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## Expression of SNMP-1 in olfactory neurons and sensilla of male and female antennae of the silkmoth *Antheraea polyphemus*

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**Abstract** SNMP-1 (sensory neuron membrane protein 1) is an olfactory-specific membrane-bound protein which is homologous with the CD36 receptor family. Previous light level immunocytochemical studies suggested that SNMP-1 was localized in the dendrites and distal cell body of sex-pheromone-specific olfactory receptor neurons (ORN); these studies further suggested SNMP-1 was expressed in only one of two to three neurons in male-specific pheromone-sensitive trichoid sensilla. To better understand the expression and localization of SNMP-1, an immunocytochemical study was performed using electron microscopy to visualize the distribution of SNMP-1 among the neurons of several classes of olfactory sensilla of both male and female antennae of the silkmoth *Antheraea polyphemus*. SNMP-1 antigenicity was primarily restricted to the receptive dendritic membranes of ORNs of all sensilla types examined and was observed in cytosolic granules, but not plasma membranes, of the cell soma. Mean labeling densities ranged from 1 to 16 gold particles per micrometer of dendrite circumference; dendrites of trichoid and intermediate sensilla showed significantly higher labeling densities than those of basiconic sensilla. Larger dendrites of trichoid sensilla showed significantly higher mean labeling densities (13–16/μm) than smaller

diameter dendrites (3–7/μm). Immunofluorescence studies using baculovirus expressed SNMP-1 and multiphoton photon laser scanning microscopy (MPLSM) indicated that rSNMP-1, which was post-translationally processed to the in vivo molecular weight, was inserted into the plasma membrane in a topography presenting extracellular epitopes. These studies suggest SNMP-1 is a common feature of the ORNs, is asymmetrically expressed among functionally distinct neurons, and possesses a topography which permits interaction with components of the extracellular sensillum lymph.

**Keywords** Baculovirus · CD36 · Pheromone · Receptor · MPLSM · *Antheraea polyphemus* (Insecta)

### Introduction

Insects detect a range of odors using diverse classes of olfactory sensilla on the antennae; each sensillum typically contains two to three olfactory receptor neurons (ORNs). Studies have shown that the different classes of sensilla are endowed with a variety of olfactory-specific proteins, including extracellular odorant-binding proteins (OBPs) and odorant-degrading enzymes (ODEs) (e.g., Vogt and Riddiford 1981; Vogt et al. 1985, 1999; Rybczynski et al. 1990; Laue and Steinbrecht 1997; Hekmat-Scafe et al. 1997; Rogers et al. 1999; Sandler et al. 2000), and neuronal odorant receptor proteins (ORs) (Clyne et al. 1999; Gao and Chess 1999; Vosshall et al. 1999). All three types of proteins are thought to interact with odor molecules within the extracellular compartment that surrounds the olfactory neuron and fills the sensillum: OBPs transport odor molecules to the ORs, and ODEs degrade odor molecules (see Fig. 8A). OBPs and ORs exist as multi-gene families, and ODEs exist as unrelated proteins (e.g., esterase, aldehyde oxidase; Rybczynski et al. 1990). Unique combinations of members of all three protein families are apparently expressed among the different classes of olfactory sensilla contributing to the overall odor-sensitive phenotypes of the sensilla.

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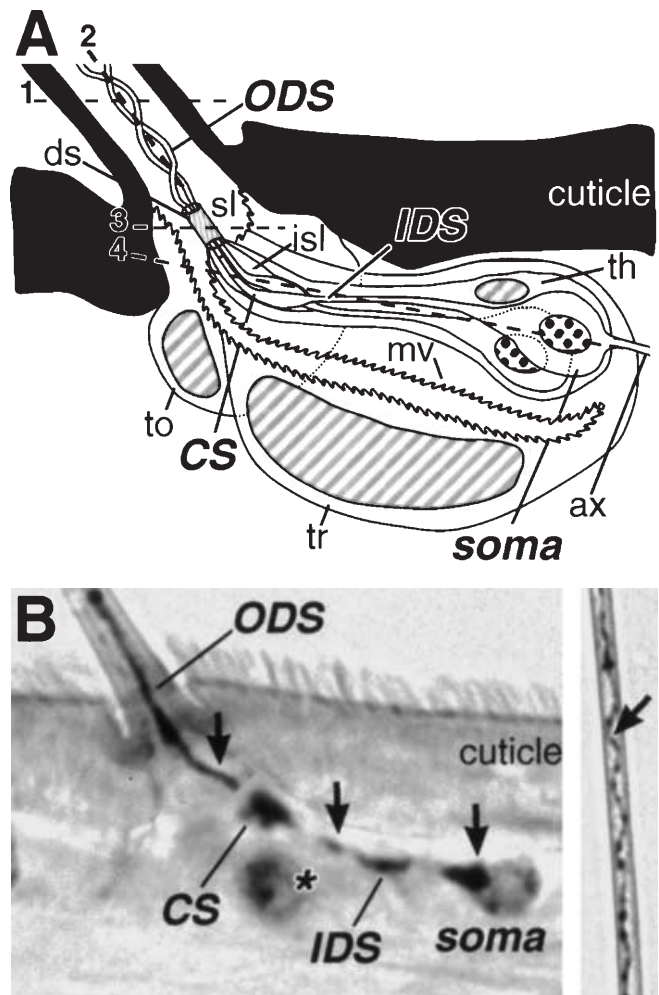
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SNMP-1 is an olfactory-specific protein (67 kDa) uniquely expressed in ORNs (Rogers et al. 1997). SNMP-1 was purified from olfactory receptor membranes of the silkworm *Antheraea polyphemus*, directly sequenced in part, and subsequently cloned. In *A. polyphemus*, SNMP-1 expression is antennal specific, and is present in both male and female antennae, though more highly expressed in males. *SNMP-1* mRNA expression was observed to initiate at around 50% of adult development, but to substantially increase near the end of adult development and continue into adult life, coinciding more with olfactory function than with antennal formation. SNMP-1 is homologous with the CD36 receptor family, which is represented in multiple phyla as two-transmembrane domain proteins which predominately recognize proteinaceous ligands. While the expression profile of SNMP-1 is somewhat consistent with that expected for an OR, its two-transmembrane domain topography fails to support this role; insect ORs are thought to be seven-transmembrane domain proteins coupled to G-protein-mediated second messenger cascades (Clyne et al. 1999; Gao and Chess 1999; Vosshall et al. 1999). Nevertheless, the temporal and spatial pattern of SNMP-1 expression suggest this protein also plays a central role in odor reception.

Initial immunocytochemical studies employing light microscopy indicated that SNMP-1 protein was localized in the dendrite and cell body but was absent from the axons of olfactory neurons of pheromone-specific sensilla (sensilla trichodea); the protein was distributed more distally than proximally within the cell body (see Fig. 1; Rogers et al. 1997). Localization to the olfactory dendrites indicated that SNMP-1 resides in the region also occupied by membrane proteins that transduce odor signals (e.g., ORs, G-proteins, ion channels; see Zufall and Hatt 1991; Laue et al. 1997). Interestingly, antigenicity appeared to be restricted to only one of the two to three neurons per sensillum. This asymmetric distribution of SNMP-1 among the multiple neurons of a sensillum led to hypotheses that there might be additional SNMP genes expressing in the other neurons and that SNMP-1 may contribute to defining the identity of these neurons.

To obtain a higher resolution picture of the distribution of SNMP-1, immunocytochemical studies were performed employing electron microscopy. Four morphologically distinct classes of sensilla were compared in male antennae, and two classes were compared in female antennae. Only male antennae possess the sex-pheromone-sensitive type I and II sensilla trichodea, while both male and female antennae possess sensilla basiconica and a class here termed "intermediate." A statistical analysis was performed comparing SNMP-1 densities in membranes of different cells within a sensillum, as well as between the respective sensilla classes. Also, in order to confirm that the SNMP-1 clone contained the sequence determinants which control membrane insertion, SNMP-1 was expressed in cultured insect cells using the baculovirus expression system. Fluorescent immunocytochemical analysis was performed on non-permeabilized and permeabilized cells using multiphoton laser scan-



**Fig. 1** SNMP-1 expression is restricted to the olfactory receptor neurons of *A. polyphemus* antennae. **A** Schematic drawing of a typical sensillum showing two bipolar ORNs and three auxiliary cells. Surrounding non-sensory epithelium is not shown. The neuronal cell bodies (i.e., soma) are situated at the base of the sensillum lumen and are surrounded by the thecogen (*th*), tormogen (*to*), and trichogen (*tr*) auxiliary cells. A ciliary segment (*CS*) partitions the dendrites into the outer and inner dendrite segments (*ODS* and *IDS*, respectively). The *ODS* of each neuron enters the cuticular sensillum hair and is surrounded by the sensillum lymph (*sl*); the axons (*ax*) innervate the deutocerebrum in the brain (1–4 sectional planes used for EM-level immunocytochemistry; see text) (modified from Keil 1992). **B** Light-microscopic immunolocalization of SNMP-1 in male *A. polyphemus* sex-pheromone-sensitive olfactory sensilla (see Rogers et al. 1997). Staining is apparent in only one (arrows) of the two or three ORNs that inhabit these sensilla. SNMP-1 is expressed in the soma, *CS* and *IDS* of ORNs (left and right panels) and is clearly expressed in the *ODS*, which innervates the sensillum hair (right panel). The asterisk marks an SNMP-1-immunopositive neuronal cell body associated with a different sensillum (*ds* dendrite sheath, *isa* inner sensillum lymph, *mv* microvilli, *sl* outer sensillum lymph)

ning microscopy (MPLSM) to partially characterize the membrane association of expressed protein. These studies provide strong support for SNMP-1 localization in a central region of odor processing, and establish an assay for the further characterization of SNMP-1 function.

## Materials and methods

### Electron-microscopic-level immunocytochemistry

*Antheraea polyphemus* were obtained from pupae from various breeders, kept at 10°C after adult emergence and used when 3–8 days old. Antennae from two males and one female were cryofixed by being rapidly plunged into cooled propane (–180°C) and freeze substituted in pure acetone or acetone with 1% or 3% glutaraldehyde added. Tissue was embedded in LR white resin and sectioned with a Reichert Ultracut ultramicrotome; sections were collected on Formvar-coated copper grids (for details, see Steinbrecht et al. 1992, 1995).

Immunolabeling was performed according to Steinbrecht et al. (1992, 1995). Grids were successively floated on 30- $\mu$ l drops of each of the following solutions: 50 mM glycine in phosphate-buffered saline (PBS, pH 7.4) (2 $\times$ 10 min at room temperature, RT); blocking solution (0.05% gelatin, 0.5% bovine serum albumin, BSA, 0.025% Tween-20 in PBS; 1 $\times$ 10 min and 1 $\times$ 1 h at RT); and SNMP-1 polyclonal antiserum (see Rogers et al. 1997) (1:12,000 antiserum in blocking solution; 1 h at RT followed by 16 h at 4°C); controls used preimmune serum derived from the same rabbit from which the antiserum was obtained (1:100 in blocking solution). Following washes in glycine/PBS (6 $\times$ 5 min), SNMP-1 antibody was labeled with goat anti-rabbit IgG secondary antibody conjugated with 10 nm colloidal gold particles (BioCell, UK; 1:20 in glycine/PBS; 1.5 h at RT) and washed in gelatin/PBS (2 $\times$ 5 min at RT), glycine/PBS (2 $\times$ 5 min), PBS (2 $\times$ 5 min), and water before treatment with uranyl acetate (2% weight/volume in water; 15 min at RT).

Sections were examined in a Zeiss EM10A electron microscope at 80 kV and the negatives were scanned with a flatbed scanner. Dendrite circumferences were measured using the NIH Image program (version 1.58, National Institutes of Health; customized by Steve Barrett, Surface Science Research Centre, University of Liverpool). Labeling densities were calculated as the number of gold particles per micrometer circumference of a dendrite membrane. The total numbers of sensilla examined and the numbers in which positive labeling was observed are indicated in Table 1; only one section was examined per sensillum.

Dendrite circumference and SNMP-1 circumferential labeling density for individual ORNs in each labeled section were compared using Student's *t*-test (assuming equal variances) implemented in Microsoft Excel 97 (v. SR2). In the text, subscripts for "T" (e.g., T<sub>23,27</sub>) indicate the number of dendritic profiles analyzed for each of the two dendrite or sensillum types being compared. Subscripts for "P" (e.g., P<sub>A/B</sub> or P<sub>I/II</sub>) identify the dendrite or sensillum types being compared, and indicate the dendrite size class (A, B), sensillum type (I, II, Int, Bas), and sex (m, f) where appropriate.

### Localization of SNMP-1 protein expression in cultured insect cells

Recombinant baculovirus encoding full-length SNMP-1 coding region (base pairs 1–1568, Genbank accession number U95026) was generated using the Bac-To-Bac Baculovirus Expression System (Life Technologies) following recommended protocols. SNMP-1 baculovirus was generated by chemical transfection of recombinant bacmid DNA into a monolayer of *Sf9* cells; bacmid DNA contained full-length SNMP-1 coding region inserted downstream of the pPolh baculovirus-specific promoter (Bac-To-Bac baculovirus expression system). Following a 72-h incubation at 27°C of the bacmid-transfected cells, recombinant baculovirus was harvested from the culture medium, titered (1 $\times$ 10<sup>6</sup> pfu/ml), and reamplified to a final concentration of 1 $\times$ 10<sup>8</sup> pfu/ml. Media containing amplified virus were centrifuged (1000 $\times$ g, 5 min, 4°C) and the supernatant was stored in 1-ml aliquots at –75°C until use. All cell culture experiments were performed at 27°C and used log phase cells (>95% viability and 24–36 h doubling times) unless noted otherwise. *Sf9* and High Five cells maintained in suspension cultures were seeded in 24-well culture plates (0.6 $\times$ 10<sup>6</sup> cells/well) and infected with SNMP-1 baculovirus at a multiplicity of infec-

tion (MOI) of 10 for 1 h. Media containing virus were replaced with 500  $\mu$ l fresh media (supplemented with 10  $\mu$ g/ml gentamicin) and incubated for 12, 24 or 72 h (for Western blots, Fig. 6A, 12 h not shown) or 60 h (for immunofluorescence, Fig. 6B–D). Cells were then washed with 1 ml ice-cold TRIS-HCl buffer (62.5 mM, pH 6.8) and lysed at RT with 200  $\mu$ l lysis buffer (62.5 mM TRIS-HCl, pH 6.8, 2% SDS). Cell lysate was frozen immediately on dry ice and stored at –20°C until use.

For Western blot analyses, cell lysates (5 of 200  $\mu$ l) and *A. polyphemus* antennal branch homogenates (two antennal equivalents; see Rogers et al. 1997 for protein isolation) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12%), transferred to nitrocellulose membrane (Trans-Blot Cell, Bio Rad; BA-S NC, Schleicher and Schuell), and incubated with SNMP-1 antiserum (1:5000, Rogers et al. 1997) for 1 h at RT. The membrane was then incubated with goat anti-rabbit IgG horseradish peroxidase conjugate (ICN; 1:5000, 2 h at RT) and stained (VIP substrate; Vector). Membranes were blocked and antiserum applied in PBST (PBS containing 0.1% Tween 20) containing 3% non-fat dry milk; all washes were in PBST.

Expressed SNMP-1 protein was localized in High Five cells by indirect immunofluorescence using SNMP-1 antiserum. High Five cells were seeded on 5<sup>2</sup>- to 7<sup>2</sup>-mm glass coverslips (22<sup>2</sup>-mm no. 1 coverslips cut into quarters) placed in wells of a 24-well culture plate (0.5 $\times$ 10<sup>6</sup> cells/well) and incubated with either SNMP-1 or wild-type baculovirus (MOI of 10) as described above, or with media alone (minus virus control), all for 60 h. For non-permeabilized treatments ("intact cells," Fig. 6B), cells were incubated in primary antiserum before fixation and permeabilization. Cells were washed twice with 500  $\mu$ l fresh media and incubated immediately with blocking solution (1% BSA, 5% normal goat serum in culture media; 1 h at 27°C), followed by incubation in primary antiserum (either SNMP-1 antiserum or control preimmune serum diluted 1:2500 in culture media containing 1% BSA and 1% normal goat serum; 1 h at 27°C), fixation in paraformaldehyde (4% in PBS at RT), and permeabilization in prechilled (–80°C) 70% methanol (5 min at –20°C). For permeabilized treatments ("permeabilized cells," Fig. 6C), cells were exposed to primary antiserum after fixation, permeabilization and re-equilibration in PBS. Following the treatments above, cells were washed several times with PBS, and incubated with rhodamine-red-X-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories; 1:200 in PBS; 1 h at RT), and washed and visualized in PBS.

Rhodamine-coupled secondary antibody was visualized by MPLSM (Coherent TiS laser, Mira/Verdi; BioRad MRC 1024ES; Nikon TE300 inverted microscope). Using a  $\times$ 60 oil 1.4 n.a. Nikon objective lens and a  $\times$ 2.0 digital magnification, a Z-series was taken at 1- $\mu$ m increments and signal averaged using the Kalman filter (three passes/section). Images were processed through Confocal Assistant and Adobe Photoshop, and printed by dye sublimation.

## Results

### Sensillum nomenclature

*Antheraea polyphemus* antennae are sexually dimorphic. Each male antenna has approximately 60,000 pheromone-sensitive trichoid sensilla (sensilla trichodea) and 10,000 basiconic sensilla (sensilla basiconica) (Keil 1984; Meng et al. 1989), while each female antenna has approximately 12,000 basiconic sensilla and lacks trichoid sensilla (Boeckh et al. 1960). Trichoid and basiconic sensilla can be distinguished by several structural properties and by their position on the antenna (reviewed by Steinbrecht 1987). Trichoid sensilla have a thick cuticular wall that tapers toward the tip of the hair, a relatively low

**Table 1** Mean circumference of dendritic profiles and the mean circumferential SNMP-labeling densities are indicated for individual types of sensillum. Drawings of typical cross sections are to scale. *A* and *B* refer to two size classes of dendrites in sensilla trichodea and intermediate sensilla. *X* designates dendrites in sensilla basiconica, where specific neuronal relationships were undetermined. Values associated with *A* (*A'* for intermediate sensilla) and *B* refer to individual dendritic profiles. Values associated with *combined* (or *X*) refer to the sum of all dendritic membrane in a sensillum cross section. Combined values were obtained by first determining the total circumference and mean labeling density for all dendrite profiles within each cross section, and then determining the mean values for all labeled cross sections of a given sensillum type. Unlabeled cross sections were not considered

Sex	Sensillum Type (total # / # labeled)	Dendrite Class	n	Mean Dendrite Circumference $\mu\text{m}$ (SD)	Mean Label Density gold/ $\mu\text{m}$ (SD)
Male	trichodea I (23/23)	A	23	1.7 (0.2)	13.3 (3.3)
		B	27	0.8 (0.2)	3.1 (3.0)
		combined	50	2.7 (0.6)	10.2 (3.0)
	trichodea II (7/7)	A	7	1.2 (0.3)	15.7 (5.6)
	B	9	0.7 (0.1)	7.1 (5.2)	
	combined	16	2.0 (0.5)	12.1 (3.9)	
	intermediate (4/2)	A'	6	0.9 (0.2)	10.8 (4.8)
		combined	6	2.6 (0.4)	10.5 (5.4)
	basiconic (5/3)	X combined	37	7.5 (3.4)	5.9 (2.5)
Female	intermediate (8/8)	A'	22	1.0 (0.2)	9.5 (7.7)
		combined	22	2.7 (0.3)	9.8 (5.0)
	basiconic (9/7)	X combined	49	2.7 (1.2)	0.9 (0.7)

pore density, and ORNs with unbranched dendrites. In contrast, basiconic sensilla are much shorter, have a non-tapering and thinner cuticular wall, and contain ORNs with branched dendrites. Previous studies of male *A. polyphemus* identified two subtypes of trichoid sensilla distinguished by their relative length and position on the antennal branch, and a single type of basiconic sensilla (Boeckh et al. 1960; Keil 1984; Meng et al. 1989).

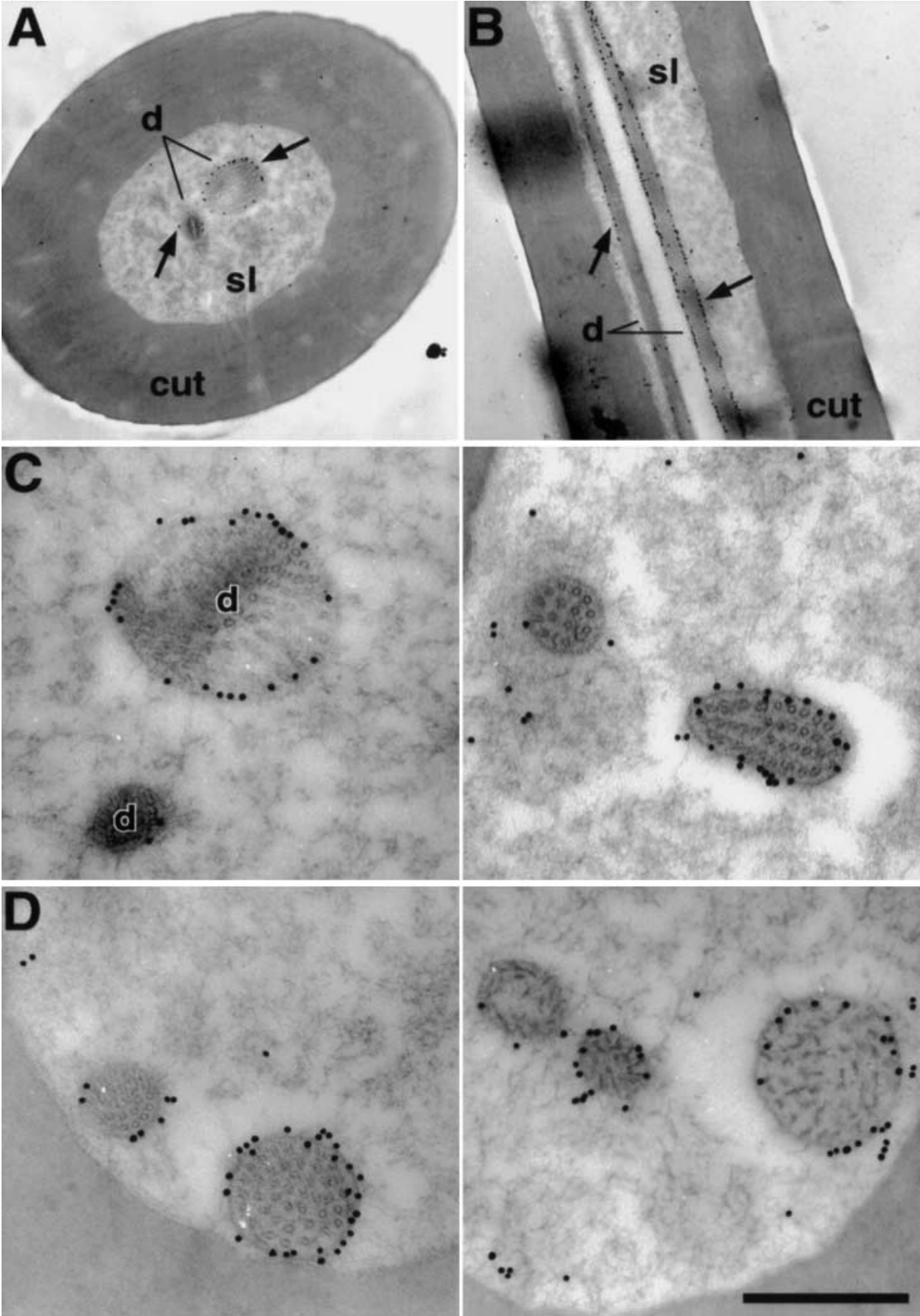
In this study, consistent with previous reports, only two recognizably distinct subtypes of trichoid sensilla were observed, and are referred to as "trichoid I" (large subtype) and "trichoid II" (small subtype) sensilla. Basiconic sensilla were recognized as short, thin-walled sensilla with branched dendrites. However, olfactory sensilla were often observed with a thick cuticular wall and unbranched dendrites similar to trichoid sensilla, but with the characteristic length and location of basiconic sensilla. These sensilla resembled the so-called "intermediate" sensilla of the silkmoths *Bombyx mori* (Steinbrecht 1973) and *Antheraea pernyi* (Steinbrecht et al. 1995; Steinbrecht, unpublished observations), and are therefore referred to here as "intermediate" sensilla.

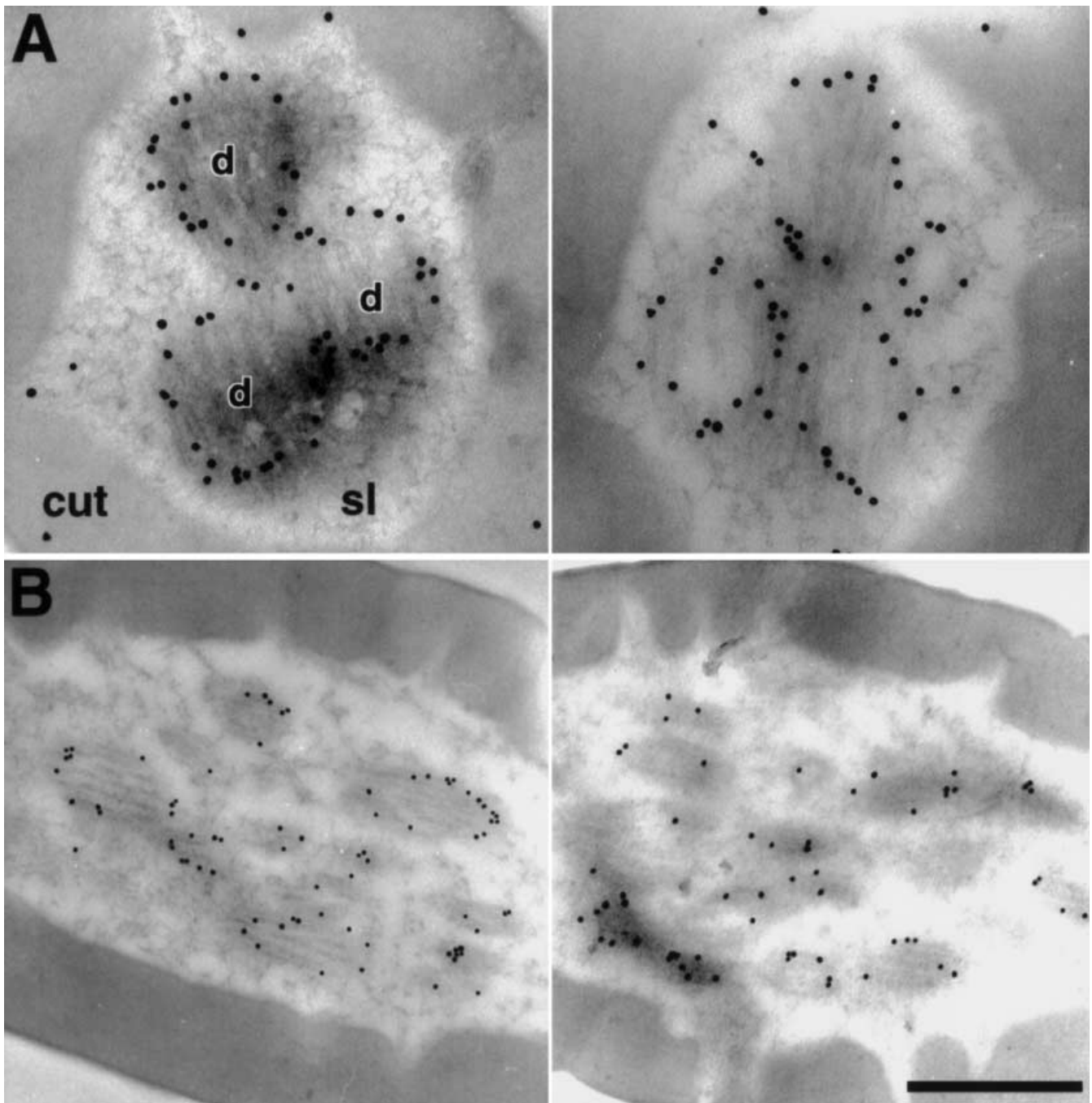
#### Electron-microscopic-level immunocytochemical localization of SNMP-1 in *A. polyphemus* antennae

SNMP-1 polyclonal antiserum (Rogers et al. 1997) was used to detect SNMP-1 protein distribution in ultrathin sections of olfactory sensilla. The locations of these sec-

tions are indicated in Fig. 1A. Sections were either transverse (position 1, Fig. 1A) or longitudinal (position 2, Fig. 1A) along the outer dendritic segments (ODS) in the hair region of the sensillum, or transverse at either the ciliary segment (CS) (position 3) that separates the inner and outer dendritic segments or at the level of the cellular layer and inner dendritic segment (IDS) (position 4, Fig. 1A). Bound SNMP-1 antibodies were labeled using secondary antibody conjugated with 10-nm gold particles and visualized by electron microscopy. Sensilla from two male antennae and one female antenna were examined; gold particle densities were determined for individual neuron profiles. The number of sensilla and neurons analyzed is presented in Table 1, along with a summary of the results.

**Fig. 2A–D** SNMP-1 expression in sex-pheromone-specific male trichoid olfactory hairs. Cross sections (**A**, **C**, **D**) and a longitudinal section (**B**) of sex-pheromone-sensitive olfactory hairs labeled with SNMP-1 antiserum are shown. **A**, **B** Low magnification of individual trichoid I sensilla. **C**, **D** Higher magnification of two different trichoid I (**C**) and trichoid II (**D**) olfactory sensilla. In all sections of trichoid I and II sensilla, SNMP-1 immunoreactivity was localized to the membranes of the dendrites (*d*) of ORNs; minimal label was observed in the sensillum lymph (*sl*) or on the thick cuticular wall (*cut*). Longitudinal sections of trichoid I sensilla reveal label along the entire length of the outer dendrite segment (**B**). Trichoid sensilla typically possess two or three ORNs with unbranched dendrites; SNMP-1 immunoreactivity was consistently more abundant in one of the two or three dendrites in each section. Scale bars 1.25  $\mu\text{m}$  (**A**), 2.5  $\mu\text{m}$  (**B**), 0.5  $\mu\text{m}$  (**C**, **D**)

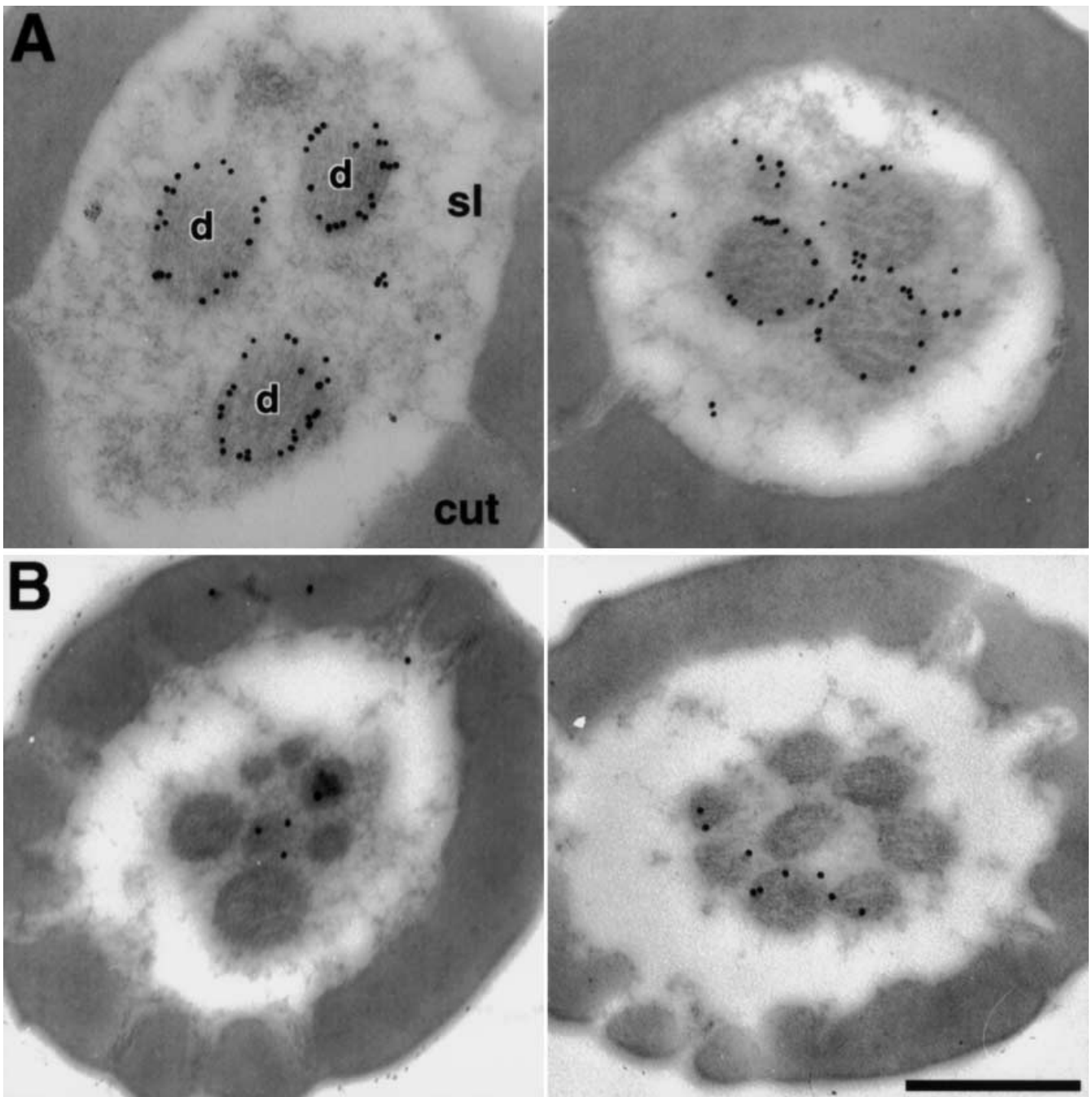




**Fig. 3A, B** SNMP-1 expression in male intermediate and general-odorant-sensitive basiconic olfactory hairs. Two different cross sections of male intermediate (**A**) and basiconic (**B**) olfactory hairs labeled with SNMP-1 antiserum are shown. SNMP-1 immunoreactivity was localized in the dendrite membranes of ORNs. In labeled sections of intermediate sensilla, heavy SNMP-1 immunoreactivity is evident on all dendrites per sensillum. Unlike trichoid and intermediate sensilla, the dendrites of basiconic sensilla divide into several branches. Labeled sections of basiconic sensilla show SNMP-1 immunoreactivity on several dendrite branches per section (*d* dendrites, *sl* sensillum lymph, *cut* cuticle). Scale bars 0.4  $\mu\text{m}$  (**A**), 0.8  $\mu\text{m}$  (**B**, left), 1  $\mu\text{m}$  (**B**, right)

#### Trichoid I and II sensilla, male

Twenty-three trichoid I sensilla and seven trichoid II sensilla were examined. Most contained two neurons, but a small number contained three neurons (five type I, three type II). In all sections of both sensillum types, gold particles were observed in close association with the membranes of outer dendritic segments of ORNs (Fig. 2). SNMP-1 immunoreactivity is apparently distributed along the entire length of the outer dendritic segment; strong immunoreactivity was observed in longitudinal and cross sections taken along the mid-region of the sensilla (Fig. 2) as well as in cross sections taken from the

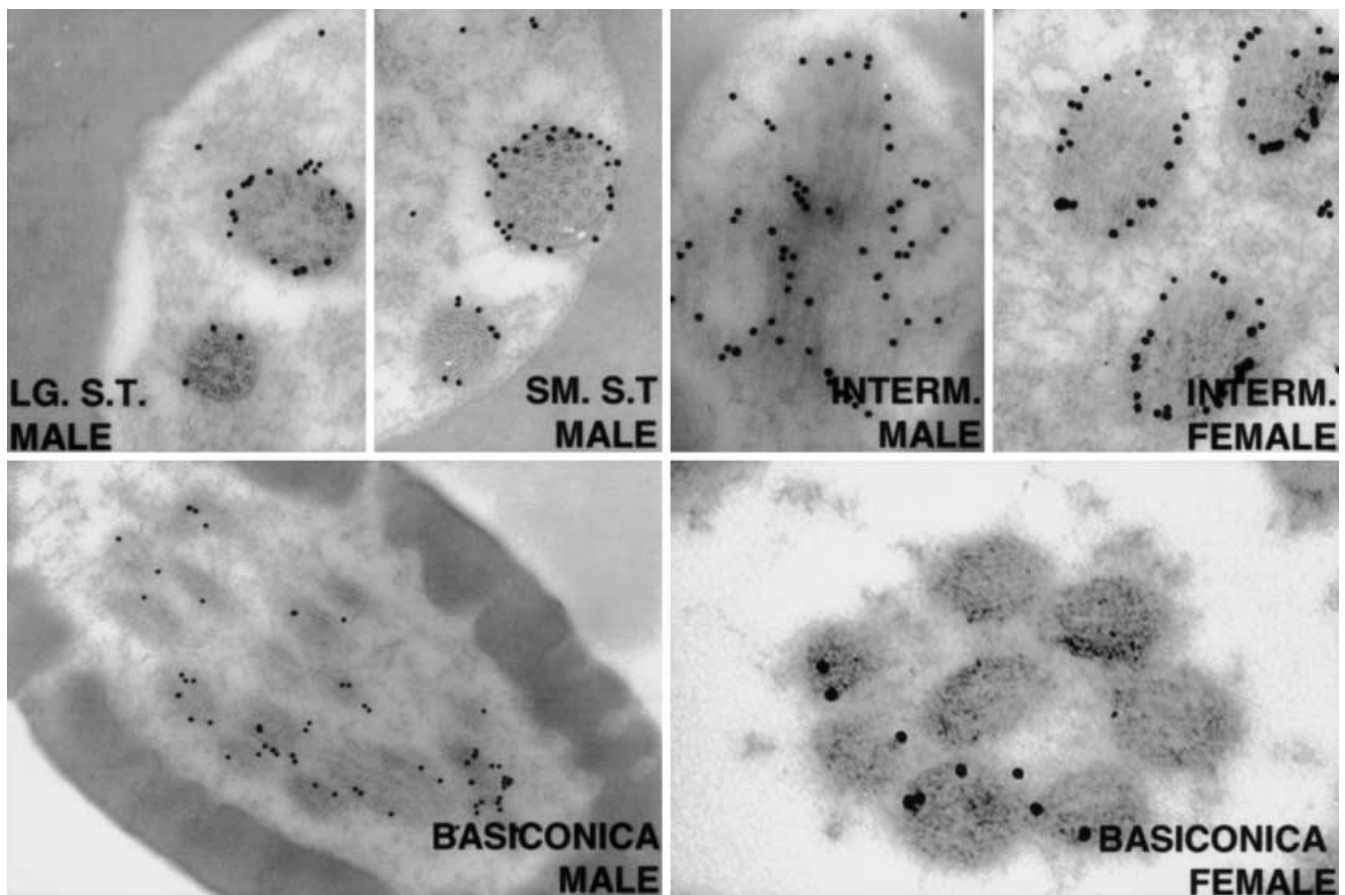


**Fig. 4** SNMP-1 expression in female intermediate and general-odorant-sensitive basiconic olfactory hairs. Two different cross sections of female intermediate (**A**) and basiconic (**B**) olfactory hairs labeled with SNMP-1 antiserum are shown. SNMP-1 immunoreactivity was localized to the dendrite membrane of ORNs. In labeled sections of intermediate sensilla, heavy SNMP-1 immunoreactivity was evident in all dendrites per sensillum. Only weak SNMP-1 immunoreactivity was detected in labeled sections of basiconic sensilla (*d* dendrites, *sl* sensillum lymph, *cut* cuticle). Scale bars 0.63  $\mu$ m (**A**, **B**)

sensillum base (see Fig. 5A) and tip (data not shown). Antiserum specificity was confirmed by labeling sections with preimmune serum derived from the same animal used to generate the SNMP-1 antiserum (data not shown).

No preimmune immunoreactivity was observed in sections labeled with the same concentration used in the SNMP-1 antiserum studies (1:12,000). Labeling could be detected at extremely high concentrations of preimmune serum (1:100), but in this case the sensillum lymph was heavily labeled while the dendrite membranes remained only weakly labeled (0–3 gold particles/dendrite).

Dendrite circumference and SNMP-1 circumferential labeling density was calculated for individual ORNs in each section, and these values were compared by pairwise statistical analyses (Table 1). Two populations of dendrites were distinguished based on membrane circumference and referred to as types A and B; type A dendrites are signifi-



**Fig. 5** Summary of SNMP-1 expression among different morphological and functional classes of olfactory sensilla. Large (*LG*) and small (*SM*) sensilla trichodea (*S.T.*) of male antennae, and intermediate (*INTERM.*) and basiconic sensilla of male and female antennae are represented. The large and small sensilla trichodea specifically respond to sex pheromone and the sensilla basiconica are broadly tuned and respond to general odorants such as volatile plant compounds. The functional properties of the intermediate sensilla remain uncharacterized. Note that SNMP-1 expression is much greater in neurons of sensilla trichodea and the intermediate sensilla compared to basiconica sensilla, suggesting a possible correlation between SNMP-1 expression and the functional properties of olfactory sensilla and their ORNs

cantly larger in diameter than type B dendrites (*t*-test: trichoid I,  $T_{23,27}=15.3$ ,  $P_{A/B}<0.001$ ; trichoid II:  $T_{7,9}=4.43$ ,  $P_{A/B}<0.001$ ). SNMP-1 expression was consistently 2–4.3 times higher in type A dendrites than in type B dendrites in both trichoid I and trichoid II sensilla, when compared within a given sensillum cross section (Table 1). No significant difference in total SNMP-1 expression was observed between trichoid I and trichoid II sensilla (i.e., summing densities for all dendritic cross sections within a section) (*t*-test:  $T_{23,9}=1.15$ ,  $P_{I/II}=0.13$ ). It has been known for some time that there are two classes of dendrites within trichoid sensilla of *A. polyphemus*, distinguished both by diameter and odor specificity (e.g., Steinbrecht 1980; Keil 1984; Kaissling 1986). The differential expression of SNMP-1 between these dendrites is consistent with their asymmetric anatomical and physiological properties.

#### Intermediate and basiconic sensilla, male

Four intermediate sensilla and five basiconic sensilla were examined. In sections of both sensillum types, gold particles were observed in close association with the membranes of the outer dendritic region of ORNs (Fig. 3). However, in contrast to trichoid I and II sensilla, not all male intermediate and basiconic sensilla were labeled. Membrane-associated SNMP-1 immunoreactivity was detected in only two of four male intermediate and three of five male basiconic sections (Table 1). Variable labeling may mean differential expression of SNMP among these sensilla types, or may simply be a procedural artifact.

The two labeled intermediate examined each contained three unbranched dendrites deriving from different neurons. However, unlike the trichoid sensilla, dendrites of intermediate sensilla were of uniform diameter, and are here referred to as comprising a single type designated as A'. Gold particle densities were statistically consistent for all dendritic profiles within a given sensillum, and were statistically similar to densities observed in the type A dendrites of the type I and II trichoid sensilla (Table 1; *t*-test: trichoid I,  $T_{23,6}=1.53$ ,  $P_{A/A'-m}=0.07$ ; trichoid II,  $T_{9,6}=1.67$ ,  $P_{A/A'-m}=0.06$ ).

Male basiconic sensilla contained an average of 12 dendrite profiles ( $n=3$ ; 10, 14, 13 profiles) (Table 1). The branched nature of these dendrites and their uniform size made it impossible to relate any dendrites to individual neurons, and the dendrites are here referred to as type X.

The total membrane circumference per basiconic section was consistently more than twice that of the other sensillum types. Gold particle densities were 5.9 particles/ $\mu\text{m}$ , similar to those found in the type B dendrites of the type I and II trichoid sensilla.

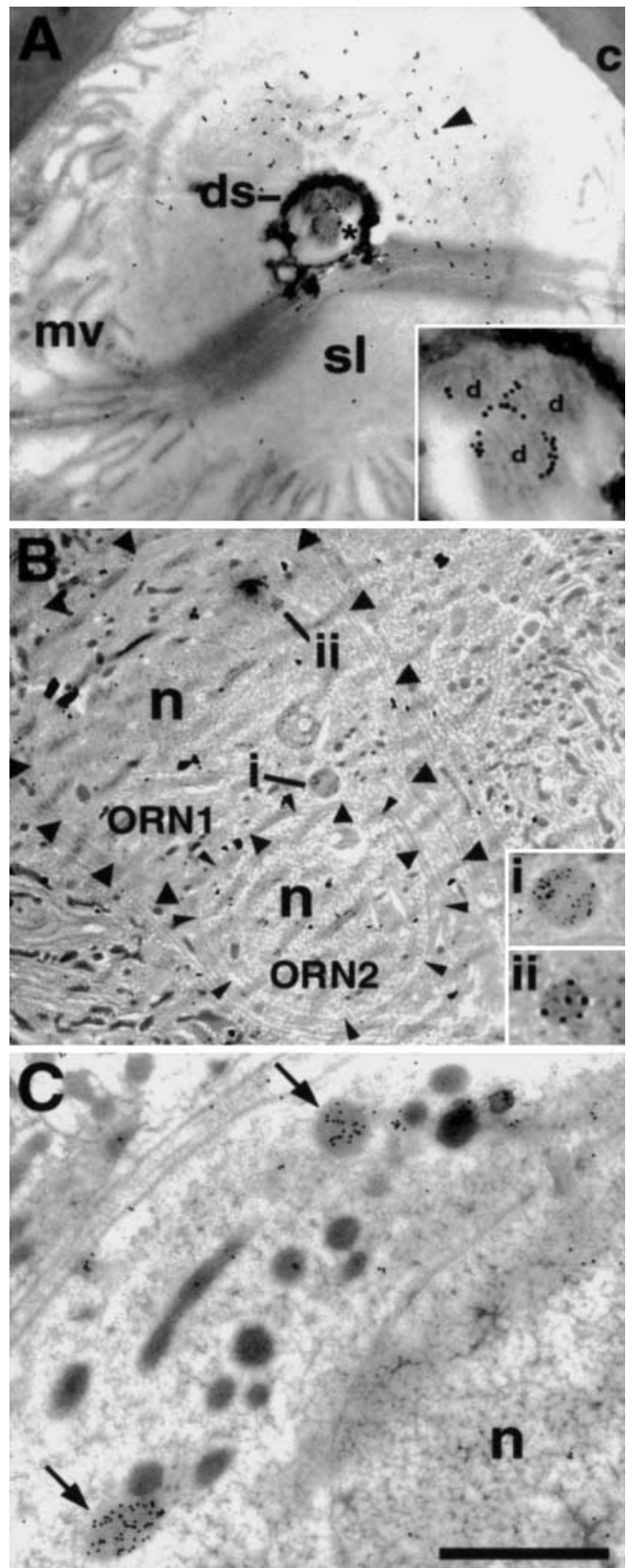
#### Intermediate and basiconic sensilla, female

Eight intermediate sensilla and nine basiconic sensilla were examined. All sections of female intermediate sensilla analyzed were labeled, and seven of nine sections of basiconic sensilla analyzed were labeled (Fig. 4, Table 1). Female intermediate sensilla were unbranched and of uniform size, and are here designated as type A'; two sensilla contained two neurons and the remainder three neurons. The mean circumference of these dendrites was nearly identical to that observed in males, as was the density of gold particles within the dendritic membranes (Table 1;  $t$ -test:  $T_{6,22}=1.19$ ,  $P_{A'-m/A'-f}=0.12$ ). All dendrite profiles showed similar labeling densities within a given section.

In female basiconic sensilla, the branched nature of the dendrites prevented relating dendritic profiles to a given neuron; the dendrites are here referred to as type X. An average of six dendrite profiles was observed per section ( $n=8$ ; 4, 4, 4, 7, 7, 7, 8, 8 profiles), much lower than that observed for male basiconic sensilla (Table 1). Also, only one to three membrane-associated gold particles were observed per section (Fig. 4), giving a labeling density of around 1/ $\mu\text{m}$ , a significantly lower value than that determined for male basiconic sensilla (Table 1;  $t$ -test:  $T_{3,6}=3.29$ ,  $P_{\text{bas-m, bas-f}} < 0.05$ ). Profiles of all sensillum types, both male and female, are compared in Fig. 5.

#### Trichoid I sensilla below the hair base

Sections of trichoid I sensilla within the antennal branch (i.e., below the hair base, positions 3 and 4 in Fig. 1A) were analyzed. SNMP-1 labeling was observed in the membranes of ORNs bundled within the dendritic sheath



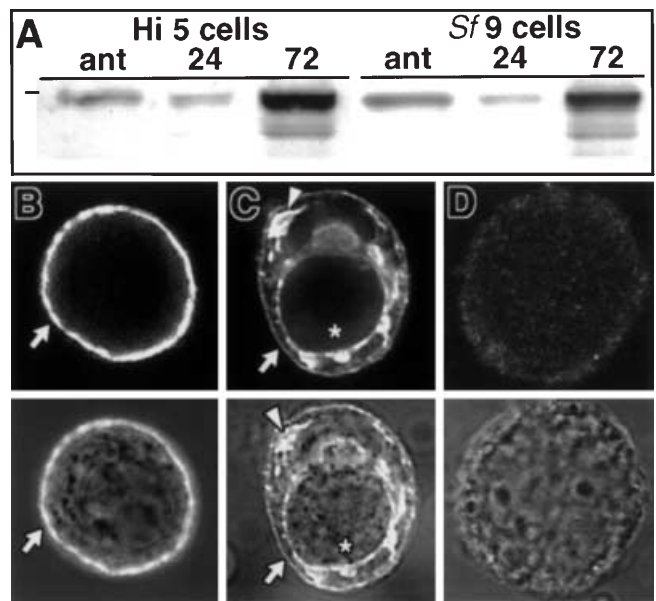
**Fig. 6A–C** SNMP-1 expression below the base of trichoid I olfactory hairs. Cross section (A) and longitudinal sections (B, C) showing labeled ORNs below the base of the olfactory hairs. A The dendrite sheath (*ds*) surrounds the dendrites of ORNs (*asterisk*) and the tormogen cell, which displays numerous microvilli (*mv*), borders the sensillum lymph cavity (*sl*). Outer dendrite segments of ORNs are labeled; the microvilli of the tormogen cell are not labeled. Labeling of the sensillum lymph (*arrowhead*) is most likely artifact considering its asymmetric distribution and the consistent lack of sensillum lymph labeling observed in the hairs (see Figs. 2, 3, 4). *Inset*: Higher magnification of outer dendrites (*d*) showing characteristic membrane SNMP-1 labeling. B *Arrowheads* (*broad* and *narrow*) mark the borders of a pair of ORNs (ORN1 and ORN2). ORN1 contains two labeled vesicles (*i* and *ii*) that appear to differ in size and labeling density; label is not apparent in the nuclei (*n*). *Inset*: Higher magnification of these labeled vesicles. C Higher magnification of another ORN showing densely labeled cytoplasmic vesicles (*arrows*). *Scale bars* 1.6  $\mu\text{m}$  (*inset* 0.53  $\mu\text{m}$ ) (A); 4.2  $\mu\text{m}$  (*inset* i 2.1  $\mu\text{m}$ , ii 1.2  $\mu\text{m}$ ) (B); 0.84  $\mu\text{m}$  (C)

(Fig. 6A), at the base of the outer dendritic segment just distal to position 3 in Fig. 1. No labeling was observed within the sensillum lymph either surrounding dendrites within the dendritic sheath or between the microvillar fingers of support cells. However, some additional labeling was observed in a halo surrounding the dendritic sheath (arrowhead, Fig. 6A), and this labeling is unexplained. Heavy SNMP-1 labeling was also observed associating with granules within cytoplasm of the cell soma (Fig. 6B, C); these granules varied in size and density and may represent stages in the post-translational processing of SNMP-1. No labeling was evident in the axons of ORNs, the cytoplasm of auxiliary cells, or non-sensory structures (i.e., epithelial cells and hemolymph) (data not shown).

#### Characterization of baculovirus-expressed SNMP-1 in cultured lepidopteran cells

In order to confirm that the SNMP-1 clone contained the sequence determinants which control membrane insertion, recombinant baculovirus containing the full-length coding region of *A. polyphemus* SNMP-1 was constructed and used to express SNMP-1 protein in monolayer cultures of *Sf9* and High Five cells. Cells were infected with recombinant baculovirus and assayed for SNMP-1 expression by Western blot analysis and fluorescent immunocytochemistry (Fig. 7). In both cell types, SNMP-1 expression was low at 24 h and increased dramatically by 72 h (Fig. 7A). The predicted MW of SNMP-1 is 59.9 kDa based solely on its primary structure and the apparent MW of bacterially (i.e., unmodified post-translationally) expressed protein (Rogers et al. 1997). However, baculovirus-expressed SNMP-1 is approximately 66 kDa, identical to native protein isolated from *A. polyphemus* male antennae, suggesting that the expressed protein is correctly processed post-translationally.

To examine the distribution of SNMP-1 within cultured cells, High Five cells were infected with SNMP-1 baculovirus for 60 h and labeled with polyclonal SNMP-1 antiserum followed by rhodamine-conjugated secondary antibody. MPLSM of intact (non-permeabilized) cells revealed numerous cells with a bright and continuous ring of fluorescence around the cell perimeter, but no fluorescence within the ring (Fig. 7B). This labeling pattern is indicative of an integral membrane protein with epitopes accessible to the extracellular space, since the antibodies cannot penetrate non-permeabilized plasma membranes. Infected cells that were permeabilized and fixed before antibody treatment, therefore permitting entry of SNMP-1 antibodies, showed both perimeter and intracellular fluorescence surrounding a non-fluorescent nucleus (Fig. 7C). Cytosolic staining appeared concentrated in structures which may represent endoplasmic reticulum and vesicular structures participating in the post-translational processing of SNMP-1. Negligible background fluorescence was detected in cells expressing SNMP-1 but incubated with preimmune serum (Fig. 7D), or in wild-



**Fig. 7A–D** Characterization of baculovirus-expressed SNMP-1 in cultured Lepidopteran cells. **A** Western blot of native (*ant*) and baculovirus expressed SNMP-1 at 24 and 72 h postinfection. SNMP-1 expression increases dramatically after 24 h. Note that native and expressed SNMP-1 are identical in apparent molecular weight. Marker (*top left*) represents 69 kDa. **B, C** Immunofluorescent detection of expressed SNMP-1 protein in viral-infected intact (**B**) and permeabilized (**C**) High Five cells. *Top panel* Fluorescent image alone. *Bottom panel* Transmission light image overlaid with the fluorescent image above. In both intact and permeabilized cells, SNMP-1 protein was detected around the cell perimeter (*arrows*), presumably indicating plasma membrane localization. Numerous SNMP-1-positive cytoplasmic structures were apparent in permeabilized cells (*arrowhead*); no labeling was seen in the nucleus (*asterisk*). **D** Negative control of a permeabilized cell infected with SNMP-1 baculovirus and reacted with preimmune serum

type-virus-infected or -uninfected cells incubated with SNMP-1 antiserum (data not shown).

## Discussion

### SNMP localization within olfactory receptor neurons

This study provides the first direct evidence of the specific association of SNMP-1 with the receptive membranes of ORNs, as well as a quantitative view of the relative abundance of the protein in different neuron and sensillum types. The techniques employed are exactly those previously used to demonstrate the localization of extracellular OBPs in the receptor lymph (e.g., Laue and Steinbrecht 1997); therefore, any non-membrane-associating SNMP can be assumed to have been visualized if it were present. In a previous immunocytochemical study employing light microscopy, SNMP-1 appeared distributed throughout the dendrite, and also in the cell body, where it appeared to be concentrated towards the distal region (Rogers et al. 1997). In the current study, SNMP-1 in the cell body is shown to be present in structures inter-

preted to be vesicles and secretory granules, but otherwise absent from the plasma membrane of the cell body, suggesting that membrane-bound SNMP-1 is entirely restricted to the dendritic region.

The expression study of SNMP-1 in cultured insect cells showed that SNMP-1 antibody could label cells under non-permeabilized conditions. While there is no assurance that this expression system represents the *in vivo* condition of the ORNs, the protein appeared correctly processed with regard to its molecular weight, and was successfully processed into the plasmalemma. Importantly, labeling under the non-permeabilized condition indicates that SNMP-1 is located within the membrane and that it presents at least some of its epitopes to the extracellular space where, if in an ORN, SNMP would have the opportunity to interact with other extracellular components of the sensillum.

#### Distribution of SNMP-1 among different olfactory receptor neurons and olfactory sensilla

Figure 5 and Table 1 summarize the distribution and densities of SNMP-1 among the dendrites of the various sensillum types. In *A. polyphemus*, SNMP-1 appears to be widely distributed among all the major antennal classes of olfactory sensilla of both male and female antennae, as well as in all neurons of these sensilla. However, there were consistent and significant differences in the amounts or densities of SNMP-1 among the neurons and sensilla. Among both trichoid I and II sensilla of male antennae, SNMP-1 levels were significantly higher in type A neurons than in type B neurons. SNMP-1 densities among neurons of the intermediate sensilla are similar to trichoid type A neurons, while densities in basiconic sensilla are similar to those of trichoid type B neurons. All dendritic profiles of intermediate and basiconic sensilla show similar levels of SNMP-1 when compared within a given section, unlike the unequal densities observed between trichoid type A and B neurons. These comparisons clearly indicate that SNMP-1 expression, if universal among ORNs, is differential between neuron and sensillum types.

Previously, SNMP-1 was shown to be expressed in both male and female antennae, but its localization was only characterized in the long trichoid sensilla (type I) of the male antennae, where it appeared to be absolutely restricted to only one of the two to three neurons present in these sensilla (Rogers et al. 1997). Expression in other classes of sensilla was inferred from Northern blot analysis of SNMP-1 expression in female antennae, but not characterized due to the difficulty of identifying these sensilla by light microscopy.

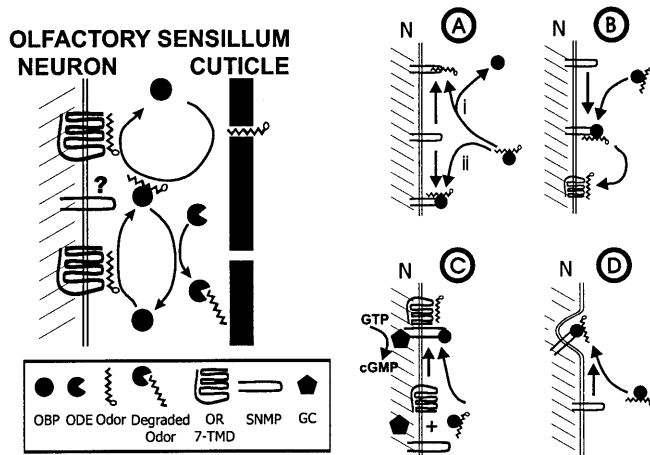
The EM data for trichoid sensilla are not inconsistent with the light-microscopy data, but do present a different result regarding SNMP-1 distribution among the ORNs, suggesting it is present in all neurons. The difference may be in the method of detection. Visualization of SNMP-1 under EM is a 1:1 relationship between primary antibody

and secondary antibody conjugated with gold particle, while visualization under light microscopy employed an enzyme-conjugated secondary antibody and was thus based on the accumulation of enzymatic reaction product, a process which is operationally stopped when staining appears sufficient. It may be that stain production by the other neurons had not progressed to a detectable level, although this lack of staining was consistently observed on multiple slides and for a large number sections (Rogers et al. 1997). However, it might also be argued that because enzymatic detection is an amplification of signal, labeling should have been more visible under the conditions of light microscopy. It may be that in these EM studies the SNMP-1 antibody is cross reacting with a highly similar SNMP homologue in the "other" neurons, and that while this immunocytochemical study is indicating the distribution of SNMP, it is not necessarily showing the expression pattern of a single SNMP-1 gene. In support of this, two SNMPS have recently been identified in the sphinx moth, *Manduca sexta* (Rogers, Krieger and Vogt, unpublished; Robertson et al. 1999). These two *M. sexta* SNMPS are about 26% identical in amino acid sequence but share conserved regions that could, in principle, represent common epitopes; both proteins are antennal specific and express in ORNs (Rogers, Krieger and Vogt, unpublished), although their expression overlap is as yet unclear. Therefore, as in *M. sexta*, multiple SNMPS may be present in *A. polyphemus* and may have been detected in the studies presented here. Nevertheless, whether these EM studies indicate the distribution of SNMP-1 specifically or SNMPS in general, they clearly suggest that SNMPS are represented in most, if not all, olfactory sensilla and olfactory neurons.

If the EM data accurately represent the distribution of SNMP-1, then they suggest that, while SNMP-1 is olfactory specific, the protein is common to all ORNs and therefore may have a functional role that is central to the olfactory reception process. Consider the olfactory-specific G-protein ( $G_{olf}$ ) in vertebrates, which is common to all olfactory neurons (Jones and Reed 1989; Sullivan et al. 1995; Laue et al. 1997). This G-protein is capable of interacting with a multitude of different ORs, and thus plays a central role in olfactory transduction. SNMP-1 may similarly have a central role in processing odor signals, participating in the transport or reception of the odor signal to or by the ORN rather than in the transduction of that signal.

#### Possible function of SNMP-1 within olfactory sensilla

Figure 8 details several models which suggest possible functions of SNMP-1. These models are based on several known features of SNMP-1 (Rogers et al. 1997). First, the olfactory-specific expression of SNMP-1 suggests the protein is involved in some functional aspect of the ORNs. Second, expression of SNMP-1 during the late developmental and adult stages suggests the protein is more involved in the odor detection properties of ORNs, rather than their earlier differentiation or growth. Third, the apparent homology between SNMP-1 and the class



**Fig. 8A–D** Possible SNMP-1 functions in olfactory sensilla. A general scheme of odor detection is shown (left), along with four schemes suggesting possible SNMP function (A–D). See text for details

of receptor proteins characterized by CD36 suggests the protein has a receptor-like function.

The CD36 gene family is a phylogenetically diverse family of membrane glycoproteins whose members are expressed in a wide variety of tissue types and appear to play diverse functional roles. Members of this family share common structural features including an N-terminal uncleaved signal peptide sequence, two transmembrane stretches near the N- and C-termini (respectively), and conserved cysteine residues and asparagine-linked glycosylation sites (reviewed by Greenwalt et al. 1992). An additional unifying theme of this family is that the members appear to bind proteinaceous ligands. Several members have been termed class B scavenger receptors because of their role in the endocytosis of low-density lipoproteins and several other modified proteins (e.g., maleylated bovine serum albumin), but not a broad array of other polyanions which are bound by the class A scavenger receptors (e.g., Acton et al. 1994; Calvo et al. 1997; Murao et al. 1997; Imachi et al. 1999). Other members are thought to be docking receptors for high-density lipoproteins or appear to be involved in the phagocytosis of several different cell types (Savill et al. 1992; Ren et al. 1995; Acton et al. 1996; Ryeom et al. 1996; Murao et al. 1997; Rigotti et al. 1997; Calvo et al. 1998). A homologue from *Drosophila melanogaster*, Croquemort, is expressed exclusively in embryonic macrophages and is required for the selective recognition and phagocytosis of apoptotic cells (Franc et al. 1996, 1999).

By homology with the CD36 receptor family, SNMP-1 is suggested to be a receptor protein with two transmembrane domains located near the N- and C-termini, and with a large extracellular domain. The presumed extracellular domain of SNMP-1 is rich in cysteine; analysis has identified disulfide bridges in bovine CD36 and suggested that SNMP1-*Apol* and the mammalian SR-B1 have a similar disulfide configuration (Rasmussen et al.

1998). Finally, and perhaps most importantly, SNMP-1 may interact primarily with proteinaceous ligands.

Figure 8 (left) details the basic elements of the proposed scheme of odor reception involving OBPs, ODEs and ORs, where odor molecules are transported to ORs by OBPs and degraded by ODEs (after Vogt et al. 1999). Figure 8A–D suggests several possible roles SNMP-1 may have within this scheme. SNMP-1 may be a novel OR, binding either the pheromone directly (Fig. 8A, pathway i) or a pheromone-PBP complex (pathway ii). While pathway “i” is suggested by previous photoaffinity-labeling experiments (Vogt et al. 1988), it currently seems unlikely based on protein structure and the recent identification of putative 7-transmembrane domain ORs from *Drosophila* (Clyne et al. 1999; Gao and Chess 1999; Vosshall et al. 1999).

A second possibility is that SNMP-1 functions as a docking protein for the pheromone-PBP complex, contributing to the off-loading of pheromone for access to the OR (Fig. 8B). Several studies have suggested the pheromone-PBP complex is highly stable and may stimulate the OR as a single unit (Prestwich et al. 1995; Kaissling 1996; Ziegelberger 1995; Steinbrecht 1997). Another recent study identified membrane properties of the ORNs that may contribute to the destabilization of the pheromone-PBP complex in *Bombyx mori* (Wojtasek and Leal 1999; Leal 2000). Destabilization of the PBP-pheromone complex by SNMP-1 would permit direct interaction between pheromone and its OR.

A third possibility is that SNMP-1 interacts with other proteins within its dendrite (Fig. 8C). SNMP could form a heterodimer with the OR, analogous to recent evidence suggesting heterodimer formation of certain membrane-bound receptor proteins, such as the GABA<sub>B</sub> (White et al. 1999) and calcitonin-receptor-like receptors (McLatchie et al. 1998). Alternatively, SNMP might interact with some intracellular protein, such as a recently identified and novel membrane-associated guanylyl cyclase (GC) expressed in the ORNs of *M. sexta* (Simpson et al. 1999). While this GC is bound to membrane, it lacks the expected membrane-associating components. SNMP might serve to anchor the GC and thus additionally serve in signal transduction.

Other possibilities include SNMP serving to OBPs (Fig. 8D), which is consistent with the scavenger properties of certain CD36 homologues but unlikely because no internalization complexes have ever been observed in EMs of insect olfactory dendrites. Also, SNMP may act in a signaling capacity with membrane proteins of other neurons of the sensillum (not shown). All of these schemes are more or less favored by current knowledge, and represent testable models for the elucidation of SNMP-1 function currently under investigation using the baculovirus-mediated expression system.

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