



RESEARCH ARTICLE

ULTRASTRUCTURAL STUDY ON OLFACTORY SENSORY NEURON IN AN AIR-BREATHING TELEOST, *ANABAS TESTUDINEUS* (BLOCH, 1792)

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ARTICLE INFO

Article History:

Received 20th September, 2024
Received in revised form
17th October, 2024
Accepted 24th November, 2024
Published online 30th December, 2024

Key Words:

Anabas testudineus, Olfaction, Olfactory neuroepithelium, OSRNs, etc.

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ABSTRACT

Olfaction represents a crucial chemosensory modality in vertebrates, mediated by specialized olfactory sensory receptor neurons. Unlike terrestrial vertebrates, the olfactory organs of fish remain in close contact with the aquatic environment, enabling them to sense waterborne chemical cues and trigger specific behaviors. This chemosensory system comprises nostrils, nasal cavities housing sensory lamellae, olfactory nerves, bulbs and brain. *Anabas testudineus* (Bloch, 1792), an air-breathing teleost (Anabantiformes: Anabantidae; IUCN Red List: Least Concern) that periodically interacts with an air-water interface, representing its dual-mode lifestyle. This study aims to explore the ultrastructure of olfactory sensory receptor neurons (OSRNs) of *A. testudineus* using electron microscopy. The olfactory organs of *A. testudineus* were dissected, the rosettes were examined under a scanning electron microscope (SEM) and transmission electron microscope (TEM) after fixation in 2.5% glutaraldehyde and Karnovsky's fixative, respectively, and subsequent histological processing. In *A. testudineus*, the oval-shaped olfactory rosette is distinguished into prominent sensory and non-sensory epithelium. The olfactory neuroepithelium is a ciliated and pseudostratified structure with ciliated supporting cells, bipolar OSRNs, basal cells, etc. A highly developed sensory epithelium equipped with abundant ciliated olfactory sensory neurons (CiOSNs), which appear dominant, and microvillous olfactory sensory neurons (MiOSNs), distributed within dense aggregations of ciliated non-sensory cells, reflecting the specific adaptive nature of the species concerned.

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Citation: Madhabi Aich. 2024. "Ultrastructural Study on Olfactory Sensory Neuron in an Air-Breathing Teleost, *Anabas testudineus* (Bloch, 1792)". *International Journal of Current Research*, 16, (12), 31063-31068.

INTRODUCTION

Olfaction is a prime chemosensory modality in vertebrates, enabling the perception of chemical signals in the environment. Fish are primarily adapted to an aquatic environment, and olfactory perceptions play a pivotal role in sustaining several life functions, such as finding food, avoiding predators, spawning, migration, reproduction, parental care, etc. [1]. In addition, peripheral olfactory organs are highly vulnerable due to direct exposure to pollutants, heavy metals, toxins, pathogens, injury, etc., making olfactory plasticity crucial [2]. The primordium of the vertebrates' olfactory system is the olfactory placode [3], an ectodermal thickening which originates during early embryogenesis. The olfactory neuroepithelium is equipped with sensory receptor neurons that detect odors in the external environment. The fish olfactory apparatus comprises of paired nostrils, olfactory chambers with rosettes, olfactory nerves, olfactory bulbs and brain, allowing them to identify a wide array of waterborne chemicals. Furthermore, teleosts are adapted to diverse habitat conditions, their olfactory system exhibited morphological, anatomical and functional differences compared to other vertebrates [4]. Several researchers had examined the olfactory organ in teleosts through morphoanatomy [5,6,7] and electron

microscopy [8,9,10,11,12]. However, the micro-morphological adaptations of olfactory mucosa in air-breathing teleosts remain under-explored. *Anabas testudineus* (Bloch, 1792) is an air-breathing, climbing perch (IUCN - Least Concern) of India and Southeast Asia. They inhabit fresh and brackish water, rivers, ponds, lakes and swamps, often surviving in hypoxic water bodies [13]. The feeding pattern of the specimen is predominantly carnivorous [14]. The olfaction of *A. testudineus* is of significant interest because it shows synchronized air-gulping behaviour, causing them to move periodically at air-water interface and can also lead an amphibious lifestyle, navigating both aquatic and semi-terrestrial habitats [15]. They are social, prefer to join in larger, unfamiliar shoals in group migration [16]. Unlike the traditional carps, the climbing perch exhibits higher market demand due to its high nutritional profile and survival rates in modern aquaculture like Recirculatory Aquaculture Systems and Biofloc, as well as consumers' inclination toward live fish [17,18]. However, the abundance of native *A. testudineus* tends to decline due to habitat loss and commercial introduction of the exotic strains. This research aims to explore the ultrastructural architecture and functional attributes of the olfactory sensory receptor neurons in *A. testudineus*, facilitating the detection of chemical

cues in their environment by scanning and transmission electron microscopy.

MATERIALS AND METHODS

Live and healthy specimens of native *Anabas testudineus* (Bloch, 1792) [length: 8 to 14 cm; weight: 11 to 47 gm] were obtained from local fishermen in Hooghly district, West Bengal, India. Fishes were acclimatized in laboratory conditions for 48 hours in well-aerated aquaria with natural foods. For study, prior to dissection, the specimens were anesthetized with buffered tricaine methanesulfonate (ms-222) solution at a dosage of 100 mg/l.

Scanning electron microscopical (SEM) study: The olfactory organ was dissected and the rosettes were removed from the dorsal head region of *A. testudineus*. Immediately, tissue fixation was done in 2.5% glutaraldehyde solution (Sigma-Aldrich, EM Grade) in 0.1 M phosphate buffer [Na_2HPO_4 and NaH_2PO_4 ; pH -7.4] for 2 hours at 4°C. The olfactory tissues were then washed in phosphate buffer (0.1 M, pH-7.4) and thoroughly dehydrated through ascending grades of chilled acetone, followed by isoamyl acetate. Afterwards, the specimens were critically point dried (CPD) using liquid carbon dioxide (CO_2) in a critical point drier (Hitachi 8CP2). The dried samples were mounted on metal stubs, coated with platinum (16 nm thick) via a sputter coater (Quorum Q150TES) and examined under a Scanning Electron Microscope (SEM, Zeiss EVO18) operated at 20 kV.

Transmission electron microscopical (TEM) study: The olfactory lamellae of *A. testudineus* were excised and promptly fixed in Karnovsky's fixative buffered with 0.1(M) phosphate buffer (PB), pH 7.4 for 12 hours at 4°C. After primary fixation, tissues were washed [0.1 (M) PB, pH 7.4 at 4°C] and underwent secondary fixation in 1% osmium tetroxide in 0.1 (M) PB, pH 7.4 at 4°C for 1 hour. The post-fixed samples were dehydrated through ascending chilled acetone gradient and embedded in resin (Araldite CY 212). The specimens were sectioned longitudinally (thickness: 70-90 nm) using an ultramicrotome (Leica EMUC7) with glass knives. The ultrathin sections were lifted on copper grids and subsequently contrasted by staining with uranyl acetate and lead citrate (1% aqueous solution). The specimens were visualized under Transmission Electron Microscope [(TECNAI, G 20 (FEI)] operated at 200 kV.

RESULTS

Anabas testudineus possesses oval shape multilamellar olfactory rosette situated on the antero-dorsolateral region of head within an olfactory cavity associated with anterior and posterior nostrils (Figs. 1A, B). Under scanning electron microscope, the olfactory lamellae are elongated structures, externally lined by two neuroepithelial layers integrated around a central core (Figs. 1B, C). The neuroepithelium is ciliated and pseudostratified comprises of olfactory sensory receptor neurons (OSRNs), columnar supporting cells, roundish basal cells, etc. (Fig. 1C). The olfactory lamellae are sharply demarcated into densely ciliated inner sensory epithelium bounded by peripheral non-sensory epithelium (Fig. 1D). The OSRNs are marked as ciliated olfactory sensory neurons (CiOSNs) those appear dominant type and microvillous olfactory sensory neurons (MiOSNs) in sensory

epithelium, remain distributed within the dense ciliation of ciliated non-sensory cells (Fig. 1E). The OSRNs are typical bipolar neurons equipped with dendron oriented upright toward lumen of olfactory cavity, a deep-seated cell body and lower narrow axon descend toward the epithelial base (Fig. 1F). Under transmission electron microscope, the dendron of CiOSNs at apical end form olfactory knob projecting into the olfactory lumen, appears a pronounced bulbous structure (diameter: 0.9 -1.3 μm) bears multiple sensory cilia emanating from its free surface (Fig. 2A). Each cilium is associated with a single basal body at the base and inner microtubules run parallel along its length (Fig. 2A). The cilium in cross-section shows central microtubules surrounded by circularly arranged outer nine pairs of microtubules (Fig. 2B).

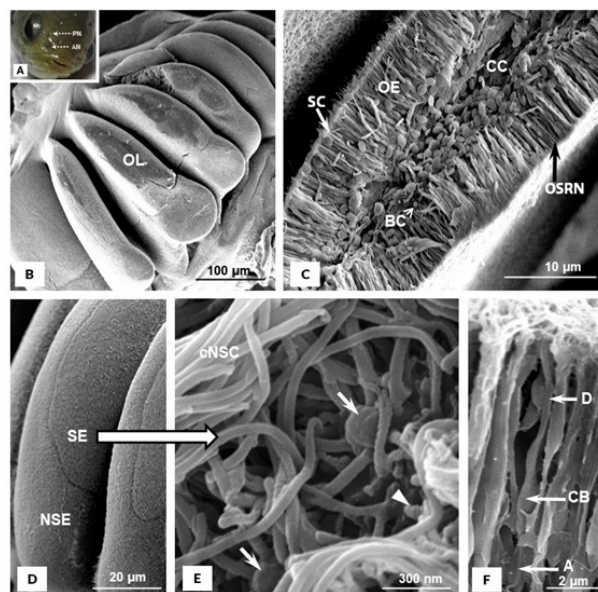


Figure 1. (A).The photograph of head of *Anabas testudineus* (Bloch, 1792) showing olfactory chamber associated with nostrils. Anterior nostril (AN), posterior nostril (PN). (B).3D structure of olfactory rosette of *A. testudineus* under scanning electron microscope (SEM). Olfactory lamella (OL). (C). Scanning photomicrograph of olfactory lamella at a cross-section shows pseudostratified olfactory neuroepithelium (OE) with olfactory sensory receptor neuron (OSRN), supporting cell (SC), basal cell (BC) and mid-central core (CC). (D). SEM micrograph of olfactory lamellae depicting the arrangement of sensory epithelium (SE) and non-sensory epithelium (NSE). (E). Ciliated olfactory sensory neurons (arrows) and microvillous olfactory sensory neurons (arrowhead) are distributed in sensory epithelium within dense cilia of ciliated nonsensory cells (cNSC). (F). SEM micrograph of a bipolar OSRN displays an apical dendron (D) directed towards epithelial surface, cell body (CB) and lower axon (A) running downward.

The approximate diameter of cilia of CiOSNs of *A. testudineus* is 0.2 μm . The CiOSNs are found to ensheath on either side by prominent supporting cells bearing glycocalyx-coated microvilli at luminal surface and marked with electron-lucent vesicles in cytoplasm (Fig. 2A). The olfactory knob of CiOSNs forms a distinct junctional complex with adjoining microvillous supporting cells at the lateral surfaces, which is an apical tight junction (length: 0.4 - 0.7 μm) followed by zonula adherens (length: 0.2 μm) (Fig. 2A). The MiOSNs are less abundant compared to CiOSNs. The dendron of MiOSNs is sharply identified with an olfactory knob at the apex oriented toward the olfactory cavity (Fig. 3A). The olfactory knob is endowed with several microvilli (8 to 11 in number),

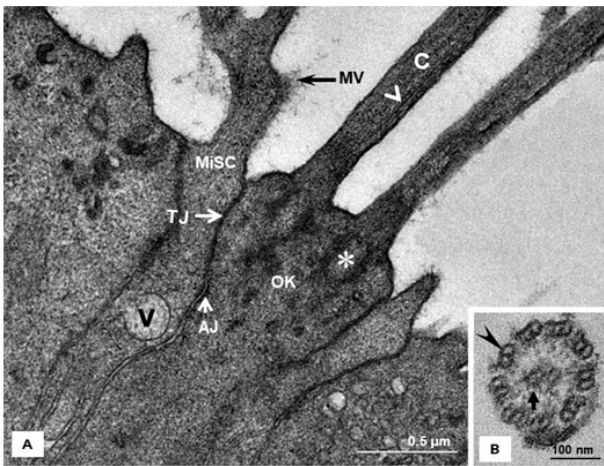


Figure 2. TEM micrographs of CiOSN ensheathed by lateral microvillous supporting cell (MiSC) in *A. testudineus*. (A). Dendritic olfactory knob (OK) bears cilia (C) with microtubules (arrowhead), basal body (asterisk), and lateral junctional complex including tight junction (TJ) and adherens junction (AJ). The adjoint MiSC represents microvilli with glycocalyx (MV) and vesicle (V) in apical cytoplasm. (B). Sensory cilia at cross-section exhibiting central (arrow) and peripheral (arrowhead) microtubules.

which are comparatively thin (diameter: 0.05 - 0.09 μm), remain in proximity, creating in a bouquet-like pattern (Figs. 3A, B). The MiOSNs are laterally flanked by prominent supporting cells, featuring apical glycocalyx-coated microvilli and vesicles in cytosol (Fig. 3A). Each MiOSN olfactory vesicle forms a well-developed lateral tight junction with neighboring supporting cells (Fig. 3A).

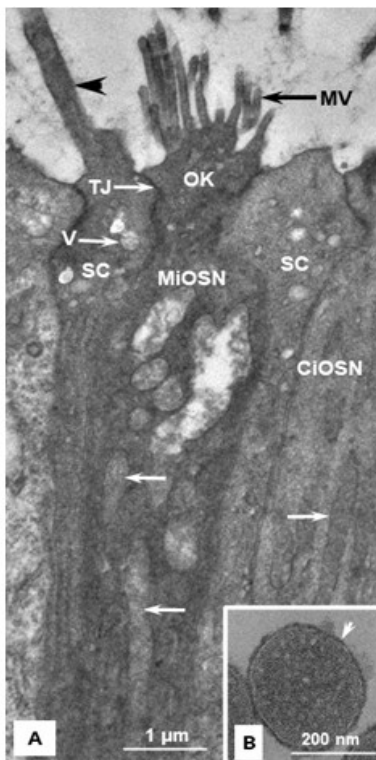


Figure 3. TEM micrographs depicting microvillous olfactory sensory neuron (MiOSN) in *A. testudineus*. (A). MiOSN shows olfactory knob (OK) projecting anterior microvilli (MV) and lateral tight junction (TJ) in sensory epithelium together with ciliated olfactory sensory neuron (CiOSN). Arrows indicate tubular mitochondria in olfactory dendritic cytoplasm. Supporting cell (SC), microvilli (arrowhead), vesicles (V). (B). Cross-sectional ultrastructure of microvilli of MiOSN.

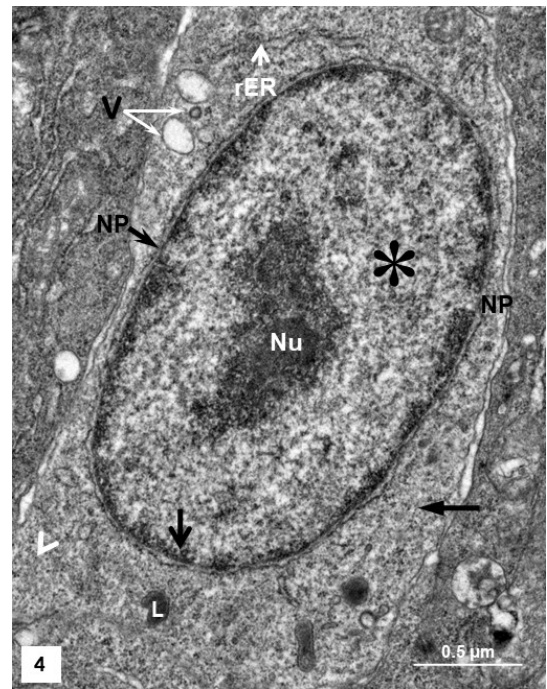


Figure 4: TEM micrograph illustrating the cell body of CiOSNs in *A. testudineus* comprises an elliptical nucleus with predominance of euchromatins (asterisk), heterochromatins (arrow), nucleolus (Nu), nuclear pore (NP), and rough endoplasmic reticulum (rER), heterogeneous vesicles (V), free ribosomes (arrowhead), polyribosome (arrow), and lysosome (L) in perinuclear cytosol.

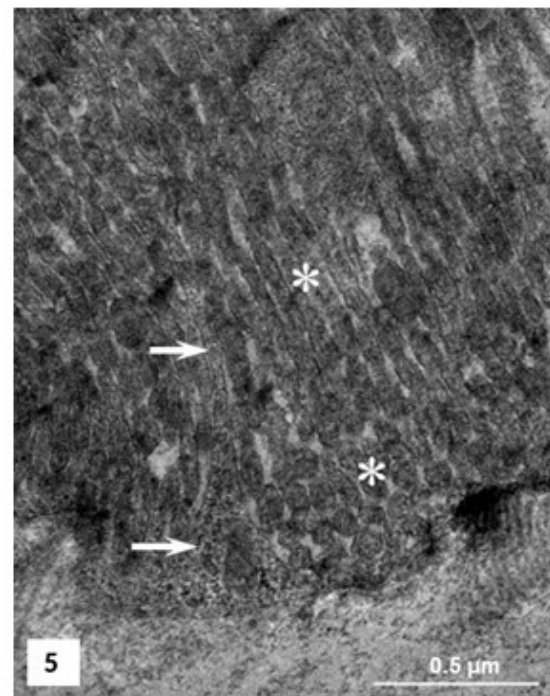


Figure 5. TEM micrograph reveals a portion of fila olfactoria consisting of unmyelinated axons (asterisks) of OSRNs wrapped by the Schwann cell processes (arrows) at base of the olfactory neuroepithelium in *A. testudineus*.

The dendron of ciliated and microvillous neurons is long, slender and characterized by an abundance of long tubular mitochondria that create a large mitochondrial mass in dendritic cytoplasm (Fig. 3A). The cell body constitutes a large, elliptical well-defined nucleus with nuclear lamina,

multiple nucleopores and a voluminous nucleolus. Nucleoplasm shows widely dispersed euchromatins and a comparatively few heterochromatins (Fig. 4). The cisternae of rER, heterogeneous vesicles *viz.*, large electron-lucent and small coated vesicles, and dense lysosomes are marked in the perikaryon (Fig. 4). Abundant free ribosomes and polyribosomes are scattered in the perikaryal cytoplasm (Fig. 4). The olfactory axons are unmyelinated, descend to the basal lamina, clustering to form fila olfactoria externally wrapped by cytoplasmic sheath of Schwann cells, and distant olfactory nerve that projects towards the olfactory bulb of brain (Fig. 5).

DISCUSSION

Fish rely heavily on olfaction to detect and discriminate aquatic chemical cues. Their olfactory organs exhibit considerable diversity, reflecting the evolutionary adaptation and ecological habitats [19]. The climbing perch, *A. testudineus* possesses well-developed olfactory system situated on dorso-lateral aspect of head, anterior to eyes. The nostrils serve as an avenue for the inflow and outflow of water during ventilation [20]. The multilamellar rosette of *A. testudineus* increased the surface area and sensitivity, effectively compensating for a lower lamellar count; oval pattern of rosette reflects the criteria of 'eye-nose' fishes [21]. The olfactory sensory epithelium in this air-breathing teleost is densely ciliated, protected by long cilia of non-sensory cells, a unique feature that supports adaptation to a semi-aquatic mode of life. The non-sensory cilia generate force by bending near its base [22], mediate the ventilation of olfactory chamber by beating, and facilitate transport of odorants over the olfactory mucosa.

The profuse density of sensory receptor neurons in olfactory mucosa of *A. testudineus* suggests a highly specialized sensory suite, underpinning distinctive behavioral modalities. Olfactory processing in vertebrates primarily begins with odorant-receptor interactions in cilia of olfactory neurons, which transduce intracellular signals by coupling to G proteins [23]. In this study, unlike non-sensory cilia, olfactory cilia are shorter and anchored to a basal body lacking striated rootlet, which is typical for sensory cilia. The olfactory mucus generated the optimal condition necessary for odorant-receptor interactions [24], and also prevents mechanical damage, desiccation during emersion. The nuclei of CiOSNs, with predominant euchromatic matrix, indicate high metabolic and transcriptional activity. The large, prominent nucleolus reflects the intensive ribosomal biogenesis, that morphologically coupled with the marked abundance of free ribosomes and polyribosomes, underscoring a highly upregulated somatic protein-building machinery. The odorant binding to the receptor of OSRNs triggers an electrical impulse, which is then transmitted to the brain [25]. Therefore, the tight adhesion in apical olfactory knob of CiOSNs and MiOSNs is indispensable during olfactory signalling as it prevents the diffusion of solutes across the extracellular spaces. In *A. testudineus*, different OSRN morphotypes coexist within sensory region with varying degrees of prevalence; ciliated neurons are the dominant type compared to microvillous variety.

The number of microvilli in MiOSNs exhibited variation among teleosts, e.g., 10 to 30 in *Dicentrarchus labrax* [26] and 40 to 70 in *Carassius auratus* [27], although this study noted a range of 8 to 11. Earlier studies in catfish demonstrated that CiOSNs responded to detect amino acids related to feeding, and predominantly to bile salts [28,29], potent semiochemicals,

which act as specific olfactory cues during spawning migration [30], playing roles in both intra-specific chemical communication and in inter-specific interactions [31]. CiOSNs also participate in responding to alarm substances [32], thus being tuned to social/alarm cues and exhibiting a multifaceted role within the fish's olfactory landscape. The microvillous neurons serve as specialists responding to amino acids and nucleotides, generally feeding stimulants [33,28]. Thus, the higher abundance of CiOSNs in *A. testudineus*, air-breathing perch, implies a high sensitivity to these specific chemical signals, suggesting that this species relies heavily on chemical maps (bile salts, amino acids) and social communication for its survival and reproductive cycles. Although crypt cell neurons are reported in a few fish species [34] that exhibit seasonal variation and respond to sex pheromones. The close association between the microvillous supporting cells and OSRNs suggests that they may act like glial cells as nourishing element of sensory receptor cells. The glycocalyx in microvilli implied the carbohydrate-rich zone provided cellular protection against mechanical and chemical damage, prevented undesirable protein-protein interactions [35]. The cytosolic vesicles indicate secretory function of microvillous supporting cells. Abundant basal cells scattered at base of the olfactory neuroepithelium of *A. testudineus* are considered to be progenitors of olfactory neurons and supporting cells [36]. Thus, the journey from a waterborne chemical to a behavioral response is a multi-step transduction process that effectively translates the language of chemistry into the language of electrical signal, which travels toward the olfactory bulb of brain. Glomeruli in the olfactory bulb serve as basic modules in information processing and the signal reaches higher olfactory centers of the brain [37], triggering specific behavioural responses. Thus, *A. testudineus*, prefers to live in groups, an air-breathing teleost exposed to air-water interface largely depends on olfactory cues, having highly developed olfactory system with a neurogenic niche comprising different olfactory neurons and progenitor cells, which are of particular interest in several neurodegenerative disorders where loss of smell is critical. Consequently, this study provides a foundational framework for further research on the characterization of bile salts in *A. testudineus*, underlying their biological context for terrestrial orientation and navigation.

ACKNOWLEDGEMENTS

The author is grateful to the authorities of the Centre for Research in Nanoscience and Nanotechnology (CRNN), University of Calcutta, for providing the scanning electron microscope (SEM) facility and All India Institute of Medical Sciences (AIIMS), New Delhi, India, for providing access to transmission electron microscope (TEM) instrumentation.

Conflicts of interest: The author declares no conflict of interest.

Funding statement: There is no funding source to disclose.

Key points

- Olfaction is crucial for fish survival in diverse aquatic habitats, where OSRNs, functional units located in olfactory neuroepithelium, act as a bridge between environmental chemical cues and the CNS. The olfactory neuroepithelium of *A. testudineus*, air-breathing perch, is of scientific interest due to its functional adaptation to the air-water interface, dual-environment orientation.

- The olfactory neuroepithelium of *A. testudineus* is ciliated and pseudostratified with sharp demarcation of sensory and non-sensory regions. The sensory epithelium is equipped with bipolar neuronal populations, viz., 1. CiOSNs: predominant cell type, projecting apical cilia (diameter: 0.2 µm) supported by microtubular internal structure, 2. MiOSNs: bearing 8-11 apical microvilli, distributed within dense aggregations of ciliated non-sensory cells, trigger different behavioural responses.
- The apical olfactory knob of OSRNs, projecting into lumen, is anchored to adjacent microvillous supporting cells by a well-defined lateral junctional complex, i.e., tight junction (0.4-0.7 µm), and a subsequent zonula adherens (0.2 µm).
- The dendritic cytoplasm is marked with a dense mass of long, tubular mitochondria, while the cell body constitutes an euchromatinized nucleus, rERs, heterogeneous vesicles, lysosomes, free ribosomes, and polysomes, underscoring a highly upregulated protein-building machinery. Unmyelinated axons descend to the basal lamina, aggregating into Schwann cell-wrapped fila olfactoria, neural bundles, then project toward the olfactory bulb for signal transmission.

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