CHAPTER TWELVE

Olfactory Cilia: Our Direct Neuronal Connection to the External World

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Contents

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Abstract

An organism's awareness of its surroundings is dependent on sensory function. As antennas to our external environment, cilia are involved in fundamental biological processes such as olfaction, photoreception, and touch. The olfactory system has adapted this organelle for its unique sensory function and optimized it for detection of external stimuli. The elongated and tapering structure of olfactory cilia and their organization into an overlapping meshwork bathed by the nasal mucosa is optimized to enhance odor absorption and detection. As many as 15–30 nonmotile, sensory cilia on dendritic endings of single olfactory sensory neurons (OSNs) compartmentalize signaling molecules necessary for odor detection allowing for efficient and spatially confined responses to sensory stimuli. Although the loss of olfactory cilia or deletion of selected components of the olfactory signaling cascade leads to anosmia, the mechanisms of ciliogenesis and the selected enrichment of signaling molecules remain poorly understood. Much of our current knowledge is the result of elegant electron microscopy studies describing the structure and organization of the olfactory epithelium and cilia. New genetic and cell biological approaches, which compliment these early studies, show promise in elucidating the mechanisms of olfactory cilia assembly, maintenance, and compartmentalization. Importantly, emerging evidence suggests that olfactory dysfunction represents a previously unrecognized clinical manifestation of multiple ciliary disorders. Future work investigating the mechanisms of olfactory dysfunction combining both clinical studies with basic science research will provide us important new information regarding the pathogenesis of human sensory perception diseases.

1. Olfaction as a Sensory Modality

Chemosensory systems, such as the olfactory and gustatory systems, give us an awareness of our immediate environment by allowing us to detect airborne and fluidborne stimuli. The gustatory and olfactory systems are thought to have evolved from the chemical sensing apparatuses, which can be found on almost all creatures in the animal kingdom (Hildebrand and Shepherd, 1997). The olfactory system is responsible for the detection of volatile chemicals dissolved in the air around us. While the olfactory system is necessary for detecting odors and crucial for our sense of taste, it also plays important roles in our quality of life, health, and safety. Dysosmia (impaired sense of smell) or anosmia (loss of ability to smell) can prevent us from detecting signs of danger such as smoke or spoiled food, and also can lead to medical problems such as weight gain and poor nutrition (Toller, 1999). Impaired olfactory function is thought to affect over 2 million Americans and over 50% of those over the age of 65 (Murphy *et al.*, 2002), however, this may be a gross underestimate given that olfactory dysfunction

frequently goes unreported (Nguyen-Khoa *et al.*, 2007). While in some cases we understand the cause of olfactory dysfunction, in at least 20% of cases the underlying etiology remains unknown (Jafek, 2000).

Inhalation of odorants across the surface of the olfactory epithelium (OE) initiates the olfactory signaling cascade, which involves the binding of odorants to receptors localized on the cilia of olfactory sensory neurons (OSNs). In the canonical pathway, activated odorant receptors (ORs) act through a stimulatory G protein-coupled mechanism to activate adenylyl cyclase type III (ACIII) and increase the ciliary concentration of cAMP. Olfactory cyclic nucleotide-gated (CNG) channels open in response to cAMP binding and allow the depolarization of the OSN that is further amplified by the Ca^{2+}-activated Cl^- channel. All of the components necessary for odorant detection are enriched in olfactory cilia, and any perturbation in the localization of these components or in cilia themselves causes impaired olfactory function. In this chapter, we will focus on olfactory cilia including structure and function, developmental formation and relation to human disease.

2. Anatomy and Organization of the Olfactory Epithelium

2.1. Gross anatomy

In humans, the olfactory and respiratory epithelia line the three bony turbinates and cartilaginous septum of the nasal passage. In other mammals such as rodents there is a larger number of turbinates (I, II, IIb, III, IV), which presumably either fused or were lost during evolution to bipedalism (Harkema *et al.*, 2006). In the human, the respiratory epithelium, which is responsible for warming, cleaning, and humidifying inspired air, lines most of the inferior and middle turbinates as well as a portion of the superior turbinate. The OE, however, is segregated from the respiratory epithelium and is responsible for the detection of volatile chemicals dissolved in the air. It lines part of the nasal septum, the remainder of the superior turbinate and potentially part of the middle turbinate with a combined surface area of 1–2 cm^2 (Bucher, 1973; Leopold *et al.*, 2000). As odorant-containing air passes across the turbinates it is exposed to the OE, which contains the sensory element of the olfactory system, the OSN.

Inspired odorants bind to ORs localized on the ciliary membrane of OSNs to stimulate the olfactory sensory cascade (Fig. 12.1E). Bundled axons from OSNs form the olfactory nerve and serve to transmit their information to synapses on mitral cells and tufted cells in the glomeruli of the main olfactory bulb (MOB) (Hinds and Hinds, 1976a,b). From the

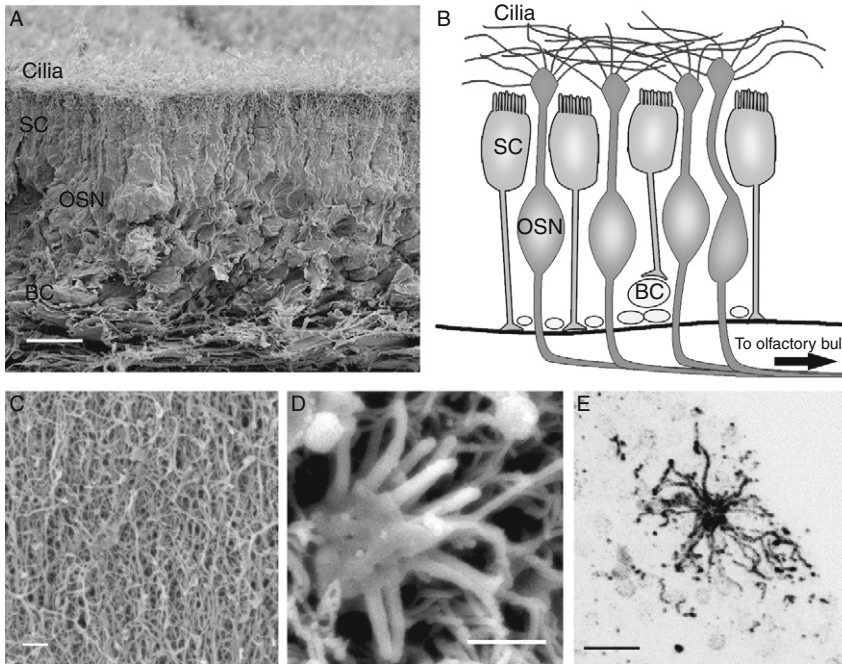

Figure 12.1 *Anatomy of the olfactory epithelium*. Mouse olfactory epithelium was dissected, fixed in glutaraldehyde, and processed for scanning electron microscopy as previously described (McEwen *et al.*, 2007). Scanning electron micrographs were captured using an Amray (Drogheda, Ireland) 1910FE field emission scanning electron microscope at 5 kV. Images were recorded digitally with Semicaps software. (A) Side view of a scanning electron micrograph at 1030× zoom from mouse olfactory epithelium (image courtesy of Wanda Layman and Donna Martin, Department of Human Genetics, University of Michigan). The layers of the olfactory epithelium are labeled on the left of the image. For (A) and (B), OSN = olfactory sensory neuron, SC = sustentacular cell, BC = basal cell. Scale bar represents 10 μm. (B) Cartoon representation of the organization of the various cell types in the olfactory epithelium. (C) Scanning electron micrograph of a surface view of the olfactory epithelium shown at 7400 × zoom. The dense meshwork of overlapping cilia across the surface of the epithelium is visible. Scale bar represents 1 μm. (D) Scanning electron micrograph of a single dendritic knob showing multiple cilia extending from the surface of the knob. Image is shown at 16,000× zoom. Scale bar is 1 μm. (E) Immunocytochemistry of mouse olfactory epithelium stained for an odorant receptor, mOR28 (antibody courtesy of Dr. Richard Axel). A 14-μm thick slice of mouse olfactory epithelium was stained as described previously (McEwen *et al.*, 2007). Numerous cilia can be observed expressing mOR28 and extending from a single dendritic knob. Scale bar is 5 μm.

MOB, olfactory information is sent to higher order centers of the brain for processing, including the amygdala, anterior olfactory nucleus, olfactory tubercle, piriform cortex, and entorhinal cortex (Haberly, 2001; Lledo *et al.*, 2005).

2.2. Cell types and ultrastructure

The main OE is a stratified epithelium composed of several cell types (Fig. 12.1A and B). The OSN is the main sensory cell, which houses the elements of the olfactory sensory cascade. OSNs are bipolar neurons with long axons projecting through the bony cribiform plate into the olfactory bulb, and relatively short dendrites terminating in a specialized ending termed a dendritic knob. The dendritic knob contains multiple basal bodies from which the olfactory cilia project into the mucous of the OE (Fig. 12.1D) (Cuschieri and Bannister, 1975a,b).

Surrounding the OSNs is a layer of supporting cells, termed sustentacular cells (Fig. 12.1A and B). The sustentacular cells contain many microvilli on their apical surface that underlie the cilia layer of the OE. Sustentacular cells have been shown to play a role in water balance, regulation of mucous ion composition (along with the Bowman's glands), drug metabolism, and purinergic modulation of odor sensitivity (Carr *et al.*, 2001; Hegg *et al.*, 2003; Kern and Pitovski, 1997; Menco *et al.*, 1998), however, their precise function remains unknown.

In addition to the OSN and sustentacular cell, there is also a population of stem cells, termed basal cells, capable of replenishing the OSN and sustentacular cell population (Fig. 12.1A and B). The basal cell layer is composed of two types of cells: the globose basal cell (GBC) and the horizontal basal cell (HBC). Although it is a matter of continued debate, it is thought that HBCs divide slowly and replenish the GBCs, which in turn allow the regeneration of new OSNs (Caggiano *et al.*, 1994; Iwai *et al.*, 2008; for review see Murdoch and Roskams, 2007).

While sustentacular cells possess microvilli, there are also five other distinct microvillous cells types in the OE that are found in much lower abundance than OSNs, sustentacular cells, and basal cells. These cells, while sharing the common feature of microvilli, are distinct in their morphological characteristics and distribution (Lin *et al.*, 2007; Moran *et al.*, 1982a,b; Rowley *et al.*, 1989).

Together, these cell types combine to form the main OE, lying on top of the basal lamina along the dorsal roof of the nasal turbinates and along the nasal septum. The basal cell layer, consisting of both GBCs and HBCs, lies immediately superficial to the basal lamina. The OSN cell bodies are arranged in a layer superficial to the basal cell layer. The OSN dendrites project through the layer of sustentacular cells where the dendritic knobs terminate near the apical surface of the sustentacular cells. Olfactory cilia project from these dendritic knobs into the mucous layer of the OE as an intermingled web (Fig. 12.1C), which lies on top of the microvilli of the sustentacular cells.

In mammals, the mucous layer bathing the olfactory cilia is a mixture of water secretions of approximately 5 μm in thickness (Menco, 1980c). The mucous is secreted primarily by the Bowman's gland, although there

may be some regulation of the mucous composition by the sustentacular cells (Menco *et al.*, 1998). In general, the nasal mucosa contains mucopolysaccharides, immune factors, metabolizing enzymes, and odorant-binding proteins (OBPs). These components serve to protect the epithelium from damage by physical stresses, exposure to toxicants, or infection.

One main functional component of the olfactory mucosa is the family of OBPs. OBPs are lipocalin family members secreted from the lateral nasal gland into the olfactory mucosa that serve to passively shuttle hydrophobic ligands through the aqueous mucous to the olfactory cilia. There are multiple homologous forms of the lipophilic OBP (OBPI-OBPIV in mice), which bind odorants in the micromolar range with distinct ligand specificity (Lobel *et al.*, 1998; Pes and Pelosi, 1995; Pevsner *et al.*, 1990). In addition to the potential chaperone role for OBPs, there have been other physiological roles suggested, such as protection from toxicants (Marinari *et al.*, 1984), prevention of saturation of the ORs (Burchell, 1991; Schofield, 1988), and even acting as a required cofactor for odorant binding to the OR (Pelosi, 1994).

2.3. Regeneration

The OSN is one of only a few types of neurons that continually regenerate throughout adult life. OSNs die through apoptotic processes and are replaced by neurons derived from the progenitor basal cells approximately every 30–90 days (Farbman, 1990; Graziadei and Graziadei, 1979b; Mackay-Sim and Kittel, 1991). This regeneration is often accelerated following insult and allows for the repair of the OE after loss/damage of OSNs through sickness, exposure to environmental toxicants, or following invasion from pathogens (Graziadei and DeHan, 1973; Graziadei and Graziadei, 1979a,b; Graziadei and Metcalf, 1971; Graziadei *et al.*, 1978; Harding *et al.*, 1977). Interestingly, even noninhalatory routes of toxicant exposure can lead to damage and regeneration in the OE through the formation of systemic reactive intermediates (Bergman *et al.*, 2002). In contrast, at least one report suggests that OSNs not exposed to toxicants may survive for over a year (Hinds *et al.*, 1984). Importantly for our topic, evidence suggests that deciliation may be an early step, or perhaps a trigger, for increased neuronal damage/death and basal cell regeneration following toxicant exposure (Calderon-Garciduenas *et al.*, 1998).

3. Structure of Olfactory Cilia

Almost any cell in the human body is capable of forming a cilium (see http://www.bowserlab.org/primarycilia/cilialist.html for a comprehensive list). Cilia are typically divided into classes based on their axonemal structure

and motility. The ciliary axoneme is most often composed of nine doublets of microtubules arranged symmetrically around a central core that either contains ((9 + 2) configuration) or lacks ((9 + 0) configuration) a central doublet of microtubules. Traditionally, cilia of the (9 + 2) configuration have been termed motile, whereas cilia of the (9 + 0) configuration have been termed nonmotile or primary cilia. Motile (9 + 2) cilia and flagella, which utilize structures called dynein arms along with the energy from ATP hydrolysis to generate movement, play important roles in fluid flow, sexual reproduction, and airway clearance. Nonmotile (9 + 0) cilia are commonly found as single primary cilia that help regulate cell-cycle progression, oncogenesis, and renal function. However, these classifications are not steadfast. For example, rare motile (9 + 0) cilia can be found in the embryonic node and allow the development of proper left–right asymmetry in the body (Okada *et al.*, 2005). Nonmotile (9 + 2) cilia can be found in sensory organs such as the inner ear and OE (Dabdoub and Kelley, 2005; Menco, 1984). Although olfactory cilia have the (9 + 2) microtubule configuration normally found in motile cilia, they lack the dynein arms and are thus rendered immotile (Menco, 1984). Interestingly, some nonmammalian vertebrates, such as goldfish and frogs (Lidow and Menco, 1984; Reese, 1965), display motile olfactory cilia, which have an axoneme resembling that of respiratory cilia in their proximal segments and are suggested to play a role in odorant clearance (Bronshtein and Minor, 1973; Mair *et al.*, 1982).

3.1. Cilia axoneme

Much of what we currently know about the structure of olfactory cilia derives from early electron microscopy studies (Cuschieri and Bannister, 1975a,b; Menco, 1980a,c; Menco and Morrison, 2003; Reese, 1965). These reports showed that the mammalian olfactory cilium is approximately 50–60 μm in length and can be divided into two distinct sections termed the proximal and distal segments. The thicker proximal segment in a (9 + 2) configuration projects 2–3 μm from the basal body with a thickness of around 0.3 μm (Menco, 1997). The thinner distal segment projects the remaining ~50 μm with a distinct axonemal configuration of 1–4 singlet microtubules, most commonly consisting of a pair of singlet microtubules (Menco, 1997).

Interestingly, the proximal and distal cilia segments may represent distinct sub-cellular compartments. Throughout development, signaling proteins display a differential distribution between these two regions. In nascent cilia, signaling proteins are more uniformly distributed between the proximal and distal segments. In mature cilia, the signaling proteins, such as $G\alpha_{olf}$, ACIII, and CNG channel, appear to preferentially localize to the long distal segment where the odorant first makes contact with the OSN (Fig. 12.1E)

(Flannery *et al.*, 2006; Matsuzaki *et al.*, 1999; Menco, 1997). This clustering of signaling molecules at the site of odorant exposure may increase the efficiency of odorant-stimulated signaling. The distal segments of the olfactory cilia are oriented parallel to the epithelial surface. Because there are many cilia (10–30) per cell and because they project 50–60 μm from the dendritic knob there is substantial overlap of cilia from different OSNs (Fig. 12.1C and D)(Menco, 1997). This intertwined mat of cilia increases the sensory surface of the OE by over 40 times thus increasing our ability to detect odorants (Fig. 12.1C) (Menco, 1992).

The ciliary axoneme is composed of polymers of α and β tubulin, which form the structural backbone for the cilium (reviewed in Rosenbaum and Witman, 2002; Scholey, 2003). These microtubules provide the roadway for molecular motors to move their cargo into and out of the cilium. Olfactory ciliary axonemes are oriented with the plus end located in the distal tip of the cilium, which means that plus end-directed motors carry cargo to the tip of the cilium, while minus end-directed motors are responsible for the return of cargo (reviewed in Rosenbaum and Witman, 2002; Scholey, 2003).

Posttranslational modifications of tubulin have been discovered to play functional roles in the regulation of cargo transport (reviewed in Hammond *et al.*, 2008). Many modifications to tubulin of the ciliary axoneme have been found, including acetylation (α), polyglutamylation ($\alpha + \beta$), polyglycylation ($\alpha + \beta$), and detyrosination (α). While all of these modifications have been detected in olfactory cilia, their precise functional relevance is poorly understood (Pathak *et al.*, 2007; Schwarzenbacher *et al.*, 2005). However, a recent study found that loss of an enzyme responsible for polyglutamylation in zebrafish caused a loss of olfactory cilia (Pathak *et al.*, 2007), indicating a role for posttranslational tubulin modifications in assembly or maintenance of olfactory cilia.

3.2. Lipid composition

The ciliary axoneme is encased in a membrane sheath formed by the lipid bilayer, which most certainly plays an integral role in olfactory signaling. There is a historic interest in the role of these lipids in the regulation of olfaction. Prior to the discovery of olfactory receptors, numerous reports hypothesized on the potential role of membrane lipids in the modulation of odorant transduction and odor recognition (Cherry *et al.*, 1970; Kashiwayanagi *et al.*, 1987, 1997, 1990; Nomura and Kurihara, 1987a,b; Russell *et al.*, 1989). The lipophilicity of odorants and the ability of various odorants to induce changes in membrane fluidity suggested that membrane lipids might play an important role in olfactory signaling (Cherry *et al.*, 1970; Kashiwayanagi *et al.*, 1987, 1990, 1997; Nomura and Kurihara, 1987a,b; Russell *et al.*, 1989). While it is now well established that odor

recognition is mediated by G protein-coupled receptors (GPCRs), this does not exclude the possibility for a role of ciliary membrane lipids in the modulation of odorant transduction. In fact, there may exist a dynamic reciprocity between odorant signaling proteins and membrane lipids in olfactory cilia such that perturbation of membrane lipids can affect olfactory signaling.

Recently, there is growing evidence for the role of lipid rafts in the organization of olfactory signaling proteins that are highly concentrated in the cilia (Brady *et al.*, 2004; Kobayakawa *et al.*, 2002; Schreiber *et al.*, 2000). In OE, Schreiber and colleagues (Schreiber *et al.*, 2000) demonstrated that the G protein and adenylyl cyclase isoforms involved in odorant signaling associate with lipid rafts. They also reported that G_{olf} and ACIII interact with the cholesterol binding protein, caveolin, and that disruption of the caveolin interaction inhibits odorant-induced cAMP production in OSNs. Additionally, the recently identified stomatin-related olfactory (SRO) protein (Goldstein *et al.*, 2003; Kobayakawa *et al.*, 2002) has been shown to associate with lipid rafts in olfactory cilia and bind both caveolin and ACIII. Importantly, anti-SRO antibodies stimulated cAMP production in fractionated cilia membranes suggesting that rafts and/or a caveolin/lipid/protein complex regulate odorant signaling (Kobayakawa *et al.*, 2002). In further support of this, early ultrastructural data from the Menco laboratory comparing olfactory cilia membranes to that of respiratory cilia led them to conclude that that the outer leaflet membranes of olfactory cilia are thicker than inner leaflets (Lidow and Menco, 1984). This is consistent with a potential enrichment of sphingolipids that are localized almost exclusively to the outer leaflet. Interestingly, the bilayer thickness of lipid raft domains will be greater than surrounding membrane due to sterol packing and the fact that raft hydrocarbon chains are longer and straighter than the acyl chains in the surrounding phospholipid regions (Tillman and Cascio, 2003). The enrichment of certain lipids is supported by work in invertebrates that has shown that the ciliary membrane of *Paramecium* is highly enriched with sphingolipids (Andrews and Nelson, 1979). Furthermore, these investigators later showed that ciliary membrane excitability in the same invertebrate model was sensitive to sterol composition (Hennessey *et al.*, 1983). Others have reported that there is an enrichment of cholesterol in the ciliary shaft, but not the necklace region, of epithelial cilia that extends during ciliogenesis (Chailley *et al.*, 1983). Perhaps another interesting feature of mammalian cilia is the distinct lipid composition at the base of the cilium. For example, Madin–Darby canine kidney cells demonstrate an annulus of condensed lipids at the base of their primary cilia (Vieira *et al.*, 2006). This peripheral evidence supports the hypothesis that the lipid composition of olfactory cilia may be specialized to support olfactory signal transduction. Surprisingly, however, there is virtually no information regarding the precise lipid composition of this important membrane structure in the olfactory system.

Nevertheless, in addition to the potential role for lipids in the nucleation of olfactory signaling complexes, the lipid composition of olfactory ciliary membranes may be important for several reasons.

Ciliary membrane lipids may be an important pharmacological consideration for the intranasal route of drug delivery. One recognized limitation of intranasal drug delivery, an approach often used to bypass the blood–brain barrier, is the temporary or permanent loss of olfactory function (Agarwal and Mishra, 1999; Illum, 2003). To improve transport across the nasal membrane, cyclodextrins (CD) are often used in drug formulations. These molecules are cyclic oligosaccharides that contain a hydrophobic binding cavity capable of incorporating a drug (Challa *et al.*, 2005). CDs exchange the drug contents of their cavity with the lipids in a plasma membrane. Importantly, these molecules are often used to deplete membranes of lipids and have been shown to disrupt lipid raft/caveolae formation. Using similar logic, it is not surprising that lipid-lowering drugs (i.e., statins) can cause anosmia and dysosmia in patients (Doty *et al.*, 2003; Weber *et al.*, 1992); this is listed as one of the manufacturer's recognized side effects on the FDA-approved product labeling for atorvastatin (Lipitor™).

Finally, one of the more clearly defined roles for lipid microdomains is as a portal of entry for viral pathogens. As discussed in detail later in this chapter, several viruses enter the body through the nasal cavity, gain access to the brain, and spread transneuronally to other parts of the central nervous system (CNS) using the olfactory system as a gateway. This suggests that the content and organization of ciliary lipids may be important not only in maintaining the integrity of olfactory signaling but also as a permissive entry site for invading pathogens.

3.3. Ciliary necklace/transition zone

At the extreme proximal end of the olfactory cilium, where the lipid membrane sheath meets the dendritic knob, there exists a region termed the "ciliary necklace." This highly ordered domain is marked by a spiraling array of membrane particles (Andres, 1969; Gilula and Satir, 1972; Menco, 1980d), which connect to the basal body just below the ciliary axoneme (Satir and Christensen, 2007). While most cilia types possess a ciliary necklace, olfactory cilia typically have more strands per cilium than their respiratory counterparts (Menco, 1980d). The formation of the ciliary necklace precedes ciliogenesis as a patch of membrane, and in malformed cilia there are still necklace-like structures (Carson *et al.*, 1981; Menco, 1980d). Interestingly, ciliary transport proteins have been found to be localized at the ciliary necklace indicating that it may serve as a cargo docking site connecting the ciliary shaft to the protein complexes at the base of the cilium (Deane *et al.*, 2001).

3.4. Basal body

The protein complex at the base of the cilium is formed by the basal body, a modified centriole that migrates to the plasma membrane prior to ciliogenesis. The basal bodies are duplicated *en masse* in the cell body of the OSN before they migrate to the dendritic knob (Cuschieri and Bannister, 1975a, b; Dirksen, 1974; Hagiwara *et al.*, 2004; Schwarzenbacher *et al.*, 2005). Basal bodies, like the ciliary axoneme, are composed of nine sets of microtubules arranged in a radial symmetry. However, unlike the axoneme, basal bodies are composed of polymers of triplet microtubules of γ tubulin rather than doublet microtubules of α and β tubulin. The basal body serves as the microtubule organizing center (MTOC) in the dendritic knob with the axonemal tubules projecting from the basal body, such that the plus ends orient toward the distal tip of the cilium (Burton, 1992).

In addition to serving as MTOCs for the ciliary axoneme, the basal bodies are associated with electron-dense satellite particles that appear to also be MTOCs (Burton, 1992). These organizing centers serve as nucleation sites for microtubules that project from the dendritic knob back through the dendrite toward the cell body (Burton and Laveri, 1985). Some of the MTOCs are connected to the basal body through a sheath of material that surrounds the basal body and thickens at its proximal end. The basal bodies and sheath are connected to the plasma membrane through nine struts which correspond to the electron-dense endings of the ciliary rootlet (Menco, 1980d).

3.5. Ciliary rootlet

The ciliary rootlet, first described over a century ago, is a cytoskeletal feature found projecting from the basal body in ciliated cells (Engelmann, 1880). Although the structural components of the ciliary rootlet are beginning to be elucidated (Yang *et al.*, 2002), still very little is known about its function. It has been proposed that the ciliary rootlet is important for the stability of sensory cilia. In these studies, photoreceptor connecting cilia from mice lacking rootletin, a component of the ciliary rootlet, displayed fragility at their basal body (Yang *et al.*, 2005). Although olfactory cilia were not examined in the rootletin-null mice, OSNs have been shown to express components of the ciliary rootlet in a localization consistent with the dendritic knob/basal body region (McClintock *et al.*, 2008; Yamamoto, 1976). Given that rootletin has been shown to affect cilia stability and that OSNs have been shown to express components of the rootlet, the prediction would be that olfactory cilia lacking a rootlet may also show fragility and become detached from the OSN more easily upon physical stresses such as sneezing.

4. Formation of Olfactory Cilia

Much of what we currently know about ciliogenesis derives from the study of lower vertebrates. In the mouse, the olfactory placode is first visible at embryonic day 9 (E9) postfertilization (Cuschieri and Bannister, 1975a,b; Menco, 1980a,b; Menco and Morrison, 2003; Schwarzenbacher *et al.*, 2005). At E10, an invagination of the olfactory placode leads to the development of the olfactory pit. At this point, two different cell types are visible: a population that is electron dense (proliferative basal cells) and those that appear light (differentiated OSNs) (Cuschieri and Bannister, 1975a,b; Menco, 1980a,b; Menco and Morrison, 2003). It is not until E11, however, that the dendrites begin to form and extend toward the apical surface. Further, the olfactory pit deepens and secondary recesses, which will eventually develop into turbinates, appear (Cuschieri and Bannister, 1975a,b; Menco, 1980a,b; Menco and Morrison, 2003). During this period of extensive proliferation, OSN growth and maturation is most pronounced in the deep recesses of the olfactory pit (Cuschieri and Bannister, 1975a,b; Menco, 1980a,b; Menco and Morrison, 2003).

The earliest detectable signs of neuronal differentiation are observed at E10, where, using electron microscopy, a small population of OSNs extending dendrites toward the apical surface can be detected (Cuschieri and Bannister, 1975a). By E11, several morphological changes occur in these OSNs, suggesting the initiation of ciliogenesis. First, in the perinuclear region of these neurons, numerous microtubules, and microfilaments have formed and extend vertically toward the apical surface (Cuschieri and Bannister, 1975a; Menco and Farbman, 1985; Menco and Morrison, 2003). Second, the terminal portion of the dendrite now extends past the apical surface and into the lumen of the nasal cavity, where it begins to swell indicating the formation of the dendritic knob (Cuschieri and Bannister, 1975a; Menco and Farbman, 1985; Menco and Morrison, 2003). Finally, and perhaps most importantly, centriole duplication has occurred and groups of centrioles are amassed in the perinuclear region of the neuron (Fig. 12.2A) (Cuschieri and Bannister, 1975a; Menco and Farbman, 1985; Menco and Morrison, 2003).

By E12, the number of OSNs with well-formed dendrites and dendritic knobs has increased markedly. The dendritic knobs in these neurons are filled with mitochondria, small coated vesicles, and numerous microtubules (Cuschieri and Bannister, 1975a; Menco and Farbman, 1985; Menco and Morrison, 2003). These microtubules are arranged in two distinct populations; one is arranged concentrically around the periphery of the knob while the other is arranged longitudinally and extends deep into the dendrite (Cuschieri and Bannister, 1975a; Menco and Farbman, 1985; Menco and Morrison, 2003). Further, the groups of centrioles observed at E11 have

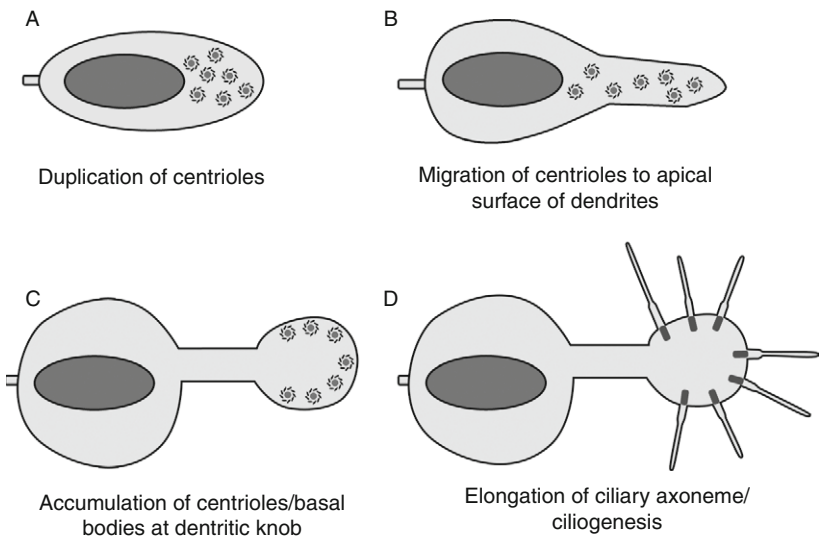

Figure 12.2 *The major steps of OSN ciliogenesis.* **Cartoon representations are shown for the major phases of ciliogenesis in the olfactory epithelium. (A) In the first step, centrioles duplicate and accumulate in the perinuclear space prior to dendrite formation. (B) The OSN dendrite extends and the duplicated centrioles migrate toward the apical surface of the olfactory epithelium. (C) The terminal ending of the dendrite extends past the apical surface of the olfactory epithelium and begins to swell, forming the dendritic knob. The centrioles accumulate at the dendritic knob and begin to arrange around the periphery of the knob, where they will become the ciliary basal bodies. (D) The axonemes of the newly formed cilia grow and extend into the lumen of the nasal cavity. Also, the peripheral structures of the basal bodies form, anchoring the cilia into the plasma membrane.**

begun to migrate out to the dendritic knob (Fig. 12.2B) and appear either as rosette-like clusters at the center of the knob or are dispersed singly around the knob periphery (Fig. 12.2C). In the OSNs with the centrioles arranged around the periphery, the first hint of ciliogenesis is occurring when a single, primary cilium of approximately 1 μm is observed extending into the nasal cavity (Cuschieri and Bannister, 1975a; Menco and Farbman, 1985; Menco and Morrison, 2003; Schwarzenbacher *et al.*, 2005). In the next stage of development, the microtubule-based axoneme of these newly formed cilia begins to elongate and the basal body, formed by the migrating centrioles, matures and is anchored at the plasma membrane (Fig. 12.2D) (Cuschieri and Bannister, 1975a,b; Dirksen, 1974; Hagiwara *et al.*, 2004; Schwarzenbacher *et al.*, 2005). By E13 or E14, multiple cilia up to 2 μm in length can be seen extending from a single dendritic knob (Fig. 12.2D). Over the next several days, olfactory cilia elongate and can reach up to 60 μm prior to birth. Intraflagellar transport (IFT), which will be discussed in more detail below, plays a key role in the growth and maintenance of cilia

(Rosenbaum and Witman, 2002; Scholey, 2003). Postnatally, the cilia will continue to grow and can reach up to 200 μm in length in some vertebrate populations (Menco and Morrison, 2003; Reese, 1965; Seifert, 1971). Due to this length, OSN cilia create a meshwork across the surface of the OE, thus increasing the surface area of the OE up to 40 times and enhancing the likelihood of odorant detection (Lidow and Menco, 1984; Menco and Morrison, 2003).

For the process of olfaction, it is important to consider not only how cilia are formed, but also when the odorant signaling molecules are expressed and localized to cilia. The majority of studies investigating the developmental expression of olfactory signaling molecules have probed for mRNA expression using either RT-PCR, northern blot, or *in situ* hybridization analysis (Margalit and Lancet, 1993; Saito *et al.*, 1998; Schwarzenbacher *et al.*, 2004, 2005; Strotmann *et al.*, 1995). Interestingly, not all of the components necessary for odor detection are expressed at the same point during development. The first proteins to be expressed are the ORs, of which a subset begin to be expressed at E11, as determined by both mRNA and protein expression (Saito *et al.*, 1998; Schwarzenbacher *et al.*, 2004, 2005). This expression occurs prior to ciliogenesis, resulting in the accumulation of the OR protein in high density at the dendritic knob (Schwarzenbacher *et al.*, 2005). Surprisingly, not all ORs are expressed at the same time in development. Two of the earliest expressing ORs identified, mOR256-17 and V1, begin to be expressed at E11 (Saito *et al.*, 1998; Schwarzenbacher *et al.*, 2004, 2005). By E12, several more receptors, mOR5, mOR14, mOR18–2, mOR37, mOR111–5, mOR124, and mOR171–24 exhibit increased expression in OSNs (Schwarzenbacher *et al.*, 2004; Strotmann *et al.*, 1995). The diversity of OR expression continues to increase over the next several days of embryonic and postnatal development (Margalit and Lancet, 1993; Saito *et al.*, 1998; Schwarzenbacher *et al.*, 2004). As for the other components of the olfactory signaling cascade, they are expressed later in embryonic development. ACIII is first detected around E15, while G_{olf} and the CNG channel are expressed at E16 and E19, respectively (Margalit and Lancet, 1993). Due to this range in developmental expression patterns, it is assumed that odor detection cannot occur until all proteins are present in olfactory cilia. However, this has yet to be determined.

The protein expression of one specific OR, mOR256–17, has been used to track ciliogenesis (Schwarzenbacher *et al.*, 2004). Specifically, as mentioned previously, the earliest expressing ORs are present prior to the initiation of ciliogenesis. mOR256–17 accumulates at the dendritic knob in high density at this stage. As cilia begin to form and elongate, the pattern of OR localization changes. At E11, when a primary cilium around 1 μm can be observed on a subset of neurons, the OR remains localized to the knob and at the very proximal portions of the cilia. As early as E12, when the cilia reach 2 μm or longer, mOR256–17 migrates almost exclusively to

OSN cilia, and, by E13, this localization is complete (Schwarzenbacher *et al.*, 2004). The physiologic relevance of OR expression prior to ciliogenesis, however, remains to be determined.

5. Intraflagellar Transport

The formation of cilia occurs through an evolutionarily conserved process termed IFT, which was first discovered in the laboratory of Joel Rosenbaum in *Chlamydomonas* (Kozminski *et al.*, 1993). Since cilia lack the necessary components for protein synthesis, cargo must be synthesized in the cell and carried into the cilia through IFT, which involves movement along microtubules by molecular motors in complex with transport molecules, called IFT particles (reviewed in Rosenbaum and Witman, 2002; Scholey, 2003, 2008). Given that the basic mechanisms of IFT are widely conserved not only between cilia types, but also often between species, we presume that these mechanisms studied in invertebrates are also acting in mammalian olfactory cilia.

Much of what we know about IFT in chemosensory cilia is from work in *Caenorhabditis elegans* where IFT can be visualized in real time using GFP-tagged motors (Orozco *et al.*, 1999). Transport in the anterograde direction, towards the distal, plus end of the cilium microtubules, has been shown to involve kinesin motors (Cole *et al.*, 1998), whereas retrograde transport back into the cell is accomplished with the cytoplasmic dynein motor (Pazour *et al.*, 1998). These microtubule-based motors utilize the energy from ATP hydrolysis to move cargo processively along the microtubules to their destination.

Work in *C. elegans* has shown that the formation and maintenance of the chemosensory ciliary axoneme and the delivery of cargo is accomplished through coordination of two kinesin motors: the heterotrimeric kinesin-II motor and the homodimeric OSM-3 (Snow *et al.*, 2004). The conservation between IFT mechanisms was shown in the mammalian kidney, where the heterotrimeric kinesin-II motor, consisting of the two motor subunits KIF3a, KIF3b, and the accessory protein, KAP3, is necessary for ciliogenesis (Lin *et al.*, 2003). However, differences are beginning to be recognized between specialized cilia types in invertebrates and mammals (Jenkins *et al.*, 2006; Ou *et al.*, 2005). Although loss of function of the OSM-3 homolog, KIF17, impaired ciliary trafficking of the olfactory CNG channel, it had no effect on cilia length as predicted by work in *C. elegans* (Jenkins *et al.*, 2006; Ou *et al.*, 2005). Future studies, however, are necessary to determine if these mechanisms are functioning directly in mammalian olfactory cilia. Interestingly, OSM-3 operates on singlet microtubules of the distal segments of *C. elegans* cilia (Ou *et al.*, 2005), and since olfactory cilia have such prominent distal segments it is likely that KIF17 is also functioning on distal

segments in the mammalian olfactory cilium. Nevertheless, the differences in kinesin-2 regulation of cilia length between the cilia of *C. elegans* and mammalian cilia highlight the need to further explore the mechanisms of IFT in mammalian olfactory cilia.

Early work using electron microscopy of frog olfactory cilia found cargo in complex with IFT particles seen as electron-dense regions along the ciliary axoneme (Reese, 1965). It is known that IFT motors associate with two distinct complexes of transport proteins called IFT proteins, named for their molecular weight. These two complexes comprise 17 highly conserved proteins, termed complex A and complex B (Cole, 2003). Complex A consists of IFT144, 140, 139, 122, and possibly 43, while complex B consists of IFT 172, 88, 81, 80, 74/72, 57/55, 52, 46, 27, and 20. Defects in either complex can impair IFT and cause a host of human diseases (reviewed in Blacque *et al.*, 2008). The precise role of the IFT complexes in mammalian olfactory cilia transport remains undefined. IFT proteins have been shown to share significant homology with Golgi-localized clathrin trafficking machinery (Avidor-Reiss *et al.*, 2004). Interestingly, the clathrin AP-1 μ adaptor, UNC-101, has been shown to be responsible for the localization of ORs to the cilia of C. *elegans* (Dwyer *et al.*, 2001).

Another complex of proteins that have been shown to be involved in cilia assembly and maintenance are the Bardet–Biedl syndrome (BBS) proteins. BBS is a pleiotropic ciliopathy that includes phenotypes such as retinal degeneration, polydactyly, obesity, anosmia, and others (discussed in more detail below). There are 12 known BBS proteins (BBS1–12), which encode proteins involved in different stages of cilia transport. While there are a variety of ciliary phenotypes associated with defects in BBS proteins, loss of function of BBS1 and BBS4 caused impaired olfactory function (Iannaccone *et al.*, 2005; Kulaga *et al.*, 2004). Interestingly, mice null for BBS1 or BBS4 may exhibit defects in olfactory cilia maintenance or assembly, although the mechanism for this defect remains unknown (Iannaccone *et al.*, 2005; Kulaga *et al.*, 2004).

A recent report suggests that there is a dynamic reciprocity between the signaling function of cilia and its structural maintenance through IFT. Work in *C. elegans* by Mukhopadhyay *et al.* has shown that the loss of activation of the sensory signaling cascade modulates the structure of the AWB neuron modified sensory cilia (Mukhopadhyay *et al.*, 2008). This sensory signaling-dependent remodeling was shown to be dependent on kinesin-II as well as BBS proteins (Mukhopadhyay *et al.*, 2008). This is similar to a previous study showing that structure of AWC neuron cilia is also linked to sensory function (Roayaie *et al.*, 1998). While structural changes have been reported in mice deprived of odorant stimulation by naris occlusion (Farbman *et al.*, 1988), it would be interesting to examine changes in cilia architecture due to loss of olfactory cues. Regardless, this suggests a feedback interaction between the ciliary proteins involved in assembly and maintenance and those participating in signaling.

6. Ciliary Proteome

Cilia contain a subset of cellular proteins which comprise a population distinct from the extraciliary compartment (Inglis *et al.*, 2006). Emerging techniques, such as bioinformatic screens or proteomic analyses, are yielding new insights into cilia-related genes novel proteins that may be involved in olfactory signaling or ciliary structure and maintenance.

6.1. Olfactory signaling through the canonical G protein-coupled pathway

Perhaps the most recognized subset of proteins enriched in the olfactory cilia is that of the canonical signaling pathway. This cascade begins when odorants dissolve in the nasal mucosa where they gain access to the OSN. The odorant binds to the OR on the cilia of OSNs to initiate the odorant detection pathway (Fig. 12.1E). The OR activates the olfactory G protein, which stimulates ACIII to increase the local concentration of cAMP. The CNG channel is activated by the increased cAMP and opens to allow Ca^{2+} influx into the OSN. This signal is amplified by the Ca^{2+}-activated Cl^- channel which binds Ca^{2+} and opens allowing Cl^- efflux from the OSN leading to further depolarization (reviewed in Ronnett and Moon, 2002). The function of the olfactory system is dependent on the ciliary localization of each of these proteins described below.

6.1.1. Odorant receptors

The discovery of the family of ORs was described by Linda Buck and Richard Axel in a groundbreaking publication in 1991 (Buck and Axel, 1991), which led to the awarding of the Nobel Prize in Physiology or Medicine in 2004. These receptors represent the most numerous member of the family of 7-transmembrane GPCRs, with humans having ~400 functional genes and ~600 pseudogenes (Gilad and Lancet, 2003). Interestingly, only one OR is expressed in any single OSN. Given the tremendous diversity of odorants and the finite number of OR genes each OR must have the ability to bind multiple odorants (Buck, 2004; Malnic *et al.*, 1999; Mombaerts, 2004, 2006). While some specific ligands have been identified (reviewed in Mombaerts, 2004), difficulties with functional expression in nonciliated heterologous systems have slowed OR characterization perhaps suggesting a requirement for cilia in OR expression.

6.1.2. Olfactory G proteins

The main olfactory G protein is a heterotrimeric stimulatory G protein comprising $G\alpha_{olf}$, $\beta 1$, and $\gamma 13$ (Jones and Reed, 1989; Kerr *et al.*, 2008). This heterotrimer provides the link between the ORs and ACIII in mature

OSNs. Interestingly, $G\alpha_s$ is also expressed in OSNs and appears precede the developmental expression of $G\alpha_{olf}$ (Belluscio *et al.*, 1998; Menco *et al.*, 1994). For reasons unknown, there is a phenotypic switch in olfactory cilia from $G\alpha_s$ to $G\alpha_{olf}$ later in the maturation of the OE.

6.1.3. Adenylyl cyclase

The olfactory heterotrimeric G protein is a stimulatory G protein that mediates downstream signaling through the stimulation of adenylyl cyclase. The major form of adenylyl cyclase in the OSN is ACIII, which was cloned by Reed and colleagues in 1990 (Bakalyar and Reed, 1990). ACIII, like most components of the olfactory signaling cascade, is critical for odorant detection as genetic deletion leads to anosmia (Wong *et al.*, 2000). ACIII is a Ca^{2+}-calmodulin stimulated isoform of adenylyl cyclase that is responsible for the elevation of intracellular cAMP in the cilia of OSNs (Bakalyar and Reed, 1990; Choi *et al.*, 1992).

6.1.4. CNG channel

CNG channels were first discovered in retinal photoreceptors and olfactory neurons, where they modulate the membrane potential in response to stimulus-induced changes in the intracellular concentrations of cyclic nucleotides (Fesenko *et al.*, 1985; Firestein and Werblin, 1989; Nakamura and Gold, 1987). Although CNG channels have now been found in many other neuronal and nonneuronal cells, their physiological roles in nonsensory tissues remain obscure (Finn *et al.*, 1996). The functional role of CNG channels in the olfactory system is firmly established. The olfactory CNG channel comprises three distinct subunits, CNGA2, CNGA4, and CNGB1b, in a 2:1:1 stoichiometry (Bonigk *et al.*, 1999; Zheng and Zagotta, 2004). CNGA2 is the only subunit capable of forming functional homotetramers, while CNGA4 and CNGB1b serve to modulate properties of the channel such as ion selectivity, nucleotide sensitivity, and Ca^{2+}/Calmodulin regulation (Kaupp and Seifert, 2002). Recently, the CNGB1b subunit has been found to be necessary for delivery of the CNG channel to cilia (Jenkins *et al.*, 2006; Michalakis *et al.*, 2006), and this trafficking was shown to be dependent on a the kinesin motor protein, KIF17, and a C-terminal "RVxP" trafficking motif on CNGB1b (Jenkins *et al.*, 2006).

6.1.5. Ca^{+2}-activated Cl^- channel

Ca^{2+} entry through the CNG channel directly stimulates the Ca^{2+}-activated Cl^- channel. Although chloride conductance is often considered an inhibitory process, in olfactory cilia there is a reverse chloride gradient resulting in an efflux of chloride with the opening of the channel. This current further

depolarizes the neuron and amplifies the signal from the CNG channel causing the generation of an action potential. While functional studies have firmly established a role for the olfactory Ca^{2+}-activated Cl^- channel (Boccaccio and Menini, 2007; Kaneko *et al.*, 2006; Kleene and Gesteland, 1991; Kurahashi and Yau, 1993; Pifferi *et al.*, 2006; Qu *et al.*, 2003; Reisert *et al.*, 2003, 2005; Reuter *et al.*, 1998), its molecular identity has remained elusive.

6.2. Signaling through noncanonical pathways

While the canonical olfactory signaling cascade has long been thought to be the main pathway involved in odor detection, recent evidence suggests that at least three other pathways exist that are uniquely involved in detecting changes in the external environment. In the OE, a subset of neurons has been shown to express the guanylyl cyclase type D (GC-D) receptor, while lacking the canonical signaling components (Juilfs *et al.*, 1997; Ma, 2007; Meyer *et al.*, 2000). GC-D expression seems to be specific to the olfactory system, where its localization is widely dispersed over multiple turbinates of the epithelium (Breer *et al.*, 2006; Fulle *et al.*, 1995). As its name implies, upon stimulation, the GC-D receptor generates cGMP, which then signals to a cGMP-responsive CNG channel subunit, CNGA3 (Leinders-Zufall *et al.*, 2007). Importantly, all of the components of this cascade are enriched in olfactory cilia, suggesting that they are involved in chemosensation (Juilfs *et al.*, 1997; Leinders-Zufall *et al.*, 2007; Meyer *et al.*, 2000). While the physiologic relevance of this system has been controversial, recent evidence suggests that, in rodents, these neurons are responsible for the detection of hormone peptides as well as natural urine stimuli (Leinders-Zufall *et al.*, 2007). These data are supported in both the GC-D and CNGA3 knockout mice, which are unresponsive to these stimuli, while canonical odor detection remains intact (Leinders-Zufall *et al.*, 2007). Perhaps more controversial is the hypothesis that these neurons act as the CO_2 sensor in the OE, which may be involved in detecting signs of danger in the external environment (Hu *et al.*, 2007).

More recently, a third subset of OSNs has been shown to express a family of receptors termed trace amino acid receptors (TAARs) (Borowsky *et al.*, 2001; Gloriam *et al.*, 2005; Lewin, 2006; Liberles and Buck, 2006). In addition to their expression in OSNs, TAARs are also expressed outside of the main OE, in the Gruenberg ganglion (Fleischer *et al.*, 2007). The topology of these receptors includes seven transmembrane-spanning segments, linking them with the GPCR family of proteins. However, sequence analysis of the TAARs shows the most similarities with dopamine and serotonin, but not odorant, receptors (Liberles and Buck, 2006). Nine subtypes of TAARs have been cloned, with eight of them being specifically expressed in the OE (Ma, 2007). Despite co-localization with G_{olf}, it

remains to be determined if TAARs couple to the canonical downstream signaling mechanisms involved in odor detection (Liberles and Buck, 2006). As with the odorant and GC-D receptors, the TAARs localize to OSN cilia, again indicating a chemosensory function (Liberles and Buck, 2006). In support of this function, three TAARs expressed in the OE have been shown to respond *in vitro* to volatile amino acids found in mouse urine (Liberles and Buck, 2006), suggesting that the TAARs are involved in detecting social cues from neighboring animals.

Finally, in most mammals and perhaps even primates, a separate organ, termed the vomeronasal organ (VNO), is involved in pheromone detection. Recent evidence suggests that the VNO can also detect small, volatile odorants (Breer *et al.*, 2006; Dulac and Torello, 2003; Ma, 2007; Sam *et al.*, 2001). The mammalian VNO possesses both ciliated and microvillar sensory neurons whose projections synapse in the accessory olfactory bulb located on the dorsal-posterior side of the MOB (Cuschieri and Bannister, 1975b). While no distinct VNO has been shown to exist in humans beyond early development (Boehm and Gasser, 1993), both of the vomeronasal receptors, V1R and V2R, are expressed in the OE (Giorgi *et al.*, 2000; Rodriguez, 2004; Rodriguez and Mombaerts, 2002; Witt and Hummel, 2006). In the rodent, both the V1R and the V2R localize to cilia of VNO neurons where they couple to either the G_i (V1R) or G_o (V2R) family of G proteins (Dulac and Torello, 2003). Unlike the canonical odorant signaling pathway, the V1Rs and V2Rs couple to a PLC-mediated pathway, producing the downstream signaling molecules diacyl glycerol and phosphatidylinositol-3-phosphate. Stimulation of this pathway leads to arachadonic acid production and, ultimately, to TRPC2 activation, converting the chemical signal into an electrical response (Liman *et al.*, 1999; Lucas *et al.*, 2003).

Taken together, these data indicate that the mammalian nose is a complex organ with multiple systems designed to detect everything from simple odors to pheromones and social cues. Despite differences between these systems, the underlying commonality remains that chemical detection, whether in the OE or the VNO, occurs at the level of the cilium. Consequently, while mutation or deletion of a single signaling protein will only affect a single pathway, disrupting proteins involved in ciliary formation or maintenance should render all of these systems ineffective.

6.3. Bioinformatics and expression profiling

While we are beginning to more fully understand the proteins involved in the signaling and maintenance of olfactory cilia, the full scope of genes involved in the various aspects of cilia function has yet to be identified (McClintock *et al.*, 2008). Recent advances in technology have vastly improved our ability to use bioinformatics as a tool to identify novel

genes involved in various cellular processes, such as cilia formation and function. Hundreds of genes present in numerous ciliated species have recently been identified to be important in cilia-related functions (Avidor-Reiss *et al.*, 2004; Blacque *et al.*, 2006; Li *et al.*, 2004; McClintock *et al.*, 2008; Pazour *et al.*, 2005; Smith *et al.*, 2005; Stolc *et al.*, 2005). While these studies are informative in identifying cilia-related genes, it is not clear that the mechanisms or gene products regulating ciliary function are entirely conserved between invertebrate and mammalian species. Only a handful of studies have concentrated on identifying cilia-related genes in mammals, with only two focusing on olfactory cilia (Klimmeck *et al.*, 2008; McClintock *et al.*, 2008; Ostrowski *et al.*, 2002; Sammeta *et al.*, 2007; Su *et al.*, 2004). Using complementary approaches, comparing olfactory cilia to other ciliated cell types and calcium calmodulin-affinity column purification from isolated cilia, these two studies have identified over 100 cilia-related genes of known and unknown function in OSNs (Klimmeck *et al.*, 2008; McClintock *et al.*, 2008). While these approaches have proved useful in identifying new gene products, perhaps the most intriguing use will be to identify changes in gene expression in known ciliopathies, which will yield insights into the underlying mechanisms of these cilia disorders.

6.4. Ciliary proteomics

While it is interesting to identify novel gene products involved in cilia function, perhaps more important is to characterize the function of these newly identified proteins. In the study using calmodulin-affinity purification from isolated cilia, not only were proteins identified from the olfactory signaling cascade, but they also identified proteins involved in cytoprotection, cytoskeletal proteins, and proteins involved in modification of ciliary proteins (Klimmeck *et al.*, 2008). In a similar study, isolated cilia preparations were subject to mass spectrometric analysis, from which 268 proteins were identified (Mayer *et al.*, 2008). Of these proteins, 49% were transmembrane proteins, 41% were cytosolic, and 10% were cytoskeletal proteins (Mayer *et al.*, 2008). Within the membrane fraction, the traditional signaling components were identified, along with ER and Golgi proteins, including proteins involved in metabolism, protein biosynthesis, and signal transduction. One such protein was PDE1C, which had been previously shown to localize to olfactory cilia and is thought to aid in odorant signal termination (Mayer *et al.*, 2008). The cytosolic fraction included such proteins as calmodulin, calreticulin and two 14–3–3 isoforms, which are known to interact with and be modulated by calmodulin. The cytoskeletal fractions included tubulin, various keratin isoforms, and actin (Mayer *et al.*, 2008). The presence of actin indicates that this preparation contains not only olfactory cilia, but also extraciliary proteins, since actin is not found in olfactory cilia. Thus, the isolation of pure populations of cilia, free of

contamination from other membranes or organelles, appears to be a limiting factor (Klimmeck *et al.*, 2008; Mayer *et al.*, 2008). Given the large number of newly identified targets that may be expressed throughout the entire OSN, the question remains if they are enriched in the cilium. Nevertheless, these proteins were detected as part of the ciliary proteome, and the challenge remains to demonstrate their function and physiological relevance. This may not prove simple as illustrated by the case of olfactory marker protein (OMP). While this olfactory-specific protein, which is expressed in the cilia and throughout the OSN, was identified over 30 years ago, its precise function remains elusive(Youngentob and Margolis, 1999; Youngentob *et al.*, 2001, 2003, 2004). Regardless, use of OMP as an identifier of mature OSNs and its OSN-selective promoter have proved invaluable for cell biological and genetic studies of olfactory function. The potential for the discovery of other such markers of olfactory cilia or function alone justifies this proteomic approach.

6.5. Regulation of ciliary protein entry

Although our knowledge of proteins localized to olfactory cilia is expanding, from a mechanistic perspective it is interesting to consider the fact that only a subset of cellular proteins is able to gain access to the cilium. Since the cilium contains a protein population distinct from the extraciliary compartment (Inglis *et al.*, 2006), there must be a barrier to diffusion that restricts entry into the cilium. This selective gate is thought to occur at the basal body through interactions with a large complex of proteins (Rosenbaum and Witman, 2002; Scholey, 2003). Recently, mutation in the cilia/centrosomal protein CEP290 has been implicated in the specific mislocalization of olfactory G proteins (McEwen *et al.*, 2007). Importantly, mutation in CEP290 did not globally alter cilia structure and all other olfactory signaling molecules tested were localized normally, indicating that in olfactory cilia, regulation of cargo entry is distinct for different proteins.

Growing interest in ciliary protein trafficking has led to the identification of amino acid sequences necessary for entry of cargo into cilia. For example, the "RVxP" motif originally identified in polycystin-2 (Geng *et al.*, 2006), was found to be necessary for the ciliary delivery of the olfactory CNG channel (Jenkins *et al.*, 2006). Additionally, several ORs were recently found to contain a ciliary targeting motif consisting of (AX[S/A]XQ) which was sufficient to drive ciliary localization of nonciliary receptors (Berbari *et al.*, 2008). The precise mechanisms by which these motifs control ciliary localization remain unknown. Interestingly, only a subset of ciliary proteins express these motifs indicating that there are multiple potential ciliary targeting motifs that most likely act through distinct ciliary entry mechanisms.

7. Olfactory Cilia and Disease

It is estimated that 3–6 million people suffer from general or clinical anosmia in the United States alone (Nguyen-Khoa *et al.*, 2007). This may be an underestimate of the true number of cases, as people do not often report lost or altered sense of smell to their physicians (Nguyen-Khoa *et al.*, 2007). Deficits in olfactory function can decrease the quality of life and be potentially hazardous (Nguyen-Khoa *et al.*, 2007). While the leading causes of smell disorders in patients occur following head trauma, upper respiratory tract infections, and chronic rhinosinusitis, olfactory dysfunction due to genetic mutations or neurodegenerative disorders affecting cilia are becoming increasingly recognized and better studied.

7.1. Olfactory ciliopathies

One of the first documented cases of a human patient with anosmia presumably due to ciliary defects was in 1975 (Afzelius, 2004; Douek *et al.*, 1975). A biopsy from this patient, who suffered from congenital anosmia, revealed that, while the global architecture of the epithelium appeared normal, his OSNs were devoid of cilia, the cause of which is unknown (Douek *et al.*, 1975). It has only been within the past 5 years that patients with deficits in olfaction due to ciliary defects have been clearly identified (Iannaccone *et al.*, 2005; Kulaga *et al.*, 2004; McEwen *et al.*, 2007). In these cases, the olfactory deficits were shown to occur in two different pleiotropic diseases, BBS and Leber congenital amaurosis (LCA).

BBS is highly pleiotropic with patients exhibiting mental disabilities, obesity, retinal degeneration, polycystic kidneys, hypertension, and hypercholesterolemia, which together may lead to premature death (Bardet, 1995; Beales *et al.*, 1999; Biedl, 1995; Klysik, 2008). The varied effects are dependent upon mutations in one of 12 members of the BBS gene family, with the most severe mutations occurring in either BBS1 or BBS10 (Beales *et al.*, 2003; Hichri *et al.*, 2005; Klysik, 2008; Mykytyn *et al.*, 2003; Stoetzel *et al.*, 2006). Several BBS proteins, BBS1–8, have been characterized as basal body proteins that are thought to regulate protein entry into the cilium (Klysik, 2008). Human mutations in two BBS proteins, BBS1 and BBS4, and genetic deletion of BBS1, BBS2, or BBS4 in mice resulted in severely impaired olfactory function (Iannaccone *et al.*, 2005; Kulaga *et al.*, 2004; Mykytyn *et al.*, 2004; Nishimura *et al.*, 2004). However, mutations in the BBS proteins do not seem to share a common underlying mechanism of olfactory dysfunction. For example, patients with mutations in BBS1 are anosmic, most likely due to a loss of olfactory cilia, as the cilia are absent in the BBS1 null mouse model (Iannaccone *et al.*, 2005; Kulaga *et al.*, 2004).

In the BBS2 null mouse, the status of olfactory cilia has not been examined, but both renal and retinal cilia are able to assemble (Nishimura *et al.*, 2004). Finally, in two different studies, patients with mutations in BBS4 exhibited decreased olfaction or anosmia (Iannaccone *et al.*, 2005; Kulaga *et al.*, 2004). The effect of these mutations on olfactory cilia formation and maintenance is controversial. Using the BBS4 null mouse model, Kulaga *et al.* show that cilia are absent in the BBS4 null mice (Kulaga *et al.*, 2004), while Iannaccone *et al.* argue that BBS4 is not involved in cilia formation and that primary cilia form normally in BBS4 null mice (Iannaccone *et al.*, 2005; Mykytyn *et al.*, 2004). Despite these differences, it is clear that mutations in BBS proteins affect cilia function and lead to olfactory deficits.

A second example of a ciliary defect leading to olfactory impairment is a recent study investigating olfactory function in patients with LCA (McEwen *et al.*, 2007). LCA, first discovered by Theodor Leber almost 140 years ago (Leber, 1869), is a congenital retinal dystrophy accounting for more than 5% of inherited retinopathies (Koenekoop, 2004). LCA can occur due to mutations in several proteins of varying function, from retinoid metabolism and phototransduction to cell-cycle progression (Koenekoop, 2004). Recent reports have also shown that LCA can be caused by mutations in the centrosomal/basal body protein, CEP290 (Cideciyan *et al.*, 2007; den Hollander *et al.*, 2006). Olfactory function was tested in the original LCA patient population with mutations in CEP290 using the Brief Smell Identification Test (B-SIT) (McEwen *et al.*, 2007). For all patients tested, mutations in CEP290 resulted in severely impaired olfactory function despite a self-described normal sense of smell (McEwen *et al.*, 2007). Using a mouse model, it was determined that the olfactory impairment was due to a mislocalization of the olfactory G protein rendering the signaling pathway nonfunctional, despite cilia remaining intact (McEwen *et al.*, 2007). Together, these studies suggest that olfactory dysfunction due to ciliary defects can occur by two separate mechanisms; (1) a complete loss of olfactory cilia and (2) a defect in protein trafficking leading to a loss in olfactory signaling.

7.2. Head trauma

In addition to age, which will not be discussed, one of the leading causes of loss of the sense of smell is head trauma, which can affect patients of any age (Costanzo and Miwa, 2006; Doty *et al.*, 1997; Reiter *et al.*, 2004). Currently, the largest incidence of head trauma occurs during a car accident (~52%), with nearly 25% of these cases experiencing deficits in olfactory function (Costanzo and Becker, 1986; Reiter *et al.*, 2004). The severity of the olfactory loss is directly correlated with the severity of the head trauma and duration of any posttraumatic amnesia (Costanzo and Becker, 1986; Reiter *et al.*, 2004; Sumner, 1964).

This loss in function is thought to occur by three main mechanisms: (1) skull or facial fractures resulting in a disruption of the sinonasal tract; (2) shearing or tearing of the olfactory nerve bundles; or (3) hemorrhaging or bruising of the brain regions within or surrounding the olfactory bulb (Costanzo and Miwa, 2006; Reiter *et al.*, 2004). Electron microscopy of an olfactory biopsy from a patient suffering from posttraumatic olfactory dysfunction revealed three major changes to the OE (Jafek *et al.*, 2002). In general, the OE exhibited large regions of disorganization, as well as an increase in axonal proliferation (Jafek *et al.*, 2002). Perhaps more relevant to the loss of olfactory function, however, was the finding that there were a decreased number of OSNs, and those that remained lacked cilia (Jafek *et al.*, 2002). In support of this finding, two other studies reported loss of olfactory cilia following traumatic head injuries (Hasegawa *et al.*, 1986; Jafek *et al.*, 1989). No matter what the cause of the olfactory dysfunction due to head trauma, the chances for spontaneous recovery are only approximately 30% (Costanzo and Becker, 1986; Doty *et al.*, 1997). Thus, it appears that simply regenerating the OE with ciliated neurons may not be enough and that proper axonal targeting and synapse formation may be equally important to restoring olfactory function.

7.3. Chronic rhinosinusitus

Chronic sinusitis is characterized by inflammation of the nasal mucosa occurring for at least 12 weeks and is associated with both allergic and nonallergic rhinitis, as well as nasal polyps (Benninger *et al.*, 2003; Raviv and Kern, 2004). Typical symptoms of chronic rhinosinusitis include nasal obstruction and mucosal discharge, which has been shown to cause olfactory dysfunction (Cullen and Leopold, 1999; Raviv and Kern, 2004; Seiden and Duncan, 2001). These anosmias affect nearly 10 million people worldwide and account for approximately 25% of all smell loss cases (Raviv and Kern, 2004; Seiden and Duncan, 2001). The mechanism of olfactory dysfunction in chronic sinusitis patients is thought to be twofold: (1) damage or alteration of the olfactory mucosa or (2) inflammation and damage to the OSNs in the epithelium (Benninger *et al.*, 2003; Raviv and Kern, 2004). Similar to head trauma, cilia were missing from dendritic knobs of OSNs in biopsies obtained from patients with chronic sinusitis (Cullen and Leopold, 1999; Douek *et al.*, 1975). Further, there appeared to be fewer neurons and axons in the damaged epithelium (Cullen and Leopold, 1999; Douek *et al.*, 1975). Patients exhibiting smell loss due to chronic sinusitis probably exhibit a combination of obstruction and neurodegeneration. Cilia loss may occur in response to the inflammation or changes in the nasal mucosa and represent the initial step in neurodegeneration.

7.4. The olfactory system as a pathogenic target

The mammalian olfactory system is unique in that it is the only region of the CNS that is directly exposed to the external environment (Doty, 2008; Doty *et al.*, 1991). It is estimated that the exposed surface of the OE, comprising the dendritic knob plus cilia, is around 23 cm^2 (Doty, 2008; Doty *et al.*, 1991). Together, this makes the OE a unique and vulnerable target for the entry of pathogens directly into the brain. Even though the OE is partially protected by the presence of the nasal mucosa as well as high levels of metabolizing enzymes, such as cytochrome P450s, evidence exists that pathogens can enter the brain through the OE (Baker and Genter, 2003; Ding and Dahl, 2003; Doty, 2008). In early twentieth century, it was shown that viruses could enter the monkey brain and that this was prevented by lesioning either the OE, the axon tracts, or the olfactory bulb (Brodie and Elvidge, 1934; Doty, 2008; Flexner, 1917; Schultz and Gebhardt, 1936). One of the major debilitating viruses shown to enter the brain via the OE was the poliomyelitus virus (Brodie and Elvidge, 1934; Flexner, 1917; Schultz and Gebhardt, 1936). Today, the list of viruses able to infect the OE has expanded and includes some major viruses, such as adenovirus, herpes simplex, hepatitis, influenza A, and rabies, as well as many others (Doty, 2008). A subset of these pathogens may enter exclusively through OSNs and specifically the cilia. For example, the olfactory cilia from a patient with sporadic Creutzfeldt–Jakob disease were positive for protease–resistant prion protein (Tabaton *et al.*, 2004). Following death, a neuropathological examination revealed nerve loss and gliosis in cerebral cortex, striatum, and cerebellum, suggesting that the olfactory cilia served as a site for pathogen entry (Tabaton *et al.*, 2004). Therefore, the OE, specifically the OSN cilia, is likely a major target for pathogenic transmission of xenobiotics directly into the brain.

8. Summary

The mammalian nose, through the use of olfactory cilia, has the capability of detecting millions of different odorants. The ability to identify these odors yields diverse information about the surrounding environment, from social cues to signs of danger. The inability to detect these changes as a result of olfactory dysfunction is frequent in the general population, affecting at least 2.5 million people in the U.S. alone. It is now clear that olfactory dysfunction is also a clinical manifestation of an emerging class of human genetic disorders, termed ciliopathies, which involve defects in ciliary assembly and/or protein transport. Given the plasticity of the olfactory system and its regenerative properties, OSNs undergo a continual process of ciliogenesis and protein transport that is critical for olfactory function.

Defects in these processes or the potential loss of OSN cilia following trauma, inflammation, or pathogen entry may all contribute to the etiology of olfactory disorders and, together, highlight the need for more understanding of the mechanisms and molecular machinery necessary for ciliary transport in OSNs.

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