Chem. Senses 33: 145–162, 2008
doi:10.1093/chemse/bjm073
Advance Access publication November 20, 2007

Proteomic Analysis of a Membrane Preparation from Rat Olfactory Sensory Cilia

Ulrich Mayer[1], Nicole Ungerer[1], Daniel Klimmeck[1], Uwe Warnken[2], Martina Schnölzer[2], Stephan Frings[1] and Frank Möhrlen[1]

[1]Department of Molecular Physiology, University of Heidelberg, Im Neuenheimer Feld 230, 69120 Heidelberg, Germany and [2]Functional Proteome Analysis, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 580, 69120 Heidelberg, Germany

Correspondence to be sent to: Frank Möhrlen, Department of Molecular Physiology, University of Heidelberg, Im Neuenheimer Feld 230, 69120 Heidelberg, Germany. e-mail: moehrlen@uni-hd.de

Abstract

The cilia of mammalian olfactory receptor neurons (ORNs) represent the sensory interface that is exposed to the air within the nasal cavity. The cilia are the site where odorants bind to specific receptors and initiate olfactory transduction that leads to excitation of the neuron. This process involves a multitude of ciliary proteins that mediate chemoelectrical transduction, amplification, and adaptation of the primary sensory signal. Many of these proteins were initially identified by their enzymatic activities using a membrane protein preparation from olfactory cilia. This so-called "calcium-shock" preparation is a versatile tool for the exploration of protein expression, enzyme kinetics, regulatory mechanisms, and ciliary development. To support such studies, we present a first proteomic analysis of this membrane preparation. We subjected the cilia preparation to liquid chromatography-electrospray ionisation (LC-ESI-MS/MS) tandem mass spectrometry and identified 268 proteins, of which 49% are membrane proteins. A detailed analysis of their cellular and subcellular localization showed that the cilia preparation obtained by calcium shock not only is highly enriched in ORN proteins but also contains a significant amount of nonciliary material. Although our proteomic study does not identify the entire set of ciliary and nonciliary proteins, it provides the first estimate of the purity of the calcium-shock preparation and provides valuable biochemical information for further research.

Key words: olfactory receptor neurons, proteomic analysis, signal transduction, sensory cilia

Introduction

In mammals, odor perception begins when odorants interact with the sensory surface of the olfactory epithelium in the nose. This surface contains a dense layer of cilia, the sensory organelles of olfactory receptor neurons (ORNs). Exposed to the inhaled air, this ciliary layer represents the only known structure where neurons are in direct contact with the external environment. The cilia detect odorants by exposing metabotropic odorant receptor proteins to the air within the nasal cavity (Buck and Axel 1991). All components of the signal transduction cascade initiated by these receptors are associated with the ciliary membrane (reviews: Schild and Restrepo 1998; Frings 2001), so that the generation of the olfactory receptor potential is indeed a function of the cilia themselves. Moreover, the constant exposure of the cilia to environmental compounds may require an effective xenobiotic biotransformation metabolism, which protects the olfactory epithelium and the ORNs from cytotoxic compounds and terminates the sensory response (reviews: Marini et al. 1998; Ling et al. 2004). The expression of xenobiotic-metabolizing enzymes in ORNs is supported by the finding that cytochrome oxidase staining, which is correlated with xenobiotic functional activity, was not only observed in epithelial supporting cells but also in ORN perikarya, dendrites and their ciliated knobs, as well as within the mucus layer that contains parts of the ORN knobs, their cilia, and supporting cell microvilli (Pataramekin and Meisami 2005). However, highest activity levels of xenobiotic-metabolizing enzymes were generally found in supporting cells, suggesting that protection of the entire sensory neuroepithelium from environmental compounds is mainly a task of supporting cells (reviews: Dahl and Hadley 1991; Thornton-Manning and Dahl 1997; Marini et al. 1998). ORNs have a very short life span. After operating for only a few weeks, the neurons undergo apoptosis and are replaced by newly differentiating

stem cells. This fascinating example of adult neurogenesis is a subject of intense research activity (reviews: Beites et al. 2005; Nicolay et al. 2006; Gheusi and Lledo 2007; Henion and Schwarting 2007) and may also profit from our proteomic analysis.

Biochemical exploration of the olfactory cilia largely relies on a membrane preparation protocol that was established by Chen and Lancet (1984). Ciliary membranes are detached from the olfactory epithelium by a ''calcium-shock'' and are subsequently collected by ultracentrifugation. The resulting preparation was partially characterized by electron microscopy and analysis of glycoprotein expression (Anholt et al. 1986). It was accepted as an in vitro model for olfactory biochemistry and, from that time on, successfully used for research in olfactory transduction. Virtually, all components of the signal transduction cascade—apart from odorant receptors and transduction channels—have been initially discovered using this preparation. In particular, adenylate cyclase type III (AC III), its stimulatory guanosine triphosphate (GTP)–binding protein G_{olf}, and the cyclic adenosine monophosphate (cAMP)-dependent phosphodiesterase PDE1C were characterized by their enzymatic activity which leads to subsequent identification by molecular cloning (e.g., Pace et al. 1985; Sklar et al. 1986; Lowe et al. 1989; Pfeuffer et al. 1989; Yan et al. 1995). Furthermore, the kinetics of odor-induced second messenger signaling (Breer et al. 1990; Boekhoff and Breer 1992), as well as various molecular mechanisms for feedback inhibition and adaptation (e.g., Boekhoff et al. 1992; Schleicher et al. 1993; Wei et al. 1998), were described based on biochemical experimentation with the cilia preparation. Finally, evidence was found for other odor-induced signal transduction cascades, which operate with IP_3, Ca^{2+}, or cyclic guanosine monophosphate as second messengers (e.g., Breer and Boekhoff 1992; Moon et al. 1998). Thus, the examination of the cilia preparation yielded many of the molecular details which support—in conjunction with the biophysical identification of transduction channels in the ciliary membrane (Nakamura and Gold 1987; Kleene and Gesteland 1991)—the current concept for vertebrate olfactory signal transduction.

For further investigations, it would be helpful to have an overview of the specific set of proteins present in the sensory cilia. Possible functions of such proteins in signal transduction, adaptation, xenobiotic metabolism, and ORN maturation could be investigated on the grounds of such information. Here, we present a first proteomic analysis of the cilia preparation from rat olfactory epithelium. We have tested the degree of cilia purification using marker proteins for the ciliary and nonciliary cell compartments. To characterize proteins present in the preparation, we have isolated membrane integral and membrane-associated proteins using cetyl trimethyl ammonium bromide (CTAB) to solubilize the proteins. After separating the proteins by two-dimensional (2D) gel electrophoresis, we identified 268 different proteins by LC-ESI-MS/MS tandem mass spectrometry. These proteins were classified according to their expected cellular and subcellular locations and functions. Our data extend the view on biochemical signal processing in olfactory cilia and promote systematic studies of olfactory signal transduction, xenobiotic metabolism, and other functions of ORNs.

Materials and methods

Animals

Preparations of olfactory epithelium and olfactory cilia were obtained from 3- to 6-month-old Wistar rats. For immunohistochemical staining, the olfactory marker protein (OMP)–green fluorescent protein (GFP) mouse line, which contains a gene-targeted insertion of the GFP reporter in the OMP locus (Potter et al. 2001), was kindly provided by Dr Jörg Strotmann (University of Hohenheim). All experiments were performed in accordance with the Animal Protection Law and the guidelines and permissions of the University of Heidelberg, Germany. Animals were killed by CO_2 inhalation and decapitation. For immunohistochemical treatment, the rostral part of the skull, containing the nasal cavity, was dissected. For membrane preparations, the head capsule was opened by a sagittal section to remove the olfactory epithelium from the dorsal posterior part of the nasal septum.

Immunohistochemistry and microscopy

Immunohistochemistry was performed as previously described (Bönigk et al. 1999). Rabbit anti-AC III (Santa Cruz Biotechnology, Inc., Heidelberg, Germany, #sc-588, 1:400) was used as primary antibody and visualized by donkey anti-rabbit Alexa 568 (Molecular Probes, Karlsruhe, Germany, A-11057, 1:500) secondary antibody. A 1:300 dilution of a 90-μM 4′,6-diamidino-2-phenylindole solution (Molecular Probes, C-7509) was used to stain nuclei. Sections were analyzed using a Nikon TE2000-E Confocal Microscope C1 system at the Nikon Imaging Center at the University of Heidelberg.

Isolation of olfactory ciliary membranes

Olfactory cilia were isolated using the established Ca^{2+}-shock method as described in Bönigk et al. (1999) and Delgado et al. (2003). Briefly, the olfactory epithelium of 5 rats was washed in ice-cold Ringer's solution (120 mM NaCl, 5 mM KCl, 1.6 mM K_2HPO_4, 25 mM $NaHCO_3$, 7.5 mM D-glucose, protease-inhibitor-Mix M [Serva, Heidelberg, Germany], pH 7.4), transferred to Ringer's solution containing 10 mM $CaCl_2$, and gently stirred for 20 min at 4 °C. The detached cilia were isolated by 3 sequential centrifugation steps for 5 min at 7700 × g. The supernatants were collected and loaded on top of a 45% sucrose solution in Ringer's solution with 10 mM $CaCl_2$ and concentrated at 100 000 × g for 30 min at 4 °C using a Beckman L-70 ultracentrifuge. Ciliary membranes represented by a yellowish

layer at the sucrose–supernatant interface were diluted in their 10-fold volume of Ringer's solution with 10 mM $CaCl_2$ and centrifuged at 100 000 × g for 30 min at 4 °C. Routinely, the resulting ciliary membrane pellet was resuspended in 10 mM Tris, 3 mM $MgCl_2$, 2 mM ethyleneglycol-bis(aminoethylether)-tetraacetic acid (EGTA), pH 7.4, and stored at −70 °C. Protein yield was measured using the amido black assay (Schaffner and Weissmann 1973) and resulted in ~250 μg per 5 rats.

Whole-tissue membrane protein purification

Olfactory epithelium was homogenized in a glass/Teflon homogenizer in 10 ml ice-cold hypotonic solution (20 mM NaCl, 1 mM ethylenediaminetetraacetic acid [EDTA], 0.1 mM EGTA, 1 mM dithiothreitol [DTT], 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], 0.02% *o*-phenanthrolin, complete protease inhibitor cocktail [Roche, Mannheim, Germany], pH 7.4). The suspension was washed by centrifugation at 300 × g for 5 min at 4 °C to separate the membranes from nuclei. The cellular and ciliary membranes were washed and collected in hypotonic solution by 2 centrifugation steps at 100 000 × g for 30 min at 4 °C. The resulting membrane pellet was resuspended in ice-cold hypertonic solution (500 mM NaCl, 1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 20 mM HEPES, 0.02% *o*-phenanthrolin, complete protease inhibitor cocktail [Roche], pH 7.4), centrifuged (100 000 × g, 30 min, 4 °C), and resuspended in 100 μl of isotonic solution (150 mM NaCl, 1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 20 mM HEPES, 0.02% *o*-phenanthrolin, complete protease inhibitor cocktail [Roche], pH 7.4).

Gel electrophoresis

1D sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) using a polyacrylamide concentration of 10% in the separation gel. For the detergent-based solubilization and separation of the ciliary membrane proteins, we applied a technique for 2D gel electrophoresis according to Hartinger et al. (1996) and Navarre et al. (2002). This protocol combines an acidic buffer and the cationic detergent CTAB in the first dimension with an SDS-PAGE in the second dimension. In both dimensions, a vertical gel with a separation length of 11 cm was used and electrophoresis was performed at 18 °C. For the first dimension, the 8% T 1.04% C resolving gel (3 M urea, acrylamide/bisacrylamide, 0.1% CTAB, 4 mM ascorbic acid, 75 mM KH_2PO_4, pH 2.1 [H_3PO_4]) and the 8% T 2.56% C stacking gel (1.66 M urea, 0.1% CTAB, 4 mM ascorbic acid, 125 mM KH_2PO_4, pH 4.1 (H_3PO_4)) were degassed and polymerized with 0.0125 mM $FeSO_4$ and 0.0012% H_2O_2. For sample preparation, 1 volume of a 2-fold sample buffer (6 M urea, 0.2% CTAB, 10% glycerol, 75 mM DTT, 0.05% pyronin Y) was transferred to 1 volume of protein solution corresponding to 80 μg protein. The denaturation occurred for 15 min at 70 °C. Electrophoresis toward the cathode was performed at 25 mA in the stacking gel and 45 mA in the separation gel. The resulting gel stripe was washed 4 times for 5 min in deionized water and then equilibrated 4 times for 15 min in an adequate volume of SDS-PAGE lysis buffer. For the second-dimension run, the stripe was positioned in a large well of the 3.8% stacking gel of a 10% Laemmli SDS gel. Electrophoresis toward the anode was performed at 45 mA.

Immunoblot

In total, 10 or 60 μg of proteins from ciliary membranes or whole olfactory tissue were loaded on 1D-SDS gels or 2D-CTAB/SDS gels, respectively, and electroblotted to polyvinylidene difluoride membranes (Machery & Nagel, Dueren, Germany) according to Towbin et al. (1979) using a semidry blotting apparatus. Evenly, protein loading of each probe was confirmed by densitometric quantification of colloidal coomassie CBB G250 stained gels. Nonspecific binding sites were blocked with 5% milk powder dissolved in phosphate-buffered saline (PBS) (8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, 130 mM NaCl, pH 7.4) overnight at 4 °C and incubated with primary antibody in MPBST (1% milk powder, 0.05% Tween 20, PBS) for 90 min. The following antibodies and dilutions were used: rabbit anti–AC III 1:100 (Santa Cruz Biotechnology, Inc., #sc-588), rabbit anti-CNGA2 1:100 (Sigma, Steinheim, Germany, #N7529), mouse anti-CNGA4 (mAB7B11, monoclonal, directed against c-terminal residues 392–575; developed by Bradley et al. (1997) and obtained from the Developmental Studies Hybridoma Bank, University of Iowa), rabbit anti-$G_{\alpha olf}$ 1:2000 (Santa Cruz Biotechnology, Inc., #sc-383), goat anti-occludin 1:200 (Santa Cruz Biotechnology, Inc., #sc-8145), rabbit anti-prohibitin 1:200 (Abcam Limited, Cambridge, UK, #ab2996), and goat anti-ezrin 1:50 (Santa Cruz Biotechnology, Inc., #sc-6409). The blots were washed 3 times with 0.1% Triton X-100 in PBS and incubated with adequate horseradish peroxidase–conjugated secondary antibodies (Sigma) in a dilution range of 1:20000–1:80000 in MPBST for 60 min. The blots were washed 3 times with 0.1% Triton X-100 in PBS and once in water. The ECL plus enhanced chemoluminescence system (GE Healthcare, Munich, Germany) was used to monitor bound antibodies.

Mass spectrometry

For mass spectrometric analysis, the ciliary membranes were resuspended in 300 μl of 10 mM Tris, 100 μM phenylmethylsulfonyl fluoridephenylmethylsulfonyl fluoride, 10 mM beta-mercaptoethanol, 3 mM $MgCl_2$, pH 8.0, and purified from nucleic acids with Benzonase (Merck, Germany). After an incubation step for 30 min at 37 °C, the suspension was diluted in 10 mM Tris, 3 mM $MgCl_2$, 2 mM EGTA, pH 7.4, and centrifuged at 100 000 × g for 30 min at 4 °C. A total of 80 μg of ciliary proteins were separated by 2D-CTAB/SDS-PAGE as described above and stained by colloidal coomassie

CBB G250 according to Neuhoff et al. (1990). The resulting gel was cut into 63 pieces and digested with trypsin (Promega, Madison, WI). The supernatant from the tryptic digestion and the extracts from steps 1 (acetonitrile [MeCN]/H_2O/formic acid [FA], 50.0/49.9/0.1, v/v/v), 2 (MeCN 100%), 3 (H_2O/FA, 99.9/0.1 v/v), and 4 (MeCN/H_2O/FA, 50.0/49.9/0.1, v/v/v) were combined, evaporated, and dissolved in H_2O/FA, 99.9/0.1, v/v.

Nanoscale LC-ESI-MS/MS analysis of the extract aliquots was performed using the CapLC capillary LC system (Waters, Eschborn, Germany) coupled to a hybrid quadruple orthogonal acceleration time-of-flight tandem mass spectrometer (Micromass, Manchester, UK). The LC-ESI-MS/MS device was adjusted with a PicoTip Emitter (New Objective, Woburn, MA) fitted on a Z-spray (Micromass) interface. Peptides were trapped on a Symmetry 300 NanoEase C18 column (Waters). Chromatographic separations were performed on a reversed-phase capillary column Atlantis C18, 3 μm particle size, 75 μm i.d., 15 cm length (Waters), with a flow rate of 200 nl/min.

For weakly stained gel slices (1–3, 5, 14, 17, 24, 28, 29, 33, 34, 37–39, 42–46, 49–51, 53, 55, 58–63; Figure 3A), the chromatography was carried out using a short linear gradient from 5% to 40% solvent B in 35 min and from 40% to 95% solvent B in 55 min (solvent A: H_2O/FA, 99.9/0.1, v/v; solvent B: H_2O/acetonitrile/FA, 5.0/94.9/0.1, v/v/v). For intensely stained gel slices (4, 6–13, 15, 16, 25–27, 30–32, 35, 36, 40, 41, 47, 48, 52, 54, 56, 57; Figure 3A), the chromatography was carried out using a long linear gradient from 0% to 55% solvent B in 2.3 h and from 55% to 100% solvent B in 3.8 h. Eluted peptides were ionized using a nanoelectrospray ion source. The capillary voltage was set to 2400 V, and the cone voltage was set to 80 V. Data acquisition was controlled by MassLynx 4.0 software (Waters). Low-energy collision-induced dissociation was performed using argon as a collision gas (pressure in the collision cell was set to 5×10^{-5} mbar), and the collision energy was optimized for all precursor ions dependent on their charge state and molecular weight in the range of 25–40 eV. MassLynx raw data files were processed with Protein Lynx Global Server 2.2 software (Waters). Deisotoping was performed using the MaxEnt3 algorithm.

Protein identification

Processed data were searched against the rat protein subdatabase (67 996 protein sequences) of the National Center for Biotechnology Information (NCBI) nonredundant database (version 071507; 5 269 953 protein sequences) using the Mascot algorithm version v2.1.0 (Matrix Science Ltd, London, UK). The mass tolerance was set to 200 ppm for precursor ions and 0.1 Da for fragment ions. No fragment ions score cutoff was applied when performing the search. Protein hits were taken as identified if 1) the sum Mascot ion score exceeded the significance threshold of 49, 2) a minimum of 1 peptide had an individual ion score exceeding the Mascot identity threshold, and 3) a minimum of 2 nonredundant peptide matches were detected. Under the applied search parameters, a sum Mascot score of >49 typically refers to a match probability of $P \leq 0.0005$, where P is the probability that the observed match is a random event. If peptides matched to multiple members of a protein family, we routinely assigned redundant peptide matches to the highest scoring protein. Proteins whose percentage of redundant peptide matches was lower than 40% were considered as identified. The following search parameters were selected: fixed cysteine modification with the carbamidomethyl side chain, variable modification due to methionine oxidation, and one missed cleavage site in the case of incomplete trypsin hydrolysis.

Bioinformatics

For annotation of protein sequences, BLAST (Basic Local Alignment Search Tool) searches (Altschul et al. 1990; Altschul and Gish 1996) at NCBI and sequence-based Harvester searches (Liebel et al. 2004) at FZK were used. Prediction of transmembrane helices was performed by using TMHMM Server v2.0 (Krogh et al. 2001) and SOSUI (Hirokawa et al. 1998). For subcellular localization, the subcellular localization database LOCATE was used (Fink et al. 2006). For analysis of the cellular source of the identified proteins, we compared the respective genes to the expression profiling of ORNs recently published by Sammeta et al. (2007).

Results

Preparation of ciliary membrane proteins

The sensory cilia contribute only a small fraction to the total membrane material of the olfactory epithelium. This is illustrated by displaying the immunosignal of AC III (Figure 1), a protein that is specifically expressed in the sensory cilia.

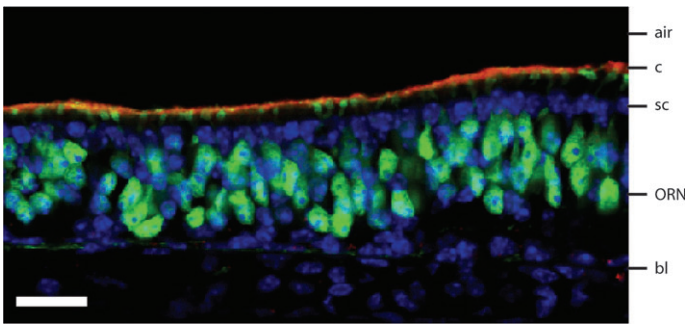

Figure 1 Polarized protein expression in a sensory epithelium. Section from the olfactory epithelium of an OMP-GFP gene-targeted insertion mouse illustrating the stratified structure of this tissue. Mature ORNs are labeled green by GFP, expressed under the promoter of the OMP (Potter et al. 2001). Nuclei are stained blue by 4′,6-diamidino-2-phenylindole. A single layer of nuclei marks the row of epithelial supporting cells (sc). At the interface to the nasal cavity, the red immunosignal originating from AC III indicates the sensory cilia (c). Ciliary knobs are interspersed as green dots among the ciliary layer. bl is the basal lamina. The scale bar indicates 20 μm.

To detach the cilia from the apical surface of the epithelium, we used the "calcium-shock" method that produces the membrane preparation that is routinely used in olfactory biochemistry. We obtained roughly 50 μg protein per animal by this method and first characterized the preparation by a membrane protein enrichment assay (Bönigk et al. 1999). The rationale for this assay is to compare the immunosignals of a protein on 2 western blots made with the same amount of protein (10 μg, verified by amido black assay) from either membranes of the entire olfactory epithelium, including cilia (Figure 2, lane I), or from the cilia preparation gained by calcium-shock treatment (Figure 2, lane II). If the calcium-shock protocol leads to a significant enrichment of ciliary membranes in the preparation, a cilia-specific protein should give rise to a much stronger signal in lane II, whereas proteins with nonciliary localization should be prominent in lane I. Accordingly, we found that 3 proteins which are known to be expressed almost exclusively in the cilia—the cyclic nucleotide-gated channel subunits CNGA2 and CNGA4 as well as AC III—display strong immunosignals in lane II and only weak signals in lane I. $G_{\alpha olf}$, a protein which is homogeneously distributed throughout ORNs (Belluscio et al. 1998) yielded signals of similar intensities in both lanes, thus serving as a control for nonpolarized expression. The protein occludin, which is a constituent of the tight junction complex between epithelial supporting cells and ORNs, was less prominent in the cilia preparation as was prohibitin, a marker protein for intracellular membranes (Mishra et al. 2006). Ezrin is a protein localized in the microvilli of epithelial cells (Elsaesser et al. 2005). Although microvilli are located in direct proximity to olfactory cilia and are in contact with agents used in the cilia-detaching procedure, ezrin was merely detected with similar intensities in both lanes. This finding indicates that the cilia preparation contains a small but detectable amount of microvilli membrane. To estimate the degree of enrichment of the analyzed marker proteins densitometrically, we used the software ImageJ 1.37S. We found that CNGA2, CNGA4, and AC III were enriched about 30-, 20-, and 50-fold, respectively, in the ciliary preparation. With occludin or prohibitin, a 50- and 30-fold depletion was measured. For $G_{\alpha olf}$ and ezrin, neither enrichment nor depletion was observed, as shown by the immunoblots. The finding that the ciliary marker proteins are 20–50 times enriched in the cilia preparation, while the microvilli marker ezrin is not detectably enriched, indicates that the preparation contains 20–50 times more ciliary material than microvillar material. In other words, the microvillar contamination of the cilia preparation is only 2–5%. Taken together, these results demonstrate that the preparation obtained by the calcium-shock method is indeed strongly enriched in ciliary membrane but contains a significant amount of nonciliary material.

2D gel electrophoresis and mass spectrometry

The cilia preparation was subjected to 2D gel electrophoresis using the cationic detergent CTAB in the first and SDS in the second dimension. CTAB is particularly suitable for the solubilization of membrane proteins (Navarre et al., 2002; Rais et al., 2004; Helling et al., 2006), whereas the conventional separation method by isoelectric focusing tends to precipitate membrane proteins (Santoni et al. 2000). Because the signal transduction molecules of the ciliary membrane are of critical interest for olfactory biochemistry, we tried to optimize the yield of membrane proteins. Figure 3A shows a 2D gel, stained with colloidal coomassie, that was obtained from the cilia preparation. Western blots showed that the ciliary marker proteins CNGA2 and CNGA4, as well as $G_{\alpha olf}$, were present on the gel (Figure 3B–D). To obtain an overview of the protein content of the cilia preparation, we divided the entire stained area of the 2D gel into 63 areas and prepared each gel block separately for mass spectrometry by in-gel

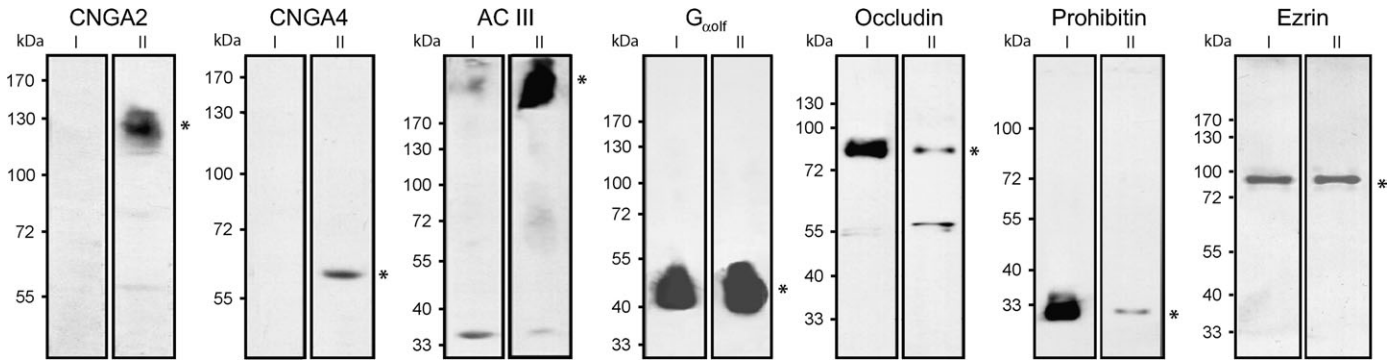

Figure 2 Determination of ciliary membrane enrichment in the cilia preparation. Each pair of western blots compares the amount of a test protein marked by asterisks between whole olfactory epithelium, including cilia (lane I) with the amount of the same protein in the cilia preparation (lane II). CNGA2, CNGA4, and AC III (left) are known to be restricted to ORN cilia. All 3 show much stronger signals in lane II, pointing to enrichment of ciliary membrane in the preparation. The diminished content of the nonciliary proteins occludin and prohibitin supports this interpretation. The ezrin signal in lane II (right) indicates no enrichment but a significant content of microvilli in the cilia preparation. $G_{\alpha olf}$ (center) serves as a control for nonpolarized protein expression. Equal protein loading (10 μg) was adjusted using amido black assays.

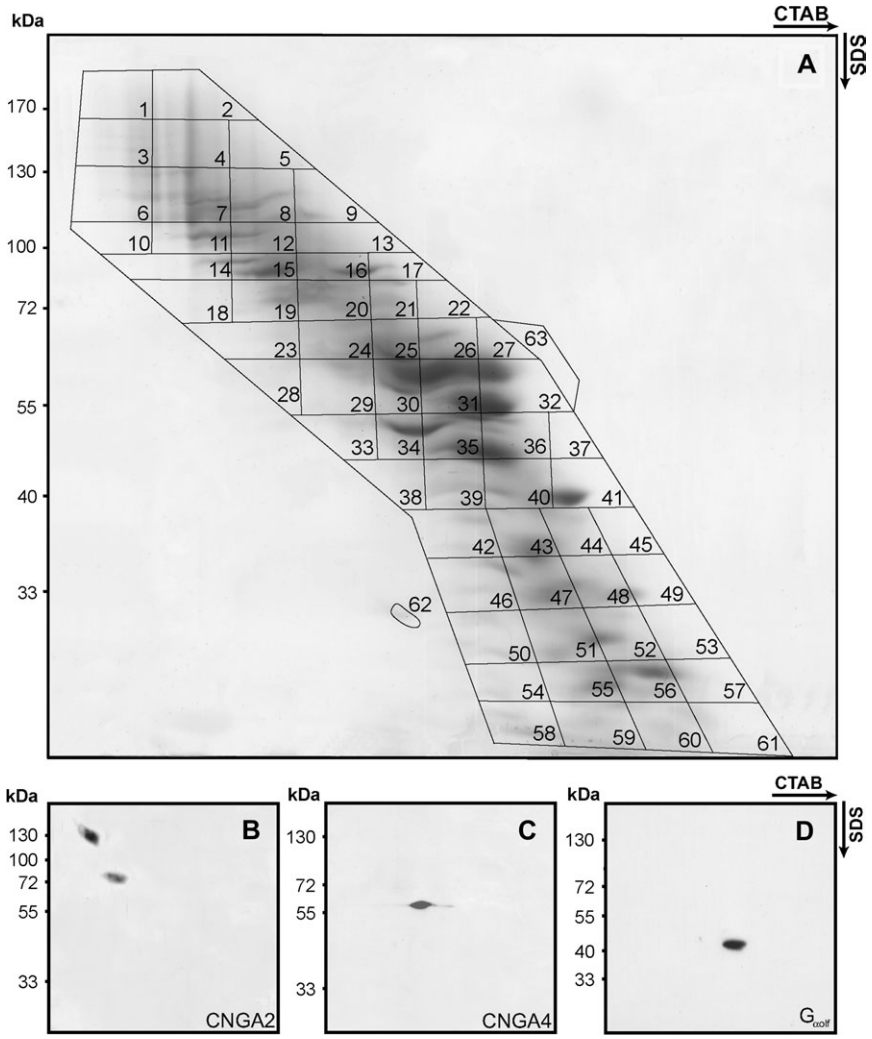

Figure 3 Separation of ciliary membrane proteins by 2D-CTAB/SDS-PAGE. **(A)** Eighty micrograms of ciliary membrane proteins, prepared using the calcium-shock method, were separated and stained with colloidal coomassie. The gel was cut into 63 fragments according to the indicated pattern. Each gel fragment was subjected separately to mass spectrometric analysis to identify the proteins present. **(B)** Western blot of a 2D gel loaded with 60 µg protein each and probed with an antibody raised against the ion channel subunit CNGA2 which is expressed almost exclusively in the ciliary membrane. The 2 signals indicate the glycosylated (~130 kDa) and the deglycosylated (75 kDa) forms of the protein. Detection of **(C)** CNGA4, another specific ciliary protein, and **(D)** $G_{\alpha olf}$, which is homogeneously expressed in the ORNs.

digestion with trypsin. Data obtained from LC-ESI-MS/MS tandem mass spectrometry of the 63 samples were searched against the NCBInr database using the Mascot algorithm for protein identification. The significance threshold for unambiguous protein identification was a minimum of 2 unique peptide fragments with a sum Mascot score of ≥50. In total, 268 distinct rat proteins could be identified in the cilia preparation (Table 1). The identified proteins were grouped according to their expected subcellular localization, and each group is ranked according to their score and to the number of identified peptides per protein. These parameters are an approximate measure for the relative quantity of each identified protein in the samples analyzed by the mass spectrometer (Corbin et al. 2003; Ishihama et al. 2005). They do, however, not reflect the relative amount of proteins in the original cilia preparation prior to CTAB treatment and electrophoresis. The original protein mix can be substantially altered due to specific interactions of each protein with the solvents used in the experiments. The complete absence of the highly glycosylated >210 kDa AC III protein from the protein list is a case in point. The complete protein list including sum Mascot ion scores per number of scored peptides, protein sequence coverage, predicted transmembrane helices, gene annotations, literature references, and short description of the cellular function is available at the authors' Web site (http://www.zoo.uni-heidelberg.de/prot/UM1031). In addition, all individual Mascot search results can be downloaded from this site.

Table 1 The 268 proteins identified in the cilia preparation

gi accession number	Score/peptides	In ORN	Membrane	Protein name
Cilia membrane				
6978543	1481/36	$+^{1}$	m	Na+/K+ ATPase alpha-1 chain
232135	519/12	$+^{1}$	ma	G-protein G(o) subunit alpha 2
6680045	515/11	$+^{1}$	ma	G-protein G(l)/G(S)/G(T) β 1
47271350	455/12	$+^{1}$	ma	G-protein G_{olf} alpha
2851469	241/5	$+^{1}$	ma	G-protein G_{olf} subunit alpha
109466657	220/4	0	m	Stomatin-like protein 3
9296968	201/6	$+^{2}$	ma	G-protein G(q) subunit alpha
6978671	192/8	$+^{1}$	m	Cyclic nucleotide-gated channel 2
149064798	186/5	0	m	Stomatin-like 3 (isoform)
6980964	185/6	$-^{2}$	ma	G-protein G(k) subunit alpha
16758252	152/5	$+^{1}$	m	Cyclic nucleotide-gated channel 4
148747253	137/4	$+^{1}$	m	Na+/K+ ATPase beta-1 chain
61557003	57/2	$+^{2}$	ma	G-protein alpha 13
Plasma membrane				
9845234	832/17	−2	ma	Annexin A2
2981437	776/15	$+^{1}$	ma	Annexin A5
55742832	550/11	$+^{4}$	ma	Annexin A4
40786479	382/7	$+^{2}$	m	NTPDase5
235879	376/8	$-^{2}$	ma	Annexin A1
58865500	279/8	$+^{1}$	ma	Stomatin
34879021	221/6	$+^{1}$	m	SPFH domain-containing protein 2
58865414	221/5	$+^{2}$	ma	Annexin A11
109485936	145/5	$+^{1}$	m	Lectin, mannose-binding 2 like
149026142	137/5	0	m	Rat brain Ca^{2+}-activated Cl^{-} channel
13124118	136/5	$+^{2}$	ma	Flotillin-1 (Reggie-2)
21489987	125/4	$+^{1}$	ma	Cl^{-} ion pump-associated 55 kDA
16930147	85/2	$+^{2}$	ma	Annexin A7
91940	83/3	0	m	Alkaline phosphatase
16758300	80/3	$+^{1}$	m	Phosphodiesterase I ENPP1
13928706	57/2	$+^{1}$	m	Neural cell adhesion molecule 1
Intracellular membrane				
6679299	565/14	$+^{2}$	m	Prohibitin
61556754	551/15	$+^{2}$	m	Prohibitin-2
4105605	523/10	$+^{1}$	m	Voltage-dependent anion channel 1
34856315	497/10	0	ma	V-ATPase B1 subunit
6647578	389/10	$+^{1}$	m	Progesterone receptor component 1
27465561	331/11	$+^{1}$	m	Sphingosine phosphate lyase 1
17105370	320/5	$+^{1}$	m	V-ATPase B2 subunit

Table 1 Continued

gi accession number	Score/peptides	In ORN	Membrane	Protein name
109493234	307/11	0	ma	V-ATPase subunit A 1
13786202	278/5	+[1]	m	Voltage-dependent anion channel 2
40786463	222/7	+[1]	ma	V-ATPase D subunit
2143900	168/5	+[2]	m	Peptidylprolyl isomerase B
4558732	157/3	+[2]	m	Voltage-dependent anion channel 3
149038022	155/6	+[1]	ma	V-ATPase D subunit
62078897	115/5	+[2]	m	Nicalin (nicastrin-like protein)
21489985	90/3	0	m	All-*trans*-retinol 13,14-reductase
67078422	86/2	+[2]	m	Thioredoxin domain-containing 1
28570188	72/2	+[2]	m	Chloride intracellular channel 6
149065272	63/2	0	m	V-ATPase alpha 4
56090369	63/2	0	m	Thioredoxin-related transmembrane 2
19924091	62/2	+[1]	m	Synaptic glycoprotein SC2
29336095	61/2	+[1]	m	Suppressor of lin-12-like protein
38303997	53/2	+[1]	m	Reticulon 3
Membrane unknown				
56605670	356/11	0	m	Leucine-rich repeat-containing 59
82654202	355/9	0	m	Methyltransferase-like 7A
109475725	297/11	0	m	Uncharacterized protein
109478347	265/8	0	m	Dehydrogenase/reductase SDR 7
62079015	197/6	+[1]	m	Uncharacterized protein
62078695	175/5	+[2]	m	Uncharacterized protein
28827824	136/3	0	m	Transmembrane protein 111
56090359	133/7	+[1]	m	Mesenchymal stem cell protein
109483169	118/3	+[1]	m	Hypothetical protein
109510077	117/2	0	m	Hypothetical protein
109507680	96/3	0	m	Similar to thioredoxin domain-containing 10
27676422	84/2	+[1]	m	Palate transmembrane protein 1
66730294	72/2	0	m	Abhydrolase domain-containing 12
Rough ER				
62641851	1202/33	+[1]	–	Alpha glucosidase 2 alpha neutral
129731	991/21	+[2]	–	Prolyl 4-hydroxylase, beta
1352384	874/21	+[2]	–	Protein disulfide isomerase-associated 3
62512124	661/13	+[2]	m	Ribophorin II
109507912	447/12	0	m	Similar to calmegin
62653339	336/12	+[1]	–	Protein kinase C substrate PRKCSH
58865778	307/5	+[2]	m	Dolichol-p_i-mannosyltransferase
13994184	234/7	+[1]	m	Glucosidase 1
6981486	141/4	+[1]	m	Ribophorin I

Table 1 Continued

gi accession number	Score/peptides	In ORN	Membrane	Protein name
34860748	113/5	+[1]	–	Dolichol-(β-D)-mannosyltransferase
71162390	86/3	+[1]	m	Signal sequence receptor alpha
33086606	67/2	+[1]	m	Ab2-417
58865476	52/2	+[1]	m	GPI-anchor transamidase precursor
149039878	50/2	+[1]	m	Lectin, mannose-binding 2
ER/Golgi				
51859516	810/23	+[1]	–	Heat-shock protein HSP 90-beta
149067319	801/20	0	–	Heat-shock protein HSP 90-beta, 1
25282419	487/15	+[1]	m	Calnexin
16758640	426/14	+[1]	m	Suppressor of actin mutations 1 like
17865351	284/8	+[1]	–	Valosin-containing protein, VCP
92022	255/6	+[2]	ma	Ras-related protein Rab-7
2463536	210/6	0	ma	Ras-related protein Rab-11B
4240462	191/6	+[2]	m	VAMP-associated protein A
58865966	172/3	+[1]	–	Endoplasmin, HSP90 beta member 1
420272	169/7	+[1]	ma	Ras-related protein Rab-14
27689505	167/4	+[2]	ma	Ras-related protein Rab-5C
27697481	118/5	+[1]	m	Transmembrane emp24 domain 4
11177880	111/4	+[1]	m	VAMP-associated protein B/C
12083645	106/3	+[2]	ma	Ras-related protein Rab-5A.
420269	102/3	+[1]	ma	Ras-related protein RAB10
51948420	92/5	+[1]	m	B-cell receptor-associated protein 31
121583768	87/2	+[1]	ma	Ras-related protein Rab-5B
109459131	86/4	+[1]	ma	Ras-related protein Rab-6A
60223069	64/2	+[1]	m	Ras-related protein Rab-18
2225894	55/4	+[2]	–	Ras homolog gene family, member A
Smooth ER/microsomal fraction				
6978743	1470/31	+[2]	m	Cytochrome P450 2A3
19924182	1450/27	+[1]	m	Cytochrome P450 2G1
9506531	1256/28	–[2]	m	Cytochrome P450 2F2
77539442	1210/33	+[2]	m	Epoxide hydrolase 1
11560022	1090/26	+[1]	m	UDP-glucuronosyltransferase 2A1
13928780	783/19	+[2]	m	P450 (cytochrome) oxidoreductase
28933457	773/20	+[2]	–	Glutathione S-transferase Mu 2
8393502	757/20	+[2]	–	Glutathione S-transferase Mu 1
31542442	683/14	+[1]	m	Cytochrome P450 1A2
56899	681/15	+[2]	–	Carboxylesterase 3
45737866	633/13	+[2]	–	Aldehyde dehydrogenase 2
57528682	533/12	+[2]	–	Rhodanese

Table 1 Continued

gi accession number	Score/peptides	In ORN	Membrane	Protein name
16758348	529/15	–[2]	–	Peroxiredoxin-6 (EC 1.11.1.15)
6978847	525/13	+[2]	m	Flavin-containing monooxygenase 1
20302049	492/13	0	–	Cytochrome b5 reductase 3
34880876	482/11	0	m	Flavin-containing monooxygenase 6
57303	461/13	+[1]	m	ATPase 2, Ca++ transporting
38454230	448/12	+[2]	–	V-ATPase E subunit
1334284	437/13	+[2]	–	Heat-shock protein 60 kDa
13929028	399/10	+[2]	m	Aldehyde dehydrogenase 3 A2
25742763	376/10	+[1]	–	Heat-shock protein 70kDa member
109492418	371/10	0	–	Similar to carbonyl reductase
1945471	277/7	+[2]	–	Paraoxonase 1
23397411	252/6	+[2]	m	Cytochrome P450 4B1
13591894	228/8	+[2]	–	Aldo-keto reductase family 1, A1
1711569	224/10	+[2]	–	Sulfotransferase 1C1
25282395	211/6	+[2]	–	Glutathione S-transferase M5
2506240	196/6	0	m	Cytochrome P450 3A9
51854237	195/5	+[1]	–	Paraoxonase 3
16758848	195/5	+[2]	–	ER protein 29
18426854	193/4	0	m	Gamma-glutamyltransferase-like 3
78099259	190/6	–[1]	m	Flavin-containing monooxygenase 3
347019	178/9	0	–	Heat-shock 70 kDa family, HSP72
53850628	169/6	+[1]	–	NADH dehydrogenase Fe-S 1
75811937	166/4	0	m	Cytochrome c oxidase subunit 2
61557037	165/6	0	m	Cytochrome b5 reductase 1
27720723	163/6	–[1]	–	Glutathione S-transferase alpha 4
149027586	158/5	+[1]	m	UDP-glucuronosyltransferase 2A2
10720174	133/6	+[1]	–	150 kDa oxygen-regulated protein r
32140186	121/3	0	–	Olfactory-specific acyl CoA synthetase
8393057	115/3	+[3]	m	Heat-shock protein 47 kDa, HSP47
16923958	113/4	+[2]	–	Peroxiredoxin-1
51948390	112/6	+[1]	m	Dehydrogenase/reductase member 8
27702072	101/3	+[1]	–	NADH dehydrogenase Fe-S 3
1906812	95/3	+[1]	–	Carbonyl reductase 1
14192935	95/2	–[1]	–	Aldehyde dehydrogenase 1 A1
11968132	89/3	+[1]	–	Perioredoxin-3
7387724	78/2	+[2]	–	Hydroxyacyl-CoA dehydrogenase 2
56606108	67/2	+[1]	m	NADH dehydrogenase Fe-S 7
20304123	62/2	+[2]	m	3-Mercaptopyruvate sulfurtransferase

Table 1 Continued

gi accession number	Score/peptides	In ORN	Membrane	Protein name
Peroxisomal				
27545384	546/12	0	m	Hydroxysteroid dehydrogenase 9
2492741	489/12	+[1]	–	Hydroxysteroid dehydrogenase 4
13929034	223/8	+[1]	m	Solute carrier family 27 member 2
15375324	101/3	+[1]	m	ATP-binding cassette subfamily D 2
81892292	80/6	+[1]	m	Hydroxysteroid dehydrogenase 12
417242	78/2	+[1]	m	Long-chain acyl-CoA synthetase 6
236058	61/2	0	–	3-α-hydroxysteroid dehydrogenase
Mitochondrial				
54792127	1237/21	+[2]	m	ATP synthase beta
40538742	983/19	+[1]	m	ATP synthase alpha
32189350	530/14	+[1]	m	Solute carrier family 25 member 5
6980972	502/13	+[1]	–	Glutamate oxaloacetate transaminase 2
109483901	482/17	0	–	Acyl-CoenzymeA dehydrogenase 11
55741544	376/12	+[1]	m	Ubiquinol cytochrome c reductase 2
42476181	343/9	+[1]	–	Malate dehydrogenase
10637996	329/11	+[1]	–	Mitochondrial aconitase
149053212	296/9	+[2]	m	Solute carrier family 25 member 11
109488254	282/9	0	m	Solute carrier family 25 member 35
728931	275/7	+[1]	m	ATP synthase gamma
20302061	249/7	+[1]	–	ATP synthase O subunit
32996721	239/5	+[1]	–	NADH dehydrogenase 1α 10 like
47718004	222/6	+[1]	m	Solute carrier family 25 member 3
19705465	217/5	+[1]	m	ATP synthase B
57527204	210/7	+[2]	–	Electron transfer flavoprotein alpha
56090293	202/4	+[1]	–	Pyruvate dehydrogenase beta
19173788	185/8	+[2]	m	Solute carrier family 25 member 10
51948476	170/6	+[1]	m	Ubiquinol-cytochrome c reductase 1
19424338	145/3	+[1]	–	Trifunctional enzyme subunit beta
57114330	143/4	+[1]	ma	Ubiquinol-cytochrome c reductase
149023155	137/3	+[1]	–	Sulfide quinone reductase
60688426	136/4	+[1]	–	NADH dehydrogenase 1 α 9
20806141	121/3	+[1]	m	Solute carrier family 25 member 3
16758446	111/2	+[1]	–	Isocitrate dehydrogenase 3 alpha
6981180	111/3	–[2]	m	Amine oxidase [flavin-containing] B
12621120	100/3	+[1]	m	Sideroflexin-3
135757	98/2	+[1]	–	Acetyl-CoA acetyltransferase
109484025	71/3	+[2]	–	Glycerol-3-p_i-dehydrogenase 1 like

Table 1 Continued

gi accession number	Score/peptides	In ORN	Membrane	Protein name
8392833	65/3	+[2]	–	Acetyl-coenzyme A dehydrogenase medium chain
Cytoskeleton				
5174735	1422/26	0	–	Tubulin beta 2
38328248	1248/22	+[2]	–	Tubulin alpha 1
71620	826/15	+[1]	–	Actin beta
109507063	602/15	+[2]	–	Actin gamma 1
52138521	438/17	–[2]	ma	Ezrin
149036390	406/12	+[1]	m	Mitofilin, heart muscle protein
51854227	396/12	+[2]	–	Gelsolin
55742755	346/8	0	ma	Alpha 1 catenin
34862422	279/6	–[2]	ma	Similar to cytoskeleton-associated 4
56912235	263/5	0	–	Keratin complex 1, acidic, gene 4
13591902	252/5	+[1]	–	Actinin alpha 1
57012378	225/8	0	–	Keratin, type II Kb26
57012382	215/8	0	–	Keratin, type II Kb25
7106439	195/8	+[2]	–	Tubulin, beta 5
57012436	177/4	0	–	Keratin, type I cytoskeletal 10
17902245	153/6	–[2]	ma	Ezrin
68534953	121/5	–[2]	–	Keratin, type I cytoskeletal 18
57012430	121/2	+[2]	–	Keratin complex 1, acidic, gene 2
57114290	103/2	0	–	Keratin, type II Kb2
81891716	101/3	0	–	Keratin, type II cytoskeletal 1
20799322	93/2	+[2]	–	Tubulin beta 3
587518	89/3	–[2]	–	Keratin 18
78097100	81/4	–[2]	–	Microtubule-associated protein 1
30352203	72/2	0	–	Cytokeratin-8
11024674	68/3	+[2]	ma	Solute carrier family 9 isoform 3 1
109486136	66/2	+[2]	–	Actin-related protein 2/3 complex 2
Cytosol: metabolism				
6981146	445/13	+[2]	–	Lactate dehydrogenase
6978809	304/6	+[1]	–	Enolase 1, alpha; Crystallin
62653546	289/9	0	–	Glyceraldehyde-3-p_i-dehydrogenase
56188	117/3	0	–	Glyceraldehyde-3-p_i-dehydrogenase
16757994	113/4	+[1]	–	Pyruvate kinase isozymes M1/M2
12002054	111/2	+[2]	–	Transaldolase
56090564	106/4	+[2]	–	Aldose 1-epimerase
480806	98/5	+[1]	–	Glycogen phosphorylase
109475867	82/2	0	–	Hexose-6-p_i-dehydrogenase
54261608	76/2	+[1]	–	UDP-glucose pyrophosphorylase 2

Table 1 Continued

gi accession number	Score/peptides	In ORN	Membrane	Protein name
62079055	71/6	$-^{2}$	–	NADP+-isocitrate dehydrogenase
8393381	69/3	$+^{2}$	–	Glucose-6-p_i-1-dehydrogenase
15100179	63/2	$+^{1}$	–	Malate dehydrogenase 1
Cytosol: unknown				
109471674	346/11	0	–	Hypothetical protein
62660728	330/10	0	–	SEC14-like protein 3
84781654	238/6	0	–	Sperm flagellar protein 1
28461157	217/6	$+^{2}$	–	Crystallin, lambda 1
60097947	135/6	$+^{1}$	–	SEC14-like protein 2
34867100	122/5	$+^{1}$	–	Uncharacterized protein
62945328	85/2	$+^{2}$	–	Similar to NipSnap2 protein
109500044	85/6	0	–	Hypothetical protein
34866113	80/2	0	–	Tetratricopeptide repeat protein
18266692	80/3	$+^{2}$	–	Selenium-binding protein 2
Cytosol: signal transduction				
23618922	749/21	$+^{1}$	–	Phosphodiesterase 1C
488838	490/7	0	–	Calcium-binding protein 1
56388799	379/8	$+^{1}$	–	Creatine kinase, brain
6756041	331/8	$+^{1}$	–	14-3-3 protein zeta/delta
13928824	203/7	$+^{2}$	–	14-3-3 protein epsilon
11693172	188/7	$+^{1}$	–	Calreticulin
56605938	177/7	$+^{1}$	–	Thioredoxin domain-containing 4
34856626	164/6	$-^{1}$	–	Reticulocalbin 1 precursor
62643032	142/7	$+^{1}$	–	Calcyphosine like
12018304	112/3	0	–	KPL2 protein
34856103	78/4	$+^{2}$	–	Nodal modulator 1
25282405	66/3	$-^{2}$	–	Plunc
Ribosomal				
70850	377/13	$+^{2}$	–	40S ribosomal protein S3
11693176	291/7	$-^{2}$	–	60S acidic ribosomal protein P0
13385036	251/7	$-^{2}$	–	60S ribosomal protein L15
62642554	204/10	0	–	Similar to 60S ribosomal protein L7a
28460696	189/6	$+^{2}$	–	Elongation factor 1-alpha 1
109458180	147/4	$+^{1}$	–	Similar to 40S ribosomal protein S5
13592057	147/5	$-^{2}$	–	60S ribosomal protein L18
8393296	113/4	$+^{2}$	–	Eukaryotic elongation factor-2
27660180	83/2	$+^{2}$	–	60S ribosomal protein L7
27665858	67/5	0	–	Similar to 40S ribosomal protein S9
2920825	63/2	$+^{2}$	–	40S ribosomal protein S2

Table 1 Continued

gi accession number	Score/peptides	In ORN	Membrane	Protein name
12621122	54/2	$+^{2}$	–	60S ribosomal protein L14
Nucleus				
5902076	363/11	$+^{2}$	–	Splicing factor, arginine/serine-rich 1
92559	264/9	$+^{2}$	–	Nucleolin
8393519	161/7	$+^{1}$	–	H2A histone family, member Y
109504563	70/3	0	–	Splicing factor, arginine/serine-rich 3
84781668	62/2	$-^{2}$	–	Similar to splicing factor, arginine/serine-rich 7
Secreted				
109465410	709/15	$+^{2}$	–	Chitinase 3-like 4
109480584	260/7	$+^{2}$	ma	Carboxypeptidase M
13540638	90/2	0	–	Aminopeptidase PILS
149033753	87/2	0	–	Albumin
913986	70/2	$-^{2}$	–	Apolipoprotein E

Each protein is identified by its NCBI gi accession number. Proteins are categorized according to their subcellular localization derived from the literature or bioinformatic protein resources. The proteins of each category are ranked by decreasing sum Mascot ion score and number of identified peptides. The respective protein expression in ORNs was derived from a GeneChip expression profiling study (Sammeta et al., 2007): + indicates gene expression in ORNs, – indicates lack of gene expression, and 0 indicates that no information about gene expression was available. The following abbreviations are used: $+^{1}$ indicates GFP^{+}/GFP^{-} ratio ≥ 1.3, GFP^{+} signal > 150; $+^{2}$ indicates GFP^{+}/GFP^{-} ratio 0.5–1.2, GFP^{+} signal > 150; $+^{3}$ indicates GFP^{+}/GFP^{-} ratio ≥ 1.3, GFP^{+} signal < 150; $+^{4}$ indicates GFP^{+}/GFP^{-} ratio ≥ 0.5, GFP^{+} signal < 150; $-^{1}$ indicates GFP^{+}/GFP^{-} ratio < 0.5, GFP^{+} signal < 150; $-^{2}$ indicates GFP^{+}/GFP^{-} ratio < 0.5, GFP^{+} signal > 150. Transmembrane proteins are indicated by m, membrane-associated proteins by ma. ATP, adenosine triphosphate. Detailed information on all 268 identified proteins is available at http://www.zoo.uni-heidelberg.de/prot/UM1031.

Bioinformatic analysis of the identified proteins

Figure 4 summarizes distinctive properties of the identified protein set. Molecular weights of 10 to >100 kDa were detected (Figure 4A), illustrating that the CTAB/SDS gel covers a range that represents a considerable fraction of relevant protein sizes. Figure 4B shows that the protein set contains acidic, neutral, and basic proteins with isoelectric point (IP) values ranging from 4 to 12. Importantly, there was no systematic codependence of molecular weight and IP (Figure 4C), demonstrating that no IP range was excluded from any range of protein sizes by the separation method. To identify membrane proteins, we used the prediction algorithms for transmembrane helices of the TMHMM Server v2.0 (Krogh et al. 2001) and the SOSUI (Hirokawa et al. 1998) server for classification of integral membrane proteins. Of the 268 proteins identified in the cilia preparation, 83 (31%) are integral membrane proteins with 1–10 predicted membrane-spanning domains (Figure 4D).

Cellular source and subcellular localization of the identified proteins

To predict the cellular source of the 268 proteins identified in this proteomic study, we compared the genes encoding these proteins to the list of genes recently published in Sammeta et al. (2007), which predicts with great accuracy whether a gene is expressed primarily by ORNs. For 57 proteins (21%), no corresponding gene entry could be found. Of the remaining proteins, 190 (90%) were found to be expressed in ORNs with, respectively, >99% ($P = 0.0001$, 110 proteins) and >83% ($P = 0.05$, 80 proteins) confidence (Table 1, column "In ORN"). This clearly demonstrates that ORNs are the primary cellular source of the cilia preparation.

Using the curated subcellular localization database LOCATE (Fink et al. 2006) and peer-reviewed publications, we categorized all proteins identified in the cilia preparation according to the classification criteria of the Gene Ontology Consortium (Ashburner et al. 2000). We assigned the identified proteins to 3 main groups that are subdivided into different categories (Figure 5A). Of these proteins, 49% could be assigned to the membrane fraction, which includes integral membrane proteins as well as membrane-associated proteins. The remaining 51% are composed of soluble proteins (41%) and proteins belonging to the cytoskeleton (10%). The high percentage of membrane proteins clearly demonstrates the suitability of the calcium-shock method combined with 2D-CTAB/SDS-PAGE for purification of membrane proteins. Within the membrane protein fraction, components of the olfactory signal transduction cascade were robustly identified, including GTP-binding proteins and 2 subunits

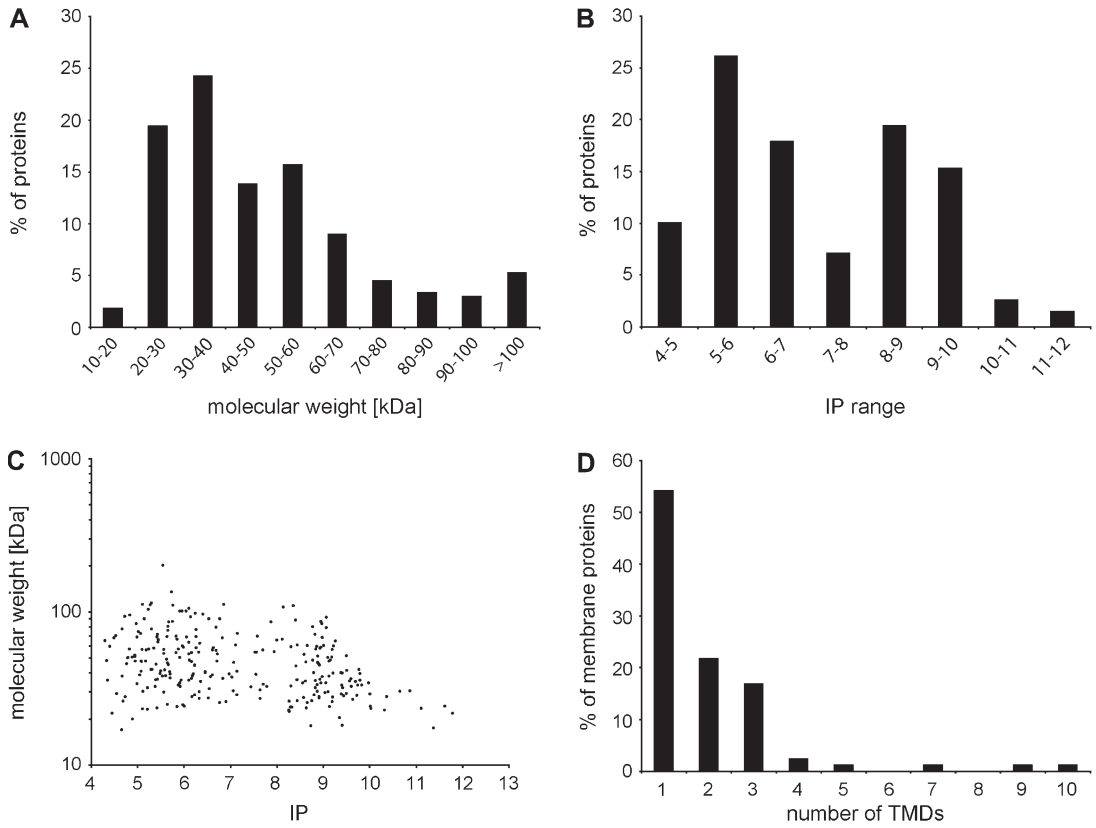

Figure 4 Characterization of the protein set identified in the cilia preparation. The proteins separated by 2D-CTAB/SDS-PAGE and identified by mass spectrometry cover a broad range of molecular weights **(A)** and IP. **(B)** No systematic relation between these parameters is apparent **(C)**, demonstrating that the full range of IPs is represented in all groups of molecular weights. **(D)** Of the identified proteins, 31% are predicted to be integral membrane proteins. The histogram illustrates the distribution of numbers of predicted transmembrane domains (TMDs) among the protein set.

of the cAMP-gated transduction channel (CNGA2 and CNGA4). The high identification scores of these components are in good accordance with the immunoblot analysis and demonstrate the effective enrichment of ciliary membranes. The absence of AC III from the protein list most likely indicates that the predominant glycosylated form of this protein (>210 kDa, Figure 2) did not enter the separation gel. In addition to ciliary proteins, we also detected proteins belonging to organelle membranes, indicating contributions from the endoplasmic reticulum (ER), mitochondria, the Golgi system, and cytosolic vesicles. Although density-gradient centrifugation was applied to the isolated cilia, a high proportion of soluble proteins were identified which originated from mitochondria, ER, ribosomes, and cytosol. These proteins are mainly involved in metabolism, xenobiosis, protein biosynthesis, and signal transduction processes. The latter category contains the strongly enriched cAMP-dependent phosphodiesterase PDE1C, which is a key component of the signal transduction cascade in olfactory cilia. Thus, our data demonstrate that the enriched cilia preparation resulting from calcium-shock treatment contains a number of proteins that are not associated with the primary transduction process.

Discussion

Sensory cells with polarized protein expression patterns present a special challenge to research. The proteins involved in signal transduction are usually restricted to specific sensory organelles, such as the photoreceptor outer segment or the olfactory sensory cilia. Biochemists need to separate these organelles from the rest of the cells in order to dissect and to study the relevant proteins. Based on deciliation procedures for gill tissue of the mollusc *Aequipecten irradians* (Link 1973), Rhein and Cagan (1980) developed an isolation method for ORN sensory cilia that uses a calcium-shock treatment followed by centrifugation to detach and harvest the cilia from the olfactory epithelium. Chen and Lancet (1984) and Anholt et al. (1986) improved this technique and established the cilia preparation for biochemical experimentation with ciliary membranes. Examination of membrane structures by electron microscopy revealed that the ciliary membrane was largely detached from the axoneme and reorganized in vesicles of 100–500 nm diameter (Anholt et al. 1986). This preparation was considered to be contaminated to some extent with membranes from other organelles. But, in the absence of specific ciliary markers, it

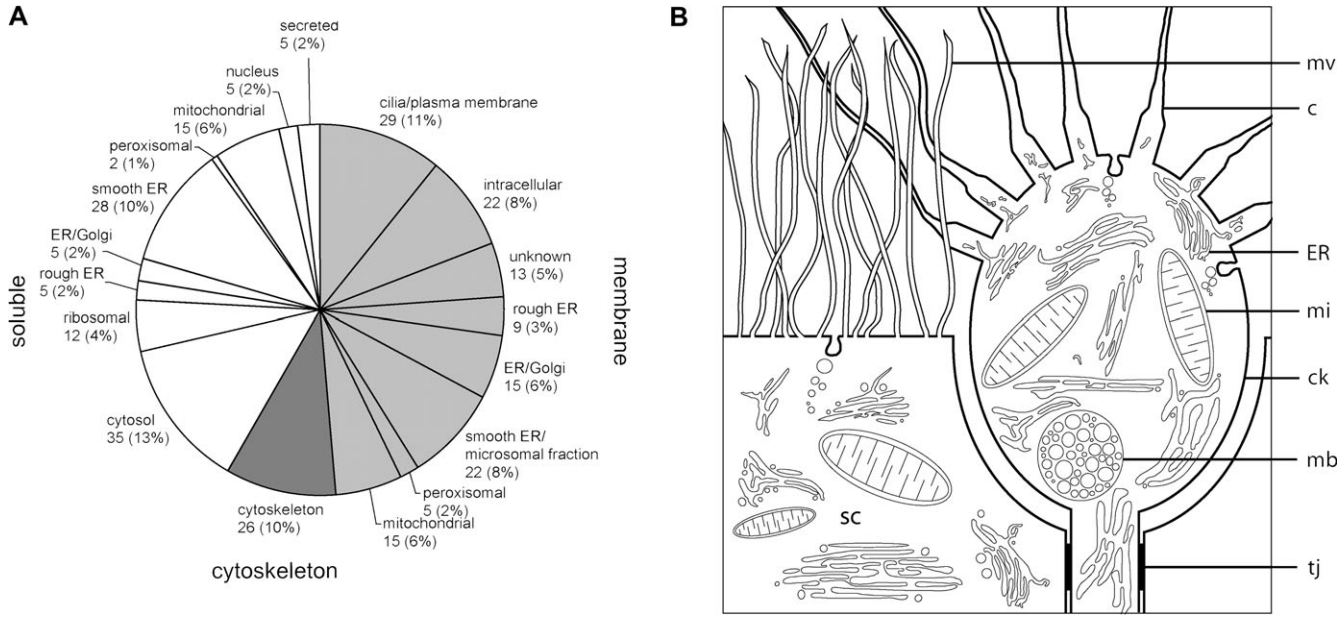

Figure 5 Proteins identified in the cilia preparation. **(A)** Graphical representation of the predicted subcellular distribution of all 268 proteins identified in the cilia preparation. Numbers of proteins are given for each category with the percentage (relative to 268 proteins) in parentheses. A detailed list of all proteins is available at http://www.zoo.uni-heidelberg.de/prot/UM1031. **(B)** Schematic representation of the proximal segments of olfactory cilia (c) with the ciliary knob (ck) and of an epithelial supporting cell (sc) with its microvilli (mv). Indicated are the subcellular structures discussed in the text: mitochondria (mi), ER, and tight junctions (tj). Modified from the electron microscopic study of Andres (1969).

was not possible to determine its purity. Nevertheless, the cilia preparation was successfully applied in many biochemical studies and yielded information of critical importance for olfactory research. Further biochemical investigations will benefit from a catalogue of proteins present in the cilia preparation because these proteins represent a significant part of the protein inventory involved in sensory function, biotransformation, and other tasks performed by ORNs.

Of our identified proteins, 211 (78%) could be assigned to the GeneChip expression profiling of OMP–GFP cells (GFP^+) versus all nonfluorescent olfactory cell types (GFP^-) (Sammeta et al. 2007). One hundred and ninety (90%) of them are in fact expressed in ORNs with 83% confidence. Many of the proteins listed in the specific subcellular categories ''cilia membrane,'' ''plasma membrane,'' ''intracellular membrane,'' ''rough ER,'' ''ER/Golgi,'' ''peroxisomal,'' or ''mitochondrial'' even meet more stringent criteria (GFP^+/GFP^- ratios ≥1.3, GFP^+ signals >150; Sammeta et al. 2007), where ORN expression is supported with 99% confidence. In contrast, most of the biotransformation enzymes, ribosomal proteins, cytosolic proteins, or cytoskeleton proteins have intermediate GFP^+/GFP^- ratios (0.5–1.2) and GFP^+ signals above 150, indicating that these genes are expressed in ORNs as well in other cell types. In other words, some of these proteins may, therefore, partly originate from contaminating epithelial supporting cells or other olfactory cell types. Ezrin, for example, a cytoskeletal protein expressed in supporting cells but not in ORNs (Elsaesser et al. 2005; GFP^+/GFP^- ratio = 0.1), points to the presence of small amounts of microvillar material in the preparation. In addition, the xenobiotic-metabolizing enzymes are thought to be expressed in ORNs and epithelial supporting cells but with much higher expression levels in the latter (Dahl and Hadley 1991; Thornton-Manning and Dahl 1997; Pataramekin and Meisami 2005). Nevertheless, in conjunction with the immunoblot experiments it could be stated that the preparation is strongly enriched in ORN membranes and the contribution of other cell types appears to be small.

The detection of odorants involves odorant-binding proteins, olfactory receptor proteins, GTP-binding proteins, AC III, transduction channels, as well as various regulatory proteins (reviews: Frings 2001; Ronnett and Moon 2002). Many of the signal transduction molecules could be identified in our data set with robust peptide signals. They have been localized to the distal segment of the cilia (Menco 1997). Apart from the axoneme, the distal cilia contain no discernible structures, suggesting that this largest part of the cilia is specialized for the transduction process. In contrast, the proximal segment and the ciliary knobs show various subcellular structures (Figure 5B; Andres 1969) that are important for the interpretation of biochemical data obtained from the cilia preparation. Knobs and proximal cilia contain smooth endoplasmic reticulum (sER), and about 50 of our identified 268 proteins are localized to that compartment (sER/microsomal fraction). Most of these proteins are biotransformation enzymes involved in the xenobiotic metabolism as well as various stress-induced proteins (e.g., 4 heat-shock proteins). This collection of cytoprotective proteins may reflect the

precarious situation of a neuron, which is exposed to every chemical compound present in the inhaled air. Mitochondria within the ciliary knob supply adenosine triphosphate for the transduction process because no mitochondria are present in the distal segments of the cilia themselves. Notably, the gene expression profiling (Sammeta et al. 2007) indicated that most of the 30 mitochondrial proteins (GFP^+/GFP^- ratios ≥1.3, GFP^+ signals >150) found in the cilia preparation originated most likely from ORNs. Ultrastructural and light microsopic studies have demonstrated vesicle trafficking in the ciliary knobs (e.g., Mashukova et al. 2006). Thus, sER, endosomes, multivesicular bodies, and other vesicular structures are present in the apical regions of both ORNs and supporting cells (Andres 1969) and are possible sources for the microsomal and peroxisomal proteins in our data set. But, again, the gene expression profiling of these proteins points most likely to an ORN origin. The presence of 35 cytosolic proteins, mainly enzymes of the glycolytic metabolism and various signal transduction processes, may be interpreted as a consequence of vesicle formation and trapping of cytosol during the calcium-shock treatment (Anholt et al. 1986). Finally, a small number of nucleus- or rough ER–associated proteins (in total 19 proteins; 7%) was detected. Considering that the olfactory epithelium undergoes a continuous turnover and that a sizeable fraction of ORNs is in the process of apoptosis at any time, this contamination with somatic proteins appears small.

In summary, this study revealed that the cilia preparation routinely used in olfactory biochemistry is indeed highly enriched in ciliary over nonciliary membranes. Our proteomic analysis provides a window into the set of proteins present in the cilia preparation and suggests that the cilia are detached close to their base at the dendritic endings of the ORNs. Many proteins originating from sER and mitochondria (in total 80 proteins), but much fewer from rough ER and nucleus (in total 19 proteins), are present in the preparation. This suggests that part of the apical segment of the ORNs (ciliary knobs plus cilia) is collected in the preparation, whereas somatic and dendritic materials are largely excluded. For biochemical analyses, it should be taken into account that the preparation contains proteins related to different physiological tasks, which include signal transduction, biotransformation, metabolism, neurogenesis, and apoptosis.

Funding

Deutsche Forschungsgemeinschaft (Fr 937/8); Studienstiftung des Deutschen Volkes (D.K.).

Acknowledgements

We are grateful to Dr Tore Kempf (DKFZ Heidelberg) for valuable help with the protein analysis and to Dr Urban Liebel (Karlsruhe Research Center) for assistance with the FZK Harvester. We thank the Nikon Imaging Center at the University of Heidelberg for access to confocal microscopy.

References

Altschul SF, Gish W. 1996. Local alignment statistics. Methods Enzymol. 266:460–480.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol. 5:403–410.

Andres KH. 1969. Der olfaktorische Saum der Katze. Z Zellforsch. 96: 250–274.

Anholt RRH, Aebi U, Snyder SH. 1986. A partially purified preparation of isolated chemosensory cilia from the olfactory epithelium from the bullfrog, Rana catesbeiana. J Neurosci. 6:1962–1969.

Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, et al. 2000. Gene ontology: tool for the unification of biology. Nat Genet. 25:25–29.

Beites CL, Kawauchi S, Crocker CE, Calof AL. 2005. Identification and molecular regulation of neural stem cells in the olfactory epithelium. Exp Cell Res. 306:309–316.

Belluscio L, Gold GH, Nemes A, Axel R. 1998. Mice deficient in G_{olf} are anosmic. Neuron. 20:69–81.

Boekhoff I, Breer H. 1992. Termination of second messenger signaling in olfaction. Proc Natl Acad Sci USA. 89:471–474.

Boekhoff I, Schleicher S, Strotmann J, Breer H. 1992. Odor-induced phosphorylation of olfactory cilia proteins. Proc Natl Acad Sci USA. 89: 11983–11987.

Bönigk W, Bradley J, Müller F, Sesti F, Boekhoff I, Ronnett GV, Kaupp UB, Frings S. 1999. The native rat olfactory cyclic nucleotide-gated channel is composed of three distinct subunits. J Neurosci. 19:5332–5347.

Bradley J, Zhang Y, Bakin R, Lester HA, Ronnett GV, Zinn K. 1997. Functional expression of the heteromeric "olfactory" cyclic nucleotide-gated channel in the hippocampus: a potential effector of synaptic plasticity in brain neurons. J Neurosci. 17:1993–2005.

Breer H, Boekhoff I. 1992. Second messenger signalling in olfaction. Curr Op Neurobiol. 2:439–443.

Breer H, Boekhoff I, Tareilus E. 1990. Rapid kinetics of second messenger formation in olfactory transduction. Nature. 345:65–68.

Buck L, Axel R. 1991. A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. Cell. 65:175–187.

Chen Z, Lancet D. 1984. Membrane proteins unique to vertebrate olfactory cilia: candidates for sensory receptor molecules. Proc Natl Acad Sci USA. 81:1859–1863.

Corbin RW, Paliy O, Yang F, Shabanowitz J, Platt M, Lyons CE, Root K, McAuliffe J, Jordan MI, Kustu S, et al. 2003. Toward a protein profile of *Escherichia coli*: comparison to its transcription profile. Proc Natl Acad Sci USA. 100:9232–9237.

Dahl AR, Hadley WM. 1991. Nasal cavity enzymes involved in xenobiotic metabolism: effects on the toxicity of inhalants. Crit Rev Toxicol. 21: 345–372.

Delgado R, Saavedra MV, Schmachtenberg O, Sierralta J, Bacigalupo J. 2003. Presence of Ca^{2+}-dependent K^+ channels in chemosensory cilia support a role in odor transduction. J Neurophysiol. 90:2022–2028.

Elsaesser R, Montani G, Tirindelli R, Paysan J. 2005. Phosphatidyl-inositide signalling proteins in a novel class of sensory cells in the mammalian olfactory epithelium. Eur J Neurosci. 21:2692–2700.

Fink JL, Aturaliya RN, Davis MJ, Zhang F, Hanson K, Teasdale MS, Kai C, Kawai J, Carninci P, Hayashizaki Y, et al. 2006. LOCATE: a mouse protein subcellular localization database. Nucleic Acids Res. 34:D213–D217.

Frings S. 2001. Chemoelectrical signal transduction in olfactory sensory neurons of air-breathing vertebrates. Cell Mol Life Sci. 58:510–519.

Gheusi G, Lledo P-M. 2007. Control of early events in olfactory processing by adult neurogenesis. Chem Senses. 32:397–409.

Hartinger J, Stenius K, Högemann D, Jahn R. 1996. 16-BAC/SDS-PAGE: a two-dimensional gel electrophoresis system suitable for the separation of integral membrane proteins. Anal Biochem. 240:126–133.

Helling S, Schmitt E, Joppich C, Schulenborg T, Mullner S, Felske-Muller S, Wiebringhaus T, Becker G, Linsenmann G, Sitek B, et al. 2006. 2-D differential membrane proteome analysis of scarce protein samples. Proteomics. 6:4506–4513.

Henion TR, Schwarting GA. 2007. Patterning the developing and regenerating olfactory system. J Cell Physiol. 210:290–297.

Hirokawa T, Boon-Chieng S, Mitaku S. 1998. SOSUI: classification and secondary structure prediction system for membrane proteins. Bioinformatics. 14:378–379.

Ishihama Y, Oda Y, Tabata T, Sato T, Nagasu T, Rappsilber J, Mann M. 2005. Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. Mol Cell Proteomics. 4:1265–1272.

Kleene SJ, Gesteland RC. 1991. Calcium-activated chloride conductance in frog olfactory cilia. J Neurosci. 11:3624–3629.

Krogh A, Larsson B, von Heijne G, Sonnhammer ELL. 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol. 305:567–580.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227:680–685.

Liebel U, Kindler B, Pepperkok R. 2004. 'Harvester': a fast meta search engine of human protein resources. Bioinformatics. 20:1962–1963.

Ling G, Gu J, Genther MB, Zhuo X, Ding X. 2004. Regulation of cytochrome P450 gene expression in the olfactory mucosa. Chem Biol Interact. 147:247–258.

Link RW. 1973. Comparative isolation of cilia and flagella from the lamellibranch mollusc, *Aequipecten irradians.* J Cell Sci. 12:345–367.

Lowe G, Nakamura T, Gold GH. 1989. Adenylate cyclase mediates olfactory transduction for a wide variety of odorants. Proc Natl Acad Sci USA. 86:5641–5645.

Marini S, Longo V, Mazzaccaro A, Gervasi PG. 1998. Xenobiotic-metabolizing enzymes in pig nasal and hepatic tissues. Xenobiotica. 28:923–935.

Mashukova A, Spehr M, Hatt H, Neuhaus EM. 2006. Beta-arrestin2-mediated internalization of mammalian odorant receptors. J Neurosci. 26:9902–9912.

Menco BPhM. 1997. Ultrastructural aspects of olfactory signaling. Chem Senses. 22:295–311.

Mishra S, Murphy LC, Murphy LJ. 2006. The prohibitins: emerging roles in diverse functions. J Cell Mol Med. 10:353–363.

Moon C, Jaberi P, Otto-Bruc A, Baehr W, Palczewski K, Ronnett GV. 1998. Calcium-sensitive particulate guanylyl cyclase as a modulator of cAMP in olfactory receptor neurons. J Neurosci. 18:3195–3205.

Nakamura T, Gold GH. 1987. A cyclic nucleotide-gated conductance in olfactory receptor cilia. Nature. 325:442–444.

Navarre C, Degand H, Bennett KL, Crawford JS, Mørtz E, Boutry M. 2002. Subproteomics: identification of plasma membrane proteins from the yeast Saccharomyces cerevisiae. Proteomics. 2:1706–1714.

Neuhoff V, Stamm R, Pardowitz I, Arold N, Ehrhardt W, Taube D. 1990. Essential problems in quantification of proteins following colloidal staining with coomassie brilliant blue dyes in polyacrylamide gels, and their solution. Electrophoresis. 11:101–117.

Nicolay DJ, Doucette JR, Nazarali AJ. 2006. Transcriptional regulation of neurogenesis in the olfactory epithelium. Cell Mol Neurobiol. 26:803–813.

Pace U, Hanski E, Salomon Y, Lancet D. 1985. Odorant-sensitive adenylate cyclase may mediate olfactory reception. Nature. 316:255–258.

Pataramekin PP, Meisami E. 2005. Cytochrome oxidase staining reveals functionally important activity bands in the olfactory epithelium of newborn rat. J Neurocytol. 34:257–268.

Pfeuffer E, Mollner S, Lancet D, Pfeuffer D. 1989. Olfactory adenylyl cyclase. Identification and purification of a novel enzyme form. J Biol Chem. 264:18803–18807.

Potter SM, Zheng C, Koos DS, Feinstein P, Fraser SE, Mombaerts P. 2001. Structure and emergence of specific olfactory glomeruli in the mouse. J Neurosci. 21:9713–9723.

Rais J, Karas M, Schagger H. 2004. Two-dimensional electrophoresis for the isolation of integral membrane proteins and mass spectrometric identification. Proteomics. 4:2567–2571.

Rhein LD, Cagan RH. 1980. Biochemical studies of olfaction: isolation, characterization, and odorant binding activity of cilia from rainbow trout olfactory rosettes. Proc Natl Acad Sci USA. 77:4412–4416.

Ronnett GV, Moon C. 2002. G proteins and olfactory signal transduction. Annu Rev Physiol. 64:189–222.

Sammeta N, Yu T, Bose SC, McClintock TS. 2007. Mouse olfactory sensory neurons express 10,000 genes. J Comp Neurol. 502:1138–1156.

Santoni V, Molloy M, Rabilloud T. 2000. Membrane proteins and proteomics: un amour impossible? Electrophoresis. 21:1054–1070.

Schaffner W, Weissmann C. 1973. A rapid, sensitive and specific method for the determination of protein in dilute solution. Anal Biochem. 56:502–514.

Schild D, Restrepo D. 1998. Transduction mechanisms in vertebrate olfactory receptor cells. Physiol Rev. 78:429–466.

Schleicher S, Boekhoff I, Arriza J, Lefkowitz RJ, Breer H. 1993. A β-adrenergic receptor kinase-like enzyme is involved in olfactory signal termination. Proc Natl Acad Sci USA. 90:1420–1424.

Sklar PB, Anholt RRH, Snyder SH. 1986. The odorant-sensitive adenylate cyclase of olfactory receptor cells. J Biol Chem. 261:15538–15543.

Thornton-Manning JR, Dahl AR. 1997. Metabolic capacity of nasal tissue interspecies comparisons of xenobiotic-metabolizing enzymes. Mutat Res. 380:43–59.

Towbin H, Staehelin E, Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA. 76:4350–4354.

Wei J, Zhao AZ, Chan GCK, Baker LP, Impey S, Beavo JA, Storm DR. 1998. Phosphorylation and inhibition of olfactory adenylyl cyclase by CaM kinase II in neurons: a mechanism for attenuation of olfactory signals. Neuron. 21:495–504.

Yan C, Zhao AZ, Bentley JK, Loughney K, Ferguson K, Beavo J. 1995. Molecular cloning and characterization of a calmodulin-dependent phosphodiesterase enriched in olfactory sensory neurons. Proc Natl Acad Sci USA. 92:9677–9681.

Accepted October 4, 2007