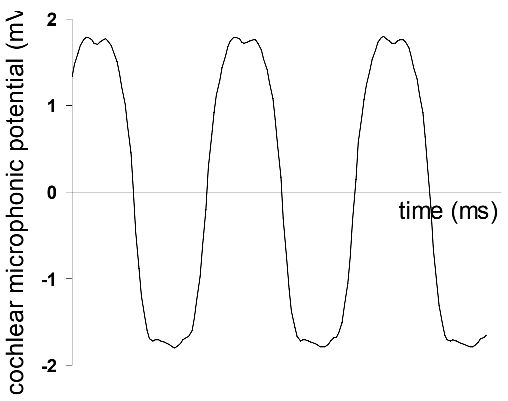


Figure 3.1: A transfer curve from an isolated outer hair cell from the apical turn, recorded by Kros et al. (1992) (see text).



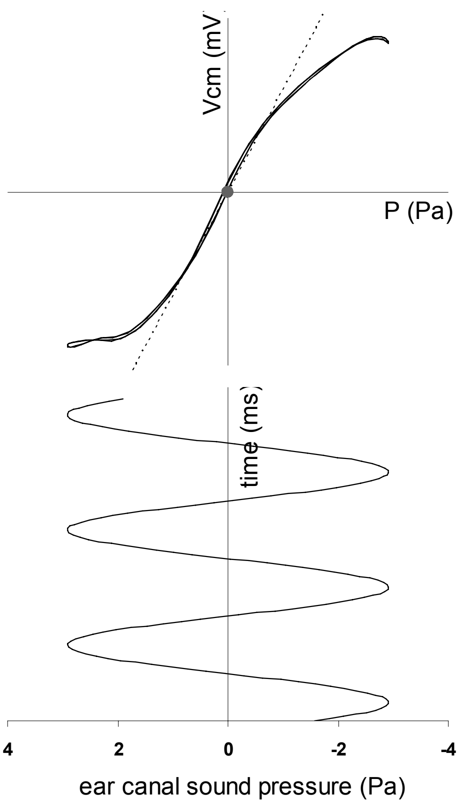


Figure 3.2: A Lissajous figures generated by plotting the 207 Hz CM waveform against the phase-shifted ear-canal sound-pressure waveform.

Cody and Russell (1987) carried out such microelectrode experiments and found an absence of DC receptor potentials for tones above 2 kHz in basal turn OHCs *in vivo*, suggesting that the OHCs operate relatively symmetrically around the steepest part of their transduction curves. Kros et al. suggested the difference between their asymmetric transfer curves and the symmetry implied in Cody and Russell's results may have been due to an external force acting on the hair bundle (such as from the insertion of the hair bundle into the TM), rather than an intrinsic property of the hair bundle itself.

Regardless of the *cause* of the symmetry, and because Boltzmann analysis of CM waveforms is necessarily carried out *in vivo* where OHC transfer curves are relatively symmetric, the 1st-order Boltzmann function produces an adequate description of OHC MET. Patuzzi and Moleirinho (1998) calculated that any errors arising from the fitting of a 1st-order Boltzmann function to CM waveforms produced by a 2nd-order transfer curve would be minimal in comparison to the changes in the parameters they observed experimentally.