MASTERARBEIT

Titel der Masterarbeit

„Neurogenesis in *Nucula tumidula* and *Kurtiella bidentata* (Mollusca: Bivalvia) as revealed by immunocytochemistry and confocal laserscanning microscopy"

verfasst von

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Für meine Eltern

die immer für mich da sind

Content

Abstract

Bivalvia is a class of marine and freshwater molluscs that includes clams, oysters, mussels and scallops. The majority of bivalves are filter or suspension feeders and, in contrast to other molluscs, lack a head and radula. To date only very few studies are concerned with the ontogeny of the nervous system in bivalves. In order to shed light on shared and divergent neural features among bivalves, this study provides a description of neurogenesis of representatives of both major bivalve clades, the protobranch *Nucula tumidula* and the autobranch *Kurtiella bidentata*. The larval development of serotonin-, FMRFamide- and α-tubulin-like immunoreactive (lir) components of both species was examined using immunocytochemistry and confocal laserscanning microscopy (CLSM). In *Nucula tumidula*, two serotonin-lir flask-shaped cells are part of the apical organ of the early pericalymma larva. In the late pericalymma three serotonin-lir cells are visible in this region, which degenerate subsequently during metamorphosis together with the test cells of the larva. The shape and the ontogenetic fate of these apical cells and other morphological features, such as the apical tuft, are similar to those of the scaphopod trochophore larva. In contrast, FMRFamide-lir components could not be revealed in any larval stage. In *Kurtiella bidentata* FMRFamide-lir and serotonin-lir elements are first present in the late veliger stage. There are three roundish apical cells with eight neurites projecting into the velum as well as the paired neurites of the visceral nerve cords present. These paired neurites are connected to two cells, the putative *anlagen* of the visceral ganglia. This study highlights the different modes of neurogenesis present in autobranch versus protobranch bivalves. The striking similarities in neurogenesis of the basal bivalve *Nucula* and the scaphopod *Antalis* may support a close bivalve-scaphopod relationship (Diasoma concept), but additional data are necessary to further test for this assumption.

Zusammenfassung

Zur Klasse der Bivalvia (Muscheln) gehören verschiedene marine und limnische Vertreter, wie z.B. Sandmuscheln, Pfahlmuscheln, Jakobsmuscheln oder Austern. Die Mehrheit der Muscheln sind Filtrierer oder Suspensionfresser und besitzen, im Vergleich zu anderen Mollusken, keinen Kopf und keine Radula. Bis heute sind nur wenige Studien über die Ontogenese des Nervensystems bei Muscheln vorhanden. Um Aufschluss über die gemeinsamen und abweichenden neuronalen Merkmale innerhalb der Bivalven zu geben, liefert diese Studie eine Beschreibung der Neurogenese von Vertretern der zwei Hauptgruppen der Bivalven, der protobranchen *Nucula tumidula* und der autobranchen *Kurtiella bidentata*. Die larvale Entwicklung von Serotonin-, FMRFamid- und acetylierten α-Tubulin-enthaltenden immunoreaktiven Komponenten werden mittels Immunozytochemie und konfokaler Laserscanning-Mikroskopie in beiden Vertretern untersucht. In *Nucula tumidula* sind zwei Serotonin-enthaltende flaschen-förmige Zellen Teil des Apikalorgans der frühen Pericalymmalarve. In der späten Pericalymma werden drei Serotonin-enthaltende Zellen sichtbar, welche während der Metamorphose zusammen mit den Hüllzellen der Larve degenerieren. Die Form und das ontogenetische Schicksal von diesen Apikalzellen, sowie anderer morphologischer Merkmale, wie der Apikalschopf, sind der Trochophoralarve der Scaphopoden (Kahnfüßer) sehr ähnlich. Im Gegensatz konnten in keinem larvalen Stadium FMRFamid-enthaltende Komponenten erkannt werden. In *Kurtiella bidentata* sind die ersten FMRFamid- und Serotonin-enthaltenden Elemente in der späten Veligerlarve vorhanden. Es sind drei rundliche Zellen mit acht Neuriten, die in das Velum ziehen und paarige Neurite der viszeralen Nervenstränge klar erkennbar. Diese paarigen Neurite sind mit zwei Zellen, den vermutlichen Anlagen der Viszeralganglien, verbunden. Diese Studie beleuchtet die unterschiedlichen Modi der Neurogenese in autobranchen und protobranchen Muscheln. Die markanten Ähnlichkeiten der Neurogenese des basalen Bivalven *Nucula* und des

Scaphopoden *Antalis* könnten eine enge Verwandtschaft zwischen Bivalven und Scaphopoden (Diasoma Konzept) anzeigen. Trotzdem sind noch zusätzliche Daten notwendig, um diese Annahme weiter zu überprüfen.

Introduction

Bivalvia is the second largest class-level taxon within Mollusca. The aquatic animals are laterally compressed with two shell valves, which are dorsally interconnected by a ligament. *Nucula tumidula*, a marine protobranch bivalve mollusc, belongs to the Nuculidae and is widely distributed in the Eastern Atlantic, primarily at bathyal depths of 500-2.000m (Malm, 1861). *Kurtiella bidentata*, a marine autobranch bivalve mollusc, belongs to the Montacutidae and is widespread in mud-filled crevices, in sublittoral and shelf mud and muddy sand down to a depth of 200m, sometimes associated with ophiuroid-dominated communities, but also with sipunculans and polychaetes (Ockelmann and Muus, 1978).

Cleavage in both groups is spiral, the first divisions being asynchronous and unequal. First, a ciliated blastula develops, followed by a gastrula that develops through invagination by epiboly (Strathmann, 1987; Zardus and Martel, 2002). The prototroch, a ciliary band, is formed in the early trochophore stage. At the animal pole a bundle of cilia develops and forms the apical tuft. The telotroch is formed at the vegetal pole from a cluster of ciliated cells (Strathmann, 1987; Zardus and Martel, 2002). After the trochophore stage most marine bivalves develop into a free-swimming veliger and successively into a pediveliger larva (Giribet, 2008). The veliger larvae of autobranch bivalves, such as *Kurtiella bidentata*, have an external velum, which consists of two semicircular ciliated lobes that can be withdrawn into the bivalve shell when the organism is not swimming or feeding. The velum is normally resorbed or discarded at metamorphosis or may be ingested (Gustafson and Lutz, 1992).

In contrast, the larvae of protobranch bivalves differ considerably from the veliger-type larvae of autobranch bivalves. The lecithotrophic pericalymma larvae of protobranch bivalves, such as *Nucula tumidula*, are characterized by a ciliated epithelial covering (test or calymma) that surrounds all definitive adult structures, including the shell (Gustafson and Reid, 1986). The

test cells are cast off at metamorphosis during which the emerging juvenile commences a benthic existence (Zardus and Morse, 1998).

The typical cells of the apical region in many lophotrochozoan larvae are the sensory flask-shaped cells, sometimes supplemented by non-sensory peripheral cells. The flask-shaped cells are usually associated with a ciliary tuft at the anterior pole of the larva, altogether they form the apical organ (Croll and Dickinson, 2004; Wanninger, 2008). Serotonin-lir flask-shaped cells are present in the apical organ of most lophotrochozoan taxa, but FMRFamide-lir flask-shaped cells are less common (Gianordoli, 2013). Also a serotonin-lir nerve ring underlying the prototroch is common in most lophotrochozoan larvae. In Platyhelminthes two serotonin-lir cells in the apical organ from which one axon per cell emerges and runs in posterior direction are present. There, they form contact with such a serotonin-lir nerve ring, which is connected to six serotonin-lir cell bodies that are associated with six ciliated lobes of the larva. While the larval lobes are reduced during metamorphosis, the fate of the larval serotonin-lir nervous system is uncertain (Wanninger, 2008).

Also in all investigated species of the clade Annelida, like larval polychaetes, a serotonin-lir nerve ring underlying the prototroch and an apical organ with a ciliated tuft are present. The number of associated serotonin-lir and FMRFamide-lir cells varies from none to four (Wanninger, 2008).

In molluscs the first immunocytochemical signals are also present in the apical region and/or in the cerebral region of late trochophore larvae (Croll and Dickinson, 2004; Wanninger, 2008). Many of the developing larval neural structures integrate in the adult nervous system, but the flask-shaped cells of the apical organ cease to express immunocytochemical signal or undergo apoptosis during metamorphosis (Croll and Dickinson, 2004).

In many existing studies the nervous system of adult bivalves and other molluscs in general has been described well (e.g. Bullock and Horridge, 1965; Morse and Zardus, 1997; Haszprunar and Götting, 2007), but only little is known about larval nervous systems. This

reflects the general poor knowledge available on molluscan neurogenesis, whereby by far the most comprehensive data stem from gastropods (Page, 2002; Croll, 2006; Page, 2006; Wollesen et al., 2007; Page, 2009; Kristof and Klussmann-Kolb, 2010).

The present work provides a detailed description of nervous structures of the larvae of a protobranch and an autobranch bivalve. The neurogenesis in the larvae of *Nucula tumidula* from the trochophore until after metamorphosis and in *Kurtiella bidentata* from the early veliger until the late veliger stage was examined. Antibodies against serotonin, FMRFamide and acetylated α-tubulin were applied and for the analysis a confocal laserscanning microscope was used. This study fills significant gaps in our knowledge on neurogenesis in one of the major molluscan lineages and thereby contributes to the discussion concerning putative plesiomorphic and apomorphic neural characters in Bivalvia, Conchifera and the entire Mollusca.

Material and Methods

Animals and immunocytochemical staining

Adult *Nucula tumidula* and *Kurtiella bidentata* were extracted from sediment samples collected with a hyperbenthic sled at 180-220m water depth on muddy seafloor in Hauglandsosen (Bergen, Norway) and transported to and maintained in bowles with seawater from the native habitat at the Department of Integrative Zoology, University of Vienna. Adults were harvested in November and December 2012 and in December 2013. Spawning of adult bivalves was thermo-stimulated by heat-shocks. Released oocytes were rinsed in UV-sterilized, filtered sea water (8°C). The oocytes were fertilized and water was changed every two days. The fertilized eggs were transferred into Petri dishes with natural sea water and antibiotics (0.06g Penicillin G (Sigma Aldrich, Brøndby, Denmark) and 0.05g Streptomycinsulfate (Sigma Aldrich, Brøndby, Denmark) in 1.000ml sea water). The larvae were collected at specific time intervals. Specimens were relaxed in 4% $MgCl_2$, fixed in 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB) for 30-45 minutes at room temperature and were then rinsed thrice in 0.1M PB and stored in 0.1M PB + 0.1% NaN_3 at 4°C. For decalcification, the late-stage larvae were incubated in 0.05 EGTA overnight at 4°C. Permeabilization of the specimens was performed by rinsing them three times (30 minutes each) in PBT (0.1M PB + 2% Triton X-100) and non-specific binding sites were blocked overnight in 0.1M PB (pH 7.3) with 2% Triton X-100, 0.25% bovine serum albumin (Roth; Karlsruhe, Germany) and 10% normal goat serum (NGS; Invitrogen; Molecular Probes, Eugene, OR, USA) (=blockPBT) at 4°C. The larvae were double-labelled by incubating them for 24h in anti-Serotonin (Immunostar; Hudson, WI, USA) or anti-FMRFamide (Biotrend; Cologne, Germany) and anti-acetylated α-tubulin antibodies (Sigma; St. Louis, MO, USA) at 4°C with a dilution of 1:900 (for anti-acetylated α-Tubulin) and 1:300 (for anti-Serotonin and anti-FMRFamide) in 0.1M PB, 2% Triton X-100, 0.25% bovine serum albumin and 10%

NGS (=block PBT). Afterwards, the larvae were washed for a minimum of 4x30 minutes in PBT (0.1M PB, 2% Triton X-100) at RT. Subsequently, the larvae were incubated overnight at 4°C in a 1:1.500 dilution of goat anti-rabbit Alexa Fluor 568 (Invitrogen; Molecular Probes, Eugene, OR, USA), goat anti-mouse Alexa Fluor 633 (Invitrogen; Molecular Probes, Eugene, OR, USA) and the cell nuclei were stained with Hoechst (Sigma; St. Louis, MO, USA) in blockPBT. Prior to mounting in Fluoromount-G (Southern Biotech; Birmingham, AL, USA) or Vectashield (Vector Laboratories; Burlingame, CA, USA), the stained larvae were rinsed 5x15 minutes in 0.1M PB at RT. The stained specimens mounted in Fluoromount-G (Southern Biotech; Birmingham, AL, USA) on glass slides were stored at 4°C for at least three to four days prior to analysis to allow for penetration of the mounting medium. The stained specimens mounted in Vectashield (Vector Laboratories; Burlingame, CA, USA) were examined immediately. Negative controls were made by omitting either the first or the second antibody or both and yielded no fluorescence signal.

Analysis and digital image processing

Specimens were analyzed using a Leica TCS SP5 II confocal laserscanning microscope (CLSM) (Leica Microsytems; Wetzlar, Germany). The optical sections between 0.3 and 0.5µm of the whole mounts were generated and digitally merged to maximum projections. Then, snapshots of the stacks of maximum projections were made and exported as TIFF images for illustrations.

Fiji (ImageJ; Bethesda, MD, USA) was used to analyse the confocal stacks for the depiction of the 3-dimensionality of the larvae and to accentuate the parts of interest. The adjustments and optimization of contrast and brightness of the images were performed with LAS AF (Leica Microsystems), Fiji (ImageJ) and Adobe Photoshop CS6 (Adobe Systems; San Jose, California, USA). For the arrangement into plates, Adobe Photoshop CS6 (Adobe Systems;

San Jose, California, USA) was used. The schematic line drawings were created with Adobe

Illustrator CS5 (Adobe Systems; San Jose, California, USA).

Results

General aspects of larval development in *Nucula tumidula* and *Kurtiella bidentata*

In *Nucula tumidula* the almost roundish one day old trochophore larva is ciliated on the whole surface and lacks a distinct apical tuft (Figure 1A). In the three days old pericalymma larva the individual ciliated bands become weakly visible (Figure 1B). In the six and seven days old larva the rows of ciliated test cells are clearly recognizable (Figure 1C, D). The larva has three ciliated bands in the median region and ciliated fields at the anterior and posterior pole. The surface of the ciliated field on the posterior pole is larger than that on the anterior pole (Figure 1C, D, F). In the posterior region there is the mouth opening and the anlage of the gut with a single tube, which leads into the stomach (Figure 1C). In the nine days old pericalymma larva three dominant bands of cilia and the ciliated fields are visible (Figure 1F). In all stages there is a ciliated apical tuft visible (Figure 1B, C, D, F). The 15 days old larva of *Nucula tumidula* undergoes metamorphosis and shows the degenerating ciliated test cells at the anterior part of the now clearly visible juvenile mussel with the ligament interconnecting the shells (Figures 2C, D; 3C, D; 4B). From this stage on the gut is U-shaped with a mouth opening and an anus (Figure 2D). The postmetamorphic stage shows almost no cilia at the outer surface, inside only the gut is visible (Figure 3C, D).

In *Kurtiella bidentata* the 15 days to 24 days old veliger larva shows a very prominent ciliated velum, the ligament of the shells, the mouth, the curved U-shaped gut and the anus (Figures 5A; 6C, D). The ciliated apical tuft is clearly discernable (Figure 6C).

Development of the serotonin-like immunoreactive (serotonin-lir) nervous system in *Nucula tumidula*

In one day to five days old larvae no serotonin-lir signals are recognizable. The first serotonin-lir cells appear in the six days to seven days old pericalymma larva in the apical region. Two sensory, flask-shaped cells with a ciliary tuft form the apical organ. These cells are interconnected and form a horseshoe-like arrangement (Figures 1E; 2A, B; 3A; 4A). In nine days and 12 days old pericalymma larvae there are three interconnected flask-shaped cells visible. In two of them serotonin is expressed stronger than in the third one (Figure 2A, B). In 15 days old larva, at the beginning of metamorphosis, three serotonin-lir cells in the degenerating test cells are distinguishable. Two cells are flask-shaped and still interconnected. The third cell is roundish and has no connection to the other cells anymore (Figures 2E, F; 3A; 4B). Later, the serotonin-lir cells fuse during the degeneration of the test cells (Figure 3B). Surprisingly, in early juveniles after metamorphosis no serotonin-lir signal was present (Figure 3E).

The pericalymma larvae and the postmetamorphic stage of *Nucula tumidula* show no FMRFamide-lir signals during their development.

Development of the FMRFamide-like immunoreactive (FMRFamide-lir) and the serotonin-like immunoreactive (serotonin-lir) nervous system in *Kurtiella bidentata*

In the 15 days old veliger larvae three interconnected FMRFamide-lir cells in the apical region are recognizable. From these oval apical cells four arrow-like neurites project towards the cilia in each half of the extended velum. Two paired neurites of the visceral nerve cords project towards the posterior pole of the larva and form contact with two roundish to oval, serotonin-lir cells, the putative *anlagen* of the visceral ganglia (Figures 4C, D; 5B, C).

However, no serotonin-lir components were found at this developmental stage. In the 16 days to 24 days old larvae there are always the same three interconnected FMRFamide-lir and serotonin-lir apical cells present (Figure 6A, B, E). The 16 days old veliger larva shows only one serotonin-lir neurite that projects in direction of the velum (Figure 5D). In the 17 days old larva also paired serotonin-lir neurites of the visceral nerve cords are recognizable (Figure 6A). In the 21 days old veliger larva one serotonin-lir cell, most likely the *anlage* of the visceral ganglia, is visible (Figure 6B). In the 24 days old veliger are again paired FMRFamide-lir cells, the putative *anlagen* of the visceral ganglia, at the posterior end of the visceral nerve cords distinguishable (Figure 6E).

Acetylated α-tubulin only labelled the cilia, no signal was expressed in the nervous system in any of the two species investigated.

Discussion

Comparison of serotonin-lir and FMRFamide-lir neurogenesis of *Kurtiella bidentata* larvae and *Nucula tumidula* larvae with other bivalves

To date, there are only scarce data of bivalve neurogenesis available. Most data concern the neuronal development of serotonin-, FMRFamide-lir and catecholamine-containing cells of *Mytilus trossulus* (Voronezhskaya, 2008), the catecholamine-containing cells in larval and postlarval *Mytilus edulis* and *Placopecten magellanicus* (Croll et al., 1997) and the development of serotonin- and FMRFamide-lir cells of *Lyrodus pedicellatus* (Gianordoli, 2013). In all investigated species an immunoreactive apical organ was found. In *Mytilus trossulus* the apical organ contains four serotonin-lir and five FMRFamide-lir flask-shaped cells and in *Mytilus edulis* two flask-shaped cells were found. Also in the veliger of *Placopecten magellanicus* a pair of catecholamine-containing, flask-shaped apical cells was found (Croll et al., 1997). In contrast, in the teridinid *Lyrodus pedicellatus* three serotonin-lir, two FMRFamide-lir flask-shaped cells and two non-sensory peripheral cells are present (Gianordoli, 2013). The investigated veliger larva of the autobranch bivalve *Kurtiella bidentata* shows three serotonin-lir and FMRFamide-lir apical cells. In all investigated autobranch bivalves the cerebral ganglia develops at the base of the previous apical organ. In *Mytilus trossulus* the serotonin-lir signal is restricted to the cerebral ganglia and no other serotonin-lir structures were found (Voronezhskaya, 2008). The pleural ganglia in *Lyrodus pedicellatus* larvae show only an FMRFamide-lir signal, no serotonin-lir was observed. In most bivalves, the pleural and cerebral ganglia fuse in the late veliger stage to a combined cerebro-pleural ganglion (Gianordoli, 2013). In *Lyrodus pedicellatus* and *Mytilus edulis* the pedal ganglia express a serotonin-lir and an FMRFamide-lir signal, in *Mytilus trossulus* they express only an FMRFamide-lir signal (Croll et al., 1997; Voronezhskaya, 2008; Gianordoli, 2013).

In the 15 days to 24 days old veliger larva of *Kurtiella bidentata* no serotonin-lir or FMRFamide-lir signal was found in the region of cerebral, pleural and pedal ganglia. However, paired neurites of the visceral nerve cords with two visceral cells, likely the *anlagen* of the visceral ganglia, were present. Also in *Lyrodus pedicellatus* an FMRFamide-lir and serotonin-lir signal appears in the visceral region (Gianordoli, 2013). In *Mytilus trossulus* only an FMRFamide-lir signal was found in this region and in *Mytilus edulis* no immunorective visceral ganglia are present (Croll et al., 1997; Voronezhskaya, 2008). The peripheral innervation, such as the eight FMRFamide-lir neurites of the velum in the 15 days old veliger of *Kurtiella bidentata* is only expressed in *Mytilus edulis*. In *Mytilus trossulus* an FMRFamide-lir signal appears in the region, where the foot would later develop and in *Lyrodus pedicellatus* FMRFamide-lir and serotonin-lir innervation of the foot is distinguishable (Voronezhskaya, 2008; Gianordoli, 2013). The cerebro-pedal and cerebro-visceral connectives of the ganglia are developed in all specimens, but in *Mytilus trossulus* no serotonin-lir connectives are present. In *Kurtiella bidentata* only paired FMRFamide-lir and serotonin-lir neurites of the nerve cords were found.

It is also mentionable that the pedal strands of the late veliger larva of *Mytilus trossulus* seem to be interpretated in a wrong way by Voronezhskaya et al., because these strands proceed exclusively into the visceral region and not into the foot region. In truth the assumed pedal strands are visceral strands.

In the early pericalymma larvae of the protobranch bivalve *Nucula tumidula* only two serotonin-lir interconnected, flask-shaped apical cells and no FMRFamide-lir cells are present. Later in the older pericalymma and during metamorphosis three serotonin-lir apical cells are visible. Differently to the autobranch bivalves mentioned before, such as *Mytilus edulis* and *Placopecten magellanicus* (Croll et al., 1997), the cerebral ganglia of *Nucula tumidula* do not develop in the region of the previous apical organ. In the pericalymma of *Nucula tumidula* the whole apical cells degenerate with the test cells during the development

and the adult nervous system seems to develop entirely independently of the apical organ. But the *anlagen* of the cerebral ganglia could also exist, although there are no immunoreactive signals present. Also in the pericalymma and postmetamorphic stage of *Nucula tumidula* no serotonin-lir or FMRFamide-lir signal could be found in the region of cerebral, pleural and pedal ganglia.

The comparison within this work between the representatives of the two major bivalve clades, the protobranch bivalve *Nucula tumidula* and the autobranch bivalve *Kurtiella bidentata*, shows, that there are many differences in morphology and neuronal development. In the veliger larva of *Kurtiella bidentata* there are three interconnected serotonin-lir and FMRFamide-lir apical cells and also other neuronal structures, such as neurites and visceral nerve cords, detectable. In the pericalymma larva of *Nucula tumidula* only serotonin-lir signals, such as three serotonin-lir apical cells, are present.

Comparison of serotonin-lir and FMRFamide-lir neurogenesis of bivalves with other Conchifera (gastropods, scaphopods) and Polyplacophora

In Scaphopoda (Wanninger and Haszprunar, 2003), Gastropoda (Kempf et al., 1997; Page, 2002; Croll, 2006; Page, 2006; Wollesen et al., 2007; Page, 2009; Kristof and Klussmann-Kolb, 2010) and in Polyplacophora (Friedrich et al., 2002; Voronezhskaya, 2002) the first immunoreactive signal always appears in the apical organ. In all studies the apical organ differs in complexity and structure.

In basal gastropod molluscs, such as the patellogastropod *Tectura scutum*, the apical organ of the veliger larva consists of three large roundish cells that generate a very long apical ciliary tuft, two cells that generate a bilateral pair of shorter ciliary tufts, and an apical ganglion. Putative sensory neurons forming the ganglion give rise to neurites that extend to the apical surface of the larva and to basal neurites that contribute to a neuropil (Page, 2002). Serotonin-lir signals are expressed by a medial and two lateral neurons, all having an apical projection,

and also by neurites within the neuropil and by peripheral neurites that run beneath the ciliated prototrochal cells (Page, 2002). In the larval abalone *Haliotis kamtschatkana* one to three serotonon-lir cells of the apical organ are present (Page, 2006). The neritimorph gastropod *Nerita melanotragus* has special cilia-filled pockets embedded within the apical ganglion of the apical organ. These so-called "sensory cups" are cassettes of multiple cells: one supporting cell and up to three multiciliated sensory cells. There are also two serotonin-lir non-sensory neurons within this apical organ (Page, 2009).

In higher evolved gastropod molluscs, such as *Phestilla sibogae* (Croll, 2006), *Aeolidiella stephania* (Kristof and Klussmann-Kolb, 2010) and other nudibranchs (Kempf et al., 1997) three serotonin-lir flask-shaped and two non-sensory round cells are present. Also in the mid-veliger stage of *Lyrodus pedicellatus* (Gianordoli, 2013) the same serotonin-lir cell cluster appears and two FMRFamide-lir flask-shaped plus two round cells are present. In *Aeolidiella stephania* (Kristof and Klussmann-Kolb, 2010) again three FMRFamide-lir flask-shaped and two round cells are detectable.

In Polyplacophora more flask-shaped apical cells and peripheral cells are obviously present. In *Mopalia muscosa* three to eight serotonin-lir flask-shaped sensory cells and two to four non-sensory cells are present, in this study there is also an FMRFamide-lir apical organ present, but no concrete cell number is mentioned (Friedrich et al., 2002). In *Ischnochiton hakodadensis* there are up to 14 serotonin-lir flask-shaped apical cells and six non-sensory apical cells detectable. There are also four to six FMRFamide-lir flask-shaped cells and four non-sensory cells in the apical organ present (Voronezhskaya, 2002). In *Kurtiella bidentata* three FMRFamide-lir and serotonin-lir apical cells are present. In the early pericalymma larva of *Nucula tumidula* two, later three, only serotonin-lir flask-shaped cells of the apical organ are developed.

In the early pericalymma larva of *Nucula tumidula* only two serotonin-lir apical cells are present and in the trochophore larva of the scaphopod *Antalis entalis* the neuronal

development also starts with the establishment of two flask-shaped serotonin-lir cells in the apical organ (Wanninger and Haszprunar, 2003). The somata of these cells merge at their bases in both groups. Later in the 9 days old pericalymma a third serotonin-lir cell develops. In the older *Antalis entalis* larva the fully established apical serotonin-lir nervous system consists of four central cells, which are connected to two lateral non apical cells. In comparison to larvae of other clades of the Mollusca the serotonin-lir complex of the apical organ of the bivalve *Nucula tumidula* and the scaphopod *Antalis entalis* is relatively simply organized. During metamorphosis of the protobranch bivalve *Nucula tumidula* all three apical cells degenerate with the test cells and are no longer part of the nervous system of the juvenile mussel.

During development of *Nucula tumidula* no larval FMRFamide-lir signals were found in the larval nervous system. The first signal of the neurotransmitter FMRFamide in *Antalis entalis* is found in the *anlage* of the future cerebral nervous system of the juvenile. Thus, there are neither distinct larval FMRFamide-lir bearing neuronal components in the scaphopod *Antalis entalis* nor in the protobranch bivalve *Nucula tumidula*. As in the vast majority of indirect developing mollusks, the larval neuroanatomy in *Antalis entalis* shows a typical mosaic character with a combination of already present adult serotonin-lir and FMRFamide-lir as well as strictly larval (serotonin-lir) neuronal features (Wanninger and Haszprunar, 2003). In the early postmetamorphic stage of *Nucula tumidula* no serotonin-lir or FMRFamide-lir signals are expressed, while in *Antalis entalis* both neurotransmitters are present in the early juvenile. In the trochophore larva of the scaphopod *Antalis entalis* early neuronal development starts at the apical pole and proceeds in anterior to posterior direction (Wanninger and Haszprunar, 2003). Interestingly the neurogenesis of the protobranch bivalve *Nucula tumidula* shows more similarities with the nervous development of scaphopods than with other bivalves.

In contrast to the results in *Kurtiella bidentata* no FMRFamide-lir cells appear in the apical organ and no peripheral larval FMRFamide-lir nerve elements were found in *Antalis entalis*. In this species, all the FMRFamide-lir neurons from earliest detection belong exclusively to the developing adult nervous system.

Conclusion

In summary, the neurogenesis of the pericalymma larva of the protobranch bivalve *Nucula tumidula* is different to the common and frequent neuronal development of the typical molluscan larvae, like the veliger larva of the autobranch bivalve *Kurtiella bidentata*. The results of this work show surprising similarities during neurogenesis of the basal protobranch bivalve *Nucula tumidula* and the scaphopod *Antalis entalis*. Parallel to these neural conditions no distinct, truly larval muscle, contrary to Gastropoda or autobranch bivalves, has been identified in the protobranch bivalve *Nucula tumidula*. Also in the scaphopod *Antalis entalis* no truly larval muscle has been found, only young adult muscles could be identified (Wanninger and Haszprunar, 2002). This may indicate that the last common ancestor of scaphopods and bivalves had a lecithotrophic larva with a simple nervous system comprising two to four serotonin-lir (but not FMRFamide-lir) flask-shaped cells in the apical organ and was devoid of specific larval retractor systems. This would imply that the complex larval retractor muscles commonly found in gastropod and autobranch bivalve larvae evolved independently. Moreover, the similarities of the larval neuromuscular anatomy of protobranch bivalves and scaphopods as revealed here may support a scaphopod-bivalve sister group relationship, thus reviving a classical hypothesis in molluscan phylogeny, the so-called Diasoma concept. However, additional data, particularly on other protobranch bivalves, are necessary to further substantiate this assumption.

Acknowledgement

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Figure Legends

Figure 1: Serotonin-like and acetylated α-tubulin-like immunoreactivity in *Nucula tumidula* larvae from trochophore to nine days post fertilization. Serotonin-like immunoreactivity is in bright-yellow and acetylated α-tubulin-like immunoreactivity in magenta. Images A to F are projections of scans through the entire specimen. Insert in F presents a sagittal section through the projection of an autofluorescence scan. All images are in lateral view; apical is always up. **(A)** Trochophore larva (scale bar 25 μm). The whole larva is covered with ciliated cells (cc). **(B)** A four days old pericalymma larva (scale bar 25 μm). On the surface three ciliated bands (cb) and an apical tuft (at) are established. **(C)** A six days old pericalymma larva (scale bar 25μm). On the anterior pole there is a small ciliated field (cf) with an apical tuft (at) and on the posterior pole a bigger ciliated field (cf) recognizable. Between there are three ciliated bands (cb) present. The larval mouth (lm) is connected with the stomach (st) through the gut (g). **(D)** A seven days old pericalymma larva (scale bar 25 μm). At the anterior pole there is a small ciliated field (cf) with an apical tuft (at) and on the posterior pole a bigger ciliated field (cf) with the larval mouth (lm) recognizable. Between there are three ciliated bands (cb) present. The typical test cells (tc) are very massive. **(E)** A six days old pericalymma larva (scale bar 25μm). The two flask-shaped interconnected cells (*arrowheads*) of the apical organ are present. Also the test cells (tc) and the stomach (st) are visible. **(F)** A nine days old pericalymma larva (scale bar 25μm). Anterior there is a small ciliated field (cf) with an apical tuft (at) visible. The larval mouth (lm) is centrally located in the bigger ciliated field (cf) on the posterior pole. Between there are three dominant ciliated bands (cb) present. The small additive image shows a projection of a sagittal section through an autofluorescence scan of the same larva (scale bar 10 μm).

Figure 2: Serotonin-like and acetylated α-tubulin-like immunoreactivity in *Nucula tumidula* larvae from nine days to 15 days post fertilization. Serotonin-like immunoreactivity is in bright-yellow and acetylated α-tubulin-like immunoreactivity in magenta. Images A, C, D and E are projections of scans through the entire specimen. Image B shows a detail of A and image F shows a detail E. All images, instead of D, are in lateral view; D is in frontal view. The apical side is up in all aspects. **(A)** A nine days old pericalymma larva (scale bar 25 μm). Three nearly flask-shaped cells (*arrowheads*) of the apical organ are expressed. Also the stomach (st) of the larva is detectable. **(B)** Detail of apical region of nine days old larva (scale bar 10 μm). Two of the flask-shaped cells of the apical organ (*arrowheads*) are clearly distinguishable; the third one is only weakly visible. **(C)** A 15 days old pericalymma during metamorphosis (scale bar 25 μm). The larva casts off the degenerating ciliated test cells (tc). **(D)** A frontal view of a larva during metamorphosis (scale bar 25μm). At the anterior pole the degenerating test cells (tc) are visible. Posterior the larval mouth (lm) and the U-shaped gut (g) of the young mussel are observable. **(E)** The larva during metamorphosis (scale bar 25 μm). The larva casts off the degenerating ciliated test cells (tc) with two flask-shaped and one roundish apical cell (*arrowheads*). Also the ligament (li) of the young mussel is visible. **(F)** Detail of the apical region of the larva during metamorphosis (scale bar 10 μm). Two interconnected flask-shaped cells and one roundish apical cell (*arrowheads*) are recognizable.

Figure 3: Serotonin-like and acetylated α-tubulin-like immunoreactivity in *Nucula tumidula* larvae during metamorphosis to the postmetamorph stage. Serotonin-like immunoreactivity is in bright-yellow and acetylated α-tubulin-like immunoreactivity in magenta. All images are projections of scans through the entire specimen and, except for A, are in lateral view; A is in fronto-lateral view. The apical side is always up. **(A)** The larva during metamorphosis (scale bar 25 μm). The larva casts off the degenerating test cells (tc)

with two flask-shaped and one roundish apical cell (*arrowheads*). **(B)** A larva at the end of the metamorphosis (scale bar 25 μm). The larva casts off the rest of the degenerating test cells (tc) with the fused apical cells (*arrowheads*). Also the ligament (li) of the young mussel is visible. **(C)** The larva at the almost finished metamorphosis (scale bar 25 μm). There is only a rest of the degenerating ciliated test cells (tc) visible. The gut and the ligament (li) are recognizable. **(D)** The postmetamorph juvenile (scale bar 25 μm). Only few remaining degenerating test cells (tc) are present. The gut is clearly distinguishable. **(E)** The young adult mussel (scale bar 25 μm). No serotonin-lir or FMRFamide-lir signals are expressed. The ligament (li) is visible on the dorsal side of the mussel.

Figure 4: Schematic drawings of serotonin-lir neurogenesis of *Nucula tumidula* larvae and serotonin- as well as FMRFamide-lir neurogenesis of *Kurtiella bidentata* larvae.

A is a frontal view, C a dorso-ventral view and B, D are lateral views; apical is always up. **(A)** The seven days old pericalymma larva of *Nucula tumidula* in frontal view. The first serotonin-lir cells are visible in the apical region, where two interconnected flask-shaped cells with a ciliary apical tuft form the apical organ. Anteriorly and posteriorly two ciliary fields are visible. In this stage the test cells also form three lateral rows of ciliary bands. The gut with the larval mouth and the roundish stomach is fully developed. **(B)** During metamorphosis of *Nucula tumidula* the larva casts off the degenerating ciliated test cells with two merged, flask-shaped and one roundish apical cell. The newly developed mussel with the shell is visible. **(C, D)** In the dorso-ventral and the lateral view of the 15 days old veliger larva of *Kurtiella bidentata* there are three interconnected FMRFamide-lir/serotonin-lir apical cells and four neurites projecting into each half of the extended velum visible. Connected with these apical cells there are paired neurites of the visceral nerve cords distinguishable. At the end of these neurites two cells, the *anlagen* of the visceral ganglia are visible. Also the shell with the ligament is well developed. (The 24 days old veliger larva is not illustrated in these schematic

drawings, because the general and nervous structure remains the same as in the previous stage. Only the neurites projecting into the velum are missing.)

Abbreviations: ac, apical cells; at, apical tuft; cb, ciliary bands; cf, ciliary fields; g, gut; li, ligament; lm, larval mouth; n, neurites; s, shell; st, stomach; tc, test cells; ve, velum; vg, *anlagen* of the visceral ganglia; vnc, visceral nerve cords

Figure 5: Serotonin-, FMRFamide-, and acetylated α-tubulin-like immunoreactivity in *Kurtiella bidentata* larvae from 15 days to 16 days post fertilization. Serotonin-like and FMRFamide-like immunoreactivity is in bright-yellow and acetylated α-tubulin-like immunoreactivity in magenta. All images are projections of scans through the entire specimen and all are in lateral view. The apical side is always up. Image C shows a detail of B. **(A)** A 15 days post fertilization veliger larva with an extended velum (scale bar 25 µm). Anteriorly the prominent ciliated velum (ve) is visible. The U- shaped gut (g) opens with the larval mouth (lm) and terminates with the larval anus (la). The ligament (li) is present. **(B)** A 15 days post fertilization veliger larva (scale bar 25 µm). Three interconnected FMRFamide-lir apical cells (*arrowheads*) and four neurites (*arrow*) projecting into each half of the velum (ve) are recognizable. Connected with these apical cells there are paired neurites of the visceral nerve cords (vnc) distinguishable. At the end of these neurites two cells, the *anlagen* of the visceral ganglia (vg), are visible. Also the ligament (li) is detectable. **(C)** Detail of the apical region of a 15 days old veliger (scale bar 10 µm). Three interconnected FMRFamide-lir apical cells (*arrowheads*) and four neurites (*arrow*) projecting into the velum (ve) are present. Connected with these apical cells there are paired neurites of the visceral nerve cords (vnc) observable. **(D)** A 16 days post fertilization veliger larva (scale bar 25 µm). Three interconnected serotonin-lir apical cells (*arrowheads*) and one neurite (*arrow*) projecting into the velum (ve) are recognizable. The ligament (li) is present.

Figure 6: Serotonin-, FMRFamide-, and acetylated α-tubulin-like immunoreactivity in *Kurtiella bidentata* larvae from 17 days to 24 days post fertilization. Serotonin-like and FMRFamide-like immunoreactivity is in bright-yellow and acetylated α-tubulin-like immunoreactivity in magenta. All images are projections of scans through the entire specimen and all are in lateral view. The apical side is always up. Image C is a projection of an autofluorescence scan and image D a projection of a brightfield scan. **(A)** A 17 days post fertilization veliger larva with a retracted velum (scale bar 25 μm). The velum (ve) is clearly visible on the anterior side. Centrally two interconnected serotonin-lir apical cells (*arrowheads*) are present. Connected with these apical cells there are paired serotonin-lir neurites of the visceral nerve cords (vnc) distinguishable. Also the ligament (li) and the larval anus are detectable. **(B)** A 21 days old veliger (scale bar 25 μm). Three interconnected serotonin-lir apical cells (*arrowheads*) and the *anlagen* of the visceral ganglia (vg) are recognizable. The larval mouth (lm) and larval anus (la) are also present. The ligament (li) and anterior the velum (ve) are visible. **(C)** A 24 days post fertilization veliger larva (scale bar 25 μm). The ciliated velum (ve) shows a dominant apical tuft (at). The gut (g) opens with the larval mouth (lm) and ends with the larval anus (la). The ligament (li) is also observable. **(D)** A 24 days old veliger larva (scale bar 25 μm). The ciliated velum (ve) is slightly contracted. The gut (g) opens with the larval mouth (lm) and ends with the larval anus (la). The ligament (li) is present. **(E)** A 24 days post fertilization veliger (scale bar 25 μm). The velum (ve) is retracted. Three interconnected FMRFamide-lir apical cells (*arrowheads*) are recognizable. Connected with the apical cells there are paired neurites of the visceral nerve cords (vnc) distinguishable. At the end of these neurites two cells, the *anlagen* of the visceral ganglia (vg), are visible. Also the ligament (li) is detectable.
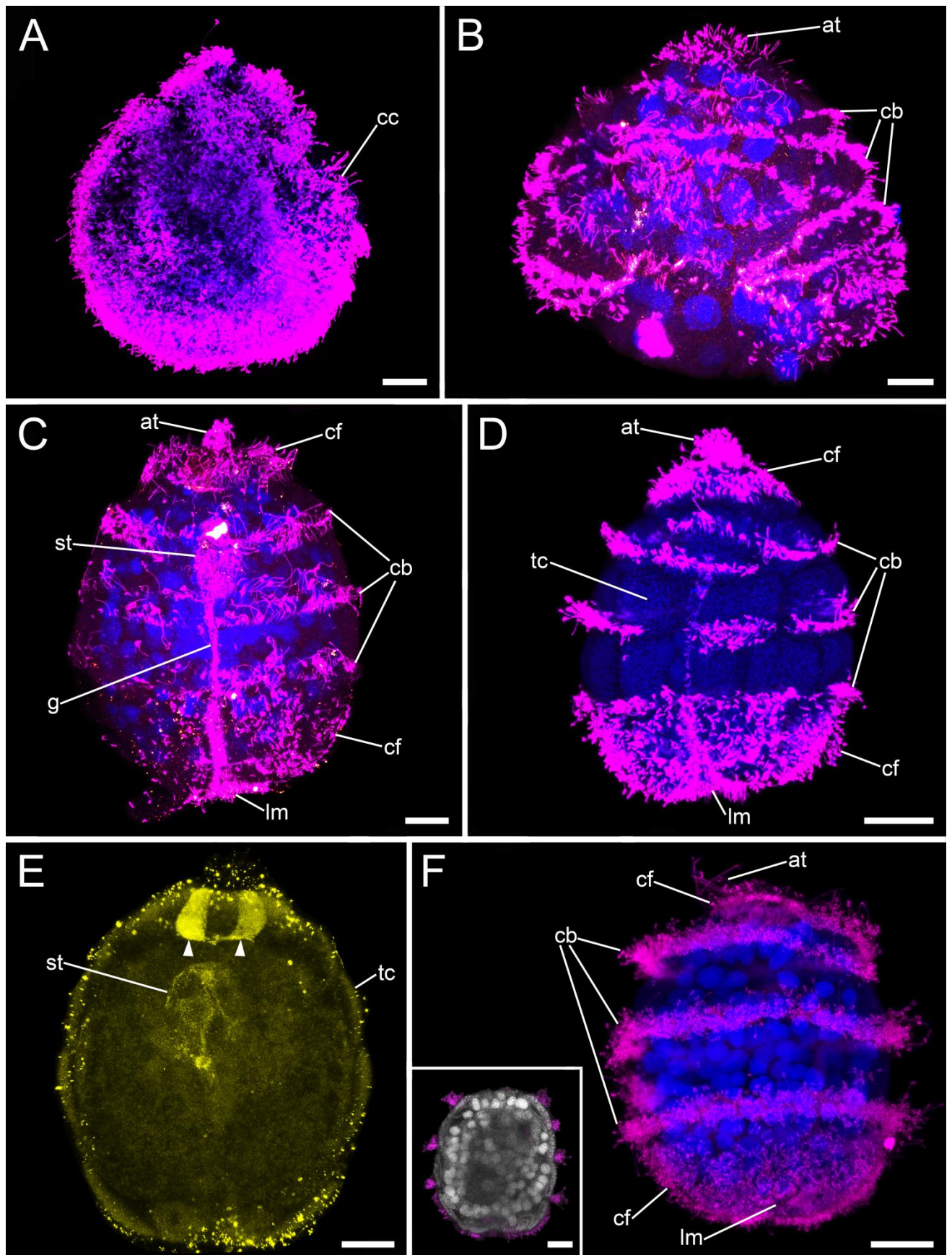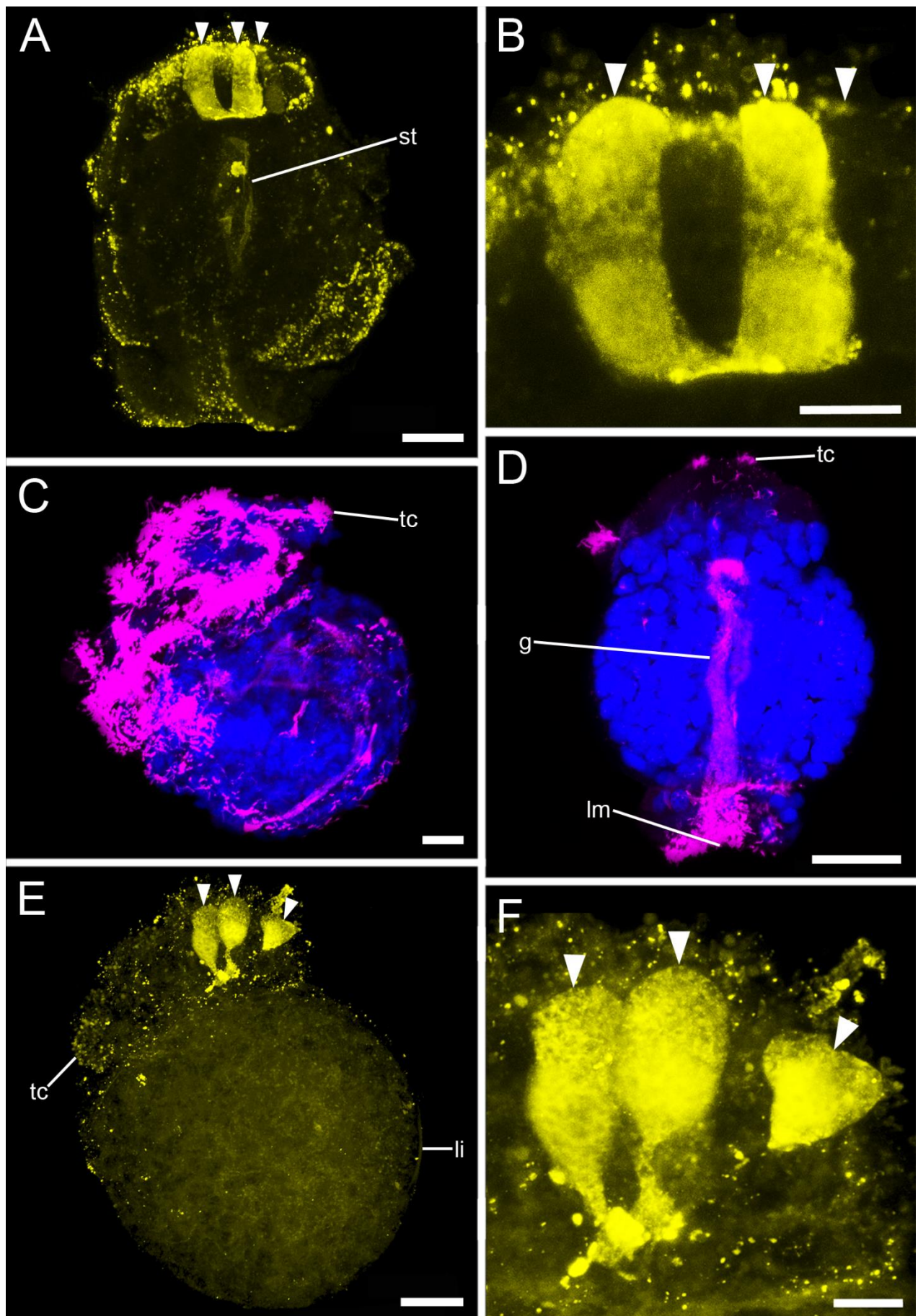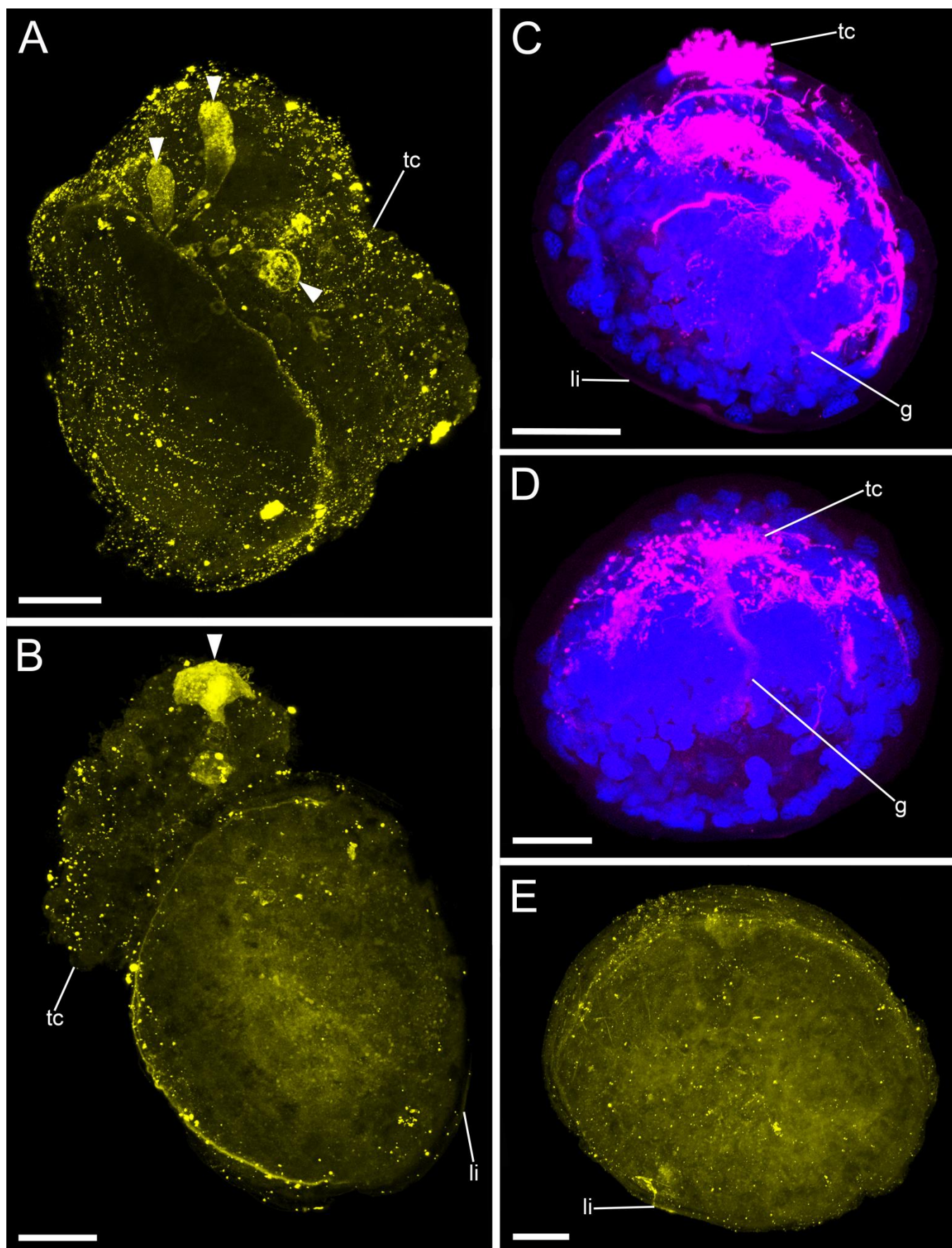
Appendix

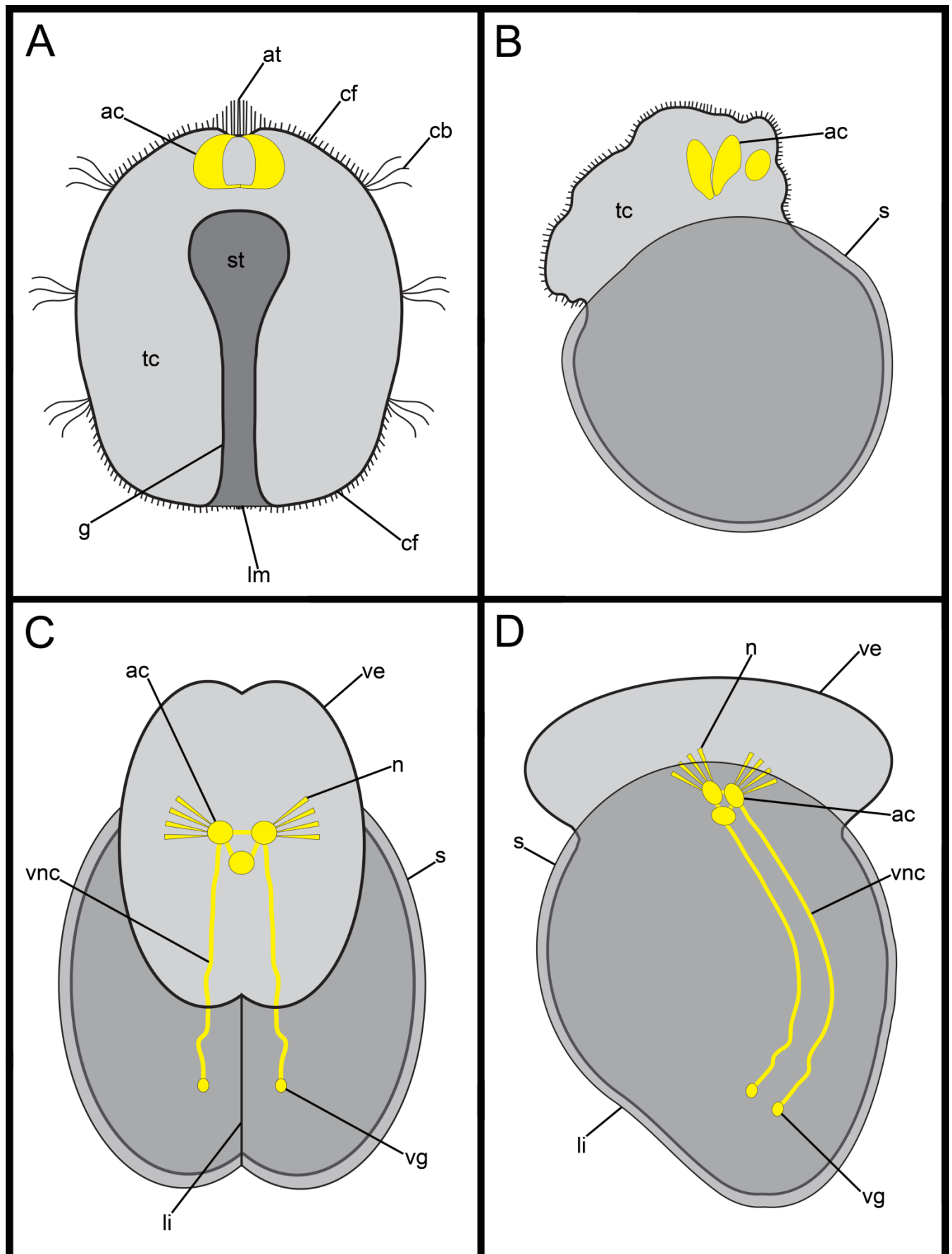Figure 2

Figure 3

Figure 4

Figure 5

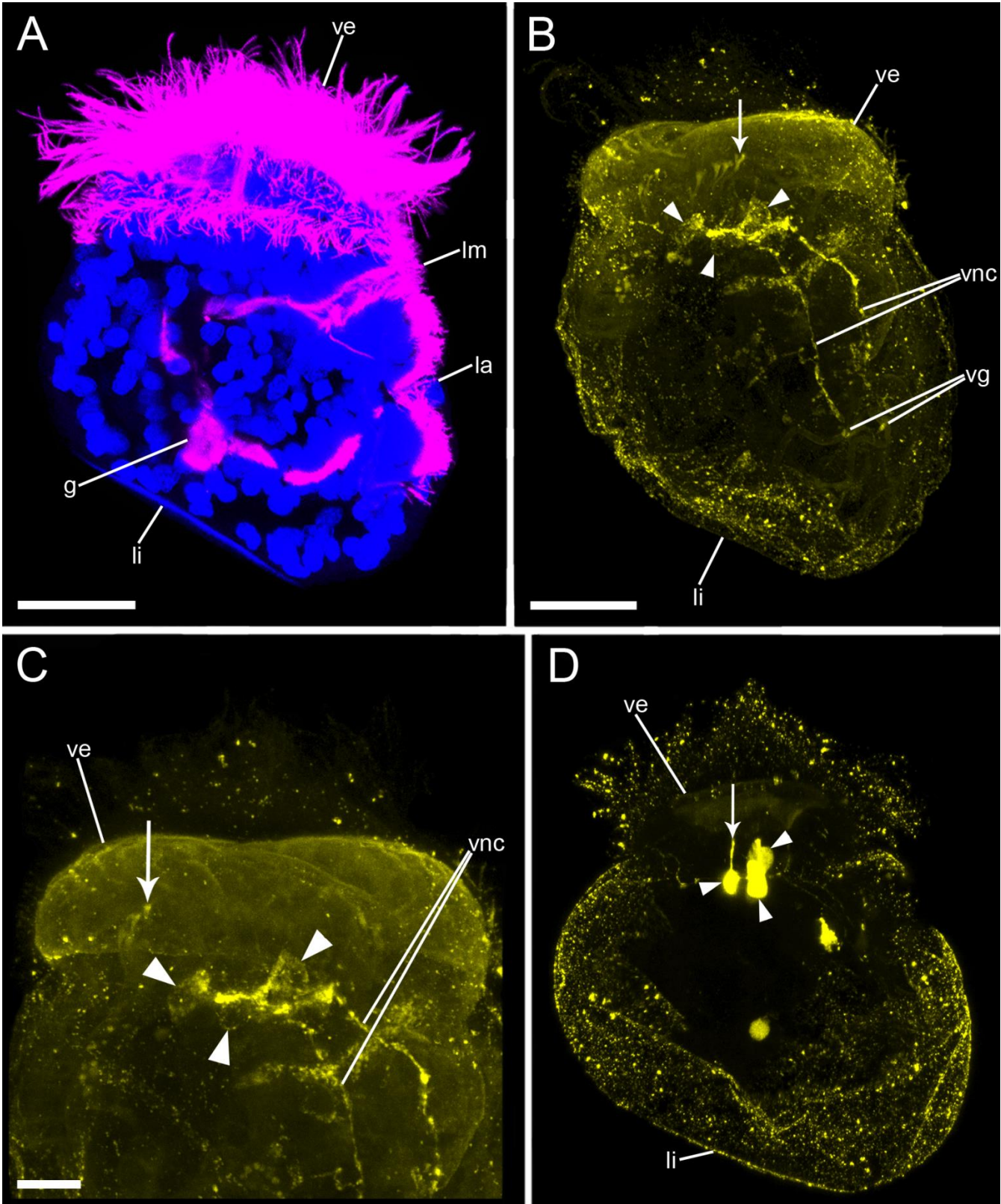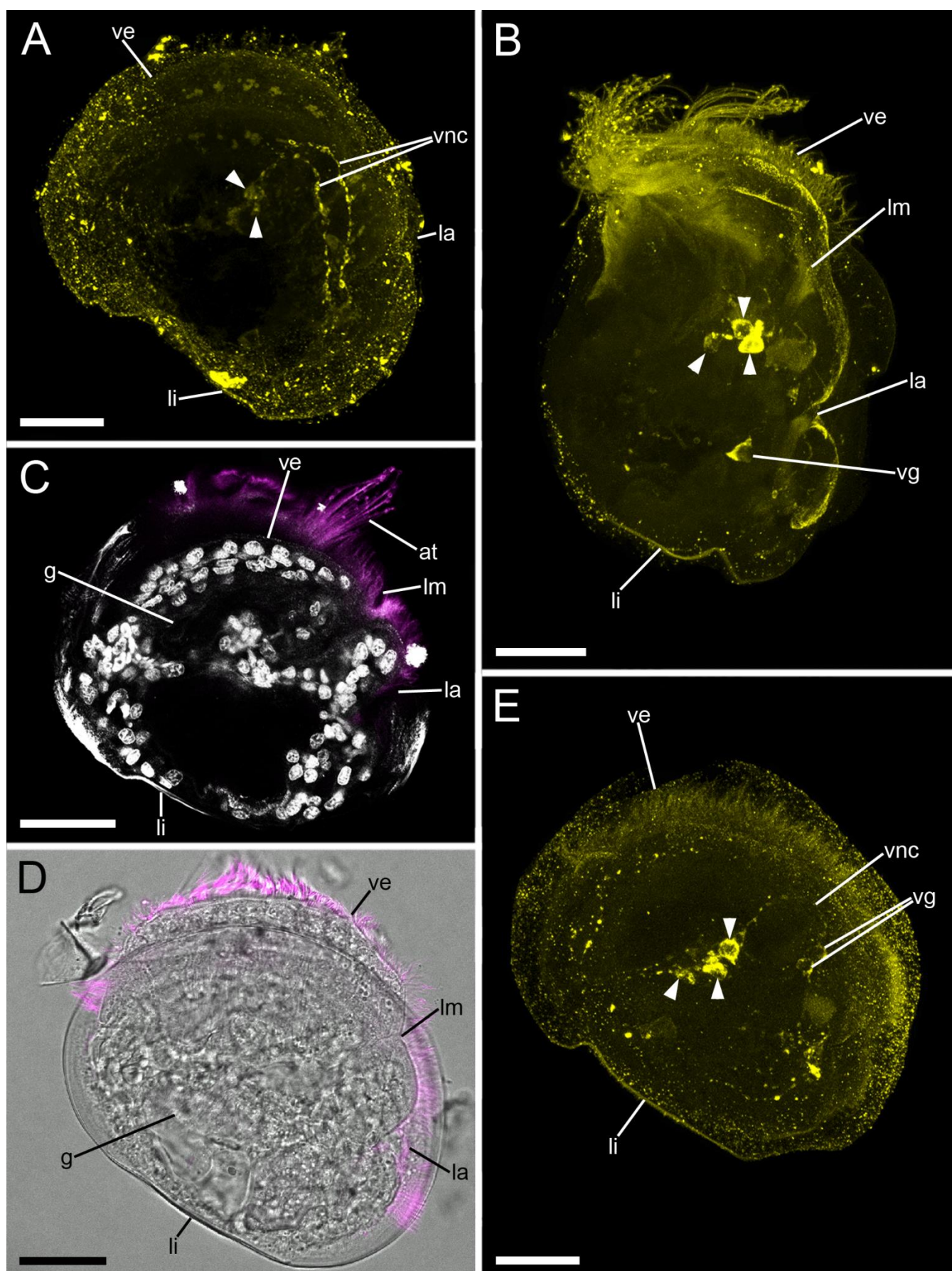Figure 6

Supplementary information

Curriculum Vitae

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Bachelorstudium Biologie

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- Bachelorarbeit: Histologie und Verteilung von Drüsenzellen sowie histochemische Untersuchungen in verschiedenen Epidermisregionen von *Gobiodon histrio* (Valenciennes, 1837) und *Gobiodon* sp. 3 (Actinopterygii: Gobiidae)
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  Anatomie und Physiologie des Menschen

- Masterarbeit: Die Entwicklung des larvalen Nervensystems bei *Nucula tumidula* und *Kurtiella bidentata* (Mollusca: Bivalvia) unter Verwendung der Immunocytochemie und der konfokalen Laserscanning-Mikroskopie

- Abschluss: Herbst 2014

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03/14 Fahrgast- und Verkehrszählungen bzw. Verteilen von Fragekarten im Verkehrsverbund Ost-Region, Intra-Performance® M. Bauer KG

| 08/14 | Pflegepraktikum im Bezirksaltenheim Enns, |
| | Sozialhilfeverband Linz-Land |

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10/10 - 01/11	„Tierbeobachtungen – Verhaltensbiologisches
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11/10 - 01/11	„Histologisches Projektpraktikum"
03/13 - 04/13	„Submikroskopische Anatomie und Präparationstechnik
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ÜBUNGEN

„Chemische Übungen für Biologen"

„Chemisches Rechnen für Biologen"

„Baupläne der Tiere 1"

„Baupläne der Tiere 2"

„Bestimmungsübungen heimischer Tiere"

„Kenntnis mitteleuropäischer Lebensräume"

„Das Verhalten der Tiere"

„Übungen zur Physiologie der Tiere 1"

„Übungen zur Physiologie der Tiere 2"

„Musealtechnik"

„Entomologisches Laboratorium"

„Mikroanatomie der Wirbeltiere"

„Spezialpraktikum Zuckmücken (Chironomidae)"

„Zoologisches Labor Praktikum"

KOMPETENZEN

Methoden

Mikroskopie:

      Light microscopy

      Confocal laserscanning microscopy

      Transmission electron microscopy

      Scanning electron microscopy

Schneidetechniken:

      Ultra-thin sectioning

      Semi-thin sectioning

Färbetechniken:

      Immunocytochemie

      Histologische Färbetechniken

Molekularbiologische Techniken:

       DNA - Extraktion

       PCR

       DNA – Sequenzierung

Software skills:

       3D Rekonstruktion mit IMARIS

       Adobe Illustrator CS5

       Adobe Photoshop CS6

       MS Office

Computer-Führerschein: ECDL Standard

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Englisch: in Wort und Schrift

Latein: Maturaniveau

Tauchen

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Führerschein

PKW

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