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Neurochemical diversity in the central olfactory pathway of the crustacean *Parhyale hawaiensis* (Amphipoda): evolutionary implications

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Abstract

In mandibulate arthropods, the primary olfactory centers, termed olfactory lobes in crustaceans, are typically organized in distinct fields of dense synaptic neuropils called olfactory glomeruli. In addition to olfactory sensory neuron terminals and their postsynaptic efferents, the glomeruli are innervated by diverse neurochemically distinctive interneurons. The functional morphology of the olfactory glomeruli is understudied in crustaceans compared with insects and even less well understood and described in a particular crustacean subgroup, the Peracarida, which embrace, for example, Amphipoda and Isopoda. Using immunohistochemistry combined with confocal laser scanning microscopy, we analyzed the neurochemistry of the olfactory pathway in the amphipod *Parhyale hawaiensis*. We localized the biogenic amines serotonin and histamine as well as the neuropeptides RFamide, allatostatin, orcokinin, and SIFamide. As for other classical neurotransmitters, we stained for γ-aminobutyric acid and glutamate decarboxylase and used choline acetyltransferase as indicator for acetylcholine. Our study is another step in understanding principles of olfactory processing in crustaceans and can serve as a basis for understanding evolutionary transformations of crustacean olfactory systems.

KEYWORDS
allatostatin, crustaceans, GABA, histamine, olfactory system, orcokinin, peracarida, RFamide, serotonin, SIFamide

1 | INTRODUCTION

In the brains of malacostracan crustaceans such as crayfish, crabs, and spiny lobsters, the olfactory lobes (OLs) are the primary sensory centers that process olfactory input (Derby & Weissburg, 2014; Harzsch & Krieger, 2018; Schachtner et al., 2005; Schmidt & Mellon, 2010). They are structured in discrete synaptic neuropils, the olfactory glomeruli, that are radially arranged around a fibrous core that is made up by the afferent and efferent neurites that supply the glomeruli (Derby & Weissburg, 2014; Harzsch & Krieger, 2018; Schachtner et al., 2005; Schmidt & Mellon, 2010). These fundamental units of olfactory processing are sites where axons from olfactory sensory neurons synapse with local olfactory interneurons and projection neurons (PNs) that target certain other brain regions. Odorants are detected by ionotropic olfactory receptor molecules associated with the dendrites of the olfactory sensory neurons within the aesthetascs, specialized, unimodal sensilla on the 1st antennal pair (Derby et al., 2016; Hallberg & Skog, 2010; Schmidt & Mellon, 2010; Wicher

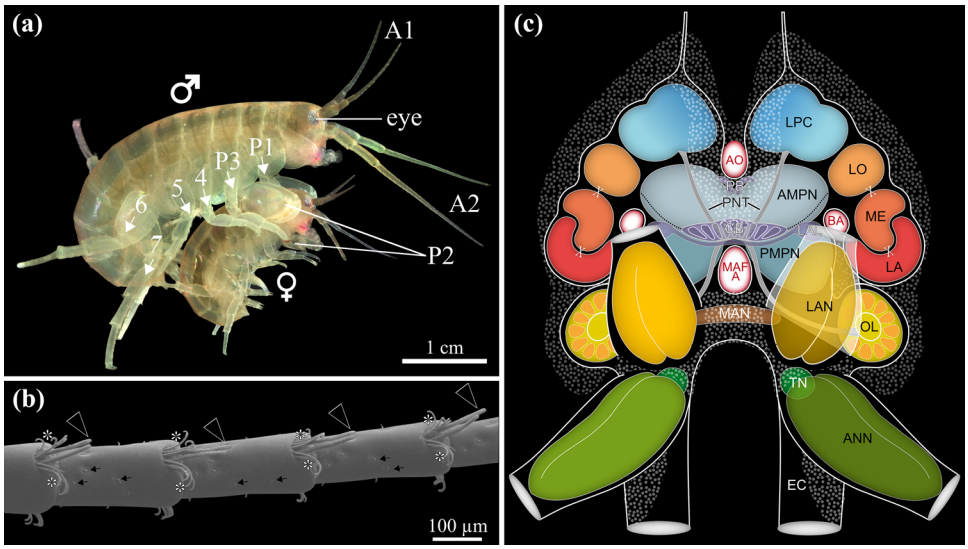

FIGURE 1 *Parhyale hawaiensis* in precopula, 1st antenna, and brain scheme. (a) Picture of a male and a female *P. hawaiensis* in precopula (modified from Wittfoth et al., 2019, creative commons license). (b) Scanning electron microscopy image of 1st antennae of *P. hawaiensis* (modified with permission from Krieger et al., 2021). Arrowheads indicate rod-shaped aesthetascs, asterisks show sensilla hairs and arrows indicate different types of mechanosensory sensilla. (c) Schematic representation of the brain of *P. hawaiensis* (modified from Wittfoth et al., 2019 creative commons license). A1, antenna 1; A2, antenna 2; AMPN, anterior medial protocerebral neuropil; ANN, antenna 2 neuropil; AO, anterior aorta; BA, brain artery; CB, central body; EC, esophageal connective; LA, lamina; LAL, lateral accessory lobe; LAN, lateral antenna 1 neuropil; LO, lobula; LPC, lateral protocerebrum; MAF A, myoarterial formation a; MAN, medial antennae 1 neuropil; ME, medulla; OL, olfactory lobe; P1–7, pereopods 1–7; PB, protocerebral bridge; PMPN, posterior medial protocerebral neuropil; PNT, projection neuron tract; TN, tegmentary neuropil.

& Große-Wilde, 2017). PNs connect the OL to the lateral protocerebrum (LPC) that comprises higher multimodal neuropils, the mushroom body, and the terminal medulla (review Harzsch & Krieger, 2021). A second, parallel chemosensory pathway is present that originates from bimodal, combined chemo- and mechanosensory sensilla on the first antennal pair, the axons of which target a distinct primary sensory area in the brain, the lateral antenna 1 neuropil (LAN) (Derby & Weissburg, 2014; Harzsch & Krieger, 2018; Schachtner et al., 2005; Schmidt & Mellon, 2010). The presence of such a dual chemosensory pathway driven by bimodal sensilla and aesthetascs on the first antennal pair was suggested to be a feature of the malacostracan ground pattern (Kenning et al., 2013).

Many different neurotransmitters are present in the local olfactory interneurons of crustaceans. So far, the presence of "classical" neurotransmitters such as acetylcholine (ACh), γ-aminobutyric acid (GABA), L-glutamate, and nitric oxide was shown in crustaceans, as well as the presence of biogenic amines such as serotonin, dopamine, histamine, and octopamine and diverse neuropeptides (summarized in table 1, reviewed in Harzsch et al., 2021; Schachtner et al., 2005). Different neurotransmitters provide the basis for a regionalization of the olfactory glomeruli of decapod crustaceans into cap, subcap, and base along their long axis (Harzsch et al., 2012; Harzsch et al., 2021; Polanska et al., 2020). Notably, the neurochemistry of olfactory glomeruli is best understood in decapod crustaceans such as crayfish, crabs, and lobsters (Table 1), whereas large gaps in our knowledge exist for nondecapod Malacostraca.

With our study on *Parhyale hawaiensis* (Dana 1853) (Peracarida, Amphipoda, Hyalidae; Figure 1a), we aim to improve our understanding of olfactory systems in non-decapod crustaceans. This species is an epibenthic marine amphipod occupying tropical and subtropical intertidal marine habitats such as estuaries and mangroves (Shoemaker, 1956; Tararam et al., 1978). It reproduces year-round (Alegretti et al., 2016) and is considered an emerging crustacean model species (Rehm et al., 2009; Stamataki & Pavlopoulos, 2016). Multiple genetic tools were established for this species such as CRISPR-mediated gene editing (Martinet al. et al., 2016), gene knockdown (Liubicich et al., 2009; Özhan-Kizil et al., 2009; Vargas-Vila et al., 2010), and a sequenced genome is available (Kao et al., 2016). Furthermore, a detailed atlas of its brain anatomy was published (Figure 1c; Wittfoth et al., 2019) as well as a description of its visual system (Ramos et al., 2019) and of the first antennae with rod-like aesthetascs and different types of sensilla (Figure 1b; Krieger et al., 2021). A comparison of available information on the olfactory system in *P. hawaiensis* (Wittfoth et al., 2019) with that on representatives of basal malacostracan taxa such as Leptostraca (Kenning et al., 2013), Mysidacea and Euphausiacea (Johansson, 1991, Johansson & Hallberg 1992b, Moreau et al., 2002), marine Isopoda (Harzsch et al., 2011; Kenning & Harzsch, 2013), Stomatopoda (Derby et al., 2003), Dendrobranchiata (Meth et al., 2017), and Stenopodidea (Krieger et al., 2012) suggests that concerning number and shape of olfactory glomeruli, *P. hawaiensis* falls within the range represented by these basal lineages. All of the aforementioned taxa feature between 60 and 90 spherical olfactory glomeruli, as opposed to members of the Reptantia, which feature several hundreds to in excess of thousand wedge-shaped olfactory glomeruli (review Harzsch & Krieger, 2018).

We used immunohistochemistry to explore the glomerular structure in the OL of this amphipod and its neurochemical diversity. This study gives an overview of the functional morphology of the central olfactory pathway in this species and provides new information on the

TABLE 1 Neurochemistry of local olfactory interneurons in selected crustacean taxa. *Abbreviations*: 5-HT, serotonin; ChAT, choline acetyltransferase; FaRPs, FMRFamide-related peptides; GABA, γ–aminobytric acid; GnRH, gonadotropin-releasing hormone-like peptides; SCPB, small cardioactive peptide B; TH, tyrosine hydroxylase; TRP, tachykinin-related peptide.

	Spiny lobster (Achelata)	Clawed lobster (Homarida)	Crayfish (Astacida)	Hermit crabs (Anomala)	True crabs (Brachyura)	Peracarida
5-HT	Schmidt & Ache, 1997	Beltz et al., 1990; Beltz, 1999; Helluy et al., 1993; Langworthy et al., 1997	Mellon & Alones, 1993; Sandeman et al., 1988; Sandeman & Sandeman, 1994; Sandeman et al., 1995; Sandeman & Sandeman, 1987	Harzsch & Hansson, 2008; Polanska et al., 2020; Harzsch et al., 2021	Antonsen & Paul, 2001; Johansson, 1991; Kotsyuba & Dyachuk, 2021; Krieger et al., 2010, 2015	Johansson & Hallberg, 1992b; Thompson et al., 1994; Moreau et al., 2002; Helluy & Thomas, 2003; Kenning & Harzsch, 2013; **Present report**
ChAT	?	?	?	?	Kotsyuba & Dyachuk, 2021	**Present report**
TH	?	?	?	Kotsyuba, 2012	?	?
GABA	Wachowiak et al., 1997; Wachowiak & Ache, 1997; Orona et al., 1990	?	?	?	?	**Present report**
Dopamine	Schmidt & Ache, 1994, 1997	Schmidt, 1997	?	?	?	?
Histamine	Orona & Ache, 1992; Wachowiak & Ache, 1997	Langworthy et al., 1997	?	?	?	**Present report**
Nitric oxide	?	Benton et al., 2007; Scholz et al., 1998	Johansson & Mellon, 1998	?	Kotsyuba et al., 2010; Kotsyuba & Dyachuk, 2021	?
Allatostatin	?	?	Yasuda-Kamatani & Yasuda, 2006	Harzsch & Hansson, 2008; Krieger et al., 2012; Krieger et al., 2010; Polanska at al. et al., 2012, Harzsch et al. 2021	Krieger et al., 2010, 2015	**Present report**
Enkephalin	?	?	Ollivaux et al., 2002	?	?	?
FaRPs	Schmidt, 1997; Schmidt & Ache, 1994, 1997	Kobierski et al., 1987	Yasuda-Kamatani & Yasuda, 2006	Harzsch & Hansson, 2008; Krieger et al., 2012; Polanska et al., 2012; Schachtner et al., 2005; Harzsch et al. 2021	Kotsyuba & Dyachuk, 2021	Johansson & Hallberg, 1992b; Harzsch et al., 2011; Kenning & Harzsch, 2013; **Present report**

(Continues)

TABLE 1 (Continued)

	Spiny lobster (Achelata)	Clawed lobster (Homarida)	Crayfish (Astacida)	Hermit crabs (Anomala)	True crabs (Brachyura)	Peracarida
GnRH	?	?	?	?	Saetan et al., 2013	?
Orcokinin	?	?	Yasuda-Kamatani & Yasuda, 2006	Polanska et al., 2020	?	**Present report**
Proctolin	?	?	?	?	Wood et al., 1996	?
SCP_B	Schmidt & Ache, 1997	Langworthy et al., 1997	?	?	?	?
SIFamide			Polanska et al., 2007	Krieger et al., 2010	?	**Present report**
Substance P	Schmidt & Ache, 1994; 1997	Langworthy et al., 1997	Sandeman et al., 1990; Schmidt, 1997	Schmidt, 1997	Schmidt, 1997	?
TRP	?	?	Johansson et al., 1999; Yasuda-Kamatani & Yasuda, 2006	?	?	?

localization of various neurochemicals in the OL including the biogenic amines serotonin and histamine, the neuropeptides RFamide (part of the FMRFamide family), A-type allatostatin, orcokinin and SIFamide, and the "classical" neurotransmitters GABA, and its catalyzing enzyme glutamic acid decarboxylase (GAD), as well as choline acetyltransferase (ChAT), the enzyme catalyzing ACh. In combination with labeling of presynaptic proteins (synapsins), our study provides novel insights into the diverse neurochemistry of the central olfactory pathway and structural regionalization of the olfactory glomeruli in cap and base regions. We discuss our findings with regard to the evolution of the olfactory systems in crustaceans.

2 | MATERIAL AND METHODS

2.1 | Experimental animals

P. hawaiensis were reared in aquaria with aerated artificial seawater (32 PSU) at approximately 25°C, under a 12/12 h dark/light cycle. Animals were fed three times a week with JBL NovoCrab. For all experiments, large, sexually mature males where used. Our description is based on a minimum of 10 OLs per marker set.

2.2 | Immunohistochemistry

Immunohistochemical labeling was carried out for eight neurotransmitters (Table 2). Costaining for neuropils was done with an antiserum directed against a family of presynaptic proteins, the synapsins. For labeling nuclei, the DNA markers Hoechst, POPO-1, and Sytox green were used (table 2).

Animals were chilled at 4°C, then decapitated, and their heads were fixed overnight in 2 or 4% paraformaldehyde (PFA) (Carl Roth, 0335.2, lot 511179307) with 5 % Glucose (Sigma, G-8270, lot 116H1200) in phosphate-buffered saline (PBS) at 4°C. Subsequently, their brain was dissected. The brain tissues were then washed repeatedly with PBS with 1% Triton X-100 (PBS-Tx; Sigma–Aldrich; X100-500ML, lot STBJ5677), 1.5% dimethyl sulfoxide (DMSO; Carl Roth; A994.2, lot 391310628) and 1% normal goat serum (Millipore; S26-100ML, lot 3603058) for serotonin staining, and 1% bovine serum albumin (BSA; Sigma; A5253-250G, lot SLBL7392V) for all other neurotransmitters. DMSO was used for better permeabilization of the cell membranes to increase the penetration of the antibodies. Five washing steps were carried out PBS-Tx and DMSO, 2 × 15 min, 2 × 30 min, 60 min. For antihistamine staining, heads were first incubated in 4% N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDAC; Sigma–Aldrich E6383) for 4 h at room temperature (RT) and then fixed for 4 h in 2% PFA with 5% glucose. Brains were subsequently dissected. Brains were incubated for 3.5 days in the primary antisera at RT and gentle agitation.

After incubation with the primary antisera, the specimens were washed with PBS-Tx with NGS for 2 × 10 min, 2 × 20 min, then incubated in the secondary antisera for 2.5 days at RT and gentle agitation.

TABLE 2 Antisera and reagents used for double labeling experiments.

Primary antisera	Final dilution	Manufacturer	RRID
Rabbit anti-Asn13-orcokinin, polyclonal	1:1000–1:2000	Generous gift of Heinrich Dircksen (Stockholm University)	AB_2315017
Rabbit anti-5-hydroxtryptopamin (serotonin), polyclonal	1:1000	Immunostar, #20080	AB_572263
Rabbit anti-FMRFamide, polyclonal	1:2000	Immunostar, #20091	AB_572232
Rabbit anti-A-type Dip allatostatin I, polyclonal	1:2000	abcam, #ab53956	AB_879536
Rabbit anti-histamine, polyclonal	1:1000	Progen, #16043	AB_2892841
Rabbit anti-SIFamide	1:1500	(Janssen et al., 1996)	AB_2569992
Rabbit anti-GABA, polyclonal	1:1000	Sigma, #A2052	AB_477652
Rabbit anti-GAD, polyclonal	1:2000	Sigma, #G5163	AB_477019
Mouse anti-ChAT, monoclonal	1:500	Developmental Studies Hybridoma Bank #4B1	AB_528122
Mouse anti-synapsin 3c11	1:10	Developmental Studies Hybridoma Bank #3C11	AB_528479
Secondary antisera			
Alexa Flour488 goat anti-mouse IgG (H+L), polyclonal	1:500	Invitrogen, cat# A11008	AB_143165
Cy3-AffiniPure goat anti-mouse IgG (H+L), polyclonal	1:500	Jackson Immuno, cat# 115-165-003	AB_2338680
Cyanina3 goat anti-rabbit IgG (H+L), polyclonal	1:500	Invitrogen, cat# A10520	AB_2534029
DNA marker			
Hoechst 33258	1:10,000	Thermo Fisher cat# H1398	NA
Hoechst 33324	1:10,000	Thermo Fisher cat# 62249	NA
Sytox Green	1:10,000	Invitrogen cat# S7020	NA
POPO-1	1:10,000	Thermo Fisher P3580	NA

Brains were washed and infiltrated in a graded three-step PBS:glycerol series (1:1, 1:3 PBS:glycerol). Brains were then mounted on slides in glycerol or mowiol (2.4 g mowiol, 6 g glycerol, 6 mL H_2O, 12 mL 0.2 M Tris–HCl; Carl Roth; lot 204203539).

In control experiments, primary antisera were replaced by PBS-Tx, and all labeling was blocked.

Antisera were chosen to represent a wide range of neurotransmitters including monoamines, peptides, inhibitory, and "classical" neurotransmitters that are known to be present in the crustacean central olfactory pathway (Table 1). Describing a large variety of neurotransmitters can serve as an anatomical basis of the supposed model system *P. hawaiensis*, as this species is severely lacking in anatomical descriptions of its brain. The individual markers were chosen for two main reasons: (1) already existing, well-established protocols, which were easy to adapt, and (2) preliminary results that had indicated successful labeling with the specific antiserum in *P. hawaiensis*.

2.3 | Specificity of the antisera

2.3.1 | Synapsin

The monoclonal anti-*Drosophila* synapsin SYRNOF1 antibody (Developmental Hybridoma Bank (DSHB) Hybridoma Product 3C11, anti-SYRNOF1 as deposited to the DSHB by E. Buchner, University Hospital Würzburg, Germany; supernatant) was raised against *Drosophila melanogaster* GST–synapsin fusion protein and recognizes at least four different synapsin isoforms (70, 74, 80, and 143 kDa) in western blots of *D. melanogaster* head homogenates (Klagges et al., 1996). In a western blot analysis of brain tissue of *D. melanogaster* and the crustacean *Coenobita clypeatus*, the antibody stained a strong band around 80–90 kDa in both species (Harzsch & Hansson, 2008). No further experiments for the specificity of this antiserum in *P. hawaiensis* were conducted so that we will refer to the labeling as "synapsin-like immunoreactivity" (synapsin-like IR).

2.3.2 | Serotonin

The biogenic amine serotonin (5-hydroxytryptopamine; 5-HT) is conserved in invertebrates and has been described in the brains of many insects and crustaceans (reviewed in: Harzsch et al., 2005; Kutsch & Breidbach, 1994; Schachtner et al., 2005). Serotonin plays a role in the development of the olfactory pathway, as serotonin depletion in *H. americanus* inhibits the branching of olfactory PNs (Sullivan et al., 2000). We used a polyclonal antiserum raised in rabbit against serotonin coupled to BSA with PFA. Preadsoprtion experiments with 10–25 μg serotonin coupled to BSA per milliliter of diluted antiserum

eliminated all specific staining (Harzsch et al., 2021). According to the manufacturer, using the horse radish peroxidase labeling method, 5-hydroxytryptophan, 5-hydroxyindole-3-acetic acid, and dopamine did not react with the 1:20,000 diluted antiserum. Since we did not analyze the specificity of the antiserum any further, the term "serotonin-like IR" will be used.

2.3.3 | Histamine

The biogenic amine histamine plays an inhibitory role in the crustacean central olfactory pathway (Bayer et al., 1989; McClintock & Ache, 1989). In the lobster *Panulirus argus*, histamine is present in olfactory tissues, including the OL, and can be localized in the cap regions of the glomeruli as well as in local olfactory interneurons (Orona et al., 1990). The polyclonal antihistamine antiserum used in our study was raised in rabbit against histamine conjugated to human serum albumin. For specific staining, a prefixation of the tissue with EDAC is needed. According to the manufacturer, preadsoprtion of the diluted antiserum with 10–100 μg histamine eliminated the staining, while preadsoprtion with noradrenaline, serotonin, VIP, glucagon, and histidine had no effect (Rieger & Harzsch, 2008). As we did not do any preadsorption experiments ourselves, we will refer to the labeling in our study as "histamine-like IR."

2.3.4 | Allatostatin A-like peptides

A-type allatostatins form a large family of neuropeptides sharing the YXFGLamide motif and were found in decapod and nondecapod crustaceans (Christie et al., 2008; Dircksen et al., 1999; Dircksen et al., 2011; Duve et al., 2002; Kress et al., 2016; Nässel & Homberg, 2006; Stay & Tobe, 2007; Yasuda,-Kamatani & Yasuda, 2006). The antiserum used in our experiments was raised against *Diploptera punctata* A-type Dipallatostatin I, ASPSGAQRLYGFGLamide, coupled to bovine thyroglobulin using glutaraldehyde (Vitzthum et al., 1996). It has previously been used to localize allatostatin in crustaceans (Dircksen et al., 1999; Harzsch & Hansson, 2008; Krieger et al., 2012; Utting et al., 2000). In noncompetitive ELISAs, the antiserum displayed no crossreactivity with Proctolin and FMRFamide, amongst others, additionally, preadsorption experiments with the diluted antiserum against Dipallatostatin I abolished all immunolabeling in brain sections of *Schistocerca gregaria* (Vitzthum et al., 1996). The term "allatostatin-like IR" will be used in this study, since it is possible that a polyclonal antiserum might label related peptides.

2.3.5 | Orcokinin-like peptides

Orcokinins are a conserved family of neuropeptides found in many crustaceans and insects (Bungart et al., 1994; Hofer & Homberg, 2006a, 2006b; Hofer et al., 2005. We used a polyclonal rabbit Asn^{13}-orcokinin antiserum, which was raised against glutaraldehyde-conjugate of bovine thyroglobulin and synthetic orcokinin (Bungart et al., 1994). The antiserum showed almost full reactivity withVal13-orcokinin (Dircksen et al., 2000) and showed clear staining in *C. clypeatus* and *Pagus berhardus* (Harzsch et al., 2021; Polanska et al., 2020). As we did not do any preadsorption experiments ourselves nor an analysis of the genome, we will refer to the labeling in our study as "orcokinin-like IR."

2.3.6 | FMRF amide-like peptides

The FMRFamide and FMRFamide-related peptides are distributed among numerous invertebrates and constitute a large neuropeptide family with over fifty members, all sharing the RFamide motif (reviewed in: Dockray, 2004; Greenberg & Da Price, 1992; Nässel, 1993; Homberg, 1994; Nässel & Homberg, 2006). In malacostracan crustaceans, at least 12 FMRFamide-related peptides have been identified and sequenced in crabs, crayfish, lobsters, and shrimps (Huybrechts et al., 2003; Mercier et al., 2003). Peptides of the FMRFamide family are shown to be cardioexcitatory (Greenberg & Price, 1979; Griffond et al., 1986) and different members of the peptide family can be excitatory as well as inhibitory (Papaioannou et al., 2005). The antiserum used in our experiments was raised in rabbit against synthetic FMRFamide conjugated to bovine thyroglobulin. Preadsorption of the diluted antiserum with 100 μg/ml of FMRFamide completely eliminates all immunohistochemical labeling, according to the manufacturer as well as in experiments with *C. clypeatus* (Harzsch & Hansson, 2008). Since the common denominator of crustacean FMRF-related peptides so far is the carboxyterminal sequence LRFamide, we expect the antiserum to label any peptides terminating with the sequence RFamide. For that reason, we will refer to the labeled structures in our specimens as "RFamide-like IR."

2.3.7 | SIFamide

Immunohistochemistry of the neuropeptide SIFamide in two *Procambaraus* species shows a distribution of SIFamide in the OL, accessory lobe, and the olfactory projection tract, which implies a role of SIFamide in crustacean olfaction (Polanska et al., 2007; Yasuda et al., 2004; Yasuda,-Kamatani & Yasuda, 2006). The distribution of SIFamide is described using an GYRKPPFNGSIF-antiserum (crustacean SIFamide antiserum) raised in rabbit, conjugated to BSA by the material basis set method with NH_2 (Yasuda et al., 2004). SIFamide is encoded in the genome of the amphipod *Echinogammarus veneris*, further proving the specificity of the staining (Christie, 2014). As we did not do any preadsorption ourselves nor an analysis of the genome, we will refer to the labeling in our study as "SIFamide-like IR."

2.3.8 | GABA and GAD

GABA is one of the main inhibitory neurotransmitters in the olfactory pathway in the central nervous system of vertebrates and inverte-

brates (reviewed in Freund & Buzsáki, 1996; Halasz & Shepherd, 1983; Laxmyr, 1984; Mori, 1987). GABA is synthesized through GAD from the amino acid glutamate. Like histamine, GABA is an inhibitory neurotransmitter in the olfactory pathway of crustaceans (Orona et al., 1990; Wachowiak & Ache, 1997, reviewed in Wachowiak et al., 2002). GABA acts as a presynaptic inhibitor through reduction of action potential amplitude at the axon terminal (Wachowiak & Ache, 1997, 1998; Wachowiak & Cohen, 1998; Wachowiak et al., 2002). The GABA receptor-antagonist picrotoxin increases the amplitude of hyperpolarization of odor-evoked responses in *P. argus* (Wachowiak & Cohen, 1998). The polyclonal antiserum against GABA was produced in rabbit with GABA–BSA as an immunogen. It has a wide range with positive staining in many insects, for example *Drosophila* (Seki et al., 2010), *Apis* (Ai et al., 2017; Mogily et al., 2020), *Nymphalidae* (Carlsson et al., 2013), and *Hemissenda* (Webber et al., 2017). According to the manufacturer, the antiserum showed positive binding with GABA but not with BSA in a dot blot assay.

The polyclonal antiglutamic acid decarboxylase 65/67 antiserum was raised in rabbit and stains both isoforms, GAD_{65} and GAD_{67}. According to the manufacturer, it was produced using a synthetic peptide KDIDFLIEEIERLGQDL corresponding to the C-terminal region of GAD_{67} of human origin coupled to KLH with glutaraldehyde. The antiserum was purified to provide an IgG fraction of the antiserum. Both isoforms can be found in the brains of rats and mice (Kaufman et al., 1991). The x-organ in the crayfish *Procambarus clarkii* showed immunoreactivity against GAD (Pérez-Polanco et al., 2011), the antiserum used was raised against the mammalian isoforms GAD_{65} and GAD_{67} (Chang & Gottlieb, 1988; Kaufman et al., 1991; Pérez-Polanco et al., 2011), showing that an antiserum raised against mammalian GAD can successfully stain neurons in crustacean brains. As we did not do any preadsorption experiments ourselves nor an analysis of the genome, we will refer to the labeling in our study as "GABA-like IR" and "GAD-like IR."

2.3.9 | ChAT

Only a few examples describe antisera staining for ChAT in crustaceans, for example, in the barnacle *Balanus amphitrite* (Gallus et al., 2006), and in the nervous system of *Hemigrapsus sanguineus* (Kotsyuba & Dyachuk, 2021) and *Paralithodes camschaticus* (Kotsyuba & Dyachuk, 2022). However, ChAT staining is well established in *D. melanogaster* (Seki et al., 2010; Shang et al., 2007; Yasuyama et al., 1995) and other insects like *Manduca sexta* (Clark et al., 2005; Torkkeli et al., 2005) and *Periplanta americana* (Fusca et al., 2015). The monoclonal antibody against *D. melanogaster* ChAT, isotype MIgG1 was raised in mouse. The immunogen was a recombinant fusion protein, the alternate antibody name is 4B1. It was first used against *D. melanogaster* ChAT in larval neuropils (Yasuyama et al., 1995). As we did not do any preadsorption experiments ourselves nor an analysis of the genome, we will refer to the labeling in our study as "ChAT-like IR."

2.4 | Imaging

Immunohistochemical preparations were scanned using a Leica TCS SP5 II confocal laser-scanning microscope equipped with argon-, DPSS-, and diode-lasers and operated with the Leica Application Suite Advanced Fluorescence software package (LAS AF). For the analysis, at least 10 successfully stained and imaged OLs were used per marker set. Single optical sections, as well as z-projections were globally enhanced in ImageJ (Version 2.9.0, Schindelin et al., 2012). To trace neurons, the plugin "simple neurite tracer" (Longair et al., 2011) in ImageJ was used.

2.5 | Nomenclature

We follow the neuroanatomical nomenclature proposed by Wittfoth et al. (2019), which is based on the studies by Sandeman et al. (1992) and Richter et al. (2010), with modifications as suggested by Loesel et al. (2013). We deviate from this nomenclature in that we do not distinguish hemiellipsoid body and terminal medulla (Wittfoth et al., 2019) but refer to these fused areas as "lateral protocerebrum" (Krieger et al., 2021). Additionally, we deviate from Wittfoth et al. (2019) by using the term "olfactory lobe" and addressing the soma cluster wrapped around the OL as "lateral cluster" (Figure 3). The orientation of the brain is described according to bodyaxis. Note that the neuroaxis in this animal strongly differs from the bodyaxis in that the brain is bent dorsally and backward (Wittfoth et al., 2019).

3 | RESULTS

3.1 | Overview

Synapsin-like immunoreactivity provides an overview of the characteristic brain neuropils (Figures 2a–c; compare Wittfoth et al., 2019). The visual neuropils lamina (LA), medulla (ME), and lobula (LO) can be clearly distinguished, with the lamina displaying a particularly strong signal (compare Ramos et al., 2019). The protocerebrum is composed of the confluent neuropils of the LPC and the anterior median protocerebral neuropil (AMPN) and the posterior medial protocerebral neuropil (PMPN). The central body (CB) is an unpaired, transverse neuropil at the interface between the AMPN and the PMPN. In the deutocerebrum, the LAN and OL are clearly demarcated, the former being notably larger than the latter. The tritocerebrum is primarily made up by the antenna 2 neuropil (ANN).

In decapod crustaceans, the neuronal somata are clustered in distinct groups (Sandeman et al., 1992), with cell clusters (9) and (11) comprising the somata of local olfactory interneurons, and cluster (10) the somata of the olfactory PNs. Labeling the neuronal nuclei with a DNA marker reveals that in *P. hawaiensis*, a conspicuous cell cluster is present medially to the OL close to the LAN (Figure 3a), and a second cluster more laterally. In a different optical section, however, these two clusters are confluent (Figure 3b). In our previous study (Wittfoth

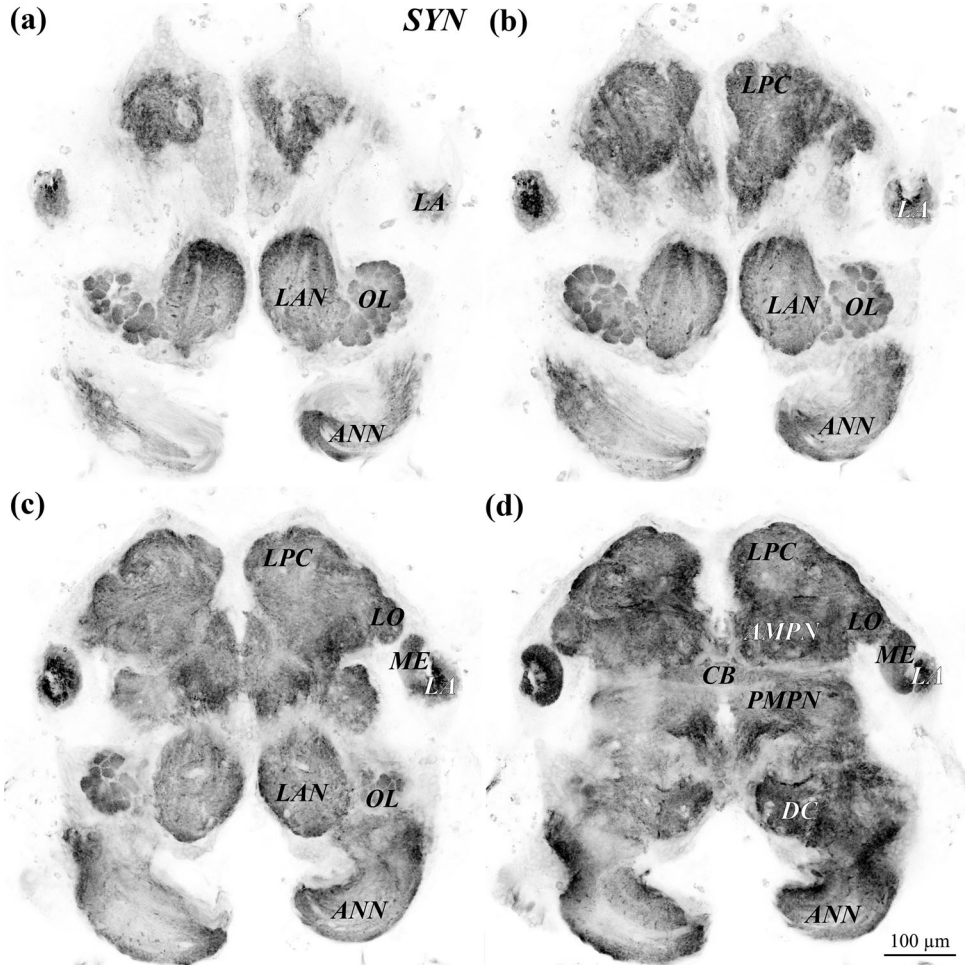

FIGURE 2 Synapsin. (a)—(d) Consecutive single optical sections (thickness 0.63 μm) of a brain whole-mount labeled with the antiserum against synapsins (black-white inverted images) to provide an overview over the brain neuropils. Dorsal is toward the top. AMPN, anterior medial protocerebral neuropil; ANN, antennae 2 neuropil; CB, central body; LA, lamina; LAN, lateral antenna 1 neuropil; LO, lobula; LPC, lateral protocerebrum; ME, medulla; OL, olfactory lobe; PMPN, posterior medial protocerebral neuropil.

et al., 2019), we had suggested a correspondence of the cell clusters in *P. hawaiensis* and decapod crustaceans. However, a reexamination of interactive supplementary Figure 6 from Wittfoth et al. (2019), which shows a reconstruction of individual neuronal nuclei from a histological section series (Figures 3c and d) confirms the presence of one more medial and one more lateral cell cluster, which are confluent and therefore cannot be clearly separated. While our data show that most somata of local interneurons are located in the lateral group, in the absence of a specific marker for the PNs, we cannot attribute their somata to either the lateral or medial group. Hence, at this point, we cannot draw any meaningful comparison of the situation in *P. hawaiensis* to the cell clusters comprising the olfactory interneurons in decapod crustaceans (Sandeman et al., 1992).

3.2 | Serotonin (5-HT)

Serotonin-like immunoreactivity (serotonin-like IR) can be found in most neuropils in the brain, with a total number of serotonin-positive somata of 64.1 ± 6 (N = 7). Between the left and right LPC, a cell cluster is situated, which embraces roughly 20 somata with strong serotonin-like IR (Figure 4a, asterisk). These somata innervate the LPC and form a characteristic commissural fiber bundle below the anterior aorta (Figure 4a, arrow). All three visual neuropils, LA, ME, and LO, are innervated by serotonergic fibers (Figure 4b). A paired cluster of approximately five somata with strong serotonin-like IR is located above the LO (Figure 4b, asterisk). Additional staining can be found in the AMPN (Figure 4a). The CB shows prominent IR in its ventral half. Another commissural fiber bundle with serotonin-like IR can be found posterior-ventrally below the CB (Figure 4a, double arrow). All neuropils in the deutocerebrum show clear serotonin-like IR (Figures 4a and c). However, the OL is the neuropil with the faintest overall staining (Figures 4a, c, and g).

In the OL, serotonin-like IR was dispersed throughout the lobe with sparse serotonin-like IR in the glomeruli and short, pronounced fibers between the glomeruli, giving the appearance of more immunolabeled fibers between the glomeruli than within the glomeruli (Figures 4f and g). Serotonin-like IR is also present in the fibrous core of the OL.

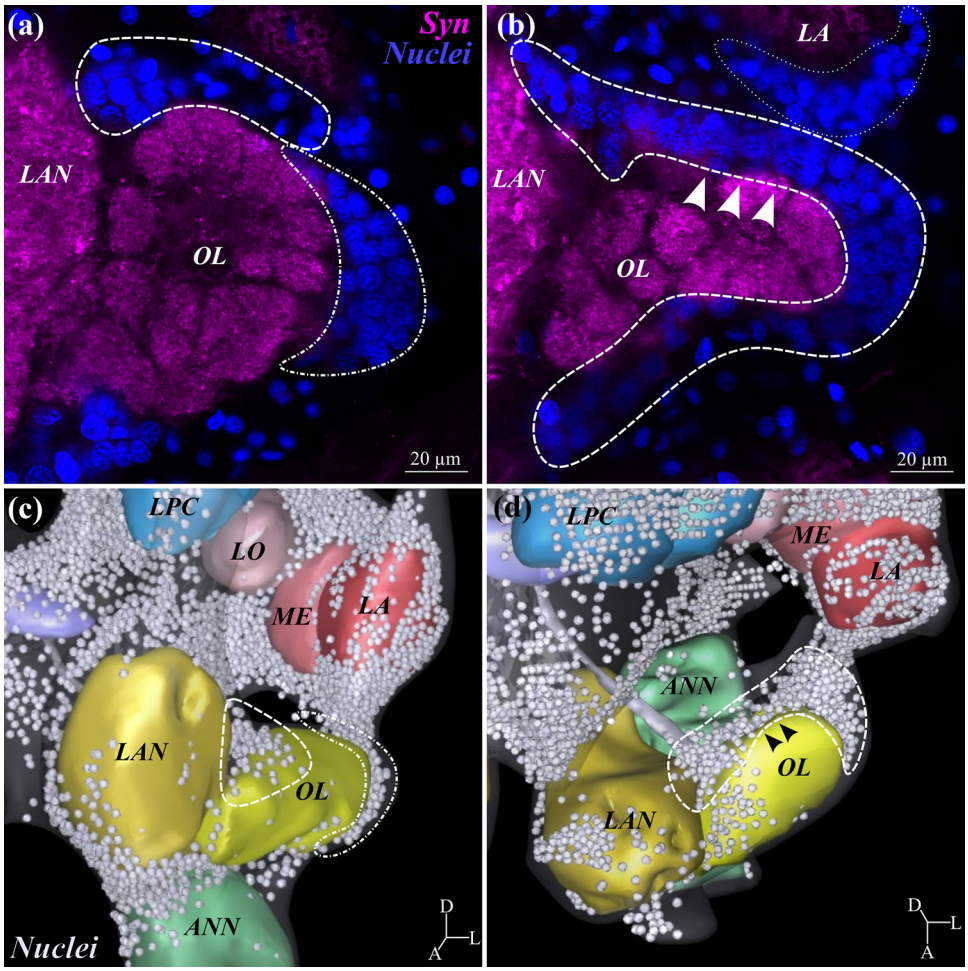

FIGURE 3 Cells associated with the olfactory lobe. (a) Single optical section (thickness 0.3 μm) showing two nuclei cluster surrounding the right olfactory lobe, indicated by a dashed and a dashed-dotted line (synapsin-like immunoreactivity in magenta, cell nuclei in blue; dorsal is toward the top). (b) single optical section (0.3 μm thickness) showing that the cell clusters are connected, indicated by arrowheads and the dashed line, the dotted line indicates cell cluster surrounding the lamina. (c) 3D rendering of the brain modified from Wittfoth et al. (2019; their additional file 6), two soma clusters are indicated with a dashed and a dashed-dotted line, dorsal is toward the top of the picture. (d) same 3D rendering turned forward showing that the two soma clusters are confluent. A, anterior (bodyaxis); ANN, antennae 2 neuropil; D, dorsal (bodyaxis); L, lateral; LA, lamina; LAN, lateral antenna 1 neuropil; LO, lobula; LPC, lateral protocerebrum; ME, medulla; OL, olfactory lobe.

One intensely stained soma with serotonin-like IR was observed in the lateral cell cluster located posterolaterally to the OL (N_{OL} = 21) (Figure 4e, asterisk). Tracing of that soma showed that it projected toward the OL as well as the surrounding tissue (Figure 4e). The neurite was subsequently lost in the deutocerebrum or the PMPN (N_{somata} = 12). Serotonin-like IR is pronounced in other parts of the deutocerebrum, especially the lateral antenna 1 neuropil (LAN; Figure 4d). Between the base of the LAN and the ANN, a large soma with intense serotonin-like IR is visible (Figures 4a and c). The soma measures 25.61 ± 4.82 μm in length and 13.01 ± 3.47 μm in width (N_{somata} = 14). This neuron sends prominent projections to the deutocerebrum as well as tritocerebrum and gnathal ganglia (GG). Next to the dorsal giant neuron, two smaller somata can be found (Figure 4a). In the antenna 1 nerve, one fiber with serotonin-like IR was found in three cases (Figure 4d, arrows).

3.3 | Histamine (HIST)

Histamine-like IR can be found in all parts of the brain with a total number of 36.6 ± 5.5 (N = 7) somata and is conspicuous throughout the deutocerebrum. The LAN is innervated by fibers with histamine-like IR, with a prominent fiber in the median division and multiple branches on both sides (Figures 5a and b). In the OL, histamine-like IR is present throughout all glomeruli (Figures 5a–c). Histamine-like IR is concentrated in the glomeruli, with neurites also present between glomeruli (Figures 5c and d). A prominent half-circle shaped fiber can be observed in the core of the OL (Figure 4c). Small, spherical immunolabeled structures (1.72 ± 0.37 μm, $N_{microglomeruli}$ = 100) are distributed throughout the whole volume of the glomeruli (Figures 5c, insert i–iii). These spherical structures overlap with synapsin staining and will be called microglomeruli. Similar to the other neuroac-

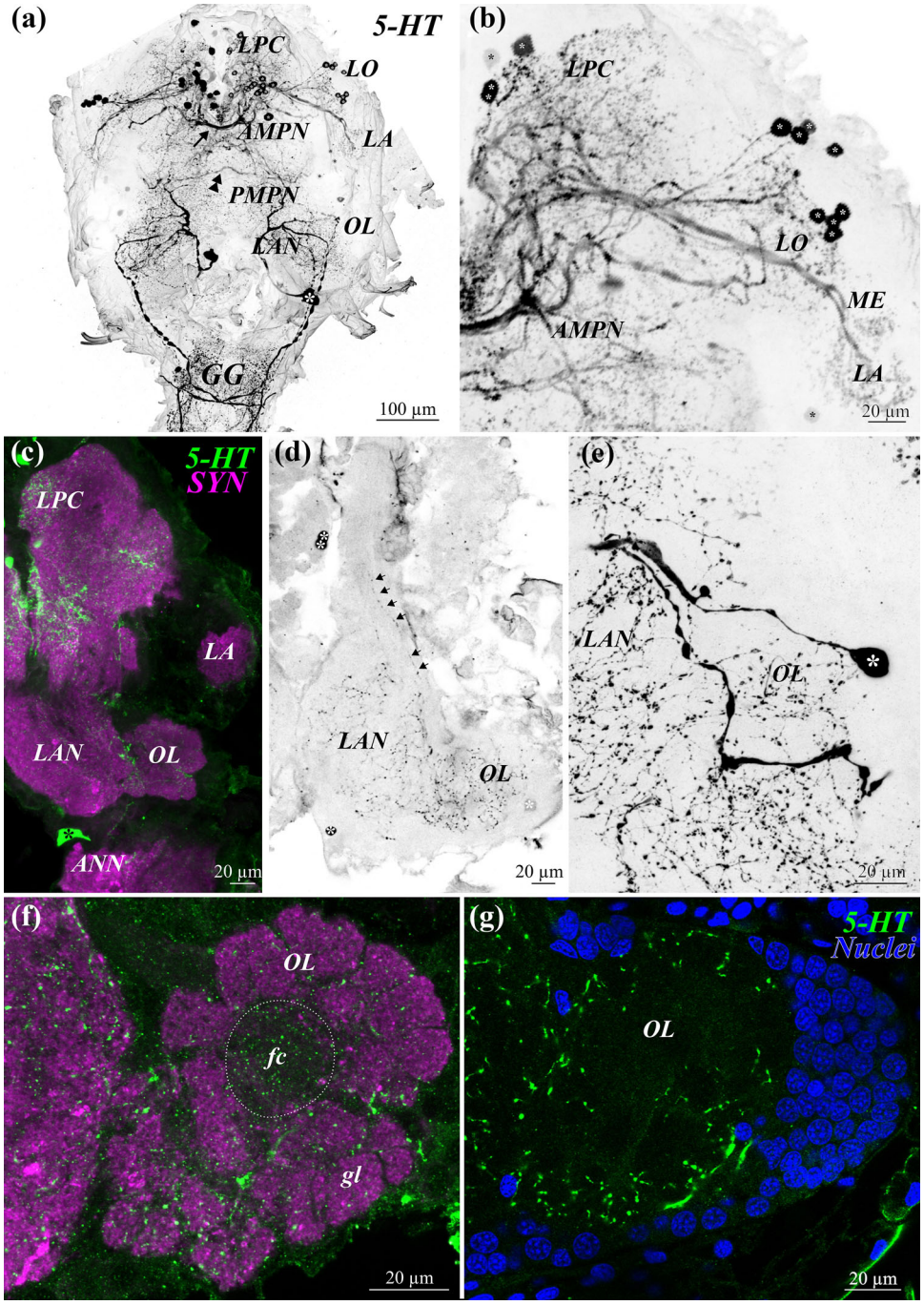

FIGURE 4 Serotonin. (a) Serotonin-like immunoreactivity (black-white inverted image), maximum projection (thickness 71.82 μm), asterisks indicate somata, arrow indicates first commissural bundle in the protocerebrum and double arrow indicates second commissural bundle below the anterior aorta. (b) Maximum projection (thickness 71.82 μm) of the protocerebrum, asterisks indicate somata. (c) Maximum projection with (thickness 12.60 μm), serotonin-like IR in green, synapsin in magenta, giant neuron indicated with asterisk. (d) Single optical section of 0.63 μm of serotonin-like IR in greyscale, arrows indicate fiber in antennae 1 nerve, asterisks indicate somata. (e) Maximum projection (thickness 25.5 μm) of serotonin-like IR showing the projection of a soma located in the lateral cluster projecting into the olfactory lobe. (f) Single optical section (0.3 μm thickness) of serotonin-like IR in green and synapsin in magenta. (g) Single optical sections (0.3 μm thickness) of serotonin-like IR in green and nuclei in blue. Dorsal is toward the top in all pictures. 5-HT, serotonin; AMPN, anterior medial protocerebral neuropil; ANN, antennae 2 neuropil; fc, fibrous core; GC, gnathal ganglia; gl, glomeruli; LA, lamina; LAN, antenna 1 neuropil; LO, lobula; LPC, lateral protocerebrum; ME, medulla; OL, olfactory lobe; PMPN, posterior medial protocerebral neuropil.

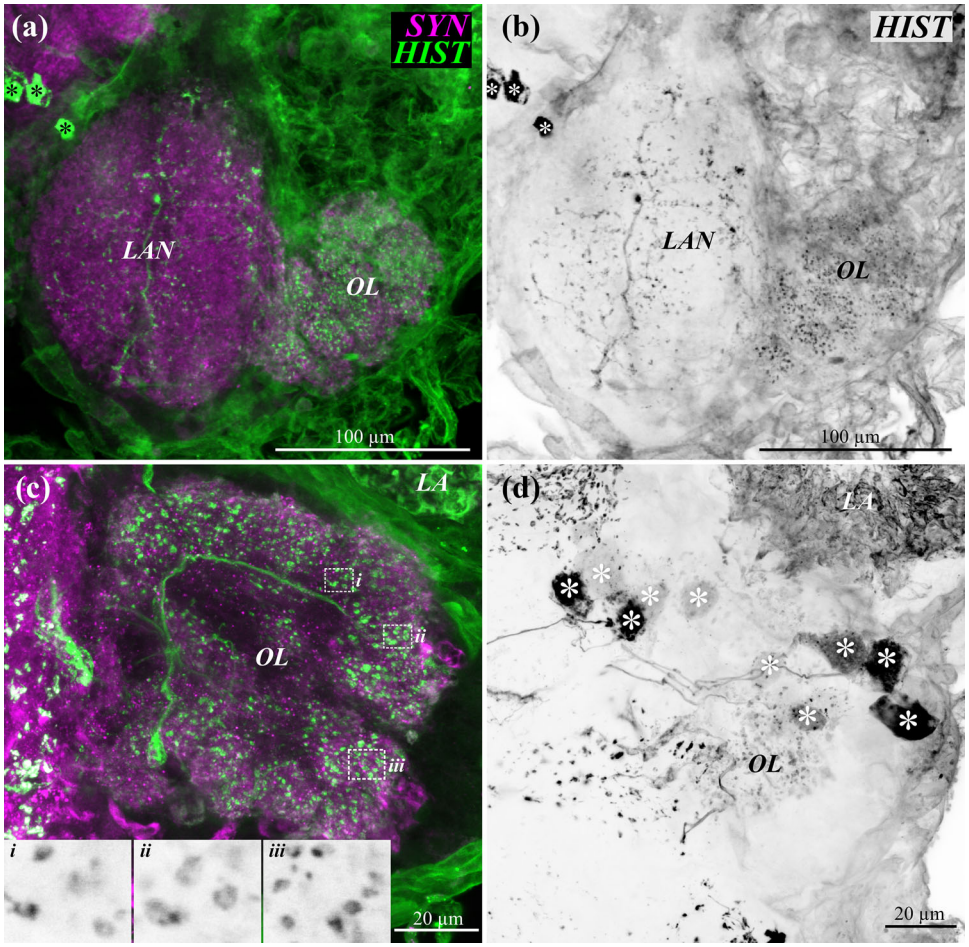

FIGURE 5 Histamine. (a) Histamine-like immunoreactivity (green) and synapsin IR (magenta) in a maximum projection (18.9 μm), asterisks indicate somata. (b) Maximum projection of histamine-like IR (thickness 18.9 μm; black-white inverted). (c) Histamine-like IR (green) and synapsin IR (magenta) maximum projection (thickness of 9.9 μm), inserts i–iii are single optical sections (thickness 0.3 μm). (d) Histamine-like IR in a maximum projection (thickness of 26.7 μm; black-white inverted), asterisks indicate somata. Dorsal is toward the top. LAN, lateral antenna 1 neuropil; OL, olfactory lobe.

tive substances described in this study, multiple local interneurons with histamine-like IR can be observed in the confluent cell cluster surrounding the OL (Figure 5d, asterisks). Out of the 12.8 ± 3.1 (N_{OL} = 14) observed somata, roughly three (2.8 ± 0.39, N_{OL} = 14) somata are intensely stained and clearly visible (Figure 5d). Those local interneurons have ipsi- and contralateral projections to the LPC (N = 2).

3.4 | Allatostatin (AST)

Allatostatin-like IR can be found in most parts of the *P. hawaiensis* brain. In total, 79.45 ± 26.33 (N = 11) somata can be counted in the nuclei rich tissue surrounding the brain, with roughly two thirds of the allatostatin-like somata located around the protocerebral neuropils (Figure 6a). The LA and ME show strong IR, whereas the LO only shows faint labeling. A small paired cluster of somata with allatostatin-like IR is located between ME and LO (cluster (6)). The LPC as well as the LPC show strong allatostatin-like IR, a paired cluster of multiple strongly stained somata can be found around the LPC (Figure 6a). A commissural fiber bundle is located under the aorta. The CB is not innervated.

In the deutocerebrum, allatostatin-like IR can be found in all neuropils. The LAN shows very little IR except for a few clearly demarcated fibers on the frontal side (Figures 6b and c). A few pronounced fibers with a dotted appearance are located in the median division of the LAN, branching off to both sides in regular distances (Figures 6b and c). Strong labeling is found in the deutocerebrum around the myoarterial formation (MAF A) and in the ANN (Figure 6a). Below the LAN, a cell cluster with few somata is located (cluster (13)). In the lateral cell cluster next to the OL are 8.45 ± 2.63 somata (N_{OL} = 22) with allatostatin-like IR (Figure 6d). One very strongly stained soma can be individually identified, the other somata cannot be differentiated from each other. Some somata project neurites into the OL whereas others extend neurites into the deutocerebrum and they are eventually lost in the PMPN (N = 3) or AMPN

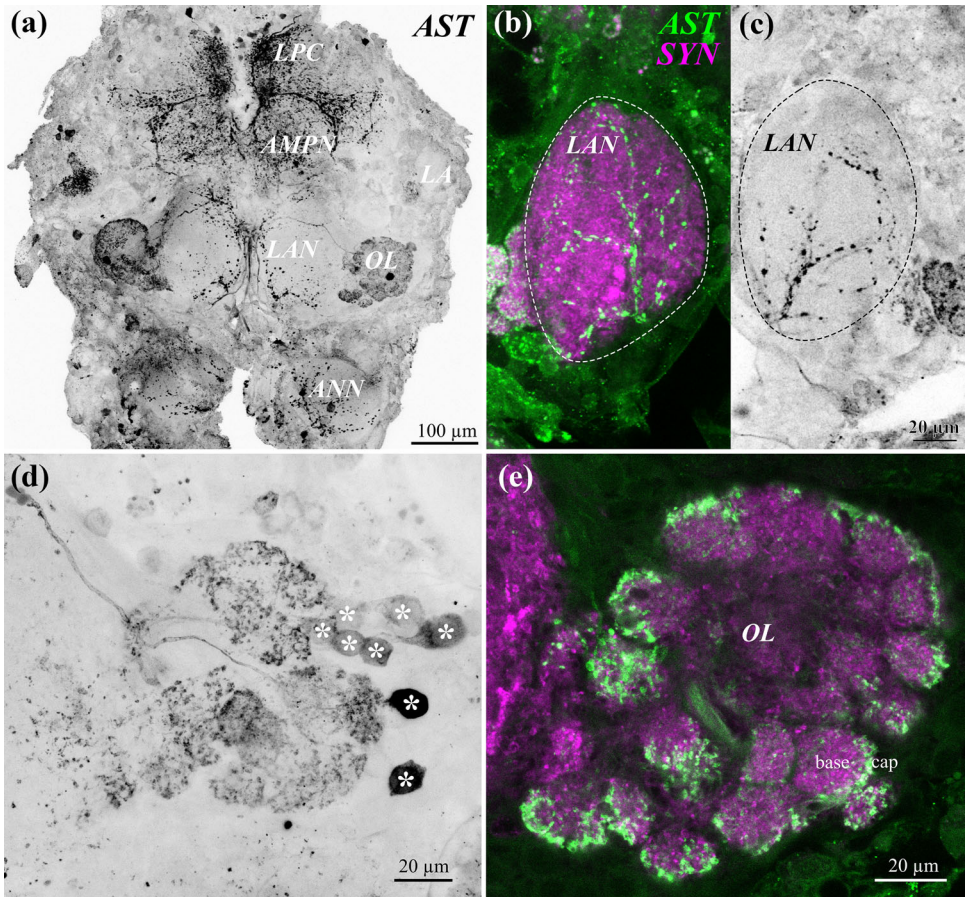

FIGURE 6 Allatostatin. (a) Allatostatin-like immunoreactivity in a maximum projection (thickness 79.38 μm). (b) Allatostatin-like IR (green) and synapsin (magenta) in a maximum projection (thickness 1.26 μm), LAN is indicated by dashed lines. (c) Maximum projection (thickness 1.26 μm), LAN is indicated by a dashed line. (d) Maximum projection (16.5 μm thickness), asterisks indicate somata. (e) Single optical section (0.3 μm thickness) of allatostatin-like IR (green) and synapsin (magenta). Dorsal is toward the top. AST, allatostatin; AMPN, anterior medial protocerebral neuropil; ANN, antenna 2 neuropil; CB, central body; LA, lamina; LAN, lateral antenna 1 neuropil; OL, olfactory lobe.

(N = 1). Allatostatin-like IR was observed in all glomeruli in the OL (Figures 6d and e). Within a glomerulus, allatostatin-like IR is regionalized in the cap region of the glomeruli. Immunoreactivity between the glomeruli is observed at the cap-level as well (Figure 6e). In the tritocerebrum, a cluster of somata is located at the base of the ANN, and fibers with allatostatin-like IR can be found within the ANN (Figure 6a).

3.5 | Orcokinin (OK)

Orcokinin-like IR is distributed in all major regions of the brain. In the protocerebrum, the LA shows strong orcokinin-like IR (Figure 7a). The LPC as well as the AMPN show labeling with a dotted appearance. The CB exhibits orcokinin-like IR with a striped appearance (Figure 7a). In the deutocerebrum, IR is visible in the OL, structured in glomeruli, and dotted fibers in the LAN (Figure 7a). In the tritocerebrum, the ANN neuropil shows orcokinin-like IR. Multiple local olfactory interneurons with orcokinin-like IR are visible in the lateral cell cluster located posterolaterally to the OL (Figures 7d and g, asterisks). Projections of these neurons could be traced up to the glomeruli, and innervating them (N_{somata} = 11, N_{OL} = 4). Within the OL, all glomeruli show orcokinin-like IR throughout the neuropil (Figures 7b and c). However, orcokinin-like IR is also visible in between glomeruli, mainly at the cap-level of the glomeruli (Figure 7c). As observed in the histamine-like IR, we found small, ring-shaped structures, which form balls and barrels in the x axis of 1.74 ± 0.61 μm diameter ($N_{microglomeruli}$ = 100). They overlap with synapsin staining and are distributed through the whole volume of the glomeruli (Figures 7b, c, e, and f). They vary in size with the smallest measuring 0.95 μm and the largest measuring 4.25 μm.

3.6 | RFamide (RF)

As described in Wittfoth et al. (2019), RFamide-like IR is present in the brain of *P. hawaiensis*, especially in the neuropils associated with the olfactory pathway. Multiple somata with RFamide-like IR

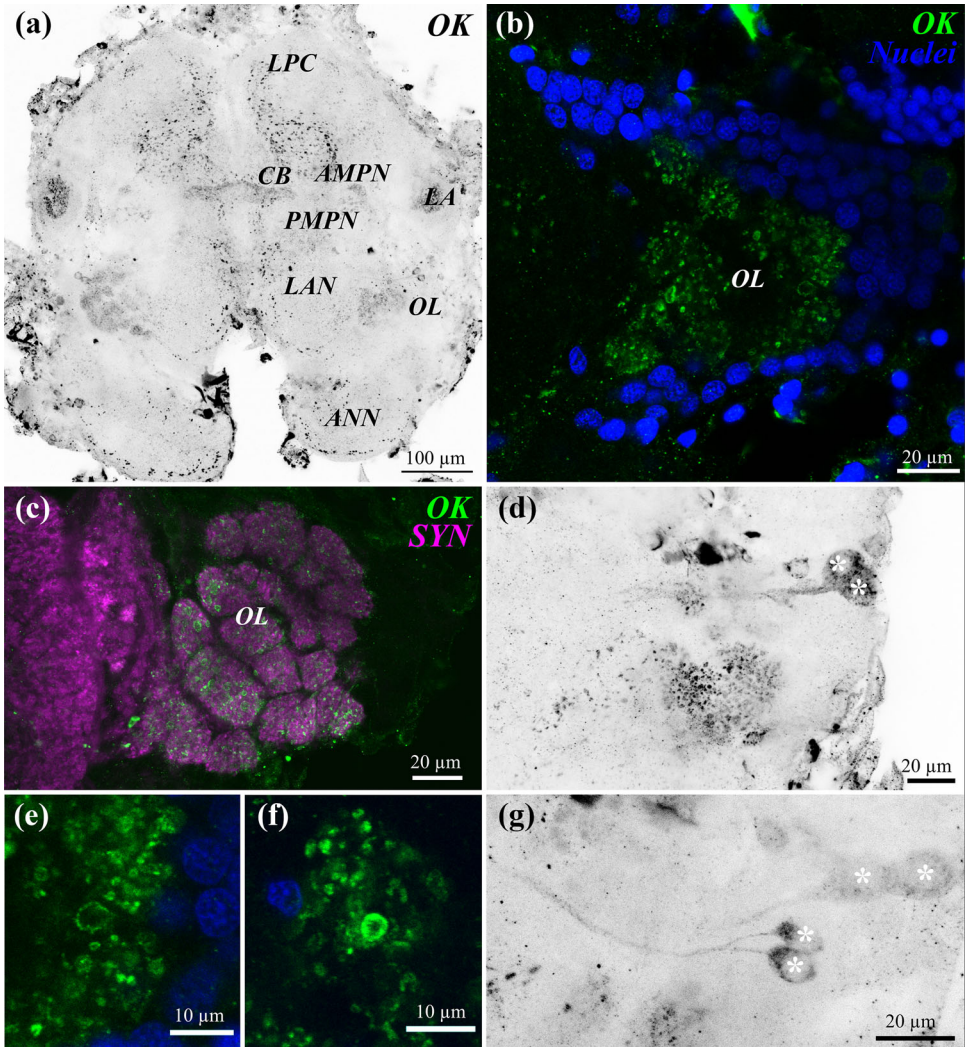

FIGURE 7 Orcokinin. (a) Orcokinin-like immunoreactivity in a maximum projection of the whole brain (thickness 27.09 μm, black-white inverted image). (b) Overlay of orcokinin-like IR (green) and nuclei (blue) in a single optical section (thickness 0.3 μm). (c) Overlay orcokinin-like IR (green) and synapsin (magenta) in a single optical section (thickness 0.3 μm). (d) Maximum projection (thickness 4.2 μm), asterisks indicate somata. (e) and (f) Single optical section (thickness 0.3 μm). (g) Maximum projection (thickness 4.5 μm), asterisks indicate somata. Dorsal is toward the top. AMPN, anterior medial protocerebral neuropil; ANN, antennae 2 neuropil; LA, lamina; LAN, antenna 1 neuropil; LPC, lateral protocerebrum; OK, orcokinin; OL, olfactory lobe; SYN, synapsin; PMPN, posterior medial protocerebral neuropil.

are located in the lateral cell cluster next to the OL (Figures 8a and b). Those somata send projections to the OL. The RFamide-like IR in the OL is clearly visible in all glomeruli, as well as between glomeruli (Figures 8b and d). Contrary to the serotonin-like IR, the RFamide IR is concentrated in the glomeruli (Figures 8b and d). Dispersed through the complete volume of the glomeruli are spherical structures (microglomeruli) in the size range of 1.94 ± 0.78 μm ($N_{microglomeruli}$ = 100; Figure 8d and inserts i–iii). The microglomeruli extend along the *z* axis into three dimensional structures like barrels and balls. The RFamide-microglomeruli structures overlap with synapsin, most likely indicating complex synapses with RFamide as neurotransmitter. Fibers with RFamide-like IR can be detected in the nerve to antenna 1 in some brains (N = 3; Figures 8a and c, arrowheads).

3.7 | SIFamide (SIF)

SIFamide-like IR is present in the entire brain of *P. hawaiensis*, with the olfactory pathway being no exception (Figure 9a). The OLs on either side of the deutocerebrum have SIFamide-like IR throughout all parts of their glomeruli (Figures 9a, b, and d), SIFamide-like IR is more pronounced within the glomeruli compared with the fibrous core. One intensely stained soma in the lateral cluster innervates the OL, targeting multiple glomeruli (N_{OL} = 14; Figure 9c). Similar to the histamine-like, RFamide-like and orcokinin-like IR, small, spherical structures are distributed throughout the glomeruli, again called microglomeruli (1.47 ± 0.3 μm, $N_{microglomeruli}$ = 100; Figure 9, inserts i–v). These microglomeruli are more commonly found in the wider cap area of the glomeruli. One symmetrical large soma was clearly visible in

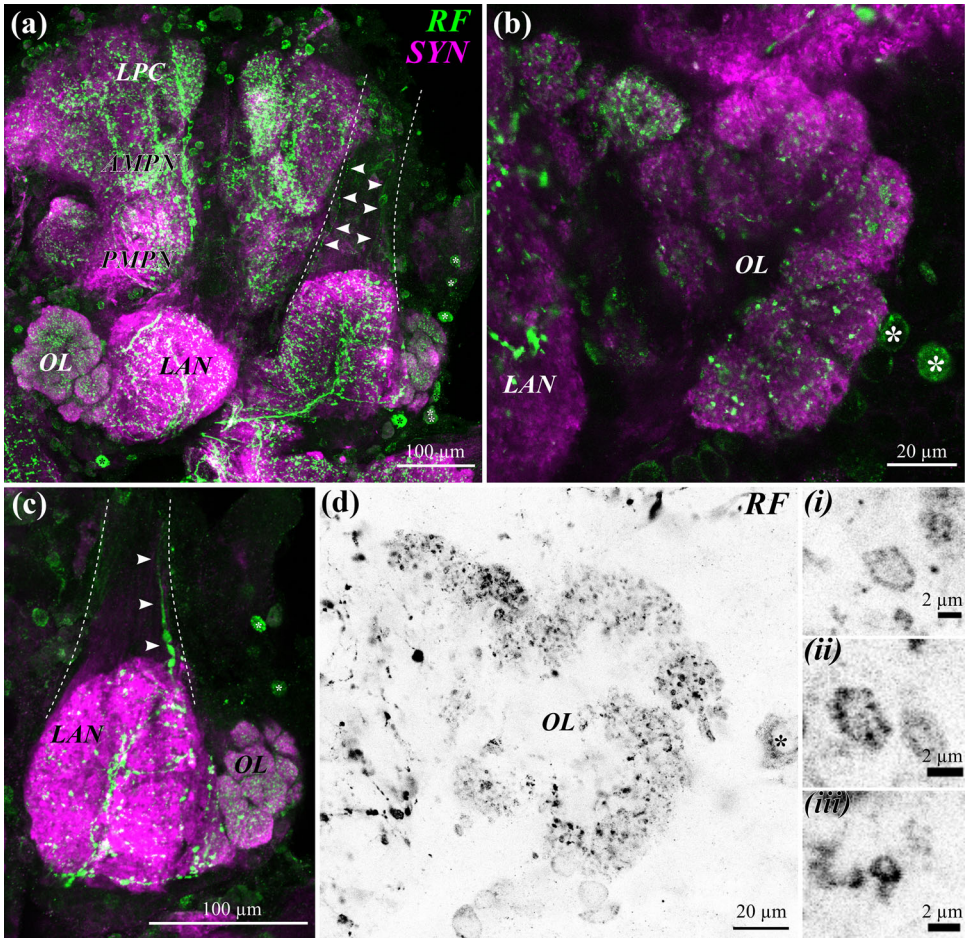

FIGURE 8 RFamide. (a) RFamide-like immunoreactivity (green) and synapsin IR (magenta) in a maximum projection (thickness 11.52 μm), somata are indicated by asterisks, arrowheads indicate RFamide-like IR in the antennae 1 nerve, indicated in dashed lines. (b) Overlay of RFamide-like IR and synapsin in a single optical section (thickness 0.3 μm) of the olfactory lobe, asterisks indicate somata. (c) Maximum projection (thickness 16.2 μm) of RFamide-like IR and synapsin, dashed lines indicate antenna 1 nerve, arrowheads indicate RFamide-like IR in fiber and asterisks indicate somata. (d) Single optical section of the OL (thickness 0.3 μm), inserts i–iii are also single optical section (thickness 0.3 μm). Dorsal is toward the top. AMPN, anterior medial protocerebral neuropil; LAN, lateral antenna 1 neuropil; LPC, lateral protocerebrum; OL, olfactory lobe; PMPN, posterior medial protocerebral neuropil; RF, RFamide; SYN, synapsin.

the anterior part of the deutocerebrum between the base of the ANN and LAN, measuring 17.98 ± 3.14 μm in length and 13.43 ± 2.49 μm in width (N_{soma} = 24) (Figure 9a).

3.8 | GABA and GAD

GABA-like IR as well as GAD-like IR is visible throughout the entire brain and GG (Figures 10a and b). The IR of both stainings generally overlapped, gave the same distribution of neurotransmitter and somata, and is therefore grouped together. Numerous somata in all cell clusters surrounding the brain are visible (Figures 10a and b). A symmetrical pair of giant somata at the base of ANN on the esophageal connective to the GG was observed (Figure 10b, asterisk). In the OL, IR was observed in all glomeruli and in multiple somata in the lateral cluster (Figures 10c–e). Pronounced fibers were visible between the glomeruli (Figures 10d and e, highlighted in insert).

3.9 | Choline acetyltransferase (ChAT)

Since an immunohistochemical staining for ACh is usually unreliable, a staining for ChAT, the enzyme responsible for the synthesis of ACh was carried out. Clearly visible dots around the nuclei are interpreted as soma with ChAT, and such somata can be found surrounding the whole brain (Figures 11a and b, inserts i and ii). A symmetrical pair of a giant somata was found in the tritocerebrum (Figures 11c1–c3). It is located in close proximity to the base of the ANN on the esophageal connective to the GG. Besides the large, with ChAT-like IR filled soma (Figure 11c1), the nucleus was also enlarged in comparison with the surrounding nuclei (Figure 11c2). Multiple somata with dots are visible around the OL (Figures 11a and b, detail in i and ii). Innervation of the glomeruli could not be identified with the current staining. In combination with SIFamide staining, a soma with both labels could be shown (Figure 11b, detail 11ii).

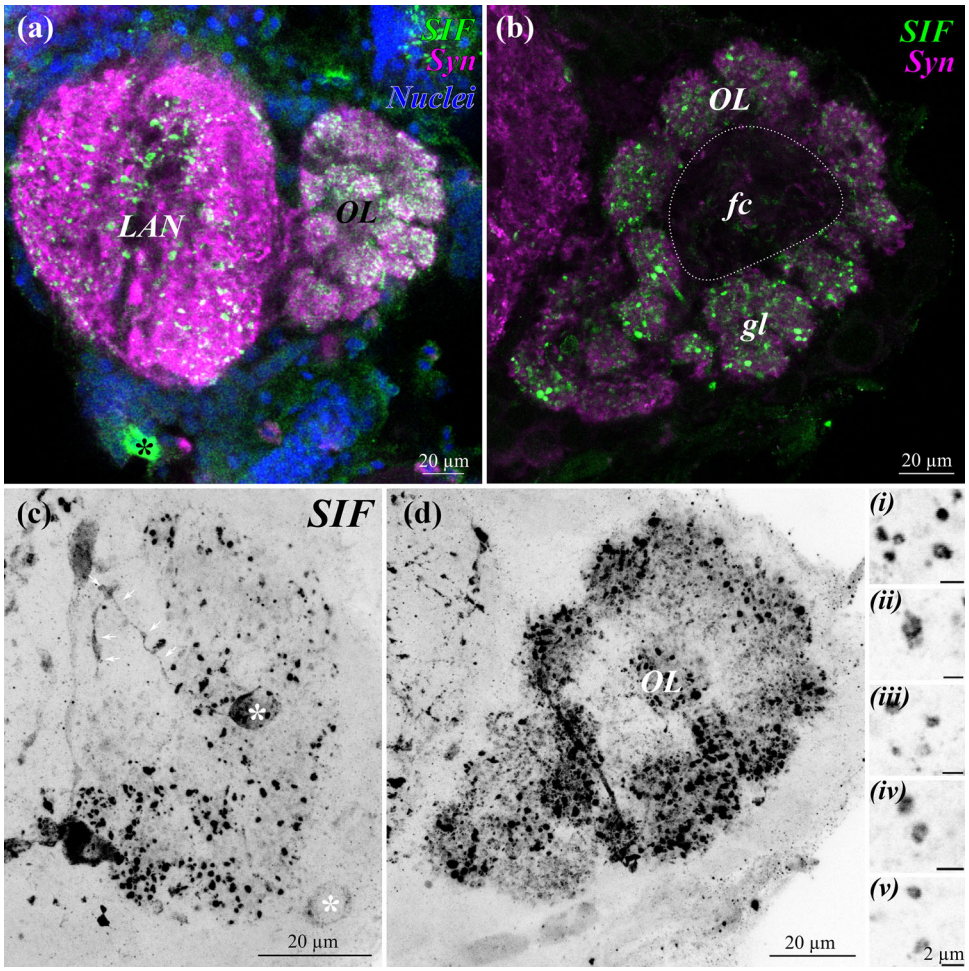

FIGURE 9 SIFamide. (a) SIFamide-like immunoreactivity (green), synapsin IR (magenta) and nuclei (blue) in a maximum projection (thickness 6.93 μm), asterisk indicates soma of giant neuron. (b) Overlay of SIFamide-like IR and synapsin in a single optical section (thickness 0.3 μm). (c) Maximum projection (thickness 16.2 μm), asterisk indicates soma, arrows indicate projection of soma into the OL. (d) Maximum projection (thickness 17.7 μm) of the OL, inserts (i–v) are single optical sections (thickness 0.3 μm). Dorsal toward the top. fc, fibrous core; gl, glomeruli; LAN, lateral antenna 1 neuropil; OL, olfactory lobe; SIF, SIFamide; SYN, synapsin.

4 | DISCUSSION

4.1 | Neurochemistry of the crustacean central olfactory pathway: Comparative aspects

Compared with the other elements of the central olfactory pathway in Crustacea, the local interneurons display a high level of neurochemical diversity. So far, 16 different neuroactive substances have been reported in malacostracan crustacean's OLs, but the available information is spread out across numerous different taxa (table 1). Our study reveals the presence of many different neurochemical substances in the olfactory glomeruli of *P. hawaiensis* and taken together with information on other representatives of the Peracarida (Table 1), these findings support the suggestion that a high level of neurochemical diversity characterized the local olfactory interneurons already in a malacostracan representative close to the stem line. This claim is supported by evidence from the Hexapoda, which represent the closest relatives or even an in-group of crustaceans (Lozano-Fernandez et al., 2016; Misof et al., 2014; Reumont et al., 2012; Schwentner et al., 2017; Wipfler et al., 2019), and which display diverse populations of local olfactory interneurons (Harzsch et al., 2021; Krieger et al., 2015; Schachtner et al., 2005). Detailed information on the immunohistochemical localization of various neuroactive substances are available, for example, for the spiny lobster *P. argus* (Schmidt, 1997; Wachowiak & Ache, 1997) and the hermit crab *C. clypeatus* (Harzsch & Hansson, 2008; Polanska et al., 2012; Harzsch et al., 2021). An important aspect, common to all local interneurons that have been analyzed so far, is that individual cells innervate large ensembles of glomeruli, in some cases up to 85% of the entire glomerular array (Wachowiak et al., 1997). Therefore, possible roles of the diverse neurochemicals occurring in local interneurons comprise an action as neuromodulators for fine tuning the circuitries within the olfactory glomeruli (Schachtner et al., 2005). In the following, we will compare selected localization patterns of these decapod crustaceans to our own findings in *P. hawaiensis*.

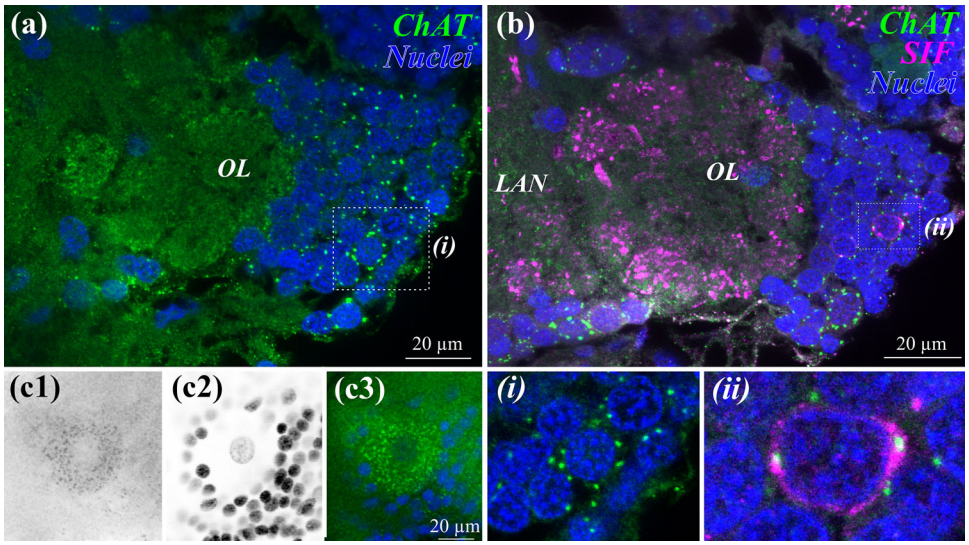

FIGURE 10 GABA and GAD. (a) Overlay of GABA-like immunoreactivity (green) and synapsin IR (magenta) in a single optical section (thickness 0.63 μm). (b) Overlay of GAD-like IR (green) and synapsin (magenta) in a maximum projection with a thickness of 3.15 μm, asterisk indicates giant soma. (c) GABA-like IR in somata indicated with asterisks, maximum projection (thickness 4.5 μm). (d) Overlay of GABA-like IR and synapsin in single optical section (thickness 0.3 μm). (e) and insect both single optical section with thickness of 0.3 μm, asterisks indicate somata. Dorsal is toward the top. ANN, antennae 2 neuropil; GABA, γ-aminobutyric acid; GAD, glutamate decarboxylase; EC, esophageal connective; LA, lamina; LAN, lateral antennae 1 neuropil; LPC, lateral protocerebrum; OL, olfactory lobe; SYN, synapsin.

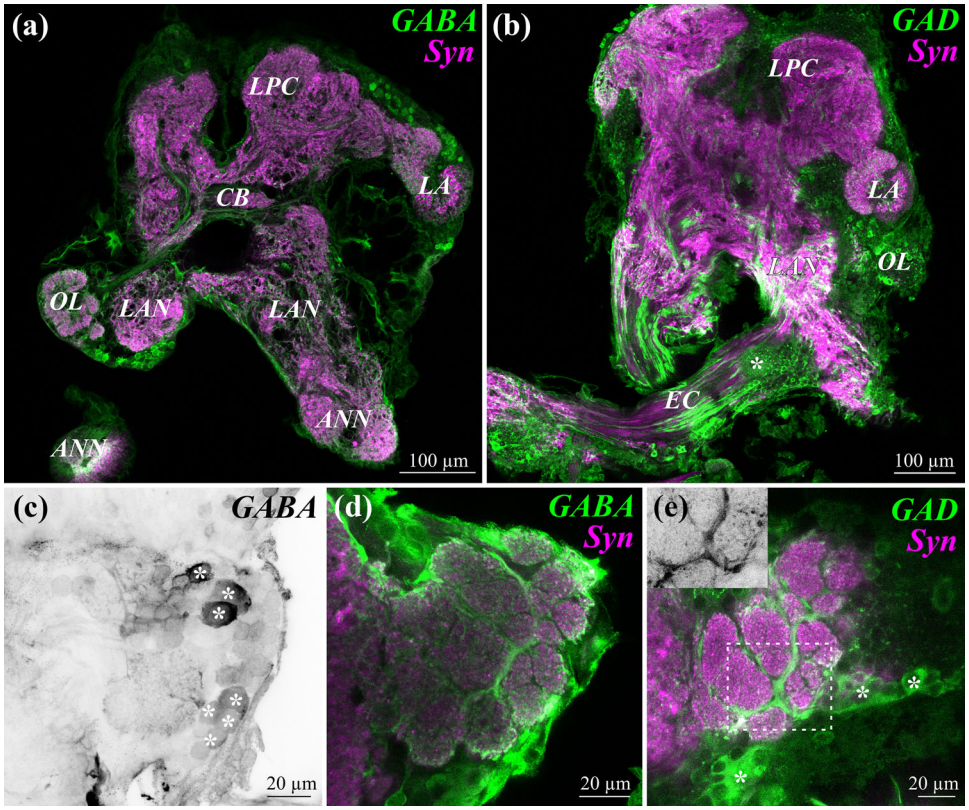

FIGURE 11 ChAT. (a) Overlay of ChAT-like immunoreactivity (green) and nuclei (blue), maximum projection (thickness 1.8 μm). (b) Overlay of ChAT-like IR (green), SIFamide-like IR (magenta), and nuclei (blue), maximum projection (thickness 2.4 μm). (c1)—(c3) Giant somata in single optical section (thickness 0.63 μm). (c1) ChAT-like IR, black-white inverted image. (c2) Nuclei, black-white inverted image. (c3) Overlay, ChAT-like IR in green, nuclei in blue. (ii) Single optical section (thickness 0.3 μm), position indicated by dashed box in (a). (ii) single optical section (thickness 0.3 μm), position indicated by dashed box in (b). Dorsal toward the top. ChAT, choline acetyl transferase; LAN, lateral antennae 1 neuropil; OL, olfactory lobe; SIF, SIFamide.

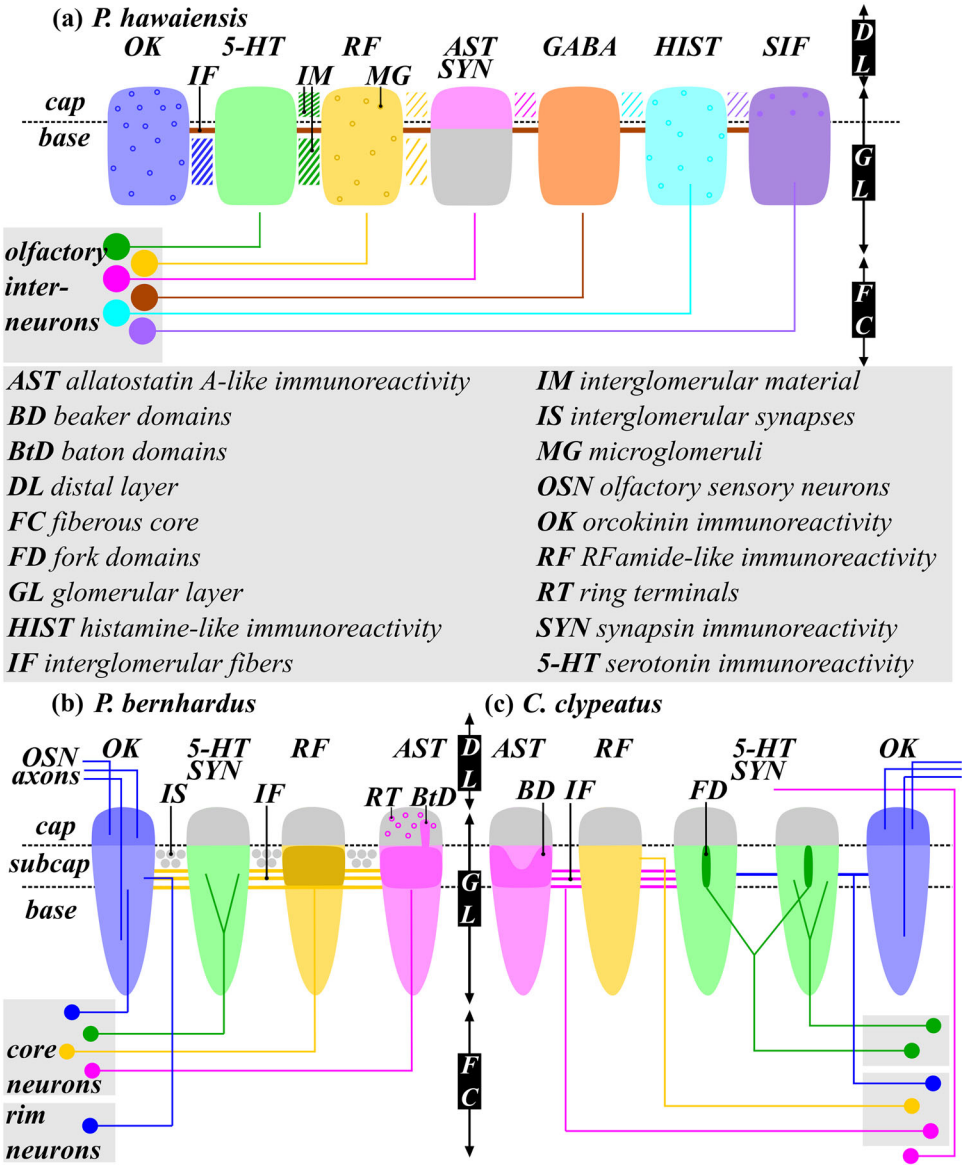

FIGURE 12 Glomerular structure. Comparison of the glomerular structure and neurochemistry in *P. hawaiensis* (a) with that in two hermit crabs (modified from Harzsch et al. 2021), *Pagurus bernhardus* (b) and *Coenobita clypeatus* (c).

The immunoreactivity of serotonin, histamine, orcokinin, RFamide, and SIFamide seems to be evenly distributed across the glomerular volume in *P. hawaiensis*. This is in stark contrast to, for example, spiny lobsters and hermit crabs, in which immunolocalization of these substances reveals striking patterns of intraglomerular regionalization (Figures 12 b and c; Schmidt & Ache, 1997; Wachowiak & Ache, 1997; Harzsch & Hansson, 2008; Polanska et al., 2012; Harzsch et al., 2021). This observation suggests that in these species, the function of olfactory glomeruli as highly complex units of local olfactory processing may be more advanced than in *P. hawaiensis* (compare discussion in the next section). Allatostatin-like peptides provide an exception because also in *P. hawaiensis*, these peptides are more strongly localized in the cap compartment than in the base, the former representing the input region of the glomeruli as targeted by the OSN axons. In *P. bernhardus*, AST is localized both in the cap and base regions, but most strongly in the subcap region, the latter also applying to *C. clypeatus* (Harzsch et al., 2021). This comparison suggests that the differentiation of a subcap region in decapod crustacean glomeruli may have emerged form a structural elaboration of the cap region in crustacean that have only a cap–base organization as *P. hawaiensis* (see next chapter and Figure 12a).

One striking observation is the frequent occurrence of microglomerular profiles in the *P. hawaiensis* olfactory glomeruli that were labeled by immunohistochemistry against RFamide, SIFamide, orcokinin, and histamine. These structures may represent, for example, complex polyadic synapses or nonsynaptic release sites. Conspicuous ring terminals recently were also observed in the glomerular cap region of *C. clypeatus* (Harzsch et al., 2021). Electron-microscopic studies are needed to reveal the nature of these structures.

In insects, a comparison and subsequent modulation of global activity across all sensory neurons was suggested to represent one essential

step for transforming the input of olfactory sensory neuron into an odor-specific combinatorial code of glomerular activity (Martin et al., 2011; Wilson, 2013; Galizia, 2014; Szyszka & Galizia, 2015). In insect olfactory systems, a network of lateral inhibitory local interneurons of which the neurites extend across the glomerular array may function as a gain control in which activity surrounding specific glomeruli is suppressed at high-odor concentrations and the interglomerular contrast is enhanced (Galizia, 2014; Szyszka & Galizia, 2015). In decapod crustaceans, a certain type of rim interneurons that primarily invade the subcap region was suggested to provide massive lateral connections of the glomerular ensemble in the OL (Schmidt & Ache, 1996) so that these authors considered rim neurons as prime candidates for the mediation of lateral inhibitory interactions through presynaptic afferent inhibition. In crayfish, these have been described as "interglomerular fibers" (Blaustein et al., 1988), and massive lateral connections were also described in hermit crabs *Coenobata clypeatus* (Polanska et al., 2020), in which they express allatostatin A-like IR (Harzsch et al., 2021). In spiny lobsters, some of the interglomerular fibers are GABAergic and hence were suggested to modulate input from OSNs and control the activity within the network (Wachowiak et al., 1997). Our immunohistochemical data on the localization of GABA and GAD support the conclusion that also in *P. hawaiensis* a network of interglomerular fibers is present, which presumably mediate lateral inhibition across this species' glomerular array. Interglomerular fibers were also reported to contain RFamide-like neuropeptides in spiny lobsters (Schmidt & Ache, 1997) and hermit crabs *Pagurus bernhardus* (Harzsch et al., 2021). This is not the case in *P. hawaiensis* suggesting a simpler organization of this specie's lateral modulatory network.

4.2 | Evolutionary aspects of the deutocerebral chemosensory pathway in Crustacea: Insights from *P. hawaiensis*

The deutocerebral antennal pair of Crustacea is equipped with specialized, unimodal olfactory sensilla (aesthetascs) as well as bimodal chemo- and mechanosensory sensilla (reviews e.g. Derby & Weissburg, 2014; Hallberg et al., 1992; Hallberg & Hansson, 1999; Hallberg & Skog, 2010; Mellon, 2007, 2012, 2014; Schmidt & Mellon, 2010). Our knowledge about the sensillar equipment in *P. hawaiensis* is very limited, however, the available evidence (Figure 1b; Krieger et al., 2021) suggests that its first antennal pair, in addition to the aesthetascs, is also equipped with numerous other sensilla, presumably bimodal and unimodal mechanosensory sensilla. It is well established that in malacostracan crustaceans, the input from the chemoreceptive aesthetascs *versus* bimodal sensilla on the first pair of antennae segregate into different primary sensory neuropils in the deutocerebrum (reviews Derby et al., 2016; Derby & Weissburg, 2014; Schmidt and Mellon, 2010). Whereas the input from the bimodal sensilla is represented in a somatotopic map in the LAN, the purely olfactory input from the aesthetascs is represented nontopographically in the OL. Based on an analysis of a basal representative of malacostracan crustaceans, Kenning et al. (2013) suggested that this dual representation of antenna 1 input characterizes the malacostracan ground patter, and our current findings in *P. hawaiensis* support this suggestion. Admittedly, in the present study we did not record any volumetric data on the neuropil sizes in the brain of *P. hawaiensis*. Nevertheless, it seems safe to say that the LAN is considerably larger in these animals than the OL suggesting that the chemosensory channel associated with the LAN seems to play a more important role than the pathway associated with the OL.

Figure 13 shows a phylogram of the Malacostraca (compiled after Scholtz & Richter, 1995; Richter & Scholtz, 2001; Wirkner & Richter, 2010) with Remipedia as an outgroup. We have plotted the available data on glomerular numbers in Malacostraca on this phylogram and have included a gallery of micrographs of olfactory glomeruli from eleven selected malacostracan taxa and the outgroup. Based on a comparative analysis, Harzsch & Krieger (2018) suggested that spherical olfactory glomeruli characterize the crustacean ground pattern, and the visualization shown in Figure 13 supports this claim. Remipedia display typical spherical glomeruli, and this shape was retained as a plesiomorphic feature in the stem line of Malacostraca as is for example represented by the Leptostraca, that possess an array of about 60 spherical glomeruli (Kenning & Harzsch, 2013). Roughly spherical glomeruli are also present in other representatives of the Malacostraca such as Stomatopoda (Derby et al., 2003), Euphausiacea and Mysidacea (Johansson, 1991; Johansson & Hallberg, 1992a, 1992b; Moreau et al., 2002), and Isopoda (Harzsch et al., 2011; Kenning & Harzsch, 2013). The present results indicate that *P. hawaiensis* is another typical representative of this stem line morphology. An evolutionary transformation of spherical across wedge shaped glomeruli and further toward columnar cones can be observed within the Decapoda. Typical columnar cones are present in representatives of the Reptantia such as crayfish, spiny lobsters and clawed lobsters (Figure 13; reviewed in Harzsch & Krieger, 2018). The tallest columns have been found in terrestrial representatives of the Anomala, the Coenobitidae (Figures 12b–c; Harzsch & Hansson, 2008; Krieger et al., 2010; Polanska et al., 2012, 2020). The Reptantia also feature glomerular numbers that are much higher than those of more basal malacostracan taxa in which glomerular numbers are below one hundred (Figure 13). An intermediate wedge shape (not shown in Figure 13) occurs, for example, in prawns (Ammar et al., 2008; Johansson, 1991; Kruangkum et al., 2013), shrimps (Meth et al., 2017), brachyuran crustaceans (Krieger et al., 2012; 2015), and some representatives of the Anomala (Krieger et al., 2012).

This trajectory of evolutionary transformation seems to coincide with a transformation of intraglomerular complexity (Figure 12). In decapod crustaceans with columnar cones such as crayfish (Blaustein et al., 1988; Sandeman & Luff, 1973), spiny lobsters (Blaustein et al., 1988; Schmidt & Ache, 1992; 1996; 1997), and hermit crabs (Harzsch & Hansson, 2008; Krieger et al., 2010; 2012; Polanska et al., 2020; Harzsch et al., 2021), it has been well documented that the glomeruli are regionalized in three longitudinal compartments, cap, subcap, and base, and further in concentric compartments. The glomerular cap region primarily represents an input compartment (axons of OSNs), whereas in the base, neurites of the PNs assemble as output

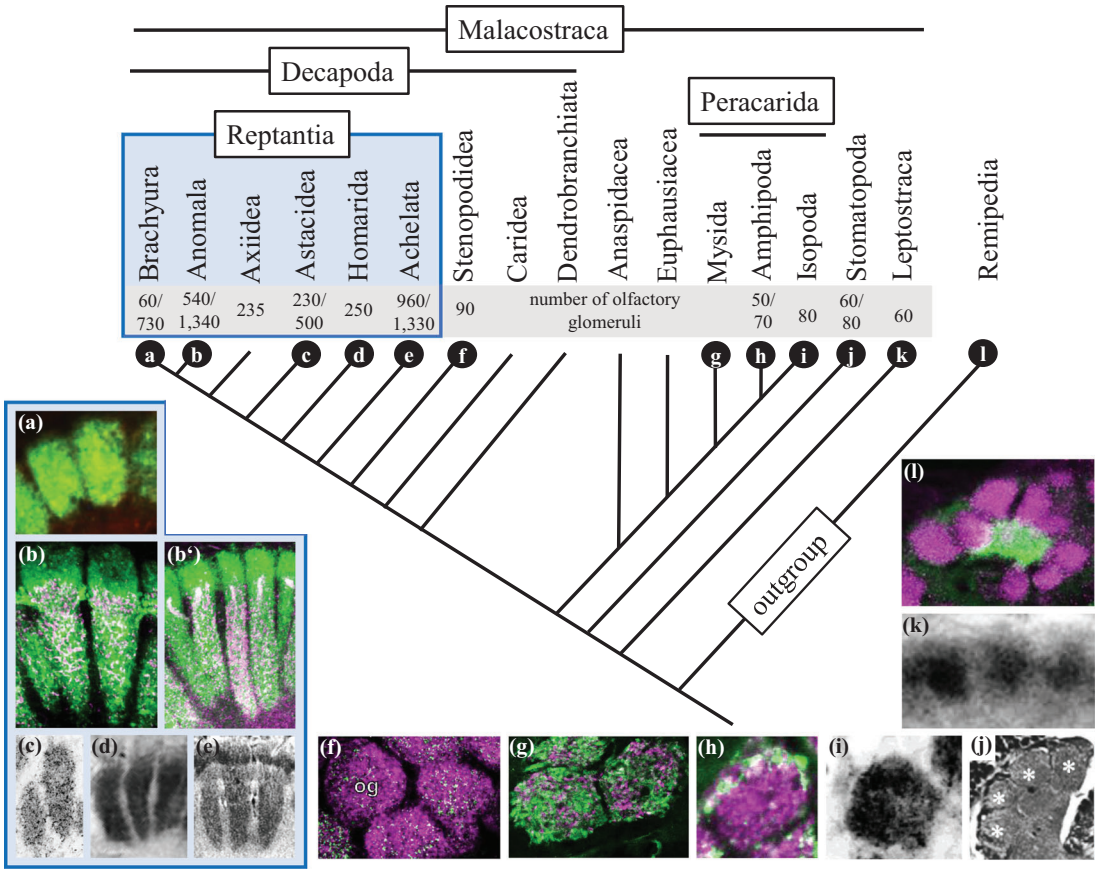

FIGURE 13 Comparative aspects: features of the central olfactory pathway plotted on a phylogram of the Malacostraca (compiled after Scholtz & Richter, 1995; Richter & Scholtz, 2001; Wirkner & Richter, 2010) and Remipedia as a malacostracan outgroup. The numbers of olfactory glomeruli were compiled after Beltz et al. (2003), Derby et al. (2003), Harzsch & Krieger (2018), and the present study. The image galery of the olfactory glomeruli shows that Remipedia as an outgroup and basal malacostracan lineages feature spherical glomeruli and glomerular numbers below one hundred. Members of the Reptantia (highlighted in light blue) such as hermit crabs, crayfish, spiny lobsters and clawed lobsters feature up to 1,340 elongate glomeruli shaped like columnar cones. Intermediate wedge shape glomeruli (not shown) occur in prawns (Caridea; Ammar et al., 2008; Johansson, 1991; Kruangkum et al., 2013) and shrimps (Dendrobranchiata; Meth et al., 2017). (a) *Carcinus maenas* (Harzsch, unpublished); (b) *Pagurus bernhardus* (Harzsch et al. 2021); (b') *Coenobita clypeatus* (Harzsch et al. 2021); (c) *Procambarus virginalis* (Kuemmerlen, unpublished); (d) *Homarus americanus* (modified from Beltz et al., 2003); (e) *Panulirus argus* (modified from Schmidt & Ache, 1996); (f) *Stenopus hispidus* (Krieger et al., 2020); (g) *Neomysis integer* (Kuemmerlen, unpublished); (h) *Parhyale hawaiensis* (this study); (i) *Idotea baltica* (modified from Harzsch et al., 2011); (j) *Neogonodactylus oerstedii* (modified from Derby et al., 2003); (k) *Nebalia herbstii* (modified from Kenning et al., 2013); (l) *Speleonectes tulumensis* (Harzsch, unpublished).

channel of the system. The subcap region, in addition to providing lateral (likely inhibitory) interglomerular connections (Polanska et al., 2020; Wachowiak & Ache, 1997), may take a central computational role in modulating the information transfer from OSNs to PNs (reviewed in Derby & Weissburg, 2014; Schachtner et al., 2005; Schmidt & Mellon, 2010; Harzsch & Krieger, 2018; Harzsch et al., 2021). In malacostracans with spherical or wedge-shaped glomeruli, the longitudinal compartmentalization is less complex. Only cap and base regions are visible in Leptostraca (Kenning et al., 2013), Isopoda (Kenning & Harzsch, 2013), and prawns (Ammar et al., 2008; Schachtner et al., 2005), at least with the histological methods used in these studies, immunohistochemistry combined with confocal laser-scan microscopy. The current study provides yet another example for a species with spherical glomeruli and only cap—base regionalization suggesting that *P. hawaiensis* represents an early stage of glomerular evolution within the Malacostraca.

4.3 | CONCLUSIONS

As is typical for malacostracan crustaceans, the amphipod *P. hawaiensis* displays dual chemosensory pathways associated with the input of the sensilla on its first antennal pair, but the LAN-associated pathway seems to be more dominant than the OL-associated pathway. With an estimated number of 50–70 olfactory glomeruli and the number of olfactory interneurons in the lower hundreds (Kümmerlen, preliminary data), the olfactory pathway of *P. hawaiensis*, compared with reptant malacostracan crustaceans (Figure 13; and see e.g. Harzsch & Krieger, 2018), displays a moderate level of complexity as far as numbers of neuronal elements are concerned. Furthermore, the spherical shape of the glomeruli and the simple intraglomerular differentiation of the cap–base type suggests that this animal's olfactory system can serve as a valid example for a crustacean olfactory system with a comparatively low level of structural complexity. Number

and shape of the glomeruli fit into the range represented by other members of the malacostracan stem line (Figure 13) suggesting that *P. hawaiensis* may serve as a valid representative featuring an olfactory system close to the ground pattern of Malacostraca. However, this species' local olfactory interneurons display a high level of neurochemical diversity. The fact that relatively few interneurons of a given neurochemical class seem to innervate all glomeruli suggests a prominent global modulatory function so that "computing with complex chemistry" is an important principle of basal crustacean's olfactory systems.

Which implications do these findings have for our understanding of structural modifications in the evolutionary trajectory toward the decapod crustaceans? Decapods such as crayfish, clawed, and spiny lobsters and hermit crabs have increased the number of olfactory interneurons by three orders of magnitude compared with *P. hawaiensis*, and increased the number of olfactory glomeruli up to one thousand (Figure 13; and see Harzsch & Krieger, 2018 for these comparative numerical aspects). Decapods also seem to have evolved morphologically more complex types of interneurons (rim and core type) that potentially allow the intraglomerular neuronal networks to execute more demanding computational tasks. Furthermore, the structural differentiation of the glomeruli toward the cap–subcap–base organization suggests that the olfactory glomeruli in decapods function as more complex units of local olfactory processing than in *P. hawaiensis*.

AUTHOR CONTRIBUTION

S. H. and K. K. conceived the study. S. R. and K. K. performed the immunohistochemical staining, K. K. scanned and analyzed specimens labeled for all neurotransmitters except SIFamide. S. R. did the scanning and analysis for SIFamide, K. K. and S. H. drafted the manuscript. S. R. assisted in drafting the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

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

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