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Andreas Wanninger · Demian Koop · Lindell Bromham · Erin Noonan · Bernard M. Degnan

Nervous and muscle system development in *Phascolion strombus* (Sipuncula)

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Abstract Recent interpretations of developmental gene expression patterns propose that the last common metazoan ancestor was segmented, although most animal phyla show no obvious signs of segmentation. Developmental studies of non-model system trochozoan taxa may shed light on this hypothesis by assessing possible cryptic segmentation patterns. In this paper, we present the first immunocytochemical data on the ontogeny of the nervous system and the musculature in the sipunculan *Phascolion strombus*. Myogenesis of the first anlagen of the body wall ring muscles occurs synchronously and not subsequently from anterior to posterior as in segmented spiralian taxa (i.e. annelids). The number of ring muscles remains constant during the initial stages of body axis elongation. In the anterior-posteriorly elongated larva, newly formed ring muscles originate along the entire body axis between existing myocytes, indicating that repeated muscle bands do not form from a posterior growth zone. During neurogenesis, the *Phascolion* larva expresses a non-metameric, paired, ventral nerve cord that fuses in the mid-body region in the late-stage elongated larva. Contrary to other trochozoans, *Phascolion* lacks any larval serotonergic structures. However, two to three FMRFamide-positive cells are found in the apical organ. In addition, late larvae show commissure-like neurones interconnecting the two ventral nerve cords, while early juveniles exhibit a third, medially placed FMRFamidergic ventral nerve. Although we did not find any indications for cryptic segmentation, certain neuro-developmental traits in *Phascolion* resemble the conditions found in polychaetes (including echiurans) and myzostomids and support a close relationship of Sipuncula and Annelida.

Keywords Evolution · Development · Segmentation · Confocal microscopy · Phylogeny

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A. Wanninger (✉)
Department of Cell Biology and Comparative Zoology,
Institute of Biology, University of Copenhagen,
Universitetsparken 15
2100 Copenhagen Ø, Denmark
e-mail: awanninger@bi.ku.dk
Tel.: +45-35-321240
Fax: +45-35-321200

D. Koop · E. Noonan · B. M. Degnan
School of Integrative Biology, University of Queensland,
Brisbane, Queensland, 4072, Australia

L. Bromham
Centre for the Study of Evolution, School of Life Sciences,
University of Sussex,
Falmer, Brighton
BN1 9QG, UK

Introduction

Molecular phylogenies of the Metazoa consistently support the placement of nearly all bilaterian triploblastic animals into one of three superphyletic groups—Deuterostomia, Ecdysozoa, or Lophotrochozoa—with the latter two considered sister protostome clades (e.g. Halanych et al. 1995; Aguinaldo et al. 1997; Giribet et al. 2000). However, the interrelationships of the various phyla within these giant clades largely remain unresolved. Recent phylogenetic trees that combine morphological and molecular data have not necessarily shed further light on interphyletic relationships, as these phylogenetic trees are largely the result of the re-evaluation of existing data matrices rather than being based on new ontogenetic or morphological characters (Jenner 2001, 2003).

Despite the lack of a solid metazoan phylogeny, debates concerning ancestral body plan features are ongoing, not only with respect to the last common ancestor (LCA) of Metazoa but also regarding the LCA of the various metazoan supraphyletic assemblages such as Bilateria, Ecdysozoa, and Lophotrochozoa. Reconstructions of these hypothetical LCAs have contributed to numerous debates on metazoan body plan evolution such as the relationship of dorsal–ventral axes and the origin of segmentation (for recent reviews, see Davis and Patel 1999; Holland 2003).

To date, Annelida (including Echiura) is the only taxon within Lophotrochozoa for which segmentation has been demonstrated, although there are clear examples of serial organ repetition in the body plans of other trochozoans (e.g. polyplacophoran molluscs). In recent bilaterian reconstructions, annelid-like concerted repetition of several organ systems along the anterior–posterior axis has been proposed to have been present in the clade that gave rise to the extant trochozoans and lophophorates, if not the protostomes and bilaterians altogether (e.g. Balavoine and Adoutte 2003; Prud'homme et al. 2003). This is in contrast to views that segmentation in annelids and arthropods is the result of convergent evolution (Eernisse et al. 1992; Abouheif et al. 1997; for a review on the rivaling hypotheses, see Davis and Patel 1999; Seaver 2003). Assuming a segmented trochozoan ancestor implies secondary loss of segmentation in a number of phyla, including Entoprocta, Mollusca, Nemertea, Platyhelminthes, and Sipuncula (Haszprunar and Wanninger 2000; Friedrich et al. 2002; Wanninger and Haszprunar 2002a; see also Nielsen 2004).

Sipuncula describes a phylum of worm-shaped, coelomate trochozoans with a retractable anterior introvert, usually bearing a characteristic tentacle crown, and a thickened trunk region. Early developmental patterns include spiral cleavage and a so-called "molluscan cross". Larval development is usually via a lecithotrophic trochophore-like and a planktotrophic pelagosphera larva, but variations from this pattern do occur (see Rice 1985). Sipuncula have previously been allied with Mollusca on the basis of morphological and developmental characters (e.g. Scheltema 1993). However, phylogenetic analyses of mitochondrial DNA data suggest that sipunculans are more closely related to Annelida than to Mollusca (Boore and Staton 2002; Staton 2003; Jennings and Halanych 2005). To shed light on this discussion from a developmental–morphological perspective, and to provide new data to the discussion about the evolution of segmentation, we investigated the development of the central nervous system and the musculature in *Phascolion strombus* (Montagu 1804) using immunocytochemical and muscle-specific markers.

Materials and methods

Animals

Adults of *P. strombus* typically inhabit empty shells of gastropod (*Turritella*) or scaphopod (*Dentalium*) molluscs. Several hundred individuals were collected during the months of September and October 2002 and 2003, respectively, by dredging on hard-bottom substratum at around 30-m depth at the mouth of the Gullmarfjord near Gasö Ränna in the vicinity of the Kristineberg Marine Research Station (Skagerrak, Swedish West Coast). Following the description of Åkesson (1958), the adults were placed in plastic tanks or small aquaria (25–100 individuals each, depending on the size of the container). The water was changed twice daily by running it through a 75-μm mesh that was checked for spawned gametes and early embryos. The first individuals spawned 2 weeks after collection, and after 4 weeks, embryos had been found in all containers. The collected embryos were transferred into small glass dishes containing 0.2 μm Millipore-filtered seawater (MFSW) and cultured at ambient seawater temperature (12–16°C). To prevent microbial or fungal infections, 50 mg streptomycin sulfate and 60 mg penicillin G were added per litre MFSW to some of the cultures.

Adult colonies of the serpulid annelid *Filograna implexa* were collected at low tide from the reef crest off Heron Island (Great Barrier Reef, North Queensland, Australia) from 0.2- to 1-m depth, and juvenile and adult individuals were obtained in the laboratory by crushing their calcareous tubes.

Reference specimens of both species used in this study, *P. strombus* and *F. implexa*, were preserved in 70% ethanol and deposited at the Zoological Museum, University of Copenhagen [registration numbers ZMUC-SIP-1 (*P. strombus*), ZMUC-POL-1778–1780 (*F. implexa*)].

Scanning electron microscopy

Larvae were fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS) for 2–4 h at room temperature or overnight at 4°C, washed three times for 15 min in PBS containing 0.1% sodium azide (NaN_3), and stored at 4°C. From the beginning of anterior–posterior elongation onwards, all individuals were relaxed by adding drops of $MgCl_2$ to a final concentration of 3.5% to the MFSW prior to fixation.

Stored animals were washed in distilled water, postfixed in osmium tetroxide (1% in distilled water for 1–2 h at room temperature) followed by another wash in distilled water, and dehydrated in a graded acetone series. After critical point drying, the samples were mounted on scanning electron microscopy (SEM) stubs, sputter-coated with gold, and observed with a LEO 1430VP scanning electron microscope.

Immunolabelling and confocal laser scanning microscopy

Larvae and juveniles were relaxed, fixed, and stored as described above. In the following, the samples were permeabilized in PTA (0.1 M PBS containing 0.1% NaN_3 and 0.1% Triton X-100) for 1 h at room temperature and incubated in a blocking solution (6% normal goat serum in PTA) overnight (18–24 h) at 4°C. Both antibodies were diluted in blocking solution and applied at a 1:800 (anti-serotonin; Calbiochem, Cambridge, USA) or a 1:400 (anti-FMRFamide; DiaSorin, Stillwater, USA) final working concentration (v/v). Incubations in the primary antibody solutions were carried out at 4°C for 24 h and were followed by four washes in blocking solution over 6 h at 4°C and by application of a tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit secondary

antibody (1:100 dilution in blocking solution; Jackson ImmunoResearch, West Grove, USA) for 20–24 h at 4°C. The samples were then washed in PBS (four changes over 12–20 h at 4°C), dehydrated in a graded ethanol series, and mounted on glass slides using a clearing medium consisting of a 2:1 mixture of benzyl benzoate and benzyl alcohol.

For F-actin labeling, the stored samples were washed in 0.1 M PBS without NaN_3 (3×15 min at room temperature), permeabilized for 1 h in PBS containing 0.2% Triton X-100, and stained in a 1:40 dilution of Oregon Green 488 phalloidin (Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. After that, the samples were washed again 3×15 minutes and mounted in Vecta Shield mounting medium (Vector Laboratories, Burlingame, CA, USA) on glass slides.

Analysis and digital image acquisition of the fluorescence preparations was performed on a Leica DM IRBE microscope equipped with a Leica TCS SP confocal unit. Thus, image stacks of optical sections were recorded as Z-projections with a 1-µm step size, which can be analysed individually or as merged whole-mount projection images with greater focal depth.

Definitions and terminology

The terms "seriality", "metamerism", and "segmentation" are often used synonymously, thus frequently causing confusion in discussions about the subject. In this paper we define seriality as any kind of repetitive arrangement of body elements along the anterior–posterior axis, irrespective of their ontogenetic formation pattern. In contrast, in many polychaete annelids, metameric structures form subsequently (one after another) from a pre-anal growth zone, leading to an annelid-like segmented body plan. Thus, "segmented" describes a body plan that is formed by several metameric units which originated from a pre-anal growth zone. This is e.g. exemplified in the nervous system of polychaetes, where one pair of ganglia is found in each segment. In contrast, adult sipunculans show a straight, single ventral nerve cord without such ganglia (Fig. 1).

Results

Embryogenesis and larval development in *P. strombus*

Phascolion strombus is an unequal cleaving sipunculan that develops inside a thick egg hull. Cleavage is of the spiral, holoblastic type (data not shown). Development is very heterogeneous even within a single culture. Accordingly, we refer to developmental stages that are easily recognized by external morphological characters rather than to exact time points after fertilization. Important landmarks of *Phascolion* development include the onset of swimming [at around 12 h post-fertilization (hpf)], the start of anterior–posterior elongation ("teardrop stage", accomplished at approximately 48 hpf), the fully elongated larva (about 60 hpf), and the beginning of metamorphosis, which

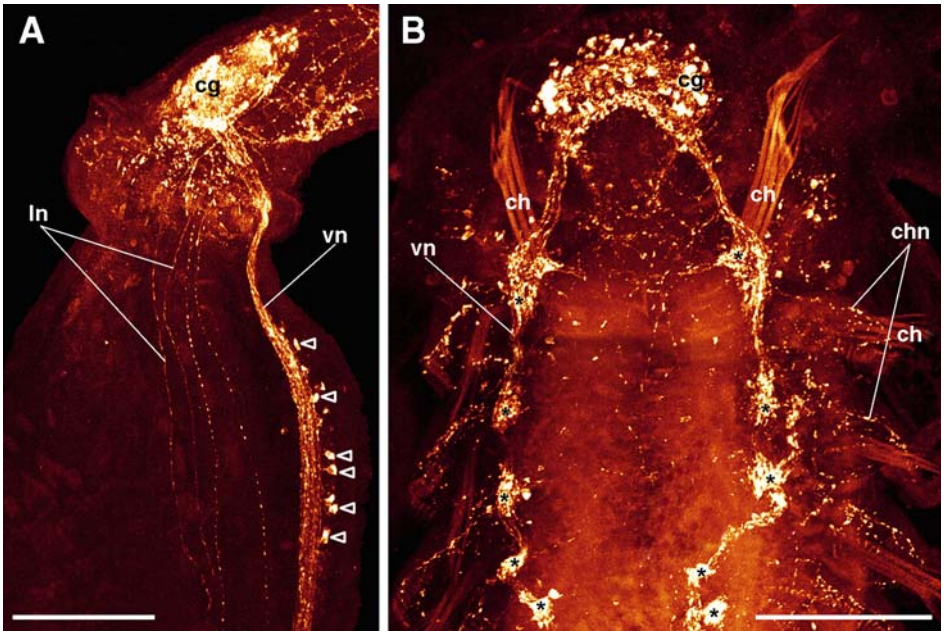

Fig. 1 Visualization of FMRFamide immunoreactivity in subadult *P. strombus* (Sipuncula) and *F. implexa* (Annelida, Polychaeta) illustrates the different neuroanatomy in unsegmented and segmented trochozoan "worms". *Scale bars* equal 100 µm. **a** *P. strombus*, lateral left view. The cerebral ganglion (*cg*) and the single ventral nerve cord (*vn*) are heavily stained. Additional FMRFamidergic neuronal elements include clusters of cell bodies, which are randomly distributed along the ventral side of the ventral nerve cord (*open arrowheads*) and four longitudinal lateral nerves (*ln*). **b** *F. implexa*, ventral view, showing the prominent cerebral ganglion (*cg*), the paired ventral nerve cord (*vn*) with segmental ganglia (*asterisks*) as well as laterally situated nerves (*chn*) innervating the chaetae (*ch*)

is marked by a gradual change from swimming to crawling behaviour of the larva (from around 72 hpf onwards).

Prior to swimming, the spherical larvae rotate around their anterior–posterior axis at the bottom of the culture dish by means of their prototroch cilia that penetrate the egg membrane. In the young trochophore-like larva, the area of the ciliated prototroch covers almost the entire surface of the larva, leaving very little space for both the pre-trochal (episphere) and the post-trochal region (hyposphere). The apical ciliary tuft is only weakly developed (Fig. 2a). Swimming is accomplished by metachronal ciliary beats of the prototroch. The larva of *P. strombus* remains within the eggshell; hatching does not occur. Instead, the shell layers are incorporated in the formation of the cuticle (see Åkesson 1958). After the trochophore-like stage, which lasts at least for 2 days, the larva starts to elongate in anterior–posterior direction, resulting in an enlargement of the hyposphere (teardrop stage) (Fig. 2b,c). Within a few hours, the body elongation increases dramatically, and an anterior larval secretory organ (cf. Åkesson 1958: Figs. 5, 18), as well as a number of epidermal papillae known as epidermal organs, which are scattered all over the visceral body region, are formed. Metamorphosis is a gradual event starting with the shedding of the prototroch, which also marks the beginning of the benthic lifestyle. Remarkably, almost all individuals reached at least the initial stages of metamorphosis, although no potential metamorphic cues were added to the cultures. The completion of metamorphosis takes up to several weeks in *P. strombus* (Fig. 2d,e). The larval secretory organ and the epidermal organs persist at least throughout the earlier periods of metamorphosis. Development is entirely lecithotrophic until the completion of metamorphosis.

Neurogenesis

Ontogeny of the FMRFamide-positive nervous system

The earliest FMRFamide-positive signal is expressed in the early trochophore-like stage immediately after the onset of swimming. The anlage of the future cerebral ganglion, as well as two to three FMRFamidergic cells of the larval apical organ, appear synchronously (Fig. 3a). At the teardrop stage, i.e. when elongation along the anterior–posterior axis starts (see above), the paired rudiment of the ventral nerve cord appears in the ventro-median region of the larva (Fig. 3b). During subsequent development these nerve strands become more solid and grow in an anterior direction until they form contact with the cerebral ganglion, which elaborates dramatically. In addition, a peripheral nerve net is formed in the visceral part of the elongated larva, and several FMRFamide-positive cell clusters, mostly associated with the ventral nerve cords, are found (Fig. 3c,d). In late-stage larvae, three commissure-like nerves that interconnect the two ventral nerve cords become visible in the visceral part of the animal (Fig. 3d). The FMRFamidergic ventral nervous system retains its paired nature in the early juvenile stage, and both cords form an anterior loop and connect to the cerebral ganglion. Between the two ventral neural cords an addi-

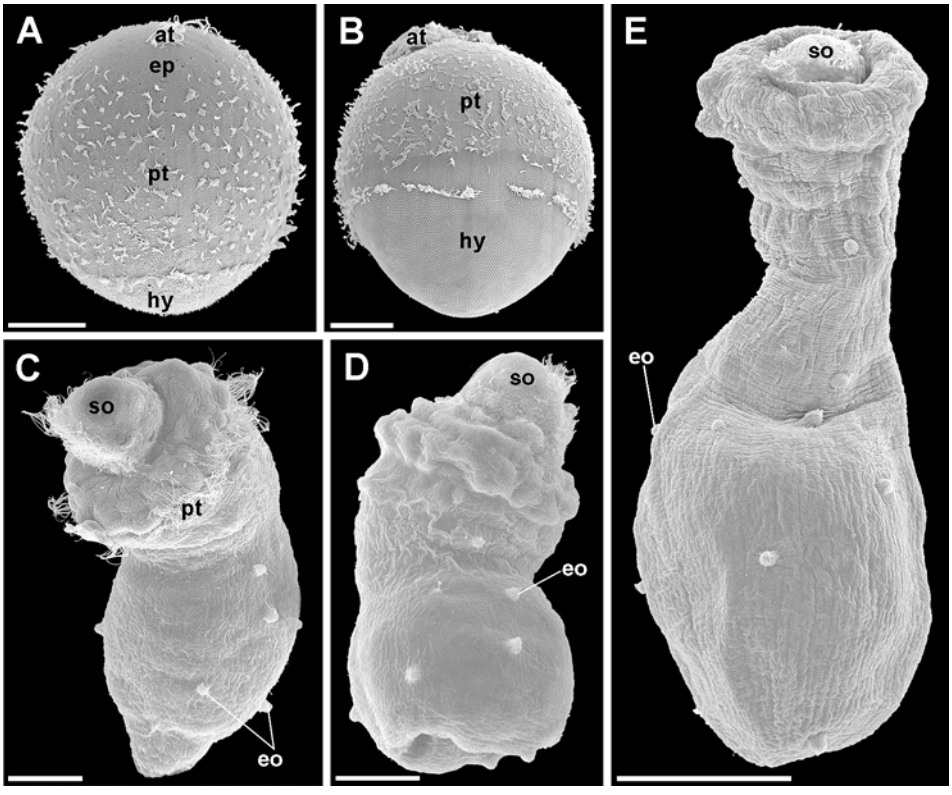

Fig. 2 Scanning electron microscopy micrographs of the larval development in *P. strombus*, anterior faces upwards. *Scale bars* equal 25 μm. **a** Early trochophore-like larva at the beginning of the free-swimming stage. Note the spherical appearance of the larva with the ciliated prototrochal area (*pt*) covering almost the entire body surface, while the episphere (*ep*), bearing the apical tuft (*at*), and the hyposphere (*hy*) are hardly discernable. **b** Larva at the teardrop stage during the elongation of the hyposphere. **c** Larva with elongated body bearing epidermal protrusions known as epidermal organs (*eo*). Note the prominent, ciliated, anteriorly situated larval secretory organ (*so*). **d** Late larva at the beginning of the benthic phase during an early stage of metamorphosis. The prototroch has already been shed while the larval secretory organ and the epidermal organs still persist. **e** Early bottom-dwelling juvenile

tional median longitudinal nerve, which expresses FMRFamide, runs along the anterior–posterior axis (Fig. 3e).

Ontogeny of the serotonergic nervous system

We detected the first serotonin-positive signal in swimming trochophore larvae at the teardrop stage. As with FMRFamide, the ventral nervous system appears as a paired cord that runs from the base of the anlage of the cerebral ganglion into the median region of the larva. There, a cluster of serotonergic cells is found, and the first peripheral nerves start to form (Fig. 4a). We did not find any serotonergic elements associated with the larval apical organ at any stage of development, nor does *P. strombus* exhibit a serotonergic prototroch nerve ring. In larvae during early stages of body elongation a fusion zone of the paired ventral nerve cord appears in the visceral part of the larva (Fig. 4b). From this fusion zone the ventral nerve cord grows posteriorly as a single nerve strand as the larva continues to elongate (Fig. 4c,d). In early juvenile specimens the two separated nerve cords are only apparent in the anterior-most region of the animal where they form the connection with the cerebral ganglion. The peripheral serotonergic nervous system is elaborated and appears as a dense nerve net. Additional serotonergic cells are found in the ventral region in close association with the ventral nerve cord (Fig. 4e).

Myogenesis

The anlagen of the circular muscles of the body wall musculature of *Phascolion* appear simultaneously as numerous muscle bands in the early trochophore larva before the onset of anterior–posterior elongation. At the same time, the rudiments of the longitudinal retractor muscles appear (Fig. 5a). During the early and mid-phases of elongation, the number of circular muscle fibres remains constant (Fig. 5b–d). As such, the longitudinal growth of the larval body does not correlate with an increase in larval body wall musculature. In addition, the two pairs of dorsal and ventral retractors are established (Fig. 5c). It is not until the late larval and early juvenile phases that additional circular muscle fibres are added. At the same time, the longitudinal muscle bands that underlie the circular muscles are formed (Fig. 5e–g). The formation of new circular body wall muscle fibres does not occur from a posterior

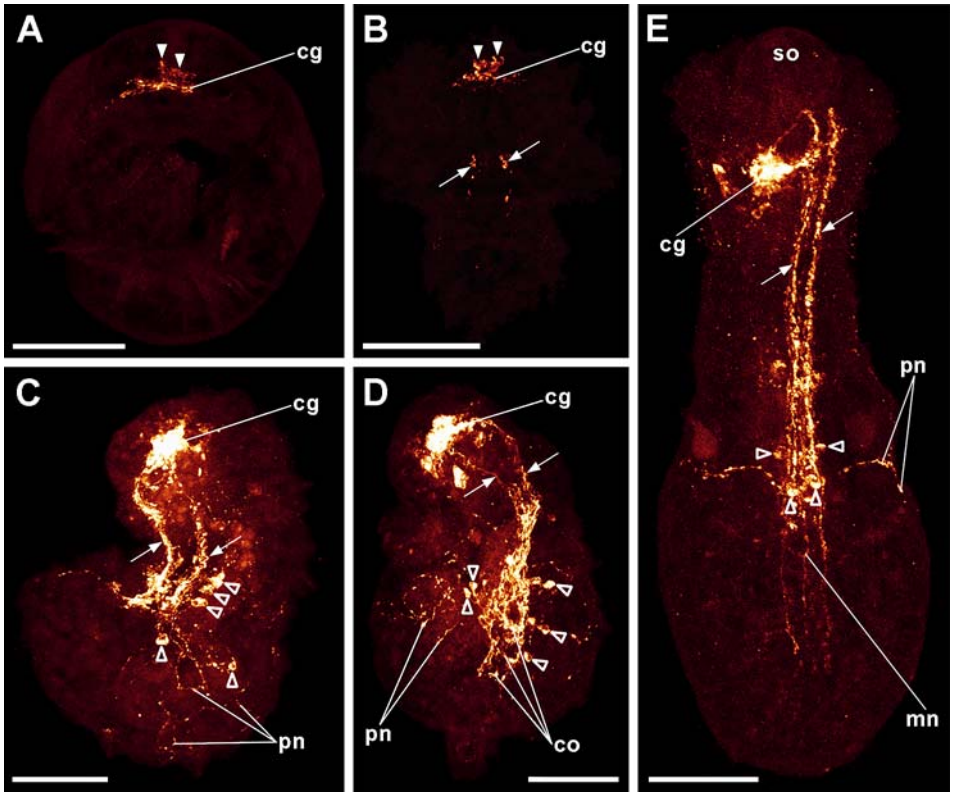

Fig. 3 Confocal laser scanning microscopy micrographs of the ontogeny of the FMRFamidergic nervous system in *P. strombus*; ventral view with anterior facing upwards in all aspects. *Scale bars* equal 50 μm. **a** Early trochophore with two FMRFamide-positive cells in the apical organ (*arrowheads*) and in the adjacent anlage of the cerebral ganglion (*cg*). **b** Larva at the teardrop stage showing the first signal in each of the two ventral nerve cords (*arrows*). **c** Elongated larva expressing strong signal in the cerebral ganglion and in the paired ventral nerve cord. In addition, the FMRFamidergic ventral cell clusters (*open arrowheads*) and the first neurons of a peripheral nerve net (*pn*) are found. **d** Late-stage larva. Note the presence of three commissure-like nerves (*co*) that interconnect the two lateral ventral nerve cords. **e** Early juvenile with both ventral FMRFamidergic nerve cords still being distinct. Note their loop-like connection to the cerebral ganglion. In addition, a median nerve (*mn*), situated between the two ventral nerve cords, is visible. The larval secretory organ is indicated as *so*

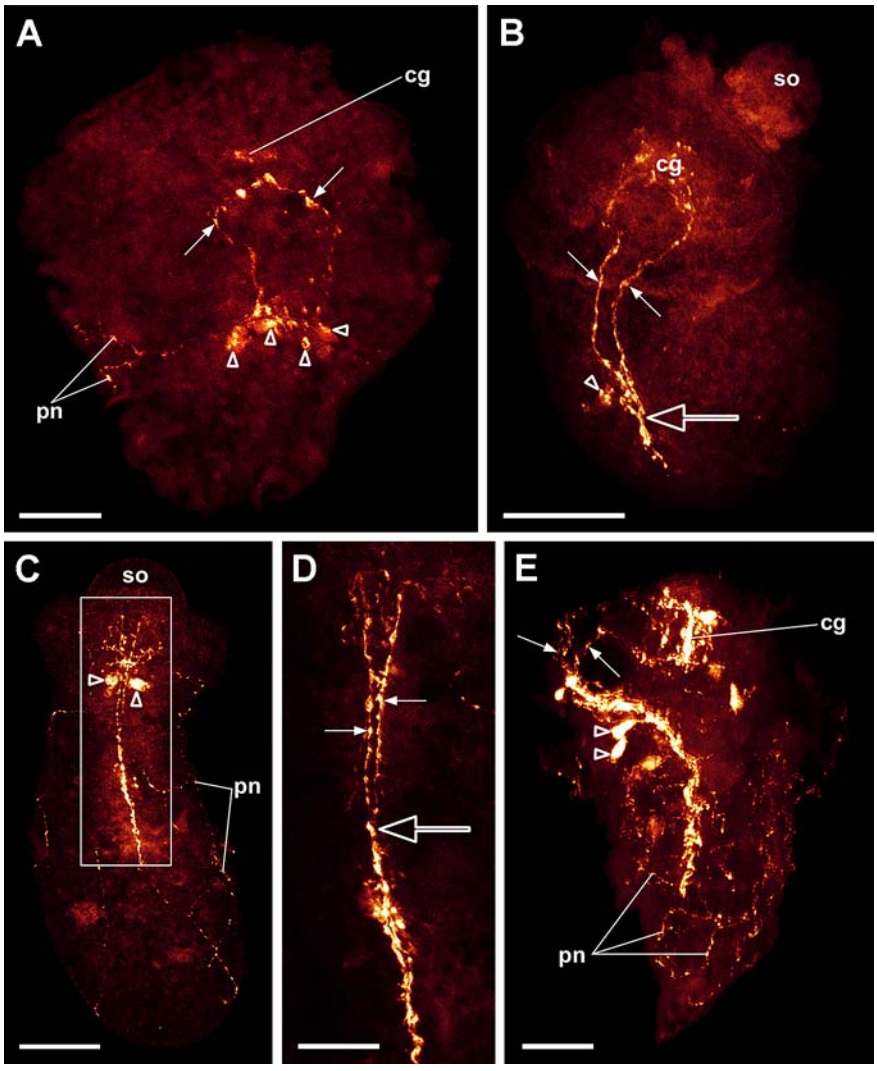

Fig. 4 Confocal laser scanning microscopy micrographs of the ontogeny of the serotonergic nervous system in *P. strombus*, anterior faces upwards. All aspects are in ventral view except for **e**, which is a lateral left view. *Scale bars* equal 25 μm in **a** and **d** and 50 μm in **b**, **c**, and **e**. **a** Teardrop-stage larva with the first rudiment of the cerebral ganglion (*cg*), the two distinct ventral nerve cords (*arrows*), a cluster of serotonergic cells in the mid-body area (*open arrowheads*) behind the stomodaeum, and few peripheral nerves (*pn*). Note the absence of signal in the apical region. **b** Elongated larva with well-developed larval secretory organ (*so*), depicting the area in the visceral body part where the two serotonergic ventral nerve cords fuse (*open arrow*). **c** Late larva illustrating the paired nature of the ventral nerve cord in the anterior and its singularity in the visceral body region. *Boxed area* is enlarged in **d**. **d** Same specimen as in **c**, with focal depth being restricted to the region of the ventral nerve cord(s) in order to emphasize the region of fusion of the ventral nervous system. **e** Early juvenile specimen with pronounced cerebral ganglion, serotonergic cell cluster, and peripheral nerve net. The dualism of the ventral nerve cord is only retained in the anterior loop that forms the connection to the cerebral ganglion

growth zone. Instead, new muscles are added throughout the entire anterior–posterior axis between the already existing circular muscles (Fig. 5h).

Discussion

Comparative neurogenesis and myogenesis in Trochozoa

Immunocytochemical data of entire developmental sequences (i.e. from early larval to post-metamorphic stages) are still missing for most trochozoan phyla except for a few annelid (mainly echiuran) (Hessling 2002; Hessling and Westheide 2002) or mollusc taxa (Dickinson et al. 1999; Friedrich et al. 2002; Voronezhskaya et al. 2002; Wanninger and Haszprunar 2003). However, the anatomy of the larval nervous system has been documented in at least some Trochozoa (for a review, see Hay-Schmidt 2000), thus enabling a comparison of certain features of the trochozoan larval nervous system.

Compared to the situation found in most other trochozoan taxa, neurogenesis in *P. strombus* shows distinct reductions and modifications. As in molluscs and annelids, the anlage of the cerebral ganglion forms at the base of the cells of the apical organ, lending support to the

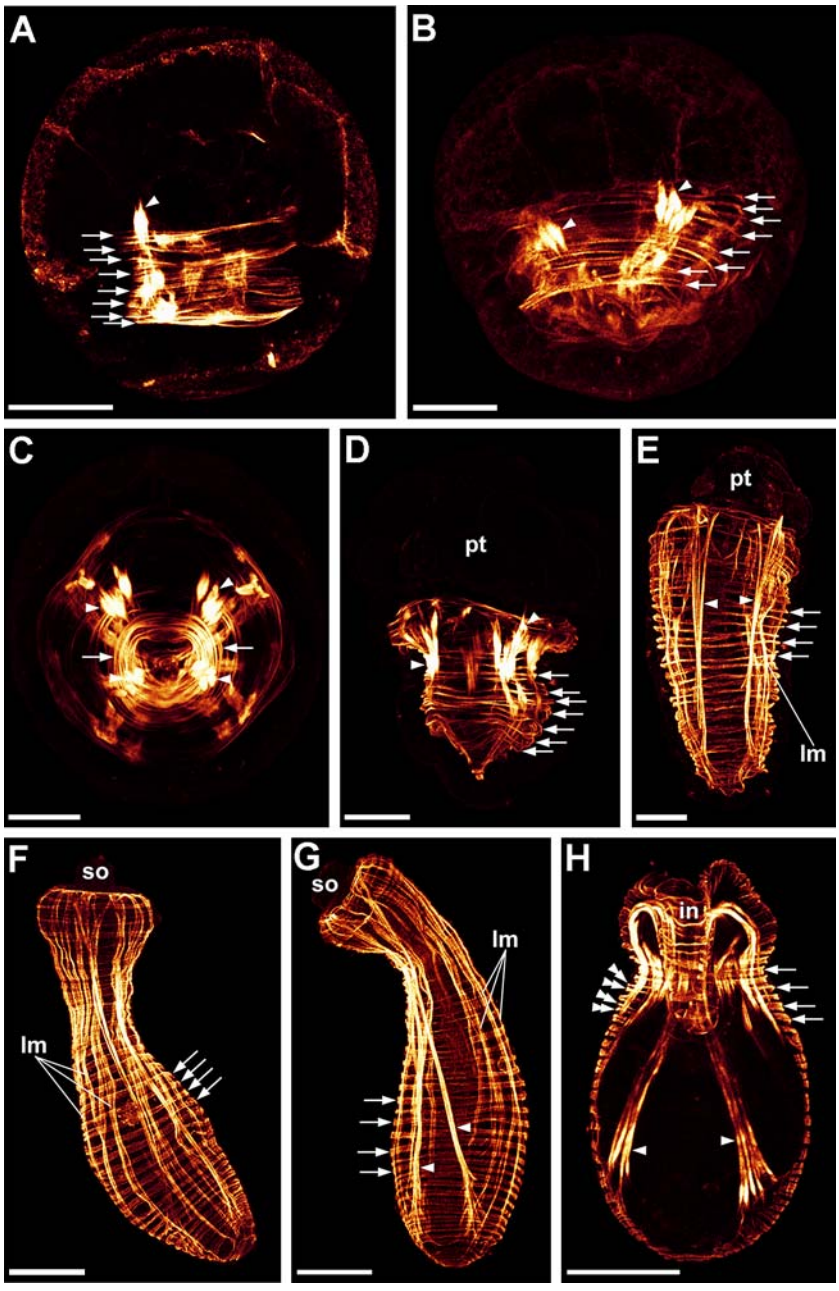

Fig. 5 Confocal laser scanning microscopy micrographs of myogenesis in *Phascolion strombus* with anterior facing upwards, except in **c**, where dorsal faces upwards. *Scale bars* equal 25 μm. **a** Early trochophore larva prior to the onset of elongation showing the first rudiments of the body wall ring muscles (*arrows*) and one of the putative longitudinal retractor muscles (*arrowhead*). The numerous ring muscles appear synchronously. **b** Trochophore at the beginning of elongation (teardrop stage), showing the same number of circular muscles (*arrows*) as in **a** as well as two retractor muscle anlagen (*arrowheads*). **c** Posterior view of a trochophore during elongation demonstrating the circular character of the body wall muscles. Note the four longitudinal retractor muscle rudiments. **d** Larva slightly further elongated as in **b** and **c** but still retaining the same number of circular muscles; *pt* marks the region of the prototroch. **e** Elongated larva with new ring muscles added. Note the prominent ventral retractor muscles and the developing longitudinal musculature of the body wall (*lm*). **f** Early juvenile in dorsal view with body wall musculature being comprised of ring muscles (*arrows*) and underlying longitudinal muscles (*lm*). Note the larval secretory organ (*so*) at the anterior pole of the animal. **g** Same stage as in **f** seen from ventral, showing the paired ventral retractor muscle (*arrowheads*). **h** Ventral view of a juvenile with retracted introvert (*in*). Focus is on the paired ventral retractor. Note the paired arrangement of some of the body wall ring muscles caused by newly formed intermediate muscle fibers (*double arrowheads*)

assumption that the larval apical organ plays an inductive role in the formation of the adult cerebral nervous system. In *Phascolion*, however, the larval neuronal features are much less prominent as e.g. in polychaete or mollusc larvae. While there are only very few (two to three) weakly stained FMRFamide-positive cells, serotonergic cells in the apical organ and a serotonergic prototroch nerve ring are entirely missing. Accordingly, *Phascolion* exhibits no

other components of a true larval nervous system except for these FMRFamidergic cells of the apical organ. These reductions, and the lack of expression of serotonin, may be due to the relatively short larval phase of only about 3 days in *P. strombus* compared to other sipunculan species, which often have a planktonic phase ranging from several weeks up to more than 6 months (Hall and Scheltema 1975; Rice 1985; Jaeckle and Rice 2002). In the polychaete *Phyllodoce maculata*, the larval apical organ contains numerous FMRFamide- and serotonin-positive cells (Voronezhskaya et al. 2003). Similar conditions are found in the polyplacophoran molluscs *Mopalia* and *Ischnochiton* (Friedrich et al. 2002; Voronezhskaya et al. 2002), while the FMRFamidergic components of the apical organ are missing in the scaphopod mollusc *Antalis entalis* (Wanninger and Haszprunar 2003). Despite the presence of an apical ciliary tuft, the pilidium larva of nemerteans seems to lack an apical organ altogether (Lacalli and West 1985; Hay-Schmidt 1990), but its derived mode of development, with the juvenile developing within the larva that then dies without undergoing metamorphosis, renders a direct comparison with other trochozoans difficult. According to recent investigations, it appears that direct developing palaeonemerteans, which express a modified vestigial prototroch, represent the ancestral mode of nemertean development (Maslakova et al. 2004). However, no immunocytochemical data on neuro- or myogenesis in these taxa are available to date.

Together with the scaphopod mollusc *Antalis* and the echiuran *Bonellia*, *Phascolion* is the only trochozoan representative known to lack a larval serotonergic prototroch nerve ring. This, as well as the dramatic reductions (in *Phascolion*) or entire absence (in *Bonellia* and *Urechis*) of certain neuronal components in the larval apical organ, indicates independent secondary losses of these typical larval features that may be due to an abbreviated larval phase in the life cycles of these taxa.

In the segmented Trochozoa, serially repeated organs along the anterior–posterior axis are formed from a pre-anal growth zone, with anterior segments and their respective organ systems forming earlier in ontogeny than more posterior ones (although modifications from this pattern do occur). With respect to the nervous system and the musculature, this holds true for annelids, including echiurans (Hill and Boyer 2001; Hessling 2002; Hessling and Westheide 2002), but not for molluscs (Haszprunar and Schaefer 1997; Friedrich et al. 2002; Wanninger and Haszprunar 2002a,b) or platyhelminths (Ladurner and Rieger 2000). Due to their coiled, U-shaped gut, the anal opening is not situated posteriorly, but in the anterior region of the trunk in sipunculans. Our data, presented herein, show no indication for the existence of a growth zone in *P. strombus* neither in the pre-anal area nor in the posterior body region. While the existence of a pre-anal growth zone has not unambiguously been shown for echiurans (Hessling 2003), their pattern of nervous system formation and molecular data indicate that Echiura may nest within the polychaetes (McHugh 1997, 2000, Bleidorn et al. 2003a,b; Jördens et al.2004; but see Siddall et al. 1998) or at least be closely related to Annelida (Staton 2003; Boore 2004). Ladder-like commissures interconnecting the ventral nerve cords in several segmented or non-segmented trochozoan phyla, such as basal molluscs, flatworms, or annelids, may or may not be plesiomorphic for Trochozoa. Their mere presence, however, is not indicative of a segmented body plan, since their ontogeny in polyplacophoran molluscs or platyhelminths occurs in a "random-like" pattern and not in an anterior–posterior direction (Ladurner and Rieger 2000; Friedrich et al. 2002; Voronezhskaya et al. 2002). Accordingly, the presence of a pair of ventral nerve cords with interconnecting commissure-like neurones in the *Phascolion* larva does not indicate ancestral segmentation in sipunculans but may rather be interpreted as vestiges of a paired, ladder-like nervous system in a hypothetical sipunculan stem species. Fusion of the ventral nerve cords during sipunculan ontogeny has been known from earlier studies (Rice 1973, 1985) and has recently been confirmed for echiurans (Hessling 2002; Hessling and Westheide 2002).

A median nerve that is positioned between the two ventral nerve cords, as observed in juvenile *Phascolion*, has also been found in certain polychaetes and in the myzostomid *Myzostomum cirriferum* (Müller and Westheide 2000, 2002; Eeckhaut et al. 2003), although the expression of neurotransmitters in this nerve cord varies. While *Myzostomum* and the dinophilid and dorvilleid polychaetes investigated so far show positive immunoreactivity against α-tubulin, only the dinophilid *Dinophilus gardineri*, but not *Trilobodrilus axi*, contains both serotonin and FMRFamide. Similar to the condition found in the dorvilleids *Parapodrilus psammophilus* and in larval *Ophryotrocha gracilis*, *T. axi* lacks FMRFamide expression. In contrast, we only found FMRFamide, but not serotonin, in *Phascolion* (Fig. 3), while *Myzostomum* lacks both serotonin and FMRFamide altogether. It has been shown earlier that neurones can change transmitter expression during ontogeny (Witten and Truman 1996; Ierusalimsky et al. 1997), which, however, does not preclude homology of the respective neurological structure. Instead, the fact that dinophilids and dorvilleids are considered progenetic polychaetes strengthens the hypothesis that a median nerve was part of the ground plan of a proposed hypothetical annelid–sipunculan ancestor and has been conserved in various taxa such as e.g. in certain polychaetes and myzosyomids.

Regarding the question about possible cryptic segmentation in Sipuncula, the pattern of muscle formation in *Phascolion* corroborates the data on neurogenesis. In contrast to the condition found in polychaetes, where the circular body wall musculature corresponds to individual segments and is formed subsequently in anterior–posterior direction, a large number of circular muscles are formed simultaneously in the early trochophore larva of *P. strombus* (cf. Hill and Boyer 2001 and herein). The number of these muscle fibres remains constant even throughout the first stages of body elongation and only increases during the later larval and early juvenile stages. Thus, there is no correlation between the increase in body length in

Phascolion and the number of circular body wall muscles. This mechanism resembles the condition described recently for polyplacophoran molluscs, where the repetitive dorso-ventral muscles also appear at the same time during early larval development (Wanninger and Haszprunar 2002a), indicating the differences in ontogenetic mechanisms underlying body plan patterning in segmented vs non-segmented trochozoans. If new circular muscle fibres of the body wall are added in *Phascolion*, they appear to be formed along the entire length of the body axis and are not established in anterior–posterior direction.

The existence of the outer ring and inner longitudinal muscles within the body wall musculature has been described for several taxa and seems to be conserved within the Trochozoa. In addition, some groups, such as worm-shaped molluscs, have additional, interspersed oblique (diagonal) muscle strands (Haszprunar and Wanninger 2000; Wanninger and Haszprunar 2002a). Although we did not trace myogenesis beyond the early juvenile stages, it appears that the paired dorsal and ventral retractor muscles fuse during the subsequent development of *Phascolion* (Åkesson 1958; see also Schulze and Rice 2003). In contrast to several other trochozoans, such as molluscs (Wanninger et al. 1999; Haszprunar and Wanninger 2000; Wanninger and Haszprunar 2002a,b) or polychaetes (Wanninger, unpublished), *P. strombus* larvae lack a distinct prototroch muscle ring.

Evolutionary considerations

The current lack of a robust metazoan phylogeny renders any interpretations of ontogenetic data difficult. However, several recent independent molecular phylogenetic studies based on mitochondrial DNA sequence data indicate that Sipuncula may cluster with Annelida rather than with Mollusca (Boore and Staton 2002; Staton 2003; Jennings and Halanych 2005). Although our data do not provide any evidence for ancestral segmentation in Sipuncula, the existence of a distinct ventral median nerve is shared with dinophilid and dorvilleid polychaetes as well as with myzostomids and hirudineans (Payton 1981; Müller and Westheide 2000, 2002; Eeckhaut et al. 2003). If the assumption that such a median nerve cord represents an evolutionary conserved character proves to be correct (see Müller and Westheide 1997, 2002), the hypothesis of Sipuncula being closely related to Annelida would gain additional morphological support. Accordingly, with respect to the evolution of segmentation, two scenarios with an equal ad hoc probability are possible: (1) sipunculans represent the basal (unsegmented) condition, and segmentation evolved in Annelida after the annelid–sipunculan split; or (2) the LCA of the annelid–sipunculan lineage was segmented, and sipunculans have secondarily lost this feature. To further assess these evolutionary issues and to shed new light on the molecular mechanisms underlying body plan patterning in these worm–shaped taxa, comparative gene expression data can potentially prove highly informative.

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