Masters of communication: The brain of the banded cleaner shrimp *Stenopus hispidus* (Olivier, 1811) with an emphasis on sensory processing areas

Jakob Krieger | Marie K. Hörnig | Renate E. Sandeman | David C. Sandeman | Steffen Harzsch

University of Greifswald, Zoological Institute and Museum, Cytology and Evolutionary Biology, Greifswald, Germany

**Abstract**

The pan-tropic cleaner shrimp *Stenopus hispidus* (Crustacea, Stenopodidea) is famous for its specific cleaning behavior in association with client fish and an exclusively monogamous life-style. Cleaner shrimps feature a broad communicative repertoire, which is considered to depend on superb motor skills and the underlying mechanosensory circuits in combination with sensory organs. Their most prominent head appendages are the two pairs of very long biramous antennules and antennae, which are used both for attracting client fish and for intraspecific communication. Here, we studied the brain anatomy of several specimens of *S. hispidus* using histological sections, immunohistochemical labeling as well as X-ray microtomography in combination with 3D reconstructions. Furthermore, we investigated the morphology of antennules and antennae using fluorescence and scanning electron microscopy. Our analyses show that in addition to the complex organization of the multimodal processing centers, especially chemomechanosensory neuropils associated with the antennule and antenna are markedly pronounced when compared to the other neuropils of the central brain. We suggest that in their brains, three topographic maps are present corresponding to the sensory appendages. The brain areas which provide the neuronal substrate for these maps share distinct structural similarities to a unique extent in decapods, such as size and characteristic striated and perpendicular layering. We discuss our findings with respect to the sensory landscape within animal’s habitat.

**Abbreviations:** A1, antennule; A2, antenna; A\text{NV}, antennular (antenna I-) nerve; A\text{Nh}Nv, antennal (antenna II-) nerve; AMPN, anterior medial protocerebral neuropil; AnN, antennal (antenna II-) neuropil; as, aesthetasc; ASTir, allatostatin-like immunoreactivity; CB, central body neuropil; (#), number of cell (somata) cluster; CN, columnar neuropil; DC, deutocerebrum; Fl, (antennal) flagellum; HN, hemielipsoid body neuropil; HN A, B, C, sublobes of the HN; cs, cuspidate seta; iCh, inner visual chiasm; La, lamina; Lo, lobula; LoP, lobula plate neuropil; LAN, lateral antennular (antenna I-) neuropil; L\text{AN} & m\text{AN}, lateral and medial lobe of the LAN; L\text{AN}Nv & m\text{AN}Nv, lateral & medial LAN-nerves; If, lateral foramen; IFI, lateral (antennular) flagellum; LL, lateral lobe; IPC, lateral protocerebrum (HN + TM); MAN, median antennular (antenna I-) neuropil; M\text{AN}Nv, MAN-nerve; Me, medulla; mf, medial foramen; mFl, median (antennule) flagellum; mPC, median protocerebrum; NUC, nuclear counterstaining (using HOECHST); OL, olfactory lobe; O\text{LN}v, OL-nerve; oCh, outer visual chiasma; OC, (o)esophageal connective; og, ofactory glomerulus; PB, protocerebral bridge neuropil; PC, protocerebrum; PMPN, posterior medial protocerebral neuropil; OG, olfactory globular tract; O\text{GTN}, olfactory globular tract neuropil; PT, protocerebral tract; R, retinula cell(s); Sc, (antennal) scaphocerite; ss, simple setae; S\text{YN}ir, synapsin-like immunoreactivity; TC, tritocerebrum; TM, terminal medulla (medulla terminalis); TN, tegumentary neuropil; T\text{NV}, tegumentary nerve; VN, visual neuropils (La + Me + Lo + LoP); V\text{NV}, visual nerve; VT, visual tract.

1According to Sandeman et al. (1992).

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2019 The Authors. *The Journal of Comparative Neurology* published by Wiley Periodicals, Inc.
1 | INTRODUCTION

The banded cleaner shrimp *Stenopus hispidus* (Olivier, 1811) is one of the most popular and thus intensely traded species of marine ornamental shrimps with a pan-tropic distribution (Dudoit et al., 2018; Healy & Yaldwyn, 1970). Its other common names include coral banded shrimp, white-banded shrimp, red-banded shrimp, boxer shrimp, bandana shrimp, or barber pole shrimp. This iconic species is famous for its cleaning behavior (Becker, Curtis, & Grutter, 2005; Jonasson, 1987) of different species of coral reef fish, but scientific reports with behavioral observations are scarce (Spotte, 1998) and video recordings are only available from private aquarists and fish-keepers (see e.g., youtube). The banded cleaner shrimp is highly territorial and features monogamous pair formation (Johnson, 1969). The phylogenetic position of Stenopodidea within Malacostraca is uncertain with regard to the Decapoda, because stenopodid shrimps share characters of euphausiids such as the sperm morphology but also characters (referring the internal and external morphology, larval development and behavior) indicating a closer relationship to reptants (reviewed in Goy, 2010). Two recent phylogenetic analyses (Schwentner et al., 2018; Wolfe et al., 2019) concordantly consider Stenopodidea to be the sister-group to all other Caridea (see phylogram in Figure 1a). The first neuroanatomical description of its central brain was provided by Sandeman and coworkers (Sandeman, Scholtz, & Sandeman, 1993) and information on the visual neuropils and the neuropils of the lateral protocerebrum (IPC) (those parts of the brain situated within the eyestalk) was added by Sullivan and Beltz (2004) (reviewed in Sandeman, Kenning, & Harzsch, 2014) and recently by Wolff, Thoen, Marshall, Sayre, and Strausfeld (2017). Here, we provide a more detailed description of its brain and primary sensory organs associated with the deuto- and tritocerebrum—the antennules and antennae—in *S. hispidus*. In this study, a broad spectrum of state-of-the-art neuroanatomical methods was used including scanning electron microscopy (SEM), X-ray micro-computed tomography (μCT), histological preparations using conventional staining methods, and immunohistochemical labeling combined with imaging techniques such as brightfield and fluorescence microscopy as well as confocal laser scanning microscopy (clsm) and 3D reconstructions.

2 | MATERIAL AND METHODS

Animals were obtained via the KN-Aquaristik Versandhandel GmbH (Pinneberg, Germany) and Mrutzek Meeres-Aquaristik GmbH (Ritterhude, Germany) and kept in seawater aquaria under constant conditions (temperature of 28 °C and a salinity of 30 PSU). Before processing, animals immersed in seawater were chilled in the freezer (−20 °C) for a few minutes and subsequently decapitated. Eight animals were processed (for μCT, n = 3 [1x ♀; 1x ♂; 1x unsexed]; for immunohistochemistry, n = 4 [2x ♀; 2x ♂]; for silver impregnated paraffin section, n = 1 [sex unknown]) and their antennal appendages were used for paraffin histology as well as for fluorescence microscopy followed by scanning electron microscopy (3x ♀; 3x ♂).

2.1 | Nomenclature

The neuroanatomical nomenclature used in this manuscript is based on Sandeman, Sandeman, Derby, and Schmidt (1992) and Richter et al. (2010) with modifications adopted from Harzsch and Hansson (2008), Kenning and Harzsch (2013), Loesel, Wolf, Kenning, Harzsch, and Sombke (2013), Krieger et al. (2015), Schmidt (2016) and Harzsch and Krieger (2018). For simplification, the neuroanatomical descriptions are limited to only one side of the brain and hold true for all specimens studied if not stated otherwise. The description of brain components is given from anterior to posterior along the neuraxis which is ventro-dorsally flexed almost 90° of the body axis, resulting in an upright position of the brain within the cephalon and the anterior part of the brain situated most dorsally relating to the body axis.

For a consistent terminology, here we suggest avoiding the term “optic neuropils” (Hanström, 1925; Sombke & Harzsch, 2015) as well as “optic lobes” (Kenyon, 1896). Even if the Greek “optiké” and the Latin term “visus” have the identical meaning, nowadays, “optic” in the field of vision research refers to the physically refractive components of the eye for the reception of light. To emphasize the perceptive character of these neuropils, we suggest using the term “visual neuropils” which is consistent with, for example, the visual cortex in mammals, formerly also termed “optic” cortex (Spiller, 1898). All post-retinal components that are related to vision, such as the “optic tract” and the “inner” as well as the “outer optic chiasm” should be consequently renamed, too. Here, we suggest to use “visual tract” (VT) and the “inner” (iCh) as well as the “outer visual chiasm” (oCh) accordingly. However, for all preretinal components that are related to vision, the term “optic,” as for example in the dioptric apparatus of the ommatidia, should be maintained. We also discourage the commonly used terms “eyestalk neuropils” (Polanska, Yasuda, & Harzsch, 2007); “optic ganglia” (Kress, Harzsch, & Dircks, 2016; Medan, Berón de Astrada,
Scarano, & Tomsic, 2015), or “eyestalk ganglia” (Bliss & Welsh, 1952; Harzsch & Dawirs, 1996; Techa & Chung, 2015), usually summarizing the visual neuropils and/or the neuropils of the TM/HN-complex, because in some malacostracans (e.g., Birgus latro, Petrolisthes lamarckii, or Callianassa australiensis; see e.g., Sandeman et al., 1993) these neuropils are together located more proximal to the central brain and not in the eyestalks, and thus are part of the central brain. This is also the case in most peracarids which lack eyestalks. Furthermore, visual neuropils together with the neuropils of the IPC do not fulfill the definition of a “ganglion” (see Richter et al., 2010).

The term “esophageal connective” and the corresponding abbreviation OC (British English) are maintained here for simplicity. The syncerebral brain mass excluding the neuropils of IPC and visual neuropils within the eyestalks (see Krieger et al., 2015) is termed “central brain” throughout the text according to Schmidt (2016).

**FIGURE 1** Phylogenetic position of Stenopodidea and relationship within Malacostraca. The phylogenetic topology of Malacostraca in a after Schwentner et al. (2018) is combined with illustrations modified after Harzsch and Krieger (2018). Photographs were modified from Meth, Wittfoth, and Harzsch (2017) *Penaecus vannamei* (Dendrobranchiata), the photograph of *Pestarella thyreena* (Axilidea) was provided from Hans Hillewaert (2006) under a creative commons license (CC BY-SA 4.0), all other photographs were provided by A.-K. Rath (née Richter) and M. K. Hörnig. Drawings were modified from Kenning and Harzsch (2013) *Saduria entomon* (Isopoda), and Krieger et al. (2015) (*Carcinus maenas*—Brachyura); *Pagurus bernhardus* (Anomala) was redrawn from our own photographs and *Cherax* (Austacea) after https://australianadaptations.wikispaces.com/as under a creative common license (CC BY-SA 3.0). Other species: *Nebalia bipes* (Leptostracaea); *Euruposquilla massavensis* (Stomatopoda); *Neomyis integer* (Mysida); *Leptochelia dubia* (Tanaidacea); *Diastylis glabra* (Cumaacea); *Caprella mutica* ( Amphipoda); *Anaspides tasmaniae* (Anaspidacea); *Euphausia superba* (Euphausiacea); *Stenopus hispidus* (Stenopodidea) in (a–c); *Pasiphaea sivado* (Caridea); and *Paribaccus antarcticus* (Achelata). Dotted rectangle in (a) depicts a portrait of a male individual of *S. hispidus* displayed in a higher magnification in (b). The brain of a male specimen which was manually segmented with the 3D-reconstruction software AMIRA and illustrated within the head-region (indicated by the dotted rectangle in b) is visualized by using different transparencies in (c). This visualization is based on a volume rendering (“Volren”) representing the manually segmented (labeled) brain (orange color map) and surrounding tissues of the whole μCT of the head of a male cleaner shrimp (gray color map) simultaneously. Note that in macro-photographs of *S. hispidus*, the antennules as well as the antennae are not shown in their full length due to injury and space constraints. Abbreviations: A1, antennule; A2, antenna; A1Nv, antennular nerve; A2Nv, antennal nerve; eye, compound eye; IF1, lateral flagellum; IPC, lateral protocerebrum; mFl, medial antennular flagellum; mFi, medial antennular flagellum; OC, esophageal connective; VNs, visual neuropils [Color figure can be viewed at wileyonlinelibrary.com]
According to the following publications, we consider the primary afferents of the retinula cells the "visual nerve" (or formerly "optic nerve," see e.g., Brusca, 1981; Schmitz, 1989; Kenning, Müller, Wirkner, & Harzsch, 2013; Loesel et al., 2013; Kress et al., 2016; Ramm & Scholtz, 2017), because: (a) In crustacean taxa, where the visual neuropils are close to the central brain, a comparably long bundle of axons of the retinula cells is present which matches the definition of a "nerve" (see Richter et al., 2010; compare with e.g., Birgus latro in Krieger, Sandeman, Sandeman, Hansson, & Harzsch, 2010). (b) It is comparable with the "optic nerve" in chelicerates (Babu & Barth, 1984; Chamberlain & Barlow, 1982) and myriapods (e.g., Petykó, Zimmermann, Smola, & Melzer, 1996). The protocerebral tract (PT) was formerly also termed Nervus opticus or "optic nerve" in crustaceans and insects (see e.g., Mangerich, Keller, & Dirksen, 1986). However, in the logic of the older terminology, even eyeless arthropods (e.g., Remipedia) would feature an optic nerve because they possess lateral protocerebral neuropils, therefore this term is misleading. Consequently, and because most of its fibers are not associated with the visual system, Sandeman et al. (1992) renamed this connection the PT.

The PT connects the terminal medulla with the median protocerebrum and only a small portion of axons within is represented by the olfactory globular tract (OGT) which connects the deutocerebrum with the IPC. The VT according to Sandeman et al. (1992) and as used here, represents the connection between the visual neuropils and the neuropils of the IPC.

2.2 | X-ray microscopy and 3D reconstruction

Two adult specimens of S. hispidus were anesthetized by chilling them at −18 °C immersed in sea water for a few minutes and subsequently killed by transferring them into plastic tubes containing Bouin’s solution (10% formaldehyde, 5% glacial acetic acid in saturated aqueous picric acid). After 30 min, the antennules, antennae, maxillipeds, pereiopods, and the pleon of each specimen were removed, and finally, the cephalothorax was transferred back into fresh Bouin’s fixative for 3 days in a refrigerator (4 °C). After fixation, the specimens were gradually dehydrated in an ascending ethanol series at RT (30, 50, 60, 70, 80, 90, 96%, and 3× in 99.5% ethanol; 30 min for each step). For enhancing the contrast, samples were incubated in iodine solution (2% iodine resublimated [Cat. #X864.1; Carl Roth GmbH, Karlsruhe, Germany] in 99.5% ethanol) for 24 hr in the fridge. Iodine was subsequently washed out of the samples for 3× 10 min with ethanol and the samples were dried with an fully automatic critical point dryer Leica EM CPD300. For more details on the μCT-preparations, see also Sombke, Lipke, Michalik, Uhl, and Harzsch (2015) with slight modifications after Krieger and Spitzner (2020).

The micro-computed tomographic scans were performed with a lab-based X-ray-microscope (Xradia MicroXCT-200; Carl Zeiss Microscopy GmbH, Jena, Germany). The dried samples of S. hispidus were mounted on plastic welding rods using hot glue and were scanned with a 4x (30 kV, 200 μA, 1 s) and a 10x (40 kV, 200 μA, 3 s) objective. For all scans, Binning 2 was applied (summarizing four pixels for noise reduction). Projections obtained by the tomography were reconstructed using the software XMReconstructor (Carl Zeiss Microscopy GmbH, Jena, Germany). To avoid subsequent information loss, Binning 1 (full resolution) was applied for the following reconstruction resulting in image stacks of 993 × 993 pixels and a pixel size of about 4.8 μm (4x) and 1.9 μm (10x), respectively.

Volume reconstruction and visualization was carried out using Amira 5.6.0 and 6.0.1 (FEI Visualization Science Group, Burlington, VT; RRID: SCR_007353) on the basis of μCT-data. Brain structures such as neuropils, nerves, and cell clusters were segmented manually for volumetric analysis and visualization. 3D surfaces corresponding to the segmentation were generated using unconstrained smoothing (Amira: SurfaceGen). Voxel data of the reconstructed neuropils were extracted by using Amira’s material statistics tool. The overall appearance of specific brain structures within the cephalon could be visualized based on μCT by using the Amira Volren-module being connected to manually segmented labels and different transparencies.

2.3 | Macro-photography

Overview images were taken with a Canon EOS 70D, equipped with a Canon MP-E 65 mm macro objective and Canon Macro Twin Lite MT-24 EX flash. Polarization filters were used in front of light sources and lenses to reduce reflections and enhancing color contrast. The specimens were photographed in ethanol and fixed with a cover slip. To generate consistently sharp images, several single photographs were taken along the z-axis and subsequently fused with Combine ZM 1.0 (Hadley, 2008). Further image processing was done with Adobe Photoshop CS3 (RRID: SCR_014199).

2.4 | Scanning electron microscopy of sensory appendages

For SEM, the antennules and antennae of a total of six specimens of S. hispidus were fixed in 70% ethanol cut into pieces of 20 mm length and dehydrated in a series of graded alcoholic solutions (70-99.5%). After critical point drying, the samples were mounted on a standard pin stub equipped with adhesive carbon tabs and a small amount of ACHESON silver paint (Cat. G301, G3347, and G3692; Plano GmbH, Marburg, Germany) and examined with a Zeiss EVO LS10 (Carl Zeiss, Germany) and Zeiss EM902 (Carl Zeiss, Germany) using a working distance of 20 μm. The images were captured using a Zeiss Ultra 55 field emission SEM at 15 kV (Carl Zeiss, Germany). To improve the contrast and enhance the details, the brightness and contrast was adjusted in Adobe Photoshop CS6 (RRID: SCR_014199).

2.5 | Immunohistochemistry

Specimens of S. hispidus were anesthetized by chilling them at −18 °C immersed in sea water for a few minutes followed by
decapitation and dissection of the brain including the brain parts situ-
ated within the eyestalk in phosphate buffered saline (PBS, 0.1 M, pH 7.4). The brains were fixed overnight in 4% PFA (paraformalde-
hyde) in 0.1 M PBS at 4 °C. After fixation, the brains were washed in several changes of PBS and mounted in agarose with low gelling temperature (Cat. A9414; Sigma-Aldrich Chemie GmbH, Munich, Germany) and then sectioned (100 μm) with a vibratome (Hyrax V50; Carl Zeiss, Oberkochen, Germany). After preincubation for 1.5 hr in PBT (PBS + 0.3% Triton X-100 + 1% bovine serum albumin) to improve antibody penetration, the sections were incubated in the primary antisera: monoclonal mouse anti-SYNOF1 (3c11 anti SYNOF1; Developmental Studies Hybridoma Bank, University of Iowa; deposited by E. Buchner, University Hospital Würzburg, Germany; supernatant; RRID: AB:2313867) in PBS (1:100) and poly-
clonal rabbit A-type Dip-allatostatin I (Jena Bioscience, abd-062; RRID: AB:2314318) at room temperature overnight. The sections were then washed in several changes of PBT for 1 hr and incubated in the secondary antibodies conjugated to Alexa Fluor 488 (Alexa Fluor 488 goat anti-rabbit IgG Antibody, Invitrogen, Thermo Fisher Scientific; Waltham, MA; RRID: AB:10374301) and Cy3 (Cy3-conjugated AffiniPure Goat Anti-Mouse IgG Antibody, Jackson ImmunoResearch Laboratories Inc.; West Grove, PA; RRID: AB:23380000) overnight at room temperature. In addition, we used HOECHST 33258 (Cat. 14530; Sigma-Aldrich Chemie GmbH, Munich, Germany) as a nuclear counterstain to visualize cell clusters of neuronal somata. Finally, the tissues were washed in several changes of PBT for 2 hr and embedded in Mowiol 4–88 (Cat. 07132; Carl Roth, Karlsruhe, Germany).

For whole mount preparations coupled with immunolabeling, we used the protocol after Ott (2008) based on a zinc-formaldehyde (ZnFA) fixation. For this method, two adult specimens of S. hispidus were processed by chilling animals being submersed in seawater for several minutes at ~20 °C until movement stopped. By substituting the seawater with formalin (4% formaldehyde in seawater) the ani-
mals were immediately killed and the brains were subsequently dis-
sected in HEPES-buffered saline (HBS) to avoid precipitation of zinc phosphate (which would occur in PBS). The brains were fixed in ZnFA (Cat-No. 15675; Electron Microscopy Sciences, Hatfield, PA) for 20 hr at room temperature on the shaker. After subsequent wash steps for 3 × 15 min in HBS, the brains were postfixed and dehydrated in Dent’s fixative (20% dimethyl sulfoxide [Cat-No. 20385; Serva Electrophoresis, Heidelberg, Germany] and 80% methanol) for 2 hr on the shaker followed by transfer into 99% metha-
nol. The samples were then gradually rehydrated in TRIS-buffer with decreasing grades of methanol (90, 70, 50, 30%, and finally pure TRIS-buffer for 15 min each). Samples were preincubated for 4 hr in PBS-TX at room temperature followed by the primary antisera (as listed above) for 4 days at 4 °C. After subsequent wash-
ing for 4 × 30 min in PBS-TX at RT on the shaker, incubation in the secondary antibodies (as listed above) was carried out for another 2.5 days at 4 °C. The brains were then washed for 2 × 1 hr in PBS-
TX followed by dehydration in a graded series of ethanol (30, 50, 70, 80, 90, 96, and 2 × 99.5% for 30 min each).

### 2.6 Antibody reporting

The monoclonal anti-SYNOF1 synapsin antibody (DSHB Hybridoma Product 3C11; anti SYNOF1 as deposited to the DSHB by E. Buchner, University Hospital Würzburg, Germany; supernatant) was raised against a Drosophila melanogaster GST-synapsin fusion protein and rec-
ognizes at least four synapsin isoforms (70, 74, 80, and 143 kDa) in western blots of D. melanogaster head homogenates (Klagges et al., 1996). Sullivan, Benton, Sandeman, and Beltz (2007) mention a single band at ~75 kDa in a western blot analysis of crayfish brain homoge-
rate. Harzszech and Hansson (2008) conducted a western blot analysis comparing brain tissue of D. melanogaster and the hermit crab Coenobita clypeatus (Anomala, Coenobitidae). The SYNOF1 serum provided identical results for both species and it stained one strong band between 80 and 90 kDa and a second weaker band slightly above 148 kDa, suggesting that the epitope that SYNOF1 recognizes is strongly conserved between D. melanogaster and C. clypeatus (see Harzsch & Hansson, 2008). Similar to the fruit fly, the antibody consistently labels brain structures in other major subgroups of the malacostracan crustaceans (e.g., Harzszech, Anger, & Dawirs, 1997; Beltz et al., 2003; Krieger et al., 2012; Kenning et al., 2013; Meth et al., 2017) in a pattern that is consistent with the assumption that this antibody labels synaptic neu-
ropils in crustaceans. In the following, the term “synapsin-like immu-
noreactivity” is used to indicate that the antibody most likely recognizes the same isoforms of synapsins as have been shown for the land hermit crab Coenobita clypeatus. However, we used the anti-SYNOF1 syn-
apsin antibody as a morphological marker to label neuropils within the brain of S. hispidus.

The A-type allatostatins represent a large family of neuropeptides that were first identified from the cockroach Diploptera punctata; they additionally share the C-terminal motif -YXF-GLamide (Christie, Stemmoller, & Dickinson, 2010; Nääs & Homberg, 2006; Stay & Tobe, 2007; Stay, Tobe, & Bendena, 1995). In the shore crab Carcinus maenas (Brachyura), almost 20 native A-type allatostatin-like peptides were identified from extracts of the thoracic ganglia (Duve et al., 1997). Shortly afterwards, various other A-type allatostatin-like peptides were isolated from the Eastern Crayfish Orconectes limosus (Astacidae: Dircksen et al., 1999). Meanwhile, A-type allatostatin peptides have been discovered in a wide range of malacostracan crustaceans, including Brachyura (e.g., Huybrechts et al., 2003), Astacidae (e.g., Cape, Rehm, Ma, Marder, & Li, 2008), the prawns Panaeus monodon (Duve, Johnsen, Scott, & Thorpe, 2002), Macrobrachium rosenbergii (Yin, Yang, Cao, & Yang, 2006) and also in the shrimp Panaeus vannamei (Ma et al., 2010; Meth et al., 2017). Christie (2016) identified a total of 29 peptides with the C-terminal motif -YXF-GLamide, in the latest analysis on the peptidome of the shore crab. The polyclonal rabbit allatostatin antiserum used in the present study was raised against the Diploptera punctata A-
type Dip-allatostatin I, APSGAQRLYGFGLamide, coupled to bovine thy-
roglobulin using glutaraldehyde (Vitzthum, Homberg, & Agicola, 1996). It has previously been used to localize A-type allatostatin-like peptides in crustacean and insect nervous systems (e.g., Kreissl, Strasser, & Gal-
izia, 2010; Polanska, Tuchina, Agricola, Hansson, & Harzszech, 2012). In the following, the term “allatostatin-like immunoreactivity” is used to
indicate that the antibody most likely binds to various related peptides within this peptide family.

In addition to malacostracans, both antisera chosen to have proven to be valuable morphological markers for the nervous system in a variety of other arthropods such as spiders (Loesel, Seyfarth, Bräunig, & Agricola, 2011; Steinhoff et al., 2017), insects (Heuer, Kollmann, Binzer, & Schachtner, 2012; Loesel & Heuer, 2010), and myriapods (Schendel, Kenning, & Sombke, 2018; Sombke, Harzsch, & Hansson, 2011).

In control experiments for secondary antibody reactivity, in which the primary antibodies were replaced with PBS-TX, no neuronal labeling was detected.

2.7 | Histology

For investigating the internal organization of sensory neurons associated with the olfactory sensilla (aesthetasc), we analyzed sagittal Azan-stained paraffin sections of the antennules of three specimens. After fixation in 70% ethanol, the paraffin embedding procedure involved dehydration of the samples in a series of graded alcohol solutions (70–99.5% ethanol), followed by xylene, and two consecutive infiltrations of paraffin (1 and 2 hr) that was kept at 59–61 °C during this process. The samples were then embedded in fresh paraffin. Sections were cut in a sagittal plane at a thickness of 6 μm using a microtome (Leica RM 2145; Leica Microsystems, Wetzlar, Germany), stained with Azan after Geidies (1954), and embedded in Roti-Histokit (Cat. 6638.2; Carl Roth, Karlsruhe, Germany).

For the visualization of somata of olfactory sensory neurons, one antennule was dissected and the lateral and medial flagella were removed distally. The sample was fixed in 70% ethanol for 24 hr at room temperature and was stored at 4 °C. The antennule was then rehydrated in decreasing grades of ethanol (50 and 30%) for 30 min each, and finally twice in PBS. It was subsequently incubated with 0.5 μl SYTOX™ Green (Thermo Fisher Scientific—Invitrogen™ Cat. No. S7020) in 1 ml PBS-TX for 24 hr at room temperature and dehydrated again in a graded series of ethanol (30, 50, 70, 80, 90, 96, and 2 × 99.5% for 30 min each).

For silver impregnations of the central brain, the anterior of the cephalothorax was removed from the animals and the exposed brains were fixed in cold, aged alcoholic Bouin’s fixative for 2 to 3 days after which they were washed and dissected free from the surrounding tissues. They were dehydrated in an ethanol series, cleared in xylene and embedded in paraffin wax. 10 μm serial sections were mounted on glass slides and stained using a modification of the Holmes-Blest silver impregnation method (Blest & Davie, 1980), in which impregnation times were increased up to 24 hr and toned with a 2% gold solution. For further details, see Sandeman et al. (1993).

3 | RESULTS

3.1 | Gross morphology of the cephalon and sensory structures

The banded cleaner shrimp features a shrimp-like body. Its carapace with anteriorly protruding rostrum, covers the cephalon and pereion (Figure 1b). From dorsal to ventral, the sensory cephalic appendages are: a pair of stalked, moveable eyes of the reflective superposition type (Richter, 2002) with ~1,400 squarish facets per eye (Figure 1b,c), the remarkably long pair of biramous antennules ("antenna I" or "first antenna": Figures 1b and 2), and the paired uniramous antennae ("antenna II" or "second antenna") each of which bears a prominent scaphocerite (SC; Figure 1b) on the second antennal peduncle. The scaphocerite is almost as long as the carapace. Although we recorded the sex of most individuals, no apparent sexual dimorphism within the brain or sensory appendages could be revealed based on our analyses, and further insights into this topic are beyond the scope of this manuscript.

3.2 | Sensory appendages associated with the brain

With about 120 mm length, the antennule (A1) as well as the antenna (A2), both extend anteriorly over more than twice the body-length of
the animal (60 mm) and can sample across almost the entire dorsal hemisphere of the animal. The antennule (Figure 1a,b) is proximally composed of three basal joints (peduncles) and further distally, two flagella: the (dorso-) lateral flagellum (lFl) and the (ventro-) medial flagellum (mFl), both pointing anteriorly (Figure 2a), however both flagella can be moved separately to some degree, mainly along the dorsoventral axis. Each flagellum consists of about 250 annuli (on the lFl: 268 and on the mFl: 244; \( n = 1 \)). On the dorsolateral side on the most proximal annuli, the antennule is armored with 18 robust cuticular spines (one or two per annulus; Figure 2a,b) pointing distally. Exclusively on the proximal annuli 7–18 of the lateral flagellum, about 84 ± 11 (average ± SD; ranging from 70 to 96; \( n = 6 \); 81 ± 13 in 3 x ♀) and

**FIGURE 2** Antennule of *Stenopus hispidus*. The proximalmost part of the antennule showing the peduncle and the lateral flagellum (lFl) bearing the aesthetasc (as) on its medial side and several armoring spines (white and black arrowheads) on its lateral side (a-c, g) as well as the medial flagellum (mFl) revealed by a scanning electron micrograph of a male specimen in (a). In image section (b), a bright-field micrograph shows the aesthetasc (as) and cuspidate setae (cs) in higher magnification on annuli 14 to 18 on the lFl of an unsexed specimen. A black-white inverted maximum projection of a confocally scanned lFl reveals the ellipsoidal clusters of olfactory sensory neurons (OSNs) in addition with integument cells (flattened cells on top and bottom) labeled with the nuclear marker Sytox™ Green (green) in (c) and in higher magnification in (d) of an unsexed specimen. The scanning electron micrograph of a female specimen in (e) displays the aesthetasc flanked of three hair-like sensilla (asterisks) on the anterior margin of each annulus. The bright-field micrograph in (f) displays a longitudinal paraffin section of a female specimen stained with Azan through the lFl revealing the aesthetasc (orange) as well as the ellipsoidal clusters of OSNs (purple) beneath the cuticle (blue) corresponding to (c) and (d). The scanning electron micrograph in (g) shows several armoring spines on lateral side of lFl in greater detail and a few cuspidate setae (cs) [Color figure can be viewed at wileyonlinelibrary.com]
86 ± 9 in 3× d) unimodal chemosensilla (Figures 2a–c.e and 3a–d; the so-called aesthetascs are identifiable but are absent on the median flagellum contradicting the description by Goy (2010). The aesthetascs (as) which mediate olfaction in crustaceans (Derby, Kozma, Senatore, & Schmidt, 2016) are arranged in parallel rows of two to three aesthetascs per row (the number increases gradually from distal to proximal thus ranging from two to six rows per annulus) at the medioventral side of the IFI in *S. hispidus*. However, several pores of

**FIGURE 3** Scanning electron micrographs of the antennular sensilla. SEM-micrographs of a lateral flagellum (IFI) of a male specimen show numerous pores at the insertion region of aesthetascs (as) in (a) and in higher magnification (dotted rectangle in a) is shown in (b), whereas no apical pores on the aesthetascs (as) could be identified as shown from a IFI in a female specimen in (c) and in higher magnification (dotted rectangle in c) in (d). Note that the IFI in (c) and (d) is orientated upside-down (aesthetascs face upwards) in contrast to the other panels. Fluorescence micrographs using the cuticular autofluorescence display the arrangement and distribution of cuspidate setae (cs) for the middle region each of the IFI in (e); the median flagellum (mFl) in (f); and the antennal flagellum (Fl) in (g) of an unsexed individual of *S. hispidus*. SEM-micrographs of the antennular IFI (in h) and mFl (in i) of a male specimen display each a cuspidate seta (cs) in addition of a simple seta (ss) in proximity to cuticular pores of unknown function.
unknown function occur in a crescent-shaped arrangement at the cuticular bulge of the IFI under which the aesthetasc originate (shared socket; see Figure 3a,b). These aesthetasc rows are occasionally flanked by very slender hair-like sensilla (simple setae according to Garm & Watling, 2013) reaching up to a third of the aesthetasc length (Figures 2e and 3c). Each aesthetasc is about 500 μm long and features about a dozen of annulus-like cuticular constrictions (Figures 2e and 3a,c) and is devoid of any pores, based on SEM analyses (Figure 3c,d). Since in our preparations, the aesthetasc rows were always typically distorted at their distalmost half (Figures 2a,b,e and 3c), we assume that this fragility is linked to a tapering of cuticular thickness toward the tip. Furthermore, each aesthetasc is associated with several somata further proximally. Most likely, the majority of these somata belong to the olfactory sensory neurons (based on three azan-stained paraffin sections as well as one whole-mount preparation using a fluorescent nuclear dye (Figure 2c,d, and f). In addition to the comparatively few aesthetasc rows in S. hispidus, both antennular flagella also bear about 3,200 (IFI: 1,880 and mFl: 1,350; n = 1) cuspidate setae (according to Garm & Watling, 2013) of a uniform morphology (cs; Figures 2b, c,e,g and 3e,f,h,i). In contrast to the aesthetasc rows, the cuspidate setae are distributed over the whole length and surface of each flagellum. Each cuspidate seta is articulated in small depressions or pits in the cuticle (infracuticular articulation), has a compact appearance (about 15 μm diameter at the setal base), tapers distally, and is about 50 μm long. In addition, each cuspidate seta is associated with one long and slender setule (sensory bristle according to Brandt, 1988) branching off on the distal half of the seta and exceeding its length by about 50 μm (see Figure 3h,i). The robust cuspidate seta (L-W ratio of about 4.5) does not possess a terminal pore. Based on its outer morphology, the cuspidate seta resembles those of the "sensory spines" described as mechanosensory sensilla in isopods such as Aega antarctica (Brandt, 1988). The cuspidate setae are occasionally flanked by one or two simple setae of almost the same length as of the setule in which a terminal pore could not yet be identified in our preparations. The antenna extends rectangularly to the body axis pointing laterally and its range of movement covers more than half of the posterior hemisphere of the animal. The uniramous flagellum (Fl) consists of 281 annuli (n = 1) and is slightly longer than both flagella of the antennule. Approximately 3,000 cuspidate setae, featuring the identical outer morphology of those present on both antennular flagella (Figure 3e,f), are present on the flagellum of the antenna (Figure 3g). Along both antennular and antennal flagella, these sensilla are located close to the borders between annuli and in the middle of each annulus (Figure 3e–g). Due to their dominating number and distribution along both sensory appendages, it seems likely that these cuspidate setae represent bimodal chemomechanosensilla being involved in the so-called “distributed chemoreception” system based on their presence on different body regions in malacostracans (after Derby et al., 2016). Articulating on the second joint of the antenna, a large, movable, and paddle-like scaphocerite (SC) is present which points anteriorly in parallel to the antennule (Figure 1b).

3.3 Overall organization of the central brain (scheme)

The brain of Stenopus hispidus (Figures 4, 5, and 6) is subdivided into an anterolateral portion, the visual neuropils and the neuropils of the IPC (Figures 4–6a,b, 7) which is located within each eyestalk (Figure 1c), and a mediodorsal portion within the cephalothorax, the central brain (Figure 6c–d) consisting of the median protocerebrum (mPC; Figure 4, 6a, c–d, 8), deutocerebrum (DC; Figures 4, 6a,c–d, 9, and 10) and tritocerebrum (TC; Figures 4–6a,c–d, 9a, and 10a–f). The neuraxis of the central nervous system is flexed ~90° dorsally with respect to the body axis anteriorly to the bilaterally paired esophageal connective (OC) resulting in an upright position of the brain within the cephalon (Figure 5). The entire brain, which is ensheathed in a layer of connective tissue, stretches over a width of about 3 mm including the portion located within the eyestalks. The neuropils of the central brain extend to ~1 mm in length, width, and depth. Primary afferents originating distally from retinula cells (R) in the retina of the stalked compound eye, form the visual nerve (VNv) which targets the visual neuropils proximally. This consists of the visual neuropils (Figure 4): lamina (La), medulla (Me), lobula (Lo), and lobula plate neuropil (LoP) which are connected via several fiber bundles summarized as the VT to higher-order neuropils in the IPC (Figures 4, 6a–b, and 7), the tripartite hemiellipsoid body (HN consisting of neuropils A, B and C) and the terminal medulla (TM, or medulla terminalis). The IPC is connected via the PT (Figure 8c) to the anterior part of the median protocerebrum of the central brain, a tract which extends over a length of about 0.4 mm. The median protocerebrum consists of the paired anterior and posterior medial protocerebral neuropils (AMPN and PMPN) and the unpaired central complex which is composed of the protocerebral bridge (PB) and the central body (CB) located posteriorly at the transition of anterior and posterior medial protocerebral neuropils (Figures 4, 6a,c–d, 8, and 10d). In addition, the unpaired CB is connected to each of the paired lateral lobes (LLs after Utting and coworkers [Utting, Agricola, Sandeman, & Sandeman, 2000]) which are embedded in the posterior medial protocerebral neuropils) via a tract posteriorly but anterior to the cerebral artery. The deutocerebrum is innervated by several fiber bundles that form the antennular nerve (ANv) from the antennule (Figures 1c, 4, 5, 9a, and 10a,b), and consists of the unpaired median antennal neuropil (MAN; Figures 4, 6a,d, and 10c–d1), the bipartite lateral antennal neuropil (LAN; Figures 4–6a,c, 8a, 9a, and 10a–d–e–g), and the olfactory lobe (OL, synonymous with the olfactory neuropil ON; Figures 4–6a,c–d, 8a,c, 9, and 10a–f). Fibers of the densely packed cell bodies of projection neurons (also termed globuli cells in Mellon and Alones, [1993]) with their somata in cell cluster (10) enter the olfactory lobe dorsolaterally via the lateral foramen (lf; Figure 9b,c). These neurons send their projections via a median neuropil (mf; Figure 9b,c, and e) primarily into the most proximal neuropil of the hemiellipsoid body (HN C) but also into the terminal medulla (Sullivan & Beltz, 2004) of the lateral protocerebra of both brain hemispheres via the OGT (Figures 4, 5a,c, 8a,c, and 10c–d) which results in a formation of a chiasm dorsal to the central body. Median to the medial foramen at the position of the OGT, a small ellipsoid neuropil can be identified as the olfactory globular tract neuropil (OGTN; Figure 4, 6c, and 8c) revealing synapsin-like immunoreactivity (SYNir). Further
posteriorly, from ventral to dorsal, the bilaterally paired tritocerebral neuropils are: the antenna-II-neuropil (AnN; Figure 4–6a,c, 9a, 10a–f1) being associated with the antenna (second antenna or antenna II; Figure 1a,b, and 3g) via the antenna-II-nerve (AIINv; Figures 1c, 4–6 a, 9a and 10a), the columnar neuropil (CN; Figures 4, and 6a,d) which points toward the esophageal connectives (OC; Figures 1c, 4–6a, 9a, and 10a, and the tegumentary neuropil (TN; Figures 4,and 6a,d) which receives input via the tegumentary nerve (TNv; Figures 4 and 6a).

3.4 | Protocerebrum

3.4.1 | Visual neuropils

From the superposition compound eye in S. hispidus (Richter, 2002), primary afferents (summarized as visual nerve VNv, formerly called optic nerve) of the retinula cells (R) from the 1,400 squarish facets (Figures 1c, 5a–c, and 7a,d) target the distalmost visual neuropil, the lamina (La; former lamina ganglionaris) within the eyestalk (Figures 4, 6a, and 7a,d,e). The cup-shaped lamina is organized into columnar cartridges showing weak synapsin-like immunoreactivity (SYNir) but strong allatostatin-like immunoreactivity (ASTir) in at least two transverse layers (data not shown). Fibers connecting the lamina to the next proximal visual neuropil, the medulla (Me; formerly: outer medulla or medulla externa), form a chiasm (outer visual chiasm; oCh). In contrast to the lamina, the ellipsoidal or kidney-shaped medulla shows clear SYNir (Figure 6b) and at least four transverse bands, in addition to several dozens of longitudinal layers with clear ASTir (Figure 7e). In cross-sections, the medulla appears to consist of synaptic subunits which are arranged in a grid-like manner. Further proximally, the inner visual chiasm (iCh) is the result of crossing fibers connecting the medulla to the lobula (Lo; former: inner medulla or medulla interna). The lobula, showing distinct SYNir, is more spherical than the kidney-shaped medulla and is
transversally layered from distal to proximal showing at least nine clear layers of ASTir. It is proximally also connected to the terminal medulla. Dorsally adjacent to the lobula, the spindle-like lobula plate neuropil (LoP) is identifiable in μCT scans and shows SYNir in immunohistochemically labeled sections. Several neurites interconnect the lobula plate distally to the medulla and proximally to the terminal medulla. The anterior half of the visual neuropils in addition with a posterior portion of the terminal medulla is surrounded by a confluent cluster of cell somata (corresponding to cell clusters (1), (2), (3), (4), and (6)). Within this cluster and especially in proximity to the medulla, lobula and the terminal medulla, few cell somata show clear ASTir.

3.5 | Lateral protocerebrum

3.5.1 | Higher-order neuropils (HN/TM-complex) of the IPC

Proximal to the visual neuropils, the higher-order neuropils, namely the postero-dorsally located hemiellipsoid body (HN) in addition with the antero-ventral terminal medulla (TM) together form an almost spherical complex mass of neuropils, neurites and somata clusters of 500 μm in length, width, and depth (Figure 7a–e). Because these neuropils receive multimodal input from primary processing units such as the visual neuropils as well as the olfactory neuropils via the OGT,
FIGURE 6  Overview of the visual neuropils (VNs) and the neuropils of the lateral protocerebrum (IPC) and central brain in S. hispidus. The general structuring of the cleaner shrimp's brain is depicted in a schematic drawing in (a). Micrographs of specific regions (dotted rectangles in a) are shown in the panels (b to d). Panel (b) shows the lateral protocerebral neuropils (note that the lamina was destroyed in this preparation due to dissection) of a male specimen in a frontal vibratome section labeled against anti-SYNORF1 synapsin (SYN; black-white inverted fluorescence micrograph). Two opposed hemispheres of the central brain are displayed equally-scaled based on a silver-impregnated paraffin section of an unsexed specimen (c) and an almost frontal vibratome-section of a female specimen triple labeled against anti-SYNORF1 synapsin (SYN; magenta) and anti-allatostatin (AST; green) in addition with the nuclear counterstain HOECHST (NUC; blue) in (d). The line drawing on the insets in (c) and (d) show the orientation of frontal sections through the central brain (dotted lines) from a lateral view. Other abbreviations: 6, 10, and 16; cell clusters (6), (10), and (16); ANv, antennular nerve; AaNv, antennal nerve; AMPN, anterior medial protocerebral neuropil; AnN, antennal neuropil; CB, central body; CN, columnar neuropil; HN (B, and C), hemiellipsoid body neuropils B, and C; ILAN and mLAN, lateral and medial lobe of the lateral antennular neuropil; Lo, lobula; LoP, lobula plate; MAN, median antennular neuropil; Me, medulla; mPC, median protocerebrum; OC, esophageal connective; OGT, olfactory globular tract; OGTN, olfactory globular tract neuropil; OL, olfactory lobe; PB, protocerebral bridge; PMPN, posterior medial protocerebral neuropil; R, retina of ommatidia; TM, terminal medulla; TN, tegumentary neuropil; TNv, tegumentary nerve [Color figure can be viewed at wileyonlinelibrary.com]
FIGURE 7  The visual neuropils and the neuropils of the lateral protocerebrum in S. hispidus. 3D reconstruction of the neuropils of the visual neuropils and the neuropils of the lateral protocerebrum based on manual segmentation (surfaces) in virtual slices based on μCT of the eyestalk are shown from posterior (a) and anterior (b) of an unsexed specimen; in addition of a frontal vibratome-section triple labeled against anti-SYNORF1 synapsin (SYN; magenta) and anti-allatostatin (AST; green) and the nuclear counterstain HOECHST (NUC; blue) of hemiellipsoid body (HN) and terminal medulla (TM) in a slightly sagittal orientation of a female specimen (rectangle in a) in (c) showing the “lateral protocerebral complex” (arrowheads in a to c) after Sullivan and Beltz (2004). A virtual section of the same μCT of an unsexed specimen is displayed in (d) and opposed to a frontal vibratome-section (using the same labeling as in c) of the hemiellipsoid body (HN) and terminal medulla (TM) of a female specimen in (e) (note that the lamina was destroyed in this preparation due to dissection) and of higher magnifications (dotted rectangles in e and f) in (f) and (g). Other abbreviations: asterisk, central region of the HN sublobes; 5; cell cluster (5); HN (A, B, and C), hemiellipsoid body neuropils A, B, and C; La, lamina; Lo, lobula; LoP, lobula plate; Me, medulla; TM, terminal medulla; TN, tegumentary neuropil. The scale bar in (c) represents 250 μm [Color figure can be viewed at wileyonlinelibrary.com]
they are considered integrative brain centers or association centers. The hemiellipsoid body in *S. hispidus* is composed of three closely spaced, but distinct lobular neuropils (HN A, HN B, and HN C; Figure 7a–f) which were first described by Sullivan and Beltz (2004). The dorsal-most neuropil of the lPC, the comparably large neuropil HN A lies anteriorly adjacent to the largest hemiellipsoid body neuropil HN B posteriorly, whereas neuropil HN C is positioned slightly medioventrally at the boundary of HN A and HN B and thus primarily receives input from the deutocerebral projection neurons via the OGT. Each of the three sublobes features a cortex showing a specific regionalization revealed by SYNir and ASTir. The densest synaptic profiles are located in the periphery of each cortex and SYNir becomes weaker toward its center. The centers of all three sublobes, which are free of any SYNir and ASTir, consist of dense fibers that...
become visible by X-ray microscopic tomograms (asterisks in Figure 7d,f) presumably originating in the somata cluster (5) displaying weak or no ASTir too. At the periphery, in higher magnifications, a network of synaptic profiles with regular swellings resembling microglomerular structures (2 to 3 μm in diameter) becomes identifiable by SYNir which is colocalized by ASTir (arrows in Figure 7g) in three clear strata (Figure 7e) of which the centralmost shows the weakest allatostatinergic signal intensity (Figure 7g). At the periphery of the cortex, allatostatinergic neurites appear to interconnect the microglomeruli peripherally (Figure 7g). Immunohistochemically labeled sections revealed that cluster (5) is almost devoid of ASTir (Figure 7c,e). Cell cluster (5) which houses densely packed somata of small globuli

**FIGURE 9** Legend on next page.
cells (a term introduced by Holmgren (1916) for interneurons in poly-
chaetes, onychophorans, and euarthropods in mushroom-body-like
structures and now known as Kenyon cells in insect mushroom bod-
ies, see Strausfeld (1976)) This cluster (5) covers anteriorly more
than a half of the HN A and further ventrally also parts of the large
terminal medulla, where few somata can be identified showing distinct
AST ir. The medioventral compact terminal medulla shows strong but
diffuse SYN ir, a network of strong AST ir and its entire volume slightly
exceeds the volume of all three hemiellipsoid neuropils together
(Table 1). At the proximal margin of the terminal medulla, a neuropil
becomes visible showing clear SYN ir and extending through the ter-




i nal medulla into a region at the boundary of all three hemiellipsoid
body neuropils (HN A, HN B, and HN C; arrowhead in Figure 7a–c)
which Sullivan and Beltz named "lateral protocerebral complex"
(Sullivan & Beltz, 2004). From a medio-dorsal perspective, this neu-
ropil has a knob-like shape at the margin of the terminal medulla and
extends into the hemiellipsoid body neuropils forming a strong
curvature.

3.6 | Median protocerebrum (mPC)

Each IPC is connected medioventrally to the ipsilateral portion of
the anterior medial protocerebral neuropil (AMPN) via a prominent fiber
bundle the PT (Figure 8c) in addition to the smaller OGT (Figure 8a)
originating from the lateral deutocerebrum. In sections, the OGT con-
necting the deutocerebrum to the IPC constitutes a subpopulation of
fibers of the larger PT which represents the connection between the
mPC and the IPC. Anterodorsally to the anterior medial protocerebral
neuropil, a dense cell cluster becomes visible (cell cluster (6); Fig-
ures 8a–c,e, 9a). Given the limitations imposed by tissue shrinkage,
range of cell diameters and domination of the smaller cells, we esti-
mate the number of cell somata to lie between 3,500 and 10,000 cells
within this cluster (Figure 8c). In addition, several cells show distinct
AST ir within cluster (6). Further anteriorly, few allatostatinergic neu-
rites project ventrally to destinations within the deutocerebrum as
well as the tritocerebrum. In silver impregnated histological sections,
several projections can be identified targeting different regions within
the anterior and posterior medial protocerebral neuropils. 3D recon-
struction revealed that cell cluster (6) is anteriorly fused and posteri-
orly bifurcates giving it a V-shaped appearance when viewed from
dorsal (Figures 8a, 9e). In frontal sections, between cluster (6) and the
AMPN, two minute bilaterally paired neuropils appear which can be
identified as representing the arms of the V-shaped protocerebral
bridge neuropil (PB; Figure 8a–c,e). Both arms of this neuropil taper
towards the midline, barely connecting to each other (Figure 8e). In
addition, they are intensely interconnected by fibers displaying strong
AST ir (Figure 8e). The anterior and posterior medial protocerebral
neuropils of almost the same size together constitute a large confluent
neuropil. Both show strong SYN ir and are interwoven by distinct
allatostatinergic neurites. Both neuropils can only be differentiated
from each other dorsally by the position of the central body
neuropil and by the presence of peripherally bilateral constrictions in
the same horizontal plane resulting in a butterfly shape of the median
protocerebrum in frontal sections (Figures 6a,c,d and 8b,c,e). The
spindle-shaped central body shows distinct SYN ir and strong AST ir
(Figure 8e,f), and can be clearly identified in μCT (Figure 8b and 3D-
reconstruction in Figure 8a). The lateral lobes (LL), which have been
described and illustrated for S. hispidus previously by Sandeman and
coworkers (2014), are connected medially by commissural neurites
that are positioned ventrally and in parallel to the central body
(Figures 4a and 8c).

3.7 | Deutocerebrum

The deutocerebrum, which is associated with the antennule (synonym
for antenna 1), is the most partitioned brain region in S. hispidus. It
consists of the median antennular (antenna I-) neuropil (MAN), the
bipartite lateral antenna-I-neuropil (LAN), the spherical olfactory lobe
(OL; Figure 9), and the minute OGTN. The deutocerebrum receives
input from the antennular nerve (A1,Nv) which is already arborized into
eight distinct neurite bundles within the basal antennular joint. Based
on μCT (Figures 9a and 10a,b), all four neurite bundles can be tracked
in manual segmentation in virtual slices based on μCT is shown in (a)
highlighting the lateral deutocerebrum and antennal neuropils (AnN—green).
(b) shows a manually segmented 3D-reconstruction of the olfactory glomeruli (og) in the OL of a female specimen based on a whole-mount
preparation of the brain of S. hispidus double-labeled against anti-SYNORF1 synapsin (colored surfaces) and the nuclear counterstain HOECHST
white). (c) displays a frontal silver-impregnated paraffin-section of the OL and its olfactory glomeruli (dotted rectangle in c is shown in d at a
higher magnification). (e) displays a maximum projection of a confocal image stack of the OL based on a vibratome-section triple labeled against
anti-SYNORF1 synapsin (SYN; magenta) and anti-allatostatin (AST; green) and the nuclear counterstain HOECHST (NUC; blue) revealing
allatostatinergic neurites of projection neurons (black arrowhead) exiting the medial foramen (mf) via the olfactory globular tract in a male
specimen. The dotted rectangle in (e) is displayed at a higher magnification in (f) (hiding the green channel) showing the substructure of og into a
base, subcap, and cap region. Panels (g) (same orientation as in e) and (h) (orientation according to the dotted parallelogram in e) show
interglomerular contacts (arrowheads) in virtual sections from confocal scans of two more preparations of a female specimen using the same
labeling as shown in (e). Other abbreviations: 6, 10, 9/11; cell clusters (6), (10), (9/11); A1,Nv, antennular nerve; A2,Nv, antennal nerve; ILANVv and
mLANVv, lateral and medial nerves innervating the ILAN and mLAN, lateral and medial lobe of the lateral antennular neuropil; If, lateral foramen;
IPC, lateral protocerebrum; MAN, median antennular neuropil; MANNVv, median antennular nerve; mPC, median protocerebrum; OC, esophageal
connective; OLNv, aesthetasc nerve innervating the OL. All scale bars represent 50 μm as shown in (b) [Color figure can be viewed at
wileyonlinelibrary.com]
to their specific target neuropils which are from lateral to median for each hemisphere: one nerve innervating the median antennular (antenna I-) neuropil (MANNv), one nerve the olfactory lobe (OLNv), and two nerves (ILANNv and mLANNv) innervating the lateral (ILAN) and the medial lobes (mLAN) of the lateral antennular neuropil (LAN). The unpaired median antennular neuropil is confluently interconnected between the posterior medial protocerebral neuropil of the median protocerebrum anterodorsally, the lateral antennular neuropil laterally, and the tritocerebrum posterodorsally, resembling an inverted “U” in frontal sections (Figure 10c–d1). The transition between posterior medial protocerebral neuropil and median antennular neuropil can be identified by the presence of the cerebral artery penetrating the central brain in an anterodorsal course. In some decapods, the median antenna antennular neuropil receives input from the statocyst on the basal antennular peduncle (which is lacking in Stenopodidea [Goy, 2010]) and other mechanosensory sensilla of the...
FIGURE 10  Deuto- and tritocerebral neuropils processing chemomechanosensation in *S. hispidus*. A 3D reconstruction as volume rendering ("Volren") of the central brain based on manual segmentation in virtual slices based on μCT of a male specimen from ventral (see arrowhead in pictogram) is shown in (a), a μCT-based visualization of the central brain of an unsexed individual at higher magnification is shown in (b) and illustrated as surfaces from posterior in (c) (see arrowhead in pictogram). (d) shows a frontal (see pictogram) silver-impregnated paraffin section through the central brain of an unsexed specimen revealing the median deutocerebrum (dotted rectangle in d at higher magnification in d1). In a silver-impregnated paraffin section further anterior (see pictogram) in (e), the antennal neuropil (AnN) of the tritocerebrum (TC) is revealed. (f) displays a slightly horizontal vibratome section (see pictogram) of the antennal and antennal chemomechanosensory processing centers triple labeled against anti-SYNORF1 synapsin (SYN; magenta) and anti-allatostatin (AST; green in f and black in a black-white inverted single channel in f1) and the nuclear counterstain HOECHST (NUC; blue) and the lateral (ILAN) and medial lobe (mLAN) of the lateral antennal neuropil (LAN) of a female specimen and of another female specimen at a higher magnification in (g). Other abbreviations: 6, 10; cell clusters (6), (10); AνN, antennal nerve; AνN, antennal nerve; AMPN, anterior medial protocerebral neuropil; DC, deutocerebrum; ILANv and mLANv, lateral and medial nerves of the LAN; IF, lateral foramen; IPC, lateral protocerebrum; MAN, median antennal neuropil; MANNv, median antennal nerve; mPC, median protocerebrum; OC, esophageal connective; OGT, olfactory globular tract; OL, olfactory lobe; Pet, protocerebral tract; VNs, visual neuropils. All scale bars represent 100 μm as shown in (d) [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1  Morphometric measurements of substructures of the brain in one individual of *S. hispidus*

| Neuronal structure(s) | Abbreviation | Relative volume (%) | Sensory organ and corresponding sensilla | Estimated number |
|-----------------------|--------------|---------------------|----------------------------------------|-----------------|
| Visual neuropils (La + Me + Lo + LoP) | VNs | 11.29 13.95 15.45 | Compound eye (ommatidia) | (1,400) |
| Lamina | La | 1.78 2.89 3.22 | Retinula cells | *11,200 |
| Medulla | Me | 4.86 5.90 6.41 |
| Lobula | Lo | 4.57 5.09 5.70 |
| Lobula plate | LoP | 0.08 0.07 0.12 |
| Lateral protocerebrum | IPC | 46.81 44.16 46.80 |
| Hemiellipsoid body | HN | 21.92 21.55 22.71 |
| Terminal medulla | TM | 24.89 22.61 24.09 |
| Median protocerebrum | mPC | 14.10 13.56 13.56 |
| Protocerebral bridge | PB | Na Na 0.03 |
| Central body | CB | 0.05 0.10 0.11 |
| Other neuropils | AMPN +PMPN | 14.05 13.46 13.52 |
| Deutocerebrum | DC | 11.88 11.77 9.55 | Antennule |
| Medial antennular neuropil | MAN | 0.47 0.49 0.48 |
| Olfactory lobe | OL | 2.10 2.39 2.25 | IFI—aesthetascs | 84 |
| Olfactory glomeruli | og | | **89 | |
| Lateral lobe of LAN | ILAN | 4.72 4.14 3.38 | IFI—cuspidate setae | 1,876 |
| Medial lobe of LAN | mLAN | 4.58 4.75 3.44 | mFI—cuspidate setae | 1,342 |
| Tritocerebrum | TC | 15.92 16.57 14.63 | Antenna |
| Antennal neuropil | AnN | 8.13 5.58 6.25 | FI—cuspidate setae | 3,091 |
| Other neuropils | CN + TN | 7.79 10.99 8.38 |

Note: Volumetric data (based on μCT of three specimens; one male; one female and one unsexed specimen) as well as the number of ommatidia (based on a male specimen) were generated based on a 3D reconstruction of a μCT. The relative volumes of neuronal structures refer to both hemispheres whereas the estimated numbers of sensory organs and corresponding sensilla refer to only one hemisphere. *The number of retinula cells results from the ommatidia count multiplied by eight retinula cells according to the tetracorneate ground pattern after Melzer, Diersch, Nicastro, and Smola (1997). **The number of olfactory glomeruli (og) results from the volume of one olfactory lobe (OL; SYNir signal of a confocal scan of one whole-mount preparation) divided by the average volume of 20 randomly chosen og therein. The number of aesthetascs is an average of a total of six individuals (three males and three females; of one lateral flagellum per individual only) manually counted based on scanning electron micrographs. The number of cuspidate setae is estimated based on the number of annuli (counted in one individual were all three flagella were intact) multiplied by the average number of cuspidate setae per annulus (based the number of cuspidate setae counted on nine annuli in different flagellar regions [apically, medially, and basally]).

Abbreviations: IFI, lateral antennular flagellum; mFI, medial antennular flagellum; FI, antennal flagellum.
antennal base (Sandeman et al., 1992). Furthermore, antennular motoneurons project from the median antenna antennular neuropil into the antennule (Sandeman et al., 1992). In S. hispidus, the median antennular neuropil shows distinct SYNir and weak ASTir (Figure 6d). Neurites with strong ASTir traverse it dorsally. Anterolateral to median antennular neuropil, the lateral antennular (antenna-I-) neuropil originates and projects anteriorly (Figure 9a). Ventral between the olfactory lobe and the lateral antennular neuropil, a cluster (16) of very large somata can be identified in silver-impregnated sections (cluster (16); Figure 6c) of which neurites project into the median as well as into the lateral antennular neuropils. The lateral antennular neuropil is distally and horizontally bifurcated into two large elongated lobes (the lateral; ILAN and medial lobe; mLAN; Figure 10a–c,f, and g) fused only by a thin proximal junction. Both lobes show strong SYNir in a longitudinally striated pattern (Figure 10e–g). In addition, also perpendicularly synaptic regions can be identified which are interwoven by allatostatinergic neurites (Figure 10f,g). The lateral antenna antennular lobes have almost the same size ($V_{\text{LAN}} = 1.02 \times 10^7 \mu m^3$ and $V_{\text{ILAN}} = 1.04 \times 10^7 \mu m^3$) about 500 μm in length. In horizontal sections, both lobes share a high degree of mirror symmetry (Figure 10g) revealed by morphological labeling. Anterolateral to the position where the lateral lobe connects to the medial antennular lobe, the olfactory lobe becomes visible in frontal sections (Figures 6c,d, and 9). This conspicuous spherical structure measures about 230 μm in diameter and consists of about 80 to 110 peripherally arranged neuropils, the olfactory glomeruli (og) showing clear synaptic profiles whereas the central part of the olfactory lobe is free of SYNir but shows distinct neurite bundles with ASTir (Figure 9e). The olfactory glomeruli range from small rather elongated ($V_{\text{og}} = 1.1 \times 10^4 \mu m^3$) to large ($V_{\text{og}} = 7.8 \times 10^4 \mu m^3$) rather spherical shapes (Figure 9b–h). Each glomerulus is substructured into a cap, a subcap and a base region towards the center of the olfactory lobe (Figure 9d,e). The cap, being peripherally targeted by primary olfactory afferents from the antennular lateral flagellum, shows strong SYNir and weak ASTir, the subcap is characterized by a stronger ASTir and weaker SYNir whereas the base displays strong SYNir and weaker ASTir again (Figure 9e,f). In higher magnifications, interglomerular connections between adjacent olfactory glomeruli become visible in particular at the periphery (cap-region) by SYNir and ASTir (Figure 9g,h). Posterovertral to the olfactory lobe, a cell cluster (CC) of thousands of almost equally sized somata (diameter of about 7 μm) of projection neurons becomes visible, called cell cluster (10). From this cluster, fibers with distinct ASTir enter the OL through the lateral foramen (lf), whereas dorsomedially, neurite bundles forming the OGT exit the olfactory lobe via the medial foramen (mf) (Figure 9e). Here, the palisade-like arrangement of olfactory glomeruli is interrupted (Figure 9b–c,e). In addition to the OGT, silver impregnated histological sections revealed that fibers of local olfactory interneurons, housing their somata (diameter of about 10 μm) in the anteromedial cell cluster (9), interconnect the OGT neuropil (OGTN) from where they project into the olfactory lobe via the mf (Figure 6c). The minute elliptical OGT neuropil is situated in close proximity of the OGT between the median foramen and lateral antennular neuropil. An accessory lobe (AcN) as present as a prominent structure in Astacidea, or rather minute compared to the size of the OL in Achelata, Axiidea, Anomala, and Brachyura (Sandeman et al., 1993) could not be detected in any of our preparations.

### 3.8 | Tritocerebrum

Beginning anteriorly, the tritocerebrum is composed of the prominent and elongated antennal (antenna-II-) neuropil (AnN; Figures 9a and 10a–d,e–f1) associated with the antenna (synonym for antenna 2), the inconspicuous tegumentary neuropil (TN; Figure 6d) and further posterior, of an unstructured columnar neuropil (CN; Figure 6d), projecting into the esophageal connectives (OC). With a volume of about $1.9 \times 10^7 \mu m^3$, the antennal neuropil is about the same size as the whole lateral antennular neuropil (ILAN + mAN) and is ventrally orientated perpendicular to the lateral antennular neuropil. In close proximity where the antennal neuropil is connected to its corresponding antenna-II-nerve (ANv), the ANv is bent by almost 90° anteriorly feeds into the antennal peduncle (base) (Figures 5 and 9a). Its structure highly resembles that of both lobes of the lateral antennular neuropil, featuring conspicuous longitudinal synaptic layers in addition with perpendicularly orientated synaptic cartridages and allatostatinergic neurites (Figure 10f–g). The tegumentary neuropil is a small elliptical neuropil dorsolateral to the antennal neuropil which receives input from the comparably thin tegumentary nerve (TNv) which can be identified in μCT-scans as well as in silver impregnated histological sections (not shown). The tegumentary nerve projects posterolaterally for 400 μm, then bends by 90° and further projects dorsally.

### 4 | DISCUSSION

#### 4.1 | General

The brain organization (Figure 4) and its innervation from the primary sensory centers in *Stenopus hispidus* comprise all elements of the suggested malacostracan ground pattern (Kenning et al., 2013; Sandeman et al., 2014). The schematic illustrations showing the brain of *S. hispidus* given in Sandeman et al. (2014), however, underestimated the size of the IPC compared to the central brain, perhaps because a previous study referring to *Stenopus* among other malacostracans has focused on the central brain only (Sandeman et al., 1993). The volume of both lateral protocerebrum taken together are larger than the central brain in the cleaner shrimp. The multimodal association centers are composed of the hemiellipsoid body and the terminal medulla dominate the brain (Figure 4 and Table 1). The gross brain anatomy is comparable to that of the dendrobranchiate Pacific White Shrimp *Penaeus vannamei* Boone, 1931, as described by Meth et al. (2017) although specific neuropils differ considerably in shape and size. In the Pacific White Shrimp, the lamina is thinner but stretches like a cup over a larger area than in the banded cleaner shrimp whereas all other visual neuropils such as the medulla, lobula, and lobula plate are of
comparable sizes in both species. The IPC is more pronounced in *S. hispidus* and its separation into a terminal medulla and a hemiellipsoid body with complex substructures are absent in *P. vannamei* (Meth et al., 2017). Hence, the organization of the stenopodan hemiellipsoid body into columnar and calycal neuropils including microglomeruli resembles that of representatives of Caridea (Sayre & Strausfeld, 2019) more closely than that of the Dendrobranchiata (Meth et al., 2017; Sullivan & Beltz, 2004). The lateral antennular neuropil in *P. vannamei* is smaller and features neither a striated pattern nor a clear bifurcation as in *S. hispidus* (ILAN + mLAN). A small lateral antennular neuropil is in good concordance with the comparably small size of the antennule which in *P. vannamei*, does not protrude further than the rostrum (Figure 1a and in Meth et al., 2017) and in our view, reflects a size relationship between primary sensory structures and their associated neuropils. Consequently, in *P. vannamei*, the antenna (synonym for antenna 2) and its tritocerebral processing unit, the antennal neuropil are comparably large so that it dominates the central brain (Meth et al., 2017).

When comparing the stenopodan brain including its primary sensory organs, to those of dendrobranchiate or caridean shrimps, many more similarities can be identified with the caridean shrimps. In members of Caridea and Stenopodidea, the aesthetascs on the lateral antennular flagellum are present only on the most proximal annuli (aesthetasc region) whereas parts of the flagellum (Zbinden et al., 2017) including the distal part of the lateral flagellum is free of aesthetascs (Zhang, Cai, Liu, & Lin, 2008). In addition, like in stomatopods (Derby, Fortier, Harrison, & Cate, 2003), some caridean shrimps, such as, for example, *Palaeon elegans* and *Macrobrachium rosenbergii* De Man, 1879, possess a “third” flagellum as an additional ramus of the lateral flagellum. One of these bears aesthetascs along its entire length. The other is free of aesthetascs (Hallberg, Johansson, & Elofsson, 1992; Zbinden et al., 2017). The study by Hallberg and coworkers (1992) revealed that the first six larval stages of *Macrobrachium rosenbergii* possess a uniramous antennule bearing a few apical aesthetascs. The medial flagellum, and the additional ramus on the lateral flagellum appear later during development. A limited regionalization of aesthetascs on the proximal part of the lateral flagellum are found only in members of Euphausiacea, Mysida, and Lophogastrida within Malacostraca (Hörning et al. unpublished data). Conversely, the regionalization of the lateral flagellum into a proximal aesthetasc region can also be arranged like in spiny lobsters (Achelata), in which only the distal part of the lateral flagellum bears aesthetascs (Grüntert & Ache, 1988). The bilobed structure of the associated lateral antennular neuropil as a feature of the malacostracan brain ground plan (Hanström, 1947; Kenning et al., 2013) was shown for Leptostraca (Kenning et al., 2013), Stomatopoda (Derby et al., 2003), Caridea, Euphausiacea, Anaspidacea, Eureptantia, (Sandeman et al., 1993, 2014; Sandeman & Scholtz, 1995), and also Amphipoda (Ramm & Scholtz, 2017; Witttoth, Harzsch, Wolff, & Sombke, 2019) but seems to be absent in members of the Isopoda (Harzsch et al., 2011; Kenning & Harzsch, 2013). In these isopods, the antennule is uniramous, suggesting that a biramous antennule is represented in a bilobed lateral antennular neuropil like in *S. hispidus* where each sublobe (ILAN and mLAN) is connected to an individual nerve (Harzsch et al., 2011; Kenning & Harzsch, 2013).

The banded cleaner shrimp possesses well-developed and easily accessible elements of the primary olfactory pathway. Each antennule features ~90 aesthetascs on its lateral flagellum (Figure 2a–c,e, and f), each being associated with about 100 to 120 olfactory sensory neurons (Figure 2c.d, and f). Their primary afferents target the olfactory lobes which are again substructured into ~90 olfactory glomeruli (Figure 9b) resulting in a surprisingly congruent numerical relationship of roughly 1:1 (Table 1).

In summary, the neural architecture of *S. hispidus* supports its phylogenetic position according to recent phylogenomic analyses (Meth et al., 2017; Sullivan & Beltz, 2004). The lateral antennalar neuropil, the antenna (synonym for antenna 2) and its tritocerebral processing unit, the antennal neuropil are comparably large so that it dominates the central brain (Meth et al., 2017).

4.2 The hemiellipsoid body and possible genealogical relations to insect higher order neuropils

Decapod crustaceans display a rich repertoire of complex behavioral patterns related to finding food, shelter, and mating partners, kin recognition and brood care, orientation and homing, and are also known for complex social interactions such as communal defensive tactics, the occupation of common shelters, cooperative behavior during seasonal long-distance migration and the establishment of dominance hierarchies (reviews Duffy & Thiel, 2007; Breithaupt & Thiel, 2011; Derby & Thiel, 2014; Thiel & Watling, 2015). Higher order integrative brain centers are suggested to play a major role in learning and memory as well as to provide the neuronal substrate for more sophisticated processing underlying such behaviors (review in Sandeman et al., 2014). These authors suggested that in particular, those behaviors that require an active exploratory component and involve 3D spatial perception (be it visual, tactile, or olfactory), require the involvement of more than one of such higher order brain centers. Higher order neuropils receive input mostly from second or third order neurons but are not targeted by any primary sensory afferents and contain interneurons that respond to the stimulation of several different sensory systems. In the malacostracan brain, the (bilaterally paired) terminal medulla, hemiellipsoid body, accessory lobe, and the unpaired central complex seem to function as higher integrative centers, all four distinct neuropil areas which display a high level of complexity and are notable for their substantial volume (Sandeman et al., 2014). The IPC and in particular, the hemiellipsoid body of *S. hispidus* (Figure 7) is exceptional in comparison to all other malacostracans studied as has already been noted by Sullivan and Beltz (2004). The size of the IPC is enormous in relation to the central brain (Figure 4 and Table 1) and also in comparison to other malacostracans studied. The total neuropil volume of the terminal medulla and hemiellipsoid body together (of both hemispheres) represented 45.9 ± 1.5%, and together with the visual neuropils 59.5 ± 2.4% of the total brain volume (average ± SD; n = 3; Table 1). All three sublobes are characterized by peripheral microglomerular synaptic networks, clearly regionalized into...
an outer dense and inner loose cortex of synaptic microglomeruli, interspersed with peripheral ASTir profiles in different intensities resulting in a highly ordered pattern (Figure 7d–g). This feature of the cortices resembles the organization of the calyx region of several hexapod mushroom bodies (Groh & Rössler, 2011), higher order multimodal neuropils in the hexapod protocerebrum (Fahrbach, 2006; Farris, 2005; Galizia, 2008; Martin et al., 2011; Strausfeld, 1998; Strausfeld, Sinakevitch, Brown, & Farris, 2009). Such similarities include as a regional distribution of different microglomerular densities and an average size of microglomeruli between 2 and 3 μm in diameter in S. hispidus (based on one female specimen; compare e.g., 3.2 μm in workers of Apis mellifera Linnaeus, 1758). As recently described for the mushroom body of bumblebees which features an inner loose and outer dense collar (Kraft, Spaethe, Rössler, & Groh, 2019), we also identified a similar regionalization in the hemiellipsoid body of S. hispidus. Furthermore, the HN possesses a complex of neuropils that resembles that of the corpo allungato of the stomatopod Gonocacytus bredini Manning, 1969 and was named “lateral protocerebral complex” by Sullivan and Beltz (2004). Based on its position, shape, and pattern of immunohistochemical labeling, the lateral protocerebral complex resembles that of the insect mushroom bodies’ peduncle; in addition, its knob-like terminal (arrowheads in Figure 7a–c) resembles that of the ventral lobe like in the stick insect Sypiloidea syplus (Westwood, 1859) which features strong SYNir and an inner fiber layer displaying ASTir (Heuer et al., 2012).

Discussing possible genealogical relationships of insect mushroom bodies and the crustacean hemiellipsoid body/terminal medulla-complex has entertained generations of arthropod neuroanatomists for almost 100 years (see references in Strausfeld, Hansen, Li, Gomez, & Ito, 1998). Whereas Strausfeld and coworkers (Strausfeld et al., 1998) and Harzsch (2006) were hesitant to assign a homology of these protocerebral centers, a study on the hemiellipsoid body of the hermit crab Coenobita clypeatus identified possible similarities of the neuronal core circuits in this neuropil with those in the insect calyx and peduncle (Wolff, Harzsch, Hansson, Brown, & Strausfeld, 2012). More recently, a reanalysis of the brain in Remipedia, the potential sister group to Hexapoda, also suggested structural similarities of their hemiellipsoid bodies and insect mushroom bodies (Stemme, Illife, & Bicker, 2016). In addition, Maza and coworkers (2016) proposed such a genealogical aspects of social behavior, for example, by pair formation (Becker et al., 2005; Johnson, 1969, 1977; Jonasson, 1987; Wolff et al., 2017). Cleaner shrimps have to master identifying a variety of host species and in particular, discriminating interspecific behaviors such as the willingness of a host to be cleaned, even if it is a potential predator. These skills suggest sophisticated learning capabilities with regard to communication which might be reflected in a very pronounced hemiellipsoid body. However, since it is very territorial (Chockley & Mary, 2003), S. hispidus is not considered to display sophisticated navigational skills which are often but not exclusively linked to the need of spatial learning and thus effective multimodal association centers like the mushroom bodies in honey bees or ants (Hexapoda) (Groh, Fleischmann, Grübel, Wehner, & Rössler, 2017; Menzel & Müller, 1996; Webb & Wystrach, 2016), or the hemiellipsoid bodies in mantis shrimps (Stomatopoda) (Wolff et al., 2017), spiny lobsters (Achalata)
(Boles & Lohmann, 2003; Steullet et al., 2002), or robber crabs (Anomala) (Krieger et al., 2010, 2012). A highly territorial lifestyle, as reported for the banded cleaner shrimp, is a behavior essentially dependent on the realization of good place memory, so that spatial learning could play a major role for these animals. Wolff and coworkers (2017) observed that insects with elaborate navigational skills display elaborate mushroom bodies and suggested that place memory is likely to be processed in the crustacean hemiellipsoid body as well as in the insect mushroom body. This hypothesis fits well with the expansion of the hemiellipsoid body in crustaceans such as the coconut crab Birgus latro (Krieger et al., 2010) for which a seminomadic behavior characterized by territorial phases coupled with phases involving elaborate navigational skills have been reported (Krieger, Grandy, et al., 2012). A similar correlation between comparably large hemiellipsoid bodies (original pictures from M. Schmidt; modified from Schmidt & Ache, 1996; combined and equally scaled in Harzsch & Krieger, 2018) and lifestyles involving elaborate spatial memory (review in Sandeman et al., 2014) can be assigned also to the seminomadic spiny lobster Panulirus argus. Deep hydrothermal vent shrimps Rimicaris exoculata live in an extreme, lightless habitat characterized by steep temperature gradients. A recent study showed that these animals also feature sophisticated hemiellipsoid bodies (Machon et al., 2019), so that these authors suggested that an excellent place memory may be essential for avoiding the dangerously hot vent chimneys and memorizing emission sites of hydrothermal fluids rich in those chemicals on which their endosymbiont bacteria depend.

### 4.3 Structural and numerical similarities of the primary deuto- and tritocerebral chemomechanosensory pathways

Due to its specific behavior in advertising and cleaning different fish species, the banded cleaner shrimp requires extraordinary communicative skills. In addition to lateral body swaying, S. hispidus is reported to perform vigorous whipping movements with both its antennules and antennae in presence of potential client fish (Becker et al., 2005) suggesting that at least for luring client fish, the very long flagella of antennules and antennae play a major role. Once attracted, the cleaner shrimp keeps contact with its host fish mainly using the walking legs and claws but only occasionally with its antennules or antennae (Sandeman et al., 2014). In addition to the mammalian neocortex (Kaas, 1997; Penfield & Boldrey, 1937), topographic representations of different sensory modalities in the central nervous system can be found, for example, in the visual (Heinze & Homberg, 2007), as well as in the gustatory and mechanosensory system (Loesel et al., 2013; Nishino, Yokohari, & Mizunami, 2005) of hexapods, and in the chemosensory pathway of the pectine organs in scorpions (Drozd, 2019). These examples show that somatotopic maps are a conserved principle for neuronal circuits in a variety of organisms. Nevertheless, for neuropils processing identical sensory modalities, it is not particularly surprising that more peripheral input needs more computational and hence neuronal substrate in the central brain. The positive correlation between the number of ommatidia and volume of optic lobes (visual neuropils) in males and females (Rein, Zöckler, & Heisenberg, 1999) as, for example, could be shown in Drosophila, supports the idea of topographical representation of a sensory organ. However, the strong correlation between the number of cuspidate setae and their corresponding targets in the brain of S. hispidus is remarkable. Due to the quantitative analysis of only a single specimen (not shown), the morphometric relationship has to be considered a hint and future studies using more replicates have to prove or disprove if the exact relationship holds statistically true.

The median antennular neuropil (MAN) is well identifiable and it is likely that it is served by a separate neurite bundle (MANN; Figures 9a and 10b) of neurons situated somewhere on the antennule being traceable in μCT. The MAN receives statocyst input (of descending interneurons) in both Brachyura and Astacidea as well as afferents from sensilla at the antennular base in Brachyura and Ateleata. Furthermore, branches of antennular motoneurons were identified in Brachyura (Sandeman et al., 1992 and references therein). However, in S. hispidus which is lacking a statocyst on the antennular peduncle (Goy, 2010), the well identifiable median antennular neuropil must process either antennular nonstatocyst input and/or is an additional center of antennular motor control besides the LAN.

### ACKNOWLEDGMENTS

We cordially thank Caroline Viertel for the animal husbandry in the laboratory. We express our gratitude to Erika Becker for performing the paraffin histology and to Mathes Kenning for providing the SEM-image of the antennule of one specimen of S. hispidus. Images of several animals in Figure 1 were kindly provided by Ann-Christin Rath and Rebecca Meth. We gratefully acknowledge Martin Winter for providing preliminary data from an internship as well as Andy Sombke for his supervision during that time. We express our gratitude to two anonymous referees for their fast reviews and constructive criticism. We furthermore want to thank all people indirectly supporting our work by providing open source, open access, or low cost software, such as CombineZM/ZP. (Grant sponsor: German Science Foundation; Grant number: DFG INST 292/119-1 FUGG, DFG INST 292/120-1 FUGG). The monoclonal anti-SYNORF1 synapsin antibody (DSHB Hybridoma Product 3C11; anti SYNONRF1) as deposited to the DSHB.
by E. Buchner, University Hospital Würzburg, Germany, was obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242, USA.

CONFLICT OF INTEREST
The authors declare that they disclose any potential sources of conflict of interest.

AUTHOR CONTRIBUTIONS
J.K., M.K.H., R.E.S., and D.C.S. conducted the experiments and J.K. and M.K.H. arranged the Figures. J.K. and M.K.H. analyzed the data and drafted the manuscript. S.H. assisted in analyzing the data and drafting the manuscript. J.K. and S.H. designed the experimental setup and the study. All authors have read and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID
Jakob Krieger https://orcid.org/0000-0001-8546-3319

REFERENCES
Babu, K. S., & Barth, F. G. (1984). Neuroanatomy of the central nervous system of the wandering spider, Capiennius salei (Arachnida, Araneida). Zoomorphology, 104(6), 344–359. https://doi.org/10.1007/BF00312185
Becker, J. H. A., Curtis, L. M., & Grutter, A. S. (2005). Cleaner shrimp use a rocking dance to advertise cleaning service to clients. Current Biology, 15(8), 760–764. https://doi.org/10.1016/j.cub.2005.02.067
Beltz, B. S., Kordas, K., Lee, M. M., Long, J. B., Benton, J. L., & Sandeman, D. C. (2003). Ecological, evolutionary, and functional correlates of sensilla number and glomerular density in the olfactory system of decadop crustaceans. Journal of Comparative Neurology, 452(2), 260–269. https://doi.org/10.1002/cne.10474
Blest, A. D., & Davie, P. S. (1980). Reduced silver impregnations derived from the Holmes technique. In N. J. Strausfeld & T. A. Miller (Eds.), Neuroanatomical techniques: Insect nervous system (pp. 97–118). New York: Springer-Verlag. https://doi.org/10.1007/978-1-4612-6018-9_7
Bliss, D. E., & Welsh, J. H. (1952). The neurosecretory system of brachyuran Crustacea. Biological Bulletin, 103(2), 157–169.
Boles, L. C., & Lohmann, K. J. (2003). True navigation and magnetic maps in spiny lobsters. Nature, 421(6918), 60. https://doi.org/10.1038/nature01226
Brandt, A. (1988). Morphology and ultrastructure of the sensory spine, a presumed mechanoreceptor of Sphaeroma hookeri (Crustacea, Isopoda), and remarks on similar spines in other peracarids. Journal of Morphology, 198(2), 219–229. https://doi.org/10.1002/jmor.1051980208
Breithaupt, T., & Thiel, M. (2011). Chemical communication in crustaceans. New York: Springer. http://www.springer.com/life+sciences/ecology/book/978-0-387-77100-7
Brusca, G. (1981). On the anatomy of Cystisoma (Amphipoda: Hyperiidae). Journal of Crustacean Biology, 1(3), 358–375. https://doi.org/10.2307/1547968
Cape, S. S., Rehm, K. J., Ma, M., Marder, E., & Li, L. (2008). Mass spectral comparison of the neuropeptide complement of the stomatogastric ganglion and brain in the adult and embryonic lobster, Homarus americanus. Journal of Neurochemistry, 105(3), 690–702.
Chamberlain, S. C., & Barlow, R. B. (1982). Retinotopic organization of lateral eye input to Limulus brain. Journal of Neurophysiology, 48(2), 505–520. https://doi.org/10.1152/jn.1982.48.2.505
Checkley, B. R., & Mary, C. M. S. (2003). Effects of body size on growth, survivorship, and reproduction in the banded coral shrimp, Stenopus hispidus. Journal of Crustacean Biology, 23(4), 836–848. https://doi.org/10.1651/C-2392
Christie, A. E. (2016). Expansion of the neuropeptidome of the globally invasive marine crab Carcinus maenas. General and Comparative Endocrinology, 235, 150–169. https://doi.org/10.1016/j.ygcen.2016.05.013
Christie, A. E., Stemmler, E. A., & Dickinson, P. S. (2010). Crustacean neuropeptides. Cellular and Molecular Life Sciences, 67(24), 4135–4169.
Derby, C., & Thiel, M. (Eds.). (2014). Crustacean nervous systems and their control of behavior. Oxford, New York: Oxford University Press.
Derby, C. D., & Blaustein, D. N. (1988). Morphological and physiological characterization of individual olfactory interneurons connecting the brain and eyestalk ganglia of the crayfish. Journal of Comparative Physiology A, 163(6), 777–794. https://doi.org/10.1007/BF00604055
Derby, C. D., Fortier, J. K., Harrison, P. J. H., & Cate, H. S. (2003). The peripheral and central antennular pathway of the Caribbean stomatopod crustacean Neogonodactylus oerstedii. Arthropod Structure & Development, 32(2), 175–188. https://doi.org/10.1651/51467-8039(03)00048-3
Derby, C. D., Kozma, M. T., Senatore, A., & Schmidt, M. (2016). Molecular mechanisms of reception and Perireception in crustacean chemoreception: A comparative review. Chemical Senses, 41(5), 381–398. https://doi.org/10.1093/chemse/bjw057
Dirckszen, H., Snieke, P., Abel, B., Agricola, H. J., Buchner, K., Muren, J. E., & Nässel, D. R. (1999). Structure, distribution, and biological activity of novel members of the allatostatin family in the crayfish Orconectes limosus. Peptides, 20(6), 695–712.
Drozd, D. (2019). Topographic organization of the pectine neuropils in scorpions: An analysis of chemosensory afferents and the projection pattern in the central nervous system. Wiesbaden: Springer Spektrum. https://www.springer.com/gp/book/9783658251549
Dudoit, A., Iacchei, M., Coleman, R. R., Gaithers, W. E., Bowe, W. B., & Toonen, R. J. (2010). The little shrimp that could: Phylogeny of the circumtropical Stenopus hispidus (Crustacea: Decapoda), reveals divergent Atlantic and Pacific lineages. PeerJ, 6, e4409. https://doi.org/10.7717/peerj.4409
Duffy, J. E., & Thiel, M. (2007). Evolutionary ecology of social and sexual systems: Crustaceans as model organisms. Oxford, New York: Oxford University Press.
Duve, H., Johnsen, A. H., Maestro, J. L., Scott, A. G., Jaros, P. P., & Thorpe, A. (1997). Isolation and identification of multiple neuropeptides of the allatostatin superfamilies in the shore crab Carcinus maenas. Journal of Experimental Biology, 250(3), 727–734.
Duve, H., Johnsen, A. H., Scott, A. G., & Thorpe, A. (2002). Allatostatins of the tiger prawn, Penaeus monodon (Crustacea: Penaeidea). Peptides, 23(6), 1039–1051.
Fahrnback, S. E. (2006). Structure of the mushroom bodies of the insect brain. Annual Review of Entomology, 51(1), 209–232. https://doi.org/10.1146/annurev.ento.51.110104.150954
Farris, S. M. (2005). Evolution of insect mushroom bodies: Old clues, new insights. Arthropod Structure & Development, 34(3), 211–234. https://doi.org/10.1016/j.asd.2005.01.008
Galizia, G. (2008). Insect olfaction. In R. H. Masland, T. D. Albright, R. H. Masland, P. Dallos, D. Oertel, S. Firestein, et al. (Eds.), The senses: A comprehensive reference (Bd. 4, S. 725–769). New York: Academic Press. https://doi.org/10.1016/B978-012370880-9.00123-7
body ground pattern. The Journal of Comparative Neurology, 520(13), 2824–2846. https://doi.org/10.1002/cne.23059

Wolff, G. H., Thoen, H. H., Marshall, J., Sayre, M. E., & Strausfeld, N. J. (2017). An insect-like mushroom body in a crustacean brain. eLife, 6, 24. https://doi.org/10.7554/eLife.29889

Yin, G. L., Yang, J. S., Cao, J. X., & Yang, W. J. (2006). Molecular cloning and characterization of FGLamide allatostatin gene from the prawn, Macrobrachium rosenbergii. Peptides, 27(6), 1241–1250.

Zbinden, M., Berthod, C., Montagné, N., Machon, J., Léger, N., Chertemps, T., … Ravaux, J. (2017). Comparative study of chemosensory organs of shrimp from hydrothermal vent and coastal environments. Chemical Senses, 42(4), 319–331. https://doi.org/10.1093/chemse/bjx007

Zhang, D., Cai, S., Liu, H., & Lin, J. (2008). Antennal sensilla in the genus Lysmata (Caridea). Journal of Crustacean Biology, 28(3), 433–438. https://doi.org/10.1651/07-2876R.1

---

**How to cite this article:** Krieger J, Hörnig MK, Sandeman RE, Sandeman DC, Harzsch S. Masters of communication: The brain of the banded cleaner shrimp *Stenopus hispidus* (Olivier, 1811) with an emphasis on sensory processing areas. J Comp Neurol. 2020;528:1561–1587. https://doi.org/10.1002/cne.24831