Morphology and electrophysiology of the vestibular organ in the guinea pig
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The glycocalyx and stereociliary interconnections of the vestibular sensory epithelia of the guinea pig.


Introduction
The sensory epithelium of the vestibular end organs consists of sensory hair cells which receive stimuli transmitted by the bending of the stereocilia and kinocilium of the hair cell bundle. The stereocilia exhibit a surface coat, or so–called glycocalyx. The glycocalyx consists of glycoconjugates; such as glycolipids, proteoglycans, mucopolysaccharides and glycoproteins. This carbohydrate–rich glycocalyx is present on the free surface of cells, and is most highly developed on the luminal surface of certain epithelial cells (1). In 1963, Bennet (2) first described the cell surface coating material which he suspected to form a part of the plasma membrane.

Previous investigations have suggested that the glycocalyx may be responsible for the way the sensory cell reacts to its external environment; such as adhesion, fusion, filtration, protection against chemical injury, ion exchange and electrostatic interaction between stereocilia (3–5). The glycocalyx is also thought to be important for the cytoskeletal organisation of cells. It seems conceivable that the lateral stereociliary interconnections and tip–links are related to the glycocalyx.

The glycocalyx and stereociliary interconnections have been demonstrated in the sensory epithelia of the vestibular end organs of the guinea pig by different transmission electron microscopy (TEM) and scanning electron microscopy (SEM) techniques (3,4,6–8). Although detailed reports have appeared on the organisation of the glycocalyx, this structure has never been closely examined by freeze–fracturing and low–voltage cryo–SEM. To obtain a better understanding of the fine three–dimensional morphology of the
Morphology and electrophysiology of the vestibular organ in the guinea pig and its relation to stereociliary interconnections, this study has been performed using these direct techniques, in combination with TEM and conventional low–voltage SEM.

Materials and methods

Five healthy female albino guinea pigs (Harlan, the Netherlands) with a normal Preyer’s reflex and a weight of approximately 500 g were used in this study. Animal care and use were approved by the Experimental Animal Committee of Groningen University, protocol number 2325, in accordance with the principles of the Declaration of Helsinki. The guinea pigs were sacrificed by an intracardial injection of pentobarbital. After decapitation, the bullae were rapidly dissected out in ice–cold Hanks’ balanced salt solution (pH 7.4; 320 mosm; 0–4°C). The vestibulum was opened and the utricule was removed. The otolithic membrane was gently removed with fine tweezers.

For freeze–fracture and cryo–SEM the utricle was perfused with a solution of 1% glutaraldehyde, 2% paraformaldehyde, and 2% tannic acid (pH 7.0; osm 800–1000; >2h). After rinsing in 20% methanol used as a cryo–protectant, the tissue was rapidly frozen in supercooled nitrogen slush of –210°C. The utricle was mounted on a grid and placed in a specimen holder. For freeze–fracture, the utriculi were fractured in the CT1500 HF high resolution cryotrans. After etching for 3 minutes from –180°C to –100°C, the specimens were sputtercoated with Au–Pd (3nm) in argon/xenon. The specimens were studied in the JEOL FEG–SEM, type 6301F (–120°C, 5kV).

For SEM, the utriculi were fixated in a solution of 2.5% glutaraldehyde in 0.1M Na–cacodylate buffer (pH 7.4; 4°C; 400 Mosm) and 2mM CaCl2. Post fixation was carried out using the TAO–method (9), after which the specimens were rinsed in distilled water and dehydrated in ethanol. All specimens were critical point dried with liquid CO2 and sputtercoated with Au–Pd (2–3nm). The specimens were studied in the JEOL FEG–SEM, type 6301F operating at 2kV.

The fixation of the utriculi for TEM was the same as for SEM. After fixation, the specimens were post–fixed in 1% OsO4 with 1% K4Ru(CN)6 for 3 to 4h, carefully rinsed in distilled water, and then dehydrated in a graded ethanol series followed by propylene oxide. They were then infiltrated using a mixture of 1:1 propylene oxide and Spurr’s low viscosity resin for 1 h, and pure resin overnight. Polymerization took place at 70°C after exsiccation in a vacuum. Ultra–thin sections (100nm) of the utriculus were contrast–stained with 7%
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uranyl acetate in 70% methanol and lead citrate according to Reynolds, and examined using a Philips 201 transmission electron microscope (TEM) operating at 40 kV.

Results

A haircell–bundle broken lengthways, with the glycocalyx still partly standing as a rigid shell that once enveloped the intact stereocilia, is shown in figure 1. The stereocilia are partially broken, and some cilia are completely gone. The glycocalyx appears to be uniform in composition and density. The lateral interconnections between the cilia are present over the entire length, and appear to have the same length and density. These connections are morphologically similar, and appear to consist of the same material as the glycocalyx itself. In the transverse freeze–fracture (figure 2), the interciliary connections also appear to emerge out of the glycocalyx coat. The connections, at both the centre and the periphery, appear to be grossly oriented in the same direction. The extensive extracellular network or matrix surrounding the stereocilia has

Figure 1 In this freeze–fracture image, the glycocalyx is partly standing as a shell–like structure that was surrounding the stereocilia of the hair cell bundle of the sensory epithelium. The arrows indicate the lateral interciliary connections emerging from the glycocalyx. There are several holes in the glycocalyx, and around the glycocalyx matrix, material (asterisk) can be observed.
the same rough and rigid appearance as the glycocalyx and also seems to be of the same composition. The matrix material, surrounding the remnants of the glycocalyx in figure 1 also morphologically resembles the glycocalyx.

The TEM–figures 3a and 3b clearly demonstrate the glycocalyx after staining with the contrast enhancing K₄Ru(CN)₆. The glycocalyx is found over the entire length of the stereocilia of the sensory cells, and seems to emanate from the outer layer of the plasma–membrane. The glycocalyx is a dense coat of regular thickness and, with a somewhat woollen appearance at the outer surface at some points. The tip–links between the stereocilia are clearly protruding. In both longitudinal (figure 3a), and transverse sections (figure 3b), it seems that the tip–link is covered by a thin layer which stains similar to the carbohydrate–rich glycocalyx. The dense layer on the tip–link seems to be a continuation of the glycocalyx covering the stereocilia.

In the low–voltage SEM (figure 4), the outer surface of the cilia is shown, and the glycocalyx covering the stereocilia can be directly observed. This surface coat has a rough, but uniform structure. The tip–links emerge from the top of the stereocilia and are connected to the taller neighbour’s shafts.
As runners of the cilia, the tip–links appear to be coated the same as the stereocilia themselves, and are all arranged in the same direction.

**Figure 3a** In this TEM–figure, some stereocilia and their tip–links (T) are shown lengthways. The stereocilia are coated on their surface by the glycocalyx (arrows). The dense layer on the tip–links (small arrows) is a continuation of the glycocalyx covering the stereocilia. Because of the thickness of the section, a cilium is over-projected, leaving a dark shadow over the middle cilium (asterisk).

**Figure 3b** This transverse TEM image clearly demonstrates the glycocalyx surrounding the stereocilia. The tip–links (T) are covered with a dense layer, similar to the glycocalyx (arrows).
Discussion

This study has been performed using freeze–fracture, cryo–SEM, low–voltage SEM and TEM. Imaging of fast–frozen samples is the most direct approach for electron microscopy of organic material. Different rinsing and fixation steps are skipped, leaving the fine ultrastructure preserved to a maximum. Low–voltage electron microscopy has been used for an even more detailed and accurate description of these delicate vestibular structures.

Takumida et al. (3) showed by means of TEM that the glycocalyx was found over the entire length of the stereocilia, appearing as a fuzzy filamentous layer. We have now provided a three–dimensional illustrative morphological confirmation of these results. In the freeze–fracture (figure 1), it is clearly demonstrated that the glycocalyx is enveloping the stereocilia. The glycocalyx resembles a shell–like rigid structure separately surrounding each stereocilium and kinocilium. The holes in the glycocalyx may be due to freeze–damage.
In our freeze–fracture and cryo–SEM images, the lateral interciliary connections appeared to emerge out of the glycocalyx layer of the stereocilia. The network of connections between adjacent cilia had the same rough and rigid appearance as the glycocalyx itself. This confirms the findings of previous TEM studies (4–6), which show that the ciliary interconnections all emerge from the fuzzy layer of the glycocalyx. These structures were stained similar and therefore are considered to be morphologically identical.

Using freeze–fracture, Arima et al. (5) reported an extensive extracellular fibrous network between the stereocilia which they thought to be similar to the glycocalyx. They suggested that the role of these structures was to hold the cilia as a unit during bending. In addition, our results suggest that the connections are grossly oriented in the same direction (figure 2). The polarization of the sensory cell is dependent on the orientation of the cilia during bending. The connections, therefore, can also be considered to be important in the mechanical transduction system.

The idea that the glycocalyx also serves as an anchoring structure for the mechanical coupling between the sensory hair bundle and the otolithic membrane or cupula was suggested by Takumida et al. (4). In the freeze–fracture (figure 1), it seems that the material around the glycocalyx is morphologically identical to the glycocalyx itself. Because freeze–fracture and cryo–SEM most resemble the natural situation, this may support the idea that the glycocalyx plays an even bigger part in the mechanical transduction system.

The tip–links in the cochlea are believed to consist of two components: a central filamentous core, and a carbohydrate–rich coat, probably forming a part of the glycocalyx (9). In figure 3a and b, it is clearly demonstrated that the tip–link is covered with a dark staining layer (K4Ru(CN)6 ) that is similar to the glycocalyx on the stereocilia. The dense layer on the tip–link appears to be a continuation of the glycocalyx covering the stereocilia. In accordance with this knowledge, the tip–links in the low–voltage SEM (figure 4) appear to be coated the same as the stereocilia themselves. The surface topography of the tip–link appears here to be a continuation of the glycocalyx.

We can conclude that the glycocalyx is a surface coat, separately covering each entire stereocilium and kinocilium. Lateral interciliary connections are made up of glycocalyx, and tip–links are covered by a glycocalyx–layer. The glycocalyx, therefore, may play an important role in the mechanical transduction system of sensory hair cells.
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