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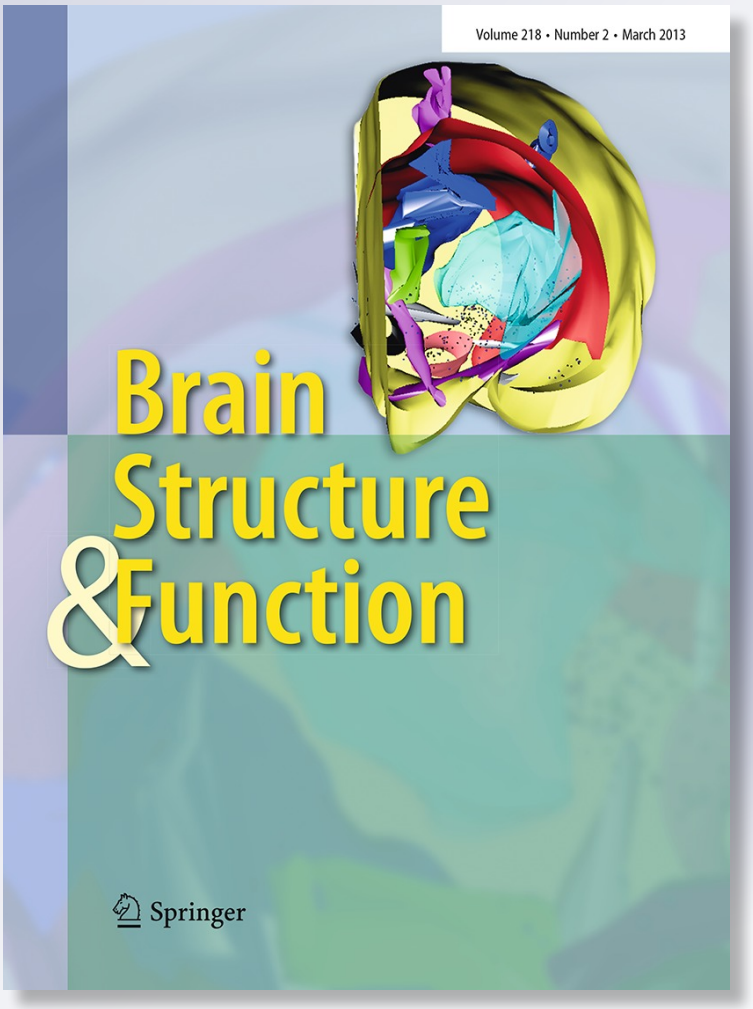

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ORIGINAL ARTICLE

Organization of the procerebrum in terrestrial pulmonates (*Helix, Limax*) reconsidered: cell mass layer synaptology and its serotonergic input system

Károly Elekes · Izabella Battonyai · Suguru Kobayashi · Etsuro Ito

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Abstract The synaptology of the cell body layer of the olfactory center, procerebrum, was investigated in two prominent terrestrial pulmonate gastropod species, *Helix pomatia* and *Limax valentianus*. In addition, the analysis of the 5-HT-immunoreactive innervation, including ultrastructural level, was performed at high resolution in *H. pomatia*. A highly complex system of synaptic and non-synaptic connections was found in the procerebrum of both species connected to local neuropil areas of different size. The procerebral (globuli) cell perikarya were richly innervated by varicosities meanwhile the axon profiles also established contacts with each other. Synaptic configurations including convergence, divergence and presynaptic modulation were also revealed. The frequent occurrence of unspecialized but close axo-somatic and axo-axonic membrane contacts referring to the modulatory forms of transmitter release were also accompanied by membrane configurations indicative of active exocytosis. In *H. pomatia*, the cell mass layer was shown to receive a rich 5-HT-immunoreactive innervation, forming a dense network around the cell bodies. At ultrastructural level, 5-HT-immunoreactive varicosities contacted both cell bodies and different unlabeled axon profiles. Our results suggest that the local neuropil regions in the cell body layer are site of local circuits, which may play a decisive role in olfactory integrative processes bound to the procerebrum. The pattern and form of the 5-HT-immunoreactive innervation of extrinsic origin suggest an overall modulatory role in the cell body layer. The results may serve a basis for considering the role of local intercellular events, connected to microcircuits, within the procerebrum cell body layer involved in oscillation activities.

Keywords Procerebrum · Axo-somatic contacts · Local neuropils · 5-HT · Ultrastructure · Immunocytochemistry · *Helix* · *Limax* · Gastropoda · Mollusca

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Introduction

The procerebrum (PC) of terrestrial pulmonates has been considered for long the center of odor processing and olfactory learning (Gelperin and Tank 1990; Chase 2000; Samarova and Balaban 2009). In both *Helix* and *Limax* it has been shown that the PC is characterized by a local field potential oscillatory activity which seems to be related to the behavioral changes caused by olfactory events and conditioning (Kimura et al. 1998; Nikitin and Balaban 2000; Kasai et al. 2006; Matsuo and Ito 2008; Matsuo et al. 2010). This synchronized oscillation is highly stable, displaying a cyclic period of 0.7 Hz but can be modulated by olfactory and gustatory stimuli as well as by the application of different signal molecules such as monoamines and neuropeptides. The exact cellular basis of this synchronized activity and the neuronal processing of odor/olfactory information has not yet been cleared completely but physiological studies indicate that both intrinsic (inside the procerebrum) and extrinsic (outside the procerebrum) mechanisms play an important role in it (Kleinfeld et al. 1994). The PC receives olfactory inputs from both (upper

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and lower) pairs of tentacles, which are equipped with sensory cells in the epithelium. From the upper pair of tentacles the odor information reaches the PC via the tentacular ganglion, following synaptic switch (Chase 2002). According to early and recent studies, other efferents may also arrive at the PC from the mesocerebrum (Bullock and Horridge 1965), the metacerebrum (Ierusamlimsky and Balaban 2010; Kobayashi et al. 2010), and the pedal ganglion (Chase 2002). This kind of efferent system suggests a complex way of regulation of olfactory processing in the PC, assuming synaptic interaction between the intrinsic and extrinsic elements as well.

The PC is a lobe of the cerebral ganglion in the terrestrial pulmonates which contains a high number of small size (5–8 μm) neurons (20–40,000 in *Helix*, and 100,000 in *Limax*), creating a cell body layer which unlike the usual ganglion structure of invertebrates is located laterally to the medially situating neuropil. The PC neurons are also called globuli cells (see in Bullock and Horridge 1965), and they are distinguished to the bursting (B) and non-bursting (NB) cells in *Limax* (Kleinfeld et al. 1994; Chase 2002), which latter represent the vast majority of the PC cell population (Kleinfeld et al. 1994; Watanabe et al. 1998). The neuropil is separated to the terminal mass and the internal mass regions, both composed of an extremely dense system of axon profiles, but displaying different structure; the internal mass neuropil has a less fine appearance of organization (Ratté and Chase 2000). Two large axon bundles can also be distinguished within the PC: one running along the borderline between the cell body layer and the medullary neuropil and composed of the interneuron fibers arriving from the tentacular ganglion, and another one located along the internal (medial) surface of the neuropil and containing efferent elements innervating the tentacle muscles.

The cyto-architecture and synaptology of the PC was analyzed in *Helix* (Zs-Nagy and Sakharov 1970; Ratté and Chase 1997, 2000; Zaitseva 2000) and in *Limax* species (Zs-Nagy and Sakharov 1970; Watanabe et al. 1998; Zaitseva 2000), following silver impregnation, electron microscopy, and intracellular biocytin labeling of individual procerebral cells, respectively. It was shown that the PC cell population was not homogeneous, and that the neuroanatomy and projection pattern of the B and NB cells was different: B cells arborized only within the lobe meanwhile NB elements projected outside the lobe. Following intracellular labeling with biocytin, the synaptic relationship of the PC neurons of *Helix aspersa* was described by Ratté and Chase (2000), including the cell body layer, terminal and internal masses, as well as outside the PC in the mesocerebrum. Although the contacts of axonal elements with PC cell bodies were briefly dealt with, little attention has been paid to the detailed synaptology of the cell mass layer. According to Ratté and Chase (2000), there was no difference either in the neuropil organization of the cell body layer nor in the frequency occurrence of the synapses observed in the different anatomical regions. At the same time, Zaitseva et al. (2000) published another paper describing the ultrastructure and synaptic contacts of the cell body layer of different terrestrial gastropod species (*Helix*, *Limax* and *Deroceras*), demonstrating different forms of axo-somatic and axo-axonic contacts. Unfortunately, the results were presented by these authors as pure ultrastructural findings, without considering them in a functional context or discussing their possible role in olfactory integrative processes. Finally, in an early study axo-somatic synapses were also described in the *Helix* and *Limax* PC by Zs-Nagy and Sakharov 1970. However the single OsO_4 fixation applied resulted in a low quality of preservation that practically hinders a comparison with the results obtained following aldehyde-osmium double fixation. Axo-somatic contacts otherwise are not a regular form of intercellular contacts either in gastropods (Elekes et al. 1985; Elekes 1991), or, in general, in invertebrates (Schürmann et al. 1989), although their role in specific actions, such as the fine tuning of nerve cell activity or the influence of cell metabolism, is not to be neglected. Therefore, based on the above-mentioned findings, we have re-investigated the fine structural organization and synaptology of the cell body layer in two prominent terrestrial pulmonate gastropod species, *Helix pomatia* and *Limax valentianus*, in order to provide a functional morphological background of signaling ways underlying olfactory processing, with emphasis on the possible role of local circuits.

A high number of neurotransmitters and modulators are present in the PC (Gelperin 1999). The effect of different signal molecules such as serotonin (5-HT), glutamate, GABA, nitric oxide and certain endogeneous molluscan neuropeptides, first of all FMRFamide, have been investigated, including the regulation of oscillatory dynamics and learning (Gelperin 1999; Inoue et al. 2001, 2004; Kobayashi et al. 2008, 2010; Matsuo et al. 2009). Earlier and recent studies, applying different histochemical and molecular techniques, demonstrated the localization of these and some other signal molecules (catch-relaxing peptide [CARP], Mytilus inhibitory peptide [MIP]) in the PC of *Helix* and *Limax* (Osborne and Cottrell 1971; Cooke and Gelperin 1988a, b; Hernádi et al. 1995; Elekes and Nässel 1990; Elekes et al. 2000; Inoue et al. 2004; Matsuo and Ito 2009; Matsuo et al. 2009; Kobayashi et al. 2010). However, the high-resolution analysis of the organization and intercellular contacts of the transmitter/modulator containing elements within the PC, including the identification of their postsynaptic targets, is still missing. In this study, we applied correlative light- and electron microscopic immunohistochemistry, in order to resolve the serotonergic innervation of the cell body layer.

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Materials and methods

Animals

Adult specimens of the garden (land) snail *Helix pomatia* and the slug *Limax valentianus* were used. Specimens of *Helix pomatia* were collected in the surrounding areas, kept thereafter under laboratory conditions and fed on lettuce. Slugs 11–14 weeks after hatching were maintained under laboratory conditions and fed on a mixed diet of rat chow (Oriental Yeast, Tokyo, Japan), wheat starch (Wako, Osaka, Japan) and vitamins (Oriental Yeast).

Electron microscopy

For fixation, the CNS from both species were isolated and pinned out in a Petri-dish and covered with a mixture of either 2.5 % paraformaldehyde and 1 % glutaraldehyde (*Helix*) or 4 % paraformaldehyde and 0.1 % glutaraldehyde (*Limax*), both diluted in 0.1 M phosphate buffer (PB). After fixation the preparations were thoroughly washed in PB, post-fixed in 1 % OsO_4 diluted in 0.1 M cacodylate buffer, dehydrated in graded ethanol and propylene oxide, and finally embedded in Araldite (Durcupan ACM, Fluka). During dehydration, block staining was performed in 70 % ethanol saturated with uranyl acetate. For orientation 1 μm semi-thin sections were stained with toluidine blue. Fifty-sixty nanometer ultrathin serial sections from the PC region were taken with an LKB Novacut ultramicrotome, stained with lead citrate, and viewed in a JEOL 1200 EX, a JEOL 1200 EXII, and a Hitachi H-7650 electron microscope.

Correlative light- and electron microscopic immunocytochemistry

The PCs were dissected from the cerebral ganglia after fixation with 4 % paraformaldehyde and 0.1 % glutaraldehyde, embedded in a mixture of 10 % gelatin and 1 % albumin (both from Sigma) and then cut into 50 μm slices with a Vibratome (Pelco). The Vibratome slices were processed for a two-step peroxidase immunocytochemistry as follows. After blocking first in phosphate buffered saline (PBS) containing 0.25 % bovine serum albumin (BSA) and then in 1 % H_2O_2, the slices were incubated in mouse monoclonal anti-5HT antiserum (Dako) diluted in PBS-BSA containing also 0.25 % Triton X-100 (PBS-BSA-TX), followed by incubation with a goat anti-mouse IgG coupled with horseradish peroxidase (HRP, Dako). Incubations lasted overnight at 4 °C. After washing in PBS and 0.1 Tris–HCl buffer, the immunoreaction was visualized by adding 0.05 % 3,3-diaminobenzidine (DAB) as chromogen and 0.01 % H_2O_2 as substrate. The development was monitored under a stereomicroscope and stopped by changing the developing solution for 0.1 Tris–HCl buffer, followed by two additional washing in PBS. The slices were post-fixed in 0.5 % OsO_4, dehydrated, and then mounted on slides in Araldite (Durcupan ACM, Fluka). After polymerization, the slices were analyzed in a Zeiss Axioplan compound light microscope attached to a Canon Powershot 650 digital or a CCD camera (Alpha DCM510 or Alpha MDC560, Hangzhou Scopetek Opto-Electric). Following light microscopy, slices displaying high quality immunolabeling were selected and re-embedded for electron microscopy. Ultrathin sections of 50–60 nm were cut, stained with lead citrate, and viewed in the electron microscopes mentioned above.

Perforated patch-clamp recordings

Perforated patch-clamp recordings were made using previously published experimental protocols (Watanabe et al. 2003). The isolated CNS was transferred to a recording chamber filled with a *Limax* saline solution containing the following (in mM): 70.0 NaCl, 2.0 KCl, 4.9 $CaCl_2$, 4.7 $MgCl_2$, 5.0 glucose and 5.0 HEPES, pH 7.0. The cerebral ganglion was isolated, and the sheath on the surface of the PC was mechanically removed with fine forceps. The posterior surface of the PC was accessible with a patch pipette. The patch-clamp recordings were performed under a differential interference contrast (DIC) microscope (BX51WI, Olympus) with a 40× water-immersion objective. The composition of the pipette solution was the following (in mM): 70.0 potassium gluconate, 5.0 $MgCl_2$, 5.0 HEPES (pH 7.6) and 100–250 μg/ml nystatin (Wako Chemicals). The resistance of the pipette was 8–12 MΩ. A patch-clamp amplifier (Axopatch 200B, Molecular Devices) was used, and the signals were recorded on a computer via an A/D converter (Digidata 1322A, Molecular Devices). Focal application of 5-HT was performed by a glass micropipette (1–2 μm tip diameter) placed beside the soma (≤50 μm), and puff duration was 50–200 ms (10–30 kPa). 5-HT was dissolved in *Limax* saline solution containing 0.05 %, Fast Green (Sigma) to allow visual confirmation. To wash out the 5-HT solution, the bath solution was perfused continuously with saline.

Results

General organization

In semi-thin sections neuropil-like regions of various size was regularly observed in the cell body layer of both *Helix* and *Limax* PC (Fig. 1a, b). Depending on the section plane, these regions appeared either typical ovoid neuropil

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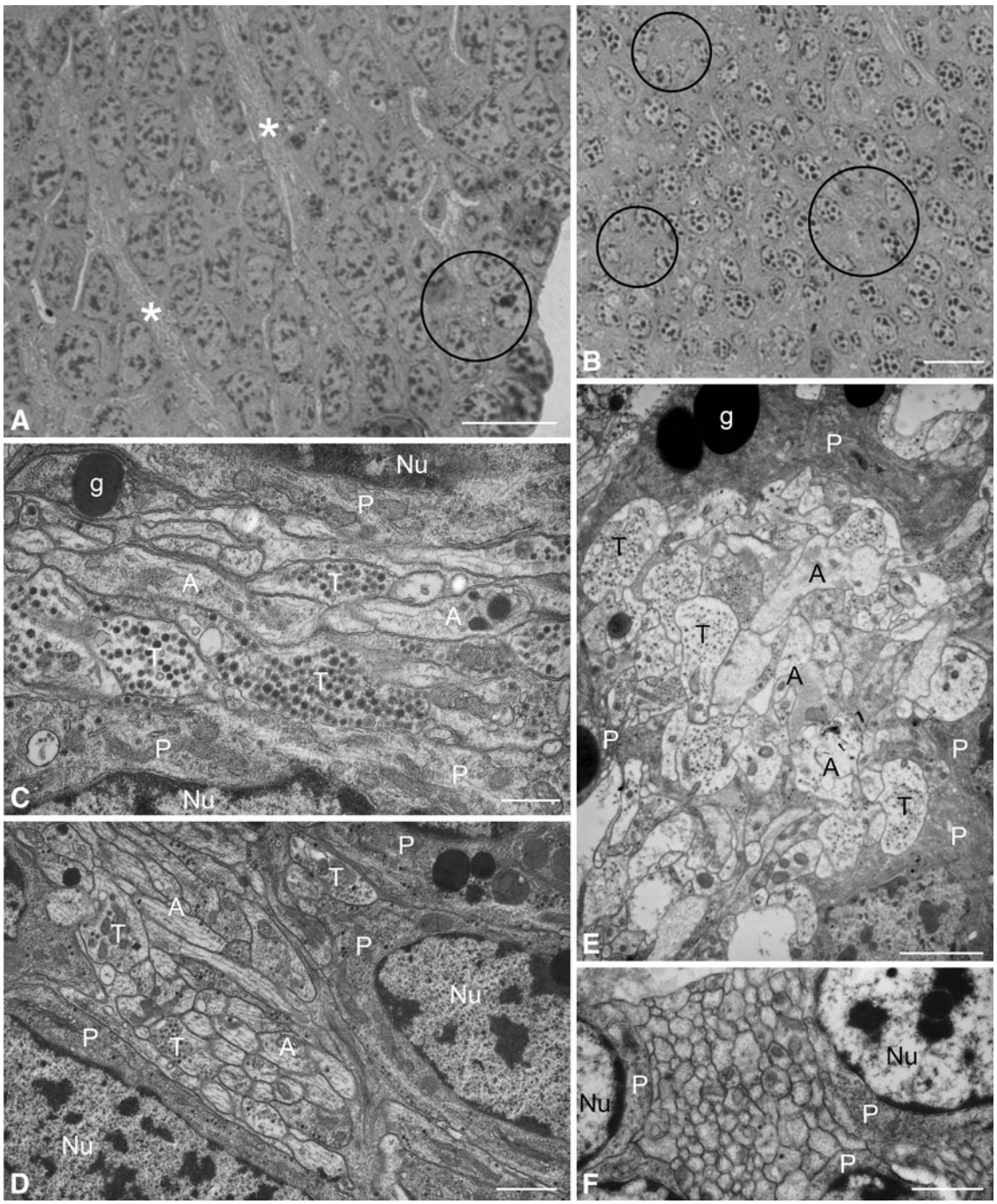

Fig. 1 Neuropil-like regions in the cell body layer of the PC of *Helix pomatia* (**a**, **c**, **d**) and *Limax valentianus* (**b**, **e**, **f**). Details of the *Helix* **a** and *Limax* **b** cell body layer seen in 1 µm toluidine blue stained Araldite sections. Note neuropil-like regions (*asterisks*, *encircled*) among the mass of procerebral perikarya containing large chromatin rich nuclei. **c**, **d** Details of typical neuropil-like regions located between cell bodies (*P*), composed of both varicosities filled with granular vesicles (*T*) and axon profiles (*A*). Note that the neuropil contacts directly the cell bodies (*P*). **e**, **f** Two types of neuropils found in the *Limax* cell body layer. In **e** a neuropil of larger size is seen surrounded by several perikarya (*P*). The neuropil consists of numerous granular vesicles containing (*T*) and electron lucent (*A*) axon profiles. In **f** an axon bundle located among cell bodies (*P*) consists of mainly electron lucent small size profiles resembling sensory axon processes. *Nu* nucleus, *g* electron-dense granules. *Scale bars* 8 µm in **a**, **b**—10 µm, 1 µm in **c**, **e** and 2 µm in **d**, **f**

regions or sometimes as elongated structures. Neuropil structures were also seen to be connected with the terminal (medullary) mass by septum-like structures.

At ultrastructural level, these local neuropil areas of the cell mass layer consisted of a highly variable number of axon profiles (Figs. 1c–f, 2). Large size neuropil structures

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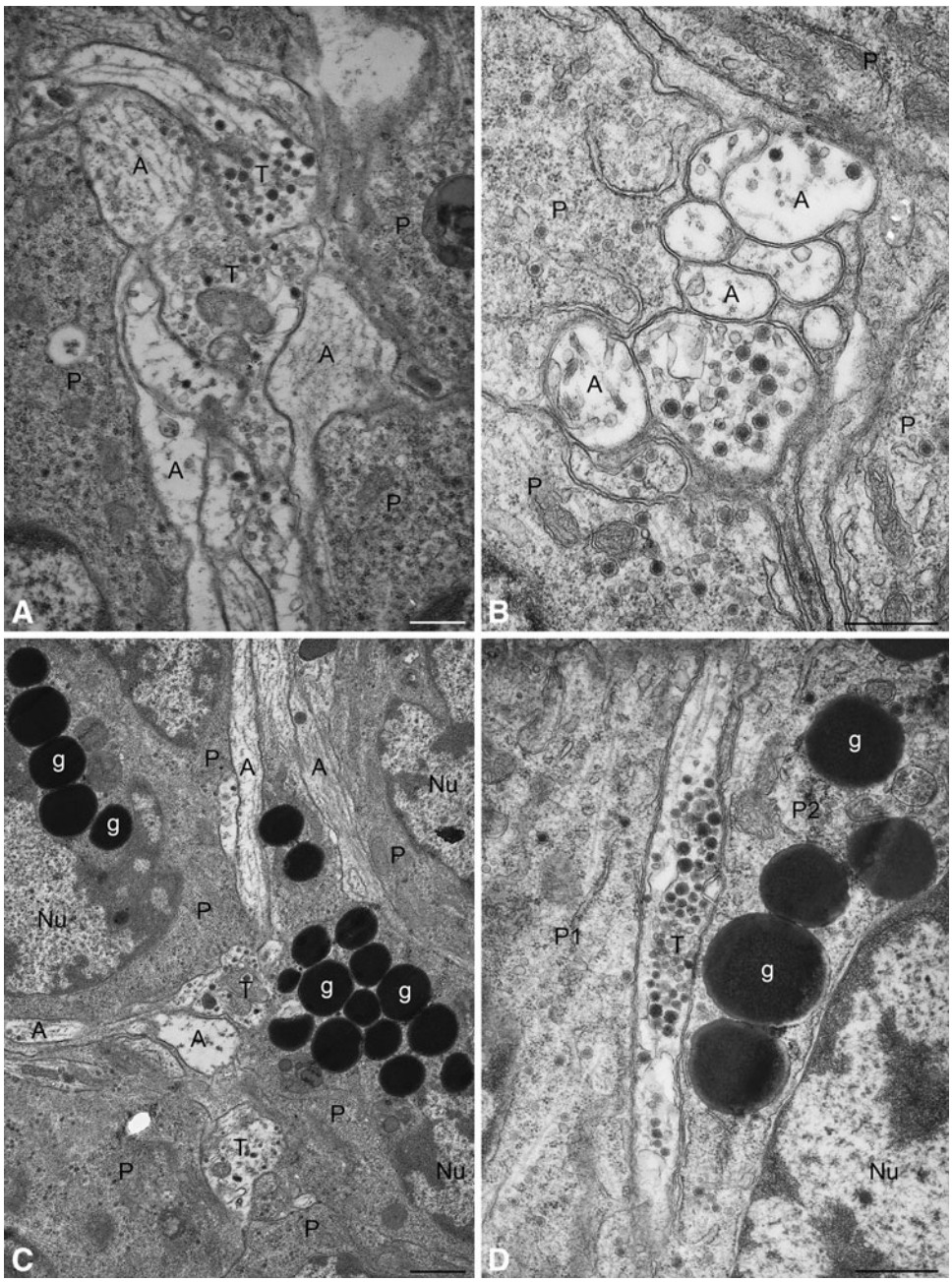

Fig. 2 Gradual axon branching/ramification in the PC cell body layer of *Helix* as demonstrated at ultrastructural level. **a** A bundle, including axons (*A*) and varicosities (*T*), is located between cell bodies (*P*). **b** A small bundle containing mainly small size axons (*A*) is surrounded by and deeply embedded in cell bodies (*P*). **c** Among cell bodies (*P*) individual processes (*A*) are running. Varicosities (*T*) are also present contacting perikarya. Note electron-dense granules (*g*) in the cytoplasm of some of the perikarya. **d** A longitudinally cut solitary axon with a varicosity (*T*) containing granular and agranular vesicles contacts simultaneously with long membrane segments two cell bodies (*P1*, *P2*). The profile was found in the course of a series of sections taken from the neuropil region shown in Fig. 1b. *Nu* nucleus, *g* electron-dense granules. *Scale bars* 1 μm in **a**, **b**, **d** and 2 μm in **c**

were surrounded by perikarya (Fig. 1c–e), meanwhile smaller units consisting only of a few axon profiles formed a more intimate relationship with the cell bodies, being partly embedded in the cytoplasm (Fig. 2a–c). Single fibers invaded deeply in among the cell bodies and their varicosities were completely surrounded by the cytoplasm (Fig. 2d). This kind of organization indicates delicate local arborization systems around the PC perikarya. In *Limax*, large bundles consisting of very thin axons were also found between cell bodies, resembling sensory axon units (Fig. 1f).

Axo-somatic contacts

Analysis of the form of axo-somatic contacts in the PC cell body layer of both species revealed both specialized synaptic and unspecialized but close membrane contacts (Figs. 3, 4). Polarized axo-somatic synapses displaying similar ultrastructural specializations described for the CNS of mollusks (Elekes 1978; Roubos and Moorer-Van Delft 1979; Bailey et al. 1979; McCarragher and Chase 1985) were found regularly both on the surface of the cell bodies and deeply embedded in the cytoplasm (Fig. 3a, b—

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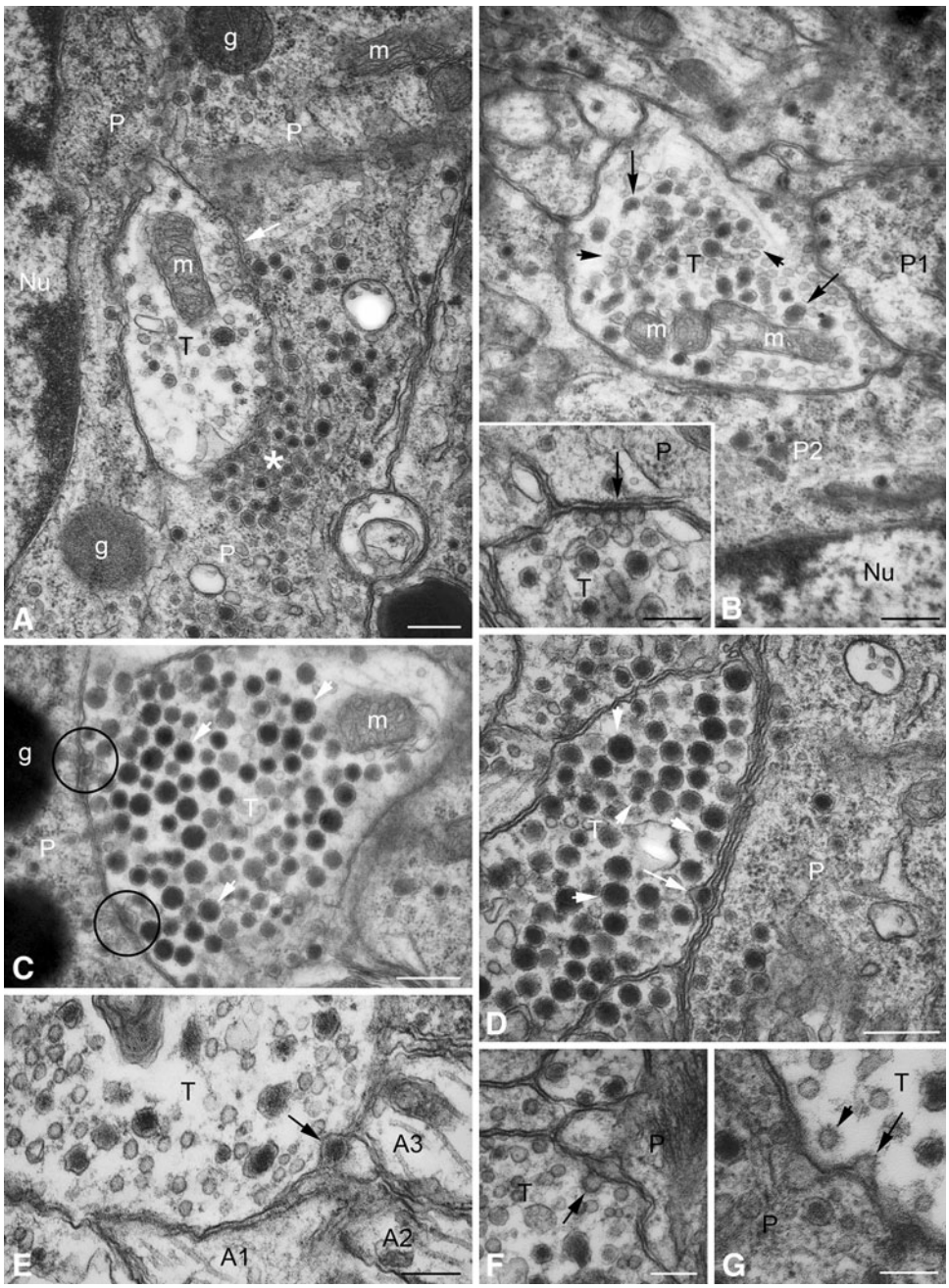

Fig. 3 Synaptic and non-synaptic connections in the cell body layer of the *Helix* PC. **a** A small ending (*T*) completely embedded in the cytoplasm establishes a synaptic contact (*arrow*) with a perikaryon (*P*). Note granular vesicles (*asterisk*) in the cytoplasm clustering opposite to the varicosity. **b** A large varicosity (*T*) contacts two globuli cell bodies (*P1*, *P2*). Note the mixed population of agranular synaptic (*arrowheads*) and granular vesicles (*arrows*). *Inset* Higher magnification view of an axo-somatic (*T*, *P*) synapse (*arrow*) established by the varicosity found after a long series of sections (*arrow*). **c**–**g** Non-synaptic release phenomena at axo-somatic contacts. **c** A large varicosity (*T*) containing numerous electron dense granules (*arrowheads*) display exocytosis at two points (*encircled*) along the membrane segment facing a cell body (*P*). **d** Exocytosis (*arrow*) of granule content from a varicosity (*T*) containing from large granules (*arrowheads*) nearby a perikaryon (*P*). **e** Exocytosis of a granule content (*arrow*) from a varicosity (*T*) facing several axon profiles (*A1–A3*). **f**, **g** Endocytotic membrane configurations (*arrows*) and a coated pit (*arrowhead*) at axo-somatic (*T*, *P*) contacts. *Nu* nucleus, *g* electron-dense granule, *m* mitochondrium. *Scale bars* 0.5 in **a**, **b**, 0.3 μm in **c**, 0.4 μm in **d**, 0.25 μm in **b** *inset* **e**, and 0.15 μm in **f**, **g**

inset). These axo-somatic synaptic contacts displayed an asymmetric clustering of a few agranular synaptic vesicles neighboring with a number of scattered large granular vesicles, pre- and postsynaptic membranes of increased electron density, presynaptic dense appositions, and intersynaptic cleft material (Figs. 3b—inset, 4c, d). On the other hand, numerous contacts were only characterized by close membrane appositions displaying no membrane specializations, but clustering of a mixed population of agranular and granular vesicles (Fig. 4a, b, e). Since the specialized synaptic contacts are rare in the gastropod CNS (Bailey et al. 1979; Elekes et al. 1983; Ratté and Chase 2000), the close but unspecialized membrane contacts may and will also be referred to as possible release sites. Indeed, along the unspecialized axo-somatic contacts exocytotic membrane configurations were also observed indicating the process of extrasynaptic release. In these cases, both omega-form membrane invaginations with dense granule contents located outside in the intercellular space (Fig. 3c–e) and coated pits and coated vesicles (Fig. 3f, g) occurred. These exocytotic structures were mainly observed in axon profiles of larger diameter and contained mostly large electron dense granules. In addition, some of them also displayed highly electron dense lipid-like granules (see

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e.g., Figs. 2c, d, 4a), indicating their origin from PC cells containing the same organelle. Whether these cells belonged to the B or the NB cell population was not possible to determine.

Synaptic configurations in the procerebrum

In the course of the analysis of the intercellular contacts within the cell body layer of the PC, simple forms of synaptic configurations referring to local integrative events were found. These were the following: (i) varicosities/boutons contacting simultaneously two or more cell bodies (Fig. 4a, e, g); (ii) varicosities/boutons contacting simultaneously cell bodies and axon profiles (Figs. 3b, 4b, f); (iii) varicosities/boutons receiving synaptic input meanwhile contacting PC cell bodies (Fig. 4c); and finally (iv) varicosities were also found establishing synaptic contacts simultaneously with more than one postsynaptic axon profile (Fig. 4d).

The synaptology of the cell body layer of the *Helix* and *Limax* PC is summarized schematically in Fig. 5, showing the forms both of the axo-somatic and axo-axonic interactions, as well as the synaptic and non-synaptic membrane configurations (Fig. 5 inset). It can be seen that local interactions may open a broad way of regulatory processes, which may have a role in the PC cells activity, and which are suggested to be taken into consideration when interpreting olfactory integrative processes.

5-HT-immunoreactive innervation and input system of the cell body layer

In *Helix* PC, the two-step peroxidase immunohistochemistry applied revealed a rich innervation established by a 5-HT-immunoreactive (5-HT-ir) network. Low- and high-magnification views showed that the cell body layer received a massive innervation by varicose axon processes, surrounding the individual cell bodies and forming frequently a perisomatic basket-like innervation (Fig. 6a, b). The varicosities were of different, smaller and larger size, and displayed intensive, dark immunostaining (Fig. 6b). Camera-lucida reconstruction of the 5-HT-ir innervation of the cell body layer of the *Helix* PC revealed the same degree of density of labeled processes (Fig. 6c), as seen otherwise in the medullary neuropil. At higher magnification the heavy, basket-like innervation pattern of practically all PC cell bodies was confirmed (Fig. 6d).

As to the *Limax* PC, Kobayashi et al. (2010) showed by fluorescence microscopy that the 5-HT immunoreactivity was exclusively confined to the neuropil region. This observation was confirmed after the application of peroxidase immunohistochemistry on Vibratome slices, since a network of faintly stained varicose 5-HT-ir fibers could only be observed in neuropil regions that were traced from the metacerebrum in the 50 μm slices (Fig. 7a). However, occasionally (one out of five PC preparations or less), at higher magnification extremely fine labeled processes could be resolved and traced in very short distances in the cell body layer (Fig. 7b). As a consequence of the weak labeling, electron microscopy failed to provide acceptable results, comparable to those obtained in *Helix*. Since the 5-HT-ir innervation of the cell body layer in *Limax* was sporadic we have tested the 5-HT sensitivity of the globuli cells by using patch-clamp technique. Following focal application of 1 mM 5-HT to the cell body layer an increased EPSP activity of the bursting cells could be recorded (Fig. 8). These increased EPSP activity may be caused also by the increased excitatory inputs from the PC neurons beside the recorded bursting cells but also 5-HT molecules coming from other, non-neuronal, for example neurohumoral source. We also found the increased spike activity in some bursting cells by 5-HT focal application (data not shown).

At the ultrastructural level 5-HT-ir varicosities were often observed to contact the procerebral cell bodies in *Helix* (Fig. 9). No synaptic membrane specialization could be found, however, it is possible that it was masked by the strong immunocytochemical reaction. Another possibility is that the very low (0.1 %) glutaraldehyde concentration applied, needed for 5-HT immunolabeling, was not optimal for the visualization of the specializations. The labeled profiles contained regularly numerous large (80–120 nm) granular vesicles (Fig. 9b, d). In the vicinity of the labeled profiles unlabeled varicosities/axons were seen, which contained also population of large granules (Fig. 9a, c) but which were clearly different in size and ultrastructural appearance when compared to those seen in the 5-HT-ir varicosities. The 5-HT-ir profiles, like the unlabeled varicosities, were also found in different configurations, contacting simultaneously more than one cell bodies (Fig. 9b), or cell bodies and unlabeled axon profiles (Fig. 9c).

A suggested scheme of the 5-HT-ir innervation of the *Helix* PC cell body layer, and its relationship to the PC cells and processes is shown in Fig. 10.

Discussion

Our results suggest that the ultrastructure and synaptology of the cell mass layer of the PC represents a functional well-defined region of the olfactory integration center both in *Helix pomatia* and *Limax valentianus*, two model species for studying the organization principles of gastropod olfaction, and so may help put the PC intercellular contacts in a new context of interpreting olfactory information processing. The results seem also to extend our general

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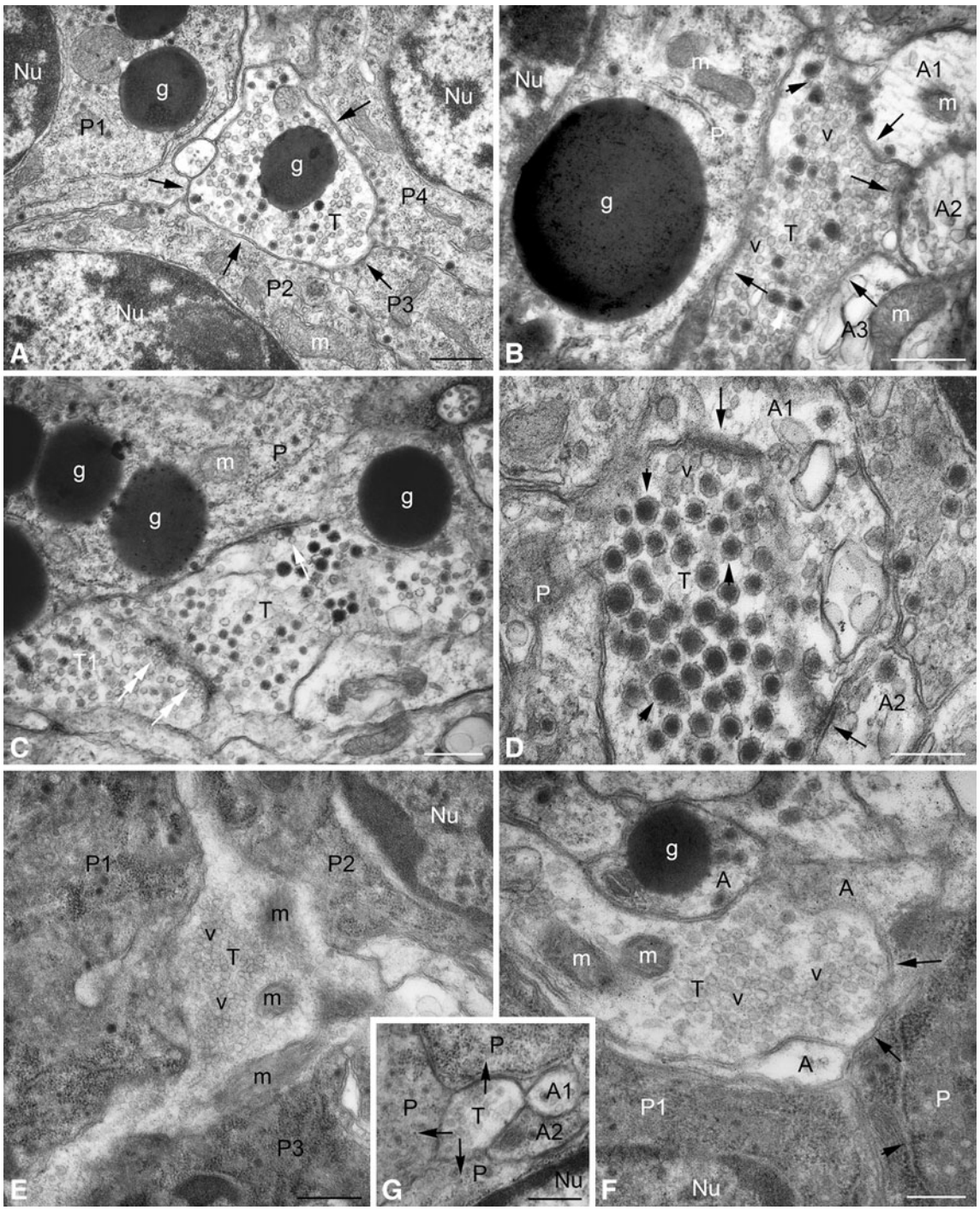

Fig. 4 Synaptic configurations in the cell body layer of the *Helix* PC. **a** A varicosity (*T*) contacts (*arrows*) simultaneously four cell bodies (*P1–P4*), indicating information divergence. **b** A varicosity (*T*) contacts (*arrows*) simultaneously a perikaryon (*P*) and three axon profiles (*A1–A3*), representing another form of synaptic divergence. **c** A varicosity (*T*) meanwhile forming a synaptic contact (*arrow*) on a perikaryon (*P*) receives an input (*double arrows*) from another varicosity (*T1*), referring to presynaptic modulation. **d** A varicosity (*T*) containing large size granules (*arrowheads*) establishes synaptic contacts (*arrows*) with two axon profiles (*A1*, *A2*). **e** A typical intersegmental varicosity (*T*) containing clear synaptic vesicles (*asterisks*) contacts simultaneously three cell bodies (*P1–P3*). **f** A large varicosity (*T*) containing almost exclusively clear synaptic vesicles (*v*) contacts a cell body (*P*) and several axon profiles (*A*). Another cell body (*P1*) is separated by a thin axon process. **g** Three small axon profiles (*T*, *A1*, *A2*) are closely attached to three cell bodies (*P*). One of the profiles (*T*) forms close membrane contacts with the cell bodies (*arrows*). *Nu* nucleus, *g* electron-dense granule, *m* mitochondrium, *arrowheads* ribosomal endoplasmic reticulum. *Scale bar* 0.5 μm in **a–d**, 0.4 μm in **e–g**

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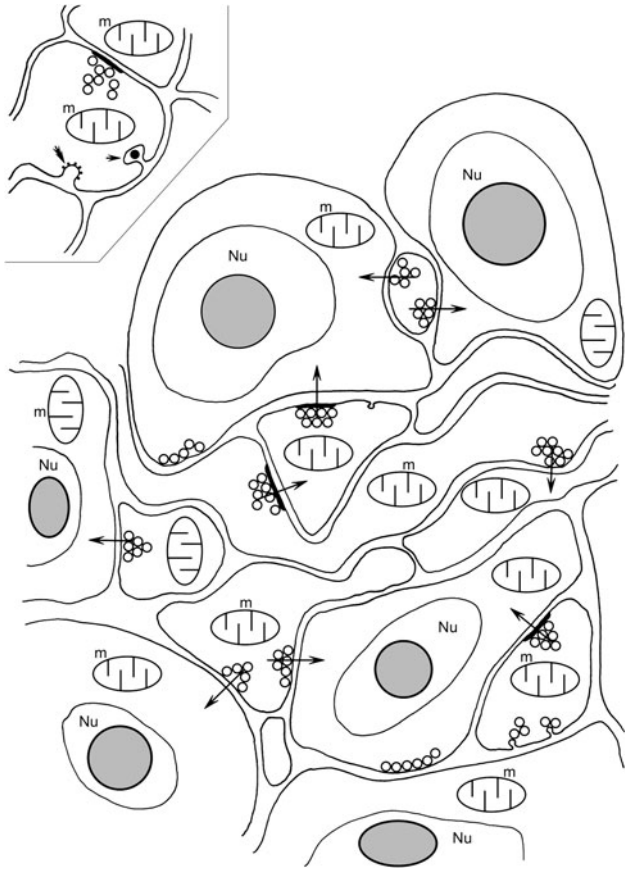

Fig. 5 A scheme summarizing the synaptology of the cell body layer of the PC of *Helix* and *Limax* shown in Figs. 2, 3, 4. *Inset* synaptic and non-synaptic membrane specializations found in *Helix* varicosities. No distinction was made between the bursting and non-bursting cells. It can be seen that the synaptic and non-synaptic contacts (*arrows*) may open a broad way of regulatory processes, influencing the activity of PC cells. *Open symbols* (*around the arrows*), synaptic vesicles, *Nu* nucleus, *shaded symbols* nucleolus, *m* mitochondrium

knowledge on PC wiring, since the majority of our current information on PC function and olfactory processing in terrestrial pulmonates comes from *Limax* (Gelperin 1999; Kimura et al. 1998; Kasai et al. 2006; Matsuo and Ito 2008; Matsuo et al. 2010), meanwhile information on structural and ultrastructural background is lacking. The high-resolution chemical-neuroanatomical and ultrastructural demonstration of the 5-HT-ir innervation provides the first morphological evidence for the involvement of 5-HT in PC processes, supporting earlier physiological studies which demonstrated the role of 5-HT in olfactory learning and memory (Gelperin et al. 1993; Gelperin 1999; Inoue et al. 2001; Kobayashi et al. 2010).

PC cell body layer synaptology

The PC cell body layer in both *Helix* and *Limax* contains neuropil regions of different size, which are characterized by a high frequency of occurrence of axo-somatic and axo-axonic contacts, displaying forms of synaptic configurations. According to our view, these observations considerably widen the interpretation possibilities concerning the modulation of integrative processes in the PC. It might be important since in the course of the only detailed study on the PC of *Helix* by Ratté and Chase (2000) concluded that the organization of the neuropil regions within the cell body layer was not different from that of the medullary neuropil. Consequently, observations obtained in the cell body layer were interpreted as a part of the entire neuropil region. Our results, however, demonstrated that the neuropils in the PC cell mass layer may represent sites of local circuits modulating the cell body activity. The gradual reduction of the number of axons participating in the formation of the neuropil regions, up to a few fibers, refer to a special role targeting the cell bodies only, and which probably conveys effects isolated from the noise of other axonal events better than in the medullary neuropil.

Our light- and electron microscopic analysis revealed that in addition to that described by Ratté and Chase (2000) complex forms of axo-somatic synaptic interaction also occur in the *Helix* and *Limax* cell mass layer. The massive axo-somatic innervation suggests that, in addition to the terminal and internal neuropil masses, the cell mass layer also represent an important level of synaptic modulation of information processing arriving from the tentacular region (sensory cells and tentacular ganglion). In addition, since the PC is supplied by diverse efferents of other CNS origin (Hanström 1925 [see in Bullock and Horridge 1965]; Ratté and Chase 2000; Chase 2002; Kobayashi et al. 2010; Ierusamlimsky and Balaban 2010), so as a consequence a part of the axo-somatic contacts and members of synaptic configurations may also be formed by these elements. We also observed axon-profiles contacting perikarya, that contained large electron dense granules. These cellular organelles occurred regularly only in the cytoplasm of a part of the PC perikarya. In contrast, even after careful analysis, axon profiles containing these dense granules could not be observed in the medullary neuropil. It suggests that the dense granules containing cells belong to PC neurons intrinsic to the cell body layer. Following intracellular labeling of PC cells in *Helix*, marked profiles synapsing with cell bodies were also found in the cell mass layer (Ratté and Chase 2000).

Serotonergic innervation of the cell body layer

The visualization of a dense perisomatic innervation of the PC cell bodies established by 5-HT-ir varicose axons is the first identifying the target sites of a given transmitter system and at the same delivers morphological evidence for the involvement of 5-HT in olfactory processing (Gelperin

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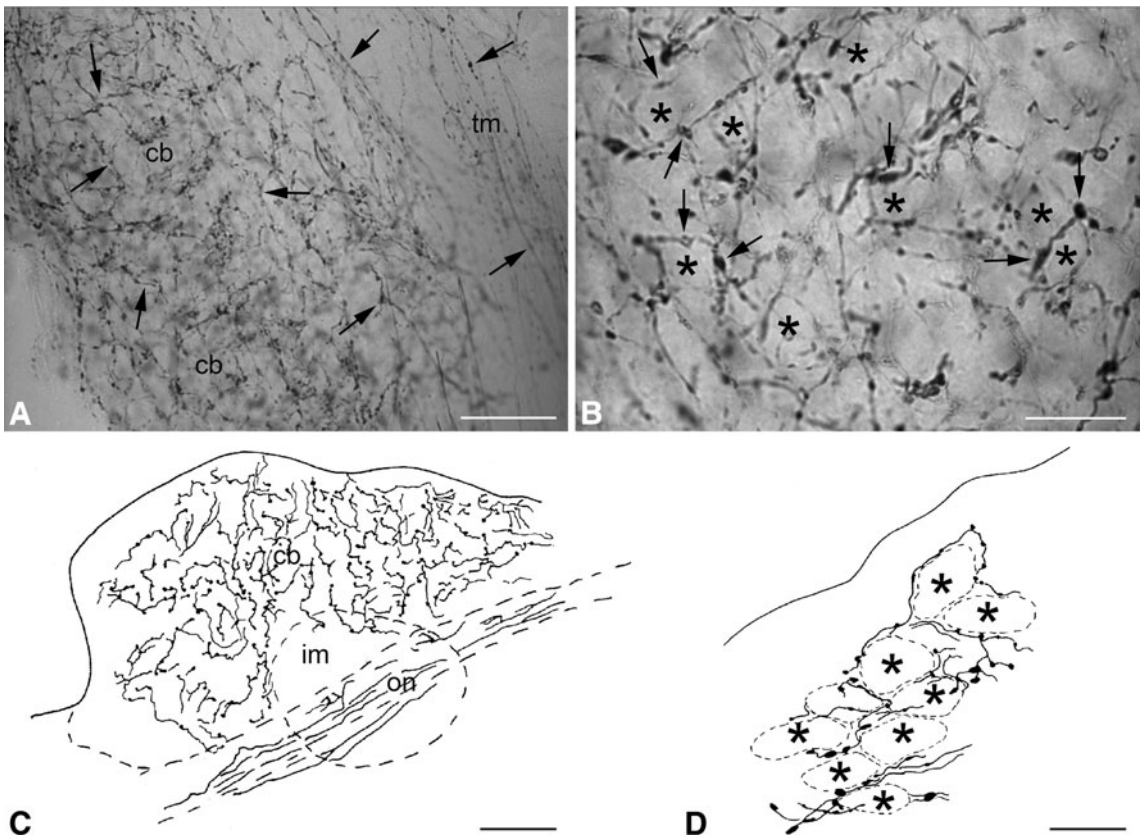

Fig. 6 5-HT-ir innervation of the cell body layer of the *Helix* PC visualized by HRP-DAB reaction. **a** Low magnification view of the PC richly innervated by 5-HT-ir fibers (*arrows*) in both the cell body layer (*cb*) and the terminal mass neuropil (*tm*). **b** Higher magnification detail from the cell body layer, showing cell bodies (*asterisks*) surrounded by varicose 5-HT-ir elements (*arrowheads*). The perisomatic basket-like 5-HT-ir innervation can well be seen. **c**, **d** Tracing of 5-HT-ir elements in the *Helix* PC cell body layer in 50 μm thick Vibratome slices. **c** Low magnification tracing in the cell body layer showing the dense 5-HT-ir innervations of the cell body layer (*cb*). **d** High magnification detail of the cell body layer revealing the innervation pattern of the individual perikarya (*asterisks*). *im* internal medulla, *on* olfactory nerve. *Scale bars* 30 μm in **a**, 15 μm in **b**, 20 μm in **c** and 8 μm in **d**

et al. 1993; Gelperin 1999; Kobayashi et al. 2010). On the other hand, our findings also shed light on possible species differences existing in this respect between closely related terrestrial pulmonate gastropods. Namely, axo-somatic 5-HT-ir contacts could be demonstrated in the PC of *Helix* but not in *Limax*, where only the terminal and internal masses displayed consequently 5-HT-ir innervation but a few thin fibers found occasionally in the cell body layer (see also in Kobayashi et al. 2010). By Inoue et al. (2004) extrinsic 5-HT-containing neurons were demonstrated in another anatomical region, the metacerebrum of the cerebral ganglion of *Limax* which innervated the PC neuropils without projecting to the cell mass layer. This difference emphasizes that in the PC of *Helix* 5-HT does have an additional level to influence the globuli cell function. Depletion of 5-HT by the serotonergic neurotoxin 5,7-dihydroxytryptamine was shown to impair odor learning in *Limax* (Shirahata et al. 2006), which effect, however, might be related to neuropil events. On the other hand, our electrophysiological data demonstrating the effect of 5-HT on the B cells of *Limax* PC may imply the possibility of another, neurohormonal source of serotonergic influence. A specific role has been attributed to 5-HT input in cellular learning models of *Aplysia* (see e.g., Kandel 2001; Glanzman 2007). It is also noteworthy that a difference between the FMRFamide-ir innervation pattern of the PC of *Helix* and *Limax* could also be established. In *Limax* the cell body layer contains a high number of FMRFamide-ir neurons innervating densely both the cell body layer and the neuropil mass (Kobayashi et al. 2010), meanwhile in the *Helix* PC, apart a few FMRFamide-ir cell bodies seen in the cell mass layer, a labeled fiber network was demonstrated only in the neuropil regions (Elekes and Nässel, 1991).

Origin of the innervation of the PC

In view of our present findings the organization and relationship of intrinsic and extrinsic innervation systems could also be re-considered regarding olfactory information processing in the PC of both *Helix* and *Limax*.

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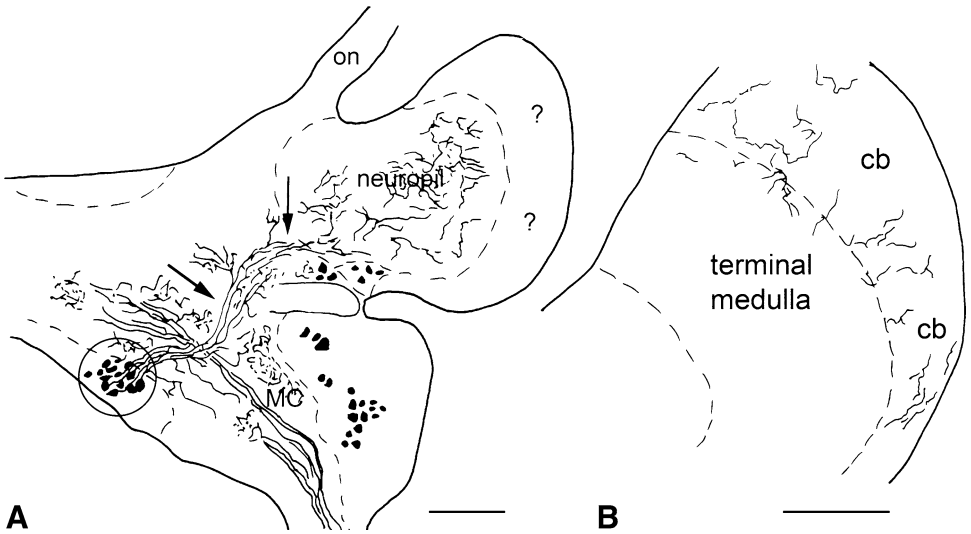

Fig. 7 Tracing of 5-HT-ir elements in the *Limax* PC in 50 µm thick Vibratome slices following HRP-DAB development. **a** Tracing of the 5-HT-ir innervation (*arrows*) from the commissural lobe (*encircled*) of the metacerebrum (MC) to the medullary neuropil. **b** Enlarged detail of the same preparation, showing labeled processes in the cell body layer (*cb*). Note their scattered presence compared to that seen in *Helix* (Fig. 6c). The success rate of finding labeled processes in the cell body layer of *Limax* PC preparations was approx. 1:5. *cpc* cerebro-pedal connective, *on* olfactory nerve. *Scale bars* 100 µm in **a** and 50 µm in **b**

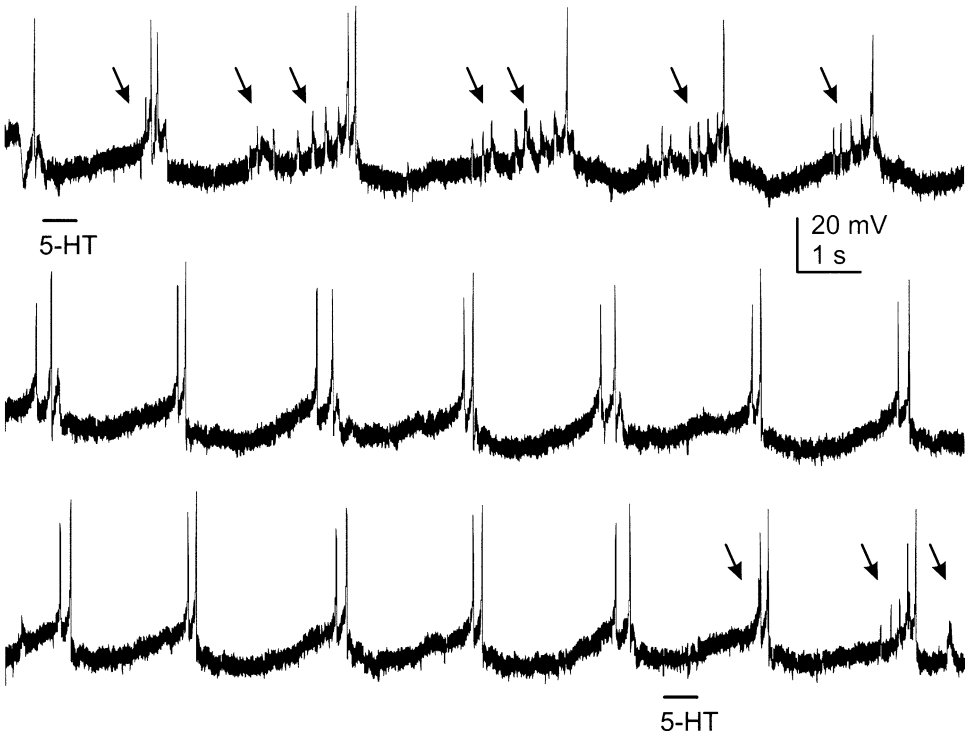

Fig. 8 Increased EPSP activity (*arrows*) following local application of 1 mM 5-HT (*horizontal bars in the upper and lower* traces of recording) on the EPSP of a bursting neuron in the *Limax* procerebrum, recorded in current-clamp mode of the perforated patch-clamp recording configuration. Middle trace of recording corresponds to the washout of 5-HT and the first part of the lower trace represents vehicle application

Connections of both intrinsic and extrinsic origin may have two levels of influence: one in the neuropil which is the conventional region of synaptic interactions, and a second one in the cell body layer of the procerebrum. This second level can be important in view of local circuits, involved in the regulation of the globulus cell activity, metabolism and synthesis as well as integrating cell body and axon activities. The simple forms of synaptic configurations demonstrated such as synaptic divergence or convergence show that the integration of olfactory information are definitely not bound exclusively to the neuropil regions of the PC. A special attention is to be paid to the configuration referring to presynaptic modulation, as a simple but basic form of learning at synaptic level (inhibition, facilitation).

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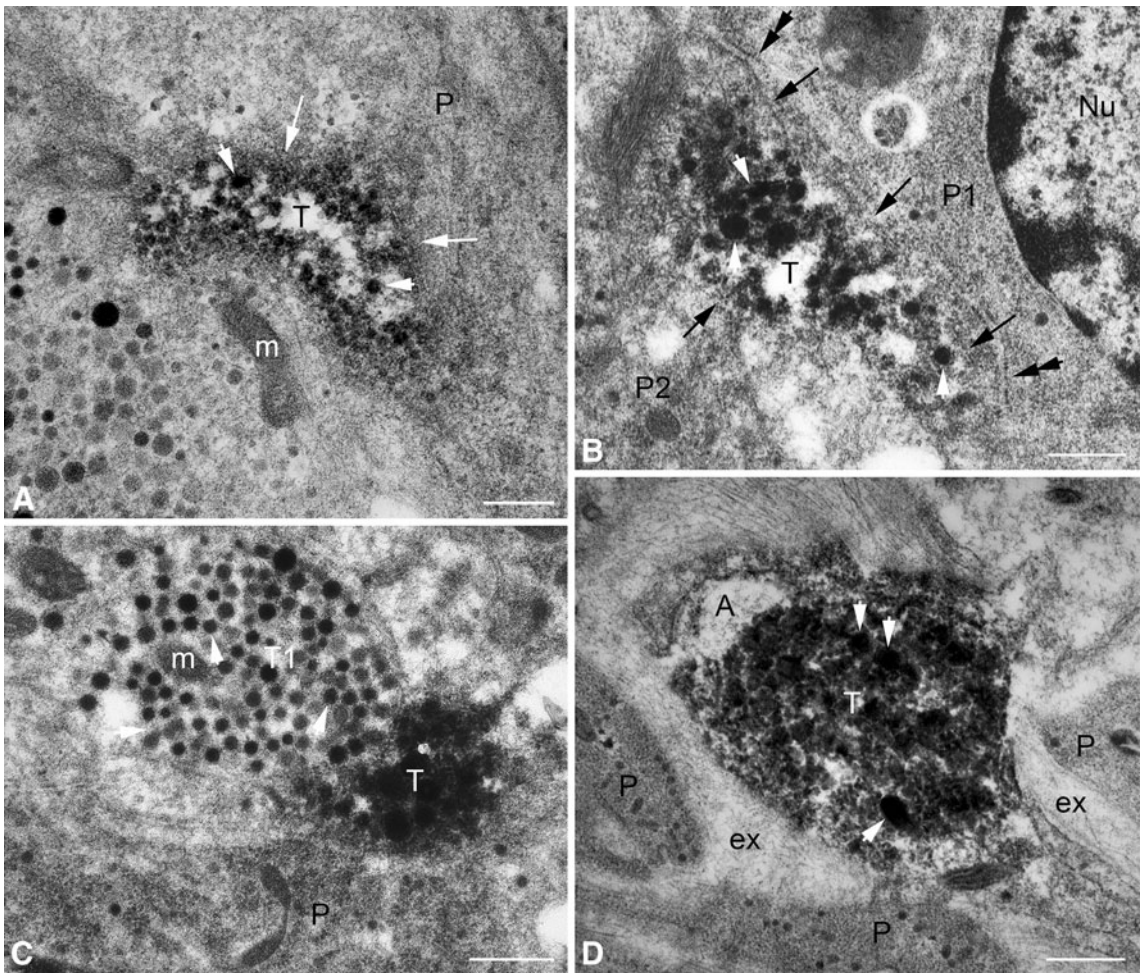

Fig. 9 Ultrastructure of 5-HT-ir elements in the cell body layer of the PC of *Helix*. **a** A labeled varicosity (*T*) containing electron dense granular vesicles (*arrowheads*) contacts (*arrows*) a perikaryon (*P*). **b** A labeled varicosity (*T*) contacts (*arrows*) simultaneously two cell bodies (*P1*, *P2*). Note large electron dense granules (*arrowheads*) in the labeled axon profile. **c** A labeled varicosity (*T*) is located between a cell body (*P*) and a large axon profile (*T1*) filled with large electron dense granules (*arrowheads*). **d** A 5-HT-ir profile (*T*) containing large electron dense granules (*arrowheads*) is located among cell bodies (*P*), but is separated by wide extracellular space (*ex*) from them. *Nu* nucleus, *m* mitochondrium, *A* unlabeled axon, *double arrowheads* ribosomal endoplasmic reticulum. *Scale bars* 0.25 μm in **a**–**d**

Presynaptic modulation was observed in both axo-somatic and axo-axonic positions, suggesting that the synaptic efferents of the procerebral cells can be modulated at both perikaryonal and axonal level. The presynaptic modulation at the axonal level involves, however, the possibility that both intrinsic and extrinsic elements of the central olfactory system can equally be the targets of these contacts. Following intracellular labeling, synaptic divergence and convergence established by marked elements were found in the internal mass of the PC of *Helix* (Ratté and Chase 2000). The pre- and postsynaptic elements involved in different synaptic configurations are possibly of PC cell origin, but they can also represent efferent (extrinsic) elements originating from other regions of the cerebral ganglion (Hanström 1925 [see in Bullock and Horridge 1965]; Ierusamlimsky and Balaban 2010), or other ganglia of the CNS (Chase 2002). According to electron microscopic immunocytochemistry, 5-HT-ir elements were also observed in synaptic configurations such as the simultaneous contact with perikarya and axon profiles in the PC of *Helix*. Accordingly, 5-HT may have an integrative (modulatory or transmitter) role either between intrinsic elements, or intrinsic and extrinsic components.

Future aspects

Our findings provide a basis for further studies in several aspects of the olfaction of terrestrial pulmonates, including (i) detailed functional and electrophysiological analysis of the globuli cells, both individually and *en masse*; (ii) distinguishing between synaptic and non-synaptic events; (iii) clarification and specification of the effect of 5-HT at cellular level; (iv) correlative studies of the neurochemical

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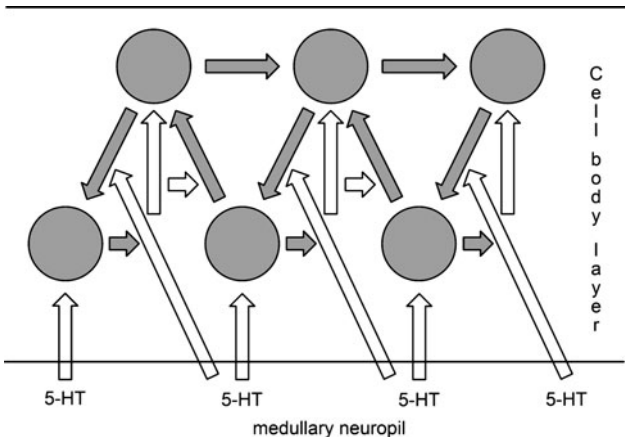

Fig. 10 A scheme representing the extrinsic 5-HT-ir innervations (*shaded arrows*) of the *Helix* PC cell body layer, relative to the contacts (*open arrows*) between the procerebral (globuli) cells, without distinguishing between bursting and non-bursting neurons. *Shaded symbols* procerebral (globuli) cells

character of the synaptic innervation pattern of the cell body layer and the whole PC; (v) identification of the synaptic contacts between the bursting and non-bursting cells, and of the procerebral targets of the serotonergic efferents.

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