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DIPLOMARBEIT

Titel der Diplomarbeit

„Analysis of the Nervous System of the Actinotroch Larva of *Phoronis muelleri* (Phoronida) based on Immunocytochemical Staining and Confocal Laser Scanning Microscopy“

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Für meine Eltern, die mich immer unterstützen

Content

Abstract

Recent molecular data place the phoronids within the protostome superclade Lophotrochozoa, where they have recently been suggested to cluster with the brachiopods and/or nemerteans. Herein, the anatomy of the nervous system and the structure of the apical organ are described for two larval stages of *Phoronis muelleri* in order to contribute to the discussion concerning the evolution of the lophotrochozoan nervous system. Specimens were investigated using immunocytochemistry and confocal laser scanning microscopy. A ventral nerve cord is common in many larval and adult spiralians. Although a ventral neurite bundle is present in the early larval development of *Phoronopsis harmeri*, such a structure is absent in both investigated stages herein. Most larval lophotrochozoans have a simple apical organ containing a few serotonin-like immunoreactive (serotonin-lir) flask-shaped cells. The complex apical organ of *Phoronis muelleri* consists of numerous serotonin-lir flask-shaped cells, bi-or multipolar serotonin-lir additional cells, and several FMRFamide-like immunoreactive (FMRFamide-lir) perikarya. Moreover, this study provides the first description of the small cardioactive peptide-like immunoreactive (small cardioactive peptide-lir) nervous system of a phoronid larva. This neuropeptide is present along the margin of the preoral lobe, in the apical organ, along the mesosome and in the tentacles as well as in the trunk and the telotroch. Moreover, a positive signal is known from molluscs, annelids and arthropods, indicating the high conservation of this immunoreactive molecule among the Protostomia and supports the notion that it was also part of the protostomian groundplan.

Zusammenfassung

Aktuellen molekularen Daten zufolge werden die Phoroniden innerhalb der Protostomia den Lophotrochozoa zugeordnet, wobei von manchen Autoren eine engere Verwandtschaft zu den Brachiopoda und/oder zu den Nemertea angenommen wird. Um ein besseres Verständnis für

die Evolution des Nervensystems der Lophotrochozoen zu bekommen, wird in dieser Studie das Nervensystem und das Apikalorgan von *Phoronis muelleri* im Detail beschrieben. Ein ventraler Nervenstrang kommt bei vielen Vertretern der Spiralia vor. Obwohl in der frühen Entwicklung von *Phoronopsis harmeri* ein ventrales Neuritenbündel vorhanden ist, konnte in dieser Studie eine solche Struktur nicht nachgewiesen werden. Ein einfach gebautes Apikalorgan mit wenigen serotonin-immunoreaktiven flaschenförmigen Zellen ist charakteristisch für die meisten Vertreter der Lophotrochozoen. Das Apikalorgan von *Phoronis muelleri* jedoch ist äußerst komplex und besteht aus zahlreichen serotonin-immunoreaktiven flaschenförmigen Zellen und zusätzlichen serotonin-immunoreaktiven bi- oder multipolaren Zellen sowie einigen FMRFamid-immunoreaktive Zellen. Weiters wird in dieser Studie zum ersten Mal das Small Cardioactive Peptide-immunoreaktive Nervensystem einer Phoronidenlarve beschrieben. Dieses Neuropeptid kommt entlang des Prosomas, im Apikalorgan, im Mesosoma und in den Tentakeln sowie im Metasoma und im Telotroch vor. Innerhalb der Protostomier gilt dieses Neuropeptid als stark konserviert. Ein positives Signal kommt bei diversen Gruppen der Mollusken, den Anneliden und den Arthropoden vor. Der Nachweis eines positiven Signals bei Phoroniden bekräftigt somit die Hypothese, dass Small Cardioactive Peptide bereits im Grundbauplan der Protostomier enthalten war.

Introduction

The phylogenetic position of the Phoronida is still highly debated. While recent molecular studies consider them as close relatives to brachiopods or nemerteans within the protostomian superclade Lophotrochozoa (Dunn et al., 2008; Hausdorf et al., 2010; Paps et al., 2010), the traditional morphological-based view is that they cluster together with brachiopods and ectoprocts within a taxon called Lophophorata, the sister group of Spiralia (Emig, 1984; Nielsen, 2002). In some recent phylogenetic studies the Phoronida are included within the Brachiopoda to form a group called Brachiozoa, which share a monophyletic clade with Nemertea or Mollusca (Hausdorf et al., 2010; Paps et al., 2010). Nevertheless, phoronids also share some features which are considered as typical Deuterostomia-like, such as radial cleavage, a regulative development as well as the still widely debated trimeric organization of the body (Nielsen, 2002).

The organization of the nervous system of adult lophotrochozoans varies between the different groups. While adult platyhelminths express an orthogonal nervous system which consists of an apical “brain” and 2 to 8 longitudinal nerve cords, a rope ladder-like nervous system with 1 to 5 ventral nerve cords is found in annelids (Wanninger, 2009). Interestingly, it was shown that the multiple ventral cords of annelids and echiurans as well as the single ventral nerve cord of adult sipunculids always results from a paired ventral nerve, which forms the first anlage of the future ventral nervous system (Wanninger, 2009). In juvenile brachiopods two ventral longitudinal neurites are present (Altenburger and Wanninger, 2010) while in adult ectoprocts each polypide possesses a ganglion with lateral nerves following the tentacle base and a peripheral nerve net (Nielsen, 2012). The typical tetraneurous nervous system of adult molluscs includes a paired ventral and lateral nerve cord together with the buccal nerves and the anterior nerve loop (Nielsen, 2012). Moreover, a tetraneurous condition of the nervous system is present in the creeping-type larva of entoprocts which is considered

basal for the phylum (Wanninger et al., 2008). It is hypothesized that the simple nervous system of adult Entoprocta results from a typical molluscan tetraneurous condition supporting the monophyletic molluscan-entoprocts clade (Wanninger, 2008). All these data of the nervous system of different Lophotrochozoa support the scenario that the last common ancestor (LCA) had at least two ventral nerve cords in its groundplan (Wanninger, 2009). Interestingly, the Acoelomorpha have traditionally been viewed as an ingroup of the platyhelminths, but based on recent molecular data they are nowadays considered as the earliest offshoot of bilaterians (Haszprunar, 1996; Ruiz-Trillo et al., 2004; Paps et al., 2009). The nervous system of Acoelomorpha consists of a nerve net which is barely concentrated anteriorly (Ruiz-Trillo et al., 1999). This simple neural organization is considered by several authors as a basal condition for Bilateria.

The body of adult phoronids can be divided in three parts: the prosome or epistome which is a fold that overhangs the mouth, the mesosome which bears the lophophore and the large metasome or trunk. The nervous system of adult phoronids consists of a ganglion which is located in the epidermis of the epistome. It gives rise to a nerve ring at the base of the lophophore and one or two lateral giant nerve fibres. Giant nerve fibres are absent in *Phoronis ovalis*. In addition, they possess a basiepidermal nerve plexus along their entire body (Emig, 1979). Compared with the organization of the nervous system of other lophotrochozoans, the nervous system of adult phoronids differs in various aspects. There are no ventral nerve cords present in the nervous system of adult phoronids.

Recently, numerous immunocytochemical studies on the development of the nervous system have been carried out on different lophotrochozoan groups (see, e.g., Hay-Schmidt, 1990a, b; Haszprunar et al., 2002; Santagata, 2002; Voronezhskaya et al., 2002; Voronezhskaya et al., 2003; Wanninger, 2005; Wanninger et al., 2005a, b; Fuchs and Wanninger, 2007; Kristof et al., 2008; Wanninger et al., 2008; Altenburger and Wanninger, 2010; Schwaha and Wanninger, 2012; Temereva, 2012; Temereva and Wanninger, 2012). These studies have

shown that the larvae of most lophotrochozoans possess a simple apical organ situated anteriorly and contain only a few serotonin-lir flask-shaped cells, often about four (Voronezhskaya et al., 2003; Fuchs and Wanninger, 2008; Wanninger, 2008; Altenburger and Wanninger, 2010; Nielsen and Worsaae, 2010). However, in polyplacophoran molluscs and in the creeping-type larva of entoprocts, the apical organ is more complex and contains 6-8 serotonin-lir flask-shaped cells and several additional peripheral cells (Voronezhskaya et al., 2002; Wanninger et al., 2008). The larva of phoronids, the so-called actinotroch larva, is sometimes considered a modified trochophore larva (Salvini-Plawen, 1980) and contains numerous flask-shaped cells in its apical organ (Hay-Schmidt, 1990a; Lacalli, 1990; Santagata, 2002; Temereva and Wanninger, 2012). However, a recent study of the larval nervous system of *Phoronopsis harmeri* has shown that four serotonin-lir flask-shaped cells differentiate simultaneously in the mid-gastrula stage (Temereva, 2012; Temereva and Wanninger, 2012). In addition, the apical organ increases in complexity during subsequent development (Temereva and Wanninger, 2012).

Another feature of the nervous system of many Lophotrochozoa is the presence of a ventral nerve cord, either in the larva or the adult or both (Kristof et al., 2008; Wanninger, 2009; Altenburger and Wanninger, 2010). For the young larva of *Phoronopsis harmeri* a serotonin-lir and a FMRFamide-lir ventral neurite bundle containing several repetitive commissures and neurons have been described (Temereva, 2012; Temereva and Wanninger, 2012). When the larva reaches the 6 primordial tentacle stage, the serotonin-lir ventral neurite bundle disappears. The FMRFamide-lir ventral neurite bundle instead is present at least until the 6 tentacle stage (Temereva and Wanninger, 2012).

The gross morphology of the nervous system of the actinotroch larva of *Phoronis muelleri* has been described previously (Hay-Schmidt, 1990a), but several details remain vague. This study herein deals not only with the analysis of the nervous system of the actinotroch larva of *Phoronis muelleri* in general, but also focuses on the detailed structure of the apical organ.

Moreover, this work presents the first data of the small cardioactive peptide-lir nervous system of the actinotroch larva. This neuropeptide was characterized from molluscan nervous tissue, where it is involved in regulation of the heart beat rate. A positive signal has been found in gastropods, bivalves, cephalopods as well as in annelids and arthropods (Lloyd et al., 1984; Mahon et al., 1985; Kempf et al., 1987; Lloyd et al., 1987; Evans and Calabrese, 1989; Candelario-Martinez et al., 1993; Fox and Lloyd, 1997; Gainey et al., 1999; Perry et al., 1999; Ohsuga et al., 2000; Willows et al., 2000; Kempf and Page, 2005; Kanda and Minakata, 2006; Ellis and Kempf, 2011). In larval and adult molluscs it is expressed in the central and peripheral nervous system and obviously plays an additional modulatory role in the control of feeding behavior (Perry et al, 1999; Ellis and Kempf, 2011). In adult arthropods the distribution of this neuropeptide is concentrated in the brain and the subesophagal ganglion while in adult annelids it is mainly expressed in the segmental ganglia (Settembrini and Villar, 2005). The present study provides new data about the distribution of the small cardioactive peptide-lir nervous system in the larva of Phoronida. Taken together, this work increases the morphological database concerning the larval neuroanatomy of Phoronida and contributes to a better understanding of the evolution of the lophotrochozoan nervous system.

Materials and Methods

Animal collection and fixation

Actinotroch larvae of *Phoronis muelleri* (Selys-Longchamps, 1903) were collected in August 2011 at the Sven Lovén Centre for Marine Sciences, Sweden. Vertical plankton tows were taken from 55 m depth (58°15.66N, 11°27.20E; 58°15.7N, 11°26.30E; 58°15.65N, 11°27.22E) and 15 m depth (58°15.02N, 11°27.23E), respectively. In addition, a horizontal tow was taken from 3-5 m depth (58°15.66N, 11°27.20E). Specimens were divided into two

developmental stages (12-16 tentacles, 18 and more tentacles). For each stage, 4-11 individuals were investigated per neurotransmitter.

The specimens were narcotized by adding 3.5% Magnesium chloride to the seawater, and subsequently fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS) and 10% sucrose (pH 7.4) for 2-3.5 hours at room temperature. After fixation they were rinsed 3-5 times in 0.1 M PBS with 0.1% sodium azide (NaN_3) for 10 minutes each (pH 7.4) and stored at 4°C.

Immunocytochemistry, data acquisition and analysis

Prior to immunocytochemical staining the larvae were washed in 0.1 M PBS with 0.1% NaN_3 and 4% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) (PTA) for 1 hour at room temperature. Unspecific binding sites were blocked overnight in a solution of 6% normal goat serum (Invitrogen, Molecular Probes, Eugene, OR, USA) in 0.1 M PBS and 4% Triton-X-100 (block-PTA). The samples were incubated for 24 hours at room temperature in block-PTA containing the primary antibodies: anti-rabbit serotonin (Immunostar, Hudson, WI, USA, dilution 1:800); anti-rabbit FMRFamide (Bio Trend, Cologne, Germany, dilution 1:1000); anti-mouse α-tubulin (Sigma-Aldrich, dilution 1:500). In case of the staining against small cardioactive peptide the samples were incubated for 48 hours at room temperature in block-PTA containing the primary antibody: anti-small cardioactive peptide (homemade, dilution 1:300). After 4 washes in block-PTA for 6-12 hours or overnight, the secondary fluorochrome-conjugated antibody (anti-rabbit Alexa Fluor 568,Invitrogen; anti-mouse Alexa Fluor 568, Invitrogen; anti-mouse Alexa Fluor 633,Invitrogen) diluted in block-PTA (serotonin: 1:300; α-tubulin: 1:300; FMRFamide: 1:100; small cardioactive peptide: 1:300) was added. The incubation lasted for 24 hours, in case of the small cardioactive peptide staining for 48 hours, at room temperature and was carried out in the dark. Multi-stainings

were made by a cocktail of the desired antibodies in the respective working concentrations. Specimens were rinsed 4 times in PBS without NaN_3 for 6-12 hours or overnight and subsequently mounted in Flouromount G (Southern Biotech, Birmingham, AL, USA) on glass slides. Controls were made by either adding only the primary antibody, only the secondary antibody, or neither of them, and rendered no signal.

Animals were viewed on a Leica TCS SP 5 II confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) with the LAS AF (Leica Application Suite Advanced Fluorescence) Leica Microsystems software. Specimens were scanned with 0.1 µm–1.5 µm step size and maximum projection images were created with the LAS AF software.

In addition, a differential interference contrast (DIC) image was taken with a Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan) equipped with a Nikon DS-Fi2 U3 microscope camera (Nikon). For a better understanding of the three-dimensional situation of the neuroanatomy and to generate 3D reconstructions, the software Imaris 7.3.1 (Bitplane, Zurich, Switzerland) was used. The figure plates were created with Adobe Photoshop CS5 software (Adobe, San Jose, CA, USA) and the line drawings with Adobe Illustrator CS5 software (Adobe).

Results

General morphology of the actinotroch larva

The body of the actinotroch larva is divided into three parts: the prosome, the mesosome and the metasome. The prosome, also called preoral lobe or hood, overhangs the mouth and includes the apical organ. The mesosome or collar region bears the tentacles. The metasome, also called trunk, is enlongated, bears the telotroch and terminates in the anus (Fig. 1, A, B).

12-16 tentacle stage

Serotonin-lir nervous system

In the preoral lobe, a U-shaped apical organ is situated (Fig. 2, A). It contains about 22 flask-shaped cells, each projecting into the neuropil (Fig. 2, C-E). Every flask-shaped cell bears a single cilium. Additionally, bi- or multipolar serotonin-lir cells are located under the neuropil and are likewise arranged in a horseshoe-like pattern (Fig. 2, A, E; Fig. 3, D). Dorso-lateral neurites emerge from the apical organ, project posteriorly and give rise to the tentacular neurite bundle, which runs under the tentacles and sends two lateral abfrontal processes into each tentacle (Fig. 2, A, B; Fig. 3, A; Fig. 4, A). The lateral part of the tentacular neurite bundle spreads towards the mesosome and sends a median frontal process into each tentacle (Fig. 2, A, B; Fig. 3, A; Fig. 4, A). Along the tentacular ridge and the frontal side of each tentacle a band of serotonin-lir perikarya is present (Fig. 2, A, B; Fig. 3, A). From the anterior part of the apical organ the median hood neurite bundle extends and runs towards the margin of the preoral lobe, where the hood margin neurite bundle with several perikarya is situated. Serotonin-lir neurites are present along the entire preoral lobe (Fig. 2, A; Fig. 4, A). The neurites of the trunk region connect the tentacular neurite bundle to the ring-shaped neurite bundle of the telotroch (Fig. 2, A, Fig. 4, A). Moreover, the telotroch is innervated by a meshwork of fine neurites (Fig. 2, A; Table 1).

FMRFamide-lir nervous system

The apical organ is located in the preoral lobe and consists of a U-shaped neuropil and a few dorso-lateral bipolar perikarya on both sides (Fig. 3, E; Fig. 5, A, C). Neurites project in dorso-lateral direction and give rise to the tentacular neurite bundle (Fig. 4, C). The posterior

part of the tentacular neurite bundle runs under the tentacles and projects into each of them on the median abfrontal side (Fig. 3, B). The lateral part of the tentacular neurite bundle spreads over the mesosome and projects into the tentacles on the frontal side (Fig. 3, B; Fig. 5, B). At the basis of each tentacle the lateral frontal processes are connected to each other via several commissures (Fig. 3, B; Fig. 5, B). Several bi-or multipolar perikarya are situated along the ventral side of the mesosome (Fig. 4, C; Fig. 5, A, B). At the anterior part of the apical organ a median hood neurite bundle develops and runs towards the hood margin (Fig. 4, C). Several perikarya are situated along the hood margin neurite bundle and some perikarya are scattered along the entire preoral lobe (Fig. 4, C; Fig. 5, A). Several neurites are scattered along the trunk region and are connected to the telotroch and its two ring-shaped neurite bundles (Fig. 4, C; Fig. 5, D; Table 2). In addition, perikarya with a granular appearance develop in the telotroch region (Fig. 4, C; Fig. 5, D, E). Some of them are connected to the nerve net of the trunk as well as to the ring shaped neurite bundles and its fine interconnecting neurites of the telotroch (Fig. 5, D, E; Table 2).

18 and more tentacles

Serotonin-lir nervous system

The serotonin-lir nervous system is similar to the one described in the previous stage (Fig. 4, B; Fig. 6, A, B; Fig. 7, A, B). The frontal organ, a sense organ situated anterior to the apical organ, contains several probably bipolar perikarya which project into the median neurite bundle of the preoral lobe (Fig. 6, C). It is connected to the apical organ as well as to some thinner neurites of the entire preoral lobe (Fig. 6, C). The number of flask-shaped cells in the apical organ increases. At the 22 tentacle stage they possess up to 37 of these flask-shaped

cells (Fig. 6, C, D). Nodes are visible along the processes of the flask-shaped cells which project into the neuropil (Fig. 6, E; Table 1).

FMRFamide-lir nervous system

The general organization of the FMRFamide-lir nervous system is similar to the one described in the previous stage (Fig. 4, D; Fig. 7, C, D; Fig. 8, A). At the tentacle tips the frontal and the abfrontal tentacular processes are connected to each other (Fig. 8, C).The signal of the apical organ increases in intensity (Fig. 8, D). The perikarya of the telotroch region disappear, while the two ring-shaped neurite bundles of the telotroch and its meshwork of fine neurites remain (Fig. 8, B; Table 2).

Small cardioactive peptide-like immunoreactiviy

On the preoral lobe, a U-shaped apical organ is situated, which contains several, probably bipolar, dorso-lateral perikarya on both sides (Fig. 3, F; Fig. 7, E, F; Fig. 9, A, B). There are several fine neurites along the entire preoral lobe (Fig. 7, E, F; Fig. 9, B). A main hood neurite bundle runs along the margin of the preoral lobe (Fig. 9, B, D). A few neurites are located in the entire mesosome. Two frontal processes project into each tentacle (Fig. 3, C; Fig. 7, E; Fig. 9, A, D). A few neurites are situated in the trunk and some of them are connected to the ring-shaped neurite bundles of the telotroch. In addition, the telotroch is innervated by several fine neurites that form a meshwork together with the two ring-shaped neurite bundles (Fig. 9, A, C; Table 3).

Discussion

Comparison of the larval nervous system within Phoronida

The serotonin-lir and FMRFamide-lir nervous system of *Phoronis muelleri* is similar to the previous description (Hay-Schmidt, 1990a), although there are some significant differences such as the absence of the serotonin-lir marginal neurite bundle of the preoral lobe and of the serotonin-lir perikarya along the tentacular ridge in the previous study which are present in the study herein (Tables 1, 2). In addition, the innervation of the tentacles differs concerning the position of the three serotonin-lir processes. Along the tentacular ridge FMRFamide-lir perikarya are present and serotonin-lir perikarya are absent in the previous study while in the present study only serotonin-lir perikarya along the tentacular ridge are detectable. Moreover, the FMRFamide-lir components of the apical organ are different. While there are 5-7 monopolar cells in the antero-lateral part of the apical organ and about 20 additional bipolar cells located behind the apical organ described in the previous study the present study shows only a few bipolar FMRFamide-lir perikarya in the dorso-lateral part of the apical organ. Another main difference is the presence of the posterior part of the FMRFamide-lir tentacular neurite bundle which projects in posterior direction and runs under the tentacles as well as some perikarya along the hood margin neurite bundle in this study herein. In addition, a nervous structure called the ‘minor nerve ring’ which connects the tentacle processes with tentacular processes of the adjacent tentacles is described (Hay-Schmidt, 1990a). In the present study the innervation of the tentacles is more complex as the tentacular processes are connected to the processes of the neighboring tentacles, to the tentacular neurite bundle and to the neurites of the mesosome. On the contrary to the previous study, the telotroch is innervated by two FMRFamide-lir ring-shaped neurite bundles. The FMRFamide-lir perikarya which are connected to the ring-shaped neurite bundle of the telotroch are only detectable in the 12-16 tentacle stage in the present study. The mentioned differences between

these two studies on *Phoronis muelleri* might result from using antibodies of different companies in a slightly modified concentration. Moreover, different technical equipments were used as specimens were viewed with an epifluorescence microscope in the previous study while a confocal laser scanning microscope was used in the present study.

Compared to the nervous system of *Phoronopsis harmeri* the serotonin-lir and FMRFamide-lir nervous system of *Phoronis muelleri* also shows some differences (Temereva and Wanninger, 2012). In early larval stages of *Phoronopsis harmeri* a serotonin-lir and FMRFamide-lir ventral neurite bundle with an associated oral nerve ring is present. This resembles a common feature in many other lophotrochozoans (Wanninger, 2009). Such a ventral neurite bundle was not detected in the current study. Moreover, the innervation of the tentacles differs slightly between these two studies. In *Phoronopsis harmeri* a serotonin-lir abfrontal loop of processes projects into each tentacle while in *Phoronis muelleri* two serotonin-lir abfrontal and a serotonin-lir frontal process as well as two FMRFamide-lir frontal and a FMRFamide-lir abfrontal process are present.

During early development of *Phoronopsis harmeri* the first serotonin-lir flask-shaped cells in the apical organ differentiate in the mid-gastrula stage (Temereva and Wanninger, 2012). During subsequent development they increase in number to about 25 in the 6 tentacle stage. In the apical organ of *Phoronis muelleri*, there are approximately 22 flask-shaped cells in the 12 tentacle stage and about 37 of those cells when the larva has 22 tentacles (Fig. 2, C, D; Fig. 6, C, D). The apical organ of *Phoronopsis harmeri* contains monopolar FMRFamide-lir perikarya and two dorso-lateral groups of several bi- or multipolar FMRFamide-lir perikarya (Temereva and Wanninger, 2012). Only two dorso-lateral groups of several bipolar FMRFamide-lir perikarya are present in the apical organ of *Phoronis muelleri*. Serotonin-lir and often also FMRFamide-lir components are found in the apical organ of many other lophotrochozoans such as annelids and molluscs (Wanninger, 2008), supporting the scenario

that the LCA of the lophotrochozoans already had, at least, serotonin-lir components expressed in its apical organ.

In addition, this study provides the first results of the small cardioactive peptide-lir nervous system in the actinotroch larva of *Phoronis muelleri* (Table 3). A positive signal is known from larval and adult molluscs, adult annelids and even adult arthropods (Lloyd et al., 1984; Kempf et al., 1987; Lloyd et al., 1987; Candelario-Martinez et al., 1993; Fox and Lloyd, 1997; Gainey et al., 1999; Perry et al., 1999; Ohsuga et al., 2000; Willows et al., 2000; Kempf and Page, 2005; Settembrini and Villar, 2005; Kanda and Minakata, 2006; Ellis and Kempf, 2011). The staining results in a weak signal where the apical organ, the marginal neurite bundle of the preoral lobe, the innervation of the tentacles and the telotroch are the most prominent nervous structures visible. Although it is hypothesized that small cardioactive peptide plays a role in the regulation of the heart beat rate, muscle modulation and ciliary activity in molluscan larva, little is known about additional functions of this neuropeptide (Ellis and Kempf, 2011). The positive signal in phoronids corroborates the assumption that it was present in the protostomian groundplan.

Comparative larval neuroanatomy of lophotrochozoans

Most lophotrochozoans, e.g., annelids, ectoprocts, the swimming type larva of entoprocts, sipunculids and platyhelminths have a simple apical organ with up to 4 associated serotonin-lir flask-shaped cells (Voronezhskaya et al., 2003; Fuchs and Wanninger, 2008; Wanninger, 2008; Nielsen and Worsaae, 2010). A simple apical organ with only a few serotonin-lir flask-shaped cells is therefore considered as basal within the lophotrochozoans (Wanninger 2009). However, a more complex apical organ containing 6-8 serotonin-lir flask-shaped cells and several additional peripheral cells is present in the larva of polyplacophoran molluscs and in the creeping-type larva of entoprocts (Voronezhskaya et al., 2002; Wanninger et al., 2008).

This derived complexity is viewed as an apomorphy of a mollusk-entoproct-clade, the Tetraneuralia (Wanninger, 2008). A simple apical organ with 1 or 2 sets of four serotonin-lir flask-shaped cells is found in the larva of craniiform and rhynchonelliform brachiopods (Altenburger and Wanninger, 2010; Altenburger et al., 2011). On the contrary, the apical organ of the "paralarvae" of planktotrophic linguliform brachiopods contains numerous serotonin-lir cells (Hay-Schmidt, 1992). The latter seemingly resembles more closely the apical organ found in phoronids, although the typical flask-shape of the serotonin-lir cells has not been described in the linguliform "paralarvae". While in some species of the short-lived lecithotrophic coronate larva of ectoprocts the apical organ consists of two serotonin-lir flask-shaped cells, others show a serotonin-lir apical commissure without serotonin-lir or FMRFamide-lir cell bodies (Shimizu et al., 2000; Wanninger et al., 2005a). However, the long-lived planktotrophic cyphonautes larva possesses a simple apical organ containing two flask-shaped serotonin-lir cells and some additional serotonin-lir perikarya (Nielsen and Worsaae, 2010). It is still debated whether the corona-type or the planktotrophic cyphonautes larva is basal for ectoprocts and therefore it is difficult to interpret these results phylogenetically. Interestingly, in the mid-gastrula stage of *Phoronopsis harmeri* four serotonin-lir flask-shaped cells differentiate simultaneously in the apical plate and subsequently increase in number (Temereva and Wanninger, 2012). The synchronous formation of four flask-shaped cells in the early development of the actinotroch larva resembles a typical simple apical organ as found in most spiralian larvae. Thus, the complex apical organ of the actinotroch larva may be interpreted as a secondary condition, corroborating the notion that the LCA of the lophotrochozoans had a simple larval apical organ with a few serotonin-lir cells (Wanninger, 2008, 2009).

Many lophotrochozoans such as annelids, echiurans, sipunculids, molluscs, entoprocts and platyhelminths, possess a ventral nerve cord (Kristof et al., 2008; Wanninger, 2009). Even in juvenile brachiopods two ventral neurite bundles with three commissures are present

(Altenburger and Wanninger, 2010). Hitherto, *Phoronopsis harmeri* is the only actinotroch larva known to have a ventral neurite bundle during early larval development. This serotonin-lir ventral neurite bundle disappears in the 6 primordial tentacle stage and is lost in subsequent stages (Table 1; Temereva and Wanninger, 2012). On the contrary, the FMRFamide-lir ventral neurite bundle is, at least, expressed until the 6 tentacle stage (Table 2; Temereva and Wanninger, 2012). In this work no ventral neurite bundle was found, neither a serotonin-lir nor a FMRFamide-lir one (Table 1, 2). Due to the documented loss of the ventral neurite bundle in *Phoronopsis harmeri*, it could be assumed that a ventral neurite bundle is only present in the very early developmental stages of phoronids and therefore was not detectable in this study. Neither in competent larva of *Phoronis pallida* (Santagata, 2002) nor in 0-6 tentacles developmental stages of *Phoronis vancouverensis* (Hay-Schmidt, 1990b) a ventral neurite bundle is present. It remains unknown whether or not a ventral neurite bundle is present in other phoronid species since only few developmental data on early phoronid neurogenesis are available. Nevertheless, the occurrence of a paired ventral nerve cord is very common in lophotrochozoans and considered basal for protostomes (Wanninger, 2009). Despite the absence of a ventral nerve cord in adult phoronids (Emig, 1979), the presence of a ventral neurite bundle during early development of *Phoronopsis harmeri* indicates that such a neural feature was also present in the LCA of Phoronida.

Serotonin-lir nerves that underlie ciliary bands are common in lophotrochozoan larvae. They are known from polyplacophoran and gastropod molluscs, polychaete annelids and nemerteans as well as from platyhelminths, entoprocts and ectoprocts (Hay-Schmidt, 1990c, 2000; Friedrich et al., 2002; Page 2002; Voronezhskaya et al., 2002; Voronezhskaya et al., 2003, Nielsen, 2005; Rawlinson, 2010). Accordingly, serotonin-lir nerves underlying the ciliary bands are considered as basal for lophotrochozoan larvae, although this feature may be secondarily reduced in certain taxa such as in larvae of sipunculids, echiurans and cycliophorans (Hessling and Westheide, 2002; Wanninger, 2005; Wanninger et al., 2005b).

The actinotroch larvae of phoronids have a preoral ciliated band that is situated along the edge of the preoral hood and a postoral ciliated band situated along the tentacles and a posterior ciliated band, the so-called telotroch (Nielsen, 1987). In *Phoronis muelleri* each of these ciliated bands are innervated by serotonin-lir and FMRFamide-lir neurite bundles. This supports the view that this condition is considered basal and that the LCA of the lophotrochozoans already had serotonin-lir and probably also FMRFamide-lir nerves underlying the ciliated bands (Wanninger, 2008; Wanninger, 2009).

Conclusion

This study demonstrates the presence of serotonin-lir, FMRFamide-lir as well as small cardioactive peptide-lir components in the larval nervous system of *Phoronis muelleri*. The complex apical organ of *Phoronis muelleri* is described in detail for each mentioned neurotransmitter. It supports the scenario that these immunoreactive substances have already been present in the groundplan of the Phoronida as well as in the LCA of Lophotrochozoa. In addition, this study provides evidence that each ciliary band of *Phoronis muelleri* is underlined by serotonin-lir, FMRFamide-lir and small cardioactive peptide-lir neurite bundles. This indicates that innervated ciliary bands have already been present in the groundplan of the Phoronida.

Moreover, this study provides the first description of the small cardioactive peptide-lir nervous system in a larval phoronid. The widespread distribution of this neuropeptide throughout the protostomians suggests that this peptide was part of the protostomian groundplan. More developmental data on the neuroanatomy of various species of Phoronida are needed in order to assess the precise phoronid neural ground pattern.

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Figure Legends

Figure 1:

Main gross anatomical features of the actinotroch larva of *Phoronis muelleri*. Lateral view of a larva with apical to the top. Scale bars equal 100 µm. (A) Differential interference contrast image of a larva showing the preoral lobe (pl), apical plate (ap), mesosome (ms), tentacles (t) with tentacular cilia (tc), the trunk (tr), telotroch (tt) and the cilia of the telotroch (ctt). (B) Nuclear staining (DAPI) of the same larva.

Figure 2:

Organization of the serotonin-lir nervous system at the 12-16 tentacle stage of the actinotroch larva of *Phoronis muelleri*. A-C: Maximum projections of double stainings for serotonin-lir (graded shades of red through white) and DAPI (blue). D is a 3D reconstruction of the dataset shown in C. E is a snapshot of an apical organ (A) Overview of the serotonin-lir nervous system of the actinotroch larva showing the apical organ (ao), the neurites in the preoral lobe (hn), the tentacle neurite bundle (tn), the lateral part of the tentacle neurite bundle (tnl) which runs along the mesosome and sends a frontal process into each tentacle, as well as neurites in the trunk (trn) which connect the tentacular neurite bundle to the neurites of the telotroch (nt). In addition, a few perikarya along the margin of the preoral lobe (mp) as well as numerous perikarya along the tentacle ridge (ptr) and in the frontal side of each tentacle have formed. Apical is to the top and scale bar equals 100 µm. (B) Detail of the tentacles with the perikarya along the tentacle ridge (ptr) continuing in the frontal side of each tentacle. The tentacular neurite bundle (tn) runs under the tentacles and sends two lateral abfrontal processes (lap) into each tentacle, while the lateral part of the tentacular neurite bundle runs along the mesosome and sends a median frontal process (mfp) into each of them. Scale bar equals 50 µm. (C) Detail of the apical organ showing the neuropil (np), which has increased in size, and about

22 flask-shaped cells (fc), as well as a few bi-or multipolar perikarya (asterisks). Each flask-shaped cell bears a cilium (sc). The tentacular neurite bundle (tn) projects in posterior direction. (a) anterior, (p) posterior. Anterior is to the top. Scale bar equals 25 µm. (D) 3D reconstruction of the dataset shown in C showing the flask-like shape of the cells. Scale bar equals 25 µm. (E) Snapshot approximately in the median of the apical organ showing one flask-shaped cell (fc) as well as some neurites (n) of the neuropil and the bi-or multipolar perikarya (asterisks) which lie under the neuropil and are also arranged in a U-shape. Lateral view with anterior to the right. Scale bar equals 25 µm.

Figure 3:

Line drawings of the neuronal components of the tentacles in A-C and the apical organ in D-F in the actinotroch larva of *Phoronis muelleri* for all analysed neurotransmitters. Scale bars equal 50 µm in A-C and 25 µm in D-E. (A) Serotonin-lir innervation of the tentacles. The tentacular neurite bundle (tn) projects posteriorly from the apical organ (ao), runs under the tentacles and sends two lateral abfrontal processes into each tentacle. The lateral portion of the tentacular neurite bundle (tnl) sends neurites along the mesosome and a median frontal process into each tentacle. Perikarya are visible along the tentacle ridge and in each tentacle on the frontal side. (B) FMRFamide-lir innervation of the tentacles showing a median abfrontal process which is formed by the tentacular neurite bundle (tn). In addition, the tentacles are innervated by two lateral frontal processes which are connected to each other (arrowheads) at the tentacle basis. (C) Small cardioactive peptide-lir innervation of the tentacles showing two lateral frontal processes in each tentacle. (D) The serotonin-lir portion of the apical organ shows the U-shape, the flask-shaped cells, each bearing a cilium and projecting into the neuropil (np), and the bi-or multipolar cells underlying the neuropil. The median neurite bundle of the preoral lobe (men) projects in anterior direction, the tentacular neurite bundle (tn) in posterior direction. (E) FMRFamide-lir portion of the apical organ

showing the U-shaped neuropil (np) and dorso-lateral perikarya on each side. The median neurite bundle of the preoral lobe (men) projects in anterior direction, the tentacular neurite bundle (tn) in posterior direction. (F) The small cardioactive peptide-lir apical organ showing the U-shaped neuropil (np) as well as dorso-lateral perikarya on each side.

Figure 4:

Line drawings of both investigated stages of the serotonin-lir (A, B) and the FMRFamide-lir (C, D) nervous system in the actinotroch larva of *Phoronis muelleri*. Apical is to the top in all aspects. Lateral right views, scale bars equal 100 µm in A and C, and 200 µm in B and D. (A) Serotonin-lir nervous system of the larva at the 12-16 tentacle stage showing the apical organ (ao), the median neurite bundle of the preoral lobe (men), the tentacular neurite bundle (tn) which projects in posterior direction and runs under the tentacles, as well as several fine neurites in the entire preoral lobe (hn) and the marginal neurite bundle of the preoral lobe (mn) with several associated perikarya (mp). The lateral part of the tentacular neurite bundle (tnl) projects more laterally, spreads over the mesosome (msn) and sends a median frontal process (mfp) into each tentacle, while the tentacular neurite bundle sends two lateral abfrontal processes (lap) into each of them. Numerous perikarya (ptr) are visible along the tentacle ridge and in each tentacle on the frontal side. Several neurites are situated in the trunk region (trn). The telotroch is innervated by two ring-shaped neurite bundles (nt). (B) Serotonin-lir nervous system of the larva with 18 and more tentacles showing the same structures as in A. The frontal organ (fo) is visible. (C) FMRFamide-lir nervous system of the larva at the 12-16 tentacle stage showing the apical organ (ao), several fine neurites (hn) along the entire preoral lobe and the marginal neurite bundle (mn). Several perikarya (hp) in the preoral lobe as well as perikarya (mp) along the marginal neurite bundle are present. Anteriorly to the apical organ the median neurite bundle (men) is visible. The tentacular neurite bundle (tn), which projects in posterior direction, runs under the tentacles and sends a

median abfrontal process (map) into each of them. The lateral part of the tentacular neurite bundle (tnl) spreads over the mesosome (msn) and sends two lateral frontal processes (lfp) into each tentacle. In addition, several perikarya (cp) are situated ventral in the entire mesosome. Several fine neurites (trn) are located in the trunk. The telotroch is innervated by two ring-shaped neurite bundles (nt). Several perikarya are visible in the telotroch region (tp). (D) FMRFamide-lir nervous system of the larva with 18 and more tentacles showing similar structures as described in C. The perikarya of the telotroch have disappeared.

Figure 5:

Organization of the FMRFamide-lir nervous system at the 12-16 tentacle stage of the actinotroch larva of *Phoronis muelleri*. Maximum projections of triple stainings for FMRFamide-lir (graded shades of red through white), acetylated α-tubulin (green) and DAPI (blue). (A) Overview of the larva showing the triple staining with the apical organ (ao), the neurites along the entire preoral lobe (hn), the marginal neurite bundle of the preoral lobe (mn), the median neurite bundle of the preoral lobe (men), neurites along the mesosome (msn), as well as the perikarya in the mesosome (cp) and the innervated tentacles (t). In addition, perikarya of the telotroch region (tp) as well as two ring-shaped neurite bundles (nt) of the telotroch (tt) and the cilia of the telotroch (ctt) are visible. Apical to the top, scale bar equals 100 µm. (B) Detail of the mesosome region shown in A. The bi-or multipolar perikarya (cp) are situated median in the mesosome. Neurites are spread over the entire mesosome (msn). The tentacles are innervated by two lateral frontal processes (lfp), which show some commissures (arrowheads) at the tentacle base, as well as a fine median abfrontal process (map). Several fine neurites are located in the trunk (trn). Scale bar equals 50 µm. (C) Detail of the apical organ showing the neuropil (np) and dorso-lateral perikarya (dlp) on both sides. Neurites (hn) are located along the preoral lobe. (a) anterior, (p) posterior. Anterior is to the bottom. Scale bar equals 25 µm. (D) Detail of the telotroch showing the two ring-shaped

neurite bundles (nt) as well as some interconnecting neurites (arrow). The perikarya of the telotroch (tp) are associated with the ring-shaped neurite bundles of the telotroch. The neurites in the trunk (trn) have increased in number. Scale bar equals 25 µm. (E) Detail of the telotroch with associated perikarya (tp). They show a granular appearance and some of them are associated with one of the ring-shaped neurite bundles (nt). Scale bar equals 15 µm.

Figure 6:

Organization of the serotonin-lir nervous system at the 18 and more tentacle stage of the actinotroch larva. Maximum projections of triple stainings for serotonin-lir (graded shades of red through white), acetylated α-tubulin (green) and DAPI (blue). (A) Overview of the serotonin-lir nervous system of the actinotroch larva showing the apical organ (ao), the median neurite bundle of the preoral lobe (men), the marginal neurite bundle of the preoral lobe (mn) with associated perikarya (mp), the tentacular neurite bundle (tn) which runs in posterior direction, the lateral part of the tentacular neurite bundle (tnl) which runs towards the mesosome. In addition, the tentacles (t) are innervated by processes (lap), and the telotroch is innervated by two ring-shaped neurite bundles (nt). The cilia of the telotroch (ctt) are visible. Apical is to the top, scale bar equals 200 µm. (B) Detail of a tentacle showing the tentacular neurite bundle (tn) which runs under the tentacles and sends two lateral abfrontal processes (lap) into each of them. Neurites of the mesosome (msn) send a median frontal process (mfp) into each tentacle. Perikarya of the tentacle ridge and in the frontal side of the tentacle (ptr) are visible. Scale bar equals 25 µm. (C) Lateral view of the apical organ shown in A. Anterior to the apical organ, the frontal organ (fo) is visible, both are interconnected by the median neurite bundle of the preoral lobe (men). The apical organ consists of about 37 flask-shaped cells (fc) that are arranged in a U-shape. Each flask-shaped cell bears a serotonin-lir cilium (sc). Bi-or multipolar perikarya (asterisks) are situated underneath. (a) anterior, (p) posterior. Scale bar equals 25 µm. (D) Dorsal view of the apical organ showing

the flask-shaped cells (fc) which are situated in a U-shape surrounding and projecting into the neuropil (np). The bi-or multipolar perikarya (asterisks) are situated under the neuropil and also in a U-shape. The flask-shaped cells appear roundish due to the dorsal view. The median neurite bundle of the preoral lobe (men) projects in posterior direction. (a) anterior, (p) posterior. Scale bar equals 25 µm. (E) Detail of the posterior part of the apical organ showing the flask-shaped cells (fc) with the serotonin-lir cilia (sc). Thickenings (arrowheads) are located along the processes they send into the neuropil. Underneath the neuropil, the cell mass of bi-or multipolar perikarya (asterisks) is situated. Lateral view, scale bar equals 15 µm.

Figure 7:

Line drawings of the distribution of the tested neurotransmitter (A-B: serotonin-lir; C-D: FMRFamide-lir; E-F: small cardioactive peptide-lir) in the 18 and more tentacle stage in the larva of *Phoronis muelleri*. Lateral view in A, C, E, dorsal view in B, D, F. Scale bars equal 200 µm. (A) Serotonin-lir nervous system of the larva showing the apical organ (ao), the frontal organ (fo), the median neurite bundle (men) which projects in anterior direction towards the margin of the preoral lobe, the marginal neurite bundle (mn) with several associated perikarya (mp) and several neurites (hn) along the entire preoral lobe. The tentacular neurite bundle (tn) projects in posterior direction and sends two lateral processes into the abfrontal side (lap) of each tentacle. The lateral part of the tentacular neurite bundle (tnl) spreads over the mesosome (msn) and sends a median frontal process (mfp) into each tentacle. Numerous perikarya are located along the tentacular ridge and in each tentacle on the frontal side (ptr). Several neurites are situated in the trunk (trn) and the telotroch is innervated by two ring-shaped neurite bundles (nt). (B) Dorsal view of the line drawing shown in A. (C) FMRFamide-lir nervous system of the larva showing the apical organ (ao), the median neurite bundle (men), which runs towards the margin of the preoral lobe, the marginal neurite bundle (mn) with several associated perikarya (mp), as well as neurites (hn) and perikarya (hp)

spread over the entire preoral lobe. The tentacular neurite bundle (tn) projects in posterior direction and sends a median abfrontal process (map) into each tentacle. The lateral part of the tentacular neurite bundle (tnl) sends several neurites towards the mesosome (msn) and sends two lateral processes into the frontal side (lfp) of each tentacle. Several bi-or multipolar perikarya (cp) are located in the mesosome. The trunk is innervated by neurites (trn) and the telotroch by two ring-shaped neurite bundles (nt). (D) Dorsal view of the line drawing shown in C. (E) Small cardioactive peptide-lir nervous system of the larva showing the apical organ (ao), a few neurites in the preoral hood (hn), the marginal neurite bundle (mn), neurites along the mesosome (msn), as well as two lateral frontal processes (lfp) in each tentacle. A few fine neurites (trn) are located in the trunk. The telotroch is innervated by two ring-shaped neurite bundles (nt). (F) Dorsal view of the line drawing shown in E.

Figure 8:

Organization of the FMRFamide-lir nervous system at the 18 and more tentacle stage of the actinotroch larva of *Phoronis muelleri*. Maximum projections of double stainings for FMRF-amide (graded shades of red through white) and DAPI (blue). (A) Overview of the FMRFamide-lir nervous system of the actinotroch larva showing the apical organ (ao), the median neurite bundle of the preoral lobe (men), the perikarya along the mesosome (cp), the tentacles (t) which are innervated by the tentacle processes (lfp, map) as well as the two ring-shaped neurite bundles of the telotroch (nt). Ventral view, scale bar equals 200 µm. (B) Detail of the telotroch showing the two ring-shaped neurite bundles (nt) as well as several neurites in the trunk (trn). Scale bar equals 70 µm. (C) Detail of a tentacle shown in A, which is innervated by two lateral frontal processes (lfp) and a median abfrontal process (map). At the tip of the tentacle these processes are interconnected (arrowheads). (a) anterior, (af) abfrontal, (f) frontal, (p) posterior. Lateral view, frontal is to the left. Scale bar equals 25 µm. (D) Detail of the apical organ shown in A. The neuropil (np) and dorso-lateral perikarya (dlp) are visible.

The median neurite bundle of the preoral lobe (men) projects in anterior direction. (a) anterior, (p) posterior. Dorso-lateral view, scale bar equals 50 µm.

Figure 9:

Organization of the small cardioactive peptide-lir nervous system at the 18 and more tentacle stage of the actinotroch larva of *Phoronis muelleri*. Maximum projections of double stainings for small cardioactive peptide-lir (graded shades of red through white) and DAPI (blue). (A) Overview of the small cardioactive peptide-lir nervous system showing the apical organ (ao), the marginal neurite bundle of the preoral lobe (mn), the lateral frontal processes (lfp) of the tentacles (t) as well as the telotroch (tt) with its ring-shaped neurite bundles (nt). Lateral view, scale bar equals 200 µm. (B) Detail of the preoral lobe showing a lateral view of the apical organ with the neuropil (np) and a few dorso-lateral perikarya (arrowheads) on both sides. In addition, the marginal neurite bundle (mn), which runs along the edge of the preoral lobe, as well as several fine neurites in the preoral lobe (double arrowheads) are visible. Lateral view, scale bar equals 50 µm. (C) Detail of the telotroch showing the two ring-shaped neurite bundles (nt) which connect to fine neurites of the trunk (arrows). Scale bar equals 50 µm. (D) Detail of the tentacle ridge region showing two lateral processes (lfp) which project into each tentacle on the frontal side. The preoral lobe with fine neurites (double arrowheads) and the marginal neurite bundle (mn) are visible. Lateral view, scale bar equals 50 µm.

Table 1: Summary of data currently available on the serotonin-lir nervous system of the actinotroch larva. Data are taken from Hay-Schmidt (1990) (*Phoronis muelleri, Phoronis vancouverensis*), Santagata (2002) (*Phoronis pallida*), Temereva and Wanninger (2012) (*Phoronopsis harmeri*) and the study herein (*Phoronis muelleri*). N/A, not applicable; +, present; -, not present; ?, unclear.

		***Phoronis muelleri* (Hay-Schmidt, 1990)**	***Phoronis vancouverensis* (Hay-Schmidt, 1990)**			***Phoronis pallida* (Santagata, 2002)**	***Phoronopsis harmeri* (Temereva and Wanninger, 2012)**			***Phoronis muelleri* (this study)**	
		10-28 tentacles	0-2 tentacles	2-4 tentacles	4-6 tentacles	10 tentacles	0 tentacles (6 days)	6 primordial tentacles (13 days)	6 tentacles	12-16 tentacles	18 and more tentacles
Serotonin	**apical organ**										
	U-shape	+	+	+	+	+	+	+	+	+	+
	flask-shaped cells	+	?	?	spindle-shaped	+	+	+	+	+	+
	other cell type (bi-multipolar cells)	+	-	-	+	+	+	+	+	+	+
	neuropil	+	+	+	+	+	+	+	+	+	+
	additional cells near AO	-	+	+	-	?	2	?	?	-	-

Serotonin		anterior median neurite bundle	+	?	?	?	+	-	-	+	+	+
		dorso-lateral neurite bundle	+	+	+	+	+	+	+	+	+	+
	preoral lobe											
		oral nerve ring	?	?	?	?	?	+	+	+	-	-
		perikarya in epistome	+	-	-	-	?	-	-	-	-	- (exception: frontal organ)
		hood margin neurite bundle	-	-	+	+	+	+	weak staining	+	+	+
		perikarya along margin neurite bundle	+	-	-	-	?	+	-	+	+	+
	mesosome											
		neurites in mesosome	+	-	+	+	+	vnc	-	-	+	+
		perikarya in mesosome	-	?	?	?	-	vnc	?	-	-	-

Serotonin	**tentacle**										
	frontal	?	?	?	?	-	N/A	-	-	1	1
	abfrontal	3	?	?	?	3		+	+	2	2
	perikarya along tentacle	-	?	?	?	-		-	-	+	+
	perikarya along tentacular ridge	-	?	?	?	-	N/A	-	-	+	+
	ventral neurite bundle	?	?	?	?	?	+	-	-	-	-
	perikarya along ventral neurite bundle	?	?	?	?	?	+	?	-	-	-
	tentacular neurite bundle	+	?	?	?	+	+	+	+	+	+
	metasome										
	neurites in trunk	+	?	?	?	+	N/A	N/A	+	+	+
	perikarya in trunk	+	?	?	?	+			-	-	-

	telotroch										
	perikarya	-	?	?	-	-	N/A	N/A	-	-	-
	neurites	+	?	?	+	+			+	+	+

Table 2: Summary of data currently available on the FMRFamide-lir nervous system of the actinotroch larva. Data are taken from Hay-Schmidt (1990) (*Phoronis muelleri*, *Phoronis vancouverensis*), Temereva and Wanninger (2012) (*Phoronopsis harmeri*) and the study herein (*Phoronis muelleri*). N/A, not applicable; +, present; -, not present; ?, unclear.

		***Phoronis muelleri* (Hay-Schmidt, 1990)**	***Phoronis vancouverensis* (Hay-Schmidt, 1990)**			***Phoronopsis harmeri* (Temereva and Wanninger, 2012)**			***Phoronis muelleri* (this study)**	
		10-28 tentacles	0-2 tentacles	2-4 tentacles	4-6 tentacles	0 tentacles (6 days)	6 primordial tentacles (13 days)	6 tentacles	12-16 tentacles	18 and more tentacles
FMRF-amide	**apical organ**			stage not investigated						
	U-shape	+	-		?	+	+	+	+	+
	monopolar cells	+	-		-	+	+	+	-	-
	other cell type (bi-multipolar cells)	+	+ (behind ao)		-	+	+	+	+	+
	neuropil	+	+		+	+	+	+	+	+
	anterior median neurite bundle	+	-		+	-	?	+	+	+
	dorso-lateral neurite bundle	only lateral	-		only lateral	+	+	+	+	+

FMRF-amide	**preoral lobe**			stage not investigated						
	oral nerve ring	?	?		?	+	+	+	-	-
	perikarya in epistome	+	-		-	+	?	+	+	+
	hood margin neurite bundle	+	+		+	+	+	+	+	+
	perikarya along margin neurite bundle	-	-		-	-	?	?	+	+
	mesosome									
	neurites in mesosome	+	+		+	+	+	+	+	+
	perikarya in mesosome	+	?		?	+	+	+	+	+
	tentacle									
	frontal	+	?		?	N/A	?	?	2	2
	abfrontal	-	?		?		?	?	1	1
	perikarya along tentacle	+	?		?		?	?	-	-
	perikarya along tentacular ridge	+	?		?		?	?	-	-

FMRF-amide	**ventral neurite bundle**	?	?	stage not investigated	?	+	+	+	-	-
	perikarya along ventral neurite bundle	?	?		?	+	+	+	-	-
	tentacular neurite bundle	-	-		-	+	+	+	+	+
	metasome									
	neurites in trunk	+	?		+	N/A	?	+	+	+
	perikarya in trunk	-	?		-		?	-	-	-
	telotroch									
	perikarya	?	?		-	N/A	?	-	+	-
	neurites	?	?		+		?	+	+	+

Table 3: Overview of the small cardioactive peptide-lir nervous system of the actinotroch larva. Data are taken from the study herein (*Phoronis muelleri*). +, present; -, not present; ?, unclear.

			Phoronis muelleri **(this study)**
			18 and more tentacles
Small Cardioactive Peptide	**apical organ**		
		U-shape	+
		monopolar cells	-
		other cell type (bi-multipolar cells)	+
		neuropil	+
		anterior median neurite bundle	-
		dorso-lateral neurite bundle	-
	preoral lobe		
		oral nerve ring	?
		perikarya in epistome	-
		hood margin neurite bundle	+
		perikarya along margin neurite bundle	-
	mesosome		
		neurites in mesosome	+
		perikarya in mesosome	-
	tentacle		
		frontal	+
		abfrontal	-
		perikarya along tentacle	-
		perikarya along tentacular ridge	-
	ventral neurite bundle		-
	perikarya along ventral neurite bundle		-
	tentacular neurite bundle		-

	metasome		
		neurites in trunk	+
		perikarya in trunk	-
	telotroch		
		perikarya	-
		neurites	+

Danksagung

An dieser Stelle möchte ich mich herzlichst bei Andreas Wanninger für die außerordentlich gute und stets verlässliche Betreuung während meiner Diplomarbeitszeit bedanken. Er war es der mir die Welt der Evertebraten näher gebracht hat und dem ich meine fachliche Entwicklung verdanke. Das Gefühl an der aktuellen Forschung teilzunehmen und einen relevanten Beitrag zu liefern ist ein weiterer Grund der die Betreuung durch Andi so unersetzlich machte.

Ein großes Dankeschön geht auch an die gesamte Abteilung der Integrativen Zoologie, ganz besonders an Thomas Schwaha, Tim Wollesen und Alen Kristof für die zahlreichen Unterstützungen, sowie an meine Kollegen Alex Kerbl, Ashwaq Batawi, Martin Moosbrugger, Barbara Schädl, Maik Scherholz und Melanie Schreiner. Es ist denkbar unvorstellbar sich an dieser Abteilung, mit diesen Leuten und deren Herzlichkeit nicht wohl zu fühlen.

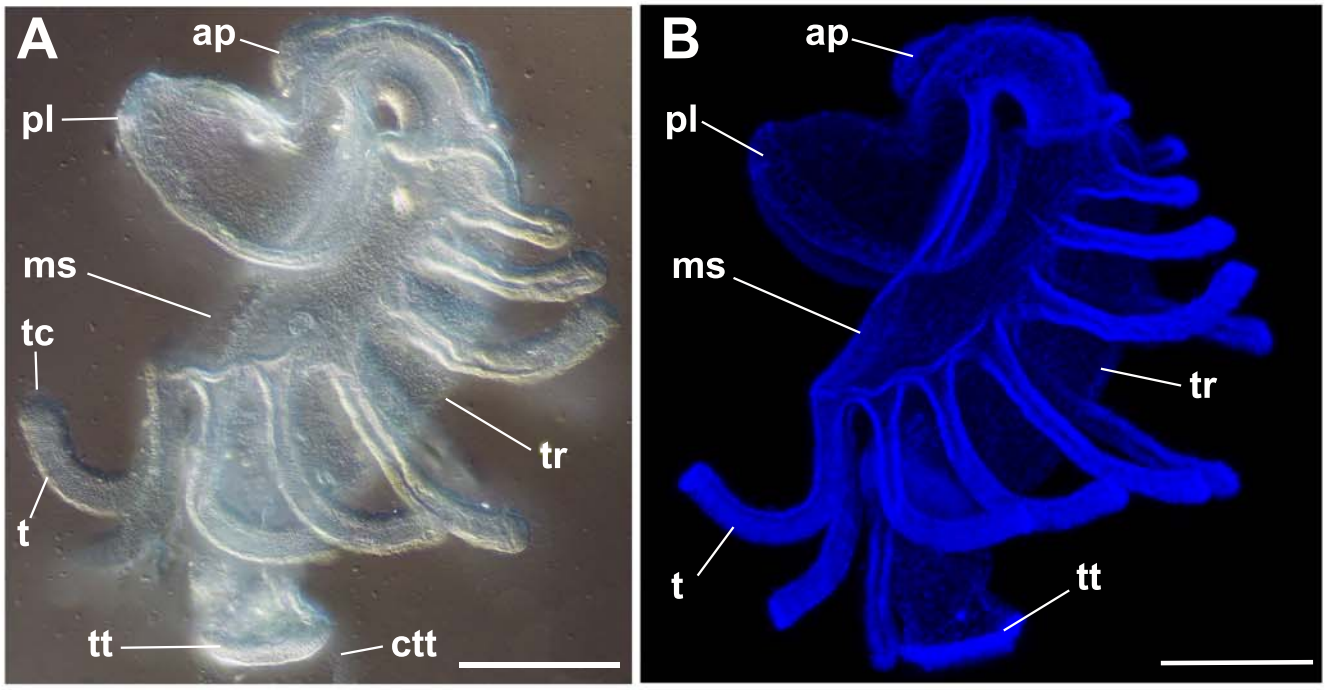
Weiters bedanke ich mich bei all meinen Freunden für das Verständnis und die guten Zeiten, ganz speziell bei Doris Bauer, Sonja Hochauer, Barbara Gratzer, Phil Kruspe und Marita Blasnig. Besonderer Dank geht an Sabine Hindinger, die mich durch all die Phasen des Studiums begleitete - ich sag nur „bello è impossibile“!

Und nicht zuletzt möchte ich Johannes Muhr und meiner Familie, insbesondere meinen Eltern für Unterstützung, Zuversicht, Geduld und Liebe danken.

Appendix

Figures

Figure 1



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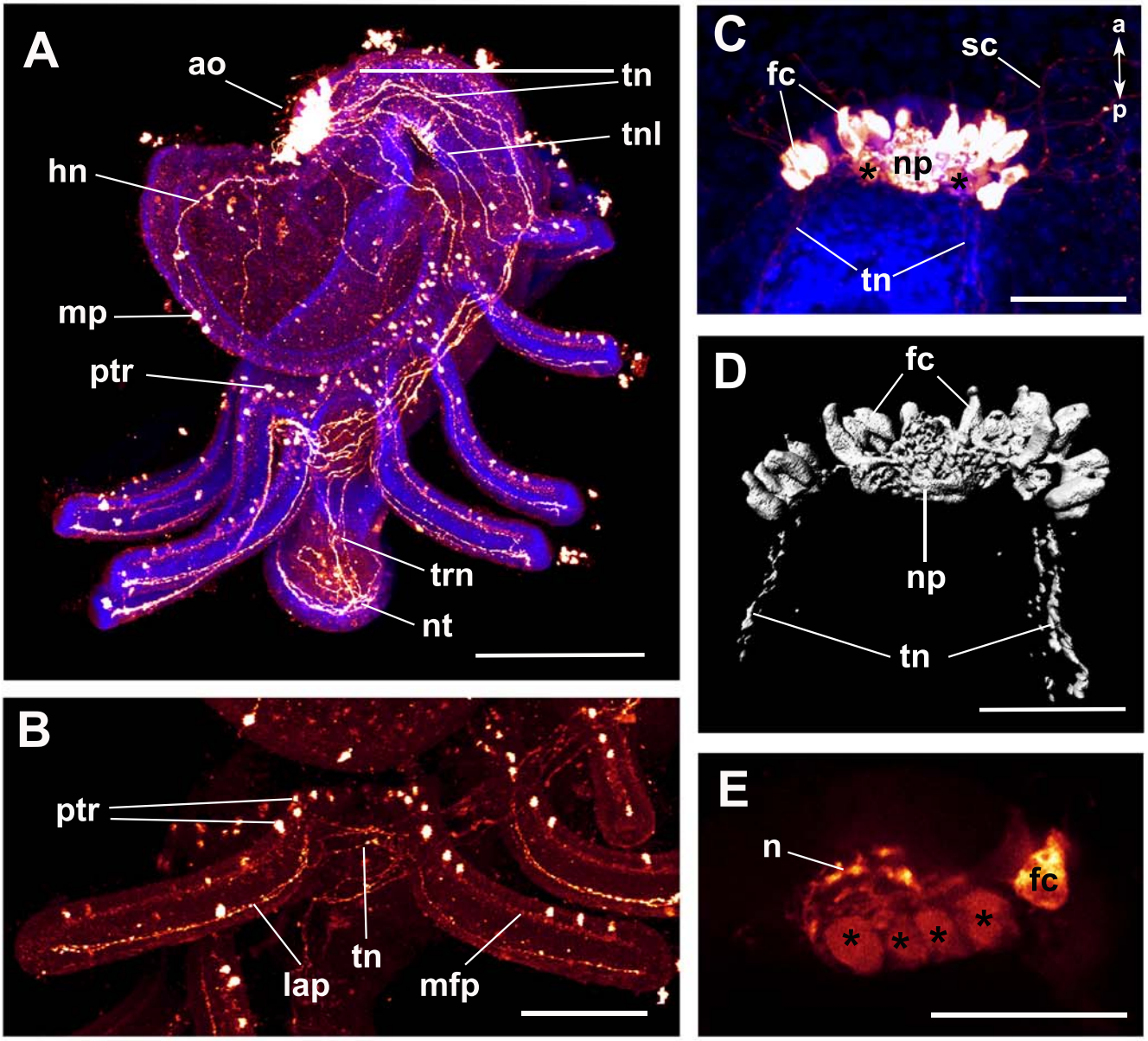
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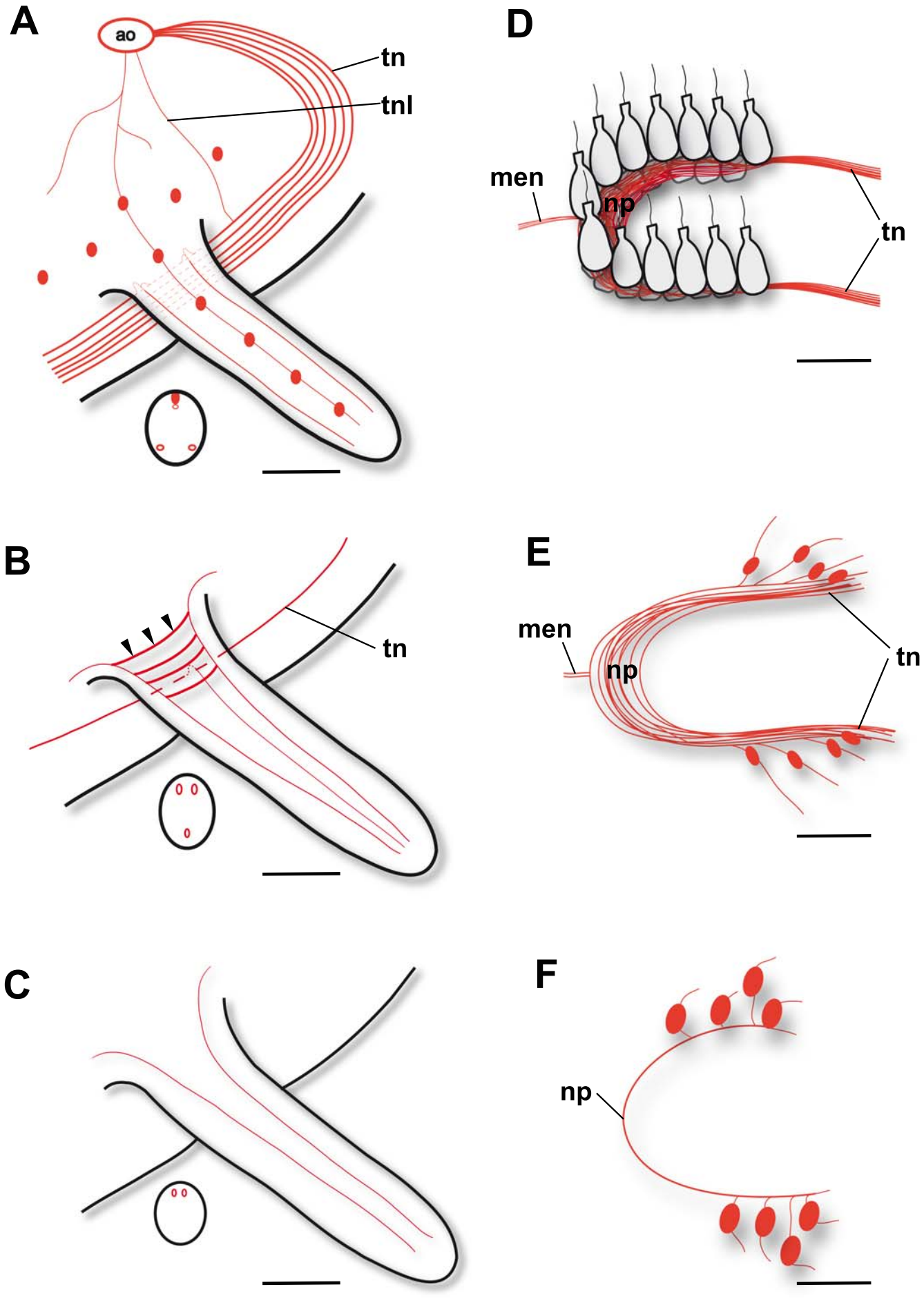
Figure 3



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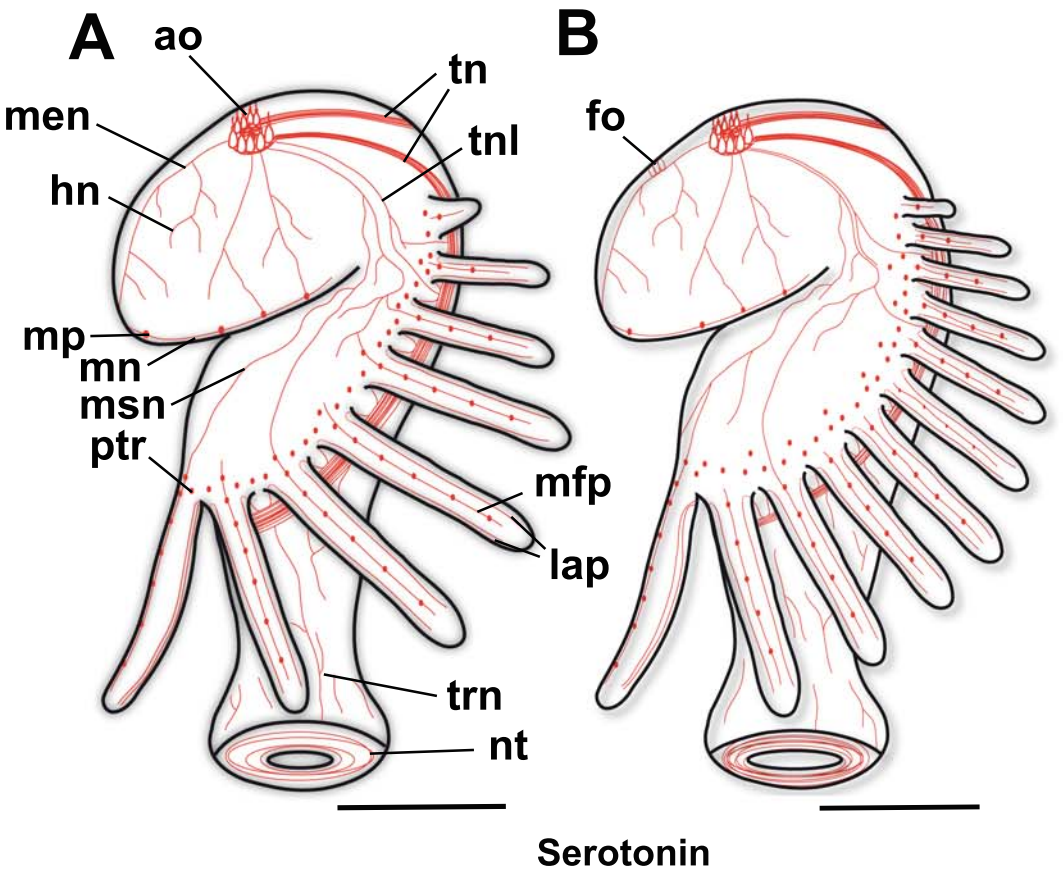
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Figure 5

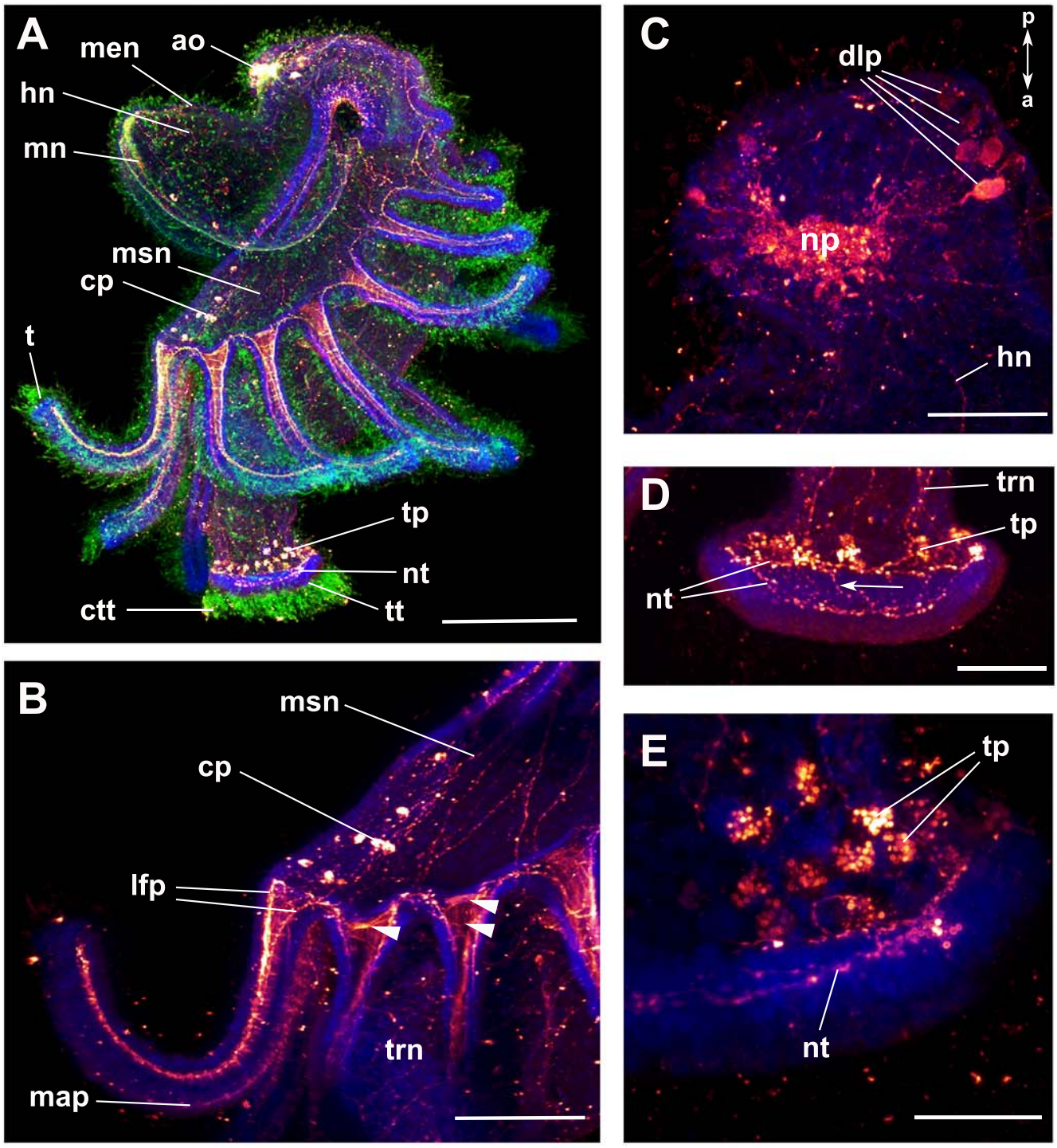
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Figure 6

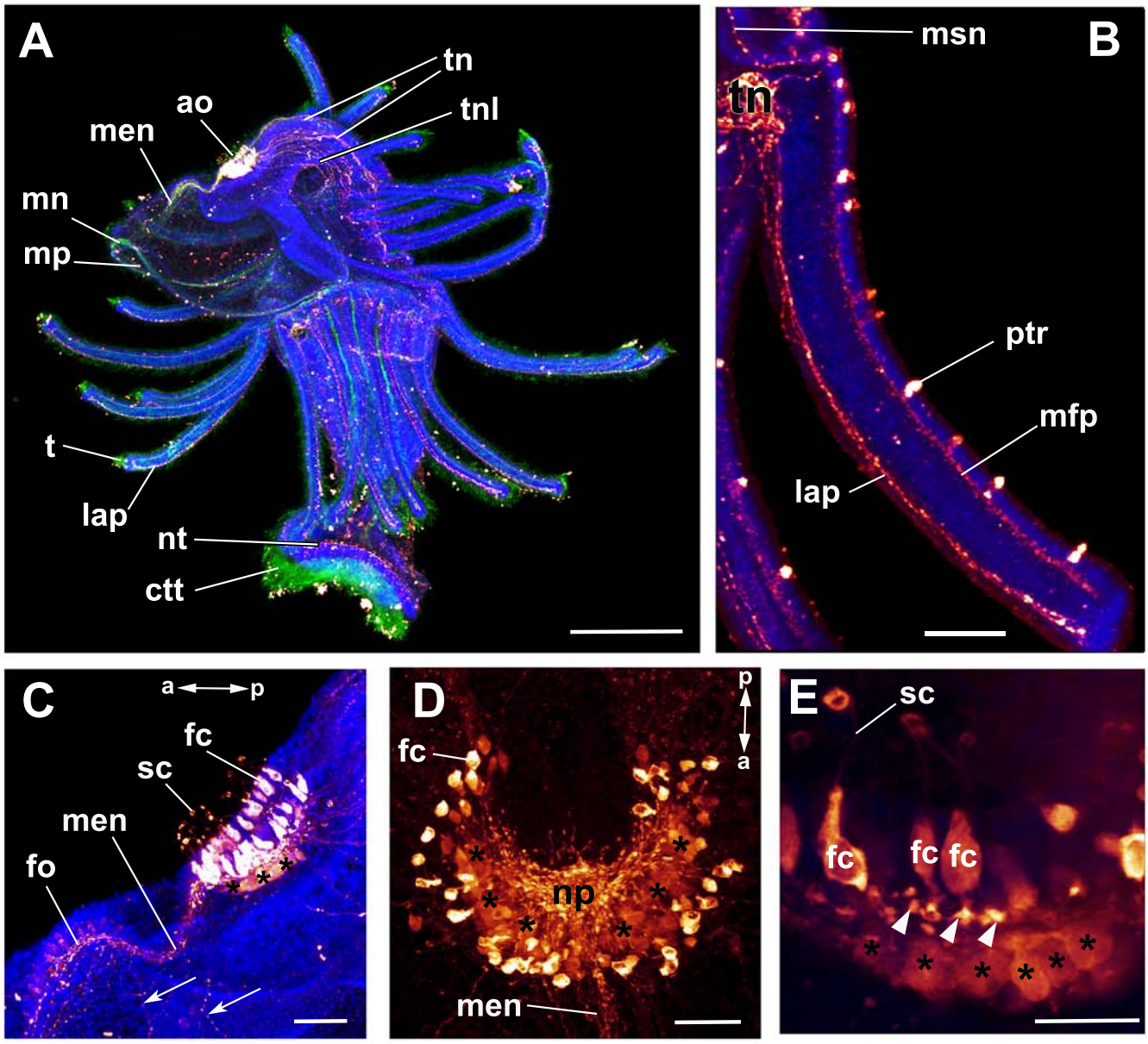
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Figure 7

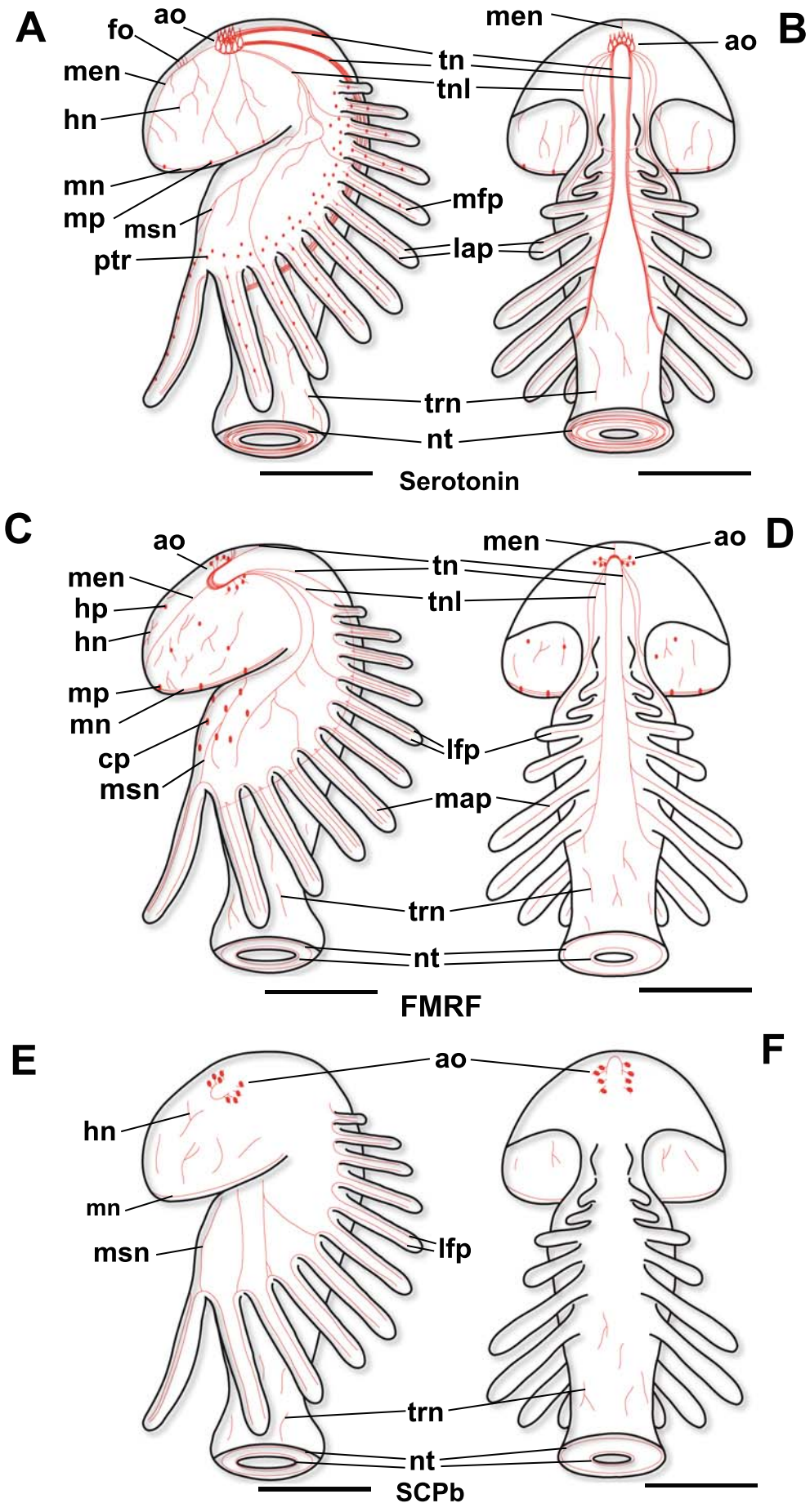
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Figure 8

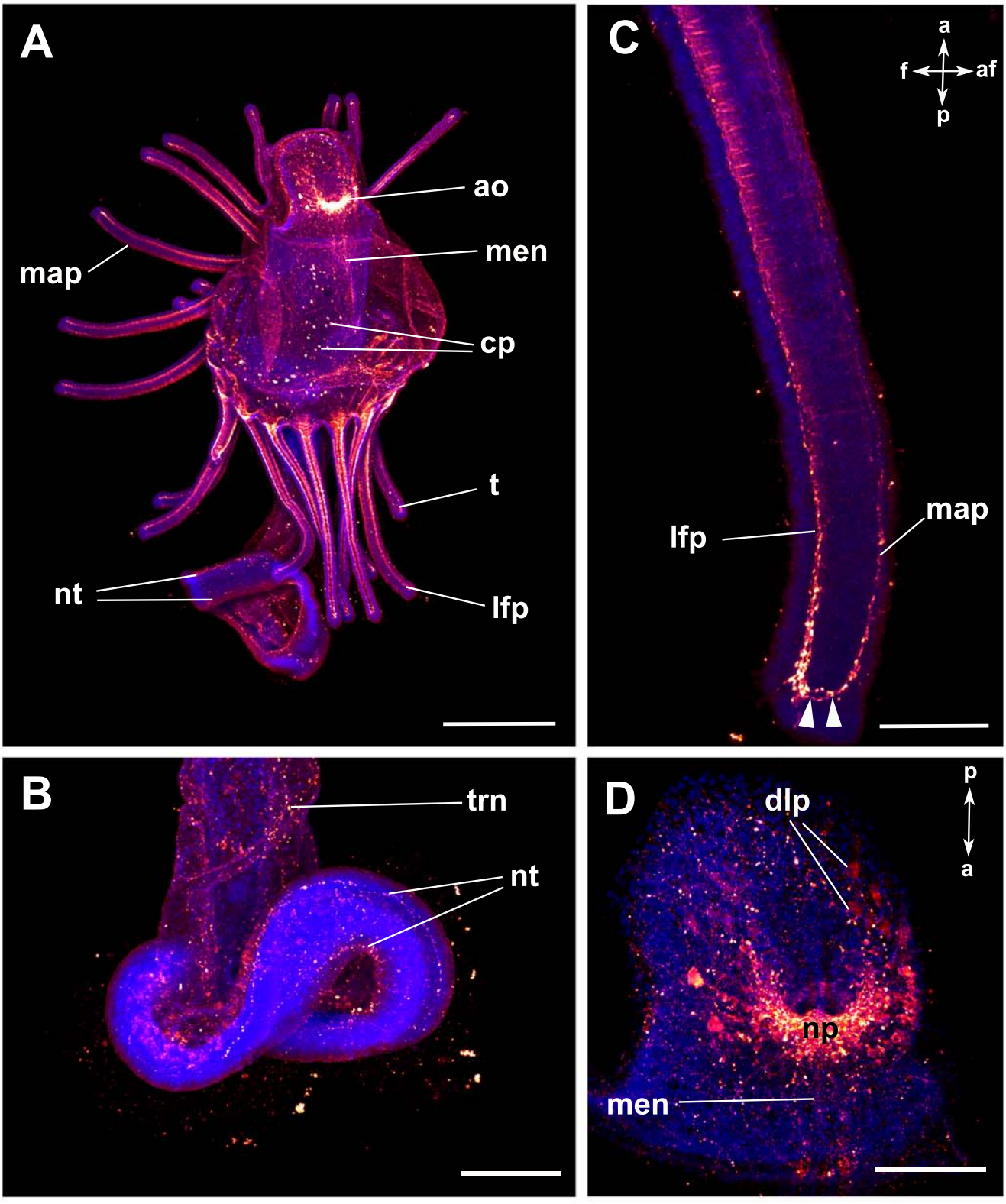
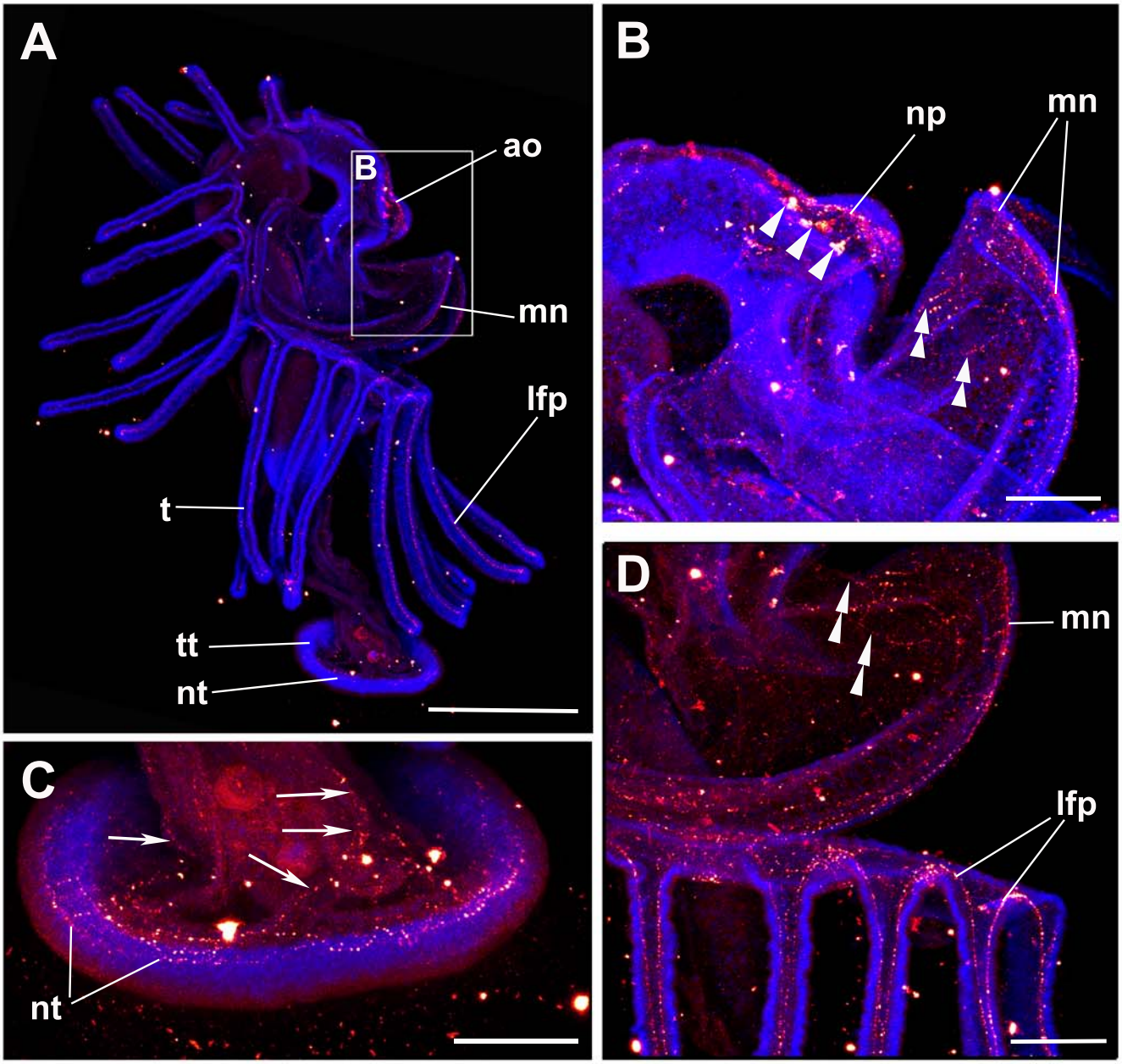


Figure 9



Supplementary Information

Curriculum vitae

Birgit Sonnleitner

Personal data

Birgit Sonnleitner

Date of birth: 19th September 1987

Nationality: Austrian

Education

since 2007	2. Diploma study of **Zoology** at the University Vienna, Austria
	(Diploma Thesis: "Analysis of the Nervous System of the Actinotroch Larva of *Phoronis muelleri* (Phoronida) based on Immunocytochemical Staining and Confocal Laser Scanning Microscopy")
2005 - 2007	Diploma study of **Biology** at the University Vienna, Austria
1997 - 2005	grammar school Wieselburg, Lower Austria

Professional Work Experience

February – May 2011	Internship at the **FIWI Vienna**, Vetmed Vienna ▪ animal monitoring during physiological experiments
September 2008	Internship at the **Zoo Schönbrunn**, Vienna ▪ animal monitoring and caring
July 2008	Internship at the Federal Agency for Water Economy; **Institute of Limnology and Fishery**, Scharfling, Upper Austria ▪ chemical analysis of water samples; inspection of fish stocks
Summer 2007	Internship at the Federal Agency for Water Economy; **Institute of Ground Water Budget**, Petzenkirchen, Lower Austria ▪ chemical analysis of soil samples

Teaching Experience

October - January 2011, 2010, 2009, 2008	**Tutor** for the "Baupläne der Tiere 1" at the University Vienna, Austria ▪ teaching and assisting in zoological practical courses

March – June 2012, 2011	**Tutor** for the "Baupläne der Tiere 2" at the University Vienna, Austria ▪ teaching and assisting in zoological practical courses
September 2009; May 2010, 2011	**Magistrate of Environmentalism** (Magistrate 22) in Vienna ▪ teaching of children (up to 11 years)

Experiences Abroad

February – July 2009	study at the University of Roma Tre, Erasmus outgoing
February 2007	field trip to Galapagos (in cooperation with University of Vienna)

Practical courses

28.08 - 08.09.2012 "Summer School on **Comparative and Functional Neuroanatomy and Neurobiology of Invertebrates** (White Sea Biological Station of Lomonosov Moscow State University)"

November 2010 – January 2011 „**Histological practical course**"

August 2009 – September 2009 "**Nature Protection** and Conservation of Marine Turtles *Caretta caretta*" (theoretical part in Vienna, 5 weeks of practical course in Turkey)

October 2009 – November 2009 "**Submicroscopic anatomy and preparation techniques**"

October 2008 - December 2008 "**Bioacoustics**"

March 2008- June 2008 "**Animal monitoring**"

Additional qualifications

Languages:

German: native language

English: fluently

Italian: basic knowledge

Advanced Education in Presentation Techniques

Methods:

- Staining Techniques:
  - Multi-labeling Immunostaining Techniques
  - Histological Staining Techniques
  - Tracer Injection Techniques (back-filling; visualization with fluorescent markers)

- Sectioning Techniques:
  - semi-thin sectioning
  - ultra-thin sectioning

- Microscopy:
  - Fluorescent and Confocal Laser Microscopy
  - Transmission Electron Microscopy
  - Scanning Electron Microscopy

- Neurophysiology Techniques:
  - registration of miniature end-plate potentials
  - analysis of regulation of calcium-dependent luminescence

EDV:

- Adobe Illustrator
- Adobe Photoshop
- 3D-Reconstruction with Imaris 7.3.1 (Bitplane, Switzerland)
- MS Office

- Apple Software

Additional Qualifications:

- Driving license (class B)

Scholarships

2010: Performance-based scholarship of the University Vienna

Hobbies

Independent Travel, Operas, Photography