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Endolymph movement visualized with light sheet fluorescence microscopy in an acute hydrops model

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ABSTRACT

There are a variety of techniques available to investigate endolymph dynamics, primarily seeking to understand the cause of endolymphatic hydrops. Here we have taken the novel approach of injecting, via a glass micropipette, fluorescein isothiocyanate–dextran (FITC-dex) and artificial endolymph into scala media of anaesthetized guinea pigs, with subsequent imaging of the inner ear using Light Sheet Fluorescence Microscopy (LSFM) as a means to obtain highly resolved 3D visualization of fluid movements. Our results demonstrate endolymph movement into the utricle, semicircular canals and endolymphatic duct and sac when more than 2.5 μl of fluid had been injected into scala media, with no apparent movement of fluid into the perilymphatic compartments. There was no movement of endolymph into these compartments when less than 2.5 μl was injected. The remarkable uptake of the FITC-dex into the endolymphatic duct, including an absorption into the periductal channels surrounding the endolymphatic duct, highlights the functional role this structure plays in endolymph volume regulation.

1. Introduction

The uncertainty of how endolymph volume is regulated in the inner ear has been a major hurdle in our understanding of Meniere's syndrome, where endolymphatic hydrops is a key feature (Naganawa and Nakashima, 2014; Pyykko et al., 2013). How disturbances in endolymph volume are 'sensed', and how this then imparts a change in fluid production is not yet clear. That said, the two main theories of normal endolymph fluid regulation include a) the influx of water and salts into the membranous labyrinth, with absorption in the endolymphatic sac (Dohlman, 1973; Lundquist, 1976; Schuknecht and Ruther, 1991), and b) local regulation of salt and water flux within the individual compartments of the inner ear (Lawrence, 1980; Lawrence et al., 1961; Salt and DeMott, 1997). A number of studies have examined endolymph dynamics, utilizing a variety of techniques (Salt, 2001). Many of these studies have injected some form of a marker into the membranous labyrinth to track fluid movements within the inner ear (Guild, 1927; Lundquist et al., 1964; Salt and DeMott, 1997), using either real-time monitoring of marker concentrations (typically via selective glass-micropipettes), or examining the marker distribution in post-mortem histological sections. However, as pointed out by Salt (2001), the process of injecting a fluid marker itself induces fluid flow. Studies where fluid markers have been iontophoresed without volume disturbances demonstrate the almost complete lack of endolymph 'flow' (Salt and DeMott, 1995). That's not to say the results of studies which injected fluid markers in volume should be dismissed, but rather they should be interpreted in regards to endolymph dynamics under non-physiological or 'disturbed' conditions, such as that which occurs with endolymphatic hydrops.

Other methods of investigating endolymph volume regulation involve experimentally altering the structures believed to be involved in endolymph regulation. The classical case of this is the obliteration of the endolymphatic sac, resulting in endolymphatic hydrops in guinea pigs (Kimura and Schuknecht, 1965). There have been many variants on this approach since then, all of which suggest that the sac plays a role in the absorption of endolymph. However, the finding that sac ablation in guinea pigs results in hydrops contrasts with results in human patients, where sac

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ablation (or variants of) can alleviate the symptoms of Meniere's in many patients (Lim et al., 2015). We still lack a clear explanation for this dichotomy.

More recently, research into endolymph regulation has focused on aquaporin expression in the inner ear (Eckhard et al., 2015; Kitahara et al., 2008), where experimental manipulation of aquaporin expression in guinea pigs may lead to mild endolymphatic hydrops (Chihara et al., 2013; Takeda et al., 2000). Other lines of research have focused on fluid transport between the various inner ear compartments, particularly across the blood-labyrinth barrier during immune activity (Floc'h et al., 2014; Hirose et al., 2014), where elevated immune activity can also result in temporary endolymphatic hydrops in guinea pigs (Takumida et al., 1998, 2008). Again, many of these studies have utilized fluid markers, using either MRI (Floc'h et al., 2014), *in vivo* fluid sampling (Hirose et al., 2014), examination of histological sections (Zhang et al., 2015), or *in vivo* examination (Zhang et al., 2015) to determine fluid dynamics in the inner ear.

The techniques for studying fluid dynamics utilized in the above studies have various advantages and disadvantages, although none provide a highly-resolved assessment of endolymph movement throughout the entire inner ear. Recently, the development of light sheet fluorescence microscopy (LSFM) has enabled researchers to examine the entire inner ear of experimental animals, *en bloc*, with sub-cellular resolution (Santi, 2011). At a simplistic level, LSFM involves illuminating a transparent sample with a thin 'sheet' of laser light, such that only a slice of the sample is illuminated, which is then imaged with a standard microscope objective and camera placed orthogonal to the light sheet. Moving the sample through the light sheet incrementally, with consecutive image acquisition, produces a Z-stack of images. The 3D resolution and field of view is determined by the optical setup and the movement range, with many LSFM setups able to image 5 mm^3 tissues, with isotropic resolution close to 5 μm (full half width maximum). A caveat of LSFM is that samples must be sufficiently transparent to enable the illumination and emitted light to pass through the sample with relatively little scatter. This is typically achieved by chemically processing fixed tissues, similar to that performed in preparation for standard light microscopy sections, with the aim of homogenizing the refractive index of the sample and the surrounding medium. A full description of LSFM and clearing techniques can be found elsewhere (Keller and Ahrens, 2015; Treweek et al., 2015).

Most researchers have used LSFM with samples that either express endogenous fluorophores, or have been stained with protein-specific or non-specific fluorophores. To the best of our knowledge, no study to date has used fluorescent agents to visualize extracellular diffusion in samples imaged with LSFM. That is, whilst various forms of fluid biomarkers (e.g. Prussian Blue, fluorescein, iodine, gadolinium, fluorodeoxyglucose etc …) have been used to visualize fluid movement or diffusion with other forms of destructive (e.g. histological sections) or non-destructive (e.g. μCT or MRI) imaging, such an approach has yet to be applied to LSFM imaging. Here, we have injected small volumes of artificial endolymph containing fluorescent agents, specifically rhodamine isothiocyanate or FITC-dex, into scala media of anaesthetized guinea pigs, with post-mortem LSFM.

Of particular interest in the present study is the movement of endolymph associated with an abrupt relief of endolymphatic pressure, observed in several studies when acutely injecting artificial endolymph into scala media (Brown et al., 2013a, 2013b; Kingma and Wit, 2009; Valk et al., 2006). It has been suggested that this event may be related to either a rupture of the membranous labyrinth, or a movement of endolymph through the morphological valves separating the utricle and semicircular canals (SSCs) from the saccule and cochlea, with conflicting evidence supporting each theory. The importance of this event is that it may indicate the mechanism underlying the acute attacks of vertigo experienced by Meniere's sufferers, providing us with a clearer understanding of this syndrome.

2. Methods

2.1. Experimental surgery & setup

Experiments were performed on 13 normal adult guinea pigs (*Cavia porcellus*) of either sex with body weights between 200 and 250 g. All animal preparation, surgery, and experimental protocol were approved by The University of Sydney's Animal Ethics Committee. The experimental setup and recording procedures used in this study are similar to those detailed in Brown et al. (2013a,b), with the primary differences being the use of FITC-dex in the injected artificial endolymph, and the use of LSFM as a post-mortem imaging technique.

Each animal was given a 0.05 ml subcutaneous injection of Temgesic (Buprenorpherine; Reckitt Benckiser, Auckland NZ) and 0.1 ml of Atrosine (0.6 mg/ml atropine sulphate; Apex Laboratories, NSW, Australia) before being anaesthetized in a chamber with 4% isoflurane. Once sedate and the foot-withdrawal reflex was absent, animals were moved to the surgical table, tracheotomized, and thereafter artificially ventilated with a mixture of oxygen and isoflurane (1-2%). The animal's temperature was maintained with the use of an electric heating pad and an infrared heating lamp. To suppress middle ear reflexes and overall muscle activity, animals were given a 0.1 ml intramuscular injection of the neuromuscular blocker Pavulon (2 mg/ml pancuronium bromide; Astra Pharmaceuticals, L.P.). To expose the cochlea, two separate methods were used. The first, a round-window approach, involved a small opening about 3 mm in diameter made in the acoustic bulla via a dorso-lateral approach, providing a direct view of the round window and the basal cochlear turn. In experiments requiring access to scala media via the lateral wall, the guinea pig was placed in a supine position and a ventral approach to the bulla was surgically made, exposing a larger view of the cochlea with access to all turns. In all experiments animals were placed in a custom-designed ear bar, which housed a low-noise microphone (ER10B, Etymotic Inc., IL, USA), and 2 separate speakers (attached via silastic tubes), forming a closed acoustic sound-field in the ear canal.

For gross-nerve response measurements, a Teflon-coated Ag/Cl electrode was placed 4 mm into the facial nerve canal. We find this location provides reasonable electrical pick-up from the VIIIth nerve, without impeding access to the round window. An indifferent Ag/Cl electrode was placed into the musculature of the neck, and a ground electrode was placed subcutaneously in the nape of the neck. Electrophysiological recordings were amplified using a multichannel bio-amplifier (ISODAM8A, WPI Inc. Sarasota, FL USA), and digitized using a National Instruments USB data acquisition device (NI 9205, National Instruments, TX, USA). Acoustic stimuli were generated using a PCIe soundcard (Xonar Essence STX II 7.1, ASUSTek Inc. China).

2.2. Endolymph injections & functional measurements

To inject endolymph with FITC-dex, a 5-10 μm tip-diameter glass micropipette with artificial endolymph (126 mM KCl, 1 mM NaCL, 25 mM $KHCO_3$, 0.025 mM $MgCl_2$, 0.025 mM $CaCl_2$, 1.4 mM K_2HPO_4 and 25 mM mannitol, with a pH of 7.4 and an osmolarity of 310–320 mOsmol, as previously described by Marcus et al., 1983) was loaded with an additional 1 mg/ml of 2000 kD FITC-dextran (Sigma Chemical Co., St. Louis, MI, USA). In two preliminary experiments, instead of using FITC-dex, the artificial endolymph