universität wien

DIPLOMARBEIT

„Neurogenesis in *Lineus albocinctus* (Lophotrochozoa, Nemertea, Pilidiophora) as inferred by immunocytochemistry and confocal laserscanning microscopy“

Verfasserin

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angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag.rer.nat.)

Wien, Oktober 2012

Studienkennzahl lt. Studienblatt:	A 439
Studienrichtung lt. Studienblatt:	Diplomstudium Zoologie
Betreuerin / Betreuer:	Univ.-Prof. DDr. Andreas Wanninger

Für meine Eltern

die immer für mich da sind

Content

Abstract

Recently, findings on the neurogenesis of different members of Lophotrochozoa enabled the reconstruction of the nervous system of the last common ancestor of this very phylum. The data led to the suggestion that the nervous system of the last common ancestor of Lophotrochozoa composed of a serotonin-like immunoreactive nerve that underlies the larval prototroch, two or more ventral nerve cords as well as of a larval apical organ. This apical organ showed few to four flask-shaped serotonin-like immunoreactive cells and several associated FMRFamide-like immunoreactive cells concentrated in the apical region. In Nemertea only few data on the neurogenesis are currently available. Only in the Pilidiophora, which exhibit “indirect” development via a pilidium larva, and in the Hoplonemertea, which develop “directly” via a planuliform larva, the neurogenesis has been investigated so far. These sparse data sets do not unambiguously allow answering the question concerning the presence of a larval apical organ in Nemertea. In order to contribute data to this issue, immunocytochemical data on the neurogenesis of the pilidiophoran *Lineus albocinctus* are presented herein. In addition to findings on the apical organ structures, this work is the first detailed study on neurotransmitter distribution during neural development in the nemertean pilidium larva and juvenile based on immunocytochemical methods. Two serotonin-like immunoreactive neurons occur in the anterior part of the apical plate and send numerous processes into all four lobes, where they form a complex subepithelial nerve net. All four larval lobes are surrounded by a marginal neurite bundle, which is associated with numerous serotonin-like immunoreactive monociliated perikarya. A serotonin-like immunoreactive oral nerve ring encircles the stomach sphincter and is associated with few serotonin-like immunoreactive conical-shaped cells. Two suboral neurites descend from the oral nerve ring and merge with the marginal neurite bundle. Of all neural structures investigated only two larval neural components are incorporated into the juvenile nervous system: the oral nerve

ring and the two suboral neurites, while the apical neurons do not contribute to the juvenile nervous system. Additionally, a complex larval FMRFamide-like immunoreactive nervous system is described in detail for the first time for Nemertea. Interestingly, no FMRFamide-like immunoreactive structures are present within the larval apical region. Furthermore, this study provides the first data on the expression of a mollusc-specific VD1/RPD2 α-neuropeptide in nemertean larvae.

The data presented here differ in several ways from previous descriptions of *Lineus albocinctus*, such as the presence of two serotonin-like immunoreactive apical neurons and the presence of a complex FMRFamide-like immunoreactive nervous system. Together with previous findings on *Micrura alaskensis*, the results of the present study confirm the presence of serotonin-like immunoreactive structures in the apical region in pilidiophoran Nemertea and are discussed together with the evolution of the larval nervous system in Lophotrochozoa.

Zusammenfassung

In letzter Zeit haben zahlreiche Arbeiten zur Neurogenese bei den unterschiedlichsten Taxa der Lophotrochozoa zur Rekonstruktion des Nervensystems des Letzten gemeinsamen Vorfahren dieses Phylums beigetragen. Das Nervensystem des letzten gemeinsamen Vorfahren der Lophotrochozoa bestand wahrscheinlich aus einem serotonergen Nerv der den Prototroch innervierte, zwei oder mehreren ventralen Longitudinalnerven sowie einem larvalen Apikalorgan. Dieses Apikalorgan bestand aus einer Konzentration von wenigen bis zu vier flaschenförmigen, serotonergen und aus einigen assoziierten FMRFamidergen Zellen. Bei Nemertea gibt es bis dato nur wenige Arbeiten zur Neurogenese. Nur bei Pilidiophora, welche sich „indirekt“ via die Pilidiumlarve, und bei Hoplonemertea, welche „direkt“ via eine planuliforme Larve entwickeln, sind Daten zur Neurogenese verfügbar. Diese spärliche Datenlage ermöglicht derzeit keine vollständige oder zufriedenstellende Aufklärung der Existenz eines larvalen Apikalorgans bei Nemertea. Um diesen Umstand zu ändern und neue Daten zur Diskussion dieser Fragestellung beizutragen, wurden immunocytochemische Untersuchungen an *Lineus albocinctus* durchgeführt und im Weiteren diskutiert. Darüber hinaus zeigt diese Arbeit die ersten detaillierten Bilder der Neurotransmitterverteilung während der Neuronalentwicklung larvaler und juveniler Nemertea mithilfe immunocytochemischer Methoden. Zwei serotonerge Neuronen befinden sich im anterioren Teil der Apikalplatte und senden zahlreiche neuronale Zellfortsätze in alle vier larvalen Loben, wo sie ein komplexes subepitheliales Nervennetz bilden. Die vier Loben sind von einem marginalen Neuritenbündel umgeben, welches mit zahlreichen serotonergen, monociliären Perikarya assoziiert ist. Ein serotonerger oraler Nervenring umschließt den muskulären Magensphinkter und ist zusätzlich mit einigen serotonergen, konischen Zellen assoziiert. Zwei suborale Neuriten ziehen vom oralen Nervenring nach posterior und vereinigen sich in der Übergangszone der lateralen Loben und des posterioren Lobus mit dem

marginalen Neuritenbündel. Die einzigen hier gefundenen larvalen Neuronal-Strukturen die in das Nervensystem des juvenilen Tiers eingebaut werden, sind der orale Nervenring und die beiden suboralen Neuriten, während die nervösen Strukturen der Apikalplatte nicht zum Nervensystem des juvenilen Wurms beitragen.

In dieser Studie wird erstmalig das komplexe larvale FMRFamiderge Nervensystem in größtem Detail beschrieben. Interessanterweise wurden keine FMRFamidergen Strukturen im Bereich der larvalen Apikalplatte gefunden. Des Weiteren wird in dieser Arbeit der erste positive Nachweis für die Expression eines Mollusken-spezifischen VD1/RPD2 α-Neuropeptids bei larvalen Nemertea erbracht. Die Ergebnisse zur Präsenz zweier serotonerger apikaler Neurone, sowie der Nachweis eines komplexen FMRFamidergen Nervensystems unterscheiden sich in fundamentaler Weise von früheren Studien an *Lineus albocinctus*. Zusammen mit den Ergebnissen zur Neurogenese bei *Micrura alaskensis* konnte nun die Präsenz zweier serotonerger Neurone im Bereich der Apikalplatte bei pilidiophoren Nemertinen bestätigt werden. Im Weiteren wird die Bedeutung dieser serotonergen Neurone bei pilidiophoren Nemertea in Bezug auf die Evolution larvaler Nervensystemen von Lophotrochozoa diskutiert.

Introduction

Nemertean development, morphology and proposed phylogenetic affinities

Adult Nemertea, also known as ribbon worms, are benthic predators and are found mostly in marine, but also in freshwater and terrestrial habitats (Turbeville 2007, von Döhren et al. 2011). They are bilaterally symmetric, unsegmented and dorsoventrally flattened. One apomorphic character for this phylum is the eversible and sometimes stylet-bearing epidermal proboscis (Stricker & Cloney 1981, Turbeville 2007). It lies within a fluid filled cavity of mesodermal origin, the so-called rhynchocoel. Unlike in other protostome invertebrates, the nemertean brain encircles the proboscis and the two frontal blood vessels, but not the foregut (Turbeville 2007, Maslakova & von Döhren 2009, Nielsen 2012).

Traditional phylogenies based on nemertean adult morphology divide the phylum into two sub-taxa: the Anopla and Enopla (Fig. 1A, Stiasny-Wijnhoff 1923). The latter clade is characterized by a single opening in the worm's anterior region that unites the digestive tract and the rhynchodaeum. Furthermore, Enopla possess an armed proboscis equipped with a single or numerous stylets. Former phylogenies suggest the monophyletic clade of Enopla to comprise the two orders Hoplonemertea and Bdellonemertea (Stiasny-Wijnhoff 1923). Recently, molecular data claim that the order Bdellonemertea resembles a specialized monostiliferous group within the Hoplonemertea, which renders Hoplonemertea a synonym for the entire Enopla (Fig. 1B, Thollesson & Norenburg 2003). Another characteristic of the Hoplonemertea is their development via a lecitotrophic (non-feeding) planuliform larva (Norenburg & Stricker 2002).

In contrast to the Hoplonemertea, members of the sub-taxa Anopla have the mouth opening separated from the rhynchodaeum (Stiasny-Wijnhoff 1923). An additional apomorphic character of the Anopla is the paired cerebral organ, which resembles a neuroglandular complex (Ling 1970). These cerebral organs lie within the anterior region, in close distance to

the brain. Due to differences in the body wall musculature across the different species of Anopla they can be divided into two orders, the Palaeonemertea and the Heteronemertea (Fig. 1A, Stiasny-Wijnhoff 1923).

This traditional phylogenetic view has recently been challenged by investigations based on 18S rDNA analysis and studies on the variations of developmental pathways found in the different nemertean taxa (Sundberg et al. 2001, Maslakova 2010a). Palaeonemertea and Heteronemertea show different developmental pathways (Fig. 1B). Palaeonemertea develop via a planuliform larva, which is probably not homologous to the planuliform larval type found in Hoplonemertea (Maslakova 2010a). Heteronemertea and Hubrechtidae in contrast develop via a pilidium larva. This apomorphic character unites them in the monophyletic clade Pilidiophora, which replaces the former sub-taxa of Heteronemertea and represents the sistergroup to the also monophyletic clade of Hoplonemertea (Fig. 1B, Norenburg & Stricker 2002, Thollesson & Norenburg 2003).

However, Palaeonemertea are suggested to be the most basal nemertean taxon based on 16S, 18S, and 28S rRNA data sets. In addition, these data suggest that Palaeonemertea are a paraphyletic taxon with respect to the remaining nemertean taxa (Fig. 1B, Sundberg et al. 2001, Thollesson & Norenburg 2003).

Recently, molecular based studies strongly support the nested phylogenetic position of Nemertea together with Mollusca, Platyhelminthes, Annelida and others within the superclade Lophotrochozoa (Giribet et al. 2000, Turbeville & Smith 2007, Dunn et al. 2008, Hejnol et al. 2009). One work based on EST data sets places Annelida as a sistergroup to a monophyletic assemblage, which comprises Nemertea, Phoronida and Brachiopoda (Dunn et al. 2008). Another current molecular study unites Nemertea together with Brachiopoda, whereby Phoronida are claimed to be part of Brachiopoda, into a monophyletic clade termed Kryptrochozoa. The name Kryptrochozoa should emphasis the development of these two

phyla via modified trochophore larvae. Annelida, Mollusca and Kryptrochozoa form the monophyletic clade Trochozoa (Giribet et al. 2009, Hejnol et al 2009).

An earlier molecular study on the mitochondrial genome led to the suggestion that Nemertea are in close relationship to various lophotrochozoan phyla. Accordingly, Nemertea might represent the sistergroup to a clade composed of Mollusca, Brachiopoda, Nematoda and Platyhelminthes based on parsimony analysis of amino acids. Another scenario based on the Bayesian analysis of amino acids claims Nemertea to be a sistergroup of Phoronida. In contrast, parsimony analysis of combined data supports monophyly of Nemertea and the gastropod genus *Haliotis* (see Turbeville & Smith 2007). Respectively, the exact sistergroup relationship of Nemertea still remains unknown and requires further investigations (Turbeville & Smith 2007, Dunn et al. 2008).

Also developmental and morphological characters of nemertean larvae support their phylogenetic position within the Lophotrochozoa. Generally, lophotrochozoan larvae show the presence of an apical ciliary tuft. Together with frequently occurring associated flask-shaped serotonin-like immunoreactive (lir) cells, the ciliary tuft forms the so-called apical organ. A small number of FMRF-amide-lir cells are involved in the formation of the larval apical organ in some phyla, such as Annelida and Mollusca. In addition, many lophotrochozoan larvae of Annelida, Mollusca and the swimming-type larva of Entoprocta exhibit a serotonin-lir neurite underlying the prototroch (Hay-Schmidt 2000, Wanninger 2008). These findings led to the suggestion that the last common ancestor (LCA) of Lophotrochozoa had an apical organ and a serotonin-lir nerve ring associated with the prototroch (Wanninger 2008). Furthermore, many lophotrochozoan representatives such as Annelida, Mollusca, Platyhelminthes and Entoprocta exhibit spiral cleavage, which is another apomorphic character for this superclade (Giribet 2003). This cleavage type is also found in Nemertea. Developmental studies have revealed that in Nemertea the ectomesoderm derives

from the 3a and 3b cell and the endomesoderm derives from the 4d cell, which are further apomorphic characters of the Lophotrochozoa (Henry & Martindale, 1998; Maslakova 2010a). Additionally, several lophotrochozoan representatives, such as certain polychaetes, some Mollusca and the swimming-type larva of Entoprocta are characterized by the development via a trochophore-like larva. It has a preoral ciliated band termed the "prototroch" (Nielsen 2012). The prototroch derives from the $1q^1$, $1q^2$ and 2q cells, which usually give rise to 24-40 large cleavage-arrested trochoblast cells in a number of trochozoan taxa (Damen & Dictus 1994, Henry et al. 2007).

Nemertean larvae do not show a typical prototroch. However, in "Palaeonemertea" the presence of a "hidden prototroch", with several large cleavage-arrested cells, which are proposed to be homologous to the trochoblast cells of other spiralians, were recently shown (Maslakova et al. 2004). The ciliated marginal band in the pilidium larva originates from hundreds of small cells that derive from the first quartet micromeres, the second quartet micromeres as well as from the 3c and 3d cells from the third quartet micromeres (Henry & Martindale 1998). These cells are not cleavage-arrested and continue to divide to form the extensive ciliary band along the four lobes (Maslakova 2010a). All these findings support the hypothesis that the ancestral Nemertea developed via a trochophore-like larval type, while the pilidium larvae with its helmet-shaped appearance resembles a derived larval form that evolved within the phylum (Ax 1995, Haszprunar et al. 1995, Maslakova et al. 2004). Currently, an unknown pilidiophoran species was found that develops via a lecitotrophic pilidium larva. The larva is uniformly ciliated with reduced anterior and posterior lobes and has a long apical tuft. Surprisingly, the larva exhibits two ciliary bands, namely a prototroch that surrounds the larval equator and a telotroch situated at the larval abapical region. Future cell lineage studies, however, might resolve the question whether or not these ciliary bands

are homologous to ciliated bands of other trochophore-like larvae (Maslakova & von Dassow 2012).

Pilidiophoran development

Nemertea display two different developmental modes. “Palaeonemertea” and Hoplonemertea exhibit “direct” development via a planuliform larva. “Direct” development in Nemertea is defined as a developmental pathway that leads to the adult bodyplan formation without a distinct feeding larval stage in between. Pilidiophora in contrast show “indirect development” via the long-lived planktotrophic pilidium larva (Fig. 1B, Norenburg & Stricker 2002).

The pilidium larva is only found in Pilidiophora and resembles one of the most striking invertebrate larval types in the marine plankton. It exhibits a pointed episphere with an apical plate that contains columnar ciliated epidermal cells, which give rise to the larval apical tuft (Cantell et al. 1982, Lacalli & West 1985). The episphere is surrounded by four lobes, an anterior, a posterior and two lateral lobes. Short cilia cover the larval epidermis, while a band of longer cilia runs along the margin of the four lobes. This so-called ciliary band is supposed to produce the feeding current (Rouse 1999, Maslakova 2010a, Nielsen 2012). The opening of the larval, thin-walled esophagus, the so-called vestibule, is situated between the two lateral lobes (Maslakova 2010b). It terminates in a blind stomach with a thick epithelium. Esophagus and stomach are separated by a muscular sphincter, which is believed to contribute to the larval mouth opening (Maslakova 2010b). Pilidium larvae are usually long-lived planktotrophic larvae and move through the water column with the apical tuft pointed forwards (Cantell 1969, Maslakova 2010b). The apical tuft together with associated neural structures within the apical plate is suggested to function as a sensory organ (Lacalli & West 1985, Hay-Schmidt 2000).

During development of the juvenile worm a set of three paired epidermal imaginal discs

appear inside the larva (Salensky 1912, Schmidt 1937, Maslakova 2010b).

First of all, the paired cephalic discs appear as invaginations from the larval epidermis at the transition zone between the anterior and the lateral lobes. They are followed by the paired trunk discs, also of epidermal origin, that invaginate from the transition of the posterior and the lateral lobes. The last pair of imaginal discs, the so-called cerebral organ discs, appears as invaginations of the lateral lobes at the level of the lobe junction, adjacent to the trunk discs with which they fuse during subsequent development (Schmidt 1937, Maslakova 2010b). In addition, two unpaired *anlagen* of probably mesenchymal origin appear (Maslakova 2010b). The proboscis *anlage* develops simultaneously with the paired cerebral organ discs (Bürger 1894, Schmidt 1937, Maslakova, 2010b). It is not clear whether the proboscis *anlage* forms from an accumulation of mesenchymal cells between the larval epidermis and the juvenile cephalic discs, or whether it develops from the pilidial epidermis (Maslakova 2010b). Subsequently, the proboscis *anlage* fuses with the cephalic discs. Together, they form the epidermal proboscis which is covered by the mesenchymal rhynchocoel. The proboscis *anlage* cells themselves are involved in formation of the mesenchymal rhynchocoel (Maslakova 2010b). The second unpaired *anlage*, the so-called dorsal *anlage*, appears between the larval epidermis and the stomach in the posterior region (Salensky 1912, Maslakova 2010b). It subsequently grows in anterior direction towards the future ventral side of the juvenile. The cerebral organ discs and the dorsal *anlage* then fuse with the trunk discs. In the following, the cephalic discs fuse with the trunk discs, forming a so-called “torus stage” juvenile, an ellipsoidal ring of juvenile mass situated around the larval stomach (Maslakova 2010b). During further development, the proboscis grows until it reaches the stomach. The trunk discs grow over the stomach, which is incorporated into the juvenile body (Salensky 1912, Maslakova 2010b). After the fusion of all discs and *anlagen*, the juvenile worm comes to lie within the larval episphere. The anterior-posterior axis of the juvenile is almost

perpendicular to the one of the larva (Maslakova 2010a). In the course of a so-called dramatic metamorphosis in Nemertea, the juvenile worm emerges from the larva and swallows the remaining larval tissue (Cantell 1966, Cantell 1969, Maslakova & von Döhren 2009, Maslakova 2010b).

There are several variations of this specialized developmental mode within the Pilidiophora, such as the non-feeding planktonic pilidium larvae of *Micrura akkeshiensis* (Iwata 1958) or the encapsulated development of the Schmidt's larvae (Schmidt 1964) and the Desor's larva (Desor 1848). The development of the juvenile via imaginal discs is also present in these specialized larval types, which indicates that the long-lived planktotrophic pilidium is the ancestral developmental mode of Pilidiophora (Maslakova 2010a).

Interestingly, several hoplonemertean species show a larval epidermis which is shed during development (Jägersten 1972, Maslakova & Malakhov 1999). This so-called transitory epidermis is formed by a relatively small number of large epidermal cells. During larval development small epidermal cells appear in clusters between the transitory epidermis cells. Subsequently, the small epidermal cells increase in number, while the large transitory epidermal cells decrease in size. In 10-day old larvae of the hoplonemertean *Paranemertes peregrina* the small epidermal cells, which at this stage form the definite epidermis of the juvenile, cover the entire larval surface. The large transitory epidermal cells completely disappear (Maslakova & von Döhren 2009). This type of epidermis is sometimes considered to be a homologous structure to the larval epidermis of Pilidiophora, which is also shed when the juvenile worm emerges from the larva (Jägersten 1972, Maslakova & Malakhov 1999).

Nemertean neurogenesis

The development of the nemertean larval nervous system has been studied with various methods in the hoplonemertean *Quasitetrastemma stimpsoni* and in different species of

Pilidiophora. TEM investigations revealed the presence of a marginal nerve that runs along the four larval lobes in undetermined pilidium larvae and in the larva of *Lineus albocinctus* (Lacalli & West 1985, Hay-Schmidt 1990). In addition, two cell types with a single cilium surrounded by a microvilli collar are present in an undetermined pilidium larva. These cells are always associated with the marginal nerve (Lacalli & West 1985). In an undetermined pilidium and in *Lineus albocinctus* an oral nerve ring encircles the sphincter between esophagus and stomach. Two suboral nerves descend from the oral nerve in both species. They merge with the marginal nerve at the transition between the posterior and the lateral lobes in an undetermined pilidium (Lacalli & West 1985). TEM investigations showed that in the larva of *Lineus albocinctus* the suboral nerve splits into an anterior and a posterior portion; both merge with the marginal nerve at the transition between the lobes. An additional pair of so-called lateral helmet nerves, shown with ultrathin sections, descends from the anterior part of the anterior lobe to the junction of the anterior and the lateral lobes, where it merges with the marginal nerve (Hay-Schmidt 1990). Interestingly, no neurons associated with the apical plate could be found in ultrathin sections of any pilidiophoran larvae investigated (Lacalli & West 1985, Hay-Schmidt 1990).

Immunocytochemical stainings of *Lineus albocinctus* and *Micrura alaskensis* revealed a serotonin-lir marginal nerve that runs along the four lobes and underlies the ciliary band. The marginal nerve is always associated with unipolar neurons in both species (Hay-Schmidt 1990, Maslakova 2010b). In *Lineus albocinctus* the neurites of these associated unipolar neurons split into two neurites, which both merge with the marginal nerve. The serotonin-lir oral nerve ring is associated with a single unipolar serotonin-lir cell (Hay-Schmidt 1990). The serotonin-lir oral nerve ring has also been reported for the larva of *Micrura alaskensis*, but was not depicted in the figure plates presented (Maslakova 2010b). An extensive subepithelial serotonin-lir nerve net is present in the larva of *Lineus albocinctus* and *Micrura alaskensis*

with numerous interconnecting serotonin-lir neurons (Hay-Schmidt 1990, Maslakova 2010b). Interestingly, apical serotonin-lir structures were only found in the pilidium larva of *Micrura alaskensis*. Thereby, two monociliated serotonin-lir neurons lie in the anterior part of the apical plate, one on each side (Maslakova 2010b). The hoplonemertean larvae of *Quasitetrastemma stimpsoni* likewise shows two apical and two subapical serotonin-lir cells, which are connected to the brain commissures and the apical plate in further developed larvae (Chernyshev & Magarlamov 2010). Generally, these findings of two serotonin-lir apical neurons in Pilidiophora and four serotonin-lir apical neurons in Hoplonemertea raise the question of the presence of a definite apical organ in the derived pilidium larva and in the LCA of Nemertea.

But not only nemertean larvae, but also juvenile worms of the pilidiophoran species *Lineus albocinctus* and *Micrura alaskensis* likewise show serotonin-lir structures. A pair of lateral nerve cords emerges from the cephalic discs and runs along the ventro-lateral side of the juvenile in both species (Hay-Schmidt 1990, Maslakova 2010b). Serotonin-lir cells are always associated with lateral nerve cord of the juvenile (Hay-Schmidt 1990, Maslakova 2010b). Several longitudinal serotonin-lir proboscis neurites are present in the juvenile worm (Hay-Schmidt 1990, Maslakova 2010b). Positive serotonin-lir stainings of ventral and dorsal commissures are found in the juvenile of *Lineus albocinctus* (Hay-Schmidt 1990). Another study on *Micrura alaskensis* revealed these structures with phalloidin stainings, which is usually used for F-actin visualization, as well (Maslakova, 2010b).

Until now, investigations on FMRFamide-lir structures are available only on one pilidiophoran species, namely *Lineus albocinctus*. One to four FMRFamide-lir cells are found in the larval episphere. They send their processes to the anterior region of the apical plate and to the junction between the anterior and the lateral lobes. In the juvenile of *Lineus albocinctus* no FMRFamide-lir structures were found (Hay-Schmidt 1990).

Despite these data on larval neuroanatomy, the development of various neurotransmitters in Nemertea, especially Pilidiophora, remains largely unknown. Likewise, the incorporation of larval neural structures into the juvenile body plan has not been investigated in a satisfying way yet. Furthermore, it is still unknown, which neural components of the juvenile develop independently from the larval neural structures. Therefore, one main aspect of this study is to asses, as to whether or not larval neural structures are incorporated into the juvenile body plan of Pilidiophora. In order to contribute data to this question and for a better comparison with other lophotrochozoan taxa, the distribution of serotonin-lir and FMRFamide-lir neurotransmitters was studied herein for different developmental stages of *Lineus albocinctus*. Moreover, immunoreactivity of the mollusc-specific VD1/RPD2 α-neuropeptide was investigated herein in early larval stages of *Lineus albocinctus*, in order to assess the presence or absence of this α-neuropeptide in a non-molluscan Lophotrochozoa.

Material and Methods

Animal collection and fixation

Lineus albocinctus has one of the most common pilidium larvae which occur between August and December in the waters of the Gullmarsfjord, Swedish west coast. In this species the anterior lobe is more prominent than the posterior one. The helmet, the anterior and the posterior lobes show a concave outline. The apical tuft can be as long as the entire height of the larva while the apical plate, build by epidermal cells, is relatively large. Also chromatophore distribution along the margin of the four lobes is considered to be a reliable identifying characteristic (Cantell 1969).

Larvae of different developmental stages were collected in August 2011 in the Gullmarsfjord by plankton tows (58°15’7”N, 11°26’30”E; 58°15’02”N, 11° 27’23”E). Animals were relaxed in a 3.5% $MgCl_2$ solution and were then fixed in 4% paraformaldehyde in 0.1M PBS

(phosphate buffered saline, pH 7.4) and 10% sucrose for approximately 1.5 to 2 hours at room temperature (RT). Afterwards, animals were rinsed in 0.1M PBS with 0.1% NaN_3 and stored in the same solution at 4°C.

Immunocytochemistry and confocal laserscanning microscopy (CLSM)

For confocal microscopy larval tissue was permeabilized for 60 min in PBT, a solution that consists of 0.1M PBS (pH 7.4), 0.1% NaN_3 and 4% Triton X-100. Non-specific binding sites were blocked in PBT with 6% goat serum (Jackson ImmunoResearch, West Grove, PA, USA) at RT overnight. Subsequently, the larvae were incubated in either of the following primary antibodies: 5-HT antibody (serotonin) raised in rabbit (ImmunoStar, Hudson, WI, USA); FMRFamide antibody raised in rabbit (Biotrend, Cologne, Germany); a mollusc-specific α-neuropeptide antibody raised in rabbit, directed against the VD1/RPD2 system (CASLOlabs, Lyngby, Denmark), first described for the pond snail *Lymnaea stagnalis* (Kerkhoven et al. 1993); or acetylated-α-tubulin antibody raised in mouse (Sigma-Aldrich, St. Louis, MO, USA); diluted by 1:800, 1:500, 1:350, and 1:400, respectively, with blockPBT (PBT with 6% goat serum) for 24 hours at RT. Specimens were rinsed four times in blockPBT for at least 6 hours. Then, they were incubated for 24 hours at RT in Alexa Fluor 568 anti-rabbit (Invitrogen, Molecular Probes, Eugene, OR, USA) or Alexa Fluor 633 anti-mouse (Invitrogen) secondary antibody, respectively, both at a dilution of 1:300 in blockPBT. DAPI (Sigma), diluted 1:400 in blockPBT, was added for staining the cell nuclei. Stained larvae were washed four times in PBS for at least six hours and then mounted in Fluoromount-G (Southern Biotech, Birmingham, AL, USA). Cover glasses were provided with clay feet to prevent squashing of specimens. Slides were stored at 4°C.

Samples were examined with a Leica SP5 CLSM (Leica Microsystems, Wetzlar, Germany) equipped with a 20 x 1.47 glycerol lense and a 63 x 1.47 glycerol lense, respectively. Stacks

of virtual sections of 0.3-0.6µm thickness were imported into the LAS AF software (Leica Microsystems) to generate projection images. Further digital image processing was done with Photoshop C5 (Adobe, San Jose, CA, USA). Illustrator C5 (Adobe) was used for creating the line drawings.

Results

Terminology

Several neuroanatomical terms, such as “nerve” and “nerve cord”, are used in different ways for various invertebrate taxa without any consensus on their exact definitions. A recently published glossary on neuroanatomical terminology defines a “nerve” as a structure of condensed axons free of cell bodies. Immunocytochemical methods, however, do not allow *in toto* visualization of entire nerves (including their cell bodies, entity of axons and dendrites) and do not allow a clear distinction between axons and dendrites. For the sake of congruency former neurobiological terms such as “nerve” and “nerve cord” are herein subsequently replaced by the adequate terms “neurite” and “neurite bundle” when the data base only on immunocytochemistry (Richter et al. 2010).

Development of serotonin-lir structures from early larva to the juvenile worm

Earliest larval stages investigated show the cephalic discs developed as small pouches at the transition between the anterior and the lateral lobes, as well as the trunk discs, which invaginate from the transition of the posterior and the lateral lobes (Fig. 2A, B; Fig. 3A; Fig. 4A). These larval stages are addressed as “larvae with incorporated trunk disc stages” in the following (see Maslakova 2010b). The cerebral organ discs, the proboscis *anlage* and the dorsal *anlage* have not developed at this stage. The prominent apical plate is present and shows two serotonin-lir cell bodies in the anterior part of the apical plate, one cell on the left and one on the right side (Fig. 2A; Fig. 3A, C). These apical neurons send processes to the

anterior, posterior and the lateral lobes, where the processes are interconnected by additional serotonin-lir multipolar interneurons (Fig. 3C, D). Beneath the apical plate, the processes of the apical neurons form a complex apical neurite plexus (Fig. 3C). Several of these processes that originate from the apical neurons merge with the marginal neurite bundle, a single compact serotonin-lir neurite bundle that runs along the four larval lobes. Serotonin-lir monociliated perikarya are always associated with the marginal neurite bundle; their number, however, increases during development (Fig. 3B).

At the junction between the esophagus and the stomach a prominent serotonin-lir nerve ring with several associated conical-shaped cell bodies is present and encircles the stomach (Fig. 2A, B, C, D; Fig. 3A, C, D). Two suboral neurites originate from the oral nerve ring and descend towards the transition between the posterior and the lateral lobes, where they merge with the marginal neurite bundle (Fig. 2A, C; Fig. 3D; Fig. 4A). The suboral neurites as well as the oral nerve ring are connected to numerous serotonin-lir cell bodies (Fig. 3C, D; Fig. 4A).

Further developmental stages show almost fused cephalic discs and more outgrown trunk discs (Fig. 2C; Fig. 4B). The processes that emanate from the apical neurons have increased in number by this larval stage (Fig. 4B). Additionally, a distinct subepithelial nerve net is present in all four lobes (Fig. 2C; Fig 4B, C). During larval development these nerve nets increase in complexity, as does the number of multipolar interneurons, which interconnect their individual neurites (Fig. 2C, D). The number of serotonin-lir cells associated with the marginal neurite bundle is higher at this developmental stage than in larvae of the incorporated trunk discs stage. The oral nerve ring appears more prominent and shows an increase in associated conical-shaped cell bodies (Fig 2A, C).

Later in development, all three pairs of imaginal discs, the proboscis *anlage* and the dorsal *anlage* of the juvenile fuse and form the juvenile worm that lies in the episphere of the larva

(Fig. 2D; Fig. 4C). The anterior-posterior axis of the juvenile is almost perpendicular to the anterior-posterior axis of the larva (Fig. 2D; Fig. 4C). The imaginal discs grow over the larval digestive tract and incorporate it into the juvenile body (Fig. 2D; Fig. 4C). Now the juvenile and the larva share the digestive tract. The larval serotonin-lir nervous system exhibits changes from the previous described developmental stage (Fig. 2C, D; Fig. 4C). The two apical neurons and their processes which form the complex nerve net are still present. The larva shows the marginal neurite bundle and its associated monociliated serotonin-lir perikarya at this stage. Two lateral neurite bundles with several associated conical-shaped cell bodies are now present along the ventro-lateral side of the juvenile worm (Fig. 2D; Fig. 4C). Interestingly, the oral nerve ring as well as the suboral neurites are now visible within the juvenile body, whereby the suboral neurites begin to merge with the juvenile lateral neurite bundle, at the position where the trunk discs originated from (Fig. 2F).

Dissected juveniles, one with almost fused discs (“torus stage”, see Maslakova 2010b) and one with the discs and *anlagen* that have entirely fused, show, that the oral nerve ring and the suboral neurites are incorporated into the juvenile nervous system (Fig. 2E, F; Fig. 4C, D, E). The suboral neurites now descend into the trunk disc portion of the lateral neurite bundles of the juvenile and are no longer connected to the larval marginal neurite bundle (Fig. 2F; Fig. 4C, D, E). Additionally, several neurites that originate from the oral nerve ring and run around the stomach along the anterior-posterior axis of the juvenile are formed *de novo* (Fig. 2E; Fig. 4C, D, E).

Two prominent lateral neurite bundles are present along the ventral side of the juvenile worm (Fig. 2D, E, F; Fig. 4C, D, E). They emerge from the posterior part of the already fused cephalic discs, transverse the cerebral organ disc region, continue laterally where they transverse the trunk discs and project into the very posterior region of the juvenile. The cephalic discs region is traversed by numerous serotonin-lir neurites connected to the lateral

neurite bundles (Fig. 2E, F). However, this region shows no serotonin-lir cell bodies associated with the lateral neurite bundle. The proboscis *anlage* exhibits several serotonin-lir neurites along its anterior-posterior axis. In the region of the cerebral organ the lateral neurite bundles express a high density of associated serotonin-lir cells (Fig. 2F). Along the trunk discs and in the posterior region of the juvenile such cells are also present, but are less in number (Fig. 2F; Fig. 4D, E; for comparison with the neural situation of the adult specimen see Fig. 4F).

Development of FMRFamide-lir structures from early larva to the juvenile worm

In larvae with incorporated trunk discs all four larval lobes are surrounded by an outer marginal neurite bundle. The lateral lobes exhibit an additional inner marginal neurite bundle (Fig. 5A, D; Fig. 6B, C). Along either side of the larval esophagus a circumesophagial neurite with few associated FMRFamide-lir cells is present (Fig. 5E; Fig. 6A). At the height of the lobe junctions the circumesophagial neurite gives rise to several peripheral lobar neurites (Fig. 5A, E; Fig. 6A). Parts of these peripheral lobar neurites and their descendants form a complex nerve net in the lateral lobes containing numerous multipolar interneurons (Fig. 5C). The neurites extend into the very distal region of the lobes, where they are connected to the FMRFamide-lir inner marginal neurite bundle via the FMRFamide-lir inner marginal interneurons (Fig. 5A, D, E; Fig. 6B). Two of the peripheral lobar neurites merge with the inner marginal neurite bundle on either side of the lobe at the lobe junctions (Fig. 5F; Fig. 6A). Additionally, a complex nerve net with several interconnected FMRFamide-lir interneurons is found in the anterior and posterior lobe (Fig. 6A). In every lobe it consists of parts of the outer marginal neurite bundle and a strand of the peripheral lobar neurites (Fig. 5F; Fig. 6A). In all four lobes distinct FMRFamide-lir cells are connected to the outer marginal neurite bundle by two neurites (Fig. 5G; Fig. 6C). These cells are termed marginal

sensory cells herein and bear a long, single cilium surrounded by a collar of microvilli (Fig. 5B, G; Fig. 6B, C). These marginal sensory cells constitute a new type of larval sensory cells for Nemertea that have hitherto been unknown. Remarkably, neither of these cells, nor any of the inner marginal interneurons, are present at the transition between individual lobes (Fig. 5F; Fig. 6A). In the apical region no FMRFamide-lir structures were found in any of the larval stages investigated (Fig. 5E; Fig. 6A).

VD1/RPD2 α-neuropeptide-lir structures in the early pilidium larva

In larvae of incorporated trunk disc stages a positive VD1/RPD2 α-neuropeptide-lir signal along the oral nerve ring and the suboral neurites is present. The suboral neurites join the inner marginal neurite bundle, which is also positively stained, at the transition between the posterior and the lateral lobes (Fig. 7A, B). In the lateral lobes the inner marginal neurite bundle splits into two neurite bundles, which seem either to be situated closely adjacent to the FMRFamide-lir inner and outer marginal neurite bundles or even may be identical to one or the other of these. An additional outer marginal neurite runs along the four lobes at the very distal border of the epidermis (Fig. 7A, B).

Discussion

General aspects of nemertean neurogenesis

Prior to this analysis, nemertean neurogenesis had been investigated with immunocytochemical methods in one single hoplonemertean species only, namely *Quasitetrastemma stimpsoni*. In this species two serotonin-lir apical cells as well as two serotonin-lir cells which lie beneath the apical cells are present. In later stages the serotonin-lir cells that underlie the apical cells are connected with the brain commissures and the two apical cells connect to the apical plate (Chernyshev & Magarlamov 2010).

In Pilidiophora several species have been investigated concerning their neurogenesis using various methods, such as TEM and immunocytochemical stainings. TEM investigations of the larva of *Lineus albocinctus* revealed the presence of a marginal nerve that underlies the ciliary band, an oral nerve that encircles the sphincter between esophagus and stomach and two suboral nerves. In addition, a pair of lateral helmet nerves descends in the anterior part of the episphere of *Lineus albocinctus* and merges with the marginal nerve at the transition between the anterior and the lateral lobes (Hay-Schmidt 1990). Ultrathin sections of an undetermined pilidium larva also showed the presence of a marginal nerve, an oral nerve ring and two suboral nerves. In addition, two cell types associated with the marginal nerve were found. Both cell types bear a single cilium, which is surrounded by a microvilli collar (Lacalli &West 1985).

Immunocytochemical stainings of the larva of *Lineus albocinctus* and *Micrura alaskensis* revealed the presence of a serotonin-lir marginal neurite bundle that underlies the ciliary band. The marginal neurite bundle is always associated with unipolar serotonin-lir cells. A serotonin-lir oral nerve ring as well as two serotonin-lir suboral neurites were found. The oral nerve ring in *Micrura alaskensis* was mentioned but not depicted (Maslakova 2010b). In late larval stages an extensive subepithelial serotonin-lir nerve net is present in both species (Hay-Schmidt 1990, Maslakova 2010b). The juvenile worm of *Lineus albocinctus* exhibits a pair of lateral neurite bundles with numerous associated serotonin-lir cells. Additionally, several longitudinal serotonin-lir proboscis neurites are present in the juvenile worm (Hay-Schmidt 1990).

In the larva of *Lineus albocinctus* one to four FMRFamide-lir cells are found in the episphere. Their processes project into the anterior region of the apical plate and to the transition between the anterior and the lateral lobes (Hay-Schmidt 1990).

In the following, the data on serotonin-lir, FMRFamide-lir and VD1/RPD2 α-neuropeptide-lir

neural structures presented herein are discussed in the light of the previous works on nemertean neurogenesis.

Development of the serotonin-lir nervous system in larval and juvenile nemerteans

Herein, the development of the serotonin-lir neural structures from the early pilidium larva to the juvenile worm of *Lineus albocinctus* is documented, whereby only parts of the previous findings based on immunocytochemical works on *Lineus albocinctus* and *Micrura alaskensis* can be confirmed (Hay-Schmidt 1990, Maslakova 2010b).

In this work a serotonin-lir marginal neurite bundle that underlies the ciliary band is found in all four lobes of *Lineus albocinctus* and corroborates previous studies on the same species as well as on *Micrura alaskensis* (Hay-Schmidt 1990, Maslakova 2010b). The marginal neurite bundle in *Lineus albocinctus* was reported to show numerous associated serotonin-lir cells, which form contact with the marginal neurite bundle via two processes (Hay-Schmidt 1990). These associated serotonin-lir cells in *Lineus albocinctus* were also found during this study, whereas the connection to the marginal neurite bundle via two processes cannot be confirmed. Interestingly, in *Lineus albocinctus*, every serotonin-lir cell associated with the marginal neurite bundle bears a single cilium. These findings on serotonin-lir structures are partly in accordance with data on *Micrura alaskensis*, which likewise exhibits a marginal neurite bundle with numerous associated serotonin-lir cells (Maslakova 2010b). However, the latter work provides no information about the innervation of these associated serotonin-lir cells, nor does it report any ciliary structures that project from these cells.

During larval development of *Lineus albocinctus* the number of serotonin-lir cells associated with the marginal neurite bundle increases, which supports previous studies (Hay-Schmidt 1990). The serotonin-lir subepithelial nerve net with its interconnected multipolar interneurons as well as the increase in complexity of the nerve net during subsequent

development is confirmed herein and likewise supports previous studies (Hay-Schmidt 1990, Maslakova 2010b). A serotonin-lir oral nerve ring is present in *Lineus albocinctus* and in *Micrura alaskensis*, although in the latter it had not been depicted so far (Hay-Schmidt 1990, Maslakova 2010b). Accordingly, the present study documents the serotonin-lir oral nerve ring and its descending suboral neurites. In addition, two serotonin-lir apical neurons are shown herein in the apical plate of *Lineus albocinctus*. These apical neurons send their processes into each of the four lobes. An apical neurite plexus that underlies the apical plate is formed by the processes of the apical neurons. These findings contradict those of previous studies, where no neural structures in the apical plate of *Lineus albocinctus* were described (Hay-Schmidt 1990). Until now the presence of two monociliated serotonin-lir apical neurons was demonstrated only for *Micrura alaskensis* (Maslakova 2010b).

During juvenile development of *Lineus albocinctus* the serotonin-lir oral nerve ring and the serotonin-lir suboral neurite are incorporated into the juvenile serotonin-lir nervous system. The incorporation of the oral nerve ring into the juvenile as well as the fusion of the suboral neurites with the lateral neurite bundles of the juvenile clearly demonstrates that larval structures form parts of the juvenile nervous system. According to an earlier study on *Lineus albocinctus*, two serotonin-lir lateral neurite bundles are present in the ventro-lateral region of the juvenile worm (Hay-Schmidt 1990). In *Micrura alaskensis* two neural structures have been interpreted as lateral neurite bundles of the juvenile, but these structures were identified by phalloidin rather than antibody staining (Maslakova 2010b).

The cephalic discs and the cerebral organ discs of *Micrura alaskensis* have been proposed to contribute to the development of the lateral neurite bundles (Maslakova 2010b). This is confirmed by the results of this study, although it has to be mentioned that former methodical approaches by use of phalloidin for visualizing neural, in particular serotonin-lir structures (Maslakova 2010b), renders these previous data doubtful. As in *Micrura alaskensis*

(Maslakova 2010b), longitudinal serotonin-lir neurites run through the proboscis *anlage* of *Lineus albocinctus*.

Development of the FMRFamide-lir neural structures in larval and juvenile nemerteans

Previous investigations on the FMRFamide-lir nervous system of pilidium larvae and juveniles have until now only been documented for *Lineus albocinctus*. Herein, the complex FMRFamide-lir neural structures are documented in detail for the same species, with numerous novel findings. A circumesophagial neurite loops around the apical part of the esophagus and descends into the abapical region of the episphere. The circumesophagial neurite is associated with few FMRFamide-lir cell bodies. Due to its position within the larva and its FMRFamide-like immunoreactivity, this circumesophagial neurite could be assumed to be the same process termed previously as “lateral helmet process” (Hay-Schmidt 1990). The present study shows that this neurite splits into several peripheral lobar neurites in the region of the lobe junctions. A complex lobar nerve net with numerous multipolar interconnected interneurons originates from the peripheral lobar neurites. In addition, the peripheral lobar neurites contribute to the inner marginal neurite bundle of the lateral lobes. The neurites of the lobar nerve net are always connected via inner marginal interneurons to the inner marginal neurite bundle of the lateral lobes. Additionally, an outer marginal neurite bundle runs along the four lobes. It is always associated with FMRFamide-lir marginal sensory cells. Due to the presence of a single cilium, the microvilli collar and the connection to the marginal neurite bundle, these cells may correspond to a cell type shown previously by TEM studies of an unknown pilidiophoran species (Lacalli & West 1985).

Throughout the different developmental stages investigated no essential changes in the FMRFamide-lir nervous system of the larva were found. No FMRFamide-lir structures were found in the region of the apical plate.

Comparative neurogenesis of Lophotrochozoa

The superclade Lophotrochozoa comprises a high diversity of different phyla with a high phenotypic plasticity of the adults and the larvae. The Phoronida, Ectoprocta and Brachiopoda have traditionally been assumed to form the monophyletic clade Lophophorata (Halanych et al. 1995). They are characterized by development via radial cleavage and by the formation of a special feeding-structure termed the lophophore. This is a ciliated tentacle apparatus, which is invaded by the mesocoelomic cavity and encircles the mouth but not the anus (Halanych et al. 1995). The Lophophorata have been considered the sistergroup of Spiralia, whereby the latter unites invertebrates that develop via spiral cleavage and a trochophore-like larva (Halanych et al. 1995, Giribet et al. 2000). Interestingly, the findings of recent molecular phylogenetic studies could not support monophyly of Lophophorata (e.g., Dunn et al. 2008, Hejnol et al. 2009). In one study based on EST data Brachiopoda represent a sistergroup to Nemertea and together with Phoronida form a monophyletic clade suggested to form a sistergroup to Annelida (Dunn et al. 2008). In another molecular study Nemertea together with Brachiopoda form the monophyletic clade Kryptrochozoa (Giribet et al. 2009). Findings on the mitochondrial genome analyzed with parsimony analysis suggest Nemertea to represent a sistergroup to a clade composed of Mollusca, Brachiopoda, Nematoda and Platyhelminthes (Turbeville & Smith 2007). Accordingly, the sistergroup relationship of Nemertea is still discussed.

Comparative morphological and developmental analyses of the nervous system of representatives of a number of lophotrochozoan phyla have contributed to reconstructing the nervous system of the LCA of Lophotrochozoa and might help answering phylogenetic questions. In Annelida, for example, an apical organ is usually present and comprises up to four flask-shaped serotonin-lir cells. Associated with the apical organ up to four FMRFamide-lir cells are found (Voronezhskaya et al. 2003, Brinkmann & Wanninger 2008, Wanninger

2008). Furthermore, the Polyplacophora, which are considered basal Mollusca, show 8 serotonin-lir and few FMRFamide-lir cells in their apical organ (Friedrich et al. 2002, Voronezhskaya et al. 2002). Some authors assume that the LCA of the Lophotrochozoa exhibited few to four serotonin-lir, flask-shaped, cells within the apical organ. Possibly, several FMRFamide-lir cells were also associated with its apical organ (Wanninger 2008). Many lophotrochozoan larvae, such as Mollusca, Nemertea, Platyhelminthes, Ectoprocta, and polychaetes show an additional serotonin-lir neurite or nerve net that underlies the larval ciliary band (Voronezhskaya et al. 2003, MacDougall et al. 2006, Brinkmann & Wanninger 2008, Wanninger 2008, Maslakova 2010b, Rawlinson 2010).

Interestingly, in Echiura, which may constitute polychaete representatives, neither serotonin-lir structures within the apical region, nor a serotonin-lir prototroch neurite is present. This might be due to the short planktonic phase of these larvae and most probably is due to a secondary loss (Wanninger 2008). The sipunculan *Phascolosoma agassizii* shows several serotonin-lir cell bodies within the apical organ and a serotonin-lir neurite is associated with the prototroch of the pelagosphera larva of this species as well (Kristof et al. 2008). Interestingly, larvae of another sipunculan *Phascolion strombus*, do not exhibit a serotonin-lir neurite associated with the prototroch, whereas both sipunculan species express two to three FMRFamide-lir cells within their apical organ (Wanninger et al. 2005, Kristof et al. 2008). Müller's larvae of the platyhelminth *Stylostomum sanjuana* exhibit two serotonin-lir cell bodies within the apical organ, sending two processes to the abapical region (Hay-Schmidt 2000). Interestingly, Müller's larva of *Maritigrella crozieri* exhibit serotonin-lir and FMRFamide signals in the apical organ region. Two serotonin-lir cells were found in the same region in another sipunculan, namely *Stylostomum sanjuana*, as well. These cells are equal to commissural cell bodies and not to apical organ cells. A serotonin-lir nerve net, rather than a condensed serotonin-lir neurite bundle, innervates the larval ciliary band

(Rawlinson 2010). The fate of the larval serotonin-lir nervous system in Müller's larvae is still unknown. Interestingly, the pilidiophoran species *Micrura alaskensis* and *Lineus albocinctus* exhibit two and the hoplonemertean *Quasitetrastemma stimpsoni* exhibits four serotonin-lir cell bodies in the apical region (Chernyshev & Magarlamov 2010, Maslakova 2010b, this study). Furthermore, a serotonin-lir marginal neurite bundle underlies the ciliary band in *Lineus albocinctus* and *Micrura alaskensis* (Maslakova 2010b, this study). Neither the two apical neurons, nor the larval neurite bundle associated with the ciliary band are incorporated into the juvenile body (Maslakova 2010b, this study).

Evolutionary implications

Recently, an apical organ that consists of two serotonin-lir apical neurons and two serotonin-lir neurons which underlie the apical neurons was found in the hoplonemertean *Quasitetrastemma stimpsoni*. This was the first work that unequivocally showed the existence of an apical organ in any nemertean larva. Furthermore, these findings suggest that the LCA of Hoplonemertea had an apical organ.

Two serotonin-lir apical neurons were found in *Micrura alaskensis* and in *Lineus albocinctus*, which might implicate that Pilidiophora exhibit a modified apical organ compared to Hoplonemertea. The serotonin-lir apical neurons in *Micrura alaskensis* show a single cilium, but are not flask-shaped and these cells lack the connection with the apical cilia. In *Lineus albocinctus* the serotonin-lir apical neurons are not flask-shaped, nor do they bear a single cilium. In addition, no FMRFamide-lir apical organ structures were found in any nemertean species investigated so far. Thus, the current data suggest that the LCA of Pilidiophora did not exhibit an apical organ as the one described above for Hoplonemertea. Many Lophotrochozoa, such as Mollusca, polychaetes, Phoronida and Entoprocta exhibit an apical organ during their larval development. In conclusion, it is more parsimonious that the LCA of

Nemertea had an apical organ. Accordingly, it must be assumed that Pilidiophora lost this neuroanatomical structure.

Only the oral nerve ring and the two suboral neurites are incorporated into the juvenile nervous system of *Lineus albocinctus*. The two lateral neurite bundles develop from the juvenile imaginal discs and most likely form the future ventral nerve cords of the adult nemertean. Furthermore, it is proposed that the incorporated oral nerve ring develops into (parts of) the adult brain commissures. This scenario requires, however, that the juvenile mouth develops secondarily. As a result, the oral nerve ring would only encircle the proboscis and not the esophagus, thus representing the situation found in adult Nemertea. In addition, the oral nerve ring and the anterior connection of the lateral neurite bundles would form a centralization in the most anterior region of the adult worm.

This work is the first that demonstrates the presence of a mollusk-specific VD1/RPD2 α-neuropeptide in a nemertean representative. In larval *Lineus albocinctus* the oral nerve ring as well as the suboral neurites show VD1/RPD2 α-neuropeptide-like immunoreactivity. In addition, two marginal neurites were found that run along the four lobes, while the inner neurite splits into two separate neurites in the two lateral lobes. Their position suggests that these two inner neurites in the lateral lobes are either situated closely adjacent to the FMRFamide-lir inner and outer marginal neurite bundles, or that they are identical to one or the other of these. The VD1/RPD2 α-neuropeptide-lir outer marginal neurite is found at the very distal region of the larval epidermis and could resemble the same marginal neurite bundle found by serotonin-lir staining. The findings concerning VD1/RPD2 α-neuropeptide reactivity in Nemertea as well as in Mollusca call for further investigations in other lophotrochozoan species to assess whether the VD1/RPD2 α-neuropeptide represents a conserved neurotransmitter for the entire Lophotrochozoa.

Acknowledgements

I am very grateful to A. Wanninger for supervising my thesis and his support throughout the time we worked together. I also want to thank T. Schwaha, T. Wollesen, A. Kristof and M. Walzl for their technical support and the exhilarant discussions. In addition, I want to acknowledge L. Rudoll (Department of Integrative Zoology, University of Vienna) for always providing any equipment needed. I am particularly grateful to my dear colleagues and fellows at the department, namely B. Sonnleitner, B. Schädl, A. Kerbl, M. Moosbrugger, M. Scherholz, M. Schreiner and A. Batawi.

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Figure Legends

Figure 1: (A) Traditional nemertean phylogeny based on adult morphology, modified after Stiasny-Wijnhoff 1923. The phylum is divided into two sub-taxa: the Anopla and Enopla. Anopla comprises Palaeonemertea and Heteronemertea. Enopla comprises Hoplonemertea and Bdellonemertea. (B) Nemertean interrelationships, modified after Thollesson & Norenburg 2003. “Palaeonemertea” is a paraphyletic assemblage where only some of its representatives constitute a basal offshoot within Nemertea. The Pilidiophora contain the Heteronemertea and the Hubrechtidae. They resemble a monophyletic clade and develop via a pilidium larva. The sistergroup to the Pilidiophora is the monophyletic Hoplonemertea. The Hoplonemertea, which in this phylogeny constitute a synonym for Enopla, include Bdellonemertea. Just as the “Palaeonemertea”, its representatives develop via a planuliform larva.

Figure 2: Serotonin-lir neurogenesis in the pilidium larva and the juvenile of *Lineus albocinctus*. Serotonin-lir is shown in graded scales of dark red to bright yellow, cell nuclei are shown in blue. All scale bars equal 100µm. Apical of the larvae faces upwards in A, C and D. The larva in B is shown from an abapical view. The anterior-posterior axis of the juvenile is almost perpendicular to the larval one. Anterior of the juvenile is to the left in E and D. The white dotted lines encircle the imaginal discs of the developing juvenile. (A) Larva with incorporated trunk disc stage. The apical plate (ap) shows two serotonin-lir neural cell bodies (arrowheads). The mouth opening (asterisk) is situated between the lateral lobes, followed by the esophagus, which terminates in a blind ending stomach (st). A serotonin-lir oral nerve ring (onr) encircles the stomach and sends two suboral neurites (son) towards the transition between the posterior and the lateral lobes. A marginal neurite bundle (mnb) runs along the four lobes. It is associated with numerous marginal serotonin-lir perikarya (msp). (B) Larva

with incorporated trunk disc stage. The marginal neurite bundle surrounds all four lobes. It is associated with marginal serotonin-lir perikarya. The oral nerve ring encircles the stomach. (C) The cephalic discs (cd) and the trunk discs (td) of the juvenile appear almost fused. The oral nerve ring appears more prominent and shows more associated serotonin-lir cell bodies. The processes of the apical neurons form a complex lobar nerve net (lnn) with numerous interconnected multipolar interneurons in all four lobes. (D) All imaginal discs, the proboscis *anlage* and the dorsal *anlage* have fused and have formed a juvenile worm (juv) that lies inside the larval episphere. The juvenile eye (ey) is visible anteriorly. The oral nerve ring and the two suboral neurites have been incorporated into the juvenile body. Two lateral neurite bundles (lnb) emerge at the ventro-lateral side of the juvenile. (E) Dissected juvenile worm with almost fused discs, proboscis *anlage* and dorsal *anlage*; dorsal view. The oral nerve ring and the suboral neurites have been incorporated into the juvenile nervous system. A neurite projects from the oral nerve ring and surrounds the stomach. Two lateral neurite bundles emerge from the fused cephalic discs and run along the ventro-lateral side of the juvenile. (pr) proboscis *anlage.* (F) Dissected juvenile worm with fused discs, proboscis *anlage* and dorsal *anlage*; ventro-lateral view. The lateral neurite bundles show a high density of associated serotonin-lir cells within the cerebral organ discs (ced), but no neural cell bodies within the cephalic disc. The suboral neurites descend from the oral nerve ring and fuse with the lateral neurite bundle. (pc) posterior cirrus.

Figure 3: Serotonin-lir neural structures in an early larval stage of *Lineus albocinctus*. Serotonin-lir is shown in graded scales of dark red to bright yellow, cell nuclei are shown in blue. Apical faces upwards in all aspects. In A the white boxes indicate the detailed views of B, C and D. Image in D is from a different specimen. (A) State of the cephalic discs (cd) and trunk discs (td). The apical plate (ap) gives rise to the apical ciliary tuft (at) and shows two

serotonin-lir neurons. The larval mouth opening (asterisk) is situated between the lateral lobes. The esophagus terminates in a blind stomach (st). An oral nerve ring (onr) encircles the stomach. Two serotonin-lir suboral neurites (son) descend from the oral nerve ring and merge with the marginal neurite bundle (mnb) at the junction of the posterior and the lateral lobes. The marginal neurite bundle is associated with numerous marginal serotonin-lir perikarya (msp). Scale bar equals 100µm. (B) Detailed view of the marginal neurite bundle of the posterior lobe. The marginal serotonin-lir perikarya bear a single cilium each (arrows) and are associated with the marginal neurite bundle. Scale bar equals 30µm. (C) Detailed view of the apical plate region and the oral nerve ring. In the apical plate two serotonin-lir neurons (arrowheads) send their processes to the anterior, posterior and the lateral lobes. Underneath the apical plate an apical neurite plexus (anp) is formed by these processes. Scale bar equals 30µm. (D) Detailed view of the oral nerve ring and the suboral neurites. The oral nerve ring gives rise to the two suboral neurites, which merge with the marginal neurite bundle at the junction between posterior and lateral lobes. The suboral neurites are always associated with conical-shaped serotonin-lir cell bodies. The lateral processes (double arrowheads) of the apical serotonin-lir neurons descend anteriorly towards the oral nerve ring into the lateral lobes. Scale bar equals 30µm.

Figure 4: Semi-schematic representations of the development of the serotonin-lir nervous system in *Lineus albocinctus*. Serotonin-lir components are shown in red in A, B and C. The juvenile serotonin-lir components are shown in dark blue in C, D and E. Apical faces upwards in A, B and C. Anterior is to the left in D, E and F. (A) Larva with incorporated trunk disc stage. The serotonin-lir nervous system consists of two apical neurons (asn), which send their processes to the anterior (anp), the posterior (pp) and the lateral lobes (lp). The oral nerve ring (onr) encircles the junction between the esophagus (es) and the stomach (st). Two suboral

neurites (son) descend from the oral nerve ring to the transition between the posterior and the lateral lobes, where the suboral neurites merge with the marginal neurite bundle (mnb). (cb) ciliary band, (msp) marginal serotonin-lir perikarya. Size of the larva is approximately 400µm from apical to abapical. (B) Serotonin-lir nervous system of a larva with almost fused cephalic discs (cd) and trunk discs (td) of the juvenile. Compared to the specimen shown in A a higher number of serotonin-lir processes, which originate from the two apical neurons and project in all four lobes, is found at this stage. These processes form a complex lobar nerve net (lnn) in all four lobes. Size of the larva is approximately 470µm from apical to abapical. (C) Larva with fused discs and *anlagen* that form the juvenile worm (juv) inside the larval episphere. The oral nerve ring and the suboral neurites (dark blue) are incorporated into the juvenile body. Two lateral neurite bundles (lnb) appear on the ventro-lateral sides of the juvenile. The stomach neurite (sn) surrounds the incorporated stomach. The larval serotonin-lir nervous system appears unchanged compared to B. (ey) eye. Size of the larva is approximately 830µm from apical to abapical. (D) Dissected juvenile; lateral left view. The oral nerve ring encircles the stomach. The two suboral neurites merge with the lateral neurite bundles. Size of the specimen is approximately 380µm from anterior to posterior. (E) Dorsal view of a dissected juvenile. The two lateral neurite bundles (lnb) run along the ventro-lateral side of the juvenile. Size of the specimen is approximately 430µm from anterior to posterior. (F) Morphology of the anterior region of an adult brain (br), the depicting rhynchocoel (rc) and the mouth opening (mo). After Turbeville 2007. The brain with its dorsal commissure (dc) and ventral commissure (vc) encircles the rhynchocoel with the inner lying proboscis (pb). The mouth opening in “Anopla” is situated posteriorly to the rhynchodaeum (rd), thus the brain encircles the rhynchocoel and not the foregut (fg).

Figure 5: FMRFamide-lir structures of larval stages of *Lineus albocinctus*. FMRFamide-lir structures are shown in graded scales of dark red to bright yellow, cell nuclei are shown in blue, cilia are shown in green. A and C; B, D and G; E and F show same specimen, respectively. Apical of the larvae faces upwards in all aspects. (A) A circumesophagial neurite (cen) proceeds from the apical part of the esophagus (es) to its distal end, where its descendants form a complex nerve net in all four lobes. A ciliary band (cb) runs along all four larval lobes. Underneath the ciliary band the outer marginal neurite bundle (omn) with associated cells is present. The lateral lobes show an additional inner marginal neurite bundle (imn). (asterisk) mouth opening, (at) apical tuft, (ll) lateral lobe, (st) stomach. Scale bar equals 100µm. (B) Detailed view of the posterior lobe. The marginal sensory cells (msc) are associated with the outer marginal neurite bundle. Arrows indicate the single cilium of the marginal sensory cells. Scale bar equals 10µm. (C) Detailed view of the lobar nerve net of the left lateral lobe. Arrowheads indicate the cell somata of the multipolar interneurons. Scale bar equals 20µm. (D) Detailed view of the inner marginal neurite bundle (imn) and the outer marginal neurite bundle (omn) of the lateral lobes. Inner marginal interneurons (imi) connect the processes of the lobar nerve net to the inner marginal neurite bundle. The marginal sensory cells are associated with the outer marginal neurite bundle. Scale bar equals 20µm. (E) Larva with incorporated trunk disc stage and a circumesophagial neurite, which proceeds towards each side of the esophagus. The circumesophagial neurite splits and its descendants form a complex nerve net in all four lobes. (ar) apical region. Scale bar equals 60µm. (F) Detailed view of the transition between the posterior and the lateral lobes. The circumesophagial neurite splits into several peripheral lobar neurites (pln), of which one on each side forms the inner marginal neurite bundle of the lateral lobes. The anterior and posterior lobes only show the outer marginal neurite bundle. At the transition between the lobes neither marginal sensory cells nor inner marginal interneurons are present. Scale bar

equals 30µm. (G) Detailed view of the marginal sensory cells. Double arrowheads indicate the microvilli collar that surrounds the single cilium of the marginal sensory cells. Two neurites (cn) connect the marginal sensory cells to the outer marginal neurite bundle. Scale bar equals 5µm.

Figure 6: Semi-schematic line drawing of the larval FMRFamide-lir nervous system in *Lineus albocinctus*. FMRFamide-lir structures are shown in red. Apical of the larva faces upwards in A and B. abapical faces left in C (A) Larva with circumesophagial neurite (cen), that descends on each side of the esophagus (es). At the level of the lobar junctions it splits into several peripheral lobar neurites (pln). In the lateral lobes some of these peripheral lobar neurites form a complex nerve net with numerous interconnected multipolar interneurons (mpi). The neurites of the lateral lobar net are connected to the inner marginal interneurons (imi) which are associated with the inner marginal neurite bundle (imn). The inner marginal neurite bundle is only present in the lateral lobes and originates from one peripheral lobar neurite on each side of the lobe. An outer marginal neurite bundle (omn) surrounds all four lobes and is always associated with the marginal sensory cells (msc). The nerve net in the anterior and posterior lobes originates from a peripheral lobar neurite and parts of the outer marginal neurite bundle. (ap) apical plate, (st) stomach. Scale bar equals 100µm. (B) Detailed representation of the distal part of the lateral lobe. The lateral nerve net (lnn) is connected to the inner marginal neurite bundle via the inner marginal interneurons. The lobar nerve net shows multipolar interneurons (mpi) interconnecting the neurites. The inner marginal neurite bundle is only present in the lateral lobes. Scale bar equals 20µm. (C) Detailed representation of the marginal sensory cell of the lateral lobe. Its single sensory cilium (sc) is surrounded by a microvilli collar (mvc). The marginal sensory cell is connected to the outer marginal neurite

bundle via two neurites. The microvilli collar lies within the epidermis (ep). The sensory cilium projects into the ciliary band (cb). Scale bar equals 5µm.

Figure 7: VD1/RPD2 α-neuropeptide-lir structures in an early larval stage of *Lineus albocinctus*. All scale bars equals 100µm. Apical faces upwards in both aspects. (A) VD1/RPD2 α-neuropeptide-lir is shown in graded scales of dark red to bright yellow, cell nuclei are shown in blue. The oral nerve ring (onr) encircles the junction between the esophagus and the stomach (st). Two suboral neurites (son) descend from the oral nerve ring towards the transition between the posterior and the lateral lobes. The four lobes are surrounded by two marginal neurite bundles. The inner marginal neurite bundle (imb) splits into two separate neurite bundles in the lateral lobes. (ap) apical plate, (asterisk) mouth opening, (omb) outer marginal neurite bundle. (B) VD1/RPD2 α- neuropeptide-lir structures are shown in red. (cb) ciliary band, (cd) cephalic disc, (es) esophagus, (td) trunk disc.

Danksagung

An dieser Stelle ist nun endlich Platz für die vielen Worte der Dankbarkeit die ich so einigen Menschen schuldig bin. Zuerst möchte ich mich bei meinem Betreuer Univ.-Prof. DDr. Andreas Wanninger für die herzliche Zusammenarbeit und die einzigartige Betreuung bedanken. Nachdem ich gezwungenermaßen die Vertiefung in die Stoffwechselphysiologie der Tiere aufgeben musste, hat er mich auf den richtigen Weg, direkt in die Invertebratenwelt geführt. Danken möchte ich ihm dafür, dass er mir gezeigt hat, dass auch Tiere ohne Wirbelsäule und Kindchen Schema unsere ganze Aufmerksamkeit verdienen.

Weiter möchte ich den Post-Docs der Abteilung für Integrative Zoologie, Dr. Thomas Schwaha, Dr. Tim Wollesen und Dr. Alen Kristof meine Anerkennung aussprechen. Die tatkräftige Unterstützung in der Dunkelkammer, am Konfokal, mit Bildbearbeitungssoftware und den anderen zahlreichen Kleinigkeiten die mir auf dem Weg zum Studienabschluss ein Hindernis waren, hätte ich ohne die drei Jungs wahrscheinlich nie aus dem Weg geräumt. Auch die vielen lustigen Stunden mit ihnen werde ich nicht vergessen.

In einem Mädchenzimmer eine wissenschaftliche Arbeit zu schreiben war ein Highlight, keiner versteht die Probleme des anderen so gut wie Alexandra Kerbl und Ashwaq Batawi und will sich noch dazu stundenlang darüber unterhalten, alles von vorne, hinten, links und rechts betrachten um am Ende alles noch mal von neuem durchzukauen. Auch die übrigen Mitglieder der Abteilung für Integrative Zoologie, die mir ein wunderschönes und lebenswertes Jahr bereitet haben, seien hiermit erwähnt, insbesondere Barbara Schädl, Martin Moosbrugger, Melanie Schreiner und Maik Scherholz.

Danke an meine Freunde an dieser Stelle, die viel Verständnis für meine wenig vorhandene Zeit entgegengebracht haben. Besonders meinem Freund Philipp danke ich für sein unendliches Verständnis und seine Geduld.

Ein ganz besonderer Dank gebührt an dieser Stelle einer meiner engsten Freundinnen, der

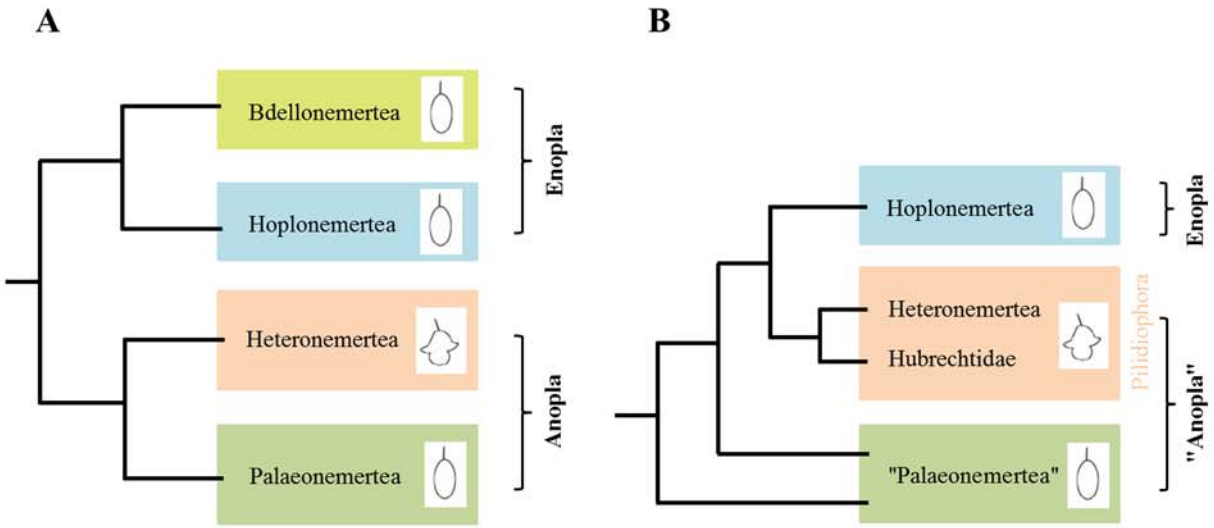
coolsten Kollegin und meiner größten Leidensgenossin; Birgit Sonnleitner. Ohne ihre unendlichen Worte des Zuspruchs, das Verständnis für die zahlreichen Auf und Abs während der Arbeit und vor allem ohne die zahlreichen Momente des Tränen-Lachens auf unserem gemeinsamen Studienweg wär ich wahrscheinlich heute noch nicht fertig mit der Arbeit und um einen der fröhlichsten Menschen, der mir je begegnet ist, ärmer. *Wir fahren nach Italien. Wir verstehen zwar kein Wort, aber lieber mal gar nix verstehen…*

Im wahrsten Sinne des Wortes unaussprechbar großer Dank gebührt meinen Eltern Jutta & Siegfried und meinem Bruder Thomas für deren Verständnis für meinen akuten Zeitmangel, die finanzielle Unterstützung während all der Jahre, dass sie immer für mich da waren und dafür dass ich mich immer und überall auf sie verlassen kann.

Appendix

Figures

Figure 1



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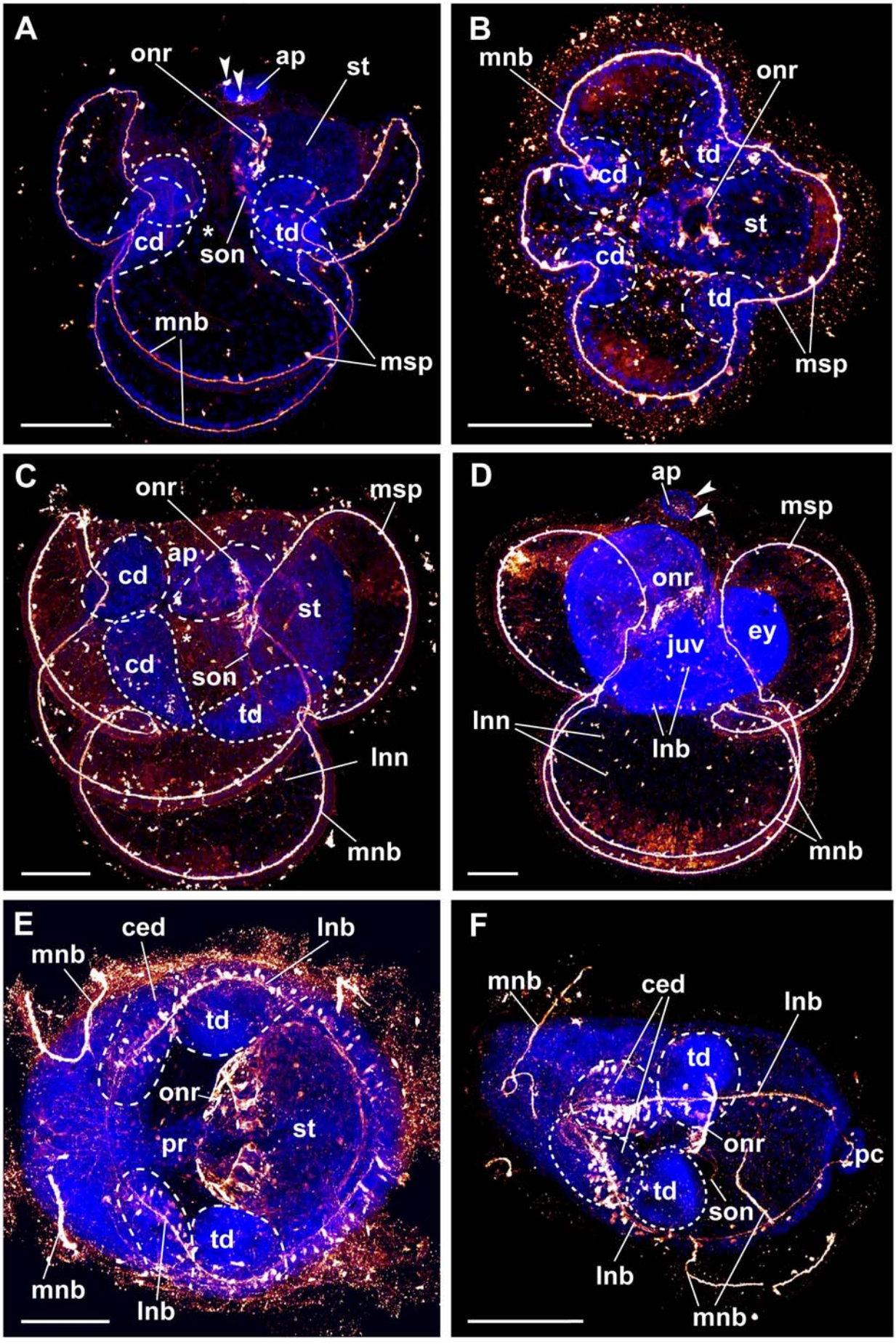
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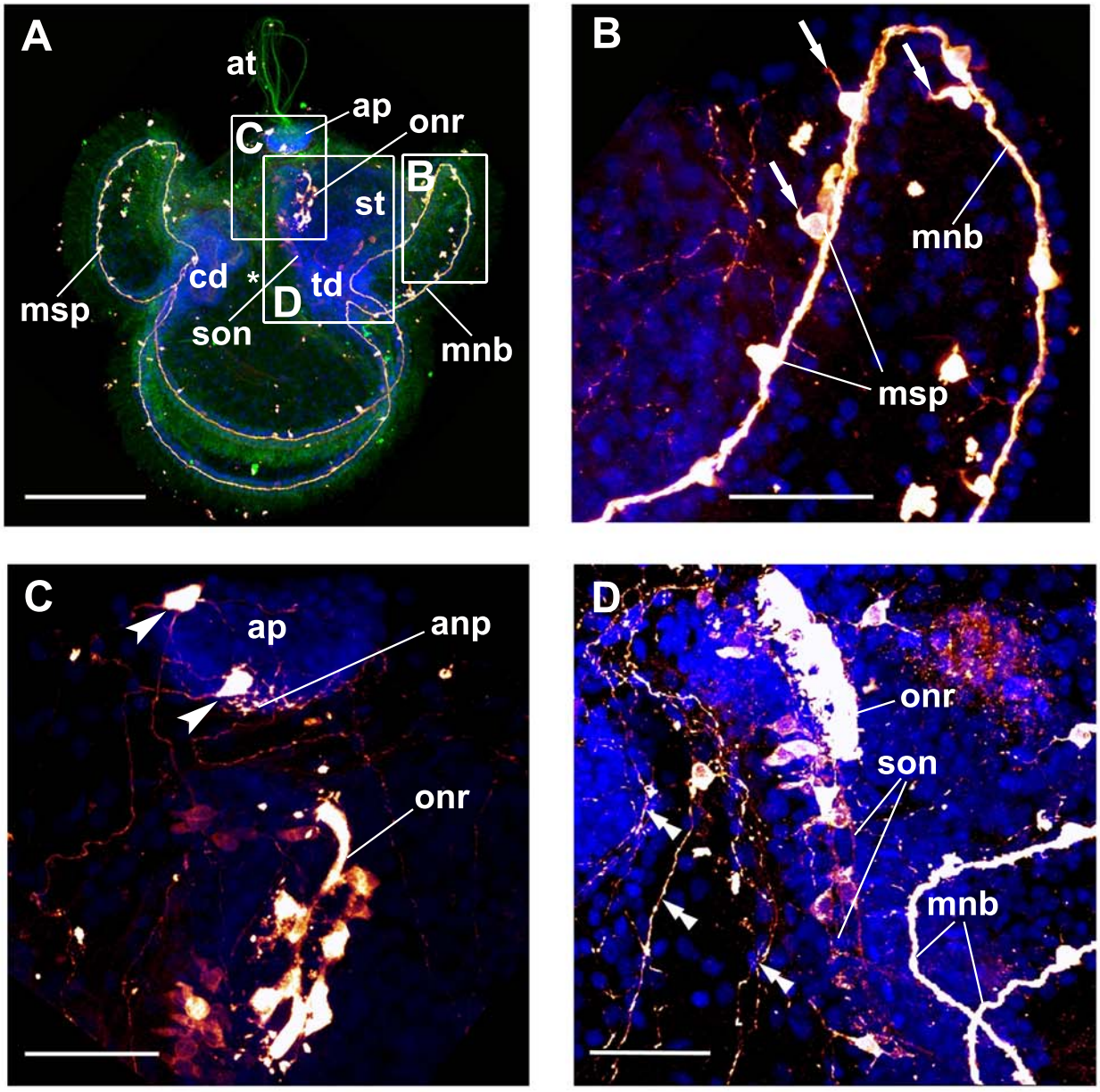
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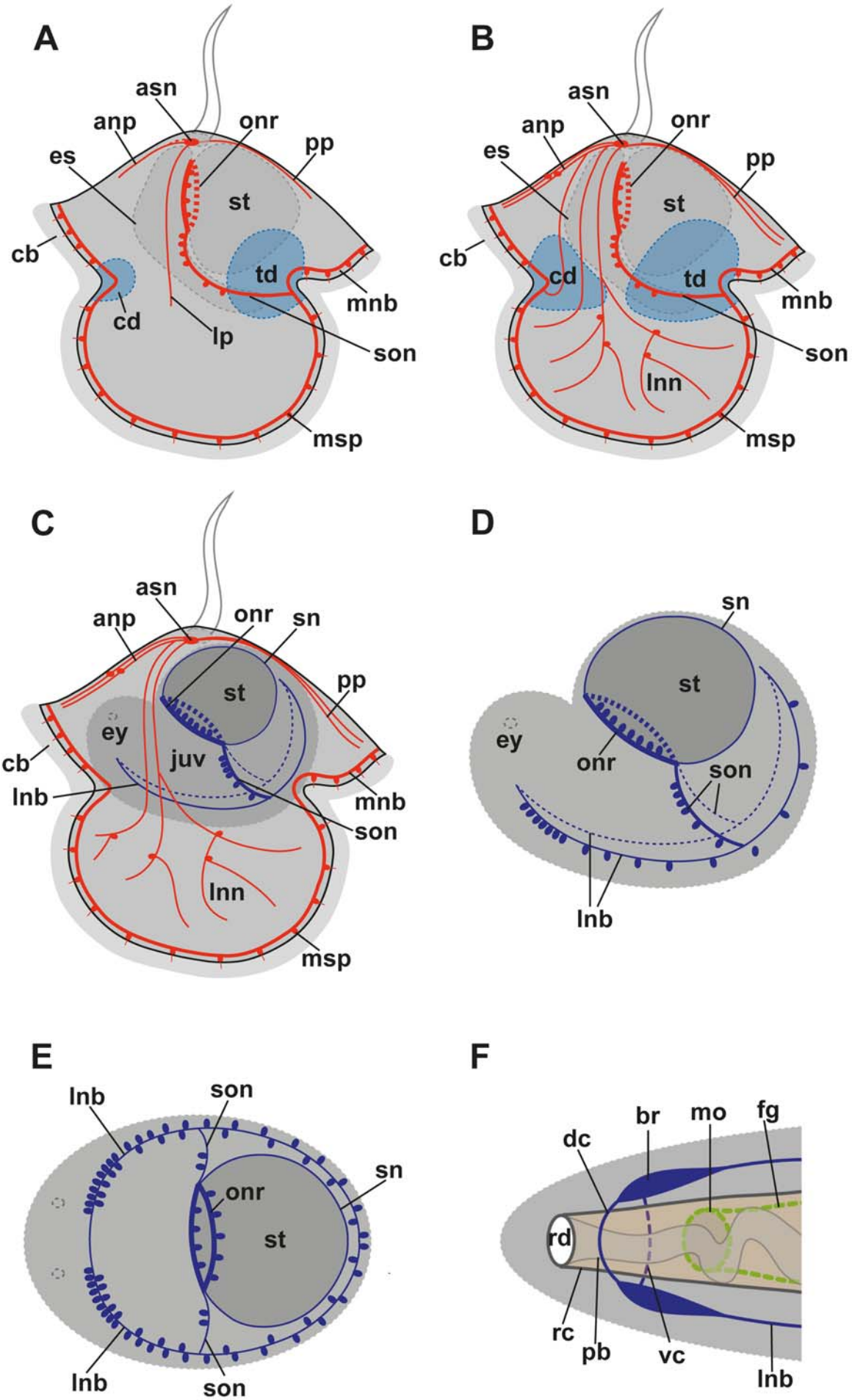
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Figure 5

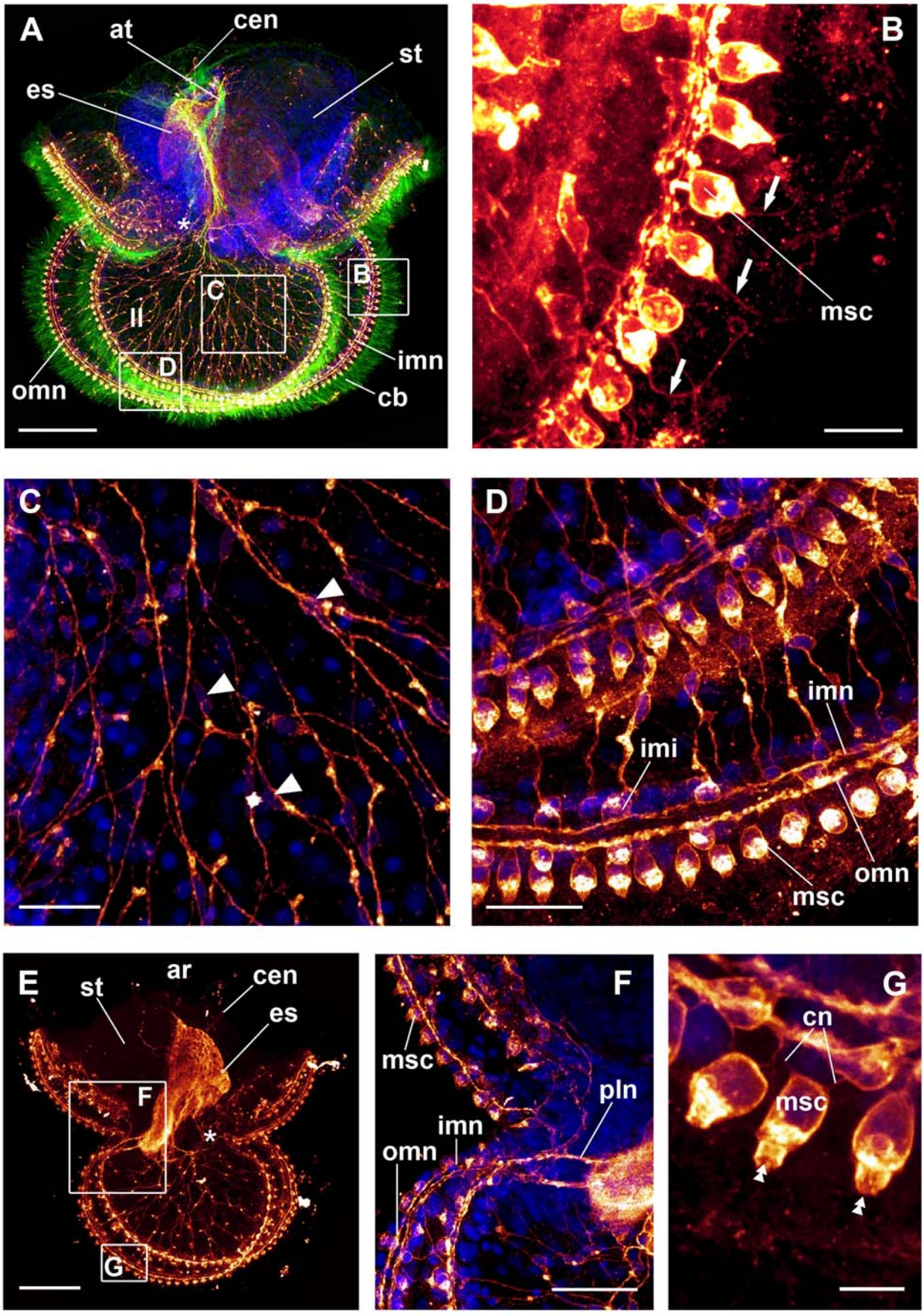
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Figure 6

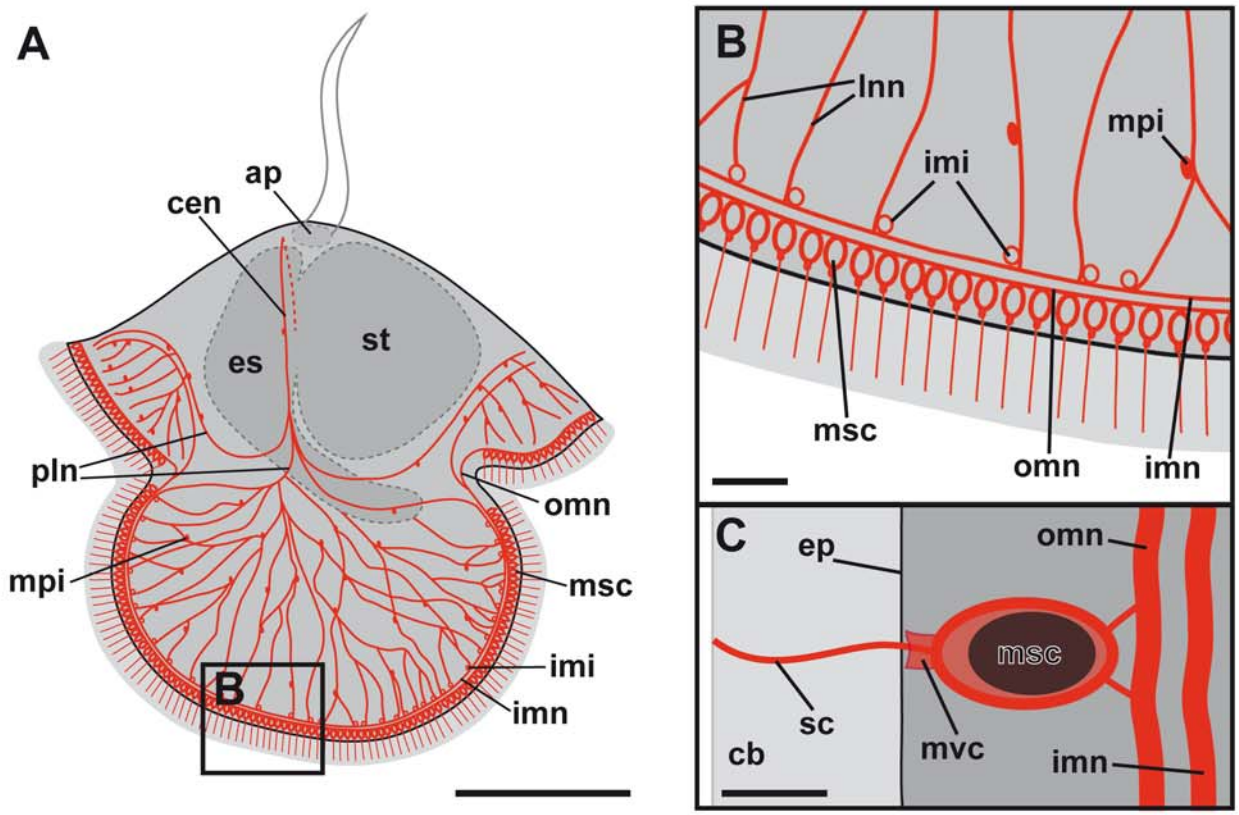
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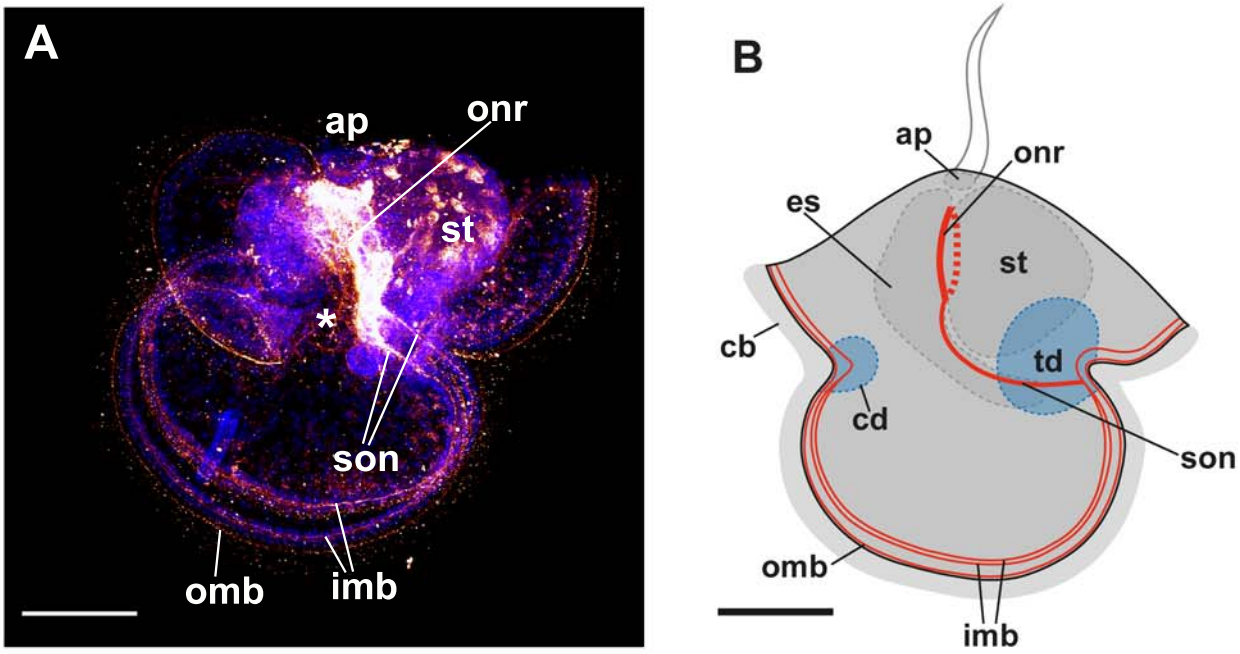
Figure 7



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AUSBILDUNG

Seit 10/05 **Universität Wien**

Diplomstudium der Zoologie

- Schwerpunkt: Morphologie und Entwicklungsbiologie bei Invertebraten
- Diplomarbeit: Die Entwicklung des larvalen Nervensystems bei Pilidiophora (Lophotrochozoa, Nemertea)
- Abschluss: voraussichtlich Herbst 2012

Diplomstudium Genetik und Mikrobiologie

09/97 – 06/05 **Bundesrealgymnasium** (Matura mit Auszeichnung)

BERUFSERFAHRUNG

Lehrtätigkeit

SS 2011, SS 2012	Tutor für den Kurs „Baupläne der Tiere 2" an der Universität Wien
WS 2008- WS 2011	Tutor für den Kurs „Baupläne der Tiere 1" an der Universität Wien

Facheinschlägige Berufserfahrung

02/11-06/11	Praktikum am FIWI Wien, betreut durch Prof. Thomas Ruf, Veterinärmedizinische Universität Wien
07/07	Praktikum am Biologiezentrum Linz in der Abteilung für Entomologie, betreut durch Dr. Gusenleitner, Linz
09/06	Praktikum am Biologiezentrum Linz in der Abteilung für Mikrobiologie und Genetik, betreut durch Dr. Pfosser, Linz

AUSLANDSAUFENTHALTE

08/12 – 09/12	Summer School on Comparative and Functional Neuroanatomy and Neurobiology of Invertebrates. White Sea Biological Station; Moscow State University, Russia
08/12	Sammelreise nach Kristineberg, Schweden
02/12	Individualreise, mit dem Rucksack durch Indien
02/10-06/10	Auslandssemester im Zuge des Programmes ERASMUS an der Universität von Rom Tor Vergata

STUDIENBEGLEITENDE TÄTIGKEIT

Seit 10/05	Nachhilfeunterricht in den Fächern Mathematik und Englisch

PROJEKTPRAKTIKA

04/12	„Experimentelle Embryologie“
11/10 - 01/11	„Histologisches Praktikum”
08/09 - 09/09	„Schutz und Arterhaltung der marinen Schildkröte *Caretta caretta*” (theoretischer Teil in Wien, 4 Wochen praktischer Kurs in der Türkei)
10/09 - 11/09	„Submikroskopische Anatomie und Präparationstechniken”
10/08 - 12/08	„Bioakustik”
03/08 - 06/08	„Tierbeobachtung im Schönbrunner Zoo”
02/07	„Übungen in Mikrobiologie und Genetik

KOMPETENZEN

Methoden

Mikroskopie:

- Confocal laserscanning microscopy
- Transmission electron microscopy
- Scanning electron microscopy

Schneidetechniken:

- Ultra-thin sectioning
- Semi-thin sectioning

Färbetechniken:

- Immunocytochemie
- Histologische Färbetechniken
- Tracer injection (back- & front-filling)

Neurophysiologische Techniken:

Untersuchungen des Miniaturendplattenpotentials

Analyse der Calcium-abhängigen Biolumineszenz

Software skills:

- 3D Rekonstruktion mit IMARIS
- Adobe Illustrator CS5
- Adobe Photoshop CS5
- Grundkenntnisse MS Office

Molekularbiologische Techniken:

- DNA- Extraktion
- PCR
- DNA- Sequenzierung
- Zellmembran - Isolation
- Enzym Assay (photometrische Bestimmung)
- Proteinbestimmung

Sprachen

Deutsch	Muttersprache
Englisch	verhandlungssicher
Italienisch	fließend in Wort und Schrift

STIPENDIEN

10/08 - 10/09	Leistungsstipendium der Universität Wien

INTERESSEN

Oper und klassische Musik, Individualreisen, biologische Sachthemen