Impact of the Usher syndrome on olfaction

Fabian Jansen¹, Benjamin Kalbe¹, Paul Scholz¹, Marta Mikosz², Kirsten A. Wunderlich², Stefan Kurtenbach¹, Kerstin Nagel-Wolfrum², Uwe Wolfrum², Hanns Hatt¹ and Sabrina Osterloh¹,*

¹Cell Physiology, Faculty for Biology and Biotechnology, Ruhr-University Bochum, 44801 Bochum, Germany and ²Cell and Matrix Biology, Institute of Zoology, Johannes Gutenberg-University, 55099 Mainz, Germany

*To whom correspondence should be addressed at: Cell Physiology, Faculty for Biology and Biotechnology, Ruhr-University Bochum, 44801 Bochum, Germany. Tel: +49 2343223529; Email: sabrina.baumgart@rub.de

Abstract

Usher syndrome is a genetically and clinically heterogeneous disease in humans, characterized by sensorineural hearing loss, retinitis pigmentosa and vestibular dysfunction. This disease is caused by mutations in genes encoding proteins that form complex networks in different cellular compartments. Currently, it remains unclear whether the Usher proteins also form networks within the olfactory epithelium (OE). Here, we describe Usher gene expression at the mRNA and protein level in the OE of mice and showed interactions between these proteins and olfactory signaling proteins. Additionally, we analyzed the odor sensitivity of different Usher syndrome mouse models using electro-olfactogram recordings and monitored significant changes in the odor detection capabilities in mice expressing mutant Usher proteins. Furthermore, we observed changes in the expression of signaling proteins that might compensate for the Usher protein deficiency. In summary, this study provides novel insights into the presence and purpose of the Usher proteins in olfactory signal transduction.

Introduction

Mammalian olfactory signaling is dependent on a complex interplay of different cascade proteins, all of which are arranged in the cilia of the olfactory sensory neurons (OSN) (1,2). These include the olfactory receptors (ORs) (3), G proteins, enzymes and ion channels. Olfactory signal transduction is initiated by the specific binding of an odorant to its related OR. Then, the G protein $G_{olf}$ is activated, which stimulates adenylyl cyclase III (AC3) (4) to convert ATP into cAMP. This, in turn, activates cyclic nucleotide-gated channels, causing an influx of Ca²⁺ and Na⁺ ions (5,6). Subsequently, the Ca²⁺-activated Cl⁻ channels are opened, leading to an efflux of Cl⁻ ions and the further depolarization of the neuron (7,8). This protein network seems to be orchestrated through scaffolding proteins, such as the multiple PDZ domain protein 1 (MUPP1) (9,10). In other ciliary sensory systems, similar protein arrangements are present, including the Usher protein complex in the inner ear or the eye. Here, the different scaffolds assemble diverse proteins that are essential for the development, morphology and function of hair cells and photoreceptors (11–14).

The Usher syndrome is a rare genetic disease in humans that is genetically and phenotypically heterogeneous. It is characterized by sensorineural hearing loss, retinitis pigmentosa and, in some cases, vestibular dysfunction. There are three different Usher subtypes (USH1, USH2 and USH3), which are differentiated by the clinical onset and severity of hearing loss and retinitis pigmentosa, as well as the presence of vestibular dysfunction (15,16). The cause of this neurodegenerative disease is linked to mutations in any of the described Usher genes. USH type 1 is linked to MYOVIIA (USH1B), USH1C, CDH23 (USH1D), PCDH15 (USH1F), USH1G and CIB2 (USH1J), and USH type 2 is linked to USH2A, USH2B (GPR98, ADGRV1 USH2C), USH2D and USH type 3 is linked to USH3A. These genes encode for Usher proteins with distinct functions, such as cell adhesion proteins (cadherin 23 and protocadherin 15), a molecular motor protein (myosin VIIa), transmembrane proteins (USH2A and VLGR1, very large G protein-coupled receptor) and scaffolding proteins (harmonin (USH1C), SANS (scaffold protein containing ankyrin repeats and SAM domain, USH1G) and whirlin (USH2D)) (16).
molecular analyses elucidated the integration of all Usher proteins in functional protein networks organized by the scaffold proteins harmonin, whirlin and SANS (16). These findings have contributed significantly to our current insights into the function of USH proteins in the inner ear and the eye, and explain why defects in proteins of different families cause very similar disorders. In the retina and the inner ear, the Usher proteins are integrated in cellular modules, such as molecular transport (11,12,17), endocytosis (18), mechanical stabilization via membrane–membrane linkages (11) and the signaling complex at the mechanosensitive tip-link complex of hair cells (19,20). Because of the structural similarities in signal transduction in cilia and the importance of G protein-coupled receptors between the visual system and the olfactory system, it has been speculated for years that the Usher syndrome may generally affect ciliary structures and may also impair the olfactory system (21). It is conceivable that olfactory signaling is also dependent on a large scaffolding network, like the Usher networks in the visual and auditory system of vertebrates or the organization of the inactivation no afterpotential D (inaD) protein in the visual system of Drosophila melanogaster (22).

This concept has previously been discussed and it has been demonstrated that the Usher protein myosin VIIa is located in the cilia of mouse OSNs (21). Furthermore, it has been implied that patients with Usher syndrome may also show an olfactory impairment (23,24), whereas another study showed that the Usher syndrome patients have a normal odor perception (25). Because of this controversy and the need for a better understanding of the olfactory system itself, it will be of significance to analyze the relevance of the Usher proteins to mammalian olfactory signaling.

In this study, we intended to describe the expression of the Usher genes in the mouse olfactory epithelium (OE) at the mRNA and protein levels. Furthermore, new, tissue-specific isoforms of these proteins could possibly be identified. Using immunohistochemical staining, we were able to localize the individual Usher proteins within the sensory cilia of the OSNs of the OE. Interactions with the most important signaling proteins could be identified via co-immunoprecipitations. In addition, we analyzed the responsiveness and sensitivity of the OE from different Usher mouse models to various odors and odor mixtures using electro-olfactogram (EOG) recordings and found differences in odor detection in the mouse models. In summary, we could show the presence and localization of Usher proteins in the murine OE and also demonstrate the significance of these proteins for odor detection.

Results
Expression analysis of the Usher proteins in the murine olfactory epithelium

To investigate whether the different Usher proteins are involved in olfactory signaling, we first analyzed their expression within the OE at the mRNA level (Fig. 1A). For this, the isolated RNA from mouse OEs was used as a template for the cDNA synthesis, which was amplified by PCR (polymerase chain reaction) using specific primer sets for seven mouse Usher orthologue mRNAs. The housekeeping gene β-actin was used as a control. We detected stable expression of harmonin, cadherin 23 (Cdh23), protocadherin-15 (Pcdh15), SANS, Ush2a, Vlgr1 and whirlin at the mRNA level (Fig. 1A). To validate the Usher protein expression in the OE, we performed western blots on lysates of the OE from wild-type C57BL/6 mice using primary antibodies that were specific for the different Usher proteins. As expected from previous studies, we detected one or more bands for most of the Usher proteins, which may represent alternatively spliced isoforms. For all Usher proteins we detected bands in the present western blots which co-migrate with previously described splice variants, namely two isoforms of harmonin (96 and 120 kDa), two isoforms of Cdh23 (370 and 55 kDa), two isoforms of Pcdh15 (214 and 76 kDa), one specific band for SANS (52 kDa), two specific signals for Ush2a (55 and 110 kDa), one isoform of Vlgr1 (687 kDa) and two isoforms of whirlin (98 and 61 kDa) (Fig. 1B). Antibody specificity was validated by pre-absorbed western blots (Supplementary Material, Fig. S1; Reiners et al. (26) and Overlack et al. (27)).

The Usher proteins are located in the ciliary region of the murine olfactory epithelium

After verifying the expression of seven different Usher genes at the mRNA and protein level, we wanted to examine the exact localization of these proteins within the murine OE. We performed immunostaining on cryosections of transgenic olfactory marker protein (OMP)–GFП hybrid mice, using specific primary antibodies for all of the tested Usher proteins (Fig. 2). The OMP–GFП mice express enhanced green fluorescent protein under control of the OMP promoter and, therefore, all OSNs are labeled (28). Also, additional co-localization experiments with acetylated tubulin as a ciliary marker (Supplementary Material, Fig. S2) were performed. Previous immunohistochemical experiments revealed that myosin VIIa is localized in the cilia of olfactory neurons of the murine OE. Here, we showed that Pcdh15 and Vlgr1 are located exclusively in the ciliary layer of the OE. SANS and whirlin are also predominantly present in the ciliary region, but are also present in the basal part of the OE. Harmonin is primarily located below the ciliary layer, Cdh23 is located throughout the apical region and Ush2a is located in distinct, small clusters along the cilia.

The Usher proteins interact with olfactory signaling proteins

Next, we wanted to elucidate the putative involvement of USH proteins in OSN function and signaling. For this, we tested the participation of Usher proteins in olfactory signaling hubs by performing co-immunoprecipitations with eight Usher proteins and a set of important olfactory signaling proteins. The antibodies against the Usher proteins pulled down protein complexes from lysates of the isolated OE from wild-type mice. The western blots revealed that the olfactory G protein Gαolf and the ATP-catalyzing AC3 were co-precipitated with the Usher proteins (Fig. 3). In addition, eight of the proteins, with the exception of whirlin, also interacted with calcmodulin (CaM) in this ex vivo approach (Fig. 3). In contrast, we did not recover any of these olfactory signaling molecules in control precipitations of the OE lysate using a normal rabbit IgG antibody. After performing reverse co-immunoprecipitations with the same proteins, we could verify the specificity of present pull-downs (Supplementary Material, Fig. S3). These findings demonstrated that the eight Usher proteins participate in protein complexes associated with olfactory signaling.

Usher mouse models show an olfactory phenotype in electro-olfactogram recordings

After demonstrating an interaction between the Usher proteins and olfactory signaling proteins ex vivo, we investigated the possible physiological effects of these Usher proteins in the OE and whether mutations in the Usher syndrome genes have an impact on olfaction. Therefore, we examined different transgenic mouse
lines with deletions, point mutations or frame-shift mutations of the Usher genes Myo7a, Ush1C, Cdh23, Pcdh15 and Ush1G as model organisms to reflect the different subtypes of the Usher syndrome. To demonstrate the potential impact of these mutations, we performed air-phase EOG recordings by applying a mixture of 100 different odorants (Henkel 100, 1:1000) through a constant stream of humidified air onto the OE. This mixture allowed us to activate a broad range of OSNs, and thus compare the EOG amplitudes as well as the rise and decay times. We performed the experiment for mice with mutations in Myo7a, Ush1C, Cdh23, Pcdh15 and Ush1G and their wild-type littermates (Fig. 4A). We detected no significant differences in the response amplitudes or the kinetics of the shaker-1 mice (USH1B, myosin VIIa-deficient). However, we observed a slight but significant difference in the decay time (115%) of the waltzer mice (USH1D, Cdh23-deficient). The Ames waltzer mice (USH1F, Pcdh15-deficient) showed a significant decrease in the decay time kinetics (57%), but no difference in the response amplitudes or rise times. We found a significant decrease in the response amplitudes of the deaf circler mutants (59 ± 4%, USH1C, harmonin-deficient) and the Jackson-shaker mutant mice (53 ± 3%, USH1G, SANS-deficient). The deaf circler mice also had a shortened decay time (83 ± 3%), while the Jackson-shaker mice showed decreased decay (90 ± 2%) and increased rise times (123 ± 3%). These data show a severe olfactory impairment in the USH1C and USH1G mouse models and, therefore, indicate a significant impact of the harmonin and SANS proteins on olfactory signaling.

We conducted additional EOG recordings with a set of single odors to focus our investigation on the physiological influence of the harmonin and SANS proteins on odor perception. Thus, we could show whether specific OSNs are influenced by these proteins or if the response of all OSNs is equally affected. After three applications during a measurement, we repeated the first odor pulse to test whether the OE remained intact (Fig. 4B).

We observed a reduction in the response amplitudes in the deaf circler mutants compared to their wild-type littermates for every odor we tested. The reduced amplitudes varied between 49 ± 5% (linalool) and 67 ± 7% (2-phenylethyl alcohol). In the
Jackson-shaker mice, the amplitudes were also reduced for all tested odors, varying between 38 ± 3% (linalool) and 59 ± 5% (acetophenone). In summary, we did not identify a single odor with a completely abolished response; however, all tested odors showed a reduced amplitude response in the two mutant mice.

Olfactory signaling protein expression is modified in the SANS and harmonin mouse models

Because we could show an olfactory impairment in the deaf circler mutants (USH1C, harmonin) and the Jackson-shaker mutants (Ush1G, SANS), we further investigated the molecular cause of this effect. As we could demonstrate an interaction between these two proteins and some of the most important olfactory signaling proteins, we compared the protein levels of these signaling components in the mutant and wild-type mice through western blot analyses. Interestingly, the protein levels of CaM (228 ± 13 and 220 ± 18%), Gαolf (177 ± 12 and 150 ± 11%) and the cyclic nucleotide-gated channel subunit A4 (CNGA4) (185 ± 17 and 169 ± 20%) were higher than in wild-type animals (Fig. 5). The protein levels of the olfactory receptor mOR-EG and AC3 were not significantly different between the mutant and wild-type mice. The β-actin protein acted as a control and showed equal amounts of protein in the wild-type and mutant mice.

Figure 2. Immunohistochemistry for the Usher proteins in the olfactory epithelium. Immunohistochemistry was performed using olfactory epithelium (OE) cryosections of the transgenic OMP-GFP (green) mice, which were stained with primary antibodies specific for the respective proteins (red). The Gαolf staining is a marker for the ciliary layer, while the annotations show the most important parts of the olfactory epithelium. CL, ciliary layer; DK, dendritic knob; D, dendrite; OSN, olfactory sensory neuron; scale = 20 µm. Myosin VIIa, Pcdh15 and VLGR1 are located exclusively in the ciliary layer of the OE. SANS and whirlin are predominantly located in the ciliary region but are also present in the basal part of the OE. Harmonin is primarily located below the ciliary layer, Cdh23 is located throughout the apical region, and Ush2a is located in distinct, small clusters along the ciliary area.
The present immunohistochemistry results presented here affirmed the expression of all analyzed Usher proteins in the OE. Utilizing transgenic OMP-GFP mice and ciliary marker antibodies, we demonstrated the expression of the Usher proteins in the olfactory neurons. Furthermore, higher magnification images of the immunostaining revealed the localization of USH proteins in cytoplasmic cell compartments and in the olfactory sensory cilia. This ciliary localization is in accord with the localization of the Usher proteins in the primary cilia of cultured cells (18) and the highly modified sensory cilium of retinal photoreceptor cells (11,33–35). As demonstrated for these other cilia, the Usher proteins may play a role in ciliogenesis, ciliary maintenance of the olfactory cilium by participating in ciliary transport processes (33–35), and/or in the regulation of endocytosis (18). In addition, the USH proteins are localized to cytoplasmic compartments where they are associated with transport complexes indicating a role in cytoplasmic transport (11,17,34,36,37). In any case, in all functional modules of the sensory cells the Usher molecules are components of protein networks that are organized by the scaffold proteins harmonin, SANS, whirlin and PDZD7, a genetic modifier of Usher syndrome (38,39). Here, we demonstrated the clustering of the Usher proteins with key molecules of the olfactory signal transduction pathway, namely $G_{\text{m}}$, AC3 and CaM. Our results indicate that these molecules are integrated into conjoined protein complexes in the OSNs. These complexes may be assembled as complexes during the transport of the signaling molecules to their ciliary destination, as previously described in photoreceptor cells (11,17,34). Furthermore, the Usher proteins may participate in organizing the signaling complexes in the olfactory cilia, as previously described in hair cells, where they are essential for the function of the mechno-sensing signaling tip-link complex (19,20). We have previously identified the scaffold protein MUPP1 as a potent scaffold for olfactory signal transduction components (9,10), and, more recently, our proteomic analysis of the interacting partners of MUPP1 revealed the binding of Usher proteins, namely SANS, VLGR1 and whirlin to the PDZ domains of MUPP1 in OSNs (40). Thus, it is tempting to hypothesize that the Usher scaffold proteins may cooperate with MUPP1 to organize the key components of the olfactory signal transduction pathway into supramolecular protein complexes. In this scenario, the Usher transmembrane and adhesion proteins are suitable for anchoring the complexes at defined membrane domains of the olfactory neurons/cilia.

This clustering of signaling molecules could increase the specificity, sensitivity, and speed of the intracellular olfactory signaling pathway, as previously showcased for the signaling complex (transducosomes) of the visual signal transduction components in the rhabdomeric photoreceptors of D. melanogaster, which are clustered by the PDZ protein INAD (22). Indeed, in this study, we identified differences in odorant-evoked decay time from the EOG recordings of the Ames waltzer and waltzer mice; these mice lack the Usher transmembrane proteins Pcdh15 and Cdh23, respectively, further supporting a role for these components in ‘processing’ olfactory signaling. Moreover, and remarkably, the deaf circler and Jackson-shaker mice showed a significant reduction in the odorant-evoked amplitude as well as a change in the response kinetics. The absence of the Usher scaffold proteins harmonin and SANS leads to a severe olfactory impairment, which is comparable to the observed phenotype in the olfactory neurons with a disruption of the MUPP1 signaling complex (9). These results further support the involvement of the Usher proteins in concert with MUPP1 in olfactory signaling. This concept is reinforced by the results from the single odor, EOG recordings. Because we did not detect a significant

**Discussion**

Here, we aimed to determine whether proteins related to the human Usher syndrome have an impact on olfactory sensory functions. We combined expression analyses at the mRNA level (RT-PCR) with western blot analyses and immunohistochemistry. Our results showed that all eight of the Usher proteins analyzed here are expressed in the murine OE which affirms previous suggestions and experimental results (21,24,29). For most Usher proteins, we observed several bands in western blots of protein lysates of the isolated murine OE, which is not surprising as previous studies have consistently demonstrated that most Usher genes are expressed in form of several alternatively spliced transcripts (27,29,30), although, we cannot entirely exclude cross-reactivity of the antibodies used in the present study. Some of these variants show tissue-specific expression, such as Cdh23 in which exon 68 is alternatively spliced in the retina and the inner ear, respectively (31), or harmonin a and b isoforms that have different scaffolding functions (32). It will be interesting to determine, which of the alternatively spliced isoforms could be found in the transcriptome of the OE. However, RNA-Seq analyses of the splice variants were beyond the scope of our present study and will be subject to future investigations.

![Figure 3](image-url)

**Figure 3.** Co-immunoprecipitations of the Usher proteins with different olfactory signaling proteins. Co-immunoprecipitations of the tested Usher proteins with the olfactory signaling proteins: $G_{\text{m}}$, adenyl cyclase III (AC3) and CaM. The murine OE lysate was immunoprecipitated with one of these proteins and detected with antibodies against the interacting protein. The input controls and normal rabbit IgG-antibody controls are included.
difference in the odor-induced amplitude responses between the
different individual odors, SANS and harmonin appear to be glo-
bal regulators of the olfactory signaling cascade as their in
fluence is not limited to specific ORs and OSNs.

Interestingly, we observed changes in the expression of the
olfactory signaling proteins in Jackson-shaker and deaf circler
mice. In accordance with the fact that the olfactory impairment
does not influence single receptors, the protein expression of
the exemplary receptor mOR-EG was not altered in both mouse
models compared to wild-type mice. Surprisingly, we observed
an increase in protein expression for the olfactory signaling com-
ponents Golf, CaM and the olfactory channel subunit CNGA4. One

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**Figure 4.** EOG recordings from the transgenic Usher mouse models. (A) EOG recordings with the Henkel 100 mixture. The Cdh23-deficient waltzer mice only showed a slight difference in the decay time (mice = 2 WT, 2 MM (mutant mice); measurements = 28 WT, 28 MM). The myosin VIIa-deficient shaker-1 mice did not show any difference compared to their wild-type littermates (mice = 6 WT, 5 MM; measurements = 84 WT, 71 MM). The Pcdh15-deficient Ames waltzer mice showed a significant reduction in decay time compared to the wild-type animals (mice = 6 WT, 5 MM; measurements = 42 WT, 35 MM). The harmonin-deficient Deaf circler mice showed a significant decrease in the response amplitude and also a decrease in the decay time (mice = 10 WT, 11 MM; measurements = 110 WT, 110 MM). SANS-deficient Jackson-shaker mice also showed a significant decrease in the response amplitude, an increase in the rise time, and a slight decrease in the decay time (mice = 10 WT, 10 MM; measurements = 100 WT, 100 MM). (B) EOG recordings with different individual odors. The deaf circler (mice = 6 WT, 5 MM; measurements = 26 WT, 26 MM) and Jackson-
Shaker (mice = 5 WT, 5 MM; measurements = 25 WT, 30 MM) mice showed only slight differences in the reduction of the response amplitude between the different
individual odors. The error bars represent the SEM. ***P ≤ 0.001; **P ≤ 0.01; *P ≤ 0.05; n.s., not significant.
explanation for these results may be a possible regulatory effect of the two scaffolding proteins on these olfactory signaling molecules. We showed a complex formation of Golf and CaM with SANS and harmonin. A direct regulatory interaction between scaffolding proteins and G proteins has previously been demonstrated (41). Another explanation is the possible self-adjustment capability of the olfactory system, which attempts to compensate for the reduced odorant response. The interactions between the signaling components could be severely impaired in the absence of their specific scaffolding protein, leading to increased expression of distinct components to compensate for this loss of function. A similar up-regulation of the olfactory signaling components was observed after stimulus deprivation of the olfactory system through unilateral naris occlusion (42). As previously reported, the visual system also attempts to compensate for the loss of a scaffolding protein by up-regulating another component (43).

In conclusion, we provide, for the first time, conclusive evidence for the expression of Usher syndrome proteins in the OSNs of the murine OE. The cytoplasmic localization of the Usher proteins and their association with the olfactory cilia enable the molecules to act in diverse functional modules of the OSNs, namely the cytoplasmic and ciliary transport systems as well as in the clustering of signaling molecules in the sensory cilia. Additionally, we functionally described olfactory sensitivity in five Usher mouse models using EOG recordings and could determine a severe olfactory impairment in the SANS (USH1G) and harmonin (USH1C) deficient, Jackson-shaker mice and deaf circler mice, respectively. Previous studies had suggested an olfactory impairment in human patients with Usher syndrome (23,44), but these were rebutted by Seeliger et al. (25). Because there were a relatively low number of subjects tested in these studies (Zrada et al. (23) 22, Seeliger et al. (25) 39) and the genetic identity of the Usher type was unknown, a more thorough analysis is necessary to confirm an olfactory phenotype in Usher patients with defined genetic defects. Our present discoveries provide novel insights into the organization of the olfactory signal transduction and indicate that olfactory function may be an interesting biomarker for certain Usher types and for monitoring efficacy of therapies in future.

Materials and Methods
Antibodies

The primary antibodies used were: Myosin VIIa (rabbit polyclonal, ab3481, Abcam, Cambridge, UK), harmonin (rabbit, 11358-1-AP,
Proteins were purified from mice by a previously described protocol (26). Cdh23 (rabbit polyclonal, 13469-1-AP, Proteintech, and rabbit polyclonal, previously described in (26,27)), Pcldh15 (rabbit polyclonal, AV50153-50UG, Sigma-Aldrich, St Louis, MO, USA), SANS (rabbit polyclonal, ab158205, Abcam, and rabbit polyclonal, previously described in (27)), USH2A/USHerin (rabbit polyclonal, previously described in (11)), VLGR1 (goat polyclonal, sc-23738, Santa Cruz Biotechnology, Dallas, TX, USA, and rabbit polyclonal, previously described in (45)), whirlin (rabbit polyclonal, ab76850, Abcam), CNGA-4 (rabbit polyclonal, ab182104, Abcam), Goil (rabbit polyclonal, sc-383, Santa Cruz Biotechnology), CaM (mouse monoclonal, sc-137079, Santa Cruz Biotechnology), AC3 (rabbit polyclonal, sc-588, Santa Cruz Biotechnology), anti-β-actin (rabbit polyclonal, ab8227, Abcam), mOR-EG (rabbit polyclonal, ab104886, Abcam). The secondary antibodies used were: horseradish peroxidase (HRP)-conjugated goat anti-mouse, donkey anti-goat and goat anti-rabbit IgGs (1:10 000) (Bio-Rad, Berkeley, CA, USA), as well as Alexa Fluor 546/660-conjugated goat anti-rabbit and donkey anti-goat (1:1000) (Molecular Probes, Eugene, OR, USA).

Transgenic mouse lines

The transgenic mouse lines used in this work included: OMP-GFP, every cell expressing the OMP also expresses GFP (23); shaker1, a point mutation is spontaneously inserted in the Myo7a gene (46); deaf circler, a spontaneous single base pair deletion is introduced in the Myo7a gene (47); Waltzer, a single G at position 3560 was deleted in the CDH23 gene (48) (#004764, The Jackson Laboratory, Bar Harbor, ME, USA); Ames waltzer, a frame-shift mutation, where an additional A was inserted after position 4521 in the Pcdh15 gene (49) (#002072, The Jackson Laboratory); and Jackson-shaker, a single base pair insertion that causes a frame-shift mutation in the Ush1g gene (50) (#000783, The Jackson Laboratory). The transgenic and wild-type mice were raised and maintained according to institutional and governmental instructions.

cDNA preparation and quantitative PCR

The RNA was isolated from freshly prepared mouse OE with the RNeasy Mini Kits (Qiagen, CA, USA). The reverse transcriptase (RT) and cDNA synthesis were performed using the iScript cDNA synthesis kit (Bio-Rad). For PCR, specific primers were designed, which amplified regions (180–250 bp) in the genes of interest. The qSYBR Green Mix (Bio-Rad) was used for amplification on a Mastercycler replexalp (Eppendorf, Hamburg, Germany). The primers used were: Ush1g_for: TGGACCTGGCAGCATCACG, Ush1g_rev: GGGGCTGAATGCGGATGTG; Ush1c_for: CTGAGGC CGAGCCTGCCCTA, Ush1c_rev: GTGGGCTCAAGCTGCGAAGAAA; MyosinVIIA_for: TGGCCAGAGCATGATTCGCC, MyosinVIIA_rev: GTTCCGGCTCCGATCTCCTG; Gad923_for: GATCCACGTAGCGCGGGC, Gad923_rev: CTCAAGGCGCAGCGGTGTG; Pchd15_for: TACGGGAAAAGGGGCGCA, Pchd15_rev: TTTGTCACCAGCTGCCG; Ush2a_for: AGCCCGCAACCAGCCAAA; GTC, VLRG1_for: CCCGGCCAGCGCTGGAATG; and Whirlin_for: AGGAGCTTGGCCTCGCTC, Whirlin_rev: TGCCCTAGGGTCTGG CCCACT.

Western blotting

The samples were loaded onto an SDS gel, separated according to size and transferred to a nitrocellulose membrane (Porablot NCL, Machery-Nagel, Düren, Germany). The blots were blocked in 5% casein (50% TBS, 5% casein in TBS, Thermo Scientific, Waltham, MA, USA) for 1 h at RT. The specific primary (1:50–1:4000) and secondary antibodies were diluted in blocking solution. For antibody validation, the antibodies against harmonin, SANS, USH2A and CDH23 were pre-incubated for 4 h at room temperature with the recombinantly expressed polypeptide containing the corresponding antigen (2 mg/ml) on a shaker (11,26,27). The washing steps were performed with TBS buffer [150 mM NaCl, 100 mM Tris-HCI, 0.12% Tween (w/v)]. Western blots were analyzed using Odyssey infra-red imaging system (LI-COR Biosciences, Lincoln, NE, USA). The interactions were detected using the ECL western blotting detection reagent (GE Healthcare, Little Chalfont, UK) to visualize the protein bands by chemiluminescence. Pictures were taken with the Fusion-Sl 3500-WL (Vilber Lourmat, Eberhardzell, Germany) imaging device.

Co-immunoprecipitations

For the co-immunoprecipitations, the OE from C57BL/6 mice (>P20) was lysated in RIPA buffer [150 mM NaCl, 50 mM Tris-HCl, 1% Nonidet, 0.5% sodium deoxycholate (w/v), 0.1% SDS (w/v) and protease inhibitors] and processed using the Capture & Release system (Catch & Release v2.0, Millipore, Billerica, MA, USA). The lysate was centrifuged at 1000g for 10 min, followed by another preparation step in RIPA buffer and centrifugation. A total of 4 mg of protein were incubated with the specific primary antibody (0.8 µg) overnight at 4°C in a special spin column. After several washing steps with the washing buffer from the kit, a denaturing elution buffer was used to elute the proteins from the spin column. Afterward, the samples were prepared for western blot analysis.

Immunohistochemistry

Cryosections through the OE of P18 mice were generated at a thickness of 12 µm. The sections were blocked for 1 h (PBS- and 1% cold-water fish skin gelatin) at room temperature and incubated with the specific primary antibodies, which were diluted in blocking solution, overnight at 4°C. After several washing steps, the sections were incubated with fluorophore-conjugated secondary antibodies for 1 h at room temperature. Then, the sections were coated with Prolong Antifade Gold (Life Technologies). The images were recorded using a confocal microscope (Zeiss LSM 510 Meta, Oberkochen, Germany) with a ×40 oil immersion objective. The image processing was performed with Corel Draw X5 (Corel Ottawa, ON, Canada).

Electro-olfactogram recordings

For the EOG, the skull of the mouse (P21–P40) was cut parasagittal to the septum. The turbinae were removed from the upper side and the skull was placed on an agarose gel (3%) inside a petri dish. The reference electrode was placed in the agarose gel and the EOGs were recorded on the septum of the OE. A constant stream of humidified air was applied to the OE (0.4 l/min). The odorants were applied to the air stream through a custom device, and the odorant pulses lasted for 100 ms. Henkel 100 (Henkel, Düsseldorf, Germany), a mixture of 100 different odors, and different individual odors were diluted in distilled water and used as a stimulus. The EOGs were recorded on a DP–301 Differential amplifier (Warner Instrument Corp., Hamden, CT, USA) using the WinEDR Software Version 2.7.9 (University Strathclyde). The peak amplitudes were measured using Clampfit Version 10.2.0.14 (Molecular Devices, MDS Analytical Technologies, Toronto, ON, Canada).
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The physiological response amplitudes generated through the EOG recordings were averaged according to the genotype. These averaged numbers were compared using two-way ANOVA and Student’s t-tests. The criterion for statistical significance was a P-value <0.05.

Supplementary Material

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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References


