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Research article

Identification and Characterization of Two "Sensory Neuron Membrane Proteins" (SNMPs) of the Desert Locust, *Schistocerca gregaria* (Orthoptera: Acrididae)

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Abstract

Pheromone-responsive neurons of insects not only require specific receptors but in addition several auxiliary components, including the "sensory neuron membrane protein," SNMP. Accordingly, SNMP is considered as a marker for neurons responding to pheromones. For the desert locust *Schistocerca gregaria*, it is known that the behavior, including aggregation behavior and courtship inhibition, is largely controlled by pheromones. However, little is known about pheromones, their receptors, and the pheromone-responsive cells in locusts. In this study, we have identified two SNMP subtypes, SNMP1 and SNMP2, and compared their phylogenetic relationship and primary structure motifs with SNMPs from other species. Both SNMPs were found in chemosensory tissues, especially the antennae. Employing double in situ hybridization, we identified and localized the SNMP-expressing cells in the antennae. Cells expressing SNMP1 were localized to sensilla trichodea but also to sensilla basiconica, which in locust respond to pheromones. One or a few cells express SNMP1 within the multineuron clusters from sensilla basiconica, whereas the SNMP2 subtype was expressed in cells surrounding the neuron clusters, possibly supporting cells. Based on the finding that SNMP1 is expressed in distinct neurons under chemosensory sensilla, it is conceivable that these cells may represent pheromone-responsive neurons of the desert locust.

Key words: desert locust, olfaction, antenna, sensory neuron membrane protein, in situ hybridization

In order to perceive olfactory cues, insects have evolved hair-like appendages, called sensilla, over the surface of the olfactory organs. The task to sense pheromone compounds is assigned to pheromone-responsive sensilla (Almaas and Mustaparta 1991, Baker et al. 2004, Hansson and Stensmyr 2011). Pheromone-responsive sensilla from moth and fly species are innervated by one to three olfactory sensory neurons (OSNs), which project their dendrites to the lymph cavity of the sensillum (Keil and Steinbrecht 1984, Steinbrecht and Gnatzy, 1984, Clyne et al. 1997). The response of the sensory neurons to volatile compounds is determined by odorant receptors (ORs; Hallem and Carlson 2006, Touhara and Vosshall 2009, Carey et al. 2010, Wang et al. 2010) residing on the dendritic membrane of the OSN (Elmore and Smith 2001, Gohl and Krieger 2006). Receptors for pheromones are members of the OR family, but they are specifically tuned to pheromonal compounds (Große-Wilde et al. 2007, Touhara and Vosshall 2009, Sakurai et al. 2015). Thus, the specific response of sensory neurons to distinct pheromones is mediated by the receptor type, such as BmOR1 for bombykol in *Bombyx mori* and DmelOR67d for 11-cis-vaccenyl acetate in *Drosophila melanogaster* (Sakurai et al. 2004, Krieger et al. 2005, Kurtovic et al. 2007). In addition to the specific OR-type, each OSN expresses a coreceptor, called Orco that is highly conserved among insect species (Krieger et al. 2003, Benton et al. 2006, Vosshall and Hansson 2011). For an adequate response to pheromones, sensory neurons not only require the specific receptor type, but as an additional molecular element the "sensory neuron membrane protein," SNMP (Benton et al. 2007, Jin et al. 2008, Li et al. 2014). SNMP is a transmembrane protein with some homology to the mammalian CD36 receptor family (Rogers et al. 1997, Nichols and Vogt 2008). Recent studies have shown that the SNMP subtype SNMP1 is coexpressed with pheromone receptors in pheromone-responsive neurons (Benton et al. 2007, Forstner et al. 2008). Thus, although the functional implications of SNMP1 in pheromone sensing are still controversial (Jin et al. 2008, Vogt et al. 2009, Li et al. 2014), SNMP1 expression seems to be an indicator for pheromone-responsive

neurons. In contrast, the expression of the SNMP2 subtype in moth is confined to supporting cells (Forstner et al. 2008, Gu et al. 2013, Liu et al. 2013, Zhang et al. 2015).

The endeavors to decipher the mechanism underlying insect pheromone reception and transduction have mainly been concentrated on two taxa, the Lepidoptera and the Diptera. The desert locust (*Schistocerca gregaria*) is a member of the taxa Orthoptera that on the phylogenetic scale represents a remote lineage relative to Lepidoptera and Diptera (Grimaldi and Engel 2005, Song et al. 2015). Moreover, the Orthoptera undergo a hemimetabolic developmental process that substantially differs from that of holometabolous insects, which were hitherto mainly used in research studying SNMP-proteins (Nichols and Vogt 2008). Locusts are unique by their behavioral plasticity to switch between a solitary and a gregarious phase (Uvarov 1966, Hassanali et al. 2004, Simpson and Sword, 2008, Wang et al. 2014), and there is evidence indicating that locust body volatiles may act as aggregation pheromones, which are involved in shaping and maintaining the gregarious phase (Chapman 1990, Byers 1991, Heifetz et al. 1996). In addition, pheromones mediating courtship–inhibition have been observed in *Schistocerca gregaria* (Seidelmann and Ferenz 2002, Seidelmann et al. 2003) and the related species *Schistocerca americana* (Stahr et al. 2013). Thus, locust antennae are supposed to have pheromone-responsive neurons. In this context, it appeared to be of particular interest to explore whether SNMPs are in fact expressed in desert locust and how SNMPs of Orthoptera may relate to SNMPs of other species with respect to structural features and expression patterns.

Materials and Methods

Animals and Tissue Collection

Schistocerca gregaria were purchased from Bugs-International GmbH (Irsingen/Unterfeld, Germany). Antennae of locust nymphs (stages 1–5), adult male antennae, adult female antennae, adult tarsi, and adult maxillary palps were dissected using autoclaved surgical scissors and were immediately frozen in liquid nitrogen. Tissues were stored at −70°C until RNA extraction.

Reverse transcription PCR (RT-PCR)

Total RNA was isolated from frozen tissues using TRIzol reagent (Invitrogen) following the manufactures protocol. For cDNA synthesis, 1 µg of total RNA was used for reverse transcription in a total volume of 20 µl employing SuperScript™ III Reverse Transcriptase (Invitrogen). Alternatively, poly $(A)^+$ RNA was purified from 100 µg of total RNA using oligo $(dT)_{25}$ magnetic dynabeads (Invitrogen) following the recommendation of the supplier and reverse-transcribed using SuperScript™ III Reverse Transcriptase. Nonquantitative RT-PCRs were employed to determine expression patterns using gene specific primers. A primer pair matching the actin gene (AEV89776) of *S. gregaria* was used to test the integrity of the cDNA preparations.

Primer pairs were the following:

SgreSNMP1 sense: 5′ CATCCAGAACATCGACGACCT 3′
SgreSNMP1 antisense: 5′ GGAGTGTCCAGGGCTAGTATCTG 3′
SgreSNMP2 sense: 5′ CTACGCTCTTCCCACCTTTCA 3′
SgreSNMP2 antisense: 5′ ACAGCGGTCCCCCGATGATTA 3′
Actin sense: 5′ AACTGGCTTGCTGCATCCTC 3′
Actin antisense: 5′ CACATCTGCTGGAAGGTGGA 3′

PCR conditions used in RT-PCR experiments were: 94°C for 1 min 40 s, then 20 cycles with 94°C for 30 s, 65°C for 30 s and 72°C for 2 min, with a reduction in the annealing temperature by 0.5°C per cycle. This was followed by 20 further cycles at the condition of the last cycling step (annealing temperature = 55°C) and a final extension step for 7 min at 72°C. PCR products were analyzed on 1.5% agarose gels and visualized by ethidium bromide staining. PCR products were purified using the Geneclean II Kit (MP Biomedicals, LLC, IIIkrich, France), cloned into the pGEM-T plasmid (Promega, Madison, USA) and sequenced using vector or gene specific primers.

To verify that the bands amplified by the SNMP1- and SNMP2-specific primers were not resulting from amplification of genomic DNA, control experiments were conducted with templates representing cDNA syntheses reactions where the reverse transcriptase have been omitted (-RT) (Supp. Fig. 1 [online only]).

Identification and Analysis of *S. gregaria* SNMP1 and SNMP2 Sequences

Previously reported insect SNMP genes (Forstner et al. 2008, Vogt et al. 2009, Gu et al. 2013, Liu et al. 2013) were used in a tBLASTx search (cut-off value 10^{-10}) as queries to identify putative SNMP genes in a nonpublished *S. gregaria* antennal transcriptome database. The identified putative *S. gregaria* SNMP genes were then utilized as queries to identify additional SNMP genes in the antennal transcriptome using tBLASTx and BLASTp; iterative BLAST searches were completed till no more candidates were identified. Verification of the BLAST outcomes as candidate SNMP sequences was further confirmed via BLASTx searches in the NCBI nonredundant protein database.

Phylogenetic Analysis

The sequence alignment was created and processed using the programs Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/; accessed February 2016) and BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html; accessed February 2016). Transmembrane domain prediction was performed using the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/; accessed February 2016). An unrooted neighbour-joining tree was constructed based on a Clustal alignment using the MEGA6 software (Tamura et al. 2013) in accordance with default settings.

In Situ Hybridization

RNA in situ hybridization probes were generated as described previously (Guo et al. 2014; Yang et al. 2012). Linearized pGEM-T vectors carrying SNMP coding sequences representing the c-terminus (902bp, SNMP1; 848bp, SNMP2) were utilized to synthesize digoxigenin- (DIG) and biotin- (BIO) labeled anti-sense and sense RNA probes using the T7/SP6 RNA transcription system (Roche, Germany). The labeled riboprobe for *Schistocerca gregaria* Orco (SgreOrco) was prepared as described previously (Yang et al. 2012).

Antennae were dissected and embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek Europe, The Netherlands), then 12 µm sections were thaw mounted on SuperFrost Plus slides (Menzel-Gläser, Braunschweig, Germany) at −21°C (Jung CM300 cryostat). Sections were stored at −70°C temporarily. RNA in situ hybridization was conducted as previously reported (Yang et al. 2012, Guo et al. 2014) with the following modifications. Section were fixed (4% paraformaldehyde in 0.1 M $NaHCO_3$, pH 9.5) at 4°C for 22 min. The following steps (at room temperature) were a wash for 1 min in PBS (phosphate buffered saline = 0.85% NaCl, 1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.1), incubation for 10 min in 0.2 M HCl and another wash for 1 min in PBS. After incubation for 10 min in acetylation solution (25% acetic anhydride freshly added in 0.1 M triethanolamine), sections were washed three times in PBS (3 min

each). 100 µl hybridization solution containing the labeled RNA in hybridization buffer (Yang et al. 2012) was placed onto the tissue section. A coverslip was placed on top and slides were incubated in a moister box at 60°C overnight (at least 18h). After hybridization, slides were washed twice for 30 min in 0.1x SSC at 60°C, then each slide was treated with 1 ml 1% blocking reagent (Roche) for 40 min at room temperature.

In single and double fluorescent in situ hybridization (FISH), visualization of hybridized probes was performed by using an anti-Dig AP-conjugated antibody in combination with HNPP/Fast Red (Roche) for DIG-labeled probes and an anti-biotin streptavidin horse radish peroxidase-conjugate together with fluorescein-tyramides as substrate (TSA kit, Perkin Elmer, MA) for biotin-labeled probes.

Sections from FISH experiments were analyzed with a Zeiss LSM510 Meta laser scanning microscope (Zeiss, Oberkochen, Germany). Confocal images stacks were processed by ZEN 2009 software. For an optimal view, otherwise non-altered images were adjusted in brightness and contrast and arranged using PowerPoint (Microsoft).

Results

Identification of Two SNMPs in *S. gregaria*

Based on the sequences of SNMPs from several insect species, we have performed iterative tBLASTx searches in a nonpublished *Schistocerca gregaria* antennal transcriptome database as well as tBLASTn exploitation against the NCBI database. These approaches have led to an initial identification of several sequences related to scavenger receptors; two of the sequences were classified into the CD36 superfamily, a subfamily of scavenger receptor B (Acton et al. 1994, Febbraio et al. 2001, Silverstein et al. 2010) and thereby were considered as candidate SNMP homologous. To verify the assumed classification, we initiated a phylogenetic relationship analysis of the two sequences with other SNMPs (Fig. 1). Given that SNMPs are divided into two subgroups (Vogt et al. 2009), we constructed an unrooted Neighbor-Joining tree (Fig. 1) based on 30 SNMP sequences (20 from SNMP1 and 10 from SNNP2) from six insect taxa. All the sequences were classified clearly into two orthologous groups and the two candidate SNMP sequences from *Schistocerca gregaria* were clustered into the SNMP1 and the SNMP2 subgroup, respectively. Within orthologous groups, members were arrayed into monophyletic lineages.

The transcriptome data suggested intact open reading frames for the newly identified SgreSNMP1 (1.5 kb) and SgreSNMP2 (1.6 kb), encoding proteins of 513 and 551 amino acids (aa), respectively. In order to gain more valid sequence information, we have conducted a sequence alignment analysis (Fig. 2). We selected 10 SNMP sequences from Lepidoptera, Diptera, Coleoptera and Hymenoptera insect species for comparison with SgreSNMP1 and SgreSNMP2 (Orthoptera). Homologous comparison revealed that members of SNMP1 subgroup are more conserved than those of the SNMP2 subgroup. SgreSNMP1 exhibited an overall amino acid identity of 40% with other SNMP1 members whereas around 30% identity was observed between SgreSNMP2 and other SNMP2 sequences. The sequence identity of 33% between SgreSNMP1 and SgreSNMP2 is in accordance with the overall identity between SNMP1 and SNMP2 proteins in general.

Utilizing the transmembrane topology prediction software TMHMM (Krogh et al. 2001), we found two transmembrane helixes in proximity to the N- and C-terminal regions. The predicted protein structure indicates a large extracellular loop in the SgreSNMPs (SNMP1, 426aa; SNNP2, 431aa). Earlier studies on SNMPs from other species have identified two domains with relatively high sequence conservation in the ectodomain of SNMP: the first motif extends 44 amino acids approximating the N-terminal

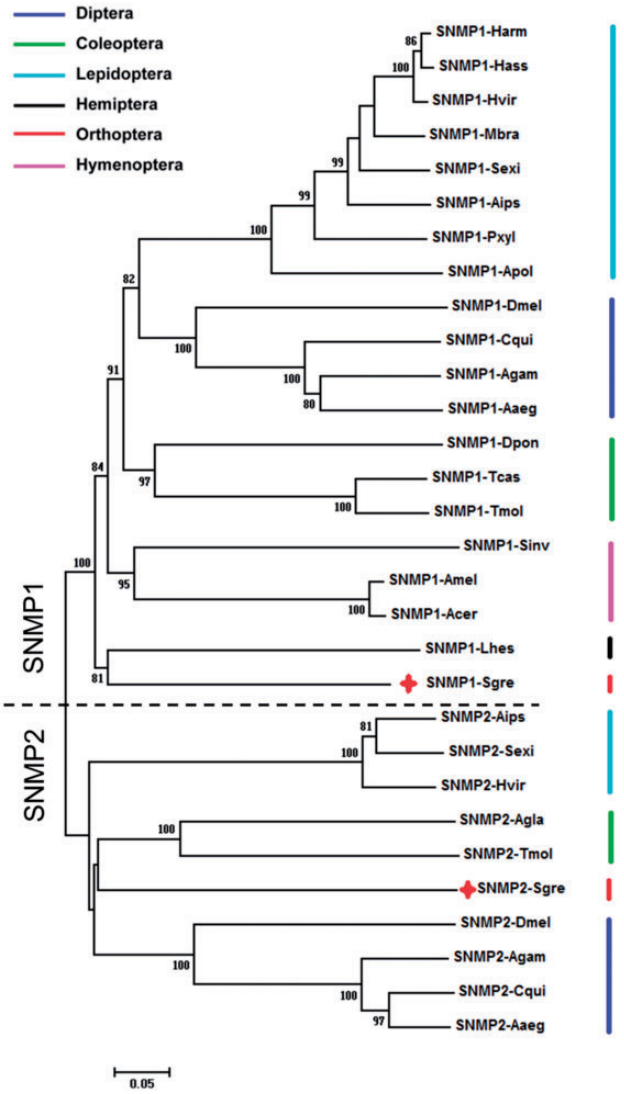

Fig. 1. Phylogenetic relationship of two putative *Schistocerca gregaria* SNMPs and 28 SNMPs from five other insect taxa. A neighbor joining tree was built using MEGA6 based on a Clustal Omega alignment of amino acid sequences deposited in Genbank. Bootstrap support values were based on 1,000 replicates and only values above 80% are shown. Branch lengths are proportional. Abbreviation and accession numbers: Aips, *Agrotis ipsilon* (SNMP1: AGF87119.1; SNMP2: AGF87120.1); Apol, *Antheraea polyphemus* (AAC47540.1); Agam, *Anopheles gambiae* (SNMP1: Q7QC49.3; SNMP2: Q7Q6R1.5); Aaeg, *Aedes aegypti* (SNMP1: Q17A88.2; SNMP2: C3U0S3.3); Amel, *Apis mellifera* (P86905.1); Acer, *Apis cerana cerana* (AGC91908.1); Agla, *Anoplophora glabripennis* (JAB63926.1); Cqui, *Culex quinquefasciatus* (SNMP1: EDS40329.1; SNMP2: AEK32389.1); Dmel, *Drosophila melanogaster* (SNMP1: AAF55863.2; SNMP2: E1JI63.1); Dpon, *Dendroctonus ponderosae* (AFI45067.1); Harm, *Helicoverpa armigera* (AAO15604.1); Hass, *Helicoverpa assulta* (ACC61201.1); Hvir, *Heliothis virescens* (SNMP1: Q9U1G3.1; SNMP2: B2RFN2.1); Lhes, *Lygus hesperus* (JAG22531.1); Mbra, *Mamestra brassicae* (AAO15603.1); Pxyl, *Plutella xylostella* (ADK66278.1); Sexi, *Spodoptera exigua* (SNMP1: AGN52676.1; SNMP2: AGN52677.1); Sinv, *Solenopsis invicta* (XP_011159295.1); Tcas, *Tribolium castaneum* (EFA02899.1); Tmol, *Tenebrio molitor* (SNMP1: AJO62245.1; SNMP2: AJO62246.1).

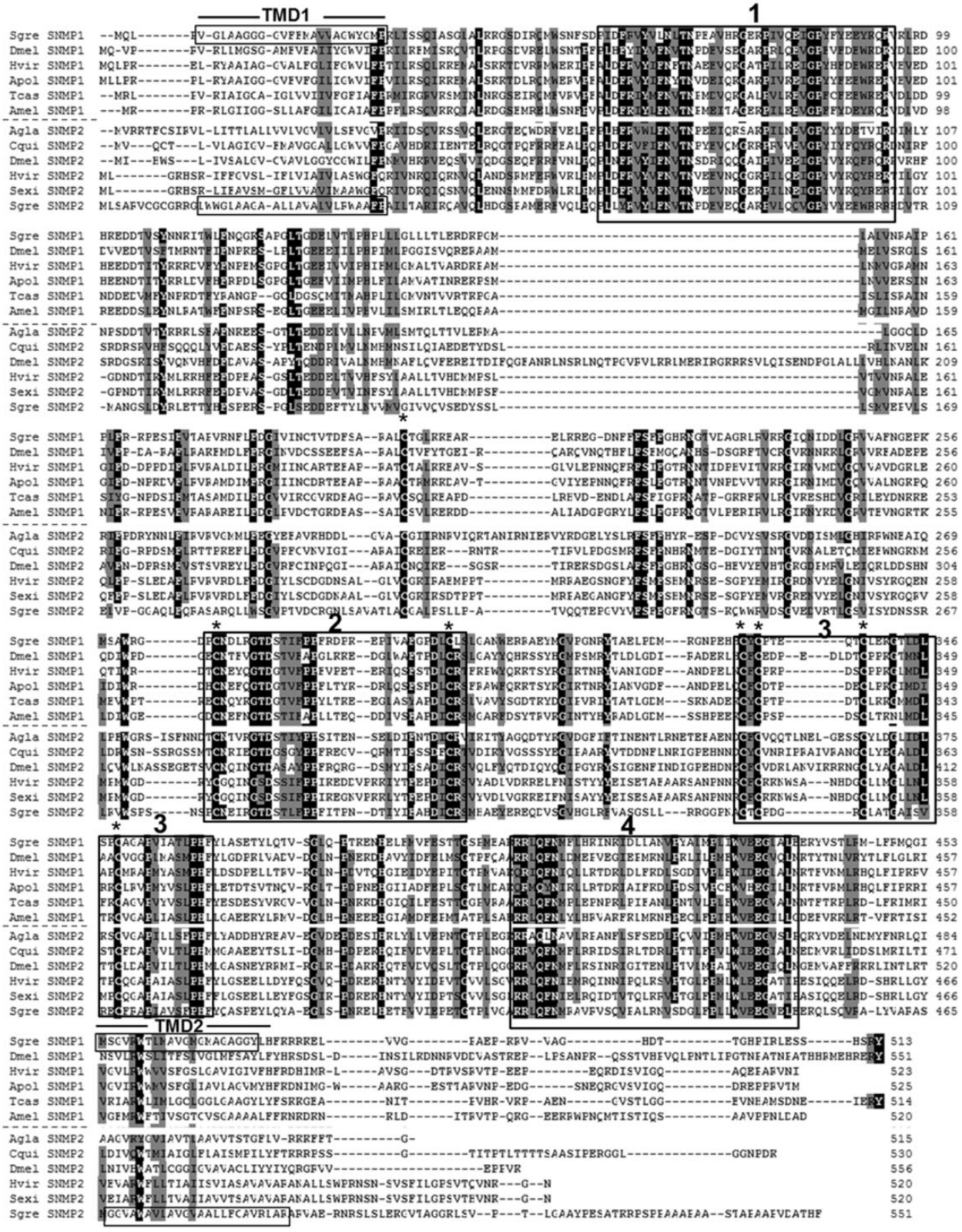

Fig. 2. Alignment of two SgreSNMP amino acid sequences with selected SNMPs from other insect species. Numbers on the right side indicate the number of last residue in a line of SNMP sequence. Residues that share more than 80% amino acid similarity are shaded in grey and identical residues are shaded in black. Predicted SgreSNMP transmembrane domains (TMD) are shown in black box. Asterisks above amino acid refer to conserved cysteine residues. Box areas below the number 1-4 propose four well conserved motifs exhibiting more than 40% sequence similarity.

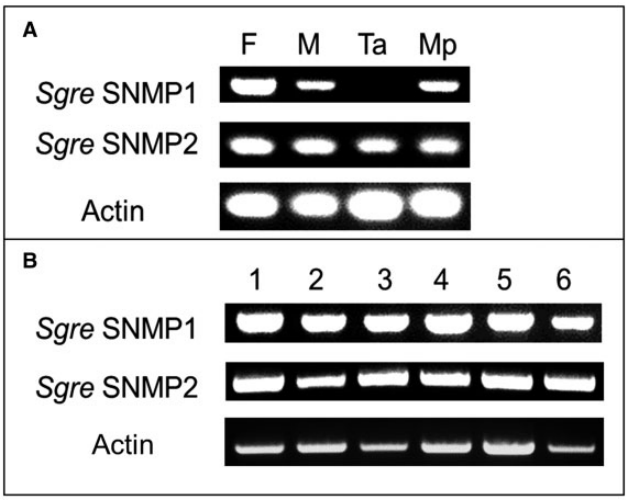

Fig. 3. Spatial and developmental expression pattern of SgreSNMP1 and SgreSNMP2. RT-PCRs were conducted with cDNA prepared from indicated tissues (A) as well as antennae from different growth stages (B). Expression of actin (AEV89776) is used as a control to reflect cDNA integrity. F, female antenna; M, male antenna; Ta, tarsus; Mp, maxillary palp; 1–5, locust nymphal stages; 6, adult locust.

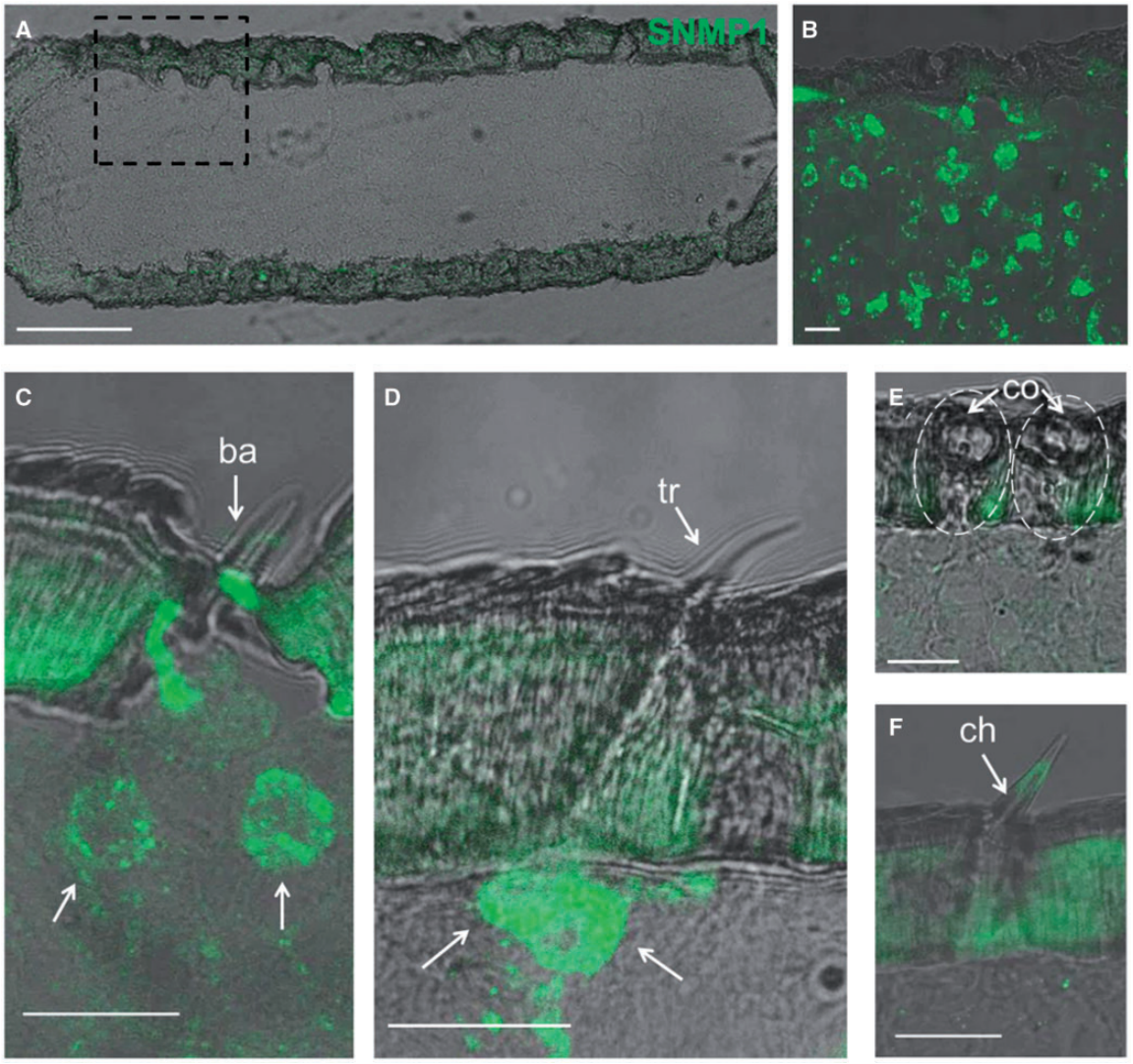

Fig. 4. Expression of SgreSNMP1 is restricted to two sensillum types on the male antenna. FISH was performed by employing a BIO-labeled probe for SgreSNMP1. (A) A section through an antennal segment at lower magnification (10×). (B) Amplified area of the dashed box in (A): numerous SgreSNMP1 expressing cells are visible. (C–F) Localization of SgreSNMP1 expressing cells in relation to four types of sensory hairs. SgreSNMP1 labeled cells (white arrows) are visible beneath a sensillum basiconicum (ba, in C) and a sensillum trichodeum (tr, in D), however, are absent from sensilla coeloconica (co, dashed area in E) and the sensillum chaeticum (ch, in F). Scale bar: A, 100 μm; B–F, 20 μm.

and the second motif contains 92 amino acids which are close to the C-terminal. For the SgreSNMPs, we identified four domains that are well conserved (over 40% sequence identity with other SNMPs); the motifs 1-3 covered the reported two conserved domains (Rogers et al. 1997); a fourth conserved motif was identified near the C-terminal following the third motif (Fig. 2). Moreover, the sequence alignment study unraveled seven conserved cysteine residues (asterisk in Fig. 2), which are located in the ectodomain (residues 190-350 in *S. gregaria*).

Expression of *S. gregaria* SNMPs

The expression profile of *SgreSNMP* genes was studied by nonquantitative RT-PCR analysis comparing several selected tissues including chemosensory organs, such as antennae, maxillary palps, and tarsi from adult locust. We found gene products of *SNMP1* and *SNMP2* in both female and male antenna, the major olfactory organ. Expression of SgreSNMP1 and SgreSNMP2 was also detected in maxillary palp, another important chemosensory organ. Using cDNA from tarsi only SgreSNMP2 was successfully amplified (Fig. 3A). Comparing antennae from different developmental stages, we found that SgreSNMP1 as well as SgreSNMP2 were expressed throughout development from the nymphal (1st to 5th) to the adult stages, with no observable variation in the expression levels (Fig. 3B).

SgreSNMP1 in Different Sensilla Types on the Antenna

To visualize where the *SNMP* genes were expressed in adult *S. gregaria* antenna, we generated riboprobes labeled with either digoxigenin (DIG) or biotin (BIO) and conducted FISH on tissue sections throughout the antenna. The specificity of the FISH results with the antisense probes was ascertained by the observation that there was no labeling in control experiments using corresponding sense probes (data not shown). Adult *S. gregaria* antennae comprise approximately 24 flagellar segments. Figure 4A depicts a longitudinal section of one segment treated with the bio-labeled SgreSNMP1 probe. Higher magnification of the area marked by the dashed box in Figure 4A revealed many intensely labeled SgreSNMP1 cells that were distributed across the antennal segment (Fig. 4B). The *S. gregaria* antennae comprise four types of sensilla, which are morphologically distinguishable (Ochieng et al. 1998) and are supposed to play different roles in chemosensation. The results of FISH-experiments indicate that labeled SgreSNMP1 cells can be assigned to sensilla basiconica (Fig. 4C) and sensilla trichodea (Fig. 4D). In *S. gregaria*, a sensillum basiconicum is innervated by up to 50 neurons, while no more than three neurons are housed in sensilla trichodea (Ochieng et al. 1998). Typically, two SgreSNMP1-positive cells were located beneath sensilla basiconica (Fig. 4C), but there were also cases with more than two SgreSNMP1 cells. Beneath sensilla trichodea, not more than two cells expressing SgreSNMP1 were found (Fig. 4D). No SgreSNMP1-positive cells were found under sensilla coeloconica (Fig. 4E) and sensilla chaetica (Fig. 4F).

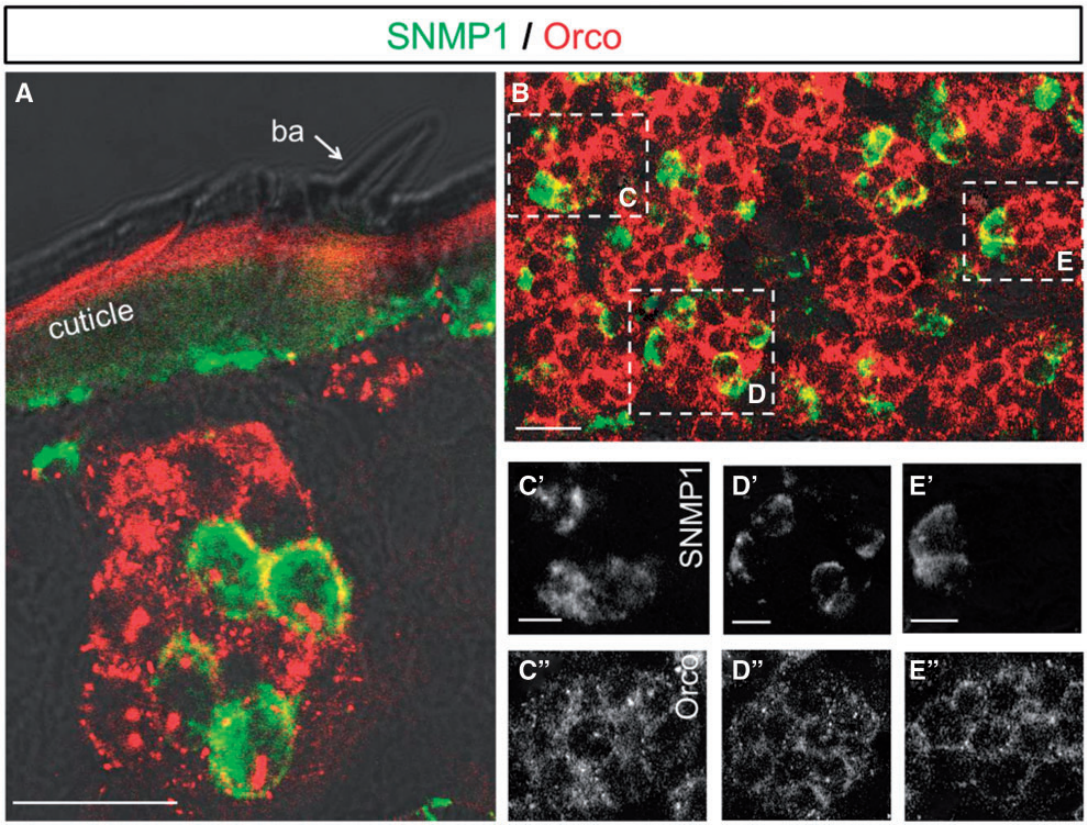

Fig. 5. Expression of SgreSNMP1 within neuron clusters on the male antenna. Two-color FISH assays were performed to visualize the expression of SgreSNMP1 and Orco in neuron clusters utilizing a BIO-labeled probe for SNMP1 and a DIG-labeled probe for Orco. (A) Some of the Orco expressing neurons in the clusters (red) also express SgreSNMP1 (green). The neuron cluster is located below a short blunt sensory hair (sensillum basiconicum, ba). (B) In almost all Orco neuron clusters one or several cells also express SgreSNMP1. (C–E) In three selected examples, the varying number of SgreSNMP1-positive cells in Orco clusters is demonstrated. The profile of SgreSNMP1 cells (C′–E′) and Orco clusters (C″–E″) were visualized by selectively blocking the red-color channel or green-color channel, respectively. Scale bar: A and B, 20 μm; C′–E″, 10 μm.

In order to explore whether the expression of SgreSNMP1 in sensilla basiconica was confined to OSNs, we performed double FISH experiments with probes for Orco and SgreSNMP1. Orco is ubiquitously coexpressed in sensory neurons expressing odorant receptors (Benton et al. 2006, Sato et al. 2008). Previous studies have shown that in the antennae of *S. gregaria* Orco expressing neurons can be assigned to sensilla basiconica and sensilla trichodea (Guo et al. 2014, Yang et al. 2012). Analyses of antennal sections treated with a specific probe for Orco revealed clusters of labeled cells; a typical example is depicted in Figure 5A showing a cluster of approximately 15 cells stained by the Orco probe (red). Out of this group, four cells were stained green by the SgreSNMP1 probe. The results indicate that only a small subgroup of the Orco-positive cells in the cluster also express SgreSNMP1. To determine what proportion of Orco-positive cells in a cluster coexpresses SgreSNMP1, tissue sections from antennal segments with numerous Orco-positive cell clusters were analyzed. The results are depicted in Figure 5B. Within each of the numerous Orco neuron clusters, the number of SgreSNMP1-positive cells varied; ranging from one to several. This is confirmed in three cases representing color-shaded profiles of SgreSNMP1 labeling (Fig. 5C'–E') and Orco labeling (Fig. 5C''–E''), separately. On the single imaging layer, the number of visible SgreSNMP1 cells ranges from 2–4, which is in line with the notion that within the multicellular clusters of the sensilla basiconica at least one cell expresses SgreSNMP1.

Localization of SgreSNMP1 and SgreSNMP2

Previous studies have shown that in moth species, SNMP2 is not expressed in sensory neurons, but in the supporting cells which surround the sensory neurons (Forstner et al. 2008). Therefore, attempts were made to explore which cell types may express SgreSNMP2. Incubating cryosections of antennal segments with a SgreSNMP2 probe led to a labeling pattern (Fig. 6A) quite different from that of SgreSNMP1 (Figs. 4 and 5). There were no labeled cells beneath the sensory hairs; instead, the periphery of neuron clusters was labeled, the region where supporting cells are located. To support this notion, we performed two-color FISH experiments with probes for Orco and SgreSNMP2; a typical result is depicted in Fig. 6B. SgreSNMP2-positive cells were

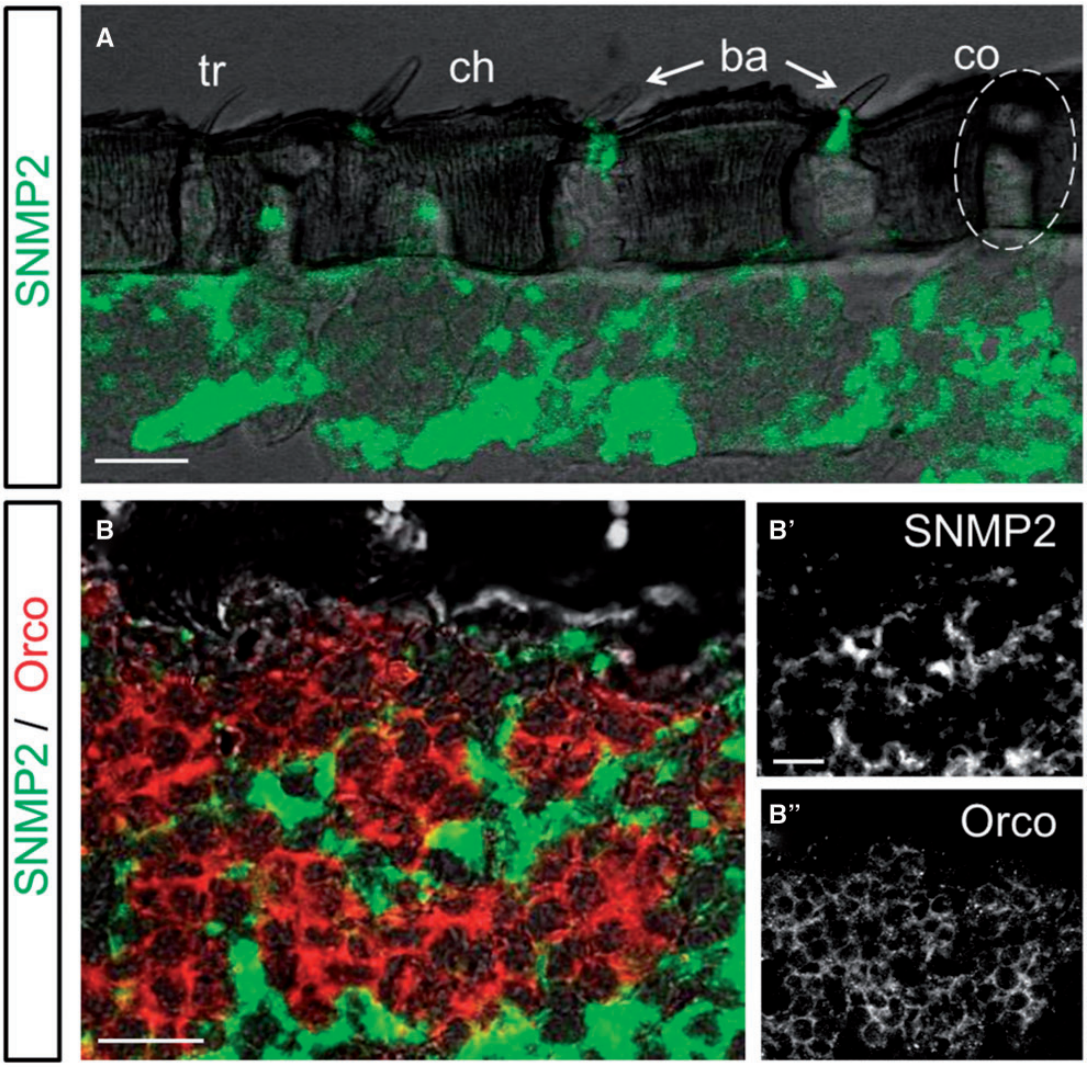

Fig. 6. SgreSNMP2 is not expressed in Orco-positive neurons. (A) FISH assays were performed on sections from male antenna using a BIO-labeled SgreSNMP2 probe. The staining for SgreSNMP2 was located relatively distant from the sensory hairs; the sensilla types are indicated; abbreviations see figure 3. (B) Employing a two-color FISH assay with a BIO-labeled probe for SNMP2 and a DIG-labeled probe for Orco revealed that the SgreSNMP2 expressing cells are located at the periphery of Orco neuron clusters. (B') and (B''): separate visualization of SgreSNMP2 cells and Orco neuron clusters by blocking red-color channel or green-color channel, respectively. Scale bar: 20 μm.

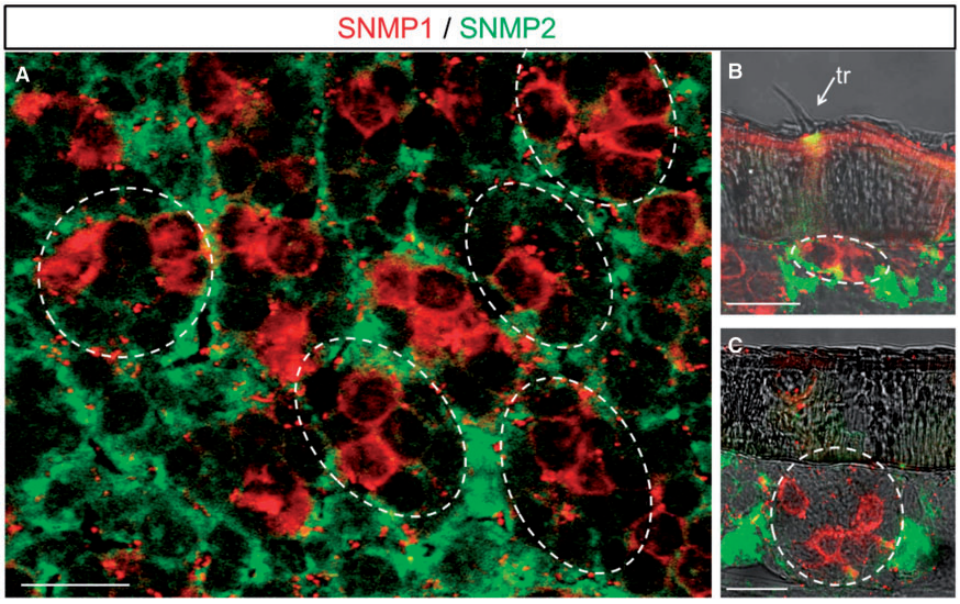

Fig. 7. SgreSNMP1 and SgreSNMP2 are expressed in spatially distinct cells. A DIG-labeled probe for SgreSNMP1 and a BIO-labeled probe for SgreSNMP2 were used in two-color FISH assays. (A) SgreSNMP1 expressing cells are encircled by SgreSNMP2 expressing cells. Dashed areas outline clusters of neurons in which some of the cells express SgreSNMP1. The sensillum trichodeum (tr, in B) and the sensillum basiconicum (in C) house SgreSNMP1 cells as well as SgreSNMP2 expressing cells; both cell types are not overlapping. SgreSNMP1 expressing cells were outlined by dash lines. Scale bar: 20 μm.

tightly encircling the Orco-positive cell clusters and none of the red cluster cells was stained green. This nonoverlapping pattern indicates that SgreSNMP2 is not expressed in neurons but appears to be expressed in supporting cells flanking neuron clusters.

Based on the results of in situ hybridization experiments, we suggested a spatially segregated expression pattern for the two SgreSNMPs. This was confirmed by two-color FISH assays using the SgreSNMP1 probe (DIG-labeled) and SgreSNMP2 probe (BIO-labeled). On tissue section of antennae, one or more SgreSNMP1-positive cells were located within a neuron cluster of neurons, identifiable as red-cells in the dashed area. The SgreSNMP2 labeling was always at the periphery of the area, never overlapping with SgreSNMP1 labeling (Fig.7A). This nonoverlapping expression profile of SgreSNMP1 and SgreSNMP2 was found beneath both sensilla trichodea (Fig. 7B) and sensilla basiconica (Fig. 7C).

Discussion

Previous studies characterizing the "sensory neuron membrane proteins" (SNMPs) focused on holometabolous insects, which undergo a complete metamorphosis from the larval to the adult stage. In this study, we have identified two SNMP subtypes of a hemimetabolous insect, the desert locust *Schistocerca gregaria*, which represents the order of Orthoptera, originating in the Carboniferous period (350–300 million years ago) and comprising more than 25,000 species, many of which are pests of economic importance (Song et al. 2015). Bioinformatics analysis of antennal transcriptome sequences from the desert locust has led to the identification of two sequences with relatively high homology to the SNMP/CD36 family (Nichols and Vogt 2008). All the identified SNMP sequences from numerous species have been categorized into two subclades (Vogt et al. 2009), and detailed analysis of the two *S. gregaria* sequences allowed to assign them to the SNMP1 and SNMP2 group, respectively. The high degree of sequence homology across SNMPs from various insect species which are far apart on the phylogenetic scale indicates a strong negative selection pressure on the primary structure of SNMPs and suggests a conserved and important function for these proteins (Nichols and Vogt 2008). The view that SNMPs fulfill a specific and important functional role is further supported by the finding that the two locust SNMP sequences were readily categorizes into one of the two subclades, indicating that each of the locust SNMP subtypes was more related to the relevant SNMP class of distantly related species than to the other SNMP subtype of *S. gregaria* protein. The conserved sequences between the members of each SNMP subfamily from different insect species, including the hemimetabolous locust suggest important physiological implications. An alignment of the previously known and the newly derived SNMP sequences revealed some characteristic motifs, such as the two transmembrane domains and a large putative extracellular loop with several cysteines that are conserved in the SNMPs as well as in the CD36 proteins and are supposed to form stabilizing disulfide bridges (Rasmussen et al. 1998, Rogers et al. 2001).

In the holometabolous moth *Agrotis ipsilon*, a characteristic age-dependent expression profile was reported for SNMP1 and SNMP2; the expression level increased quite dramatically from the day before eclosion reaching a peak at the third day (Gu et al. 2013). In contrast, in the hemimetabolous *Schistocerca gregaria* we did not find a stage where the expression was particular strong, but rather a similar level of expression for both SNMPs throughout development from nymphal to the adult stages. Visualization of cells which express SgreSNMP1 revealed that they are localized under sensilla trichoidea but also under sensilla basiconica; SNMP1 was not found to be expressed in sensilla coeloconic and sensilla chaetica. A similar spatial expression pattern has recently been reported for *Spodoptera exigua* (Liu et al. 2014), whereas in other moth species and in the fruitfly SNMP1 cells were found only at the base of sensilla trichodea but not under sensilla basiconica and sensilla chaetica (Forstner et al. 2008, Gu et al. 2013, Liu et al. 2013, Zhang et al. 2015). For the antennal sensilla of *Schistocerca gregaria*, several unique features have been described in great detail previously (Ochieng et al. 1998); most notably, the slender sensilla trichodea contain one to three sensory neurons whereas the

broader sensilla basiconica are innervated by 20 to 50 sensory neurons located below the hair base. Most of the neurons clustered in sensilla basiconica appear to be OSNs based on expression of Orco (Fig. 5). Interestingly, out of such a cluster of Orco-positive neurons only a small number (one to very few cells) expressed SNMP1. This observation indicates a molecular and probably functional heterogeneity among the olfactory neurons innervating the same sensillum, which would render the sensilla basiconica responsive to multiple chemical stimuli. The notion that SNMP1 is preferentially expressed in chemosensory neurons which are tuned to specific pheromones (Benton et al. 2007, Forstner et al. 2008) and the finding that only a small fraction of the clustered neurons express SNMP1 may indicate that these cells are responsive to pheromones. In this context, it is interesting to note that previous studies have shown that sensilla basiconica of *Schistocerca gregaria* do in fact respond to stimulation with aggregation pheromone compounds as well as to the courtship–inhibition pheromone phenylacetonitril (Hansson et al. 1996, Seidelmann and Ferenz 2002).

With respect to pheromone detection by OSN in sensilla trichodea of the desert locust, only one study reported a response of trichoid OSNs to (E,Z,)-2,6,-nonadienal. This compound was supposed as a possible female sex pheromone, but was also mentioned as a major constituent of a preferred host plant of *S. gregaria* (Ochieng and Hansson 1999).

The results of double labeling experiments indicate that in *S. gregaria* the two SNMP subtypes are expressed in different cells. The locust SNMP2 was found to be expressed in cells surrounding the Orco-positive cell clusters, suggesting that SNMP2 is not expressed in neurons but rather in the supporting cells. Thus, the spatial expression pattern of SNMP1 and SNMP2 in the hemimetabolous locust is reminiscent to that found in moth species (Forstner et al. 2008, Gu et al. 2013, Zhang et al. 2015) but different from that described for the fly (Benton et al. 2007). Although the implications of the differential expression pattern for SNMP1 and SNMP2 are unknown, the expression in different cell types, together with the low sequence similarities between the two SNMP subtypes, support the notion that SNMP1 and SNMP2 are involved in different physiological processes.

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Data Deposition

The SNMP sequences reported in this paper have been deposited in Genbank under accession numbers: KU659599 (SgreSNMP1) and KU659600 (SgreSNMP2).

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