Cell and Tissue Research (2019) 378:485–497
https://doi.org/10.1007/s00441-019-03066-y

REGULAR ARTICLE

The expression patterns of SNMP1 and SNMP2 underline distinct functions of two CD36-related proteins in the olfactory system of the tobacco budworm *Heliothis virescens*

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Received: 20 May 2019 / Accepted: 1 July 2019 / Published online: 18 July 2019
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Abstract

In insects, male and female pheromone signals are detected by olfactory sensory neurons (OSNs) expressing the "sensory neuron membrane protein type 1". SNMP1 is supposed to function as a co-receptor involved in the transfer of pheromones to adjacent pheromone receptors. In the moth *Heliothis virescens*, we previously found OSNs that project their dendrites into pheromone-responsive trichoid sensilla and are associated with cells containing transcripts for the HvirSNMP1-related protein HvirSNMP2. Like HvirSNMP1, HvirSNMP2 belongs to the CD36-family of two-transmembrane domain receptors and transporters for lipophilic compounds, but its role in the olfactory system is unknown. Here, we generated polyclonal anti-peptide antibodies against HvirSNMP2 as well as HvirSNMP1 and conducted an in-depth immunohistochemical analysis of their subcellular localization in the antenna of both sexes. In line with a function in pheromone detection, HvirSNMP1 was immunodetected in the somata and the dendrites of distinct OSNs in subsets of trichoid sensilla. These trichoid sensilla contained only one α-SNMP1-positive OSN in males and clusters of 2–3 labeled cells in females. In contrast, experiments with α-SNMP2-antibodies revealed a broad labeling of non-neuronal support cells (SCs) that are associated with OSNs in likely all trichoid and basiconic sensilla of the antenna with no differences between sexes. Detailed confocal microscope examinations of olfactory sensilla revealed SNMP2-like immunoreactivity close to the apical membrane of SCs and interestingly inside the sensillum. Together, these findings indicate a potential function of SNMP2 in pheromone- as well as general odorant-responsive sensilla and a role fundamentally different from SNMP1.

Keywords Olfaction · Moth · Pheromone detection · Sensilla trichodea · Sensory neuron membrane protein

Introduction

Olfactory systems that allow for the powerful recognition of vital chemical cues in the environment, such as plant volatiles indicating food sources or pheromones used for intraspecific communication, are of critical importance for insects' lives (Hansson and Stensmyr 2011). Proteins that mediate a sensitive and accurate detection of the relevant chemical signals are expressed by specialized olfactory sensory neurons (OSNs) and glia-like support cells (SCs). Both cell types are associated in hair-like cuticle structures, named sensilla, on the principle olfactory organs of insects, i. e., the antenna and palps (Keil 1989; Steinbrecht 1997). SCs secrete odorant binding proteins (OBPs) and chemosensory proteins (CSPs) into the sensillum lymph (Vogt 2003; Pelosi et al. 2006; Leal 2013). It is generally agreed that these small and soluble proteins take over odorant molecules from the air and transfer them across

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00441-019-03066-y) contains supplementary material, which is available to authorized users.

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the sensillum lymph towards tuned olfactory receptors residing in the dendritic membrane of OSNs projecting into the sensillum. After activation of respective odorant receptors (ORs) or ionotropic receptors (IRs) (Montagne et al. 2015; Fleischer et al. 2018), odorant molecules are supposed to be rapidly inactivated by odorant degrading enzymes or biotransformation enzymes (Vogt 2003; Leal 2013). However, the fate of the inactivated molecules, i.e., their removal from the sensillum lymph, is unknown.

Data that have accumulated over the last two decades indicate that the chemoreception process in insects involves an additional family of proteins, named "sensory neuron membrane proteins" (SNMPs) (Rogers et al. 1997; Rogers et al. 2001a). The first SNMP was identified in pheromone-responsive OSNs in the sensilla trichodea of the moth *Antheraea polyphemus* (Rogers et al. 1997). Subsequently, orthologues of the ApolSNMP1 have been identified in various insect species of the orders Lepidoptera, Diptera, Coleoptera, Hymenoptera, and Orthoptera (Rogers et al. 2001a; Nichols and Vogt 2008; Vogt et al. 2009; Jiang et al. 2016). For most insect species, two related subgroups of SNMPs, SNMP1 and SNMP2, are described displaying about 25–30% sequence identity (Forstner et al. 2008; Vogt et al. 2009). For a few insects, genes encoding a third SNMP-type have been reported. Phylogenetic analyses point out, that this type forms a separated group and appears to share a common ancestor with SNMP2s (Liu et al. 2015). Insect SNMPs belong to the family of CD36 proteins which are characterized by 2-transmembrane domains (Rogers et al. 2001a; Vogt et al. 2009). Members of the CD36-family display remarkable functional versatility. In vertebrates, this includes vital functions in the transport and reception of lipophilic compounds, lipoprotein scavenging, and cell–cell recognition (Silverstein and Febbraio 2009; Martin et al. 2011). With respect to chemoreception, studies in mice have implicated a role of CD36 in sensing fatty acids by taste cells, and recently in the detection of oleic acid by a subset of olfactory neurons (Gaillard et al. 2008; Ozdener et al. 2014; Neuhaus et al. 2015; Oberland et al. 2015). For insect CD36-related proteins other than SNMPs, such as emp (epithelial membrane protein), nina D (neither inactivation nor afterpotential D), or croquemort, functions similar to vertebrates' CD36s have been described, including lipid transport and cytoadhesion (Nichols and Vogt 2008); however, their specific functions are just beginning to be understood. Similarly, the functional relevance of the different SNMP types in the olfactory system of insects is largely unknown. Studies mainly conducted in moth species, and the vinegar fly *Drosophila melanogaster* indicate a significant role of members of the SNMP1 subfamily in the primary processes of pheromone reception. In situ hybridization experiments have demonstrated SNMP1 expression by subpopulations of antennal OSNs (Rogers et al. 2001a; Forstner et al. 2008; Zhang et al. 2015). Moreover, all OSNs which express confirmed pheromone receptors (PRs) co-express SNMP1 (Benton et al. 2007; Pregitzer et al. 2014; Zielonka et al. 2018). Inside the membrane, SNMP1 appears to be located in close proximity to a PR (Benton et al. 2007) probably being a stabilizing component in a functional complex (Ha and Smith 2009). Moreover, SNMP1 seems to be required for rapid activation and termination of pheromone-induced activity (Li et al. 2014) and was reported to contribute to the sensitivity of pheromone detection systems (Jin et al. 2008; Li et al. 2014; Pregitzer et al. 2014). Since its discovery, SNMP1s have been suggested to dock pheromone-loaded pheromone binding proteins (PBPs) near to the receptor site and to be possibly involved in the delivery of pheromones to PRs (Rogers et al. 1997; Vogt 2003). This notion has obtained some support through protein structure modeling and site-directed mutagenesis approaches indicating that SNMP1 may bind pheromones to its large tunnel-like extracellular domain and may possibly funnel/transport ligands through this domain to a PR (Gomez-Diaz et al. 2016).

Compared to SNMP1s, much less is known about the function of SNMP2s. In conflict with its name, in situ hybridization studies with antenna of the tobacco budworm *Heliothis virescens* have identified transcripts for SNMP2 in non-neuronal cells, likely the support cells that surround OSNs (Forstner et al. 2008). Similar results have been reported for other moth species and the desert locust *Schistocerca gregaria* (Forstner et al. 2008; Zhang et al. 2015; Jiang et al. 2016). While SNMP2 expression patterns have been described based on gene transcript data, little is known about the antennal topography of the protein, particularly the distribution with regard to its function. Currently, only one single study in the black cutworm *Agrotis ipsilon* has reported a localization in cells at the base of olfactory sensilla and, surprisingly, in the sensillum lymph (Gu et al. 2013). In order to evaluate the SNMP2 topography in the olfactory system of moths and to compare its localization relative to SNMP1, we have generated polyclonal antibodies against these two *Heliothis virescens* SNMP-types (hereafter named α-SNMP1-Ab and α-SNMP2-Ab) and utilized them for an in-depth immunohistochemical analysis of male and female moth antennae.

The results reveal a differential expression and distinct localizations of the two SNMP-types in the olfactory system of moths underlining the proposed role of SNMP1 in pheromone detection and indicating a more general and novel function of SNMP2.

Material and methods

Animals

Heliothis virescens pupae were kindly provided by Bayer CropScience AG, Frankfurt, Germany. Animals were sexed

and allowed to develop into adults in an incubator at 24–28 °C with an alternating light-dark cycle (LD 12 h:12 h). Tissue from 0- to 4-day-old adult males and females were used for the experiments.

Antibody production

Polyclonal anti-peptide antibodies against *H. virescens* SNMP1 (α-SNMP1-Ab) and *H. virescens* SNMP2 (α-SNMP2-Ab) were raised in rabbits by a custom service (Pineda Antibody-Service, Berlin, Germany). At first, two peptides representing amino acids 32-48 of HvirSNMP1 (NH_2-LKSQLKKEMALSKKTDV-COOH) and amino acids 33-47 of HvirSNMP2 (NH_2-NKQIQKNVQLANDSK-COOH) were synthesized. The SNMP-specific peptides, which correspond to a region in the extracellular domain near to the predicted first transmembrane domain were conjugated to the protein carrier keyhole limpet hemocyanin (KLH), and standard procedures were applied to immunize rabbits for 24 weeks. Finally, α-SNMP1-Ab and α-SNMP2-Ab were purified from the collected sera by peptide-affinity chromatography on a peptide-Sepharose 6B column.

Immunohistochemistry

Fluorescence immunohistochemistry (FIHC) was performed as previously described (Gohl and Krieger 2006; Blankenburg et al. 2015) with a few modifications. Antennae of 0–4-day-old adults were dissected and fixed for 2 h at 4 °C in a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde dissolved in phosphate buffer (1.4 mM/L KH_2PO_4, 8 mM/L Na_2HPO_4, pH 7.4). Afterwards, the antennae were rinsed three times for 10 min in phosphate buffered saline (PBS, 145 mM/L NaCl, 1.4 mM/L KH_2PO_4, 8 mM/L Na_2HPO_4, pH 7.4) and transferred into 10% sucrose solution (in PBS) for 1 h at room temperature followed by 25% sucrose solution (in PBS) over night at 8 °C. The antennae were then directly embedded into O.C.T (optimal cutting temperature compound) Tissue Tek freezing medium (Sakura Finetek Europe, Alphen aan den Rijn, The Netherlands) and frozen at − 20 °C. Cryosections (12 μm) of the antennae were thaw-mounted onto SuperFrost Plus slides (Thermo Fisher Scientific, Waltham, MA, USA) and air-dried at room temperature for at least 30 min. The sections on the microscope slides were encircled using a liquid repellent slide marker pen (Liquid Blocker, Plano, Wetzlar, Germany) and treated with PBS, 0.01% Tween20 for 5 min. The solution was replaced with 50 mM NH_4Cl in PBS for 5 min followed by a wash in PBS for 5 min. The sections were then covered with blocking solution (PBS with 10% normal goat serum, 0.5% Triton-X100), for 30 min at room temperature. Subsequently, the sections were treated with the primary antibodies diluted in blocking solution (1:100–1:200) over night at 8 °C in a humid box. After washing the slides three times for 5 min with PBS, the sections were treated with goat-anti-rabbit AF488-conjugated secondary antibodies (1:1000) (Jackson ImmunoResearch, Ely, Great Britain), goat-anti-HRP Cy3 (1:400) (Jackson ImmunoResearch), and DAPI (1:1000, Thermo Fisher Scientific) diluted in PBS, for 1 h at room temperature in a humid box. Finally, the slides were washed three times for 5 min with PBS and mounted in mowiol solution.

In control experiments, α-SNMP1-Ab and α-SNMP2-Ab were incubated for 1 h at room temperature with a 500-fold excess of the peptide used to for immunization prior to application on antennal sections.

Unspecific binding of the secondary antibodies to antennal sections was tested by omitting the primary antibodies during the FIHC procedure.

Analysis of antennal sections by confocal microscopy

Sections from FIHC experiments were analyzed on a confocal laser scanning microscope (LSM 880, Carl Zeiss Microscopy, Jena, Germany). Confocal image stacks of the fluorescence and transmitted-light channels were taken and used to obtain projections of optical planes applying the ZEN software (Carl Zeiss Microscopy). Pictures were not altered except for adjusting the brightness or contrast for a uniform tone within a single figure.

Results

In order to investigate the localization of the two SNMP types in the antenna of male and female *Heliothis virescens* on the protein level, we performed FIHC approaches on cryosections with newly generated α-SNMP1-Ab and α-SNMP2-Ab. To allow for simultaneous visualization of antennal neurons (mainly OSNs) and their extensions, a fluorescent anti-HRP antibody (hereafter named α-HRP-Ab) was utilized recognizing neuron specific glycoproteins in insects (Sun and Salvaterra 1995). In addition, DAPI staining of nuclei was performed for a better identification of cells within the tissue.

Immunolocalization of SNMP1 in male and female antenna

First, α-SNMP1-antibody (Ab) and α-HRP-Ab were used on tissue sections of the male antenna, and the immunoreactivity was visualized by confocal laser scanning microscopy. In accordance with neuronal labeling, α-HRP-immunoreactivity was detected in the somata and extensions of neurons projecting their dendrites into long sensilla trichodea. (Fig. 1a, a′, c, c′). In contrast, the α-SNMP1-Ab labeled the cell bodies of only few cells (shown in green) under these trichoid sensilla (Fig. 1a, a′, b, b′). The SNMP1-positive cells

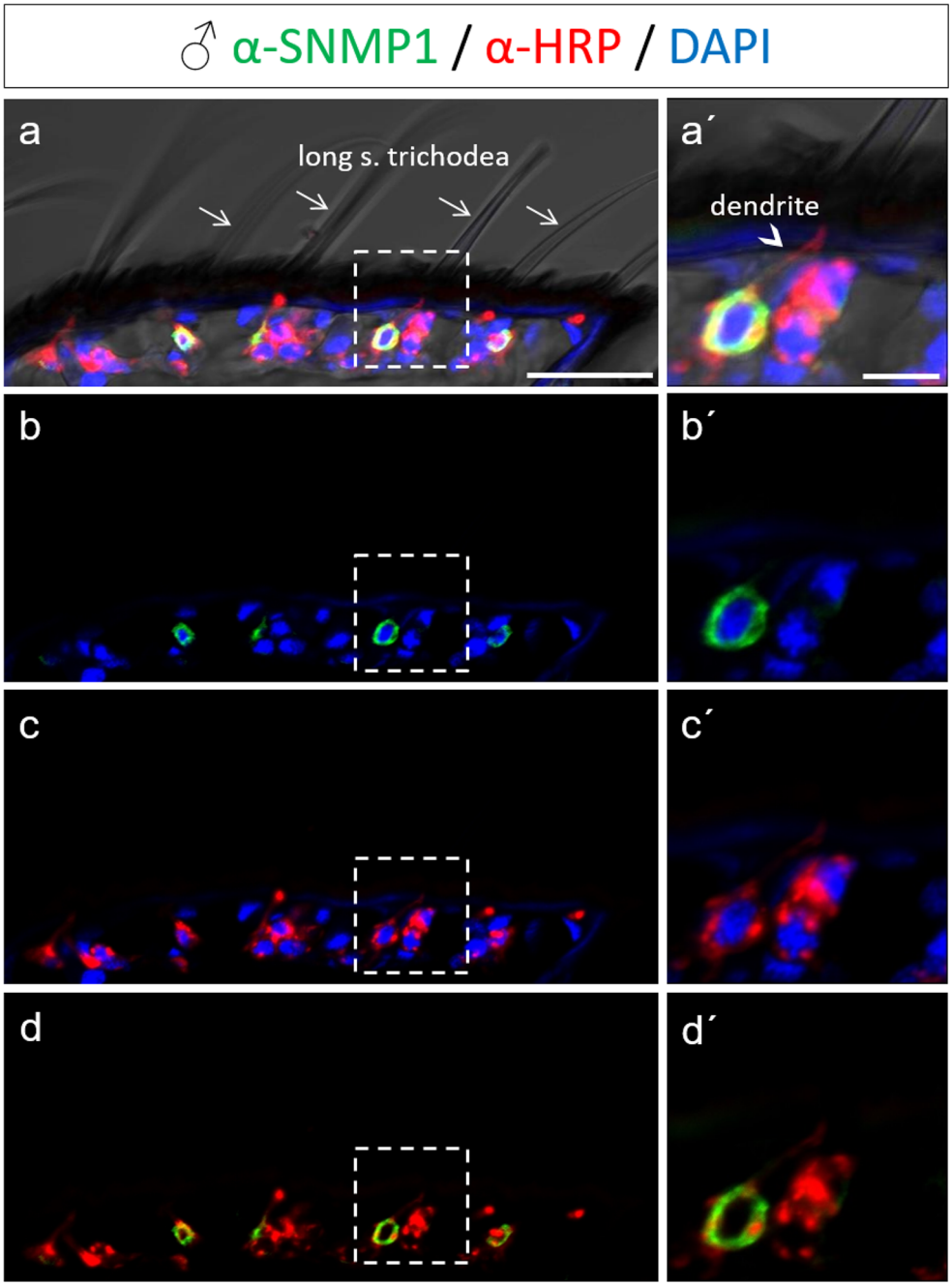

Fig. 1 SNMP1 expression in a subset of OSNs in the antenna of a *H. virescens* male. SNMP1-positive cells were visualized by FIHC in the longitudinal section using α-SNMP1-Ab (green). Neurons were identified by α-HRP-Ab (red) and nuclei were stained with DAPI (blue). Single cells within clusters of OSNs projecting into trichoid sensilla are labeled by α-SNMP1-Ab. The area boxed in **a**–**d** is shown at higher magnification in **a′**–**d′**. **a**, **a´** Overlay of the transmitted-light channel with all three fluorescence channels. **b**, **b′** Green and blue channels. **c**, **c′** Red and blue channels. **d**, **d′** Green and red channels. Long sensilla trichodea (long s. trichodea) housing SNMP1-positive cells are indicated by arrows. Images are maximum intensity projections of confocal image stacks. Scale bars, a = 20 μm; a′ = 5 μm

were co-labeled by the α-HRP-Ab (Fig. 1d, d′), validating their identity as OSNs. Only one of the 2–3 OSNs, that project into a given trichoid sensillum, was positive for SNMP1 (Fig. 1a′–d′). In several independent experiments, the confocal LSM analyses (Fig. 2) revealed partly discontinuous labeling of filamentous structures inside the sensillum by α-HRP-Ab (representing the dendrites of OSNs, Fig. 2d) and α-SNMP1-Ab (Fig. 2b–c). In contrast, SNMP1-like immunoreactivity was neither detected in sensilla basiconica (Fig. 2e–g) nor in sensilla chaetica (data not shown). In summary, these results demonstrate that in the male antenna, the SNMP1 protein is localized on dendrites of OSNs extending into trichoid sensilla.

FIHC experiments with sections of the female antenna using the α-SNMP1-Ab led to similar results i.e., labeling of distinct neurons housed in a subpopulation of trichoid sensilla (Fig. 3a–d). However, in contrast to our findings in the male antenna, all neurons (2 or 3) of a female "SNMP1-positive" sensillum trichodeum were labeled (Fig. 3a′–d′).

The specificity of the α-SNMP1-Ab to SNMP1 was underlined by FIHC experiments including the SNMP1-specific peptide that was used for the rabbit immunization

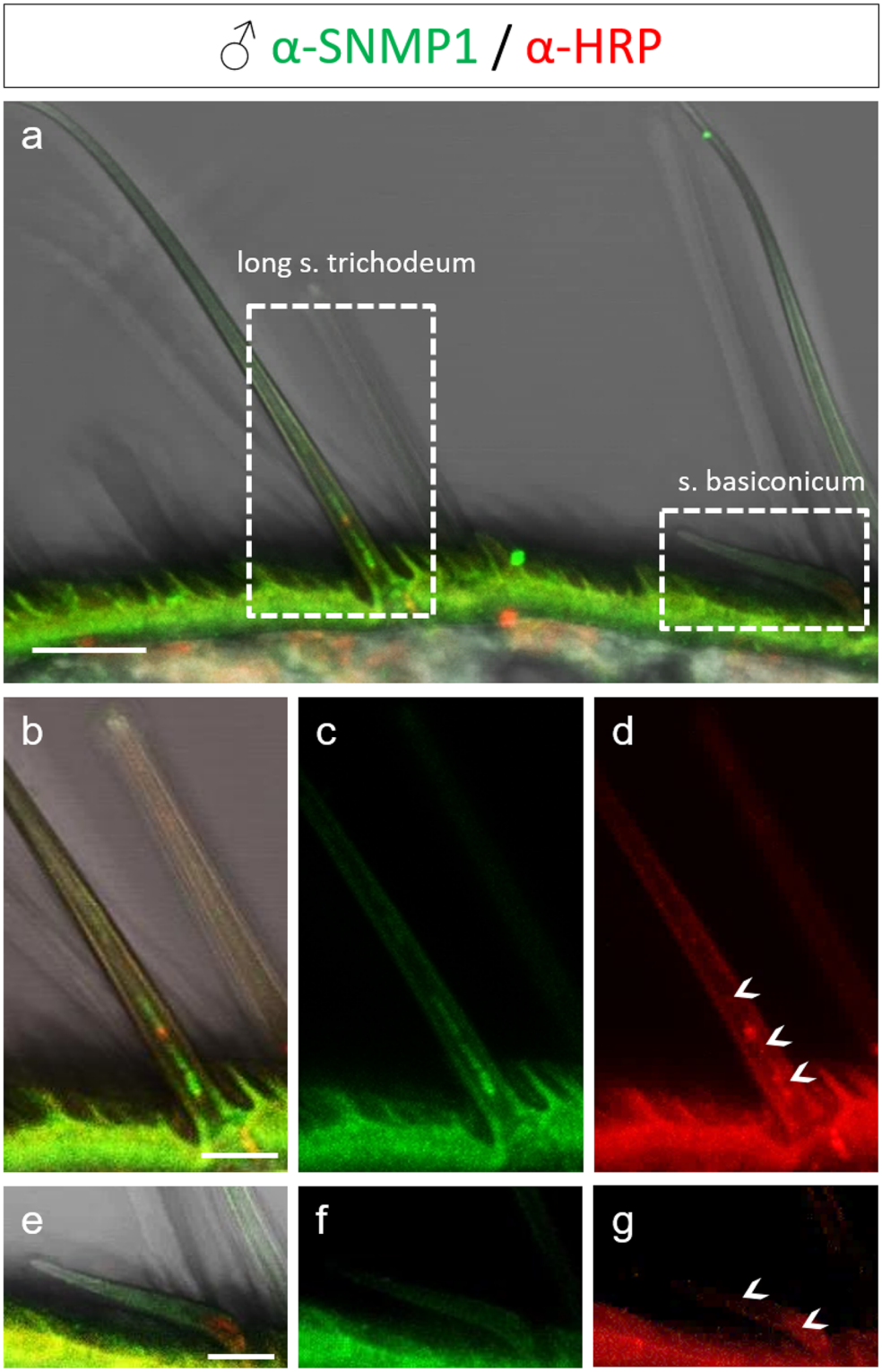

Fig. 2 Subcellular localization of SNMP1 in OSN dendrites of long sensilla trichodea. **a** FIHC using α-SNMP1-Ab (green) and α-HRP-Ab (red) on longitudinal section of the male *H. virescens* antenna. **b–d** α-HRP-Ab visualized filamentous structures inside long sensilla trichodea representing dendrites of OSNs that were also labeled by α-SNMP1-Ab. **e–f** Weak dendritic labeling by α-HRP-Ab but no labeling by α-SNMP1-Ab was detected in sensilla basiconica. A part of a long trichoid sensillum (long s. trichodeum) and a clearly distinguishable sensillum basiconicum (s. basiconicum) are boxed in **a** and shown at a higher magnification in **b–d** and **e–g**, respectively. Arrow heads in **d** and **g** denote dendritic labeling by α-HRP-Ab. Pictures show projections of confocal LSM image stacks. **a**, **b**, **e** Overlay of the transmitted-light and red and green fluorescence channels. **c**, **f** Green channel. **d**, **g** Red channel. Scale bars, a = 10 μm; b and e = 5 μm

(Fig. S1a - c). In these blocking experiments, staining of cells and their processes by the α-SNMP1-Ab was abolished (Fig. S1a) whereas the neuronal staining by the α-HRP-Ab was unaffected (Fig. S1b). Further, control experiments omitting the primary α-SNMP1-Ab but applying the fluorescent α-HRP-Ab as well as these secondary anti-rabbit Alexa 488 antibody demonstrated no tissue labeling by the secondary antibody (Fig. S2).

Together, these results indicate that the newly generated α-SNMP1-Ab specifically recognize the SNMP1 protein in

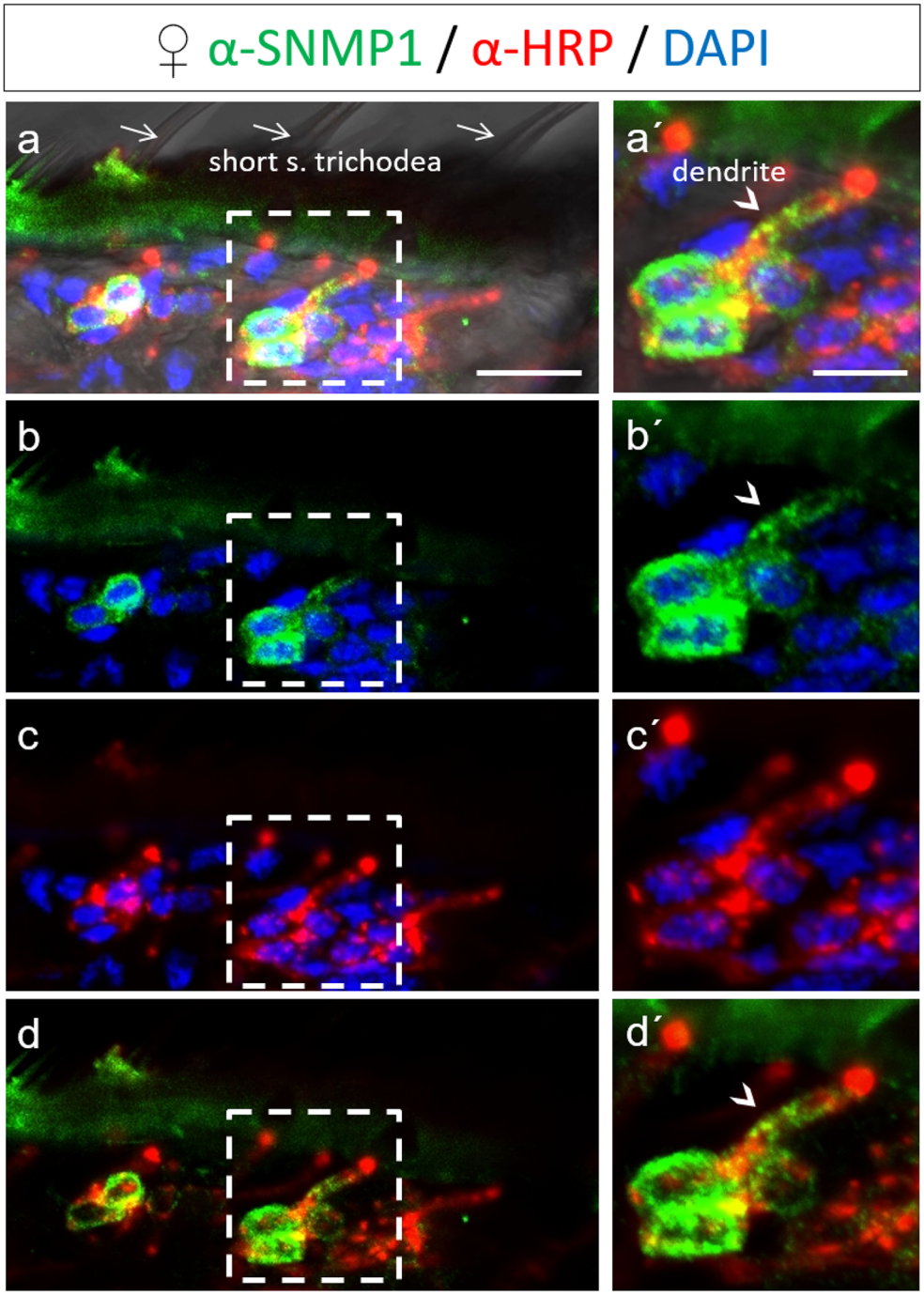

Fig. 3 Expression of SNMP1 in clusters of OSNs housed in trichoid sensilla of the female antenna. SNMP1-positive cells were visualized by FIHC in the longitudinal antennal section using α-SNMP1-Ab (green). Neurons were identified by α-HRP-Ab (red) and nuclei were stained with DAPI (blue). Clusters of two to three cells that were co-labeled by α-SNMP1-Ab and anti-HRP antibodies are visible. The area boxed in **a**–**d** is shown in **a′**–**d′** at higher magnification. Arrow heads denote α-SNMP1-Ab labeling in dendritic extensions. Pictures are projections of confocal LSM image stacks. **a, a′** Overlay of the transmitted-light channel with all fluorescence channels. **b**, **b′** Green and blue channels. **c**, **c′** Red and blue channels. **d**, **d′** Green and red channels. short s. trichodea (arrows), short sensilla trichodea. Scale bars, a = 10 μm; a′ = 5 μm

somata and dendrites of distinct populations of OSNs in the antenna. Moreover, the findings demonstrate a different expression pattern between males and females with regard to the number of SNMP1-positive OSNs per trichoid sensillum.

Immunolocalization of SNMP2 in male and female antenna

In order to assess the SNMP2 protein distribution, we next analyzed antennal sections of *H. virescens* with α-SNMP2-antibodies. In FIHC experiments with α-SNMP2-Ab and α-HRP-Ab, a complementary labeling pattern was found indicating the detection of different cell types by the two antibodies. As shown exemplarily on longitudinal sections of the male antenna (Fig. 4), SNMP2-like immunoreactivity was detected in a high number of large and elongated cells (green) that extend from deeper positions in the antennal tissue towards positions below the cuticle surface where sensilla are located (Fig. 4a, a′, b, b′). In contrast, α-HRP labeling was seen in neurons (red) characterized by rather compact somata

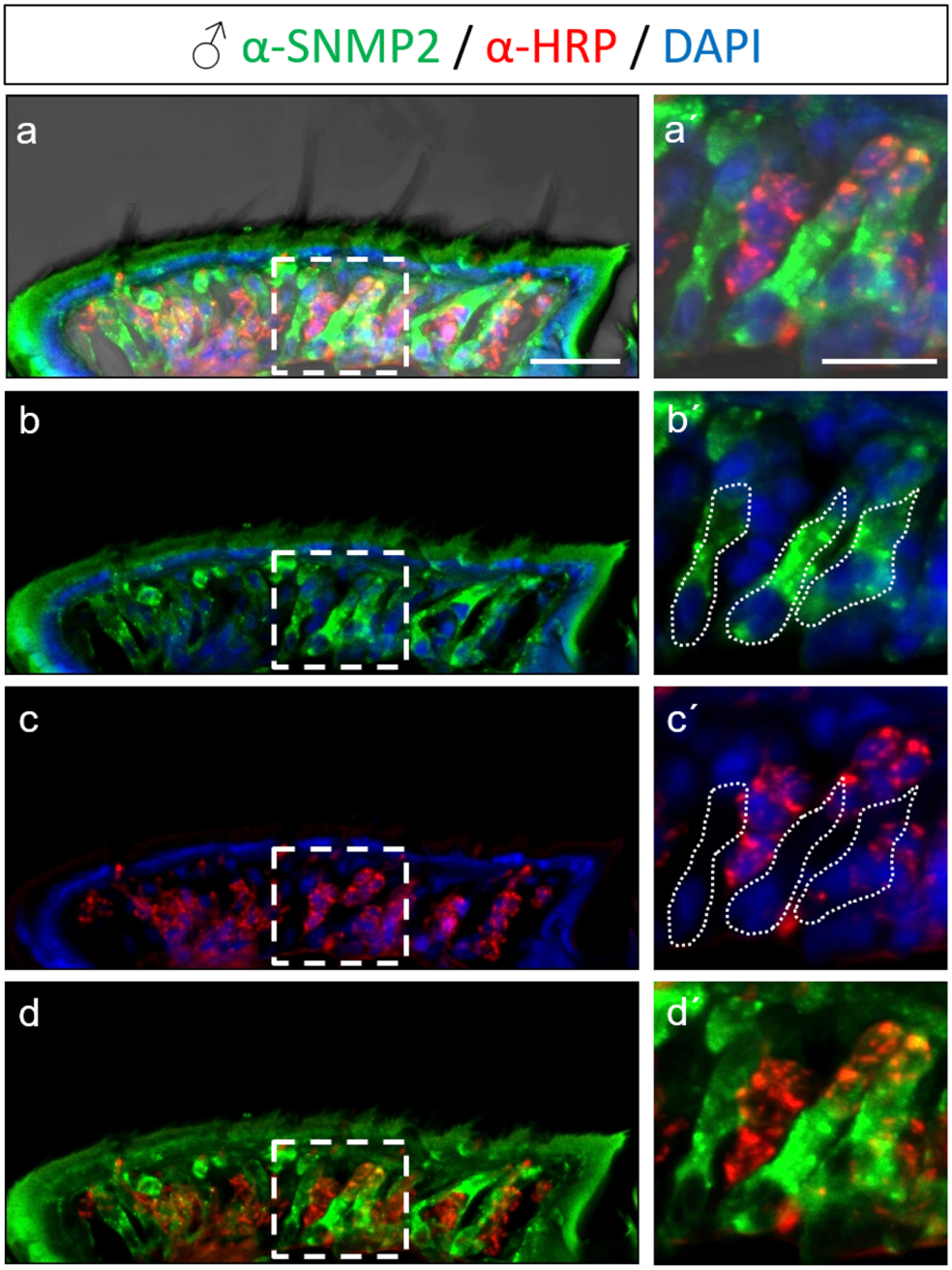

Fig. 4 Expression of HvirSNMP2 in non-neuronal support cells. FIHC on a longitudinal section of a male antenna. SNMP2-positive cells labeled by α-SNMP2-Ab (green) are closely associated with clusters of OSNs stained by α-HRP-Ab (red). DAPI staining was used to identify nuclei (blue). Magnified images of the boxed area in **a**–**d** are shown in **a′**–**d′**. The dotted lines in **b** and **c** outlines the position of individual support cells. Images show projections of confocal LSM image stacks. **a**, **a′** Overlay of the transmitted-light channel with all fluorescence channels. **b**, **b′** Green and blue channels. **c**, **c′** Red and blue channels. **d**, **d′** Green and red channels. Scale bars, a = 20 μm; a´ = 10 μm

and extensions projecting into sensilla (Fig. 4c, c′). The co-labeling experiments revealed (Fig. 4d, d′) no overlap of the α-HRP-Ab and α-SNMP2-Ab immunoreactivity, indicating the expression of SNMP2 in non-neuronal cells. Using DAPI staining (blue), SNMP2-positive cells often displayed strikingly large ellipsoidal nuclei (Fig. 4b, c). Moreover, clusters of α-HRP-Ab-labeled OSNs were regularly found closely associated with cells positive for α-SNMP2-Ab (Fig. 4a–d). Such an arrangement is typical for OSNs and support cells in olfactory sensilla (Steinbrecht and Gnatzy 1984).

Similar results were obtained in corresponding FIHC experiments with female antennal sections (Fig. S4). In both sexes, the α-SNMP2-Ab labeled numerous cells in each antennal segment. The abundancy of the non-neuronal SNMP2-positive cells in the antenna became particularly evident when the labeling by α-SNMP2-Ab and α-HRP-Ab was evaluated on horizontal sections at a plane just below the antennal surface (Fig. 5). Numerous SNMP2-positive cells are distributed over the whole segment (Fig. 5a, b). In addition, α-HRP-Ab labeling is closely associated but does not overlap with the α-SNMP2-Ab (Fig. 5c, c′).

The specificity of the α-SNMP2-Ab to SNMP2 was underlined by blocking experiments. Employing the SNMP2-specific peptide (Fig. S3) in FIHC approaches abolished the

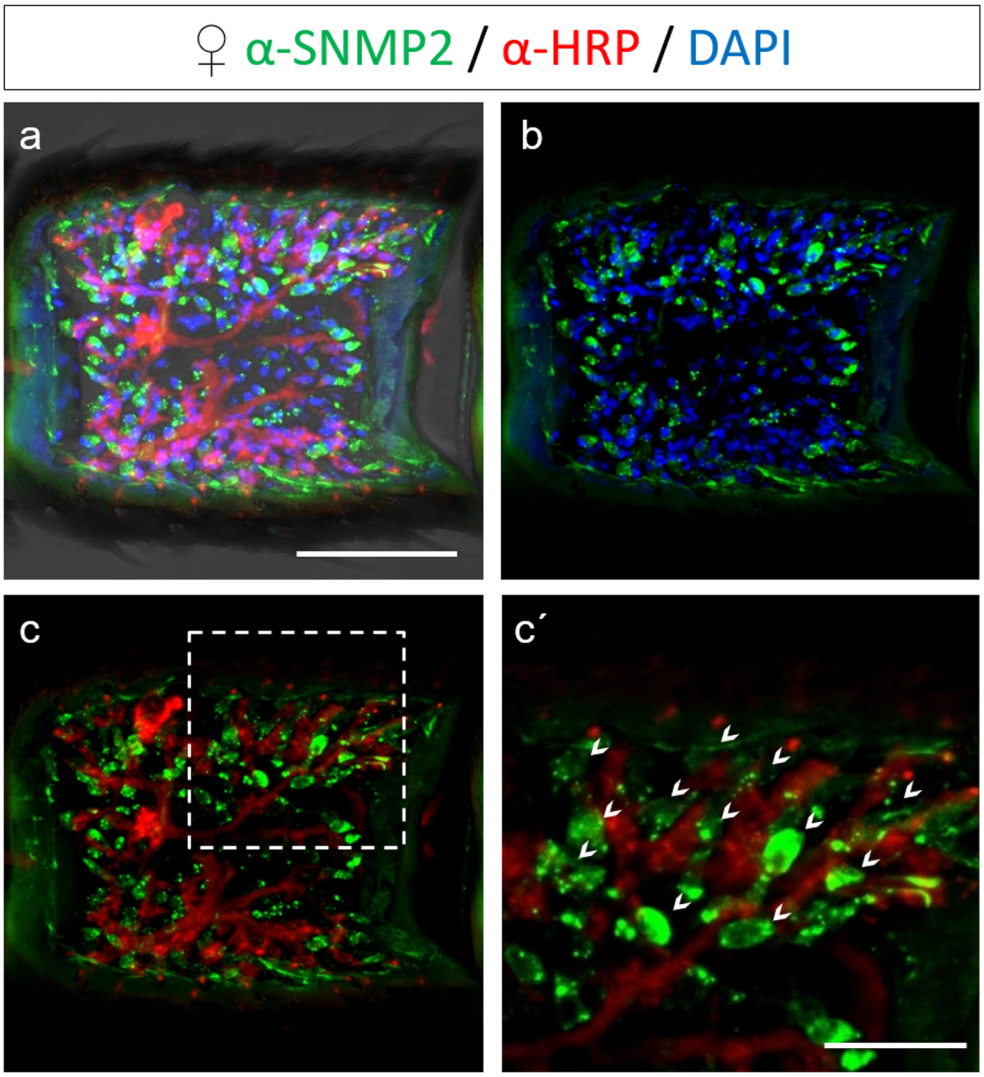

Fig. 5 FIHC on a horizontal antennal section of the antenna of a *H. virescens* female displaying the expression of HvirSNMP2 in numerous non-neuronal support cells. A complementary staining of cells by α-SNMP2-Ab (green) and α-HRP-Ab (red) is obvious. Images show projections of confocal LSM image stacks. DAPI staining of nuclei is shown in blue. The area boxed in **c** is presented in **c´** at higher magnification. **a** Transmitted-light channel overlaid with the three fluorescence channels. **b** Green and blue channels. **c**, **c′** Green and red channels. Arrow heads in **c´** denote numerous α-SNMP2-Ab-positive cells. Scale bars, a = 50 μm; c´ = 20 μm

labeling of cells by the α-SNMP2-Ab (Fig. S3 a) whereas the staining by the α-HRP-Ab was unaffected (Fig. S3 b).

The high abundance of cells displaying SNMP2-like immunoreactivity on sections of the male and female antenna of *H. virescens* indicates that in both sexes, the SNMP2 protein is expressed by support cells of numerous if not all olfactory sensilla.

Localization of SNMP2 in olfactory sensilla

In order to assess the subcellular localization of the SNMP2 protein in more detail, tissue sections co-labeled with α-SNMP2-Ab and α-HRP-Ab were analyzed focusing on specific optical planes of the area below a trichoid sensillum (Fig. 6a–d). Thereby, distinct SNMP2-positive support cells could be examined (Fig. 6b, d). Within support cells, a strong, partly dotted α-SNMP2-Ab labeling was revealed (Fig. 6d). The SNMP2-like immunoreactivity extended towards the apical region of the cell and expands to the base of the sensillum indicating that the SNMP2-expressing cell directly adjoins the inner lumen of the sensillum shaft. Interestingly, confocal LSM analyses revealed a dotted and scattered α-SNMP2-Ab labeling also within the sensillum lumen (Fig. 6d, arrows).

This observation prompted further analysis of the distribution of the α-SNMP2-Ab immunoreactivity along the sensillum shaft and its localization relative to the dendritic structures within the sensillum. As shown in Fig. 7(a–c), for a female sensillum, the dotted labeling through the α-SNMP2-Ab is dispersed over the whole length of the sensillum (Fig. 7a, b, green). In contrast, the α-HRP-Ab labeled filamentous structures representing the dendrites of OSNs extending from the base to the tip of the sensillum (Fig. 7a, c, red). The overlay of the red and green fluorescent channels illustrates that there is again no overlap of the α-SNMP2-Ab and α-HRP-Ab labeling.

To assess whether the SNMP2-like immunoreactivity within sensilla is specific to a distinct sensillum type, morphologically distinguishable trichoid and basiconic sensilla on male antenna were examined (Fig. 7d–g). Figure 7d shows a male antennal

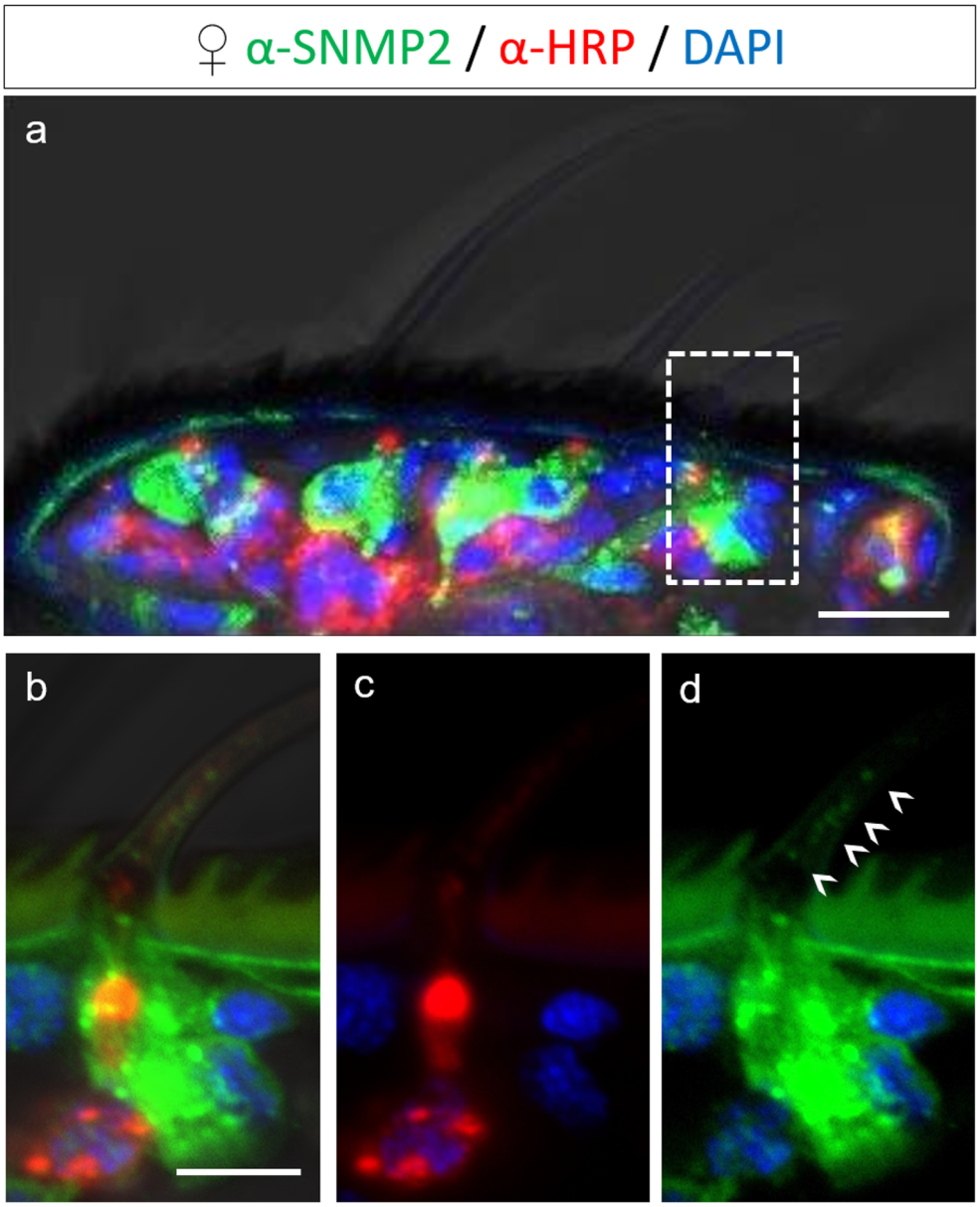

Fig. 6 Localization of HvirSNMP2-like immunoreactivity inside a trichoid sensillum of a female antenna of *H. virescens* sectioned longitudinally. Labeling by α-SNMP2-Ab is shown in green, while neuronal labeling by α-HRP-Ab is displayed in red; nuclei stained by DAPI appear in blue. The boxed area in **a** is shown at higher magnification in **b**–**d**. Strong green labeling of a single SNMP2-positive cell by α-SNMP2-Ab is apparent. The cellular labeling extends to the apical region of the SNMP2-positive cell and SNMP2-like immunoreactivity is also visible within the sensillum shaft (denoted by arrow heads in **d**). Pictures represent projections of confocal LSM image stacks. The transmitted-light channel was merged with the fluorescence channels in **a** and **b**, whereas **c** depicts only the red and blue channels and **d** shows only the red and green channels. Scale bars, a = 10 μm; b = 5 μm

section treated with α-HRP-Ab and α-SNMP2-Ab where these two olfactory sensillum types are shown side by side. The magnified images of the two sensilla types depict the filamentous labeling of the dendrites within the sensilla (red), which is clearly recognizable in the sensillum basiconicum but only partially apparent for the long sensilla trichodea (Fig. 7d, f). Similarly, a dispersed and dotted α-SNMP2-Ab immunoreactivity (green) can be readily identified in the sensillum basiconicum, whereas much less labeling was generally detected within the long sensilla trichodea (Fig. 7e, g). In control experiments employing the SNMP2-peptide (Fig. S1) or omitting the primary α-SNMP2-Ab (Fig. S2), we did not detect any dotted labeling within olfactory sensilla of male antenna. Moreover, no such labeling was found in FIHC experiments with α-SNMP1-Ab (Fig. 2), which may be valid as further control.

In summary, we found a dotted labeling by the α-SNMP2-Ab within sensilla trichodea (shown for female and males) as well as in the sensilla basiconica (shown here only for males but also found in females) of *Heliothis virescens* with no obvious differences between males and females. These results suggest that SNMP2 is present within the sensillum lymph of the two olfactory sensilla types on the antenna of both sexes.

Discussion

In the present study, we explored the protein-localization of SNMP1 and SNMP2 in the antenna of the noctuid moth *H. virescens* using FIHC with newly generated α-SNMP-peptide antibodies.

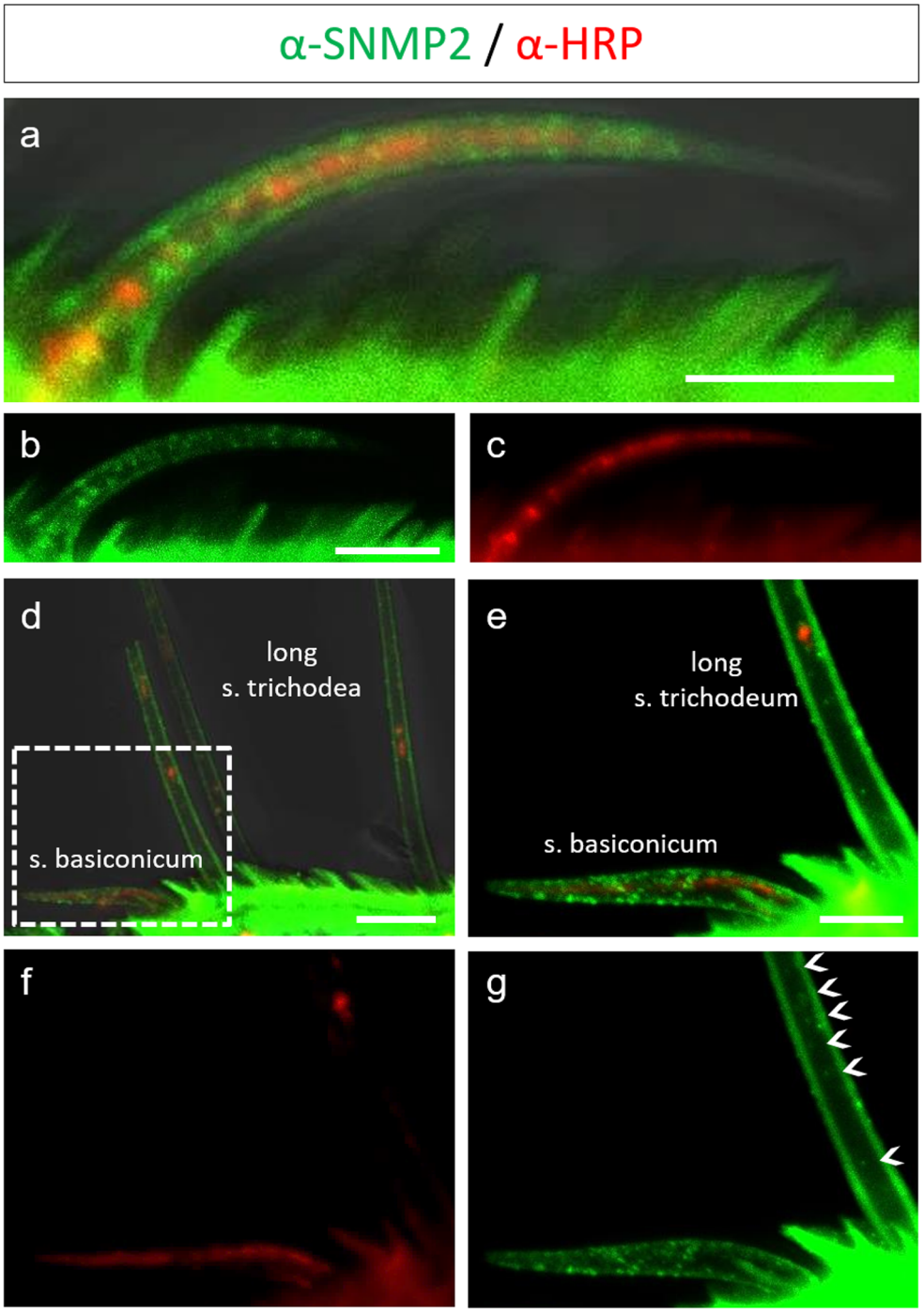

Fig. 7 Localization of HvirSNMP2-like immunoreactivity inside sensilla on the antenna of *H. virescens*. FIHC were conducted on longitudinal sections employing α-SNMP2-Ab (green) and α-HRP-Ab (red) visualizing neuronal structures. **a–c** Sensillum of a female antenna. A dispersed and dotted SNMP2-like immunoreactivity (**a**, **b**) as well as a thread-like dendritic labeling by α-HRP-Ab (**a**, **c**) is found inside the sensillum shaft. **d–g** SNMP2-like immunoreactivity and α-HRP-Ab dendritic labeling inside sensilla of the male antenna. The area boxed in **d** is shown at higher magnification in **e–g**. A dispersed and dotted α-SNMP2-Ab immunoreactivity is clearly recognizable in the sensillum basiconicum but only partially apparent for the long sensilla trichodea (denoted by arrow heads in g). Similarly, a dendritic labeling can be readily identified in the sensillum basiconicum, whereas the labeling within the long sensilla trichodea is only weak. **a**, **d** Transmitted-light channel merged with the green and red fluorescence channels. **e** Merged red and green channel. **c**, **f** Red channel. **b**, **g** Green channel. The pictures represent projections of confocal LSM image stacks. Scale bars, a, b and e = 5 μm; d = 10 μm

Our FIHC experiments with α-SNMP1-Ab visualized the SNMP1 protein in subsets of OSNs projecting into a subpopulation of the trichoid sensilla on the antenna. This finding is in agreement with recent results from fluorescent in situ hybridization (FISH) approaches with *H. virescens* antenna demonstrating SNMP1 transcripts only in a subpopulation of neurons co-expressing Orco, a marker for OSNs (Zielonka et al. 2018).

In male moths, we found only one SNMP1-positive OSN per sensillum whereas in females, all 2–3 OSNs that generally innervate a given trichoid sensillum express SNMP1 (Figs. 1 and 2). The finding of a single SNMP1-expressing OSN per male trichoid sensillum matches with the results of electrophysiological recordings from *H. virescens* antennal sensilla showing that in pheromone-responsive sensilla of males, only one of the 2–3 OSNs responds to a female sex pheromone component (Almaas and Mustaparta 1991; Baker et al. 2004). Furthermore, FISH experiments on the male antenna have demonstrated that HR13 and HR6, the receptors for the major [(Z)-11-hexadecenal] and a minor [(Z)-9-tetradecenal] female sex pheromone components of *H. virescens,* are each

co-expressed with SNMP1 in single OSNs of different trichoid sensilla (Krieger et al. 2002; Forstner et al. 2008). Overall, our data further substantiate a significant role of SNMP1 in pheromone-responsive OSNs of male *H. virescens*.

The notion that also in females SNMP1 may be involved in pheromone detection is supported by FISH-experiments demonstrating that female antennae possess OSNs that co-express SNMP1 and HR6 (Zielonka et al. 2018). Whether all of the 2–3 SNMP1-positive OSNs of a given trichoid sensillum on the female antenna respond to pheromones is presently unclear. Electrophysiological recordings from the female trichoid sensilla revealed distinct OSNs responding to male-released hair-pencil components or to (Z)-9-tetradecenal. However, these OSNs are not colocalized within the same trichoid sensillum (Hillier et al. 2006).

While we have detected SNMP1 expression in *H. virescens* only in trichoid sensilla, using the α-ApolSNMP1-antibodies on antennal sections of *Antheraea polyphemus* also led to SNMP1 detection in dendrites of OSNs in so-called intermediate sensilla and some sensilla basiconica (Rogers et al. 2001b). Similarly, in situ hybridization experiments with antennae of the beet armyworm moth *Spodoptera exigua* and the desert locust *Schistocerca gregaria* revealed SNMP1 transcripts in OSNs of trichoid and basiconic sensilla (Liu et al. 2014; Jiang et al. 2016). In view of the concept that SNMP1 expression is characteristic for pheromone-sensitive OSNs, these data suggest that within insect species pheromone detecting OSNs may be housed in morphologically different sensilla types.

In our FIHC experiments, α-SNMP2-Ab labeled non-neuronal cells in the antenna that were closely associated with OSNs projecting into sensilla hairs. This allows the conclusion that the support cells of olfactory sensilla express SNMP2. Moreover, the SNMP2-positive cells displayed strikingly large ellipsoidal nuclei, which are typical for trichogen and tormogen support cells in moth olfactory sensilla (Steinbrecht and Gnatzy 1984) further reinforcing the notion that SNMP2 is expressed by support cells. Similar α-SNMP2-Ab labeling patterns were found for female and male antenna indicating a broad expression of SNMP2 in support cells in many if not all olfactory sensilla. Thus, SNMP2 does not appear to be restricted to pheromone detecting sensilla, suggesting a more general function of the protein in all olfactory sensilla.

In-depth confocal microscope examinations of the α-SNMP2-Ab-labeling could assign SNMP2-like immunoreactivity in close proximity of the somata and the apical regions of support cells at the base of olfactory sensilla bordering the fluid-filled lumen of the sensillum. Apically, support cells, which control the composition of the sensillum lymph, comprise extensive microvilli structures (Sanes and Hildebrand 1976; Steinbrecht and Gnatzy 1984; Keil 1989). While the functional implications for SNMP2 in support cells are unclear, due to its phylogenetic relatedness to other members of the CD36-family, SNMP2 might play a role within the apical membrane of support cells. In vertebrates (Koonen et al. 2005; Levy et al. 2007; Nassir et al. 2007; Silverstein and Febbraio 2009; Pepino et al. 2014) and insects (Giovannucci and Stephenson 1999; Kiefer et al. 2002; Wang et al. 2007; Yang and O'Tousa 2007; Vogt et al. 2009), proteins of the CD36 family have been demonstrated to mediate the selective uptake of lipids, including fatty acids, carotenoids, and cholesterol into cells. Therefore, it is conceivable that SNMP2 may play a crucial role in the maintenance of the sensillum lymph by the transport of lipophilic compounds such as degradation products of pheromones and odorants (Leal 2003; Vogt 2003) out of the sensillum lymph into the support cells.

Interestingly, our FIHC experiments have revealed SNMP2-like immunoreactivity also within the sensillum lumen of trichoid and basiconic sensilla. Similarly, an electron microscopic study of the antenna of the black cutworm moth *Agrotis ipsilon*, using an anti-*Aips*SNMP2 antibody, found abundant labeling at the base as well as in the sensillum lymph of various sensillum types (Gu et al. 2013). Together, these results indicate the presence of SNMP2 within the sensillum lymph and would suggest a novel extracellular function of the protein. In general, we found much less SNMP2-like labeling in s. trichodea compared to s. basiconica. However, it needs to be determined whether this observation may reflect a functionally relevant difference in the abundance of SNMP2 in the two sensilla types or may be due to technical reasons (e.g., differences in the preservation of sensillum lymph SNMP2 of trichoid and basiconic sensilla during the FIHC-experiments).

Given the two transmembrane domain topology for SNMP2s, it remains to be determined how SNMP2s may be solubilized in the aqueous sensillum lymph. Noteworthy, the structurally related CD36 proteins in human blood plasma appear to be associated with membranous microparticles (Alkhatatbeh et al. 2011). Moreover, a soluble human CD36 protein form, termed sCD36, which is due to enzymatic cleavage of the transmembrane domains, has been reported (Handberg et al. 2006; Jimenez-Dalmaroni et al. 2009; Silverstein and Febbraio 2009). With respect to the latter and the finding that the large ectodomain of SNMPs appears to bind various lipophilic ligands (Gomez-Diaz et al. 2016), it is a tempting model that a soluble form of SNMP2 in the sensillum lymph may serve as scavenger protein capturing lipophilic waste products (such as degradation products of pheromones and odorants) and mediating their transfer to support cells at the base of the sensillum for further elimination, probably under participation of a membrane bound form of SNMP2. Thus, such a soluble form of SNMP2 may be of importance as an intermediate buffer to assure the functionality of the sensillum.

In conclusion, our data demonstrate a hitherto little noticed but remarkably broad expression of SNMP2 in support cells of possibly all trichoid and basiconic sensilla on the antenna of both sexes. This characteristic of SNMP2 together with its sensillum localization suggests a more general role of SNMP2 in processes that are required to maintain the functionality of the olfactory unit. In contrast, the results for SNMP1 indicate for both sexes expression of the protein in a distinct subset of OSNs projecting their sensory dendrites in trichoid sensilla. This topography of SNMP1 expression in accordance with the antennal distribution of OSNs expressing PRs and responding to pheromones further emphasizes a significant role of SNMP1 in the pheromone detection system of male and female *Heliothis virescens*.

Acknowledgments The authors would like to thank the insect rearing unit at Bayer CropScience AG Frankfurt for providing *Heliothis virescens* pupae. Doreen Sander is acknowledged for excellent technical assistance.

Funding This work was supported by the Deutsche Forschungsgemeinschaft, SPP1392 by a grant to J.K. (KR1786/4-2).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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