May the Palps Be With You – New Insights Into the Evolutionary Origin of Anterior Appendages in Terebelliformia (Annelida)

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Abstract

Background: Head appendages in Annelida highly contribute to the immense morphological diversity in this lophotrochozoan taxon. Nevertheless, the evolutionary origin of annelid antennae, palps, cirri and tentacles are part of vast theories and debates that took place over decades. One of these heavily discussed groups are the Terebelliformia, which bear numerous anterior tentacles originating from different regions of the head. The question, whether these tentactes are homologous to feeding palps in other annelids or if these structures evolved convergently in terebellids and the remaining taxa, was highly debated in the past.

Results: By using morphological methods including immunohistochemistry, confocal microscopy, Azan-stained serial sections and 3D-visualisation, we are able to shed new light and a fresh look on the old question of the evolutionary origin of the buccal tentacles and their associated head structures in Terebelliformia. Our investigations show that the brains of the ampharetid *Hypania invalida* and the aulophora larvae of *Lanice conchilega* (Terebellidae) consist of a dorsal, more prominent and a more slender, ventral brain region. Neurite bundles innervating the buccal tentacles split off from the ventral and dorsal root within the ventral brain region and thus originate from the dorsal and ventral root of the circumoesophageal connectives. Hence, the observed neurite bundles fulfil the morphological criteria for the innervating neurite bundles of feeding palps known from Paleoannelida.

Conclusions: We disagree with former conclusions that buccal tentacles are part of the alimentary canal. Based on the presented data, the buccal tentacles of terebelliformian taxa can be homologized with feeding palps of other Annelida.

Our comparative investigations reveal important insights into morphological changes during the evolution of anterior head appendages in Terebelliformia and Annelida in general. Nevertheless, our analyses also illustrate the gaps in knowledge and that more investigations throughout the annelid tree are necessary to explain and understand the huge diversity of annelid anterior appendages.

Background

Annelida represent a fascinating and diverse group of invertebrates. In particular the frontal end shows a variety of head appendages in terms of shape, function and number, and contributes to this immense diversity of forms within segmented worms. A remarkable and highly discussed annelid taxon in this respect are the Terebelliformia. The exact phylogenetic position of terebellifomian lineages and the topology within the taxon were under persistent discussion for a long time (Holthe 1986; Glasby et al. 2004; Nogueira et al. 2013). However, phylogenomic approaches placed Terebelliformia confidently within the Sedentaria and hence they represent the sister taxon to the Maldanomorpha (Maldanidae + Arenicolidae) (Weigert et al. 2014a; Helm et al. 2018). Recently, new analyses uncovered the phylogenetic relations within the group, and suggest a taxon of Ampharetidae + Alvinellidae and Trichobranchidae + Terebellidae (including Melinnidae) (Stiller et al. 2020). Furthermore, the authors present Pectinariidae as

sistergroup to all other Terebelliformia and therefor recover the idea of an "archaeoterebellomorph" with four pairs of branchiae and a large prostomuim (Holthe 1986).

Deeply nested within the sedentary annelids (Weigert et al. 2014b), see also Fig. 1), most members of the Terebelliformia bear numerous, well-developed anterior head appendages.

Besides highly diverse branchiael structures, numerous slender buccal tentacles can be observed throughout the family. Notably, these buccal tentacles and their evolutionary origin are part of a long-lasting and still ongoing discussion (Nilsson 1912; Binard and Jeener 1928; Orrhage 1980, 2001; Holthe 1986; Rouse and Fauchald 1997; Zhadan and Tzetlin 2002; Orrhage and Müller 2005).

The position of these buccal tentacles differs inside the Terebelliformia and reaches from tentacles surrounding the mouth opening in an arc-like frame (in Pectinariidae), over appendages originating from an evertable pharynx (in Ampharetidae) to tentacles located in a dorsal position on the anterior end (in Terebellidae and Trichobranchidae) (Zhadan and Tzetlin 2002; Stiller et al. 2020). So far, two contradictory theories about the evolutionary origin of these buccal tentacles in Terebelliformia are debated:

1) the buccal tentacles are homologous to feeding palps observed in other annelids (Nilsson 1912; Binard and Jeener 1928; Rouse and Fauchald 1997; Zhadan Anna E. 2002) or

2) the buccal tentacles evolved from buccal structures and therefore represent a convergently evolved structure non-homologous to sensory or feeding palps of other Annelida (Holthe 1986; Orrhage 2001; Orrhage and Müller 2005).

In the early 20th century, these terebelliform buccal tentacles and their associated structures were homologised with almost any kind of anterior appendage, even being rudiments of antennae (Nilsson 1912; Binard and Jeener 1928). On the other hand, Orrhage (2001) described a ribbon-like, simple brain for Terebelliformia and suggested the buccal tentacles as being an outgrowth of the upper lip. According to his descriptions, the tentacular nerves are closely associated with nerves of the alimentary canal, thus being part of it. Contradictory, Rouse and Fauchald (1997) suggested that the buccal tentacles of Terebelliformia are homologous to the palps of e.g., Sabellidae and Spionidae - a statement which also follows their Canalipalpata-Aciculata theory. Other authors used the buccal morphology to accentuate the homology to palps by the absence of pharyngeal ciliated fields on tentacles and their area of attachment (Zhadan and Tzetlin 2002). Based on this investigations, the terebelliformian tentacles are not part of the alimentary canal. Furthermore, developmental studies favour the homology with feeding palps due to the independent maturation of pharyngeal structures and buccal tentacles (Eckelbarger 1974; Cazaux 1982; Bhaud 1988).

To shed new light on this long-lasting discussion, and to unveil further details helpful for our understanding of the evolutionary origin of terebelliformian anterior appendages, we used a comparative and integrative approach. Our analyses include a variety of morphological methods such as

immunohistochemistry, serial, Azan-stained, sections and and 3D-visualization. Our comprehensive investigations provide a fresh look on a more than a hundred years ongoing discussion and uncover the evolutionary origin and homology hypotheses for anterior body appendages in terebellids and allies. Additionally, our data will critically challenge existing knowledge dealing with this topic and contribute to our understanding concerning the evolution of character complexes in annelid worms.

Results

To understand the development and anatomy of the head appendages in Terebelliformia, we used aulophora larvae of *Lanice conchilega* (Pallas, 1766) for immunohistochemistry and *Terebella labidaria* (Linnaeus, 1767) for Azan-histology (as members of the Terebellidae). Furthermore, we investigated adult *Hypania invalida* (Grube, 1860) as member of the Ampharetidae with both methods. In our descriptions of the nervous system, we refer to Richter et al. 2010 for used terms and annotations. The description of the head parts follows, if not stated otherwise, Zhadan &Tzetlin 2002.

Central nervous system

Aulophora larvae of *Lanice conchilega* and adult *Terebella lapidaria* (Terebellidae)

The aulophora larvae of *Lanice conchilega* already show all features of adult Terebellidae, although the number of anterior tentacles is highly reduced (Fig. 3A). The brain of *L. conchilega* consists of one more prominent dorsal brain region and the delicate anlage of the ventral one (Figs. 3C-F; 7D, E). Both are connected to both roots of the circumoesophageal connective (*cc*) (Figs. 3C, E, F). The ventral brain region protrudes slightly anteriorly from the dorsal one, which is well shown by confocal data but hardly perceptible in Azan-sections. Additionally, several thin longitudinal prostomial nerves connect the dorsal to the ventral region of the brain (Fig. 3F). The subepidermal ventral nerve cord is composed of two longitudinal neurite bundles. Furthermore, four pairs of serotonergic neurite bundles are visible within the ventral nerve cord (*vnc*) of aulophora larvae (Figs. 3A, B). Ventrally, in particular where the *cc* originate, groups of serotonergic cell somata form prominent aggregations (Figs. 3C, D). At regular intervals, two pairs of lateral nerves originate from the *vnc* and proceed in dorsal direction. Although, we could not trace these nerves to their final destination they seems to innervate regular emerging dorsal notopodial structures, such as chaetal muscles or branchiae (in posterior segments). Similar to *H. invalida*, four pair of nerves emerge from the *cc* and proceed in lateral direction (Fig. 3D). They branch off and innervate two lateral epidermal, serotonergic protrusions next to the heavily ciliated lateral lips (Fig. 3B). Glandular cells often catch antibodies. Hence, a strong serotonergic signal without a nervous system specific pattern in huge epidermal cells hints towards the presence of glandular structures in this region (Figs. 4C, G). The stomatogastric nervous system is composed of a lateral lip nerve (Figs. 3C; 7D, E) and a lower lip nerve (Fig. 7D, E). Both are connected to a loop-like stomatogastric nerve (Figs. 3C, D; 7D, E). The lateral lip nerve is connected to the ventral region of the brain via several thin stomatogastric nerves (Figs. 3C; 7E). Along the nerves of the lateral lip, a dense meshwork of slender neurite bundles is coating the upper mouth region (see Fig. 3C). Additionally, numerous delicate nerves originate at the lateral lip nerve and

proceed along the dorsal side of the pharynx as found for the ventral side of the stomatogastric loop-like nerve as well (see Figs. 7D, E). The lip nerves and the stomatogastric loop-like nerve are connected to the *cc* via few thin neurite bundles (see Figs. 7D, E).

Hypania invalida (Ampharetidae)

The brain of *Hypania invalida* is located subepidermally. It consists of a dorsal and ventral region similarly arranged as described for *L. conchilega* (Figs. 5A-C; 7A-C). The dorsal region of the brain (dpbr) is the more prominent part (Figs. 5A-C), linked with numerous cell bodies located dorsally to the brain. The more delicate ventral region of the brain (vpbr), better perceptible in confocal data than in Azan-sections, show a more advanced protrusion from the dorsal region compared to *L. conchilega*. It is connected to the circumesophageal connectives (cc) via a ventral brain connective (cvbr) (Fig. 5D). Additionally, it is connected to the dorsal brain region by a dorsal brain connective (cdbr) (Figs. 5D; 7B). Both parts are connected to the ventral nerve cord (vnc) (Figs. 5A, C; 6E; 7A) by the *cc* (Figs. 5A-C; 6A-C; 7A-C). The latter is composed of two major nerve strands (roots) - the ventral and the dorsal one (visible via serotonin staining, Fig. 5A). The subepidermal *vnc* is composed of two pairs of distinct serotonergic neurite bundles with cell soma concentrated in ganglia (Figs. 6E, G). Anteriorly of the transition from *vnc* to the *cc,* three pairs of neurite bundles (Fig. 5C: pn) emerge from each side of the *cc* and proceed laterally. There they branch off and become part of the intraepidermal peripheral nervous system (Fig. 5C). Anteriorly, a pair of stomatogastric nerve bundles (Figs. 5C; 7A-C) originates medially of the *cc* and fuses with the ventral root of the ventral brain region (Figs. 5B-C; 7A-C). From that point of fusion, two nerves connect a prominent stomatogastric loop-like neurite bundle which proceeds ventrally along the pharynx (Figs. 5C-E; 7A-C). Numerous delicate neurite bundles emerge from that loop-like structure and innervate the anterior stomatogastric nervous system. The dorsal branch of the *vpbr* is connected to the brain via several minute nerves, which run along the prostomium of adult *Hypania invalida* (prn) (Figs. 5B-E; 7A-C).

Anterior appendages

Aulophora larvae of *Lanice conchilega* and adult *Terebella lapidaria* (Terebellidae)

The most prominent structure observable in aulophora larvae of *L. conchilega* are the tentacles at the anterior end (Figs. 3A, B; 4A-D; 7D). The latter are located dorsally to the mouth opening. Cross-sections of the tentacles of adult *Terebella lapidaria* exhibit a prominent longitudinal invagination along the entire tentacle. This prominent structure, which is also present in the tentacles of *L. conchilega,* represents the food rim (see Figs. 4A-C, F). The (buccal) tentacles in *L. conchilega* are innervated by numerous neurite bundles (Figs. 3C, D; 7D, E), which originate in the delicate anlage of the ventral region of the brain (Figs. 3C, E, F; 7E). The ventral region of the brain is connected to both strands (roots) of the *cc* (Figs. 3C-F; 4A; 7D, E). In particular the chaplet-like appearance is obvious in serotonin-like immunoreactivity (Figs. 3C, F; 7D, E). Each anterior appendage is innervated by two pairs of neurite bundles originating from two distinct ganglia located at the base of the tentacle (Figs. 3D, F; 7D, E). The latter nerves run along the entire structure and form a prominent loop at the tentacular tip. Notably, the connection between these

ganglia is not direct. Although the respective structures are laying side by side, they are interconnected via the mentioned nerve loop of the tentacles (see Fig. 7E).

Hypania invalida (Ampharetidae)

Dorsally - at the anterior end of *H. invalida* - four pairs of tentacle-like head appendages with two prominent blood vessels are present (Fig. 6A-D). These appendages are innervated by the first four pairs of lateral neurite bundles, in every case a thick and a slender one, originating from the *vnc* and proceed dorsally (Figs. 5A, C; 7A, C). Along their course - on half way - they are connected to a crossing neurite bundle innervating the lateral organ (Figs. 5A; 7B, C). Afterwards, the lateral neurite bundles terminate in a ganglion (Figs. 5A; 7A, B). From that terminal ganglion, two main branchial neurite bundles emerge and proceed along each branchial structure forming a loop by getting interconnected by numerous ring-shaped neurite bundles (Figs. 5A; 7A).

Additionally, a second type of head appendages – normally exhibited in inverted position inside the pharynx – is present within the oesophageal cavity (see Figs. 5A; 6A-C, 6F-H; 7A, B). These so-called buccal tentacles are innervated by four neurite bundles originating from the ventral and dorsal root of the ventral region of the brain (Figs. 5A-E; 7A-C). In everted position, these tentacles can be stretched out for food uptake (shown in Figs. 5B, C; 7C).

A combination of all datasets allows for a proximation concerning the most probable location and appearance of these head appendages in everted position and their respective neuronal innervation (Fig. 7C). The assumed position and differentiation between short more proximal tentacles (Figs. 6A, B, H; 7A, C) and long distal tentacles (Figs. 6F-H; 7A, C) is mainly based on the Azan stained-sections and the 3D-reconstruction.

Discussion

The brain and major neuroanatomical properties in Terebelliformia

Based on our comprehensive investigations, the terebellid *Lanice conchilega* and the ampharetid *Hypania invalida* possess circumoesophageal connectives (cc) which are similarly connected to the brain by two strands (roots), a ventral and a dorsal one. These strands or roots of the *cc* show a clear serotonin-like immunoreactive signal, but cannot be seen in Azan-sections. Due to their general location and the patterning in anti-serotonin-staining, we assume them as being homologous to the two roots former authors described using histological sections (Orrhage and Müller 2005). Anatomical differences, in terms of course and extension of the mentioned roots in both investigated taxa, are caused by morphological transitions related to adaptive changes of the entire anterior end in Terebelliformia. Although both roots of the *cc* are closer associated in *H. invalida* than in *L. conchilega*, the transition of the latter into the two paired neurite bundles of the ventral nerve cord is comparable when observing their serotonin-like immunoreactivity. A closer examination of the neuroanatomical characteristics of both taxa shows many similarities in this respect. In anterior direction, the *cc* fuses with the dorsal, more prominent

region of the brain. Antero-ventral to the dorsal brain region, two connectives - one splitting from the *cc* (connective of ventral brain region-cvbr) and another proceeding from the dorsal brain region (cdbr) - fuse by forming the ventral, more slender region of the brain. Thereby, the ventrally-oriented brain region splits up distally and forms a distinct ventral and dorsal root, which both innervate the buccal tentacles in *H. invalida* and *L. conchilega*.

Contradictory, earlier investigations focussing on Terebelliformia describe a unified, ribbon-like brain (Orrhage 2001) and postulate the ventral and dorsal connective (cvbr and cdbr) as the two roots of the "common tract" – the latter not being part of the brain. Instead, both roots were described to innervate the lateral part of the "tentacular membrane" and the buccal tentacles.

Nevertheless, our data reveal a protrusion of a slender brain region in ventral direction. This ventral region exhibits two neuronal roots and innervates the buccal tentacles in Terebelliformia. The investigations presented herein demonstrate that nerves of the so-called "tentacular membrane" (see Orrhage 2001) are in fact loop-like neurite bundles, which connect the dorsal brain region with the dorsal root of the ventral brain region (prn). Caused by a hypothesized evolutionary transition of the dorsal lip and associated buccal tentacles from the mouth region towards dorsal in Terebellidae (as summarized in Zhadan and Tzetlin 2002), these prostomial nerves are comparable with those in the ampharetid *H. invalida.* Nevertheless, they are much shorter. The same loop-like nerves innervate the ampharetid buccal tentacles as well as the "dorsal ridge" in Terebellidae (according to Orrhage, 2001) and potentially even the "cephalic veil" in Pectinariidae.

Due to the comparable position of the brain in Terebelliformia (Zhadan and Tzetlin 2002) and similar innervation patterns – including prostomial loop-like nerves connecting the dorsal to the ventral brain region – our data generally confirm previous assumptions (Orrhage 2001; Zhadan Anna E. 2002; Orrhage and Müller 2005). Accordingly, the "cephalic veil" of Pectinariidae, the "dorsal ridge" of Terebellidae and the hood-like "tentacular membrane" of Ampharetidae should be treated as being homologous structures.

In contrast, our conclusions concerning the potential prostomial or peristomial origin of these structures differ from earlier hypotheses. Fauchald and Rouse (1997) suggested the pectinariid cephalic veil as a fusion of pro- and peristomium. Furthermore, developmental studies support the tentacles of Terebellidae as being part of the prostomium (Bhaud and Gremare 1988), whereas others suggested a peristomial origin of the latter (Zhadan and Tzetlin 2002). Only for Ampharetidae developmental and morphological studies both suggest a peristomial origin of the buccal tentacles (Cazaux 1982; Zhadan and Tzetlin 2002).

The combination of potentially homologous innervation patterns described for Pectinariidae (Orrhage 2001) and our observation concerning terebellid and ampharetid taxa, homologous structures such as the "cephalic veil", the "dorsal ridge" and the "tentacular membrane" should be defined as prostomial structures.

The earlier erroneous interpretation of terebellid tentacles as being of prostomial origin might have been caused by the developmental transition of character complexes involved into the cephalisation processes. This cephalisation is obvious for the ontogenetic transition of branchiae and their inclusion in the formation of the anterior end in Ampharetidae, Alvinellidae, Melinnidae and Terebellidae (Stiller et al. 2020), but is not responsible for the localization of terebelliform tentacles. The latter are characterized by a steady lateral addition of tentacles during ontogenesis. Both processes are independent and will be discussed in more detail below. Nonetheless, additional detailed morphological as well as comprehensive developmental analyses are necessary for a better understanding of the role of cephalisation and multiplication processes in the formation of morphological features in Annelida.

Branchiae – anterior transition during cephalisation

The ampharetid *Hypania invalida* bears four pairs of digitate branchiae grouped dorsally on the head, while the two terebellid species *Lanice conchilega* and *Terebella lapidaria* show three pairs of dichotomous branchiae serially arranged along the trunk (Capa and Hutchings 2006; Stiller et al. 2020). The putative basally-branching Pectinariidae also exhibit four pairs of branchiae along the segments II-V like shown for many other Terebelliformia (Nogueira et al. 2010, 2013). For all species investigated herein, the branchiae were easily identifiable in histological sections by the appearance of an afferent and an efferent blood vessel surrounded by the coelomic cavity.

In Terebelliformia, an ancestral number of four branchiae is assumed, while several reductions, transitions and even multiplication processes took place (Stiller et al. 2020). Due to developmental studies (e.g., Cazaux 1982) the branchiae in larval Ampharetidae occur from segment II-VI and shift towards anterior during ontogenesis. Therefore, branchia in adults are located at segment II and III (Stiller et al. 2020). All branchial appendages in Terebelliformia are innervated by a more prominent anterior and a slender posterior, segmentally arranged lateral neurite bundle originating from the anterior end of the *vnc*. They proceed in dorsal direction along the trunk musculature and terminate in a huge neuron, situated at the base of each branchia. Notably, this observed pattern is comparable to the neuronal innervation pattern known from parapodial appendages in the errant annelids *Neanthes arenaceodentata* (Moore, 1903) and *Platynereis dumerilii* (Audouin & Milne Edwards, 1833) (see Winchell et al. 2010; Starunov et al. 2017). Therefore, an evolutionary scenario including a parapodia-linked origin of the branchial structures and the later involvement in cephalization events has to be assumed. Notably, cephalisation seems to be a widespread evolutionary phenomenon in annelids, and is described for several errant as well as sedentary taxa (Åkesson 1962; Faroni-Perez et al. 2016). Such an ontogenetic transition of larval trunk-associated appendages towards anterior in adult specimens seems to represent an important mechanism. Cephalisation seem to represent one major evolutionary process responsible for the diversity of the sensorial apparatus and even physiological adaptations of the anterior end in Terebelliformia and Annelida in general. Unfortunately, we were not able to compare our observation with the neuronal innervation of the branchiae in adult Terebellidae. The branchiae in the investigated aulophora larvae of *L. conchilega* were still not fully developed and can therefore not be used for detailed interpretations.

The anterior-most appendages – a multiplications of palps?

In the ampharetid *H. invalida* each tentacle is innervated by four neurite bundles, whereas in total eight prominent neurite bundles proceed along each tentacle in late larvae of *L. conchilega*. However, in both species the tentacles are similarly innervated by neurite bundles, which originate from the ventral and dorsal root of the circumoesophageal connectives. In *H. invalida,* tentacular nerves originate from the ventral root of the more delicate ventral brain region, which is connected to the circumoesophageal connectives (cc) by the ventral brain region commissure (cvbr). Additionally, they always originate from the dorsal root of the ventral brain region, which is connected to the dorsal, prominent region of the brain by the dorsal brain region commissure (cdbr). In *L. conchilega,* nerves of the buccal tentacles originate from neurons arranged arc-like on the ventral region of the brain. They are innervated by neurite bundles of both roots of the *cc.* These neurite bundles split up directly before entering the more prominent dorsal brain region. Such an arc-like set of innervating neurons was never described for terebellids so far.

According to various authors, annelid feeding palps are defined as being innervated by nerves originating from the ventral and dorsal main neurite bundles (roots) of the *cc* (Fauchald and Rouse 1997; Orrhage 2001; Rouse and Pleijel 2001; Orrhage and Müller 2005; Purschke et al. 2014). As described for the terebellid *Pista cristata* (Müller, 1776) and the ampharetid *Amphicteis cf. gunneri* (M. Sars, 1835) (Orrhage 2001) a quite similar innervation pattern of the buccal tentacles can be assumed. Furthermore, they seem closely associated with the nerves of the alimentary canal (Orrhage 2001; Orrhage and Müller 2005). By comparing earlier investigations (see Orrhage and Müller 2005) and our results, the branching pattern of all involved neurite bundles is similar. It is shown that stomatogastric nerves only originate from the *cc* or/and its ventral root and thus belong to the ventral brain region. A pattern we can observe in *H. invalida* and *L. conchilega* as well. Accordingly, the ventral strand (root) of the *cc* and the innervation of the entire stomatogastric system are closely associated. A close neuroanatomical connection of the "common tract"/ ventral region of the brain and the stomatogastric neurite bundles seems to be a widespread phenomenon. An alimentary origin of the buccal tentacles is therefore unreasonable based on the presented data.

Furthermore, no obvious developmental connection of the pharynx and the buccal tentacles can be observed for terebelliform taxa so far (Eckelbarger 1974; Cazaux 1982; Bhaud and Gremare 1988).

According to the current knowledge (Orrhage 2001) and our presented data, a similar neuroanatomy can be assumed for Pectinariidae as being the sister taxon of all other Terebelliformia (Stiller et al. 2020). Many authors also support the homology of the buccal tentacles in all Terebelliformia investigated so far (Holthe 1986; Orrhage 2001; Zhadan and Tzetlin 2002). In conclusion, our data strongly promote a homologization of the buccal tentacles in Terebelliformia with the feeding palps in the remaining Annelida.

Accordingly, in Oweniidae (exemplarily shown for *Owenia fusiformis* (Delle Chiaje, 1844) and *Myriowenia sp.* (Hartmann, 1960)), two main neurite bundles innervate the palps or the tentacular crown. These prominent neurite bundles originate dorso-laterally and medio-dorsally from the brain (Beckers et al.

2019b). Together with data from Magelonidae - which show a much more structured brain with a clear interpretation of the palp neurite bundles coming from the ventral and the dorsal region of the brain - a homologization of the feeding palps in Paleoannelida (Oweniidae and Magelonidae) and Terebelliformia is plausible (Beckers et al. 2019a) . In Sedentaria, comparable palps and palp nerves fulfilling these neuroanatomical criteria can also be found in Orbiniidae (Wilkens and Purschke 2009), Siboglinidae (Worsaae et al. 2016; Rimskaya-Korsakova et al. 2018), Cirratuliformia (Orrhage and Müller 2005) and Spionidae/Sabellidae (Orrhage 1980; Orrhage and Müller 2005; Purschke 2016). A detailed comparison of the neuroanatomy of all mentioned taxa highly supports our hypothesis concerning the terebelliformian anterior-most head appendages as sharing the same evolutionary origin like the feeding palps of other taxa. Comparable investigations for errant annelids are still pending.

During the ontogenetic formation of the anterior end, a clear differentiation has to be made in respect of "cephalisation" and "multiplication" – not only in Terebelliformia.

In contrast to adult Terebellidae, the aulophora larvae of *L. conchilega* possesses only a few tentacles, which are arranged antero-dorsally on the head. During ontogenesis, multiplication – an increase in the number of similar structures – in this case of the tentacles, leads to a lateral increase of tentacles until adulthood. Inside the tentacular bud, loop-like nerves originate from the ventral brain region and differentiate from lateral in median direction. This multiplication process is also known from other sedentary polychaetes, such as Sabellariidae and Sabellidae (Wilson 1936; Faroni-Perez et al. 2016) and results in an anterior concentration of numerous identical structures, like e.g. feeding palps, which are therefore involved into the formation of the anterior end. In contrast, the anterior clustering of other structures – such as branchiae – is shown to be the result of ontogenetic transition of parapodia-associated structures during cephalisation (see above). Although the result of both processes – multiplication and cephalisation – highly contributes to the formation of the anterior end in annelids (or at least the realisation of the sensorial pecularities of the head), they have to be considered as independent processes that should be interpreted separately.

Conclusion

Our comparative and comprehensive approach including various morphological methods and a detailed literature revision sheds new light on the evolutionary origin of anterior head appendages in Terebelliformia.

The brains of the ampharetid *Hypania invalida* and the aulophora larvae of *Lanice conchilega* (Terebellidae) consist of a dorsal, more prominent and a more slender, ventral brain region, which seems to be the result of a brain protrusion. Neurite bundles innervating the buccal tentacles split off from the ventral and dorsal root within the ventral brain region and thus originate from the dorsal and ventral root of the circumoesophageal connectives. In this respect, the observed neurite bundles fulfil the morphological criteria for the innervating neurite bundles of feeding palps known from Paleoannelida. Furthermore, we confirm a close association of the neurite bundles of the buccal tentacles and neurite

bundles of the alimentary tract. Nevertheless, the innervation of parts of the alimentary canal by the ventral strand (root) of the circumoesophageal connectives seems to be the rule and not the exception in Annelida. Thus, we disagree with former conclusions that buccal tentacles are part of the alimentary canal. Accordingly, the buccal tentacles of terebelliformian taxa can be homologized with feeding palps of other Annelida. Additionally, our data uncover two important and independent processes during the formation and localisation of head appendages in Terebelliformia - cephalisation and multiplication. Hence, both processes result in a concentration of appendages and sensory structures around the head. Unfortunately, there is a huge lack of morphological data concerning this key features of annelid evolution and further investigations are needed to investigate both processes in related families. Furthermore, to gather a comprehensive picture concerning the evolution of head appendages in Annelida in general, additional comparative investigations of anterior appendages in other families are strongly needed.

Methods

Specimen collection

Adult specimens of *Hypania invalida* (Grube, 1860) were collected from the river Rhein near Bonn in summer 2018. Specimens were maintained together with the collected sediment in a freshwater aquarium with a 12h:12h light regime at 17°C at the University of Göttingen.

Aulophora larvae of *Lanice conchilega* (Pallas, 1766) were caught around the island of Helgoland with a plankton net and fixed by employees of the Biological Station Helgoland according to our protocols in autumn 2019.

Immunohistochemistry

Anatomical details of adult *H. invalida* and aulophora larvae of *L. conchilega* were investigated using standard immunohistochemical staining protocols. Specimens of both species were relaxed in 7% $MgCl_2$ and subsequently fixed in 4 % paraformaldehyde (PFA) in 1x phosphate buffered saline with Tween (PTW = 1x PBS: 0.05 M PB / 0.3 M NaCl / 0.6 % Tween20 (0.4% Tween20 for *L. conchilega*, pH 7,4). Fixation was performed at room temperature (RT) for 2 h for *H. invalida* and 1h for *L. conchilega*. After fixing, the specimens were washed and stored in PTW containing 0,005% NaN_3 until usage at 4°C.

For antibody staining, specimens were rinsed 2 x 5 min in PTW at RT and permeabilized in 10 µg proteinase K/ml PTW (10 min for *H. invalida* and 15min for *L. conchilega*). After 2 short rinses in glycine (2 mg glycine/ml PTW), and 3 x 5 min washes in PTW, the specimens were re-fixed using 4 % PFA in PTW containing 0.6/0.4 % Tween for 20 min at RT. Subsequently, the samples were rinsed 2 x 5 min in PTW, 2 x 5 min in THT (0.1 M TrisCl, 0.1 % Tween, pH 8,5) and blocked with 5% goat serum (Sigma-Aldrich Chemie GmbH, Steinheim, 25µl goatserum in 500 µl THT) for 2h. Afterwards, specimens were incubated with the primary antibodies against α-tubulin (Anti-acetyl α -tubuline, clone 6-11B-1, Merck, Darmstadt, 2µl

tubulin in 500μl incl. 5% goat serum) and serotonin (5-HT (serotonin), ImmunoStar Inc.,Hudson, USA, 1μl in 500μl incl. 5% goat serum) in THT for 48-72h at 4°C.

Afterwards, samples were rinsed 2 x 10 min in 1M NaCl and washed 5 x 30 min in THT.

Subsequently, the samples were incubated in the secondary antibodies goat-anti-mouse 633 (Alexa Fluor® 633 goat-anti- mouse IgG (H + L), Thermo Fisher Scientific Inc., Waltham, USA, 1μl in 500μl inlc. 5% goat serum) and goat-anti-rabbit 488 (Alexa Fluor® 488 goat-anti-rabbit IgG (H + L), Thermo Fisher Scientific Inc., Waltham, USA, 1μl in 500μl incl. 5% goat serum) in THT for 48-72h at 4°C.

After the staining, specimens were rinsed 5 x 30 min in THT and 2 x 5 min in PTW. Additionally, samples were incubated in DAPI (DAPI, Thermo Fisher Scientific Inc., Waltham, USA, 5μl in PTW) in PTW overnight at 4°C.

Subsequently, the specimens were dehydrated in an ascending isopropanol series, cleared using Murray's clear (benzyl alcohol & benzyl benzoate, 1:2) and embedded between two cover slips using DPX mounting medium (Merck, Darmstadt, Germany). The specimens were analysed with a confocal laser-scanning microscope Leica TCS SP8 (Leica Microsystems, Wetzlar, Germany). The confocal image stacks were processed with Leica AS AF v2.3.5 (Leica Microsystems) and Imaris x64 9.2.1 (Bitplane AG, Zurich, Switzerland).

Azan Staining, Histological Sections And 3d-reconstruction

For semi-thin sections and AZAN-staining specimens of *Hypania invalida* and *Terebella lapidaria* were processed as described in (Beckers et al. 2013). Accordingly, specimens were relaxed in 7% MgCl$_2$ and then fixed in Bouin's fluid for 12h, dehydrated in an ethanol series and incubated in methylbenzoat and butanol. Afterwards the samples were pre- incubated in Histoplast (Thermo Scientific, Dreieich, Germany) and embedded in Paraplast (McCormick Scientific, Richmond, USA). 5 μm thick sections were made using a Reichert-Jung Autocut 2050 microtome (Leica, Wetzlar, Germany). The sections were transferred to albumen-glycerin coated glass slides. Afterwards sections were stained with Carmalaun, differentiated with sodium phosphotungstate (5%), washed in distilled water, stained in aniline blue orange G and subsequently embedded with Malinol (Waldeck, Münster, Germany). In Azan staining, the neuropil of the nervous system stains gray, the nuclei of cell somata stain red, the extracellular matrix stains blue and the musculature stains orange (Beckers et al. 2013). Each section was digitalized at 40x magnification using a slide scanner (Olympus dotslide (2.2 Olympus, Hamburg)) and aligned using IMOD (Kremer et al. 1996) and imodalign (http://www.q-terra.de/biowelt/3drekon/guides/imod_first_aid.pdf).

For the 3D-visualization we used Amira 2019.1, Meshlab_64bit_fp v2020.6, Blender 2.83 and deep exploration 5.5. Due to the unavoidable artefacts occurring during physical sectioning and the problems coming with it to reconstruct a smooth 3D-model (see Fig. 2A) we used a new combination of freeware resulting in a much more satisfactory result for the eye (Fig. 2B).

Declarations

Availability of data and materials

All data analysed in this study are used in figures of this article. The original 3D confocal image stacks can be made available after personal contact with the corresponding authors.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Åkesson B (1962) The embryology of Tomopteris helgolandica (Polychaeta). Acta Zool 43:135–199. https://doi.org/10.1111/j.1463-6395.1962.tb00070.x
2. Beckers P, Helm C, Bartolomaeus T (2019a) The anatomy and development of the nervous system in Magelonidae (Annelida) - Insights into the evolution of the annelid brain. BMC Evol Biol 19:. https://doi.org/10.1186/s12862-019-1498-9

3. Beckers P, Helm C, Purschke G, et al (2019b) The central nervous system of Oweniidae (Annelida) and its implications for the structure of the ancestral annelid brain. Front Zool 16:1–21. https://doi.org/10.1186/s12983-019-0305-1

4. Beckers P, Loesel R, Bartolomaeus T (2013) The Nervous Systems of Basally Branching Nemertea (Palaeonemertea). PLoS One 8:. https://doi.org/10.1371/journal.pone.0066137

5. Bhaud M (1988) Change in setal pattern during early development of Eupolymnia nebulosa (Polychaeta: Terebellidae) grown in simulated natural conditions. J Mar Biol Assoc United Kingdom 68:677–687. https://doi.org/10.1017/S0025315400028794

6. Bhaud M, Gremare A (1988) Larval development of theterebellid polychaete Eupolymnia nebulosa (Montagu) in the Mediterranean Sea. Zool Scr 17:347–356. https://doi.org/10.1111/j.1463-6409.1988.tb00111.x

7. Binard A, Jeener R (1928) Morphologie du lobe préoral des polychètes

8. Capa M, Hutschings PA (2006) Terebellidae (Polychaeta) from Coiba National Park, Panamanian Pacific, including description of four new species and synonymy of the genus Paraeupolymnia with Lanicola. Zootaxa 29:1–29

9. Cazaux C (1982) Developpement larvaire de l'ampharetidae lagunaire Alkmaria romijni Horst 1919. Cahiers de Biologie Marine, 23: 143-157,7figures. 143–157

10. Eckelbarger KJ (1974) Population biology and larval development of the terebellid polychaete Nicolea zostericola. Mar Biol 27:101–113. https://doi.org/10.1007/BF00389062

11. Faroni-Perez L, Helm C, Burghardt I, et al (2016) Anterior sensory organs in Sabellariidae (Annelida). Invertebr Biol 135:423–447. https://doi.org/10.1111/ivb.12153

12. Fauchald K, Rouse G (1997) Polychaete systematics: Past and present. Zool Scr 26:71–138. https://doi.org/10.1111/j.1463-6409.1997.tb00411.x

13. Glasby CJ, Hutchings PA, Hall K (2004) Assessment of monophyly and taxon affinities within the polychaete clade Terebelliformia (Terebellida). J Mar Biol Assoc United Kingdom 84:961–971. https://doi.org/10.1017/S00253315404010252h

14. Helm C, Beckers P, Bartolomaeus T, et al (2018) Convergent evolution of the ladder-like ventral nerve cord in Annelida. Front Zool 15:. https://doi.org/10.1186/s12983-018-0280-y

15. Holthe T (1986) Evolution, systematics, and distribution of the Polychaeta Terebellomorpha, with a catalogue of the taxa and a bibliography. Gunneria 55:1–236

16. Kremer JR, Mastronarde DN, McIntosh JR (1996) Computer visualization of three-dimensional image data using IMOD. J Struct Biol 116:71–76. https://doi.org/10.1006/jsbi.1996.0013

17. Nilsson D (1912) Beiträge zur Kenntnis des Nervensystems der Polychaeten. Almquist & Wiksells

18. Nogueira JMDM, Fitzhugh K, Hutchings P (2013) The continuing challenge of phylogenetic relationships in Terebelliformia (Annelida : Polychaeta). Invertebr Syst 27:186–238. https://doi.org/10.1071/IS12062

19. Nogueira JMDM, Hutchings PA, Fukuda MV (2010) Morphology of terebelliform polychaetes (Annelida: Polychaeta: Terebelliformia), with a focus on Terebellidae. Zootaxa 2460:1–185

20. Orrhage L (1980) On the structure and homologues of the anterior end of the polychaete families sabellidae and serpulidae. Zoomorphology 96:113–167. https://doi.org/10.1007/BF00310081

21. Orrhage L (2001) On the anatomy of the central nervous system and the morphological value of the anterior end appendages of Ampharetidae, Pectinariidae and Terebellidae (Polychaeta). Acta Zool 82:57–71. https://doi.org/10.1046/j.1463-6395.2001.00070.x

22. Orrhage L, Müller MCM (2005) Morphology of the nervous system of Polychaeta (Annelida). Hydrobiologia 535:79–111. https://doi.org/10.1007/s10750-004-4375-4

23. Purschke G (2016) Annelida : Basal Groups And Pleistoannelida. In: Andreas Schmidt-Rhaesa, Steffen Harzsch GP (ed) Structure and Evolution of Invertebrate Nervous System. Oxford University Press, pp 254–312

24. Purschke G, Bleidorn C, Struck T (2014) Systematics, evolution and phylogeny of Annelida – A morphological perspective. Mem Museum Victoria 71:247–269

25. Rimskaya-Korsakova NN, Galkin S V., Malakhov V V. (2018) The neuroanatomy of the siboglinid Riftia pachyptila highlights sedentarian annelid nervous system evolution

26. Rouse GW, Fauchald K (1997) Cladistics and Polychaetes. Zool Scr 26:139–204. https://doi.org/10.1111/j.1463-6409.1997.tb00412.x

27. Rouse GW, Pleijel F (2001) Polychaetes. Oxford University Press, Oxford, New York

28. Starunov V V., Voronezhskaya EE, Nezlin LP (2017) Development of the nervous system in Platynereis dumerilii (Nereididae, Annelida). Front Zool 14:1–20. https://doi.org/10.1186/s12983-017-0211-3

29. Stiller J, Tilic E, Rousset V, et al (2020) Spaghetti to a Tree: A Robust Phylogeny for Terebelliformia (Annelida) Based on Transcriptomes, Molecular and Morphological Data. Biology (Basel) 9:1–28

30. Struck TH, Paul C, Hill N, et al (2011) Phylogenomic analyses unravel annelid evolution. Nature 471:95–98. https://doi.org/10.1038/nature09864

31. Weigert A, Bleidorn C (2016) Current status of annelid phylogeny. Org. Divers. Evol. 16

32. Weigert A, Helm C, Meyer M, et al (2014a) Illuminating the base of the Annelid tree using transcriptomics. Mol Biol Evol 31:1391–1401. https://doi.org/10.1093/molbev/msu080

33. Weigert A, Helm C, Meyer M, et al (2014b) Illuminating the base of the Annelid tree using transcriptomics. Mol Biol Evol 31:. https://doi.org/10.1093/molbev/msu080

34. Wilkens V, Purschke G (2009) Pigmented eyes, photoreceptor-like sense organs and central nervous system in the polychaete Scoloplos armiger (Orbiniidae, Annelida) and their phylogenetic importance. J Morphol 270:1296–1310. https://doi.org/10.1002/jmor.10758

35. Wilson DP (1900) The Development of the Sabellid. Development

36. Wilson DP (1936) Memoirs: The Development of the Sabellid Branchiomma vesiculosum. J Cell Sci 2:543–603

37. Winchell CJ, Valencia JE, Jacobs DK (2010) Confocal analysis of nervous system architecture in direct-developing juveniles of Neanthes arenaceodentata (Annelida, Nereididae). Front Zool 7:1–19. https://doi.org/10.1186/1742-9994-7-17

38. Worsaae K, Rimskaya-Korsakova NN, Rouse GW (2016) Neural reconstruction of bone-eating Osedax spp. (Annelida) and evolution of the siboglinid nervous system. BMC Evol Biol 16:. https://doi.org/10.1186/s12862-016-0639-7

39. Zhadan AE, Tzetlin AB (2002) Comparative morphology of the feeding apparatus in the Terebellida (Annelida: Polychaeta). Cah Biol Mar 43:149–164
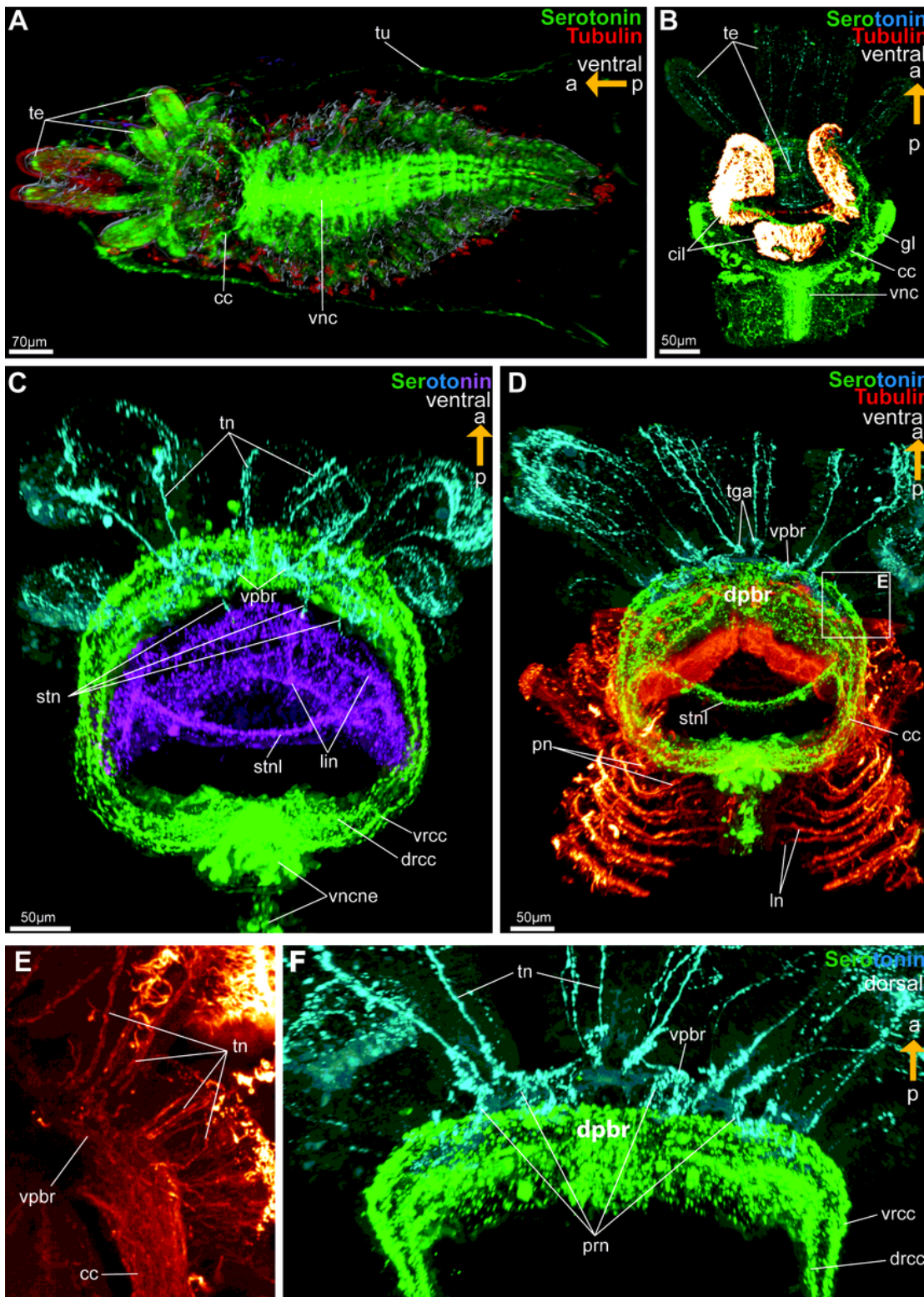
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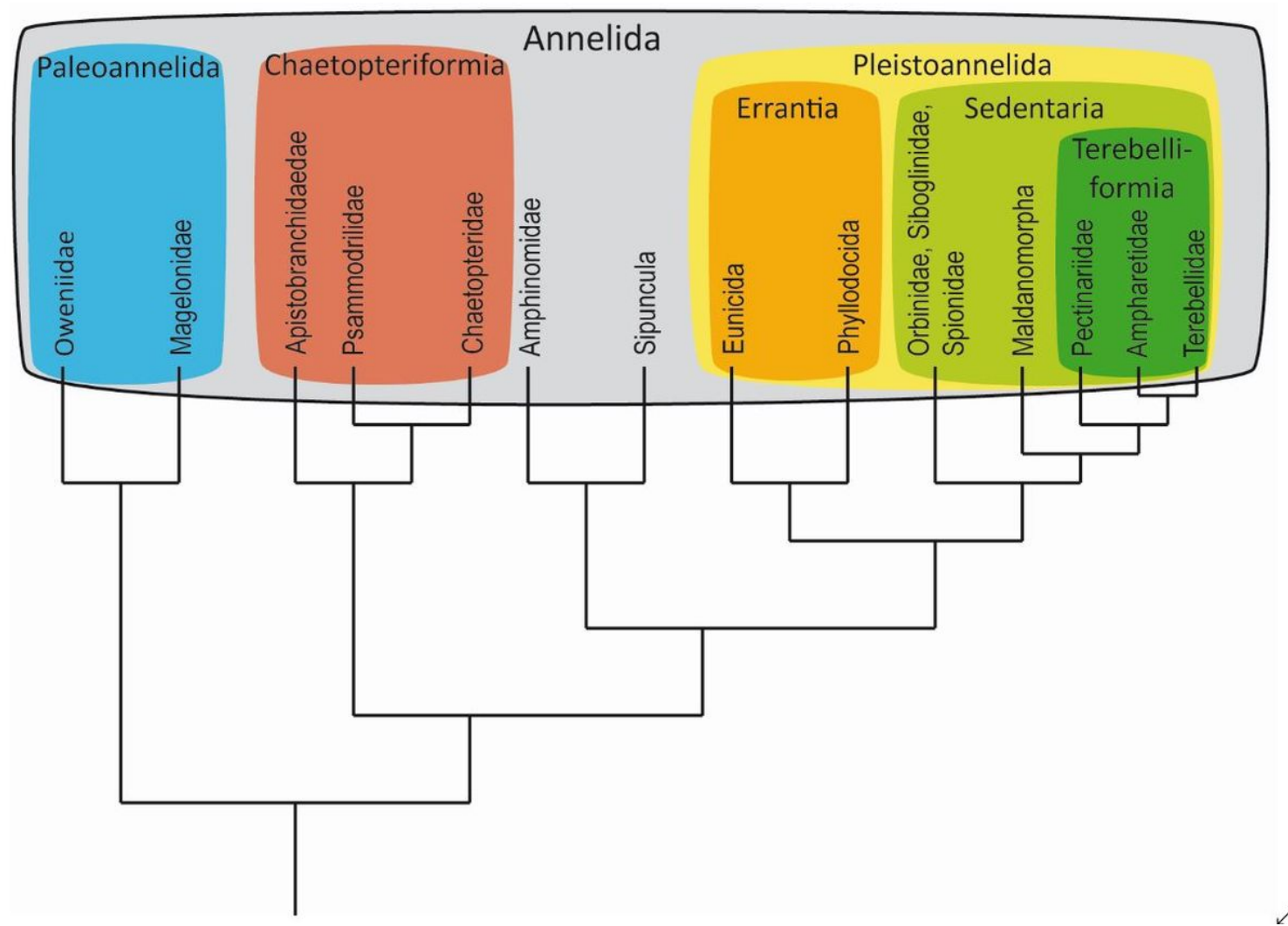
Figure 1

Combined and simplified topology showing recent hypotheses concerning relationships within Annelida. The scheme is based on different sources (Weigert et al. 2014b; Weigert and Bleidorn 2016; Stiller et al. 2020) .
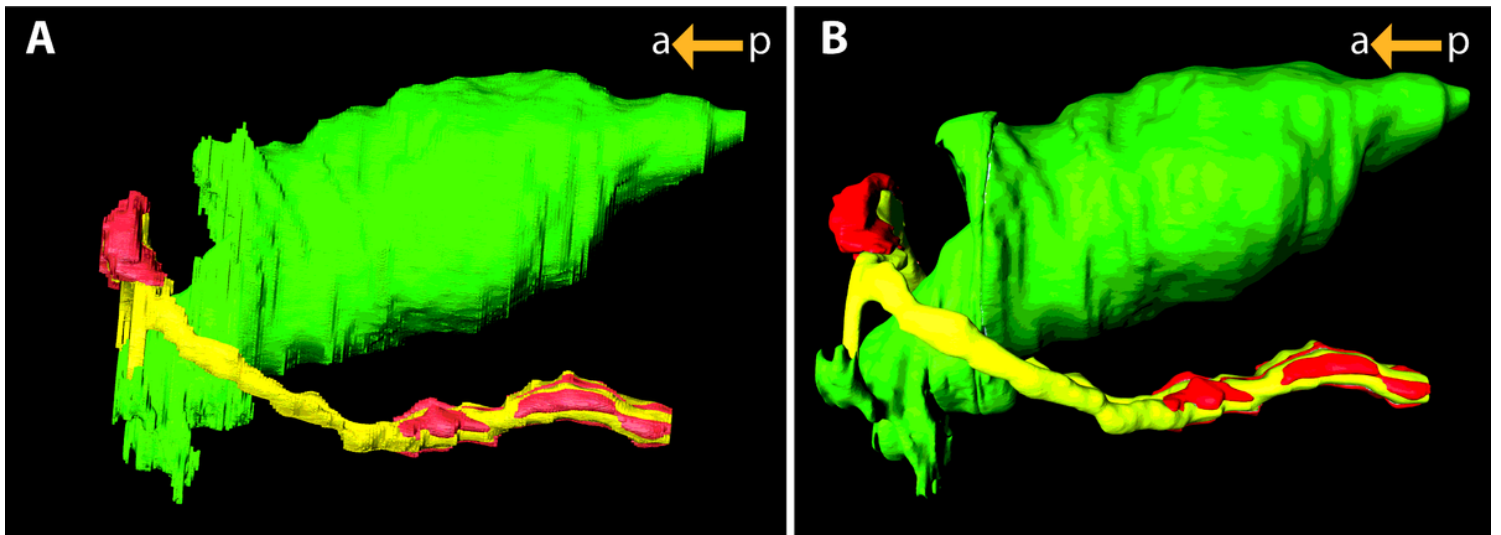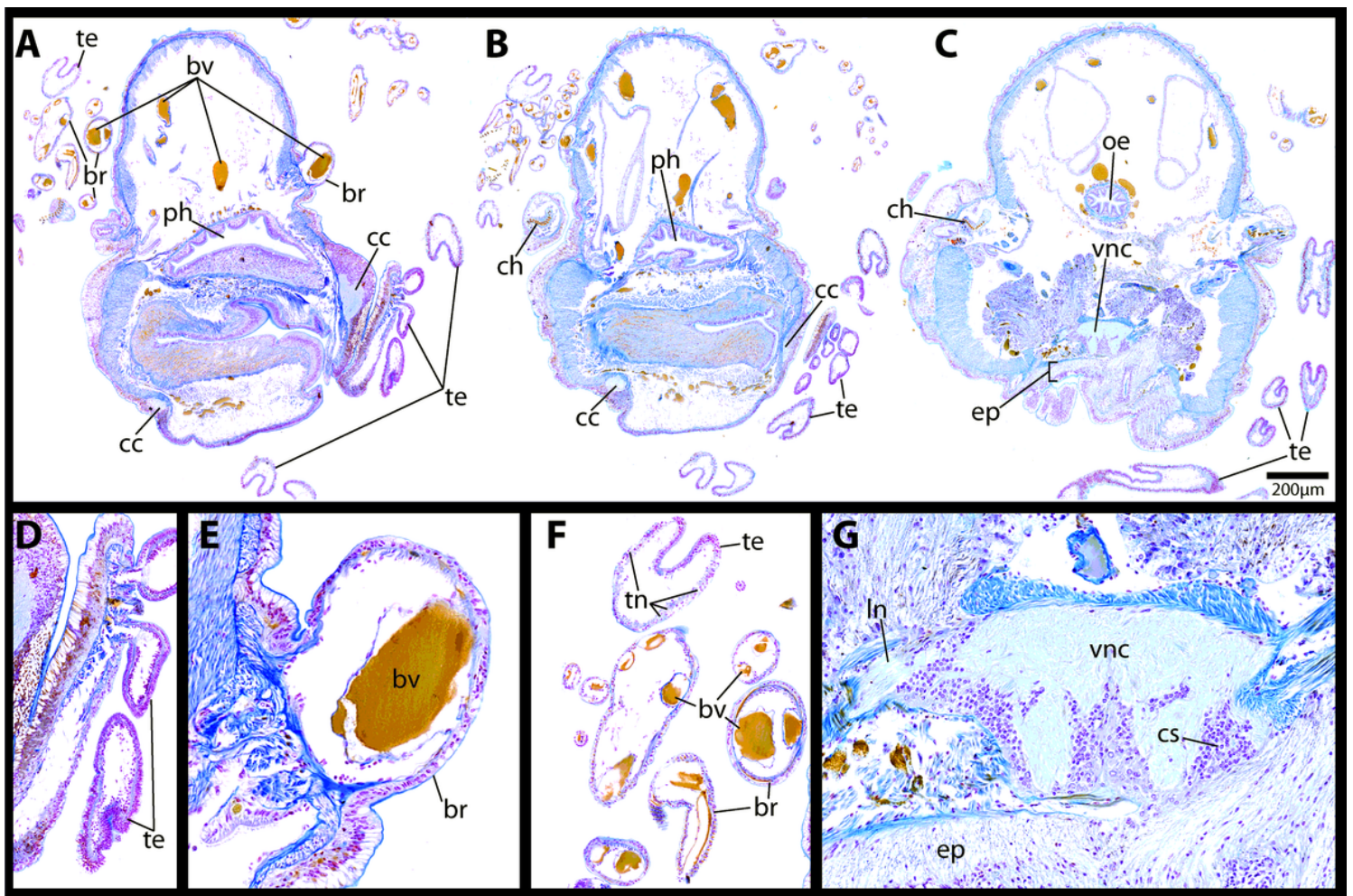
Figure 2

3D-reconstruction based on Azan-stained -sections of Hypania invalida. A. 3D-reconstruction of the brain, circumoesophageal connectives, ventral nerve cord (yellow), cell soma (red) and inverted pharynx and oesophagus (green) using Fiji and Amira. B. Same reconstruction using workflow including Blender.

Figure 3

Nervous system of the anterior region of aulophora larvae of Lanice conchilega, cLSM micrographs. A. General anatomy of aulophora larvae sitting in its tube, overall serotonergic nervous system highlighted in green and α-tubulinergic setae in red. B. Serotonergic nervous system anterior region. Central nervous system like brain and vnc are highlighted in green, tentacular nerves in cyan and α-tubulinergic cilia on the lateral and lower lips in red. C. Close-up of the serotonergic nervous system of the head region.

Ventral region of the brain and tentacular nerves are highlighted in cyan, dorsal region of the brain, cc and vnc in green and stomatogastric nerves including lip nerves in magenta. D. General anatomy of the serotonergic and α-tubulinergic nervous system of the anterior region. Ventral region of the brain and tentacular nerves are highlighted in cyan, central and stomatogastric nervous system in green and α-tubulinergic nervous system in red. E. Close-up of micrograph D. α-tubulinergic nervous system in red. On the right side innervation of a developing palp is shown. Additionally, displayed is the transition of cc to the ventral brain region. F. Close-up of the brain region, showing the prostomial nerves connecting the dorsal with the ventral brain region. cc – circumoesophageal connectives; cil – cilia of lips; dpbr – dorsal region of brain; drcc – dorsal root of cc; gl – glandular cells; ln – lateral nerves; pn – peripheral nerves; prn – prostomial nerves; stnl – stomatogastric nerve loop; te – buccal tentacles; tga – tentacular ganglion; tn – tentacular nerves; tu – tube; vncne – neurons of vnc, vpbr – ventral region of brain, vrcc – ventral root of cc

Figure 4

General anatomy of the anterior end of adult Terebella lapidaria (Linnaeus 1767), 5 μm sections, Azan-staining, light microscopic images. A. Cross-section through anterior head region from which tentacles and branchiae emerge. B. Cross-section through middle head region. C. Cross-section through posterior head region, cc united to the vnc and anterior oesophageal cavity is identifiable. D. Close-up of the cross-section through the lateral origin of buccal tentacles. E. Close-up of the cross-section of the dorsal origin

of the branchiae. F. Close-up of the cross-section through tentacles and branchiae. G. Close-up of the cross-section through the ventral nerve cord, huge glandular epidermal cells and the subepidermal vnc are shown. br – branchiae; bv – blood vessel; cc – circumoesophageal connectives; ch – chaete; cs – cell soma; ep – epidermis; ln – lateral nerve; oe – oesophagus; ph – pharynx; te – buccal tentacles; tn – tentacular nerves; vnc – ventral nerve cord
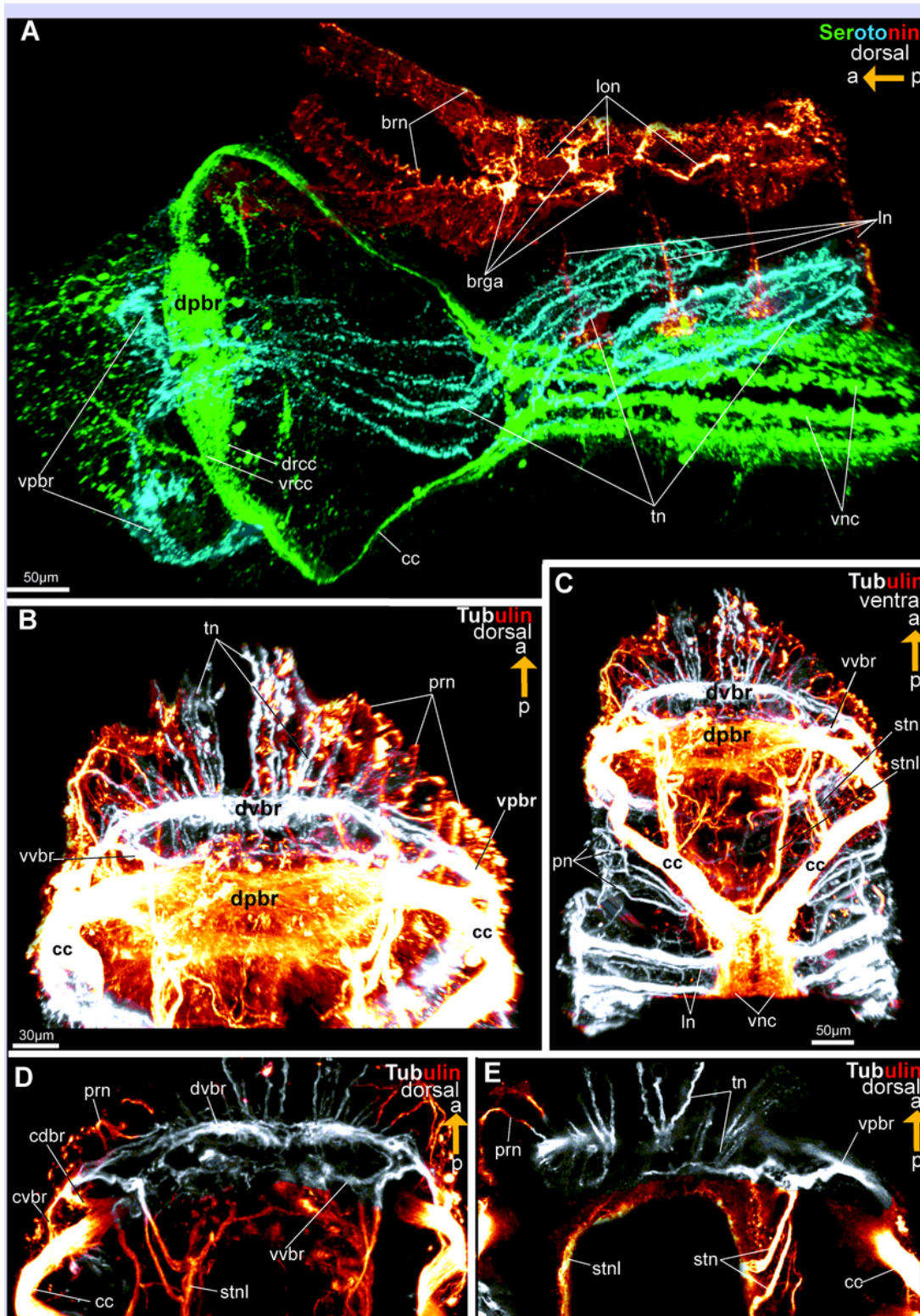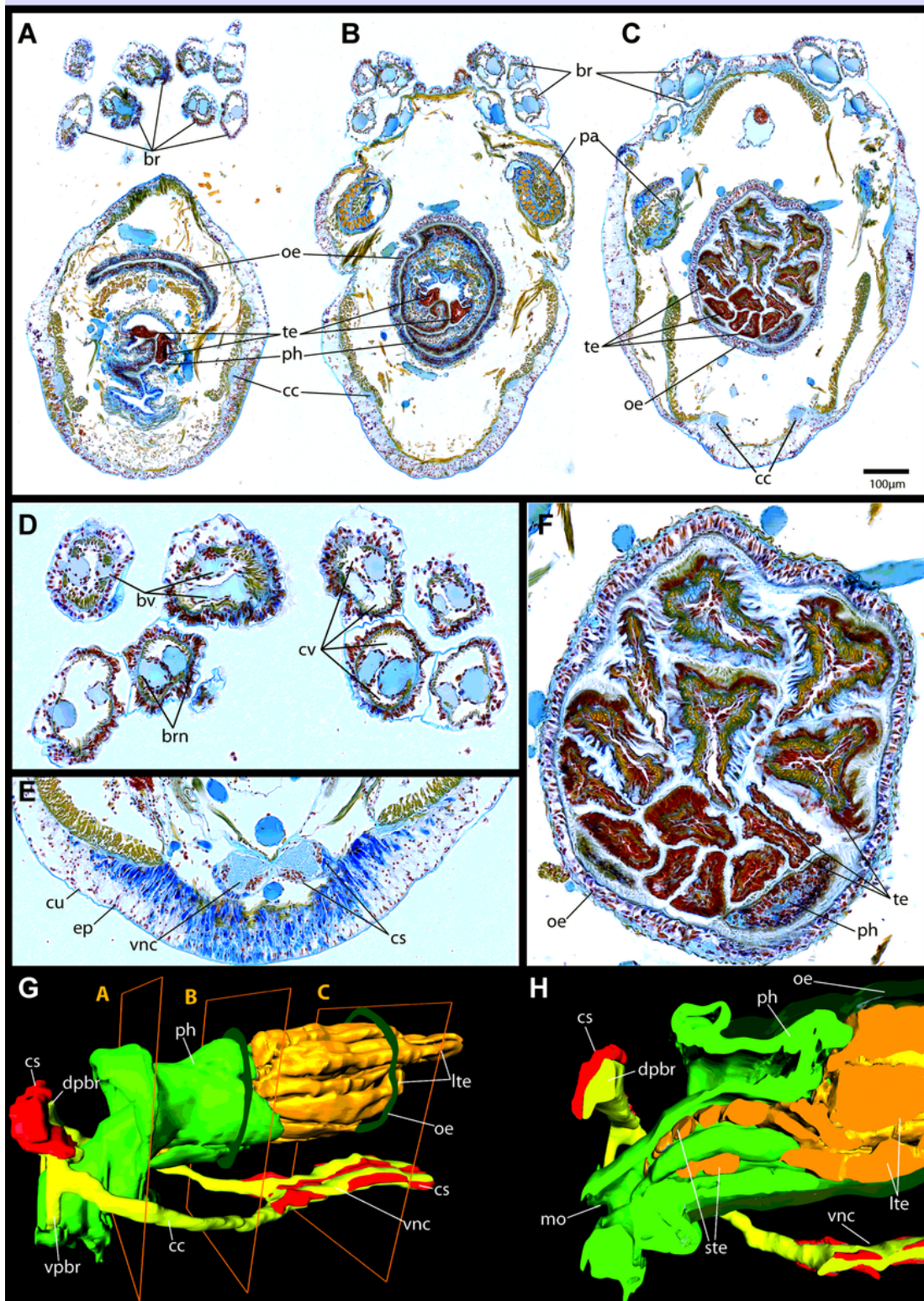

Figure 5

Anterior nervous system of adult Hypania invalida, cLSM micrographs. A. General anatomy of the serotonergic nervous system. The central nervous is highlighted in green, ventral region of brain and tentacle nerves in blue and branchial neurites in red. B. Close-up of brain region stained against acetylated α-tubulin. Ventral region of brain and tentacular nerves highlighted in white. C. General anatomy of the tubulinergic nervous system. Ventral region of brain, tentacular nerves and laterally branching nerves are highlighted in white. D. Close-up of the ventral brain region stained against α-tubulin, ventral brain region highlighted in white. Connectives of the ventral brain region to the dorsal brain region, the cc and the stomatogastric nerve loop are shown. E. Close-up of the ventral brain region stained against α-tubulin, ventral brain region highlighted in white. Tentacular nerve originate from both roots of the ventral brain region. brga – ganglion of branchiae; brn – branchae nerves; cc – circumoesophageal connective; cdbr – connective of dpbr; cvbr – connective of vpbr; dpbr – dorsal region of brain; drcc – dorsal root of cc; dvbr – dorsal root of vpbr; ln – lateral nerves; lon – lateral organ nerve; pn – peripheral nerves; prn – prostomial nerves; stn – stomatogastric nerve; stnl – stomatogastric nerve loop; tn – tentacular nerves; vnc – ventral nerve cord; vpbr – ventral region of brain; vrcc – ventral root of cc; vvbr – ventral root of vpbr
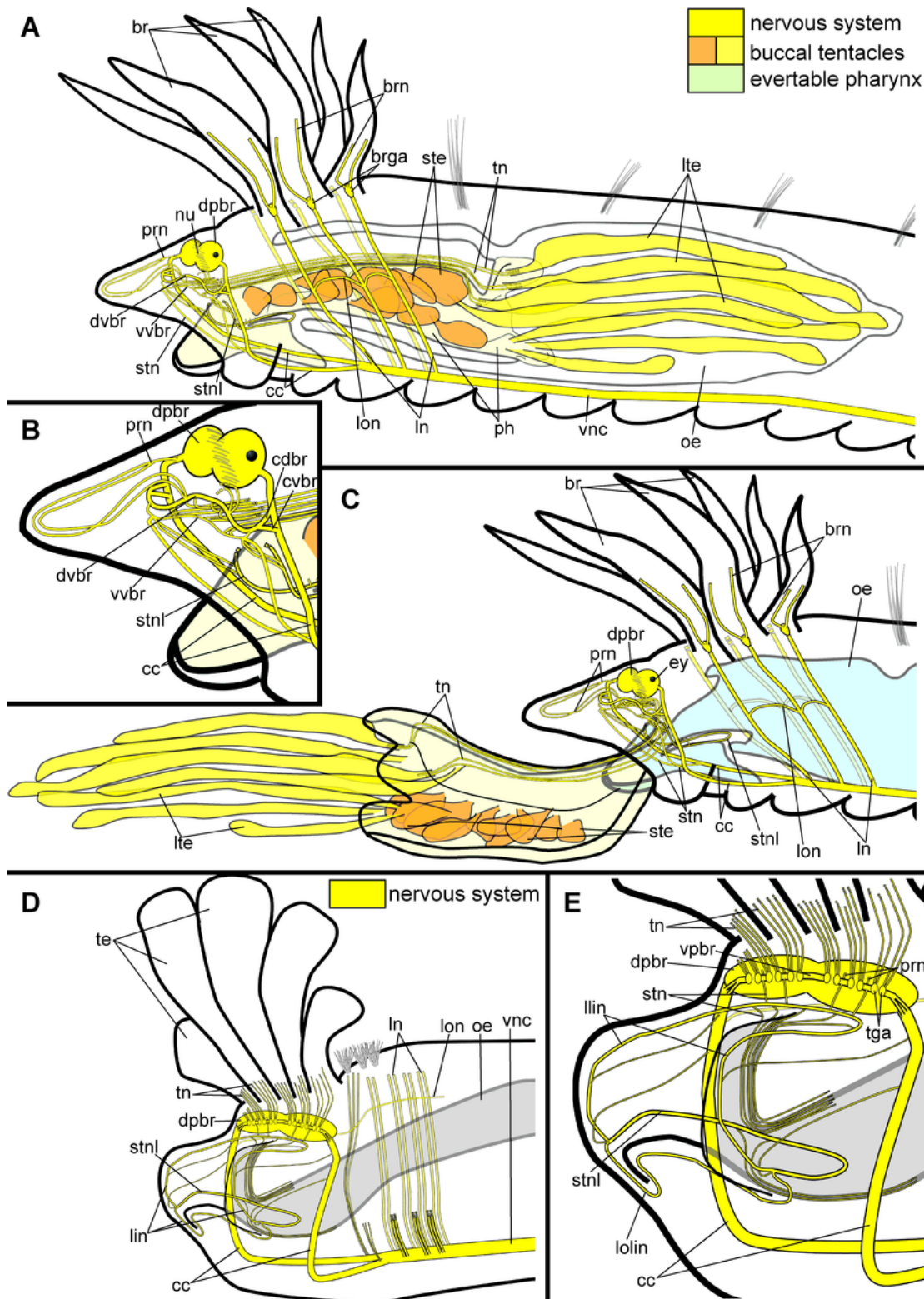
Figure 6

General anatomy of the anterior end of adult Hypania invalida, 5 µm sections, Azan-staining, light microscopic images. A. Cross-section through anterior part of inverted pharynx, see Fig. G. Short buccal tentacles situated inside the pharyngeal cavity. B. Cross-section through the posterior part of the inverted pharynx, see Fig. G. Folded pharynx located inside the oesophageal cavity associated with short tentacles. C. Cross-section through the posterior part of the oesophageal cavity, see Fig. G. Long buccal

tentacles filling the whole cavity of the oesophagus. D. Close-up of a cross-section through the branchiae with two prominent blood vessels, see Fig. A. E. Close-up of the anterior region of the subepidermal ventral nerve cord and ganglionic cell somata. F. Close-up of the posterior part of the oesophagus, see Fig. C, with numerous long tentacles located inside the oesophageal cavity. G. 3D-visualization of the anterior central nervous system and stomatogastric region, long distal buccal tentacle associated with the pharynx, oesophagus omitted. Frames show area of Parafin-sections of Fig. A, B and C. H. Digital sagittal section through the 3D-visualisation of the anterior central nervous system and the short buccal tentacles located in the middle part of the pharynx . br – branchiae; brn – branchiael nerves; bv – blood vessel; cc – circumoesophageal connectives; cs – cell soma; cu – cuticle; cv – coelom cavity; dpbr – dorsal region of brain; ep – epidermis; lte – long buccal tentacles; mo – mouth opening; oe – oesophagus; pa – palae bristles; ph – pharynx; ste – short buccal tentacles; te – buccal tentacles; vnc – ventral nerve cord; vpbr – ventral region of brain

Figure 7

Comparative schematic drawings of the overall nervous system of adult Hypania invalida (a-c) and aulophora larvae of Lanice conchilega (d, e). Nervous system is yellow, short buccal tentacles are orange and long buccal tentacles are colored in light yellow, the evertable pharynx is colored in mint green, see legend. A. Drawing of the anterior region of adult Hypania invalida, pharynx in inverted position. B. Close-up of the head region, see Fig. A. C. Drawing of the anterior region of adult Hypania invalida, pharynx in

everted position. D. Drawing of the anterior region of aulophora larvae of Lanice conchilega. E. Close-up of the head region, see Fig. D. brga – ganglion of branchiae; br – branchiae; brn – branchiael nerves; cc – circumoesophageal connectives; cdbr – connective of dpbr; cvbr – connective of vpbr; dpbr – dorsal region of brain; ey – eye; llin – lateral lip nerves; lin – lip nerves; ln – lateral nerves; lolin – lower lip nerve; lon – lateral organ nerve; lte – long buccal tentacles; nu – nuchal organ; oe – oesophagus; ph – pharynx; prn – prostomial nerves; ste – short buccal tentacles; stnl – stomatogastric nerve loop; te - buccal tentacle; tn – tentacle nerves; tga – tentacular ganglion; vnc – ventral nerve cord, dvbr – dorsal root of vpbr; vvbr – ventral root of vpbr