Association of Intracellular and Synaptic Organization in Cochlear Inner Hair Cells Revealed By 3D Electron Microscopy

Anwen Bullen¹*, Timothy West¹, Carolyn Moores², Jonathan Ashmore¹,³, Roland A. Fleck⁴, Kirsty MacLellan-Gibson⁵, Andrew Forge⁴

¹Centre for Auditory Research, UCL Ear Institute, London, WC1X 8EE
²Institute of Structural and Molecular Biology, Birkbeck College, London WC1E 7HX
³Neuroscience, Physiology & Pharmacology, UCL, London WC1E 6BT
⁴Centre for Ultrastructural Imaging, King’s College London, London WC2R 2LS
⁵National Institute for Biological Standards and Control, Potters Bar, EN6 3QG

*Corresponding Author: Email: a.bullen@ucl.ac.uk, Telephone: +442076798955

Keywords: Inner Hair Cell, Intracellular Membranes, Synapses
Abstract

The ways in which cell architecture is modelled to meet cell function is a poorly understood facet of cell biology. To address this question, we have studied the cytoarchitecture of a cell with highly specialised organisation, the cochlear inner hair cell (IHC), using multiple hierarchies of 3D electron microscopy analyses. We show that synaptic terminal distribution on the IHC surface correlates with cell shape, and the distribution of a highly organised network of membranes and mitochondria encompassing the infranuclear region of the cell. This network is juxtaposed to a population of small vesicles and represents a potential new source of neurotransmitter vesicles for replenishment of the synapses. Structural linkages between organelles that underlie this organisation were identified by high resolution imaging. Together these results describe a cell-encompassing network of membranes and mitochondria present in IHCs which support efficient coding and transmission of auditory signals. Such techniques also have the potential for clarifying functionally specialised cytoarchitecture of other cell types.
Vertebrate tissues and cells are highly structurally organised. The topographical distribution of molecules, the arrangement of macromolecular assemblies, and the disposition of subcellular organelles determine the way in which a cell works. Architectural details are therefore crucial to understanding how cells function and how they are disrupted during disease. Modern 3D EM techniques have opened new frontiers in the understanding of cytoarchitecture. In this paper these techniques are used to characterise a highly specialised cell with a well-defined function, the inner hair cells (IHCs) of the mammalian cochlea although the techniques are applicable to a wide range of sensory and neural structures where there is a rapid turnover of membrane.

IHCs are the sound sensing cells present in the organ of Corti, converting sound induced vibrations of the sensory epithelium into electrical signals that are transmitted to the postsynaptic nerve. Each cell is innervated in the infranuclear region (below the nucleus) of the cell by up to 20 afferent nerve fibres (Francis et al., 2004) with a range of sensitivities and spontaneous activity. There is evidence for an organised pattern of fibres with different physiological properties around the circumference of the basal pole of the IHC and along its basal-apical axis (Liberman, 1982; Francis et al., 2004; Liberman et al., 2011). This arrangement implies some further degree of organisation within the cell so that neurotransmitter release and vesicle turnover are directed to occur at the right place and time. This aspect of synaptic organisation is not well understood.

The ability of the IHC synapse to sustain transmission for long periods has been attributed to the presence of the synaptic ribbon, a specialised synaptic structure found as well in retinal photoreceptors and bipolar cells. Ribbons are anchored near the synapse and surrounded by synaptic vesicles. It is thought that the vesicles provide a ‘readily releasable pool’ that can be quickly recruited at the onset of sound stimulus, and that the ribbon facilitates the supply of vesicles to the synapse during prolonged stimulation (Nouvian et al., 2006; Safieddine et al., 2012). The ribbon has also been implicated in the fusion of vesicles, priming them for release and their biogenesis (Matthews and Sterling, 2008; Snellman et al., 2011; Kantardzhieva et al., 2013).
Several hypotheses have been suggested for the recycling and replenishment of vesicles at the ribbon during periods of prolonged stimulation, including both the generation of vesicles at or near the synapse, and generation and transport of vesicles from more distant sites (Heidrych et al., 2009; Neef et al., 2014). These hypotheses require membrane structures to be present in the infranuclear region of IHCs and suggest that such membrane organelles must be precisely organised for effective synaptic transmission.

Intracellular membrane systems in the infranuclear regions of IHCs have been described as ‘canaliculi’ coursing from beneath the nucleus to contact the basal plasma membrane near synapses, and linear cisternae with characteristics of rough endoplasmic reticulum (RER), associated with mitochondria and cytoplasmic vesicles (Spicer et al. 1999). As Golgi bodies have not been observed in the infranuclear region of the IHC, it has been suggested that the vesicles do not carry a protein cargo but may contain neurotransmitter (Spicer et al., 1999; Spicer et al., 2007). Membrane cisterns local to the synapse and synaptic ribbon have also been implicated in roles of membrane retrieval and recycling after synaptic stimulation (Lenzi et al., 2002; Kantardzhieva et al., 2013).

We describe here the large-scale reconstruction of the IHC infranuclear region and its associated innervation using a combination of 3D electron microscopy techniques including serial block face scanning electron microscopy (SBF-SEM) (Denk and Horstmann, 2004) and high-resolution electron tomography. By combining these methods it is possible to examine cytoarchitecture from the level of whole cell shape to macromolecular complexes, encompassing the topographical distribution of cellular contents, the structure of individual organelles and cytoskeletal elements. Such methods provide detailed structural information contextualised by the wider cytoarchitecture, and are potentially applicable to many cell types. Using these techniques, we have dissected how cell shape and complex internal organisation relate to afferent terminal arrangement and synaptic transmission.
Results

IHCs are asymmetric and non-uniformly orientated in the Organ of Corti

IHCs have a characteristic ‘flask’ shape, with a constriction in the neck region. Relative to the surface of the organ of Corti, the cell body is angled towards the centre of the cochlear spiral (the modiolus) and away from the supporting pillar cell (fig. 1A). The nucleus is located in the apical half of the cell with the afferent nerve endings forming synapses around the entire baso-lateral region below the level of the nucleus.

For this study 11 IHCs from the middle cochlear coil of two mice were examined. Figure 1 shows that the cell bodies were asymmetric in shape, rounded (bulbous) on one side and flatter on the other (fig. 1B,E). The orientation of the cell with respect to the rounded side was not consistent: for some IHCs the rounded side faced towards the pillar cell, for others it faced the modiolus. This is evident from SEM images of the row of IHCs in the organ of Corti broken along the pillar cell region (fig. 1C) but was confirmed by 3D reconstructions of segments of organ of Corti imaged by SBF-SEM (fig. 1D). The flatness of each side of the IHC was quantified using a perpendicular line placed along each side of the cell. Mean measurements from three regions of the cells (1-4µm, 5-7µm and 7-11µm) showed a consistent flatness on one side of the cell compared to the other. This side was labelled as the ‘flattened’ side of the cell (fig. 1E).

IHCs consistently showed an extensive and seemingly continuous network of intracellular membranes throughout the infranuclear region. The membranous network was asymmetrically distributed, appearing more extensive on the flattened side of the cell compared to the rounded side (white arrowheads in cells 1,2,4,5 and 8 in fig. 1B) regardless of the IHC’s orientation with respect to the modiolus. This distribution appeared particularly distinct in the region surrounding the nucleus. Examination of intracellular membranes (example from cell 9) showed that the intracellular membranes consisted of a membrane enclosing a cisternal space. This is consistent with the structure of endoplasmic reticulum (fig. 1B, white arrows cell 9).
IHC afferent terminals cluster on the flattened side of the cell

To determine whether the asymmetric shape of the IHC was reflected in an asymmetry in the innervation of the cell, the positions of synaptic structures around the IHCs were examined. Only cells where the entire basolateral plasma membrane and IHC innervation were present in the image stack were used for analysis of the positions of neurons and ribbon synapses. Eight cells (1, 2, 5, 6, 7, 8, 9 and 10) fulfilled this criterion. Afferent terminals were identified based on the presence of a synaptic density on the afferent ending and corresponding synaptic ribbon.

Each cell had between 14 and 19 afferent terminals (mean 16.1±1.9 s.e.m., n=8). There was no correlation between the orientation of the flattened side of the cell on the modiolar-pillar axis of the organ of Corti and the total number of afferent terminals synapsing on the cell (fig. 2B and Table S1). Each cell also had between 14 and 19 synaptic ribbons (mean 16.4±1.9 n=8) (Table S1). Three of the eight cells had afferent terminals with two associated ribbons; two cells had one double-ribbon synapse and one cell had two double-ribbon synapses.

For 3D reconstruction, the positions of afferent terminals were marked on cells with a representative sphere centred on the approximate centre of the synaptic bouton (Cells 2, 5, 6, 9 and 10 in fig. 2A). In addition, and to provide a more detailed view, the terminals of two cells (Cells 1 and 2 Fig. 6B, and 2A respectively) were segmented manually. A comparison between manual segmentation and centred spheres that shows the similarity of these two approaches can be seen in fig. 2A (cell 2).

Comparison of the modiolar and pillar hemispheres of the cells showed that the mean number of terminals on the pillar side was not significantly different from the modiolar side. In contrast, in seven of the eight cells examined, the majority of afferent terminals were observed on the flattened side of the cell. The mean number of terminals on the flattened side was 10.1±1 compared to 5.9±1.2 on the rounded side, a
significant difference (P <0.05). The discrepancy between the two sides was largest when the flattened side faced the pillar cell.

It has been suggested that the position of the afferent terminal on the longitudinal axis of the cell (i.e. luminal to basal cell membrane) may also be important in the arrangement of terminals around the IHC (Liberman et al., 2011), so this position was also examined in these cells. In seven of the eight cells examined the terminal furthest from the synaptic pole on the longitudinal axis of the cell was on the pillar side of the cell, with the only exception being cell 8 (Table S1). This relationship was found irrespective of the side of the cell that had the most afferent terminals.

**Fig. 2**

**Intracellular membranes and mitochondria are concentrated on the flattened side of the cell**

The concentration of afferent terminals to the flattened side of the cell, coupled with the preferential organisation of membranes and mitochondria on this side suggests a possible relationship between the synaptic sites and intracellular membrane structures. To analyse quantitatively the 3D distribution of intracellular membranes and mitochondria, stereology was used. Stereology is a method that produces statistical information about a 3D object by sampling data (in this case using a grid of points) from 2D sections. Stereological and other 3D analyses were confined here to the infranuclear region of the cell.

Within the infranuclear region, only an ER-like membrane system, mitochondria and synaptic ribbons were evident, but no other cellular organelles were observed. At a magnification that would allow the imaging of whole cells in a single image (voxel size 19 x 19 x 50 nm), smaller objects such as microtubules and membrane vesicles could not be resolved. Although Golgi bodies were visible in the supranuclear region, they were not observed in the infranuclear portion consistent with previous studies.

The distribution of the intracellular membranes and mitochondria in the infranuclear region was examined using a point-counting stereology technique. The two organelles
were examined together because in all of the datasets examined, 95-98% of mitochondria were in close association with strands of intracellular membrane (i.e. with no visible gap between the mitochondrion and membrane strand in section images) (Table S1). This showed an almost exclusive association between the two organelles in this region of the IHC, indicating that membranes and mitochondria formed complexes throughout the infranuclear region and were part of the same network.

To analyse the spatial patterning of the organelles, the whole cell was divided into hemispheres and sampled with stereology point grids at 200 nm intervals. Fig. 3H shows the orientation of cells in respect to XYZ axes and the division of the cell into hemispheres: the cell was divided into hemispheres through the centre of the cell, along the YZ plane (‘rounded’ and ‘flattened’ hemispheres with reference to the cell shape or ‘modiolar’ and ‘pillar’ hemispheres with reference to the orientation of the cell in the Organ of Corti). The cell was also divided along the XY plane (‘posterior’ and ‘anterior’ hemispheres). An example of a stereology point grid and its associated micrograph is shown for cell 2 (fig. 3A,B) in the longitudinal (A) and radial (B) sectioning planes. Points in the cytoplasm (grey), on intracellular membranes (pink) and mitochondria (yellow) are shown. Examples of stereology grids from whole cells are shown in fig.3, from cell 2 (C) and from cell 8 (D).

Eight of the nine cells showed membranes and mitochondria enriched in the flattened hemisphere of the cell, regardless of the pillar-modiolar orientation of the flattened side. The SBF-SEM images showed that the asymmetry of intracellular membrane distribution was most prominent in the region of the cell closest to the centre of the nucleus, so a more detailed analysis was also carried out on a 3 µm thick volume of the cell centred around the longitudinal midline of the nucleus and sampled every 50 nm (i.e. every SBF-SEM section). A further 2 cells, for which the datasets for whole cell analysis were incomplete were included in this analysis. Examples of partial volume grids for cells 2 and 8 are shown in fig.3, (E,F). In all 11 cells analysed, the concentration of membranes and mitochondria was greater in the flattened hemisphere of the cell. The mean results of both analyses are shown in fig. 3G. The difference in membrane distribution between the flattened and rounded hemispheres of the cell was significant in both the whole cell and partial cell volume (**P ≤0.01), indicating that
membranes and mitochondria segregate asymmetrically towards the flattened side of the cell. The distribution of membranes and mitochondria was also examined in the opposite axis of the IHC, to test if there was asymmetry in the two hemispheres facing the basal or apical directions along the organ of Corti spiral, by analysing the ‘anterior’ and ‘posterior’ hemispheres (F and B on fig.3H). The proportion of points classified as membranes and mitochondria in the anterior hemisphere (0.21±0.01 n=9 cells) was not significantly different to the posterior hemisphere (0.19±0.01). Neither of these hemispheres were significantly different to the flattened hemisphere (0.23±0.01). However, membrane/mitochondria were significantly increased in the anterior hemisphere compared to the rounded hemisphere (0.16±0.01) (P <0.05) although the posterior hemisphere and rounded hemisphere showed no significant difference. Together, these quantitative results suggested a horseshoe-shaped distribution of the membrane-mitochondria network, concentrated towards the flattened hemisphere.

**Fig. 3**

**Common features of mitochondrial distribution**

The arrangement of mitochondria suggested by stereology was confirmed by reconstruction of the entire population of mitochondria in the infranuclear region. For all cells except cell 1, mitochondria were visualised as representative spheres to show distribution. In cell 1, mitochondria were manually segmented (fig. 4A). Mitochondria in all cells were arranged in a roughly semi-circular shell distribution, concentrated on the flattened side of the cell and with a sparse central region (fig. 4A top row, fig. 4D), although this region appeared larger in the manually segmented cell than in those where spheres represented mitochondria.

The distribution of mitochondria in the cells was also compared to the distribution of afferent terminals around the cell. These reconstructions showed that the asymmetric distribution of mitochondria matched the distribution of afferent terminals as was suggested by the stereology analysis and afferent terminal reconstruction (fig. 4A bottom row).
The strong association between membranes and mitochondria (Table S1) indicates that mitochondrial reconstruction could be used to estimate the distribution of membrane. This was confirmed by the segmentation of the complete intracellular membrane population in one cell (see next section). Mitochondria (fig. 1B,C,yellow) and intracellular membranes (fig. 4C,D, pink) in this cell were manually segmented, and when compared showed almost identical distribution patterns.

**Fig. 4**

**IHC intracellular membrane system can be divided into discrete classes of cisternae**

Stereology analyses and the close association of mitochondria with membrane both indicated that intracellular membranes were concentrated on the flattened side of the cell. To probe the details of membrane arrangements, a single cell (cell 1) was chosen from the SBF-SEM stacks for full manual reconstruction of the infranuclear intracellular membranes. The membranes were modelled as tubes, and were designated as parts of a single putative membrane sheet when they lay directly above each other in adjacent SBF-SEM sections (for further details, see Materials and Methods).

Figure 5A shows a frequency histogram constructed from the estimated surface area measurements of these putative membrane sheets, showing the distribution of intracellular membrane sizes in the infranuclear region. Analysis suggested that membranes could be divided into three classes. Three very large membrane sheets were present in the sample (sheets a (orange), b (purple) and c (red) in fig. 5), which were collectively named as Type 1 membranes. These membrane sheets together comprised 36.9% of the total intracellular membrane, of which 27.9% was a single sheet, sheet c, which was positioned on the flattened side of the cell and traversed the cell from the nuclear region to the base (red in fig. 5C). The sheets, both in context with the cell body and ribbon synapse and aligned perpendicular to the Z-plane to show their continuity (insets) can be seen in fig. 5C. These sheets were not continuous
with each other or any of the other membranes, and appeared to exist as separate large membrane surfaces.

Two other clusters of membrane were also present. The histogram of membrane areas shows that they may form a single population (blue and green fig. 5A). To examine these clusters in more detail, the histogram was re-plotted without the Type 1 membranes (fig. 5B). This histogram suggests that the smaller membranes may form part of a single distribution, but could be divided into a population of a large number of very small membranes (the steeply curved region) and a small population of intermediate sized membrane sheets (the flat portion of the curve). The eight separate membrane sheets that formed the flat portion of the distribution were named Type 2 (green). The smallest separate membrane sheets, which formed the steeply curving part of the distribution, were designated Type 3 membranes (blue). Type 3 membranes comprised by far the majority of membrane sheets in the cell, containing 2073 of the total 2084 membrane sheets segmented (57% of the total membrane area). The mean membrane area for this type was 85±2 µm². Type 2 membranes had a mean area of 2383±305 µm² and comprised 6.1% of the total membrane area (insets fig. 5C).

The majority of mitochondria in the cell (68%) were associated with a single membrane type, while the rest were associated with membranes from two different types. To compare the distribution of mitochondria between different membrane types only mitochondria associated with a single membrane type were considered. Type 3 membranes, despite comprising the largest total surface area, had the lowest density of mitochondria, at 1.5 x 10⁻³ per µm². Type 2 membranes had a density of 2.5 x 10⁻³ per µm², and Type 1 a density of 3.9 x 10⁻³ per µm². Measurements from the intracellular membrane model are collated in Table S2.

Examination of membrane distribution throughout the infranuclear region confirmed the asymmetry previously shown by stereology analysis. Type 2 and 3 membranes were found around the cell, but both types were concentrated to the flattened side (fig. 5C). The position of the large Type 1 sheet c at the flattened side of the cell also contributed a large proportion of membrane. The region occupied by sheet c enclosed
36.9% of the infranuclear cytoplasmic volume, but this volume contained 68.8% of the total surface area of intracellular membranes.

Type 3 membranes were almost always the most peripheral of the membranes on the flattened side. The majority of Type 1 sheet c lay further back from the plasma membrane, behind Type 3 membrane sheets (fig. 5D). Some Type 2 membrane sheets approached the cell membrane (fig. 5E). On the rounded side of the cell the two large Type 1 membrane sheets, sheets a and b, lay close to the plasma membrane, but not close to the synaptic ribbons (fig. 5C). Views of the cell looking along the cell body towards the basolateral pole from the position of the nucleus suggested that Type 3 membranes were the closest membrane type to the cell membrane around the majority of the cell. This angle also showed concentration of membrane to the periphery of the cell, sparing the central section, similar to the arrangement previously observed in the mitochondrial reconstructions (fig. 5F,G).

Of the 19 ribbons in the segmented cell, the proximate intracellular membrane sheets of 18 of the ribbons were Type 3 membranes. One ribbon had a proximate Type 2 membrane sheet (marked by (>)) in panel 2 of fig. 5C. The distance from the closest point of the nearest membrane sheet (of each type) to the approximate centre of each ribbon were calculated; for the Type 3 the mean distance was 515±85 nm, for the Type 2 2944±511 nm and for Type 1 1714±235 nm (n= 19 for all). Type 3 membranes were significantly closer to the ribbons compared to the other two groups (P <0.05), but the difference in distance between Type 2 and Type 1 membranes was not significant. Kantardzhieva et. al. (2013) showed that membrane cisterns were mostly absent near the ribbons, but that the number of cisterns increased with increasing distance from the ribbon, reaching an asymptote at approximately 350nm and dropping off after 800nm. This seems to agree with 515nm as the mean distance from the ribbon to the Type 3 intracellular membranes.

By examining a fully reconstructed cell it was possible to understand the distribution of the intracellular membrane system in the context of the complete infranuclear region (fig. 6A,B). Intracellular membranes and mitochondria were concentrated in the regions of afferent terminal boutons, mostly at the base of the cell on the flattened
side. The gradient of membrane sizes could also be appreciated, with the smallest Type 3 membranes (blue) closest to the ribbons (* in 6A), filling the gap between the large membrane sheet and the cell membrane on the flattened side of the cell.

Fig. 5

Fig. 6

**Filamentous linkages between mitochondria and ER membranes**

The co-localisation of membranes and mitochondria seen in the infranuclear region of IHCs prompted a closer examination of the structural relationship of the two organelles in these cells. We therefore used high-resolution electron tomography to examine junctions between intracellular membranes and mitochondria.

In image slices from electron tomograms of conventionally fixed mouse tissue, structural linkages between mitochondria and intracellular membrane in the infranuclear region were clearly visible (fig. 7A,B,C). The membrane had a clear cisternal space and was studded with electron dense structures with a diameter of ~20 nm, similar to the diameter of ribosomes (Zelena, 1972). This membrane was categorised as rough endoplasmic reticulum (RER). Sampling from several sites around the IHC revealed distinct links between mitochondria and the membrane sheet. Reconstruction of these linkages showed them to be extensive, occurring along the profile of a mitochondrion and its interface with the RER membrane (fig. 7D and fig. 8E). They had a mean length of 30.9±1.3 nm (n= 44), similar to the RER-mitochondrial links described in other cell types (Rowland and Voeltz, 2012). These linkages occurred in regions of the RER that excluded ribosomes (fig. 8E and Movie S2), suggesting a specialised ER domain as seen in other cells (Csordas et al., 2006; Rowland and Voeltz, 2012).

To investigate whether the linkages observed were the result of artefacts arising from glutaraldehyde fixation, additional samples that had been processed by high pressure freezing (HPF) and freeze substitution were examined. Due to the requirement to dissect the sensory epithelia clearly from any bony constituents these samples were
obtained from adult guinea pigs, which also allowed for cross species comparisons. Tomograms from these samples showed mitochondrial membrane links (fig. 7E-G) of similar lengths to those observed in the conventionally fixed samples (mean length 27.5±4.8 nm n=10) and also occurred in regions of membrane that were devoid of ribosomes.

**Fig. 7**

**Small vesicles are linked to ER membranes and to each other at the ribbon synapse.**

The tomography of mitochondria and RER, revealed a second, unsuspected feature of IHC RER: small membrane vesicles clustered around the RER membranes. Some of these vesicles appeared tethered to the membrane sheet and to each other by thin linkages (fig. 8A-D). Guinea pig samples prepared by HPF also showed the same vesicle-membrane linkages (Fig. 8F). When these linkages were reconstructed, they showed tethered vesicles along the length of the endoplasmic reticulum. The tethers appeared to connect to the membrane surface, and occasionally to ribosomes and had a mean length of 52.9±3.3 nm (n=63) (fig. 8C, D, E and Movie S2). Vesicles were also observed tethered to each other away from membranes (data not shown). All membrane sheets examined showed these linkages, including those not directly adjacent to synapses. The membrane vesicles observed had a mean diameter of 37.3±0.6 nm (n=70). This value was not significantly different from that of the vesicles at the ribbon synapse segmented by the same method (fig. 8H) (mean diameter= 36.3±0.5 nm n=89). Linkages between the vesicles and the ribbon had a mean length of 26.8±1.8 nm (n=36) and were significantly shorter than the links between vesicles and RER membrane (P <0.05). In the HPF prepared samples, vesicles were occasionally observed with internal structure, which appeared as arms radiating from a central point (fig. 8G).

Linkages between vesicles were also observed at the synaptic ribbon (white arrows in fig. 8H, shown in red in reconstructions). Compared to the linkages between vesicles at the RER, which had a mean length of 45.4±3.0 nm (n=61), links between vesicles
at the ribbon were shorter \((\text{mean length}= 22.3\pm0.7 \, \text{nm} \, n=78)\). Links at the ribbon were significantly shorter than both those between individual vesicles and those between vesicles and RER \((P <0.05)\). Both linked and unlinked vesicles were observed at the ribbon synapse (green and purple respectively in fig. 8F and Movie S2). Vesicles around the surface of the ribbon that faced the cytoplasm were linked together, but those facing the synaptic gap appeared to have lost these linkages. Vesicles with apparently unattached tethers were also noted around the ribbons, although as the entire vesicle population of unattached vesicles surrounding the ribbon was not segmented (due to section thickness and magnification required to resolve the tethers) it was difficult to tell what proportion of these vesicles had unattached tethers.

Taken together, our data demonstrates a high degree of organisation of membranes and associated organelles proximal to the synapses, both in overall distribution and in local connectivity, with potential roles in supporting efficient synaptic transmission.

Fig. 8
Discussion
Specialisation of a cell is likely to involve not just specific structures but also tailoring of the complete cytoarchitecture to the task in question. The IHC has evolved as a dedicated sensory cell with an extraordinary capability for rapid and sustained neurotransmission of a complex signal and despite a good understanding of how component parts of IHC function, wider questions of how the whole cell is tailored to its specialised task have not been comprehensively addressed. By examining the subcellular structures of IHCs in three dimensions and in context with surrounding cellular and extracellular structures this study has shown a previously unappreciated level of internal organisation in IHCs and suggested how such organisation may be related to the cell’s function. It seems likely that such cellular organisation underpins specific function in multiple cell types and the methods used here could usefully be applied elsewhere.

Inner hair cell asymmetry

We have shown that the IHC is an asymmetric cell, and non-uniformly orientated in the organ of Corti. This non-uniform orientation may arise simply as a requirement to increase the packing density for IHCs. It has been shown previously from confocal imaging that the basolateral poles of adjacent IHCs are often staggered in adjacent radial planes (Yin et al., 2014), and we suggest that this pattern may be a result of the changing of the orientation of the flattened and rounded sides. The intracellular structure of the cell and the pattern of innervation appear to be affected by the cell’s asymmetry, with both intracellular components and afferent terminals distributed preferentially to the flattened side of the cell regardless of the cell’s orientation. Two alternate hypotheses may be proposed from these results: either factors internal to the cell affect afferent patterning; or afferent patterning affects cell shape and internal arrangement. A developmental study would be required to untangle the precise nature of this association but is beyond the scope of the current work.

The complete 3D reconstruction of an IHC infranuclear region suggested that the IHC of the mature mouse can be divided into two topographical regions: a supranuclear ‘synthetic’ pole that contains the cells machinery for protein synthesis in the form of Golgi bodies and endoplasmic reticulum; and an infranuclear ‘synaptic’ pole that
contains the ribbon synapses, and a network of endoplasmic reticulum, mitochondria and vesicles. This network is asymmetrically distributed and traverses the cell from nucleus to base. ER is known to associate with microtubules, with the microtubules acting as cell organisers (Tuffy and Planey, 2012). Although microtubules could not be detected at the resolution of the present SBF-SEM data it is likely that they were also present. Microtubule tracks in inner hair cells have been described previously as orientated longitudinally between the apex and base of the cell (Steyger et al., 1989), suggesting that they may follow similar to tracks to those described by the membranes in this study.

**Membrane organisation in the IHC**

The membrane system reconstructed in this study is most likely a snapshot of a dynamic system that changes in response to the differing energetic demands of the cell, particularly during periods of sustained neurotransmission. Membrane size distributions may represent a sheet or network of membranes that is constantly breaking and reforming. Spicer and co-workers (Spicer et al., 1999; Spicer et al., 2007) described cisternae that were broken into segments at the sites of mitochondrial contact. This was also sometimes observed in the SBF-SEM data. In the results we show three distinct categories of membrane sheet. We hypothesise therefore that the membrane system is composed of numerous small elements (Type 3 membranes) derived from the larger continuous sheets that make up the Type 1 membranes. Type 2 membranes may represent a transitional form between Type 1 and 3 membrane classes. The relative positioning of the numerous small membrane sheets (“Type 3” membranes) and Type 1 sheet c on the flattened side may also be representative of the edges of a larger membrane sheet (“Type 1”) breaking into smaller Type 3 membranes, which then project towards the synaptic ribbons. It is also tempting to consider whether the core of the Type 1 membrane sheets represent stable structures. Stability of sheet c may help to explain the consistent concentration of membrane to the flattened side of the cell, and the higher mitochondrial density of the Type 1 membranes.

Membrane cisterns in close proximity to the ribbon may be a consequence of exocytosis, formed by fusion of vesicles during repetitive stimulation. Membrane
cisterns close to ribbons have been shown to increase in size upon depletion of synaptic vesicles by stimulation, and it was proposed that they represented an important component of the vesicle recycling pathway, with vesicles reforming at the cisterns (Lenzi et al., 2002; Kantardzhieva et al., 2013). Cisterns themselves may also be competent for fusion and release of neurotransmitter (Matthews and Sterling, 2008). Although the width of the lumen of the most peripheral intracellular membrane elements described here appears larger than that of the cisterns described by those authors, it is tempting to speculate that they are the same structure. Ribbons have also been shown to attract membrane cisterns, regardless of whether the ribbon is tethered at the synapse or free floating in the cytosol (Khimich et al., 2005). Cytosolic ribbons may be attracting small membrane cisterns such as (or derived from) the ones described in this study, suggesting an attraction for cisterns that are not wholly formed by recycled vesicles. Therefore the membrane network may be altered by the stimulus state of the cell. The distribution (particularly of Type 3 membranes) in the stimulated cell is a target for future work.

**Mitochondrial organisation in IHCs**

The results of the electron tomography indicate strongly that the mitochondria-membrane structures observed represent an integrated functional complex. The linkages observed were not artefacts of chemical fixation, and were present in both species examined. Linkages between mitochondria and ER in other cell types have multiple functions, including control of lipid biosynthesis, mitochondrial division, calcium signalling and co-ordinating organelle movement (Rowland and Voeltz, 2012). In the IHC, calcium regulation in particular seems a likely linkage function, as calcium controls the release of neurotransmitter at IHC synapses and intracellular calcium levels may be particularly important in sustained signalling. Mitochondria are known to act as sequesters of calcium and to control calcium mediated neurotransmitter release in some cells (Billups and Forsythe, 2002). Work has also shown that during aminoglycoside induced hair cell death in zebrafish, elevated intracellular calcium occurs immediately after mitochondrial potential collapse (Esterberg et al., 2013). The large cellular scale of single sheets in the membrane-mitochondrial network, the sheets’ positioning around the periphery of the cell and their concentration in regions where the majority of synapses are located suggests that
the network may act by controlling calcium levels in regions local to the synapses and shuttling calcium throughout the cell. Glutamate synthesis may also be a mitochondrial function in IHCs: the enzyme phosphate activated glutaminase, a key enzyme in the synthesis of glutamate, has been shown to be restricted mainly to the mitochondria and to co-localise with glutamine and glutamate (Takumi et al., 1999). It is possible therefore that the mitochondria in the infranuclear region may also be contributing to glutamate recycling there.

**Vesicle tethering in IHCs**

The tethering of small vesicles to RER was an unexpected finding, revealed only by high-resolution electron tomography. RER is often associated with transport vesicles for the shuttling of synthesis products between the RER and Golgi apparatus but such vesicles are generally larger, 50-100 nm in diameter (Martínez-Menárguez, 2013), than those observed here tethered to the RER (ca. 36 nm). Additionally no Golgi apparatus was observed in the region of the infranuclear membrane network and vesicles were not observed either budding or fusing with the RER membrane, only connected to it by filamentous linkages. The vesicles observed were of the same diameter as putative neurotransmitter vesicles that were associated with or in proximity to ribbon synapse. Immunostaining for the vesicle glutamate uptake transporter VGlut3 in IHC shows labelling throughout the hair cell cytoplasm (Peng et al., 2013; Neef et al., 2014), which may indicate that neurotransmitter vesicles are also present throughout the cytoplasm and not just concentrated at synapses. If the vesicles do contain neurotransmitter, it is possible that the membrane network may also represent a secondary store of neurotransmitter vesicles for release during sustained synaptic transmission. Calcium sensing proteins (such as otoferlin) and their binding partners appear to label throughout the IHC cytoplasm (Duncker et al., 2013; Neef et al., 2014), suggesting they may also be involved in such a system, possibly as constituents of the linkages. It has recently been suggested that otoferlin may be part of the vesicle priming machinery in the IHC as otoferlin deletion lengthens the linkages and hence could be involved in the production of close attachment of vesicles to the synaptic active zone (Vogl et al., 2015). Thus co-ordination of a secondary vesicle store, vesicle biosynthesis and the putative calcium regulating functions of the RER and mitochondria would produce a multifunctional complex...
capable of regulating a proportion of the vesicle supply to the synapse and the release of neurotransmitter.

The presence of linkages between vesicles at the ribbon synapse raises questions about the putative role such linkages may have in synaptic transmission. At frog neuromuscular junctions it has been shown that linkages and internal vesicle structures may be important in the attachment of vesicles to a docking complex and that they may confer a preferred conformation on docking vesicles. The linkages described in this study may be analogous to the filamentous linkages described at the neuromuscular junction, which link vesicles together and to structures at the synaptic active zone (Szule et al., 2012). It was reported that internal vesicle structure was not visible after conventional fixation and room temperature staining, but only after rapid freezing and freeze substitution (Harlow et al., 2013). Harlow et. al. described the internal vesicle assembly as a bilateral structure with irregular arms radiating from the centre, which is remarkably similar to the internal structure seen in vesicles in our study. A comprehensive study of vesicles in rapidly frozen samples would be required to confirm this result, but it opens an interesting avenue of further investigation into whether the internal structure observed in the neuromuscular junction is also present in the IHC and other sensory cells and if such linkages perform a similar function in regulating vesicle docking conformation.

The cells of the vertebrate cochlea provide an ideal model system in which to evaluate the functional context of distinct populations of highly specialised cells. By combining 3D microscopy analyses it has been possible to examine and relate cellular organisation at multiple resolution levels in a specialised sensory cell. For the IHC, these observations may have significant future value in determining changes to the structures inside cells that occur after cochlea damage, for example by excessive noise or ageing. Cochleae exposed to certain noise traumas undergo selective deafferentation of IHCs, and are thought to model so called “hidden hearing loss” in humans (Kujawa and Liberman, 2009). Our preliminary work on cochleae exposed to such noise traumas suggests that the patterns of cellular arrangement described here may be disrupted in noise damaged cochleae. Early analysis of IHCs from noise damaged animals suggests a breakdown in the organisation of membranes and mitochondria within the cells, coinciding with degenerated afferent terminals and
increased numbers of abnormal mitochondrial structures. High resolution analysis of
the regions surrounding degenerated synapses, currently underway, also suggest
changes to structures local to the synapse, particularly the small membrane structures,
indicating possible cellular rearrangements in response to synaptic changes. These
results indicate the utility of these approaches in assessing cellular damage.

Conclusion

3D microscopy approaches and reconstruction on cellular scale have clear
applications in identifying the structural specialisations in a wide variety other cell
types. As our understanding of components of cellular systems improve, it is essential
to determine how they interact with each other within the system as a whole.
Multiscale resolution of cellular structures provides the vital information necessary to
understand the particular ways in which cells are organised to perform specific
functions, and how disturbances of such organisation underlies the progression of
damage ageing and disease.
Materials and Methods

Animals
Cochleae were obtained from two three month old C57/Bl6 mice and one adult tri- colour guinea pig. All work with animals was conducted in accordance with procedures licenced by the British Home Office and approved by UCL Animal Ethics committee.

Conventional Fixation
Auditory bullae were isolated from mice immediately after death. Cochleae were fixed in 2.5% glutaraldehyde and decalcified in 4% ethylenediaminetetraacetic acid (EDTA) (Sigma, UK). Post fixation staining was carried out in 1% aqueous osmium tetroxide (Agar Scientific, UK) and samples were embedded into Epon resin (TAAB, UK).

Cryo-Fixation and Freeze Substitution
After death, auditory bullae of the guinea pig were removed and placed on ice. Cochlear tissue was dissected from the bullae just prior to freezing. The time between death and freezing was approximately 30 minutes. Samples were prepared by high pressure freezing and freeze substitution as previously described (Bullen et al., 2014).

Serial Block Face Scanning Electron Microscopy
After fixation and decalcification cochleae were transferred to 0.1% tannic acid (TAAB, UK) in 0.1M cacodylate and incubated in this solution overnight at 4°C. Samples were processed for SBF-SEM following the method of Deerinck et. al. (Deerinck et al., 2010). After staining samples were dehydrated and embedded in Epon resin. Samples were examined in the Gatan 3View system (Gatan, USA) on a Zeiss Sigma FE-SEM (Zeiss, Germany), or a Gatan 3View XP system on a JEOL 7100F SEM (JEOL UK, UK). Blocks were cut at 50nm and the exposed block face imaged with a pixel size of 19nm.
Electron Tomography

200nm sections of organ of Corti were examined in a JEOL 2100 transmission electron microscope operating at 200kV (JEOL) and dual-axis images collected between at least 60º and -60º at 1º increments. Data collection was controlled by SerialEM software (Pretorius, 2011). Tomographic reconstruction was carried out by weighted back projection using the IMOD suite of programmes (Mastronarde, 1997).

Segmentation, Stereology and Modelling

Segmentation, stereology and modelling were carried out using IMOD. For modelling of membranes in SBF-SEM images a fixed threshold level that excluded cytoplasmic background was used. Point-counting stereology was carried out in IMOD. Each grid covered 11.4µm of the length of the cell (Y axis) and the complete width (up to 15.3µm) of the cell (X axis) (fig. 2L). >5000 points were classified for each stereology data set. For whole cell measurements grids were placed 200 nm apart (Z axis). Two cells (3 and 4) were not contained within a single image stack, and were excluded from whole cell analysis. For partial volume analysis grids were 50nm apart and encompassed a region 1.5 µm either side of the nucleus. Points were classified as follows: not in cell, cytoplasm for points that did not lie on either mitochondria or intracellular membrane, mitochondria, or intracellular membrane. A dynamic centre line that accounted for the asymmetry of the cell was used for division into flattened/rounded hemisphere. To define anterior/posterior hemispheres, we chose the anterior hemisphere to begin where the cuticular portion of the cell first appeared in the images and to end at the centre section of the cell, and the posterior hemisphere to begin at the next section.

Reconstruction of the whole cell was undertaken by manual segmentation. For visualisation in 3D reconstruction strands of membrane in a section were modelled as representative tubes with a radius of 50 nm, the width of the gap between one section and the next. To minimise human error the model cell was subject to two additional passes through the complete cell. Tomographic reconstructions were checked by a second expert (human) segmenter.

Measurements were taken with IMOD and ImageJ (Schneider et al., 2012). Additional specially developed software tools were used for extraction of data from
IMOD models. Statistics were calculated using SPSS (IBM, USA). Data are given as means ± s.e.m. Significance was calculated using t-tests and one-way ANOVA with Games-Howell post hoc test (where appropriate) or Kruskal-Wallis test. Images were adjusted for optimal contrast and brightness and assembled into figures using Adobe Photoshop CS6 (Adobe Systems Software Ltd, Ireland).

**Acknowledgements**

Dr Dan Clare (Birkbeck College) for assistance with HPF. Simeon Nicholls for stereology classification. Dr Hannah Armer and Dr Peter Munroe (UCL Institute of Ophthalmology) for assistance with SBF-SEM. Graham Nevill for sample preparation and Gareth Eldrett for software development. This work was funded by a project grant from the Biotechnology and Biological Sciences Research Council (BBSRC) (Grant number: BB/I02123X/1). Data contained in this paper may be accessed by contacting the corresponding author.
References


Figure 1. SBF-SEM analysis of IHCs from the middle cochlear coil of C57/Bl6 mice.

(A) Diagram of the Organ of Corti. Direction of modiolus and pillar cells are shown. (B) Whole cell sections from five hair cells. Concentration of internal membranes and mitochondria on one side of the cell is shown (white arrowheads). Images from cell 9 show the internal lumen of the membranes. (C) SEM micrograph showing IHCs viewed from the modiolar side of the cell. (D) Reconstruction of five IHCs viewed from the same position. Innervation of three of the cells is also reconstructed. Cells are non-uniformly orientated and either the rounded (purple, green) or flattened
(yellow, blue, red) side may face the modiolus. (E) Flatness of cells measured by contact of the cell membrane with a perpendicular line. Lines were placed on either side of each cell (n=11). Mean measurements of three regions showed that one side of the cell was consistently flatter than the other. Error represents s.e.m. Scale bars: B, 2 μm (Cell 9, 1 μm); C, 10 μm; D, 5 μm.

Figure 2. Distribution of afferent terminals around IHCs
(A) Distribution of afferent terminals (dark blue) on five cells. Terminals were either manually segmented (cell 2) or represented by a sphere at the approximate centre of the bouton. Comparison between manual segmentation and sphere representation is shown for cell 2. (B) Number of afferent terminals present on each cell studied. (C) Mean distribution of afferent terminals in the pillar/modiolar and rounded/flattened hemispheres of n=8 cells. **P <0.05. Error represents s.e.m. Scale bars, 2 μm. See also Table S1.
Figure 3. Distribution of membranes and mitochondria revealed by stereology analysis of IHCs.

(A, B) Stereology grid superimposed on an image of cell 2, showing the longitudinal (A) and radial (B) section. (C,D) Stereology models of whole cells (cell 2 and cell 8). (E,F) 3 µm sections around the nucleus of the same cells. Flattened side of the cell is indicated by / and rounded side by ); P and M indicate pillar and modiolar orientation. The concentration of membranes and mitochondria (pink and yellow) to the flattened side of the cell is shown. (G) Mean distribution of membranes and mitochondria between flattened and rounded hemispheres of the IHC in 3 µm section and whole cell reconstructions (n=9 and n=11 respectively). Error given as s.e.m. (H) Orientation of the IHC on the X,Y,Z-axes and orientation of division along the anterior/posterior axis (An and Po) and the rounded/flattened axis (R and F). The sides of this axis can be pillar or modiolar (P/M) depending of the orientation of the cell in the organ of Corti. Scale bars: A-F, 2 µm. See also Table S1.
Figure 4. Reconstruction of mitochondria in IHCs

(A) Mitochondrial distribution in IHCs, reconstructed using manual segmentation or representative spheres. Top row of images: The view from the nucleus to the middle of the infranuclear region is shown, overlaid on the radial section image of the cell at the centre of the infranuclear portion. Bottom row of images: mitochondrial distribution compared to the positions of the afferent terminal population of each cell (dark blue). (B) IHC mitochondria (yellow) reconstructed by manual segmentation. Orientation of the cell relative to the pillar cell (P) and modiolus (M) is shown on the image. (C) Reconstructed mitochondria shown in conjunction with reconstructed intracellular membranes (pink). (D) Mitochondria alone, and mitochondria and intracellular membrane population together viewed looking towards the basal pole of the cell from the position of the nucleus. Scale bars, 2 μm. See also Figure S1, Table S1.
Figure 5. Reconstruction and classification of IHC Intracellular Membranes

(A) Histogram of internal membrane surface areas reconstructed from Cell 1. Sizes of very large membrane sheets are shown above their respective bars. (B) Histogram of intracellular membrane surface area, excluding very large membrane sheets. (C) Membranes are shown divided into the clusters shown in the histogram. Orientation of the cell to the pillar cell (P) and modiolus (M) is shown on images. Red spheres represent positions of ribbon synapses. Insets: membranes aligned perpendicular to the Z-axis, showing their sheet characteristics. Only a portion of sheet c (red) is shown. (D) Type 3 (blue) membranes and sheet c (red) are shown together. (E) Type 2 and Type 3 membranes shown together. (F) Top section of intracellular membrane shown from the position of the nucleus, and with radial section image. (G) View through the complete length of the cell from the same position. Scale bars, 2 µm. See also Table S2 and Movie S1.
Figure 6. Complete model of membranes, mitochondria and synapse distribution in cell 1.

(A) Ribbon synapses (red spheres) are marked with (*) for clarity. (B) Reconstructions of synaptic terminals are shown (blue). Scale bars, 2µm. See Movie S1.
Figure 7. Tomographic reconstruction of linkages between rough endoplasmic reticulum and mitochondria.

(A-C) Slices from tomographic reconstructions of conventionally fixed mouse tissue showing linkages of three different mitochondria (Mt) to RER (R) (white arrows). (D) 3D reconstruction of mitochondria (blue) and membrane (green) showing the depth and arrangement of mitochondrial-membrane links (purple). (E-H) Membrane-mitochondrial links observed in HPF prepared tissue from a guinea pig. Links (white arrows) are shown between mitochondria (M) and membranes (black arrows). Images (E-G) show links in an IHC. Scale bars A-C, 50 nm; E-G, 50 nm. See Movie S2.
Figure 8. Tomographic reconstruction of vesicle-membrane and vesicle-vesicle links on endoplasmic reticulum and at the ribbon synapse.

(A-D). Linkages (white arrowheads) are shown between membrane vesicles (black arrowheads) and three different areas of the RER (R). Images show an average of 5 tomographic slices. Insets show reconstruction of these links in (C, D). (E) Reconstruction of a section of rough endoplasmic reticulum (green with ribosomes in red) with linkages to mitochondria (blue) and vesicles (yellow) showing the linkages surrounding the membrane. (F-G) Tomographic reconstructions of vesicles in HPF prepared guinea pig tissue. Images show an average of 5 tomographic slices. (F) Vesicle membrane linkages (G) HPF prepared vesicle showing apparent internal vesicle structure (black arrow). Inset shows vesicle internal structure outlined in red.

(H) Tomographic reconstruction of a mouse ribbon synapse; images show an average of 9 consecutive tomographic slices. 3D model: vesicles (green) with vesicle-vesicle links (red) and vesicles without linkages (purple) around the synaptic ribbon (blue). The synapse is viewed from four positions: through the pre-synaptic membrane (grey), from the IHC cytoplasm, and from the top and bottom of the synaptic ribbon. Scale bars A-D, 50 nm; Inset C 10nm, Inset D 20nm, E 100 nm, F 25 nm, G (and inset) 25 nm, H 50 nm. See Movie S2.