Vesicle traffic in the outer hair cell

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Abstract
The plasma-membrane marker FM1-43 was employed to reveal the relative significance of different types of endocytic and transcytic mechanisms in outer hair cells (OHCs) of the guinea-pig cochlea. A double-barrel local perfusion system was used to label independently the apical or synaptic pole of the isolated OHC to study mechanisms of vesicle uptake at the poles and of vesicle trafficking along and across the cell. Treatment with an inhibitor of macropinocytosis and phagocytosis, phenylarsine oxide, or of clathrin-mediated endocytic activity, concanavalin A, significantly reduced the dye uptake at both the apical and the synaptic poles, indicating the presence of both clathrin-independent and clathrin-mediated processes at both poles. However, measurement of uptake speed in the presence of the inhibitors suggested that clathrin-independent processes contribute more extensively to endocytosis at the basal pole than the apical pole. Treatment with an inhibitor of myosin VI, 2,4,6-triiodophenol, significantly delayed both the apical and the basoapical fluorescence signals. However, treatment with an inhibitor of kinesin, monastrol, or of dynein, ciliobrevin D, significantly delayed the signals only in the basoapical direction. The myosin VI inhibitor, but neither the kinesin nor dynein inhibitors, significantly delayed the signals to the subsurface cisternae. That is, myosin VI carries vesicles in both longitudinal directions as well as radially to the subsurface cisternae, whereas kinesin and dynein participate primarily in basoapical trafficking. This fundamental information is essential for elucidating recycling mechanisms of specific proteins involved in establishing, controlling and maintaining the electromechanical action of OHCs and, therefore, is vital for understanding auditory perception.

KEYWORDS
clathrin, dynein, guinea pig, kinesin, myosin VI

1 INTRODUCTION

Eukaryotic cells continuously recycle membrane proteins via endocytosis, exocytosis and vesicle-transport mechanisms between the plasma membrane and the intracellular organelles to maintain sensitivity to the environment (Apodaca, 2001). Coated and non-coated vesicles bud from the plasma membrane and are transcytosed along microtubules and actin

Abbreviations: Å, ångström; A, apical; Ch, channel; CilioD, ciliobrevin D; ConA, concanavalin A; DMSO, dimethyl sulphoxide; FM1-43, N-(3-triethylammonium)propyl-4-[(4-dibutylamino)styryl]pyridinium dibromide; HB, hair bundle; HBBS, Hanks’ balanced salt solution; IC, infracuticular; IHC, inner hair cell; IM, inframiddle; IN, infranuclear; M, middle; Mon, monastrol; OHC, outer hair cell; PAO, phenylarsine oxide; PM, plasma membrane; ROI, region of interest; SN, supranuclear; SSC, subsurface cisternae; TIP, 2,4,6-triiodophenol.

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filaments towards intracellular compartments. Outer hair cells (OHCs) are polarized epithelial cells in the sensory epithelium of the cochlea and are responsible for the extraordinary sensitivity, the high frequency discrimination and the wide dynamic range of hearing (Ashmore, 2008; Dallos, 2008). OHCs possess apical, lateral and basal domains with associated discrete physiological functions (Corey et al., 2017). At the apical pole, mechanosensitive channels in the stereocilia transduce displacement into receptor current (Hudspeth & Corey, 1977). At the lateral wall, prestin motor proteins (Zheng et al., 2000) in the plasma membrane (Huang & Santos-Sacchi, 1994) together with the cytoskeleton (Ludwig et al., 2001) transduce the receptor potential into somatic mechanical force (Frank et al., 1999). At the basal pole, efferent synapses modulate the output of the electromechanical transducer (He et al., 2003) and cochlear vibrations (Cooper & Guinan, 2006). There are also afferent synapses at the basal pole; they provide input to a central reflex pathway with different feedback to the OHC (Froud et al., 2015).

Rapid endocytic activity has been demonstrated at the apical pole in situ (Griesinger et al., 2004) and in isolated OHCs (Kaneko et al., 2006; Meyer et al., 2001), implying that the majority of endocytosed vesicles might be synthesized by clathrin-independent mechanisms. Although the presence of clathrin-coated pits has been demonstrated electromicroscopically at OHC synapses (Leake & Snyder, 1987; Nadol, 1983; Siegel & Brownell, 1986), the significance of that type of vesicle synthesis is still not known for OHCs. Apart from their presence in the stereocilia and cuticular plate, actin filaments have been demonstrated along the lateral wall, between the plasma membrane and the subsurface cisternae (Flock et al., 1986; Triffo et al., 2019), as well as uniformly distributed throughout the cytoplasm (Slepecky, 1989). A vast network of microtubules has also been demonstrated in the OHC soma (Furness et al., 1990). The directions of vesicle traffic associated with those actin-filament and microtubule pathways have not been described.

The amphiphilic probe \(N-(3\text{-triethylammoniumpropyl})-4\text{-4-(dibutylamino)styryl}\text{pyridinium dibromide (FM1-43)}\) enhances its fluorescence intensity upon binding to lipid bilayer and, therefore, is widely used to visualize synthesized vesicles released from the plasma membrane (Betz & Bewick, 1992). Application of FM1-43 has revealed rapid endocytic activity of hair cells of the mature guinea-pig cochlea (Griesinger et al., 2002, 2004; Kaneko et al., 2006; Meyer et al., 2001) and of the zebrafish lateral line (Seiler et al., 1986; Triffo et al., 2019), as well as uniformly distributed throughout the cytoplasm (Slepecky, 1989). A vast network of microtubules has also been demonstrated in the OHC soma (Furness et al., 1990). The directions of vesicle traffic associated with those actin-filament and microtubule pathways have not been described.

The principal purpose of the current study was to visualize and quantify microtubule and actin-filament-associated trafficking mechanisms in OHCs. After pre-incubation in various inhibitors of transcytosis, FM1-43 was applied to the apical or basal pole of the OHC, and transcytic activity was visualized with laser-scanning confocal microscopy. To establish a basis for investigating the dynamics of transcytosis, endocytic mechanisms were also studied at both poles, again using appropriate inhibitors. We show that the dye is actively taken up by endocytosis and then transcytosed, with different mechanisms being dominant in the basal or apical parts of the cell.

2 MATERIALS AND METHODS

2.1 Ethical standards

Experiments were performed in accordance with the Council Directive 2010/63EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes. Animals were bred and maintained in the Animal Facility of the Faculty of Medicine, University of Tübingen. The animal care and the study were reviewed by the committee of Animal Protection, Veterinary Service and Veterinary Medicine Department of the University of Tübingen and of the Regional Council Tübingen (reference number: 11.03.2014, 16.03.2015, 04.10.2017).

2.2 Isolation of OHCs

OHCs were isolated from the cochleae (\(N = 71\)) of adult pigmented guinea pigs (\(N = 64\); weighing 300–900 g), as adapted from Preyer et al., (1994) and described by Harasztosi et al., (2018). Animals were anesthetized with an intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (4 mg/kg). After induction, requiring approximately 10–15 min, terminal cardiac puncture was performed using the same drug mixture. Upon cessation of heart beating, animals were killed by rapid cervical dislocation. Temporal bones were dissected from the skull and stored in cooled Hanks’ balanced salt solution (HBSS; Biochrom KG, Berlin, Germany), containing (in mM) NaCl, 137; KCl, 5.4; CaCl\(_2\), 1.25; NaHCO\(_3\), 4.2; MgSO\(_4\).7H\(_2\)O, 0.81; KH\(_2\)PO\(_4\), 0.35; Na\(_2\)HPO\(_4\), 0.34; D-glucose, 5; HEPES, 5. Osmolarity of 310 mOsm/L and pH of 7.25 were adjusted with D-glucose and NaOH, respectively. All chemicals were purchased from MERCK (Darmstadt, Germany) unless otherwise stated. The bony wall of the cochlea was partially removed, and strips from the upper third of the organ of Corti were isolated in HBSS and treated for 10 min in collagenase (Type IV, 1 mg/
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OHCs were then mechanically isolated by gentle aspiration with a 100-μL Eppendorf pipette in 200-μL HBSS. Coverslips, forming the bottom of the experimental chambers, were coated with a cell-and-tissue adhesive Cell-Tak™ (Corning Inc., Corning, NY, USA) to facilitate cell adhesion. Ten minutes after the aspiration process, the chamber was filled with HBSS (2 mL). Isolated cells were used within 2 hr post-mortem. Experiments were performed at a controlled room temperature of 21.5 ± 0.5°C.

2.3 | Fluorescence labelling

The fluorescent plasma-membrane marker FM1-43 was used to study endocytosis and transcytosis. The dye was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Stock solution in the concentration of 10 mM was prepared in dimethyl sulphoxide (DMSO) and stored at −20°C. Directly before the experiments, the stock solution was diluted in HBSS to the final concentration of 10 μM.

A double-barrel perfusion system was used to apply the dye to either the apical or the basal pole of the OHC, as previously described by Harasztosi et al. (2018). The tip of the perfusion system was fabricated using a double-barrel, borosilicate-glass theta capillary of 2-mm external diameter (Harvard Apparatus, Holliston, MA, USA) pulled with a DMZ-Universal Puller (Zeitz Instruments, Augsburg, Germany). An Eppendorf GELoader was inserted and fastened with silicone glue (RAU-SIK Adhesive SI 1511, Raumedic, Helmbrechts, Germany) into each barrel for connection to the peristaltic pump (Ismatec, IDEX Health & Science GmbH, Wertheim, Germany). The flow rate was 3 μL/min for each barrel. The perfusor was positioned near the cell using a Luigs & Neumann micromanipulator (SM5, Luigs & Neumann GmbH, Ratingen, Germany).

2.4 | Endocytosis and transcytosis inhibitors

Endocytic activity was blocked with phenylarsine oxide (PAO, 40 μM), a non-specific pinocytosis and phagocytosis inhibitor (Dutta & Donaldson, 2012), or concanavalin A (ConA, 50 μM), a clathrin-mediated endocytic activity inhibitor (Luttrell et al., 1997). Transcytosis was blocked with monastrol (Mon, 50 μM), a kinesin inhibitor (Griesinger et al., 2002); ciliobrevin D (CilioD, 50 μM), a dynein inhibitor (Firestone et al., 2012); or 2,4,6-triiodophenol (TIP, 25 μM), a myosin-VI inhibitor (Bond et al., 2013). Stock solutions of PAO, Mon, TIP and CilioD were prepared in DMSO in the concentrations of 80, 17, 25 and 25 mM, respectively. A stock solution of ConA was prepared in distilled water in a concentration of 10 mM. These stock solutions were stored at −20°C and on the day of the experiment diluted in HBSS to the desired final concentration. Drug treatment started 30 min before the start of the fluorescence recordings by adding the drug solution to the HBSS-filled experimental chamber. Drugs were continuously present in the chamber during the experiments.

2.5 | Laser-scanning confocal microscopy

Fluorescence signals were imaged with a Zeiss LSM 510 confocal system (Zeiss, Heidelberg, Germany) built on a Zeiss Axioskop2 FS mt microscope (Harasztosi & Gummer, 2016). The objective was a Zeiss 40×, IR-Achroplan, water-immersion objective with NA 0.8. The pin-hole diameter was set to 1 AU. ZEN 2009 imaging software was used for data acquisition and offline analysis. Images were recorded in 1,024 × 1,024 pixel² resolution; the voxel size was 0.3 × 0.3 × 2.5 μm³. FM1-43 was excited with an argon laser of wavelength λ_ex = 488 nm. The FM1-43 signal was recorded above 505 nm using primary (HFT488) and secondary (NFT450) dichroic beam splitters followed by a long-pass filter (LP505).

2.6 | Data analysis

Regions of interest (ROIs) were assigned offline to distinct plasma-membrane, intracellular and extracellular locations using the ZEN 2009 imaging software to quantify fluorescence intensity. Before analysis of these regions, the background fluorescence signal, averaged over 60 s, was determined extracellularly at a distance of ~40 μm from the cell wall and subtracted from the fluorescence intensity of all signals. The ROI diameter was 6 μm.

For a given ROI, the threshold of the fluorescence-signal intensity was defined as 20% of the saturation level of the plasma-membrane ROI. The delay of this threshold relative to the threshold for the plasma-membrane ROI was used as an estimate of time for transcytic transport from the plasma membrane near the application region to the intracellular ROI. The distance from the plasma-membrane ROI to the intracellular ROI was plotted as a function of this delay and fitted with a linear regression line to estimate the average travel speed. Data analysis was performed with Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) and Origin 7.0 (OriginLab Corporation, Northampton, MA, USA).

2.7 | Statistical analysis

Data are presented as sample mean and standard deviation. Null-hypothesis tests were performed at the 95% level of confidence (p < 0.05).
Statistical difference between two sample means was evaluated with the t test. The test value is given as $t^*$, where the presence of the superscript * indicates that Welch’s t test for the case of unequal variances, as established by the F test, was used; the subscript ν denotes the number of degrees of freedom, which was calculated using the Welch–Satterthwaite equation.

The existence of statistical difference between multiple sample means was determined with one-way ANOVA tests and, when present, differences compared with either Dunnett’s (each-to-control) or Tamhane’s (all-to-all) multiple-comparison tests. In the case of unequal variances, as established by the F test, the Welch ANOVA test was used. Statistical significance was established at the 95% level of confidence ( $p_D < 0.05$ for Dunnett’s test, $p_T < 0.05$ for Tamhane’s test).

GraphPad Prism (San Diego, CA, USA) software was used for statistical tests. Statistical test parameters are presented in the figure legends.

3 | RESULTS

The plasma-membrane marker FM1-43 was applied to isolated OHCs to label endocytosed vesicles at either the basal or the apical pole. The dye was applied locally to cells using a double-barrel perfusion system that was developed and established to label only a single pole of isolated bipolar cells (Harasztosi et al., 2018). Local dye application enables investigation of dye uptake at a single pole and, therefore, differential evaluation of apicobasal and basoapical vesicle traffic. Data are presented for 96 OHCs of average length 70 ± 8 µm (49–86 µm), whereby for completeness and not wishing to discard earlier and valuable animal data 29 of the cells derive from two recent publications which, respectively, established the perfusion method (Harasztosi et al., 2018) and examined vesicle targets with double-labelling experiments (Harasztosi & Gummer, 2019). The use of such cells is explicitly stated in the figure legends.

3.1 | Hair-cell labelling and quantification of its onset dynamics

Figure 1 shows examples of endocytic and transcytotic activity for FM1-43 applied at the apical pole (Figure 1a–c) or the synaptic pole (Figure 1d–f). The tip of the double-barrel glass capillary was placed close to the isolated OHC (Figure 1a,d). Channel A (ChA) was filled with dye-free HBSS, while Channel B (ChB) contained 10-µM FM1-43. Fluids flowed simultaneously from the two channels, with HBBS applied to one half of the lateral wall to ensure that the dye remains focused at the other half.

After the start of perfusion, labelling ‘rapidly’ became obvious at the plasma membrane of the respective poles of dye application (Figure 1b,e, leftmost images), indicating the successful application of dye locally in the extracellular environment. Later, progressive intracellular labelling was observed towards the opposite poles (Figure 1b,e), implying apicobasal and basoapical vesicle traffic, respectively. This spatial time course to the opposite poles is essentially identical to that in our earlier report (Harasztosi & Gummer, 2019).

Fluorescence intensity was measured in ROIs positioned at the plasma membrane (PM) opposing the lumen of ChB as well as within the infracuticular (IC), apical (A), middle (M), supranuclear (SN) and infranuclear (IN) regions (Figure 1a,d). The time courses of the intensities in these ROIs are displayed in Figure 1c,f. The signal in the plasma-membrane ROI is presumed to be an indicator of dye that is applied to that extracellular region. Therefore, the time at which the plasma-membrane signal crossed a threshold intensity level (Figure 1c,f, dashed line), defined as 20% of the plasma-membrane saturation intensity level (Materials and Methods, Data analysis), served as a temporal reference to quantify the onset dynamics of the fluorescence intensity of the other ROIs.

The onset dynamics for the intracellular fluorescent signals are quantified in Figure 2 by plotting the distance of the ROIs from the plasma-membrane ROI as a function of the threshold times relative to the plasma-membrane threshold time for apical (Figure 2a) and basal (Figure 2b) dye application. Henceforth, the relative time at threshold will be simply called the ‘delay’, where the reference is the time at threshold for the plasma-membrane ROI. Data points from the same cell are given the same colour and at a similar distance are given the same symbol. For a given traffic direction, the linear regression line is constructed with slope given by the mean of the slopes for the individual cells studied for that traffic direction; fitted and constructed regression lines were forced to pass through the origin. For apical and basal applications, respectively, the slopes are 0.117 ± 0.019 µm/s (Figure 2a; $N = 7$) and 0.232 ± 0.048 µm/s (Figure 2b; $N = 6$). These values are similar to those for other cells from our recent study (Harasztosi & Gummer, 2019), with apicobasal and basoapical slopes of 0.11 ± 0.01 ($N = 8$) and 0.18 ± 0.01 µm/s ($N = 5$), respectively. The slope provides a measure of the average vesicle speed in a given direction. The basoapical speed is significantly larger than the apicobasal speed, by a factor of two.

The larger vesicle speed in the basoapical direction, originally demonstrated by Harasztosi and Gummer (2019), implies that different trafficking mechanisms might be involved in the different directions and that the amount of dye taken up at the base might be greater than that at the apex. These two possibilities are investigated here with the aid of known inhibitors of endocytosis and transcytosis. Specifically, we consider clathrin-mediated and clathrin-independent endocytosis, as well as actin- and/or microtubule-dependent transcytosis.
3.2 | Inhibition of endocytosis

To investigate different forms of endocytosis at the apical and basal poles, OHCs were pre-incubated either with PAO (40 µM), a non-specific blocker of pinocytosis and phagocytosis (Dutta & Donaldson, 2012), or with ConA (50 µM), an inhibitor of clathrin-mediated endocytosis (Luttrell et al., 1997). FM1-43 was applied to one of the poles, and the delay for that infracuticular ROI (apical application) or the infranuclear ROI (basal application) was measured for control (untreated) and treated cells. The delay data are presented in Figure 3. For control cells, delay in the infracuticular region (59 ± 16 s, \(N = 7\)) was significantly larger than in the infranuclear region (15 ± 3 s, \(N = 6\)). These values are similar to those for other cells from our recent study (Harasztosi & Gummer, 2019), with delays in the infracuticular and infranuclear regions of 45.1 ± 22.8 s (\(N = 8\)) and 13.6 ± 6.4 s (\(N = 5\)), respectively, although the variance was greater in that earlier study.

In the infracuticular region (Figure 3a), treatment with either ConA or PAO increased the delay to 125 ± 55 and 156 ± 79 s, respectively. In both cases, the increase was statistically significant. There was no statistical difference between the delay increases for the two treatments. In the infranuclear region (Figure 3b), the ConA and the PAO treatments also significantly increased the time delays to 97 ± 51 and 301 ± 109 s, respectively. However, in this region, the delay increase is much larger for the PAO treatment (286 ± 109 s) than for the ConA treatment (83 ± 51 s). These results imply that clathrin-mediated and clathrin-independent endocytic processes are operative at both poles. Moreover, at the synaptic pole, the much larger delay accompanying PAO treatment implies that pinocytosis and phagocytosis contribute more extensively to the fast uptake of FM1-43 than clathrin-mediated endocytosis.

**FIGURE 1** Cell labelling. (a,d) Differential-interference-contrast images of two OHCs showing the experimental configuration for dye application with the double-barrel perfusor to the apical (a) and basal (d) regions. The opening of the double-barrel glass capillary is positioned close to the cell with the purpose of establishing two parallel streams of laminar fluid flow; see Harasztosi et al., (2018) for details. In these two experiments, ChA was filled with dye-free HBSS and ChB with HBSS containing 10-µM FM1-43. ChA and ChB were running simultaneously. The regulated flow of HBSS through ChA was designed to prevent extracellular access of dye to the plasma-membrane region opposite the lumen of ChA, while the dye was applied through ChB for endocytic uptake from the plasma membrane at either the apical (a) or the basal (d) pole. Regions of interest (ROIs) are PM, plasma membrane; IC, infracuticular; A, apical centre; M, middle; SN, supranuclear; IN, infranuclear; HB, hair bundle. (b,e) Fluorescent images showing the temporal sequence of FM1-43 labelling, with the time of recording indicated in seconds (bottom right-hand corner). (c,f) Fluorescence intensity (F.I.) indicates signal changes in ROIs depicted in panels a and d, respectively. Dashed line shows the threshold intensity (20% of PM saturation intensity) used to determine signal-onset delays for calculating the speed of intracellular vesicle transport. The delay increases progressively from apex to base for apical application (c) and from base to apex for basal application (f), indicating intracellular vesicle transport towards the opposite pole. Notice that for basal application, there is no fluorescence signal in the hair bundle during dye application. However, after terminating the application, a signal is observed in the hair bundle (arrows in e and f), which is due to diffusion of dye throughout the extracellular fluid after terminating fluid flow from both channels. These spatial properties are essentially identical to those in our recent report (Harasztosi & Gummer, 2019). Cell lengths, 72 and 79 µm, respectively, for apical and basal dye application. Scale bar: 20 µm
3.3 | Inhibition of vesicle trafficking

To investigate the motor proteins involved in apicobasal and basoapical traffic, OHCs were pre-incubated in monastrol (Mon, 50 µM), an inhibitor of the microtubular motor protein kinesin (Griesinger et al., 2004); or ciliobrevin D (CilioD, 50 µM), an inhibitor of the microtubular motor protein dynein (Firestone et al., 2012); or...
2,4,6-triiodophenol (TIP, 25 µM), an inhibitor of the microfilament transport protein myosin VI (Bond et al., 2013). FM1-43 was applied to one of the poles and the delays for infracuticular (IC), apical (A), middle (M), supranuclear (SN) and infranuclear (IN) ROIs were measured for control (untreated) and treated cells. Specifically, referring to Figure 5a,b, a normalized delay was defined as the ratio of the traffic delay, $t_a + t_r$, from the endocytically labelled pole along the central strand to the investigated part of the SSC, divided by the delay, $t_a$, to the opposing central region from the same pole (Figure 5a,b). The normalized delay data are presented in Figure 5c,d.

The salient feature is that an increase of normalized delay was only detected for the myosin-VI inhibitor, TIP. The TIP-induced delay increase was observed in both directions, namely, from supranuclear (SN) regions for traffic from the apex to the SSC (Figure 5a,c) and from infracuticular (IC) regions for traffic from the base to the SSC (Figure 5b,d).

These results show that traffic from the cell centre towards the subsurface cisternae is highly dependent on the microfilament transport protein, myosin VI. Contributions to the traffic to the subsurface cisternae by the microtubular motor proteins kinesin and dynein were not detected.

### 4 | DISCUSSION

Fluorescence signals were recorded from control (untreated) cells and from cells pre-incubated with inhibitors of endocytosis.
or transcytosis. A dependence of endocytic and transcytic activity on cell length was not found, presumably because the cells were from the same apical turn of the cochlea, so the variation of cell length across cells was small. Control cells presented a greater abundance of endocytic activity at the synaptic pole, and basoapical traffic was faster than apicobasal traffic. Vesicle traffic depended on myosin VI in both the apicobasal and the basoapical directions, whereas a dependence on kinesin or dynein was detected only in the basoapical direction. Moreover, vesicle traffic from the cell centre towards the SSC was mainly myosin-VI dependent. This spatial and directional information is summarized in the Graphical Abstract.

4.1 Visualization of vesicle traffic

The plasma-membrane marker FM1-43 was developed for optical monitoring of vesicle recycling of living nerve terminals (Betz & Bewick, 1992). Combined with laser-scanning confocal microscopy, FM1-43 has revealed intracellular clusters of labelled vesicles in zebrafish sensory hair cells (Seiler & Nicolson, 1999) and in OHCs from the guinea-pig cochlea (Meyer et al., 2001). Optical sectioning and 3D reconstruction using two-photon confocal microscopy has demonstrated that vesicles labelled in situ at the apex of inner hair cells (IHCs) are transported in a kinesin-dependent manner to the synapse (Griesinger et al., 2002).

The aim of the present study was not to track single vesicles but to determine average vesicle speeds in the presence of endocytic and transcytic inhibitors in order to dissect the associated uptake and transport mechanisms in the OHC. The calculation of average traffic speed was based on averaged fluorescence intensity changes in distinct intracellular regions along the longitudinal and transverse axes of the OHCs.

To compare the dynamics of fluorescence signals throughout a cell, a fluorescence threshold was defined as 20% of the

FIGURE 5 Traffic to SSC. Normalized delay to the subsurface cisternae (SSC) for untreated cells (control) or cells treated with ciliobrevin D (CilioD, green), monastrol (Mon, red) or 2,4,6-triiodophenol (TIP, blue). FM1-43 was applied to the apical (a) or basal (b) poles (denoted by the green cell wall). Schematics of OHCs in (a) and (b) show the investigated putative routes for vesicle traffic to the SSC from the apex (a) and from the base (b). Normalized delay is defined as the traffic delay, $t_a + t_r$, from the endocytically labelled pole to the subsurface cisternae (SSC), via the central strand, divided by the traffic delay, $t_c$, from the labelled pole to the opposing central region. Boxed inset shows confocal-microscopic separation of the central-strand and SSC signals in the pole region that was superfused with dye-free extracellular fluid. C: Normalized delay from apex to SSC. D: Normalized delay from base to SSC. Regions of interest (ROIs) are SN, supranuclear; SSC-SN, SSC opposite the SN ROI; IC, infracuticular; SSC-IC, SSC opposite the IC ROI. Open circles in C and D are individual cell data, while numbers beneath the columns indicate the number of cells. Five of the 39 treated cells are from Harasztosi et al., (2018). Notice that in the case of apicobasal transport to the SN, compared with control (1.24 ± 0.20, N = 7), TIP treatment (1.55 ± 0.18, N = 6, $p_D = 0.009, t_{22} = 3.31$) significantly increased the normalized delay; ANOVA $F(3,22) = 4.012, p = 0.020$. In the case of basoapical transport to the IC, compared with control (1.21 ± 0.13, N = 8), TIP treatment (1.53 ± 0.19, N = 7, $p_D = 0.002, t_{24} = 3.85$) also significantly increased the normalized delay; ANOVA $F(3, 24) = 8.03, p = 0.0007$. However, CilioD (N = 6) and Mon (N = 7) each had no detectable effect for either transport direction. (For apicobasal, CilioD: $p_D = 0.816, t_{22} = 0.724$; Mon: $p_D = 0.216, t_{22} = 1.77$. For basoapical, CilioD: $p_D = 0.770, t_{24} = 0.805$; Mon: $p_D = 0.140, t_{24} = 2.01$). Cell lengths, 50–80 µm (70 ± 7 µm, N = 26) for apicobasal and 50–86 µm (71 ± 8 µm, N = 28) for basoapical transport...
saturated plasma-membrane signal. That threshold value was chosen to quantify signal delay at a place within the cell relative to the plasma-membrane delay near the dye-application region. That level is well above the noise level but still low enough to detect signals at increasingly distal locations from the application site. Choosing higher thresholds would obviously cause larger delay estimates and, therefore, slower speed estimates.

The estimated signal delays might only be partially related to synchronized vesicle traffic from one pole to the other. For example, it cannot be excluded that fluorescence signals are altered by intermediate endoplasmic reticulum compartments, which by accumulating dye molecules, could influence and presumably delay the fluorescence signal. In addition, vesicles budding from already labelled endosomes might also contribute to the observed traffic and apparently advance the signal. Therefore, caution is used when interpreting the absolute delay (speed) estimates—the estimates simply serve as indicators of the relative speeds of the different endocytic and transcytic mechanisms.

4.2 | Separation of fluorescence signals from plasma-membrane and intracellular compartments

The laser-scanning confocal-microscopic technique used in the current study, offering optical lateral and axial resolutions of ~0.3 and ~2.5 µm, respectively, is adequate for recording fluorescence signals originating from clusters of vesicles, but obviously cannot offer the spatial resolution required to visualize trafficking of individual vesicles. Moreover, for the conventional single-barrel technique, the confocal microscope cannot distinguish between plasma-membrane and SSC fluorescence signals. However, using the double-barrel perfusion technique, confocal microscopy is adequate for distinguishing between fluorescence signals from the plasma membrane, the central strand and the SSC. In the case of ROIs located in the centre of OHCs, fluorescence signals were collected from the central strand. At those ROI locations, the plasma-membrane signal was optically eliminated by the pinhole of the confocal system, which was set to reduce the sample depth to 2.5 µm. In the case of ROIs placed at the lateral plasma membrane to record SSC signals, the ROIs might theoretically have included signals from both the plasma membrane and the SSC. However, the controlled fluid flow applied by the local perfusion system continuously washes the extracellular surface and, therefore, almost certainly eliminates the extracellular plasma-membrane signal, leaving only the SSC signal. Nevertheless, directly opposite the capillary septum (dark shadow between the two channels in Figure 1a,d), the extracellular protection effect of the control flow is possibly incomplete, so in that region an SSC ROI might conceivably collect both extracellular plasma-membrane and SSC signals. However, the absence of an SSC signal in that region within the first ~100 s after dye application suggests that signal contamination from the extracellular plasma membrane was negligible.

4.3 | Clathrin-mediated endocytosis in OHCs

Different types of coated and uncoated vesicles have been observed at both the apical pole of bullfrog vestibular hair cells (Kachar et al., 1997) and synaptic regions of OHCs (Leake & Snyder, 1987; Nadol, 1983; Siegel & Brownell, 1986) using electron microscopy. Using live-cell confocal imaging, rapid endocytic activity has been demonstrated by labelling OHCs with FM1-43 in situ (Griesinger et al., 2004) and in isolated OHCs (Kaneko et al., 2006; Meyer et al., 2001). Although the involvement of clathrin-mediated processes was not investigated in those latter OHC experiments—due to the intense dye uptake—the rapid dye uptake implied that the majority of FM1-43-labelled vesicles were most probably synthesized by clathrin-independent processes (Kaneko et al., 2006). It has been reported that the dynamin inhibitor dynasore reduces the endocytic activity of IHCs, implying the presence of clathrin-mediated endocytic activity in these hair cells (Duncker et al., 2013). However, the effect of dynasore on endocytosis cannot be adequately investigated with fluorescence microscopy because dynasore can quench the FM1-43 emission (Alharazneh et al., 2011; Harasztosi et al., 2018).

Here, to unravel different types of endocytic activity at both the apical and synaptic poles of OHCs, the non-specific pinocytosis/phagocytosis inhibitor PAO (Dutta & Donaldson, 2012) and the clathrin-mediated endocytosis inhibitor ConA (Luttrell et al., 1997) were employed. Either inhibitor significantly delayed the onset of the fluorescence signal recorded in the infracuticular or infranuclear regions. For basal application of FM1-43, PAO delayed the signal onset by a factor of three compared with ConA. However, for apical application of FM1-43, the incurred delays were similar for the two inhibitors. These differences in blocking efficacy suggest that the major proportion of the vesicles at the synaptic pole is synthesized by clathrin-independent processes, whereas at the apical pole both types of synthesis appear to be equally prevalent.

4.4 | Microtubule-associated traffic

Microtubules and actin filaments guide intracellular vesicle traffic in eukaryotic cells (Apodaca, 2001). The minus end of microtubules points towards the apex of polarized epithelial cells (Bacallao et al., 1989; Meng et al., 2008). Differences
in the microtubular organization of IHCs and OHCs have been demonstrated in the cochlea using transmission electron microscopy and tubulin-associated immunofluorescence (Furness et al., 1990). In IHCs, they report that the microtubular network is mostly concentrated to the infracuticular region and that a sparsely tubular system exists adjacent to the SSC and extends between the apex and the base of the cell. In OHCs, besides a dense microtubule network in the infracuticular region, they demonstrate microtubular structures around the central strand, aligned adjacent to the SSC, around the nucleus and in the infranuclear region.

The motor protein dynein is known to follow a microtubule route to carry cargo towards the minus end of microtubules (Bhabha et al., 2016). Here, we demonstrated that the dynein inhibitor ciliobrevin D significantly increased the basoapical signal onset delays and, therefore, reduced the basoapical traffic. However, there was no observable effect of the inhibitor on apicobasal traffic. These observations are in accord with the hypothesis that in OHCs, the negative end of microtubules faces towards the apex of the cell.

The motor protein kinesin also transports vesicles along microtubules. The kinesin motor protein family includes proteins that move towards either the plus or the minus ends of microtubules (Malik & Vale, 1990). The direction of kinesin transport depends on the position of the motor domain on the kinesin molecule (Henningens & Schiwa, 1997). Kinesin, possessing an N-terminal motor domain, carries cargo towards the plus ends of microtubules, while kinesin with a C-terminal motor domain transports towards the minus ends (Endow & Barker, 2003). Griesinger et al., (2002) investigated intracellular labelling of IHCs in an explant of the organ of Corti for application of FM1-43 directed towards the apical surface. The kinesin inhibitor monastrol (Mon) significantly reduced the labelling in the basal regions of the IHCs, implying kinesin-dependent trafficking in the apicobasal direction. In contrast, for localized application of FM1-43 to isolated OHCs incubated in monastrol, the traffic speed in the basolateral direction was significantly reduced compared with untreated OHCs, but there was no detectable effect on speed in the apicobasal direction. This result implies robust kinesin-dependent transport only in the basoapical direction of the OHC and that the C-terminal of the kinesin molecules is involved in that transport.

### 4.5 Actin-filament associated traffic

Using antibody labelling at the electron microscopic level, differences have been shown in the actin-filament distribution in IHCs and OHCs (Flock et al., 1986). For OHCs, actin is present not only in the stereocilia and in the cuticular plate as in IHCs but also along the lateral wall, between the plasma membrane and the subsurface cisternae (Flock et al., 1986). In frozen-fixed OHCs, actin filaments were found to be distributed uniformly throughout the cytoplasm (Slepecky, 1989). Investigating the ultrastructure of the OHC lateral wall using electron tomography resolved tight actin–SSC links beneath the plasma membrane connected by pillars to the actin fibres (Triffo et al., 2019). Myosin VI is an unconventional protein that transports cargo towards the minus end of the actin microfilament (Wells et al., 1999), which is believed to point towards the centre of the cell (Cramer, 2000). In polarized epithelial cells, microfilaments are thought to be involved in the transport from the plasma membrane to early endosomes (Apodaca, 2001). Here, we demonstrate that in the presence of the myosin VI inhibitor TIP, the onset delays of the fluorescence-signal intensities in both the apico basal and the basoapical directions were increased. Therefore, these data indicate the involvement of myosin VI in both apicobasal and basoapical traffic. However, there was already a significant signal delay for TIP in the infracuticular ROI. Therefore, in the case of myosin inhibition, it cannot be excluded that reduced traffic was at least partially affected by the reduced number of synthesized vesicles. Since actin filaments are present throughout the OHC cytoplasm (Slepecky, 1989), it is concluded that the increased signal delays observed in the case of TIP treatment indicate the involvement of myosin VI-driven trafficking not only to the first endosome but also towards the opposite pole of the OHC. In addition, we also demonstrate that traffic from the cell centre towards the basolateral plasma membrane is also strongly dependent on myosin VI. This observation is in agreement with electron microscopic studies that demonstrated the presence of actin fibres between the plasma membrane and the subsurface cisternae of OHCs (Flock et al., 1986; Triffo et al., 2019).

### 4.6 Speed of vesicle traffic

The average traffic speeds in the apico basal and basoapical directions of 0.1 and 0.2 µm/s, respectively, concur with those obtained from our earlier double-barrel study of FM1-43-labelled OHCs (Harasztosi & Gummer, 2019). These values are smaller than the value of 0.4 µm/s reported for apicobasal speed by Kaneko et al., (2006). However, in that study, the FM1-43 was applied to the OHC with a conventional single-barrel micropipette (tip diameter ~2.5 µm), whereby the dye had extracellular access to the entire length of the cell. In such a circumstance, it is conceivable that dye from both poles could have contributed to the signal speed measured in a given direction. Supporting this assertion, the apicobasal speed presented here is practically identical to one which can be estimated from the delay times reported by Griesinger et al., (2004) from a
temporal-bone preparation of the guinea-pig cochlea, in which the apical surface of the organ of Corti, with cytoarchitecture preserved, was superfused with 5-µM FM1-43 (1 mM Ca$^{2+}$); these concentrations are similar to the ones used in the present study. Based on the reported (Griesinger et al., 2004) time difference for signal detection from the apical endosome to the synaptic cisternae (288 s) and using an OHC length of 30 µm, the average speed is estimated to be 0.1 µm/s.

Vesicle speed for axonal transport is typically ~1 µm/s (Nakata et al., 1998). A wide range of speeds has been reported for different forms of dynein, ranging from 0.05 (Mallik et al., 2004) to ~1 µm/s (Paschal et al., 1987). It has been reported that different types of vesicle-like structures leave the Golgi system at the average speed of ~0.7 µm/s (Toomre et al., 1999). Live-cell microscopy has demonstrated microtubule-dependent vesicle traffic with speeds in the range of 0.3–2 µm/s in Chinese hamster ovary cells (Mundy et al., 2002). The speeds of 0.1–0.2 µm/s estimated for the present experiments from isolated OHCs, as well as from in situ OHCs (Griesinger et al., 2004), correspond to the lower range reported in other systems. Because of the low optical resolution (250 nm) and the large size of ROIs (~6 µm), relative to vesicle size, the average speeds estimated in the present study are, in fact, for clusters of vesicles. Therefore, the speed estimates almost certainly underestimate the local speed of individual vesicles. Moreover, the relatively slow average speeds may imply that a large proportion of the vesicles are not directly transported towards the opposite pole but that intermediary intracellular organelles are involved. Improved imaging techniques will be required to address these important issues.

Ultrastructural differences at the OHC poles could also influence the dynamics of uptake and traffic. The dense meshwork of cross-linked actin filaments forming the cuticular plate appears to preclude endocytosis over most of the apical membrane (Furness & Hackney, 1990; Kachar et al., 1997; Lim, 1986). However, there is electron microscopic evidence for endocytosis in two narrow regions at the apical surface devoid of actin: (1) the membrane region between the cuticular plate and the actin circumferential ring of the membrane junctional complex (Kachar et al., 1997) and (2) the foniculium, the region occupied by the kinocilium during development (Kikkawa et al., 2008). In the basal region, endocytosis appears to be associated with the infranuclear region, where SSC are lacking and the synapses are located (Leake & Snyder, 1987; Siegel & Brownell, 1986). Therefore, the larger area available for endocytosis at the basal pole could, at least partially, be responsible for the faster speeds of basal endocytosis and basoapical transcytosis.

### 4.7 Concluding remarks

Endocytosis and transcytosis are fundamental cellular mechanisms for maintaining the functional integrity of a cell. Our data provide strong evidence that the OHC possesses clathrin-mediated and clathrin-independent endocytosis as well as microtubule-associated basoapical and microfilament-dependent bidirectional trafficking as highly organized cellular mechanisms for uptake, recycling and sorting of plasma-membrane proteins. More detailed insight into the dynamics of individual vesicles will require high-resolution live-cell imaging and molecular modelling to understand endocytic and transcytic processes, which might also be targets for cell therapy.

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### CONFLICT OF INTEREST

Authors declare no conflict of interests.

### AUTHOR CONTRIBUTIONS

C.H. and A.W.G. designed research; C.H. and E.K. performed experiments; C.H., E.K., and A.W.G. analysed data; C.H. made the figures; C.H. and A.W.G. wrote the manuscript. All authors read and approved the manuscript.

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### DATA AVAILABILITY STATEMENT

Primary data are available from the authors upon request.

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