

Antennal SNMPs (Sensory Neuron Membrane Proteins) of Lepidoptera Define a Unique Family of Invertebrate CD36-like Proteins

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ABSTRACT: SNMP1-*Apol* is an antennal-specific protein of the wild silk moth *Antheraea polyphemus*; the protein is abundantly expressed and localized to the receptor membranes of sex-pheromone specific olfactory sensory neurons (OSNs). SNMP1-*Apol* is thought to function in odor detection based on its olfactory-specific expression, localization within OSNs, developmental time of expression, and apparent homology to the CD36 family of membrane-bound receptor proteins. In the current study, SNMP1-*Apol* homologues were identified from the moths *Bombyx mori*, *Heliothis virescens*, and *Manduca sexta*. These species possess antennal mRNAs encoding proteins with amino acid sequence identities ranging from 75–80%; these proteins are collectively designated SNMP1. A second *M. sexta* SNMP homologue, previously identified and partially sequenced [Robertson et al.: *Insect Mol Biol* 8:501–518, 1999] was fully sequenced and characterized. The encoded protein shares only 26–27% sequence identity with the SNMP1

proteins, and is thus designated SNMP2-*Msex*. The SNMP sequences were used to identify 14 and four possible homologues in *Drosophila melanogaster* and *Caenorhabditis elegans* genome databases, respectively; thus, greatly expanding CD36 family membership among the invertebrate lineages. Despite their sequence difference, SNMP1-*Msex* and SNMP2-*Msex* expression is localized to OSNs and occurs simultaneously with the onset of olfactory function. These findings suggest that SNMPs play a central role in odor detection in insects, and that the CD36 gene family is widely represented among animal phyla. The SNMPs are the only identified neuronal members of the CD36 family, and as such expand the activities of this gene family into roles influencing brain function and behavioral action. © 2001 John Wiley & Sons, Inc. *J Neurobiol* 49: 47–61, 2001

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INTRODUCTION

Insects detect odors through the binding of odor molecules to receptor proteins expressed in the dendrite

cilia of olfactory receptor neurons. Odor molecules are delivered to the odor receptors by soluble odorant binding proteins (OBPs). The neurons, receptors, and OBPs are contained within cuticular hairlike structures called sensilla; these olfactory sensilla are arrayed along the antenna, which is the olfactory organ of insects. Sensory neuron membrane protein (Snmp) is an abundant 67 kDa olfactory-specific protein cloned from an adult antenna-specific cDNA library from the silk moth *Antheraea polyphemus* (Rogers et al., 1997). SNMP is uniquely expressed in olfactory neurons and is localized to receptor membranes of the dendrite cilia. SNMP is not a seven-transmembrane domain receptor, and is thus not homologous with the

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putative odor receptors of *Drosophila* (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999) or the odor receptors of *Caenorhabditis elegans* or vertebrates (e.g., Dryer, 2000 and references therein). However, SNMP is homologous with a family of two-transmembrane domain receptor proteins characterized by homology to CD36, a family of proteins whose members frequently interact with proteinaceous ligands (e.g., Acton et al., 1996; Murao et al., 1997; Calvo et al., 1997, 1998; Crombie and Silverstein, 1998; Ohgami et al., 2000). SNMP is expressed late in adult development, continuing into the adult stage when odor detection occurs, but it is not expressed early in development during organogenesis (Rogers et al., 1997). A recent EM-level immunological study of SNMP expression in adult *A. polyphemus* localized SNMP immunoreactivity to the dendrite membranes of both sex pheromone and plant-odorant sensitive sensilla, but expression was significantly higher in the former (Rogers et al., 2001). Olfactory specificity, association with the receptor membrane, differential expression among distinct functional classes of olfactory neurons, homology to CD36, and coexpression with olfactory-based behavior argue that SNMP plays a central role in odor detection.

The effort in this current project focused on transferring knowledge of SNMPs from the saturniid moth *A. polyphemus* to the sphingid moth *Manduca sexta*. This would serve to demonstrate the presence of SNMPs in more than one Lepidopteran species, and in a species, *M. sexta*, that is both an important model in insect olfaction and well suited to studies of differential and ecdysteroid regulated gene expression, as well as being amenable to viral-based manipulation viewed necessary to investigate the function of SNMP in Lepidoptera. To that end, SNMPs were first and fortuitously identified in the silk moth *Bombyx mori* and the noctuid moth *Heliothis virescens*, and the obtained sequences then used to design PCR primers for the subsequent identification of a SNMP in *M. sexta*. These SNMPs were designated SNMP1 based on sequence similarities. During this effort, a second and quite different SNMP homologue (SNMP2) was independently identified from *M. sexta* (Robertson et al., 1999), presenting the opportunity to fully characterize the expression of two divergent yet olfactory specific SNMPs from a single species. To assess the size of the SNMP/CD36 family in a single insect species, we also surveyed the recently fully characterized genome of *Drosophila melanogaster* (Adams et al., 2000), identifying 14 possible homologues in this species. This study suggests that SNMPs are at least a common feature of Lepidopteran olfactory

receptor neurons, and that the Lepidopteran SNMPs comprise a neuron-specific branch of the CD36 family of receptor proteins.

METHODS

Animals and Tissues

M. sexta were obtained as fertilized eggs (gift of Dr. L. M. Riddiford, University of Washington, Seattle), and reared at 27°C on a 16h:18h (L/D) light cycle. Adult antennae were taken from pharate adult animals within 6 h of projected emergence, except for those tissues used in the developmental study. Legs, heads, wings, and thoracic ganglia used in the tissue localization study were obtained from adult animals within 24 h of emergence. Midguts were obtained from day 4 fifth instar larvae and were dissected free of peritrophic membranes. Dissected tissue was immediately frozen on dry ice and stored at -80°C until use.

Cloning Lepidopteran SNMPs from Adult Male Antennae

SNMP-*Apol* homologues in *B. mori* and *H. virescens* were isolated by screening antennal-derived cDNA libraries (*H. virescens*, Krieger et al., 1993; *B. mori*, Krieger et al., 1996) with a Digoxigenin-labeled cDNA probe covering the full length coding region of the SNMP1-*Apol* cDNA. For screening, cDNA-carrying phage were plated on *E. coli* and phage DNA was transferred and immobilized on replicate nylon filters (Hybond N⁺ nylon transfer membranes; Amersham) using standard protocols (Sambrook et al., 1989). Filter membranes were prehybridized at 37°C for 3 h in hybridization solution [30% formamide, 5X SSC, 0.1% laurylsarcosine, 0.02% SDS, 2% blocking reagent (Roche Biochemicals), 100 mg/mL denatured herring sperm DNA], followed by hybridization at 37°C overnight in hybridization solution containing about 10 ng/mL of the DIG-labeled SNMP1-*Apol* probe. Posthybridization washes of membranes were performed twice for 5 min at room temperature and twice for 30 min at 37°C in 2X SSC, 0.1% SDS. Probes were visualized by luminous detection (CSPD; Roche Biochemicals) on X-ray film. Purification of positive clones by plaque hybridization and subcloning of cDNAs were performed using standard protocols (Sambrook et al., 1989). Briefly, phage DNA was prepared and the cDNA inserts were isolated as EcoRI fragments by restriction enzyme digestion, agarose gel electrophoresis, and GeneClean extraction (BIO 101). For sequence analysis, cDNAs were ligated into the EcoRI site of the pBluescript II SK plasmid.

To identify SNMP-*Apol* homologues in *M. sexta*, degenerate primers were designed against highly conserved amino acid regions identified by sequence alignment of the SNMP1-*Apol* and the putative *B. mori* and *H. virescens* SNMPs isolated by cDNA library screening (above); a sense primer (ARTGGAARGARAARGTNGAGG) corresponded to the EEWKEKVE motif and an antisense primer

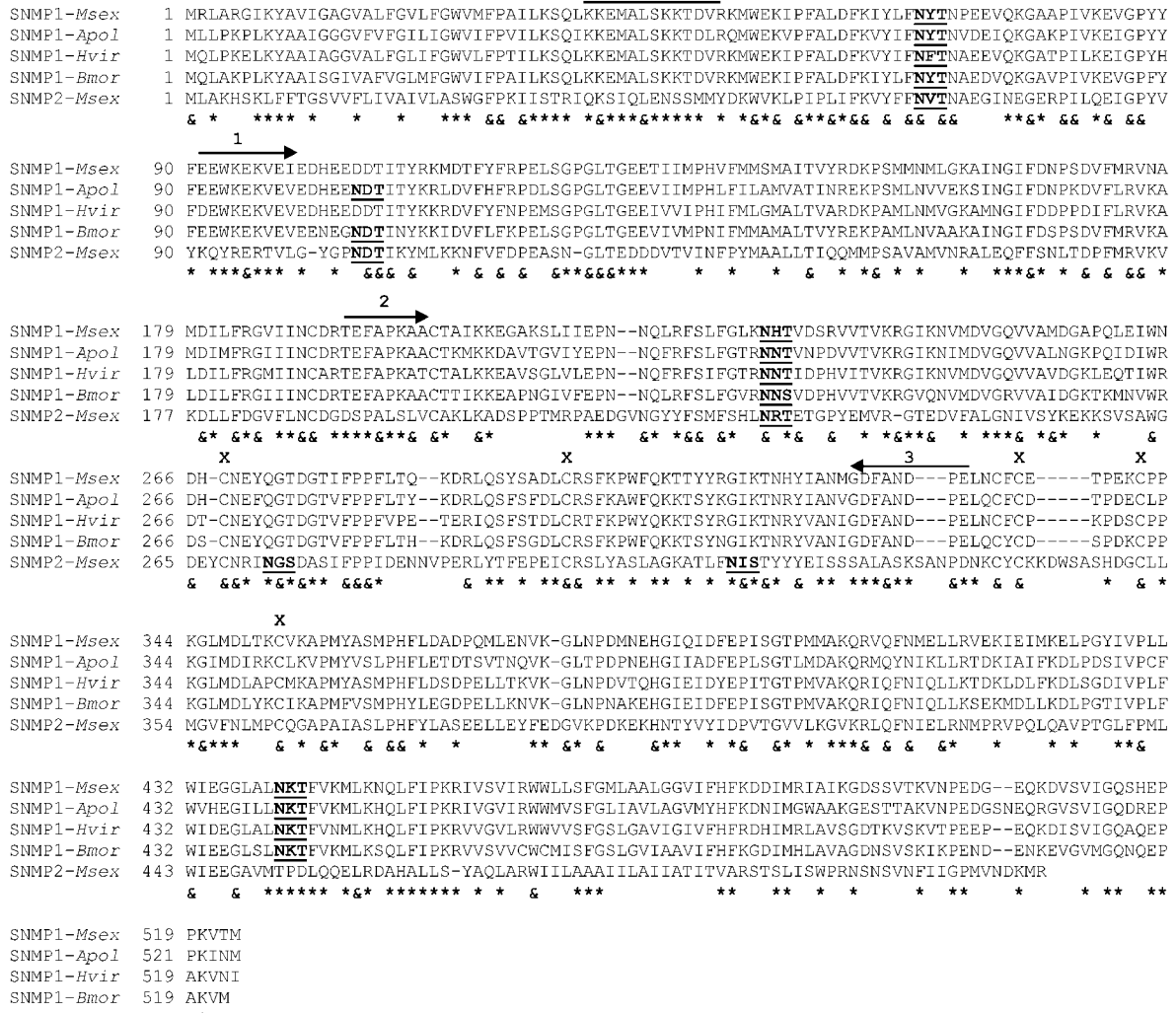


Figure 1 Amino acid sequence alignment of Lepidopteran SNMPs. Numbering is from the first amino acid in mature proteins. Amino acid residues conserved among all proteins are marked (&); asterisks indicate residues conserved among four of the five proteins. Conserved cysteins present in the Lepidopteran SNMPs and several other CD36 homologues are marked (X), as well as conserved putative N-glycosylation sites (bold, underline). Solid underline marks the highly conserved sequence near the N-terminus that was used to identify the predicted initiation codon in *SNMP1-Msex*, *SNMP1-Bmor*, and *SNMP1-Hvir*. The degenerate PCR primer sites used to clone *SNMP1-Msex* are indicated by arrows. Primers 1 (sense) and 3 (antisense) were used in the primary PCR reaction and primers 2 (sense) and 3 were used in the seminested secondary PCR reaction. Full length nucleotide sequences for all proteins are available from GenBank. GenBank accession numbers are as follows: *SNMP1-Msex* (AF323588), *SNMP1-Apo1* (U95026), *SNMP1-Hvir* (AJ251959), *SNMP1-Bmor* (AJ251958), *SNMP2-Msex* (AF323589).

(TCNGGRTTNGCRAAATC) corresponded to the DFA-NDPE motif (see Fig. 1). Antennal RNA isolations utilized the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). cDNA template was synthesized using cloned M-MLV RNase H⁻ Reverse Transcriptase (BRL) following recommended protocols and including 40 U RNasin (Promega), 5 µg total adult antennal RNA, and 500 ng of an oligo (dT₁₈) primer in a 40 µL reaction. PCR (50 µL) used *Taq* DNA polymerase (3 U;

Promega), supplied buffer containing 1.5 mM Mg²⁺, 2 mM dNTP, 5 µm each primer, and 1 µL cDNA from the above reaction. PCR was performed on a Cetus Thermocycler under oil overlay: 3 min at 94°C followed by five cycles at 94°C (30 s), 35°C (2 min), and 74°C (3 min following a 2 min ramp), and then 30 cycles as above but with a 45°C annealing temperature. PCR products of the appropriate size were gel purified (GenClean Kit II, BIO 101) and reamplified under identical conditions using a nested sense primer

(ACNGARTTYGCNCCNAAGC) corresponding to the TE-FAPKA motif and the same antisense primer (see Fig. 1). Reamplified PCR products were purified by ammonium acetate precipitation, ligated into pCR-Script vector (pCR-Script Cloning Kit; Stratagene), and sequenced. Full length SNMP1-*Msex* coding region was obtained by RACE PCR following the procedures outlined in Vogt et al. (1999) and using the 5'/3' RACE Kit (Roche Biochemicals).

A second SNMP from *M. sexta*, distinct from the SNMP1-*Msex* clone described above, was previously isolated from an expressed sequence tag (EST) library derived from adult male antennal tissue (GenBank accession number AI142164; Robertson et al., 1999). This partial SNMP sequence, referred to as pMsmB15, contains a 332 bp open reading frame. To verify the sequence of this clone and to generate template for RACE PCR reactions and sense and antisense RNA probes, the 332 bp open reading frame was amplified from adult antennal cDNA by PCR and cloned into pCR-Script vector (pCR-Script Cloning Kit; Stratagene). Two of the resulting clones were sequenced fully in both directions. Sequence discrepancies between these clones and the pMsmB15 GenBank sequence are noted as follows, referencing the numbered base position of the GenBank sequence (#AI142164), the "new" base identification, and the "old" base identification (in parentheses); a "-" indicates no base at that position: 10 G(T); 63 A(-); 175 -(G); 177 A(G); 208 A(G); 221 A(C); 334 -(A); 337 -(A); 241 A(G); 248 G(-); 253 A(C). Full length pMsmB15 coding region was obtained by RACE PCR following the procedures outlined in Vogt et al. (1999) and using the 5'/3' RACE Kit (Roche Biochemicals).

Sequencing and Identification of SNMP Homologues

Sequencing of chosen *B. mori* and *H. virescens* clones (BmSNMP22 and HvSNMP33, respectively) was performed on an ABI310 Genetic analyzer (PerkinElmer) using the dd Rhodamine Terminator Cycle Sequencing Kit. Sequence data was analyzed by the Heidelberg Unix sequence analysis resources (Husar) 5.0 software package, based on the Wisconsin package 10.0 from the Genetic Computer Group (GCG, Madison, WI). Sequencing of chosen SNMP1-*Msex* and pMsmB15 clones was performed using ABI Prism Dye Terminator cycle sequencing protocols (Applied Biosystems). SNMP1-*Msex* was sequenced at the Medical University of South Carolina Biotechnology Resource Laboratory (Charleston, SC) and SNMP2-*Msex* was sequenced at the University of South Carolina. All sequencing was performed fully in both directions. All sequences were initially characterized using the NCBI BLAST network server (Blast X algorithm, Altschul et al., 1997).

All amino acid sequence alignments were performed with the Clustal X multiple alignment program (Thompson et al., 1994). The alignment presented in Figure 1 was used to produce the pairwise comparison values in Table 1.

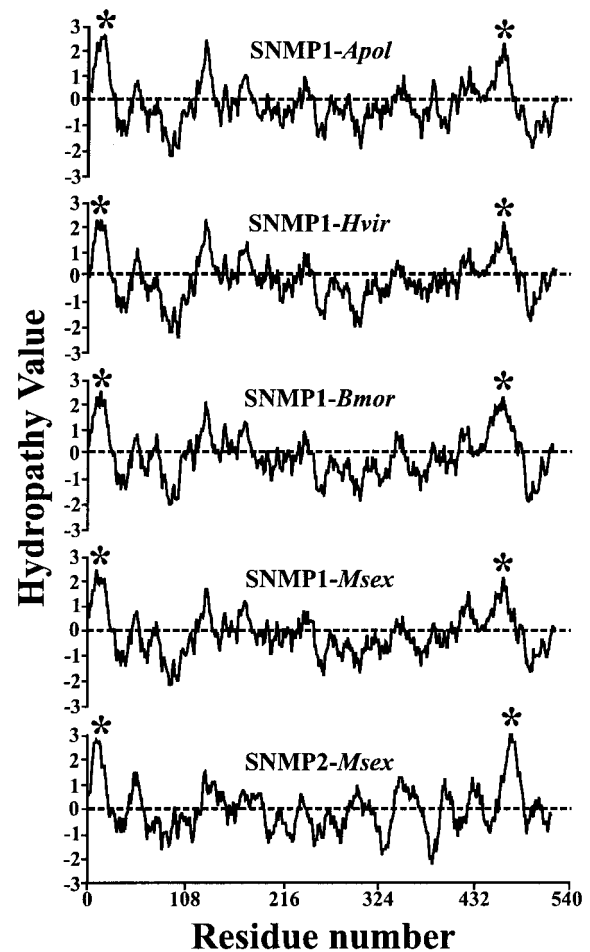


Figure 2 Hydropathy plots (Kyte-Doolittle) comparing the Lepidopteran SNMPs. Window size is 14 amino acids. Hydrophobic amino acids are scored positive and amino acid positions are indicated on the x-axes. Asterisks indicate two conserved hydrophobic regions thought to represent transmembrane domains.

Neighbor Joining Analysis

SNMP-related sequences in *Drosophila melanogaster* and *C. elegans* were initially identified by a NCBI BLAST network server homology search of the *Drosophila* and *C. elegans* genomic sequence databases. Indicated sequences were first aligned using Clustal X. A neighbor joining tree (Saitou and Nei, 1987) was constructed from this matrix using Paup (Version 4.0b1 for Macintosh) based on mean character difference (distance). The data matrix was modestly trimmed to remove hanging ends, but all other characters were included; the program calculated pairwise differences, ignoring missing characters resulting from alignment gapping. The tree presented includes nodes with 50% or greater bootstrap support based on 1000 replicates; branch lengths are proportional and indicate mean distance (percentage difference) between the sequences.

Table 1 Comparison of Lepidopteran SNMPs

Species	Probability*	% Identity [†]			
		1	2	3	4
1. SNMP1- <i>Apol</i>	—	—	—	—	—
2. SNMP1- <i>Msex</i>	<1e ⁻⁹⁹	70.4	—	—	—
3. SNMP1- <i>Hvir</i>	5e ⁻⁷⁷	67.5	70.6	—	—
4. SNMP1- <i>Bmor</i>	8e ⁻⁸²	68.2	71.8	73.2	—
5. SNMP2- <i>Msex</i>	1e ⁻⁵⁸	26.8	26.0	27.0	26.8

* Probability values from SNMP1-*Apol* amino acid homology search using NCBI BLAST network server; values <0.05 are considered statistically significant.

[†] Percentage amino acid identity values were obtained from a mean character difference matrix, which was constructed using PAUP (Version 4.0b1 for Macintosh).

Probes for Northern Blot Analysis and *in Situ* Hybridization

Digoxigenin-labeled antisense and sense RNAs were synthesized from insert DNA according to Rogers et al. (1999) and using the Genius System (Roche Biochemicals). SNMP1-*Msex* probes (antisense and sense) were *in vitro* transcribed from the full length clone (base pairs 581–997) and SNMP2-*Msex* probe was *in vitro* transcribed from the pMsmB15 EST fragment (base pairs 48–287). The control DNA probe used in the Northern blot analyses was derived from a *M. sexta* 18S rRNA clone (GenBank accession number U88190) in pBluescript II (Stratagene) and was generated by PCR according to Rogers et al. (1997).

Northern Blot Analyses

SNMP1-*Msex* and SNMP2-*Msex* antisense RNA probes were hybridized to 10 μ g of total RNA from each tissue sample. Membranes were prehybridized for 2.5 h at 65°C (5X SSC, 0.1% N-lauroylsarcosine, 2X Denhardt's solution, 0.02% SDS, 100 μ g/mL herring sperm DNA) and hybridized for 16 h with 25 ng/mL Digoxigenin-labeled antisense RNA probe under the same conditions in prehybridization solution containing formamide (50%). To show the relative amounts of total RNA present in all lanes, the same membranes were stripped at 100°C in 0.1% SDS for 15 min, washed in 2X SSC, and reprobated with the *M. sexta* rRNA DNA probe. Hybridization was at 55°C but at otherwise identical conditions. Membranes were washed at the hybridization temperature in 0.1X SSC, 0.1% SDS, and probes were visualized by luminous detection (CDP-star; Roche Biochemicals) on X-ray film (X-OMAT AR; Kodak).

Whole-Mount *in Situ* Hybridization

Whole-mount *in situ* hybridizations were done on newly emerged adult male tissue, following protocols modified from Vogt et al. (1997). Prior to whole-mount analysis, pharate antennae were bisected longitudinally, exposing the epithelial cells underlying the antennal cuticle. Tissue was immediately treated with 4% paraformaldehyde (PFA) in PBS overnight on ice. Fixed tissue was washed several

times in PBS containing 0.1% Tween 20 (PBST), dehydrated to 70% methanol, and stored at –20°C.

Tissue for *in situ* hybridization was rehydrated to PBST, digested with Proteinase K (10 μ g/mL in PBST, 2 h at 37°C), postfixed in 4% PFA in PBST (20 min, 4°C), and reacted with acetic anhydride (0.25% in 100 mM triethanolamine, 20 min, 20°C). Tissue was prehybridized for 2 h at 60°C (4X SSC, 5X Denhardt's, 500 μ g/mL herring sperm DNA, 250 μ g/mL tRNA, 2.5 mM EDTA, and 0.1% Tween-20) and hybridized 16–20 h under the same conditions with 100 ng/mL Digoxigenin-labeled probes in prehybridization solution containing formamide (50%). Following hybridization, tissue was washed in 2X SSC, 0.1% Tween-20 (1 \times 10 min, 60°C), and then in 0.2X SSC, 0.1% Tween-20 (3 \times 10 min, 60°C). For alkaline phosphatase detection of Digoxigenin-labeled probes, antennae were then incubated in AP blocking solution (100 mM Tris pH 7.5, 150 mM NaCl, and 0.1% Triton X-100 containing 5% normal sheep serum and 1% BSA) and then with alkaline phosphatase coupled anti-Digoxigenin antibody [Roche Biochemicals; 1:5000 in AP blocking solution containing 1% normal sheep serum (Sigma) and 0.2% BSA, overnight, on ice]. Following several washes (AP blocking solution minus serum and BSA), alkaline phosphatase was visualized using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) at 20°C. Staining was stopped in 10 mM Tris-HCl, 1 mM EDTA (pH 7.5, on ice). Tissue was photographed in whole mount under dark field.

For fluorescent *in situ* hybridizations, posthybridized antennae were incubated in FL blocking solution [100 mM maleic acid pH 7.5, 150 mM NaCl, 0.1% Triton X-100 containing 5% normal donkey serum, and 2% blocking reagent (Roche Biochemicals)] and then with sheep anti-Digoxigenin polyclonal antibody (Roche Biochemicals; 1:200 in FL blocking solution containing 1% serum and 0.02% blocking reagent, overnight, on ice). Following several washes with wash solution (FL blocking solution minus serum and blocking reagent), antennae were equilibrated in AP wash solution, incubated with Rhodamine Red-X-conjugated donkey anti-sheep IgG (Jackson ImmunoResearch Laboratories; 7 μ g/mL in AP wash solution, 1 h at room temperature), and washed several times. Rhodamine-conjugated secondary antibody was visualized with a confocal

microscope (BioRad 1024) using a 20X (0.50 NA) or 40X (0.75 NA) Nikon objective lens. A "Z series" of each sample was collected at 4- μ m increments using the Kalman filter (two passes/section, slow scan mode) for a smoothing effect. Chosen optical sections were projected (merged) using the confocal Assistant program (version 4.02) and imported into Adobe Photoshop (version 5.0) for final processing.

Image Processing

All X-ray film and photographic images were digitized and processed using Adobe Photoshop and printed using a dye sublimation printer.

RESULTS

Identification of *B. mori*, *H. virescens*, and *M. sexta* SNMPs from Adult Antennae

SNMP homologues of the moths *B. mori* and *H. virescens* were isolated by low stringency screening of antennal-derived cDNA libraries, using a Digoxigenin-labeled antisense RNA probe to the *A. polyphemus* SNMP1 (SNMP1-*Apol*) coding region. Sequence analysis of one of the *B. mori* cDNA clones (BmorSnmp22, 2489 bp) revealed an open reading frame of 1566 bp (522 amino acids). Sequence analysis of a *H. virescens* cDNA clone (HvirSnmp33, 2126 bp) revealed an open reading frame of 1569 bp (523 amino acids). The position of the start methionine for both sequences is suggested by an in-frame ATG located upstream of a highly conserved amino acid sequence also present in SNMP1-*Apol*; the N-terminal sequence of SNMP1-*Apol* was previously confirmed by direct amino acid sequencing (Rogers et al., 1997). The position of the termination codons are suggested by the first in-frame stop codon and alignment with SNMP1-*Apol* (Fig. 1); the size of SNMP1-*Apol* was previously determined by *in vitro* translation of transcribed RNA (Rogers et al., 1997). These positions of putative BmorSnmp22 and HvirSnmp33 initiation and termination codons yield open reading frames that are consistent in length with SNMP1-*Apol*. NCBI BLAST homology search of the full length open reading frames indicated significant sequence homology to SNMP1-*Apol* (Table 1). These clones were therefore referred to as SNMP1-*Bmor* and SNMP1-*Hvir*. Northern blot analysis using SNMP1-*Bmor* probe indicated the equivalent presence of SNMP1 mRNA in male and female adult antennae, but none was present in leg, abdomen, tho-

rax, or head (data not shown). Such confirming analysis was not performed for SNMP1-*Hvir*.

A partial sequence of a *M. sexta* SNMP was amplified from adult male antennal cDNA by PCR, using degenerate primers designed to highly conserved amino acid residues present in SNMP1-*Apol*, SNMP1-*Bmor*, and SNMP1-*Hvir* (Fig. 1). Primers derived from this sequence were used to obtain 5' and 3' sequences by RACE PCR, yielding full length sequence. Finally, primers derived from DNA sequences flanking the presumptive coding region were used to amplify full length SNMP from *M. sexta* antennal cDNA; three resulting clones were sequenced in their entirety, all yielding identical sequence within the presumptive coding region. The position of the start ATG was suggested by amino acid alignment with the other SNMP1 sequences, and the termination codon was suggested as the initial in-frame stop codon by alignment of the other SNMPs (Fig. 1). The size of the predicted coding region is 1569 nucleotides, encoding 523 amino acids. NCBI BLAST homology search indicated significant sequence homology to SNMP1-*Apol*; this clone is therefore referred to as SNMP1-*Msex*.

A second *M. sexta* SNMP (SNMP2-*Msex*) with apparent sequence homology to SNMP1-*Apol* was identified in an EST library derived from male antennal mRNA: pMsmab15 (partial sequence GenBank accession numbers AI142152 and AI142153) and pMsmab25 (partial sequence GenBank accession number AI142164) (Robertson et al., 1999). The pMsmab15 clone was generously provided by H. Robertson, and its full sequence determined. Sequence of presumptive full length coding region was obtained by 5' and 3' RACE using antennal cDNA and primers based on the pMsmab25 and pMsmab15 sequences, respectively. A clone comprising the full length sequence was finally generated using primers flanking the presumptive coding region and antennal cDNA. The location of the initiation codon could not be inferred by amino acid alignment with other SNMPs because SNMP2-*Msex* does not contain the conserved amino acid motif present near the 5' end of the SNMP1 proteins. Instead, the initiation codon was suggested by an in-frame ATG located upstream of a highly conserved N-linked glycosylation site present in several CD36 homologues (reviewed by Greenwalt et al., 1992), including SNMP1-*Apol* (Rogers et al., 1997), SNMP1-*Bmor*, and SNMP1-*Hvir* (positions 67–69 in Fig. 1). The location of the 3' terminus is suggested by the first in-frame stop codon. These presumptive initiation and termination codons yield an open reading frame of 1557 bp, encoding 519 amino acids, which is consistent in length with the

other Lepidopteran SNMPs (Fig. 1). As presented below, the sequence of SNMP2-*Msex* is significantly divergent from the other SNMPs, justifying the distinction of SNMP1 and SNMP2.

SNMP Sequence Comparison

A database homology search using the NCBI BLAST server indicated that SNMP1-*Msex*, SNMP2-*Msex*, SNMP1-*Bmor*, and SNMP1-*Hvir* all shared significant sequence similarity with SNMP1-*Apol* and several other characterized members of the CD36 family, including *Drosophila* Croquemort and epithelial membrane protein (EMP), and mammalian CD36, SR-BI, and LIMPII. Probability values derived from this search range from $1e^{-99}$ to $1e^{-35}$, where a value ≤ 0.05 is considered statistically significant (Karlin and Altschul, 1990). The Lepidopteran sequences shared the highest similarity with SNMP1-*Apol* (Table 1). Among the SNMPs, the four SNMP1 proteins are much more similar to each other (68–73% identity) than they are to SNMP2-*Msex* (26–27% identity) (Table 1).

Comparing the five SNMPs, two of four putative N-linked glycosylation sites previously noted in the SNMP1-*Apol* sequence (Rogers et al., 1997) are entirely conserved, and a third is conserved among the four SNMP1 proteins; of note are two putative N-linked glycosylation sites that are unique to SNMP2-*Msex* (bold and underlined residues, Fig. 1). Five cysteine residues are conserved throughout (“X,” Fig. 1); these cysteines are consistent within the CD36 family and are thought to contribute to disulfide bridge formation (Rasmussen et al., 1998). Hydropathy plots of the five moth SNMPs suggest that all of these proteins possess strongly hydrophobic regions near their N- and C-termini (asterisks, Fig. 2), and a relatively hydrophilic midregion encompassing approximately 90% of the protein. This hydropathy pattern is consistent with views that the CD36 family, including SNMP1-*Apol*, are two-transmembrane domain receptors containing a large extracellular loop (Rasmussen et al., 1998; Gruarin et al., 2000).

A search of the complete *Drosophila* and *C. elegans* genome sequence data bases for sequences similar to SNMP was performed using the BLAST P algorithm implemented in the NCBI BLAST network. A total of 13 *Drosophila* sequences, including EMP and Croquemort, and four *C. elegans* sequences were identified as possible homologues of the moth SNMPs. A nonrooted neighbor joining analysis of these proteins (Fig. 3) suggests that while the group is, overall, quite divergent, the Lepidopteran proteins do cluster and draw in three *Drosophila* sequences.

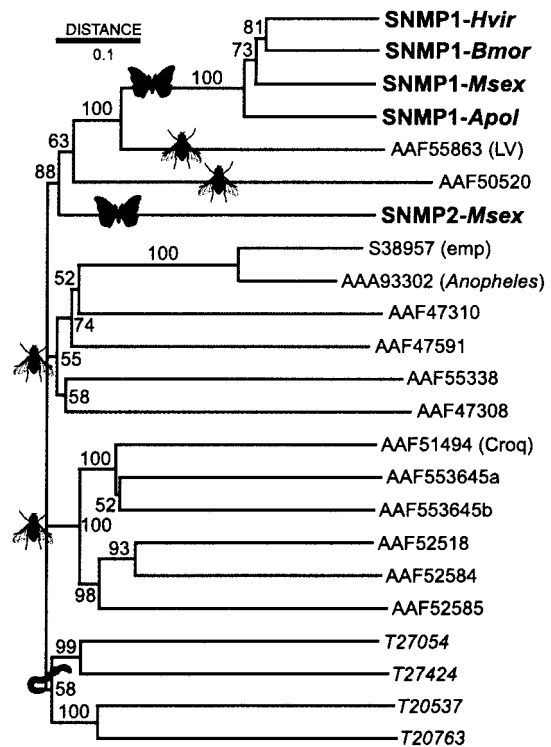


Figure 3 Neighbor joining phylogeny relating moth, *Drosophila*, and *C. elegans* SNMP homologues. Branch lengths are proportional and the scale of distance is indicated. Bootstrap support values (%) based on 1000 replicates are indicated. Sequence identities are as follows: SNMP1-*Apol* (Rogers et al., 1997), SNMP1-*Msex* (this report), SNMP1-*Bmor* (this report), SNMP1-*Hvir* (this report), SNMP2-*Msex* (Robertson et al., 1999; this report), EMP (Hart and Wilcox, 1993), Croquemort (Franc et al., 1996). All other *Drosophila* and the *C. elegans* sequences were identified by a BLAST homology search using the NCBI BLAST network server and are indicated by their corresponding GenBank accession numbers. AAF55863 is antennal specific but appears to express in support cells and not neurons (L. Vosshall, personal communication).

Overall, this analysis displayed monophyletic characteristics, with the *C. elegans* sequences remaining unassociated with the insect sequences.

Tissue Specificity and Developmental Expression of *M. sexta* SNMPs

Tissue-specific localization of SNMP1-*Msex* and SNMP2-*Msex* mRNA was examined by Northern blot analysis using Digoxigenin-labeled antisense RNA probes. Both probes hybridized to an abundant message in male and female antennae; no hybridization was detected from mRNAs isolated from leg, wing, gut, head, or thoracic ganglia (Fig. 4, top panel).

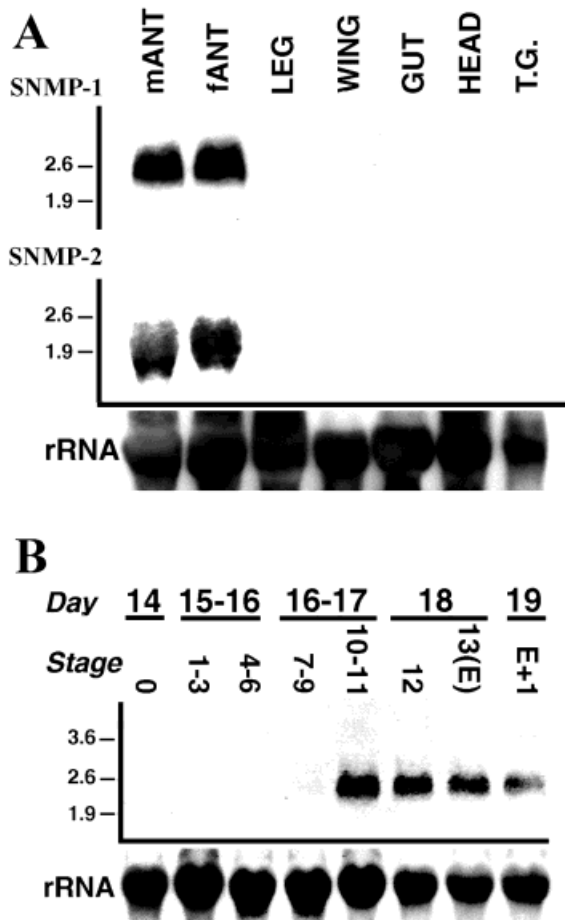


Figure 4 Northern blot analysis of SNMP expression in adult (A) and developing (B) *M. sexta*. (A) Hybridization of SNMP1-*Msex* (top) and SNMP2-*Msex* (bottom) antisense RNA probe with mRNA isolated from adult male antenna (mANT), female antenna (fANT), leg, wing, mid-gut (GUT), head, and thoracic ganglia (TG). The same membrane was hybridized consecutively with SNMP1-*Msex*, SNMP2-*Msex*, and *M. sexta* 18S rRNA (rRNA) probes, and exposed to film for the same amount of time. (B) Hybridization of SNMP1-*Msex* antisense RNA probe with male antennal RNA isolated during the final 4 days of adult development and 24 h postemergence. Individual lanes are labeled to indicate the age (day 14–19) and developmental stage (0–13) of the tissue used in the analysis. Criteria for developmental staging were based on morphological characteristics described by Schwartz and Truman (1983) and Vogt et al. (1993); these characteristics included wing pigmentation, body color, and wing and abdomen firmness, and were correlated to day number by following a group of 10 individual animals through their 18 day development. RNA markers are indicated in kilobase units; “E” refers to the time of eclosion and “E + 1” refers to 24 h after eclosion.

SNMP1-*Msex* and SNMP2-*Msex* mRNA appeared equally abundant in male and female antennae. In both male and female antennae, the SNMP1-*Msex* probe hybridized to a 2.6 kb message, whereas the

SNMP2-*Msex* probe hybridized to a 1.9 kb message. No cross hybridization between the two probes was detected.

The time course of SNMP1-*Msex* mRNA expression during the final 4 days of adult development was examined by Northern blot analysis (Fig. 4, bottom panel). SNMP1-*Msex* mRNA was first detected at very low levels 48 h before adult emergence; expression increased dramatically around 24 h before emergence, and continued at a similar level in both newly emerged animals as well as in animals 24 h postemergence. SNMP1-*Msex* mRNA expression may be slightly lower in emerged animals compared to pre-emerged (e.g., pharate) animals. However, the levels of 18S rRNA in lanes representing these periods was also slightly reduced, suggesting that the apparent decrease in SNMP1-*Msex* hybridization may in part result from lower quantities of overall target RNA present on the membrane.

***In Situ* Localization of *M. sexta* SNMPs within Male Antennae**

SNMP1-*Msex* and SNMP2-*Msex* expression was localized within the adult male *M. sexta* antenna by whole-mount *in situ* hybridization using Digoxigenin-labeled antisense RNA (Fig. 5). The adult antenna is about 2.5 cm long and is divided along its length into approximately 80 similar annular units. Antennae were bisected along their midline, producing symmetrically identical half-antennae and exposing the inner surface of the epithelial cells underlying the antennal cuticle. Images in Figure 5 thus show one-half of the sensory epithelium of an annulus, viewed from the hemolymph side. Sex-pheromone-specific olfactory sensilla are arrayed in a U-shaped region lining the peripheral regions of each annulus [four larger arrows in Fig. 5(A)], and plant volatile sensitive olfactory sensilla occupy the middle region of each annulus [two smaller arrows in Fig. 5(A)].

Hybridizations shown in Figure 5(A–C) were visualized by an alkaline phosphatase-mediated precipitation reaction. Both SNMP1-*Msex* and SNMP2-*Msex* probes yielded labeled cells throughout the sensory epithelium, associating with both sex-pheromone sensilla (larger arrows) and plant-volatile sensilla (smaller arrows) [Fig. 5(A)]. No obvious difference in the overall hybridization pattern of these two probes was observed. In *M. sexta*, the somata of olfactory receptor neurons are slightly elongated and considerably smaller than the surrounding support cells, and are located just beneath the base of the cuticular hair shaft (Sanes and Hildebrand, 1976; Keil, 1989). Higher magnification of stained cells indicates shape, size, and position, suggesting their

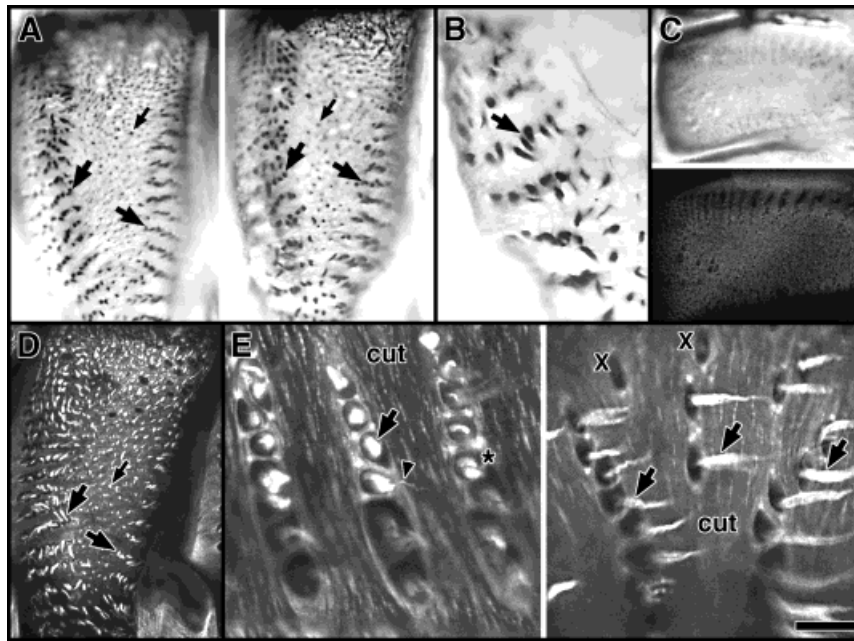


Figure 5 In situ hybridization of *M. sexta* SNMP mRNA expression in whole-mount preparations of male antennae. In all cases, the olfactory epithelium is visualized from the inner hemolymph side. (A) Hybridization of SNMP1-*Msex* (left) and SNMP2-*Msex* (right) antisense RNA probes with the olfactory epithelium of a single annulus. Arrows point to positively hybridizing olfactory receptor neurons, including the sex-pheromone-specific neurons of trichoid I sensilla (large arrows). (B) Higher magnification of sex-pheromone-specific neurons hybridized with SNMP1-*Msex* probe. (C) Negative control hybridizations using SNMP1-*Msex* sense probe. Top, colorimetric detection via alkaline phosphatase-mediated precipitation reaction; bottom, fluorescent detection via confocal microscopy. (D) Fluorescent in situ hybridization of SNMP1-*Msex* expression in the olfactory epithelium of a single antennal annulus. (E) Higher magnification of positive sex-pheromone-sensitive neurons of trichoid I sensilla. Hybridization with SNMP2-*Msex* probes yielded a similar hybridization pattern (data not shown). A sensillum that appears to possess multiple SNMP1 positive neurons is marked (asterisk), as well as a sensillum that appears devoid of SNMP1 positive neurons (X). The arrowhead marks a labeled axonal projection, cut, cuticle. Scale bar represents: (A,D) 100 μm ; (B) 50 μm ; (C) 130 μm ; (E) 16 μm .

identity as olfactory receptor neurons [Fig. 5(B)]. Hybridization using control sense probe under identical conditions showed no staining [Fig. 5(C), top panel].

Fluorescent detection of SNMP1-*Msex* probe by confocal microscopy revealed the same pattern as seen by the phosphatase detection [Fig. 5(D)]. Hybridization using control sense probe under identical conditions showed only background cuticular fluorescence [Fig. 5(C), bottom panel]. At higher magnification, labeled neurons were seen entering the base of sensillum hairs, and in some cases, labeled axonal projections were also visualized [Fig. 5(E)]. Cells labeled in the left panel of Figure 5(E) are sex-pheromone-specific neurons of trichoid I sensilla based on the shape, organization, and location of the cuticular holes that they enter (Lee and Strausfeld, 1990; Shields and Hildebrand, 1999; Rogers et al., 1999). Hybridization using SNMP2-*Msex* probe yielded similar results (data not shown). Typically, each olfactory

sensillum contains two to three olfactory receptor neurons (Keil, 1984). In many sensilla, probes appeared to label only a single neuron emerging from the sensillar hole [arrows, right panel, Fig. 5(E)]. However, in other sensilla, multiple neurons clearly appeared to be labeled [asterisk, left panel, Fig. 5(E)]. This is presumably not hybridization between SNMP1-*Msex* and SNMP2-*Msex* mRNAs, because these probes hybridized to distinctly different sized mRNAs on Northern blots under similar hybridization conditions (see Fig. 4).

DISCUSSION

Identification of a Unique Family of Invertebrate SNMP Proteins

Five Lepidopteran SNMPs are now identified comprising two apparent subclasses: SNMP1 and

SNMP2. The SNMP1s are similar in sequence (68–73% identity) and collectively different in sequence from SNMP2 (26–27% identity). All five SNMPs have similar hydrophathy profiles, consistent with their CD36 relatives, and a topology of two-transmembrane domains bounding a presumed large extracellular loop. The presumptive extracellular loop contains several conserved cysteines, which may form stabilizing disulfide bridges (Rasmussen et al., 1998), as well as several conserved putative N-linked glycosylation sites. A comparison of *in vitro* translated and native SNMP1-*Apol* showed M_w differences of 60 kDa and 67 kDa respectively, suggesting post-translational modification *in vivo* to account for the M_w shift (Rogers et al., 1997). All five proteins are of similar size, ranging between 519 (SNMP2-*Msex*) and 525 (SNMP1-*Apol*) amino acids. However, the sizes of the mRNAs vary considerably; mRNA of SNMP1-*Apol* is approximately 6 kb (Rogers et al., 1997), while those of SNMP1-*Msex* and SNMP2-*Msex* are 2.6 kb and 1.9 kb, respectively.

A search of the complete *Drosophila* and *C. elegans* genome databases using the Lepidopteran SNMPs revealed several likely CD36 homologues in both species, including ones previously identified (EMP and Croquemort). Three *Drosophila* sequences are sufficiently similar to the moth SNMPs to have been attracted to a common node with strong bootstrap support (93%). Whether any of these *Drosophila* homologues are related to olfactory function remains to be determined. One of these (AAF55863) has been characterized and appeared to be expressed in non-neuronal sensilla support cells rather than olfactory neurons (L. Vosshall, personal communication). This may mean that *Drosophila* does not use SNMP homologues in the same capacity as moths, or that one cannot rely on sequence similarity alone to assume functional equivalence between such distant species. Assuming olfactory mechanisms are conserved between Diptera and Lepidoptera, a characterization of the full complement of *Drosophila* SNMP homologues may provide significant insight into the function of these proteins in the antenna. In any event, the family of CD36 receptor proteins is clearly numerous, divergent, and broadly represented among invertebrate species.

Characterization of SNMP Expression in *M. sexta*

Both the temporal and spatial expression patterns of SNMP1-*Msex* and SNMP2-*Msex* appear nearly identical, yet the proteins share only 26% sequence identity. Messenger RNAs of both proteins are of similar

abundance based on Northern blot hybridization (Fig. 4). Both *M. sexta* proteins appear to be expressed at high levels based on hybridization sensitivities, consistent with Northern blots of SNMP1-*Apol* and observations that SNMP1-*Apol* is the most abundant protein of the receptor dendrite membranes of olfactory neurons (Rogers et al., 1997, 2001).

Previous developmental studies of SNMP1-*Apol* expression indicated mRNA was marginally detectable at about 40% of development, but expression increased dramatically shortly before adult emergence (about 90% of development) and continued at high levels for at least several days into adult life (Rogers et al., 1997). SNMP1-*Apol* was cloned from wild males attracted to females releasing sex-pheromone; *A. polyphemus* do not feed as adults and their short adult life is dedicated to reproduction. The observation that SNMP1-*Apol* expression occurred primarily during the maturational and behaviorally active stages and not during neurogenesis and sensory differentiation added support to the hypothesis that SNMP plays a central role in odor detection.

The developmental analysis of SNMP1-*Msex* (Fig. 4) indicated that expression initiates around 36 h before adult emergence (about 90% of development) and continues at least 24 h into adult life. Unlike *A. polyphemus*, *M. sexta* do feed as adults, consuming floral nectar, and therefore have a longer adult life span than *A. polyphemus*. Like *A. polyphemus*, *M. sexta* will readily mate within 24 h of adult emergence. A previous study showed that *M. sexta* antennae become electrophysiologically responsive to sex-pheromone 2–3 days before adult emergence (Schweitzer et al., 1976). Therefore, like SNMP1-*Apol*, expression of SNMP1-*Msex* coincides with the odor-detection period of antennal development and not with the neurogenic, differentiative, or morphogenic periods (Sanes and Hildebrand, 1976; Schweitzer et al., 1976), suggesting that SNMPs play roles in odor detection.

SNMP1-*Msex* expression coincides with the expression of several other olfactory specific proteins that are thought to be involved in odor detection, including the OBPs pheromone binding protein (PBP) and general odorant binding proteins 1 and 2 (GOBP1 and GOBP2) (Vogt et al., 1991, 1993), and the pheromone degrading ODE aldehyde oxidase (AOX) (Rybczynski et al., 1989, 1990; Vogt et al., 1993). The expression of the OBPs and AOX were shown to be influenced by declining ecdysteroids, which naturally occurs late during adult development; OBPs expression appeared to be strongly induced by this decline (Vogt et al., 1993). The coincidence of expression of OBPs, SNMPs, and AOX, and the acquisition of physiological responsiveness to pheromone suggest

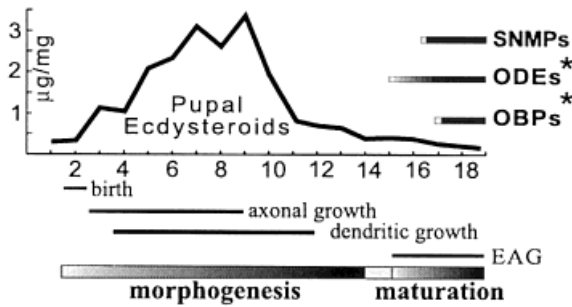


Figure 6 Summary of olfactory neurogenesis and the pupal ecdysteroid profile during adult development of *M. sexta*. Modified from Vogt (1995). The ecdysteroid profile is from Bollenbacher et al. (1981). Morphological events of the developing antenna are from Hildebrand et al. (1985). Data for ODEs and OBPs are from Vogt et al. (1993). SNMP1-*Msex* expression initiates following the completion of neuronal morphogenesis and during a period of neuronal functional maturation marked by the onset of odorant-induced electroantennogram (EAG) recordings (Schweitzer et al., 1976) and expression of the OBPs and ODEs. OBP and ODE expression is tightly regulated by the ecdysteroid 20-HE (asterisk), suggesting that SNMP1-*Msex* expression, which follows a similar developmental expression pattern, may also be ecdysteroid sensitive.

that the final days of adult development involve a maturational period during which the olfactory sensilla express genes essential to their adult function of detecting behaviorally important odors. These relationships are represented along with the pupal ecdysteroid profile in Figure 6. It is possible that declining ecdysteroids, which induce the expression of the OBPs and AOX, may regulate other maturational processes of the olfactory neurons and sensilla, including the expression of the SNMPs.

Both SNMP1-*Msex* and SNMP2-*Msex* appeared to express in neurons distributed throughout the olfactory epithelium of male antennae. In these antennae, functionally distinct classes of olfactory sensilla are segregated. Pheromone specific long-trichoid sensilla are arrayed in parallel rows of six to eight sensilla that line the outside edge of the olfactory epithelium of each annulus; plant-volatile sensitive short-trichoid and basiconic sensilla intermix fill the midregion (Lee and Strausfeld, 1990; Shields and Hildebrand, 1999). Both SNMPs expressed in neurons of both regions. Initial studies of SNMP1-*Apol* suggested it was asymmetrically expressed in only one of the two to three neurons of each pheromone-specific trichoid sensillum (Rogers et al., 1997). In a more recent study, the sensillar distribution of SNMP1-*Apol* was examined at the EM level using immunogold labeling of anti-SNMP1 binding to olfactory dendrite membranes (Rogers et al., 2001). Neurons of both pheromone-

specific trichoid and plant-volatile sensitive basiconic sensilla were labeled by anti-SNMP1, but expression was significantly higher in the pheromone-specific sensilla. Also, while all neurons of the pheromone-specific sensilla were labeled by anti-SNMP1, the density of SNMP1-*Apol* was consistently significantly greater in one neuron of each sensillum (e.g., 13 vs. 3 gold particles per μM of dendrite circumference).

In situ hybridization analysis of SNMP1-*Msex* expression by confocal microscopy [Fig. 5(C-E)] suggested that mRNA is clearly associated with neuron-like structures that emerge from the cuticular holes of olfactory sensilla and that frequently display axonlike processes oriented towards the antennal hemolymph cavity [Fig. 5(E)]. In these images, some holes appear empty, suggesting an absence of SNMP1-*Msex* expression. Some holes appear to contain multiple lobed structures, suggesting SNMP1-*Msex* expression in multiple neurons of those sensilla, in contrast with other holes that appear to contain single lobed structures, suggesting SNMP1-*Msex* expression in only a single neuron. These fluorescent studies are clearly not of the resolution of the EM level microscopic studies of SNMP1-*Apol* expression; however, they do suggest that SNMP1-*Msex* may be differentially expressed both among different sensilla and different neurons within a given sensillum.

SNMP Function

We have suggested that SNMPs function in the detection of odor because the proteins are antennal specific, are expressed in olfactory receptor neurons and are localized to the receptor membranes of those neurons, are differentially expressed among olfactory receptor neurons, and express during the functional rather than the differentiative period of antennal development (Fig. 4). SNMP1-*Apol* was initially pursued as an effort to clone an antennal-specific protein of olfactory dendrite membranes that specifically binds sex-pheromone, and was thus considered a candidate odor receptor protein (Vogt et al., 1988). While an odor receptor function for SNMP has not been strictly disproved, it seems quite unlikely in light of the identification of a family of putative odor receptors from *Drosophila* of the seven-transmembrane domain type (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999). SNMPs do not possess a seven-transmembrane motif.

SNMPs do share sufficient sequence similarity with the CD36 receptor family to suggest they are CD36 homologues having a two-transmembrane domain topology. Characterized CD36 family members are summarized in Table 2. A common feature of

Table 2 Characterized Members of the CD36 Family of Membrane Proteins

Protein	Organism	Localization	Proposed Function	Selected References
CD36	Human, bovine, rat, canine	Monocytes, macrophages, erythroblasts, adipocytes, milk fat globule membrane, endothelial cells, myocardial tissue, retinal and mammary epithelia	Cell adhesion; phagocytosis of apoptotic cells; lipoprotein receptor; signal transduction; fatty acid transporter	Oquendo et al., 1989; Greenwalt et al., 1992 (review); Abumrad et al., 1993; Ryeom et al., 1996
SR-BI	Mouse, hamster	Fat, lung, liver, ovary, adrenal gland	Lipoprotein receptor	Acton et al., 1994; Rigotti et al., 1997; Gu et al., 2000
CLA-I	Human	Adrenal gland, liver, testis, monocytes, macrophages	Lipoprotein receptor; binds apoptotic thymocytes	Calvo and Vega, 1993; Muraio et al., 1997; Pussinen et al., 2000
LIMP II	Human, rat	Lysosomal membrane of COS cells and lymphoid-derived cell lines, liver	Cell adhesion (putative thrombospondin receptor)	Vega et al., 1991; Hunziker and Geuze, 1996 (review); Crombie and Silverstein, 1998
EMP	Fruit fly	Embryonic epithelial cells, larval wing imaginal disc	Possible developmental function	Hart and Wilcox, 1993
Croquemort	Fruit fly	Embryonic macrophages	Phagocytosis of apoptotic cells	Franc et al., 1996, 1999
DdLIMP	Ameba	Vesicles and ringlike structures on cell surface	Putative receptor/transporter for phosphatidylinositides	Karakesisoglou et al., 1999; Temesvari et al., 2000
SNMP-1	Silkworm	Olfactory neurons	Odorant processing	Rogers et al., 1997, 2001

these proteins is that they utilize proteinaceous ligands. This feature has prompted us to propose several testable hypotheses of SNMP function that involve its interaction with other proteins belonging to either the extracellular space, the neuronal membrane, or the neuronal cytosol (Rogers et al., 2001). In general, odor molecules are thought to enter a sensillum via holes penetrating the cuticular wall, associate with OBPs for transport to neuronal membrane bound odor receptor proteins, and ultimately become enzymatically degraded (Vogt et al., 1999). SNMPs could interact with extracellular OBPs and either serve as a receptor for the OBP-odor complex or contribute to the off-loading of odor molecules from OBPs in proximity to the true odor receptor proteins. Alternatively, SNMPs could interact with the odor receptors in a manner analogous to the heterodimer complex formation of GABA_B receptors (White et al., 1998) or calcitonin-receptorlike receptors (McLatchie et al., 1998). Or, SNMPs might interact with intracellular proteins such as the novel membrane associated guanylyl cyclase (GC) expressed in the olfactory receptor neurons of *M. sexta* (Simpson et al., 1999).

Two very divergent yet homologous SNMPs are expressed in *M. sexta* olfactory neurons. SNMP1-*Apol* is differentially expressed among different types of olfactory sensilla, and among different neurons within sex-pheromone sensitive sensilla (Rogers et al., 1997, 2001). The odor-sensitive phenotypes of olfactory sensilla are determined by the differential expression of divergent gene families such as those encoding odor receptors and OBPs. In *Drosophila*, an estimated 32 OBPs (Kim and Smith, 2001) may deliver diverse odor molecules to at least 57 odor receptors (Vosshall et al., 2000); unique combinations of odor receptors and OBPs may determine which odor molecules a neuron responds to. We suggest that SNMPs may contribute, along with the combinatorial expression of odor receptors and OBPs, to define the odor sensitive phenotypes of olfactory neurons.

The CD36 receptor family appears to have considerable phylogenetic breadth; identified members among the vertebrate, nematode, and arthropod phyla support its distribution throughout the deuterostome and protostome lineages. The SNMPs are the only identified neuronal members of the CD36 family, and as such expand the activities of this gene family into roles influencing brain function and behavioral action. We hope this study will serve as a challenge to identify members of this family in other nervous systems to further elucidate the role of CD36 related proteins in regulating or sustaining neuronal function.

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