

New data on dinophilid neurogenesis: a variation of a common pattern

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Abstract

Background

The structure and development of the nervous system in Lophotrochozoa species is of the most important questions for comparative neurobiology. During the last decade the number of comprehensive studies on the development of serotonergic and FMRFamide systems has been skyrocketing. However, the detailed research of the earliest events of Polychaeta neurogenesis is still sparse. Polychaeta is a huge taxon within Lophotrochozoa. Its representatives are widely used as model systems in developmental and physiological investigations. Dinophilidae is a unique Polychaeta group. Its representatives combine morphological traits of different lophotrochozoan taxa. Moreover, adult dinophilids demonstrate morphological similarity to a trochophore larva. This similarity may be associated with either archaic origin of this group or neoteny. The main goal of our study is to provide a detailed description of the earliest events in *Dinophilus* neurogenesis. These data might improve our understanding of Polychaeta development and evolution.

Results

We have studied the earliest events in nervous system development in two relative species *D. gyrociliatus* and *D. taeniatus* using immunochemical labelling of serotonin, FMRF-amide related peptides, and acetylated tubulin. We used external ciliation as marker for staging. Both species go through the same developmental stages: prototroch, ventral ciliary field and ciliary bands. In both species the first neurons differentiate revealed by anti alpha-acetylated tubulin antibodies only and show no reaction with 5-HT or FMRFa antibodies. These neurons located at the anterior and posterior parts of the embryo in both species. In *D. taeniatus* embryos the anterior cell is transient and disappear just after head neuropil is constructed. On the contrary, in *D. gyrociliatus* embryos the anterior cell is not transient and remains at the same position during the whole life span of the specimen. Caudal cell is present during the whole embryogenesis in both species. Neurites of these early neurons surround the stomadeum and constitute anlagen of paired ventro-lateral longitudinal bundles. During the development the number of neurites increases and they form compact head neuropil, paired ventro-lateral and lateral longitudinal bundles, unpaired medial longitudinal bundle and transverse commissures in ventral hyposphere. Serotonin- and FMRFamide-immunoreactive neurons differentiate adjacent to ventro-lateral bundles and head neuropil, respectively, after the establishment of main structures of the nervous system at the ventral ciliary field and ciliary bands stages. Processes of serotonin-, FMRFamide- immunopositive neurons constitute the small portion of tubulin immunopositive neuropil at all described stages.

Conclusions

We announce a detailed data on the earliest events in *D. gyrociliatus* and *D. taeniatus* neurodevelopment based on anti-acetylated tubulin, serotonin, and FMRFamide-like immuno labeling. The first nerve elements demonstrate no 5-HT-IR and no FMRFa-IR, which differs from the most Polychaetes and even Lophotrochozoans, investigated so far. Moreover, these animals do not have a typical apical organ (or

perhaps do not have it at all) and the pioneer neurons of *D.gyrociliatus* are also peculiar in that they join the definitive nervous system unlike other lophotrochozoans where pioneer neurons are transient. Thus, *Dinophilus* neurogenesis demonstrates a variation of common scheme. The reported study was funded by RFBR, project number 19-3460040.

Introduction

Over the past decades the research of lophotrochozoan neurogenesis and functions of early larval neurons has made considerable progress [72–74, 69–71, 23, 67, 68, 2, 10, 26, 52, 76, 83, 92–96, 101, 15, 16, 18, 23, 85–90, 80, 21, 104]. The emergence of the first neurons and subsequent establishment of the nervous system was described in details for the main lophotrochozoan groups such as Polychaeta and Mollusca [15, 16, 18–20, 61, 84–91, 10, 25, 62, 22, 80].

The first neurons appear extremely early in embryogenesis [56, 57, 33, 34, 13, 84, 88, 89, 10, 22]. They are often located at the periphery and their emanating processes establish a layout for the developing adult (definitive) nervous system. Therefore, these early neurons may be considered as so called pioneer neurons [58, 4, 47, 76, 86, 39]. In polychaetes and nemerteans pioneer neurons were shown to be serotonin (5-HT) IR [84, 88, 90, 22, 12], while in mollusks they often express positive reaction with anti-FMRFamide antibodies [15, 16, 18–20, 25, 91, 84, 85].

Apart from the pioneer neurons, early nervous elements are often associated with sensory apical or aboral organs [29, 56, 57, 72–74, 67, 68, 69–71]. The apical organ consists of a group of flask-shaped cells reaching the surface of a larva and bearing cilia on their apices. The processes running from the basal parts of these cells form a plexus under the apical organ [56, 57, 72–74, 95, 96]. Summarizing the data from different lophotrochozoan taxa the evidence comes that the cells of the apical organ almost always have either 5-HT or FMRFamide IR [43, 72–74, 67, 68, 93–95, 51, 55, 97]. Pharmacological treatments and other approaches demonstrate that these neurons play critical role in reception of external cues [30, 53, 13, 44], regulation of developmental rates [87, 29], and morphogenetic patterning [56, 57, 72–74, 104].

Typically, the other elements of the larval nervous system (prototroch nerve, hyposphere nerve ring, and etc.) and anlage of the definitive nervous system (cerebral ganglia, ventral nerve cords, oesophageal nerve network, and etc.) emerge soon after the establishment of the first neurons [48, 49, 53, 56, 57, 67, 84, 88, 90, 96, 22].

Although many lophotrochozoans exhibit general bauplan of the nervous system, some animals have peculiar characteristics in the larval nervous system; for example, some structures might be completely lost or highly elaborated. The data obtained on the previously neglected taxa (Phoronida, Sipuncula, Acoela, Entoprocta, etc.) not only show intriguing neuromorphological patterns, but help to elucidate the origin and phylogenetic position of some questionable groups. For example, the neural systems of phoronids and brachiopods are shown to have a number of similarities with deuterostomian neural systems [69–71, 83], challenging molecular phylogeny view considering phoronids and brachiopods as

Protostomia [69]. Likewise, similarities between the neurogenesis of creeping entoproct larvae and molluscs propose a monophyletic assemblage of Mollusca and Entoprocta [92, 95, 96]. The data on Acoela neural organization support separation of this group from Platyhelminthes and allow to consider Acoela as the most primitive living Bilateria [50]. Segmental organization of the central nervous system at the larval stages of Sipuncula and Echiura (worms lacking any trace of metamerism) demonstrates the segmental origin of Sipuncula [51, 52] and Echiura [36–38].

One of the previously neglected taxa with uncertain position is Dinophilidae family (Archiannelida, Polychaeta) first described in the last half of XIX century [78, 31]. The first works depict dinophilid anatomy, histology, and embryogenesis as early as at the end of XIX – beginning of XX centuries [30, 31, 27, 40, 48, 76, 77, 31, 64, 65]. Further investigations confirm an extraordinary morphology of dinophilids, which combines characteristics of different lophotrochozoan animals such as Polychaeta (segmentation of epithelial structures, though no chaeta), Platyhelminthes (parenchymatous organization, protonephridia), Mollusca (gliding ciliary locomotion), and even trochophore larva (prototroch, ciliary bands, ventral ciliary field, and protonephridia) [4, 5, 6, 8, 35, 1, 58, 8, 78, 81, 82, 44–46]. That adult worms have larval elements suggests progenetic origin of dinophilids [98, 100, 63, 36, 103, 97, 60, 97–99, 101]. The nervous system of *Dinophilus* attracted particular attention of researches in recent times [100, 7, 63, 64, 23, 24, 44–46]. Nevertheless, the early events of neurogenesis (first differentiating neurons, pioneer neurons, the structure of the apical organ) have never been described in details.

Genus *Dinophilus* includes only two species: *Dinophilus gyrotilatus* [77] and *Dinophilus taeniatus* [31]. Our work analyzes the earliest events in neurogenesis of both species by immunochemical staining with pan-neural marker (anti-acetylated α -tubulin antibodies) as well as with antibodies to specific neuronal molecules (5-HT, and FMRFamide). Our results demonstrate that the early neurogenesis is rather similar in two *Dinophilus* species, but has significant difference from what was revealed in other Lophotrochozoa.

Materials And Methods

Culture maintaining. The culture of *Dinophilus gyrotilatus* was established from the Mediterranean Sea, Napoli Zoological station (Italy). In laboratory the animals were reared in small plastic aquaria with artificial seawater (33‰ salinity) at 21° C without aeration. Worms were fed with homogenized frozen *Urtica* leaves once a week. Water was changed every day in order to collect all developmental stages of embryos. *D. gyrotilatus* cocoons contained 1–9 large (female) embryos and several small (dwarf males) embryos. In our work we studied only female (large) embryos of *D. gyrotilatus*, so we use the term “embryo” here in relation to female embryos only.

The work on *Dinophilus taeniatus* was conducted during summer seasons at the White Sea, Pertsov White Sea biological station (Russia; 66°34'N, 33°08'E). The worms were collected in a subtidal during a low tide and were then kept in small tanks without aeration and in six-well plates at 10 °C in filtered seawater. The worms were fed with diatomic *Phaeodactylum* sp. and *Pseudinicshea delicatissima* every

other day. Water was changed every time before feeding. The cocoons of *D. taeniatus* contained up to 30 embryos the same size of both sexes.

Both *Dinophilus* species laid cocoons regularly on a regular base. The cocoons were gently removed from the aquaria using glass Pasteur pipette and placed into 30 mm Petri dishes with filtered sea water. The embryos were mechanically extracted from the cocoon and fertilization envelope with tiny needles. Then they were tested under a microscope in order to select the only embryos without any visible damages for further immunocytochemical procedures.

Immunocytochemistry. *D. gyrociliatus* and *D. taeniatus* embryos at various developmental stages were subjected to whole-mount immunocytochemical staining. The embryos were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS, 0,01 mM, pH = 7.4) at 4 °C overnight. The immunostaining was then conducted according to a protocol established for juveniles and adults of *D. gyrociliatus* [23, 24]. We used anti-acetylated α -tubulin antibodies diluted 1:5000-1:10000 in PBS containing 0,1% Triton X-100 (PBS-TX) to label ciliary bands and neural fibers. Specific neural cells were marked with anti-5HT, anti-FMRamide (polyclonal, 1:2500, Immunostar), and anti-tyrosine hydroxylase (TH) antibodies (polyclonal, 1:2500, Sigma, USA). The primary antibodies were visualized with respective secondary goat-anti-rabbit and goat-anti-mouse antibodies conjugated with Alexa-488 and Alexa-633 (1:1000, Molecular Probes).

Cell nuclei were stained with DAPI (0,25 μ g/ml). After 3×10 min washing in PBS, the specimens were mounted on glass slides in 70% glycerol for microscopic analysis and image acquisition.

Confocal scanning microscopes TCS-SPE, TCS-SP5 (Leica Microsystems, Germany) and Nikon A1 (Nikon, Japan) equipped with the appropriate set of lasers, filters, and detectors were used for the detailed study of the specimens. Stacks of optical sections taken with 0.3–0.5 μ m intervals were processed with Leica LAS AF (Leica Microsystems, Germany) and Image J (NIH, USA) to obtain two-dimensional images. For each certain preparation the optimal number of stacks was selected in order to demonstrate the structures of interest. To compose a picture, optical sections were projected into one image and then imported into the Adobe Photoshop CC; the only parameters to be changed were brightness and contrast.

Results

Relative staging

Duration of embryonic development in *Dinophilus gyrociliatus* differs from that of *Dinophilus taeniatus* significantly. *D. gyrociliatus* embryos hatch after 5 days after oviposition whereas *D. taeniatus* embryos hatch approximately two weeks after oviposition. External ciliary structures appear sequentially in anterior-to-posterior direction both species. Each stage has specific ciliary pattern. We used external ciliation as marker for staging of *D. taeniatus* and *D. gyrociliatus* embryos.

It's noticeable that during the course of development *Dinophilus* embryos curve with it's ventral ciliary field outside (Fig. 1). Thus in our Figures we have photos of curved embryos, not round or oval-shaped, especially at late stages.

In both studied species the first cilia are visualized after stomadeum emergence when embryos are at a gastrula stage. These cilia appear in the episphere above the stomadeum and form a prototroch (Fig.1 a). Then prototroch develops a circular band in ventro-dorsal direction. Prototroch extends to the dorsal side without forming a complete ring.

After the prototroch is developed, ventral ciliary field appears at the midline of the ventral hyposphere beneath the mouse opening. This ventral ciliary field elongates in rostro-caudal direction (Fig.1 b). During the further embryogenesis the ventral ciliary field becomes wider and bushier, and its cilia become longer.

After that, another ciliary band (acrotroch) appears in an episphere above the prototroch. Acrotroch also extends to the dorsal side and forms an incomplete ring. In hyposphere ciliated transversal stripes emerge under the prototroch on both sides of ventral ciliary field. In the course of development these ciliary stripes fuse at the dorsal side forming ciliary bands of the hypospere (Fig.1 c). The number of these ciliary bands increases in rostro-caudal direction, reaching five by the moment of hatching (Fig.1 c).

Thus, we suggest the following postgastrulation embryonic developmental stages in *Dinophilus*: prototroch, ventral ciliary field, and ciliary bands stages (Fig. 1 a - c). The name of the stage refers to the moment of a certain structure formation (i.e. the stage of prototroch means that the prototroch begins to develop, but no other ciliary structures have yet been formed, etc.) (Table 1). We described below the general structure of a nervous system as well as the distribution of specific neurons at these stages during early embryogenesis of *D. taeniatus* and *D. gyrociliatus*.

Table 1. The moment (hours post overposition and days post overposition) of establishment of the main external ciliary structures and hatching during embryogenesis of *D. taeniatus* and *D. gyrociliatus*.

	Prototroch	Ventral ciliary field	Ciliary bands	Hatching
<i>Dinophilus gyrociliatus</i>	20 hpo	48 hpo	72 hpo	120 hpo
<i>Dinophilus taeniatus</i>	6 dpo	9 dpo	11 dpo	14 dpo

Neurogenesis during the prototroch stage

In both *D. gyrociliatus* and *D. taeniatus* the earliest neurons develop during the prototroch stage. These earliest nerve elements construct the scaffold for nervous system. In both species first neurons demonstrate no 5-HT-LIR and no FMRFa-LIR (Fig. 2).

In *D. gyrociliatus* the first neuron appears at the periphery of anterior part of the embryo, above the mouth (Fig. 2 a, d, Fig. 8 a-a"). This cell has two lateral processes, growing on the ventral side along the prototroch (Fig. 2 a, Fig. 8 a-a"). A bit later, the projects of the first cell develop the basic structures of the

nervous system: neuropil in the episphere, two ventrolateral bundles, two commissures and two lateral bundles (Fig. 2. b, e). At the level of the first commissure another pair of neurons differentiates (Fig. 2 b). Their projects extend along the scaffold formed by the first cell (Fig. 2 b, e). Thus, the scaffold of the nervous system is build by projects of the only one cell (Fig 2. b, e, d).

In *D. taeniatum* one of the first neurons appears at the apical of anterior part of the embryo (Fig. 2 f), the other neuron is at the caudal part on the dorsal side (Fig. 2 f, g, l). Anterior cell develops lots of processes towards the head neuropile. This cell is transitory and disappears a bit later at the prototroch stage (Fig 2 g, h). The projects of the anterior cell form the basic structures of the nervous system: thin neuropil in the episphere, two ventro-lateral bundles, first commissure and two lateral bundles (Fig. 2. g, j, k). At the level of first commissure additional neurons appear (Fig. 2 g, j, k). Thus, the scaffold of the nervous system is build by projects of the only one cell (Fig 2. g-k). Caudal cell develops two lateral processes, but they are short and still on the dorsal side (Fig 2 f, l).

Neurogenesis during the ventral ciliary field stage

During ventral ciliary field stage first 5-HT-LIR and FMRFa-LIR elements differentiate, but they form only minor part of the whole nervous system in both species *D. gyrotilatus* and *D. taeniatum*.

D. gyrotilatus embryos demonstrate quite developed nervous system at the ventral ciliary field stage. The first nerve cell is still above the mouth opening, it becomes bigger (Fig. 3 a, Fig. 4 a, Fig. 5). Neuropile in the episphere contains more processes, become more dense and prominent (Fig. 3 a, Fig. 4 a, Fig. 5). No neurons are detectable in neuropil region (Fig. 3 a, Fig. 4 a, c, e, Fig. 5 b- d). The main parts of nervous system are detectable: neuropil, ventro-lateral, medial and lateral bundles, commissures (Fig. 3 a, Fig. 4 a, Fig. 5). On the ventral side two ventrolateral and lateral bundles are detectable, they contain more processes (Fig. 3 a, Fig. 4 a, Fig. 5). Additional ventral medial bundle extends in rostro-caudal direction (Fig. 3 a, Fig. 4 a, Fig. 5). At the caudal region ventral and medial bundles extend to the dorsal side of an embryo (Fig. 3 c, e, f). The caudal cell appears on the dorsal side of an embryo (Fig. 3 f, Fig. 4 f), its' processes grow towards ventro-lateral bundles and join them.

First 5-HT-LIR neurons develop at the level of the first commissure (Fig. 3 b). These neurons do not have any cilia (Fig. 3 b, c, e). The processes of these neurons extend to the main nervous structures: ventro-lateral, medial bundles and head neuropil (Fig. 3 b, c, e).

First FMRFa-LIR detectable in the anterior cell above the mouth opening (Fig. 4 b- e, Fig. 5 a-c, Fig. 8 b-b"). Moreover, the processes of the cell also FMRFa-LIR (Fig. 4 b- e). Thus, FMRFa-LIR bundles detectable in the ventro-lateral, medial and lateral nerve bundles (Fig. 4b, c, Fig. 5 d, e). It's noticeable that, the first cell has an FMRFa-LIR extension towards the surface and sensory cilia (Fig. 4 b, c, f, Fig. 5 a, b). The processes of this cell follow the main nervous structures neuropil, ventro-lateral, medial and lateral bundles. The other processes of this cell form an incomplete ring under the prototroch.

D. taeniatus embryos also demonstrate quite developed nervous system at the ventral ciliary field stage (Fig. 3g). The first cell is not detectable, thus we propose it's transient. Neuropile in the episphere contains more processes, become more dense and prominent (Fig. 3 g, i, j). No neurons are detectable in its region (Fig. 3 g, i, j). The main parts of nervous system are detectable: neuropil, ventro-lateral, medial and lateral bundles, commissures (Fig. 3 g, h). Multiple projects extend to the surface from neuropile (Fig. i, j, k). On the ventral side two ventro-lateral and lateral bundles are detectable, they contain more processes (Fig. 3 g, h, i, k, l). At the caudal region ventral and medial bundles extend to the dorsal side and meet the caudal cell projects (Fig. 3 i, l). No prototroch circular nerve is visible (Fig. 3).

First 5-HT-LIR neurons in *D. taeniatus* embryo also develop at the level of the first commissure (Fig. 3 h, i, k). These neurons do not have any cilia (Fig. 3 k). They send processes to the main nervous structures: ventro-lateral bundles and commissure (Fig. 3 i, k, l).

No FMRFa-LIR is detectable in *D. taeniatus* at this stage.

Neurogenesis during ciliary bands stage

During ciliary bands stage first 5-HT-LIR neurons become detectable in the episphere of an embryo in both *D. gyrociliatus* and *D. taeniatus* (Fig. 6). First FMRFa-LIR elements differentiate in *D. taeniatus* embryos (Fig. 7 h-k). 5-HT-LIR and FMRFa-LIR still form only minor part of the whole nervous system in both species *D. gyrociliatus* and *D. taeniatus*.

In *D. gyrociliatus* embryos nervous system revealed by tubulin immunostaining becomes more obvious and prominent. The main structures: neuropil, ventro-lateral, medial and lateral bundles contain more nerve fibers (Fig 6 a, Fig. 7 a). Additional commissures develop in rostro-caudal direction (Fig 6 a, Fig. 7 a). Additional circular bundles develop (ciliary bands innervation) (Fig 6 a, Fig. 7 a).

First 5-HT-LIR neurons appear in the head region of an embryo (Fig. 6 c, d). These four cells are located on the dorsal side of the head region just under the head neuropil (Fig. 6 c, d). Their processes intersect and go through the head neuropil (Fig. 6 c, d). Additional 5-HT-LIR neurons appear on the ventral side of the embryo (Fig. 6 e).

FMRfa-LIR elements in *D. gyrociliatus* become more prominent and easier to detect. The first cell (is still here!) and its multiple processes in the neuropil, ventro-lateral, medial and lateral bundles (Fig. 7 b- e). Also some thin projects are detectable in the neuropil, but they form only small part of the neuropil (Fig. 7 b- e).

D. taeniatus embryos nervous system revealed by tubulin immunostaining also becomes more complex. The main structures: neuropil, ventro-lateral, medial and lateral bundles contain more nerve fibers (Fig 6 f, Fig. 7 g). Additional ventral medial bundle extends in rostro-caudal direction (Fig. 6 f) Additional commissures develop in rostro-caudal direction (Fig 6 f, Fig. 7 g). Additional circular bundles (ciliary bands innervation) and media-ventral bundles develop (Fig 6 f, Fig. 7 g).

First 5-HT-LIR neurons in *D. taeniatus* embryos in the head region appear (Fig. 6 g, h, i). These four cells are located on the dorsal side of the head region just under the head neuropil, which is similar to *D. gyrociliatus* (Fig. 6 c, d). Their processes intersect and go through the head neuropil (Fig. 6 c, h). Additional 5-HT-LIR neurons appear on the ventral side of the embryo (Fig. 6 e, j). Also 5-HT-LIR reveals media-ventral bundles on the ventral side (Fig. 6 b, e j).

First FMRFa-LIR neurons in *D. taeniatus* embryos develop at apical top of the head region and send their processes through the neuropil (Fig. 7 h- k). At the caudal part of the embryo a single FMRFa-LIR cell with a short process differentiates in close proximity to the gut (Fig. h- k).

Discussion

We described the development of the nerve elements at the early embryogenesis of two dinophilid species: *Dinophilus taeniatus* and *Dinophilus gyrociliatus*. Neurogenesis in both species begins with the differentiation of the peripheral pioneering neurons. The scaffold of the central nervous system at first is visualized with only a pan-neural marker (anti-alpha-tubulin antibody); the immunoreaction to specific neuronal markers (anti-5-HT, and FMRFa-antibodies) develops later when the pattern of the central nervous system has already been established. We further discuss and compare early neurogenesis of dinophilids with that of other known lophotrochozoans. Data on the earliest events in neurodevelopment are thus on high demand from both, a developmental and an evolutionary point of view.

External ciliation - a key structure to define a stage.

External ciliation pattern is a reliable key to distinguish developmental stages during embryogenesis in polychaetes [8]. Despite the fact that duration of embryogenesis is different in *D. taeniatus* and *D. gyrociliatus* (14 and 5 days, respectively), the mode of differentiation of external ciliary structures is the same in both species: prototroch appears the first followed by ventral ciliary field and then by ciliary bands. Thus, these ciliary structures (along with absolute time scale) can be used as temporal landmarks to allow comparison of developmental stages in two *Dinophilus* species with different duration of embryogenesis.

Though dinophilids have direct development, their embryonic stages are somewhat similar to free swimming trochophores. The prototroch stage matches well with the early trochophore stage since the last is characterized by the presence of the prototroch, the first ciliary structure to develop in Spiralia [27, 28, 10, 60, 48, 53, 21]. Moreover, recent analysis of gene expression confirmed the homology of the indirect-developing annelid larval prototroch and the ciliary band of adult *D. gyrociliatus* [43, 45] developing from the prototroch [44]. The ventral ciliary field stage matches middle trochophore stage (or just trochophore) [60]. The stage of ciliary bands corresponds to late trochophore stage if to draw a parallel between a ciliary band and metatroch [39, 21].

Neurogenesis in *Dinophilus* – a variation of general template.

Early neurogenesis in vast number of lophotrochozoans often begins with the differentiation of pioneer neurons. These are transient 5-HT or FMRFamide IR cells located peripherally, the processes of which guide the differentiation of the central nervous system [14, 15, 17–19, 83–90, 11, 79]. Dinophilids demonstrate peripheral pioneering neurons at the anterior and posterior pole of the embryo at the early trochophore stage like other lophotrochozoans (Fig. 9–12). However, the perikarya of the first differentiated neurons are detected with anti-alpha tubulin antibodies only (Fig. 9). Their processes constitute the anlagen of the central nervous system: a head neuropil and the ventral neural cords. Later on, these first processes will be joined by numerous newly differentiated nervous elements (Fig. 9–12); the number of neural fibers increases drastically during the development so the central nervous system at the late trochophore stage is very similar to that of juveniles [62, 63, 75, 23, 43–45]. Therefore, the first detected neurons in dinophilids are the first elements of the central nervous system and certainly are not the typical pioneer neurons. This assumes that the pathways orchestrating differentiation of the central nervous system might be different in dinophilids and other lophotrochozoans. Moreover, the anterior pioneer neuron in *D. gyrociliatus* is not transient and seems to be present through all life long (Fig. 8). This may be regarded as paedomorphic feature in *D. gyrociliatus*.

Development of specific neural elements.

The earliest pioneer neurons develop at the stage of early trochophore in both *Dinophilus* species.

In both *Dinophilus* species the first neural cells with specific IR differentiate at middle or late trochophore stages. The first 5-HT immunopositive cells are found close to the first commissure at the middle trochophore stage (Fig. 3). Later on, an additional four 5-HT IR cells emerge within the head neuropil at the late trochophore stage (Fig. 6). Thus, 5-HT IR is localized to the central nervous system and is similar in two dinophilid trochophores; moreover, it is also reminiscent of that in other polychaeta larvae where 5-HT IR perikarya are located at the level of the first commissure as well as in a group of cells in the head neuropil [21, 61, 96]. The first FMRFamide IR cells were found in a head region in *D. taeniatus* and *D. gyrociliatus* at the late and middle trochophore stages, respectively (Fig. 4, Fig. 5, Fig. 7). The early FMRFa IR nervous system is different in the two species. A large solitary FMRFamide IR cell found just above the mouth opening in *D. gyrociliatus* trochophore resembles a cell, described in polychaete trochophores *Capitella teleta* [61] and *Phyllodoce maculata* [89]. However, in *C. teleta* and *P. maculata*, this cell is not the first differentiated FMRFamidergic neuron as is in *D. gyrociliatus*. Moreover, a similar cell was revealed in front of the cerebral ganglion in the sipunculan *Phascolosoma agassizii* [50, 51]. *D. taeniatus* embryos develop a couple of FMRFa IR neurons closer to the rostral pole (right above the head neuropil), which are similar to two FMRFa immunopositive cells observed in a head region of another sipunculan *Phascolion strombus* [93].

Pioneer neurons and their role in lophotrochozoans.

Insights in pioneer neurons of lophotrochozoans are mostly based on immunochemical study of different members. Pioneer neurons are considered the first nervous cells to be developed. Their fibers grow and construct the "skeleton" of future nervous system. Later in development other neurons use this "skeleton"

to navigate their fiber growth. Pioneer neurons are often considered to be transient and disappear soon after constructing the "skeleton".

According to existing data these neurons are either 5-HT immunopositive or FMRFa-immunopositive.

The direction of neurogenesis can be rostro-caudal or vice versa. Matching and analyzing the common modes of neurogenesis in annelids and molluscs proposes that, despite of some inclinations, the range of development in the formation of the nervous system are usually in the following way: peripheral pioneering neurons appear and their processes build a scaffold upon which the definitive nervous system will be constructed. Then develops larval nervous system, and after that the definitive nerve system appears along the pattern navigated by the pioneer neurons. After all the larval and pioneer neurons die or partly combine with the definitive nervous system.

Our results demonstrate that in terms of immunoreactivity Dinophillids' pioneer neurons are different from most lophotrochozoans.

This means, that not only 5-HT or FMRFa play important role in axon guidance during neurogenesis. Additional research are need to find the diversity of neurogenic transmitters.

Anterior cell in *D. gyrociliatus*- a special case.

The first neuron in *D. gyrociliatus* appears at the stage of early trochophore at the anterior extreme of the embryo at the midline above the prototroch and it's visualised only with anti-tubulin antibodies. No other neurons were visualised at this time by anti-tubulin immunostaining thus proposing this cell to be the first neuron. The cell has a short apical neurite and several surface cilia (Fig. 3, Fig. 8, Fig. 9–12) and two fibers, which run towards the prototroch on the ventral side.

At the stage of middle trochophore, this cell and its' fibers reveal with anti-FMRFa antibodies. Its' fibers are present in all crucial nervous system elements: neuropil, ventro-lateral, medial and lateral cords and spread in posterior direction.

At the stage of late trochophore this cell and its' fibers visualized with antitubulin and anti-FMRFa antibodies. It becomes more conspicuous and noticable.

This cell is visible even after hatching at the stages of juvenile, adult and even senior individual. It's still revealed with anti-tubulin and anti-FMRFa antibodies only. It has characteristic morphological traits and position at the periphery above the prototroch and still has surface cilia.

1. The cell bears cilia at all stages, has short anterior surface fiber and two basal fibers, running towards the prototroch
2. This cell is located at the anterior extreme of the organism between surface epithelium and head neuropil.
3. This cell is revealed with tubulin and FMRFa immunostaining.

Thus we propose that *D. gyrociliatus* pioneer neuron is not transient. *D. taeniatus* do not demonstrate such type of cell. At the stage of early trochophore there is an anterior peripheral cell, revealed with anti-tubulin antibodies only, but this cell has no cilia and located at the apical pole of the episphere, it has multiple fibers extending towards the future neuropil. In *D. taeniatus* anterior cell is not sensory, due to lacking of cilia and no surface extensions. This cell is transient and disappears just after neuropil and ventro-lateral cords are constructed.

The fact that *D. gyrociliatus* pioneer neurons present through all life span and *D. taeniatus* pioneer neurons are transient may be treated as paedomorphic feature.

As was mentioned above, polychaetes *Capitella teleta* [62], *Phyllodoce maculata* [90] and sipunculan *Phascolosoma agassizii* [51, 52] possess similar FMRFa-IR cell. But these cells do not first in course of neurogenesis of these organisms. There is no data on the ontogeny of these cells, nobody knows whether they transient or not.

Thus additional investigations and experiments required to clarify the role of this non-transient pioneer neuron in nervous system ontogeny.

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Conclusion

We revealed that the early neurogenesis of dinophilids demonstrate the same pattern as most Polychaeta and Lophotrochozoans. The first nerve elements located at the anterior and posterior poles of an embryo, their fibers construct the scaffold of future nervous system. Nervous system develops in rostro-caudal direction in both dinophilid species. We propose that such mode of neurogenesis with peripheral pioneer cells is ancestral for Annelids. First nervous cells do not 5-HT-immunopositive or FMRFa-immunopositive, which is different from most described lophotrochozoans. Anterior cell in *D. taeniatus* is transient, while in *D. gyrociliatus* anterior cell is different in morphology and detectable during the whole life span. This fact may be considered as one more paedomorphic trait in *D. gyrociliatus*. In both dinophilids the first 5-HT-immunopositive and FMRFa-immunopositive cells appear after the main scaffold of nervous system is preformed, and their fibers are only small part of the main bundles. These data add to our knowledge of how diverse the early development in Lophotrochozoa could be and to what extent evolutionary changes might affect such conservative processes like early neurogenesis.

Declarations

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Contributions

Study design: EF and EEV; Maintaining culture: EF; Specimen collection: EF and TM; Sample preparation: EF, TM; Data acquisition: EF; Data analysis: EF, EEV and TM; Lab space and resources: EEV; Manuscript writing: EF, TM and EEV. All authors contributed to manuscript revision, read and approved the submitted version.

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Ethics approval and consent to participate

Consent for publication

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Competing interests

The authors declare that they have no competing interests.

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Figures

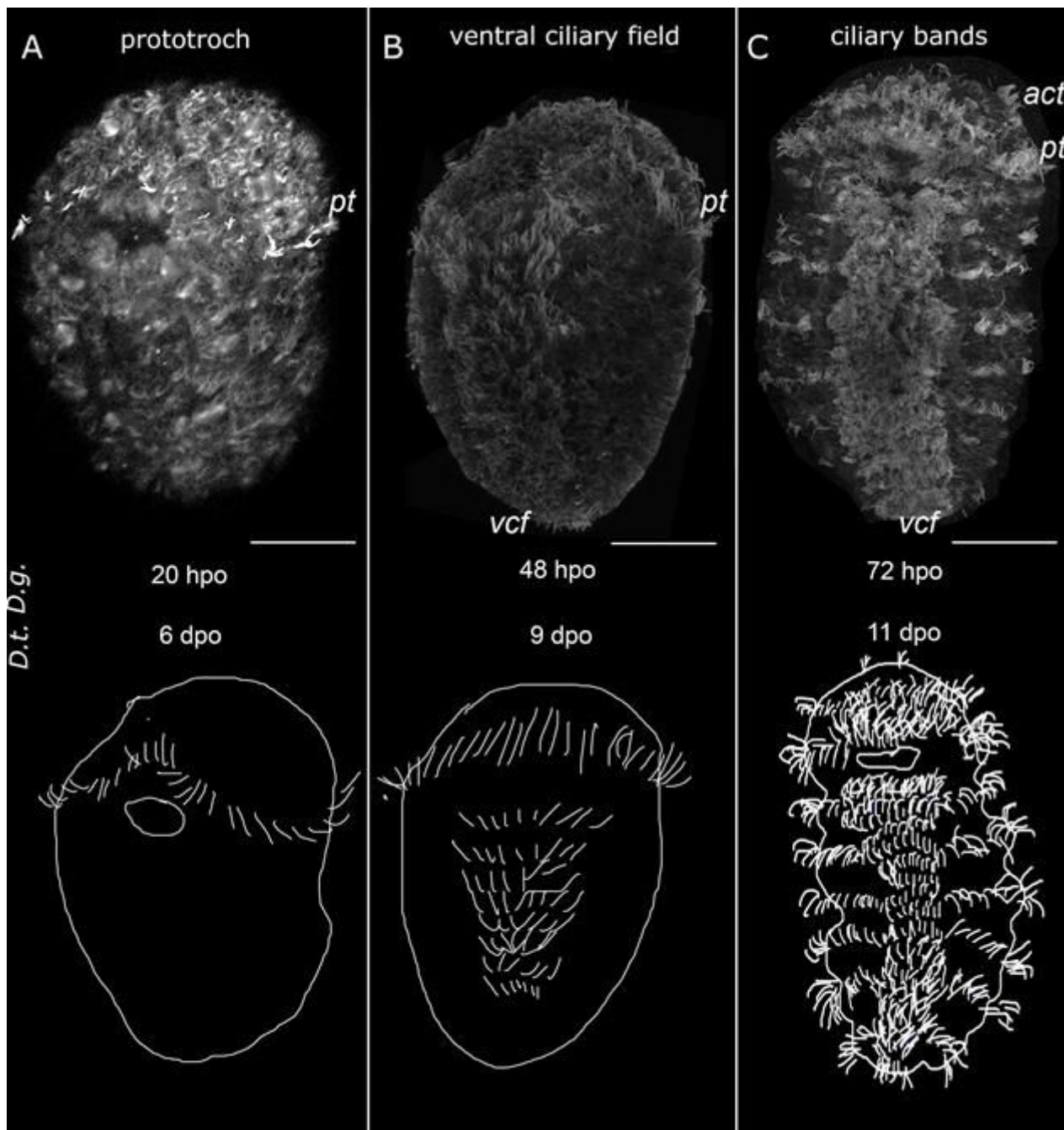


Figure 1

Relative staging and timing of *Dinophilus* embryos. Visualized by immunoreactivity with anti-acetylated tubulin. Here we define three developmental stages in both *D. gyrocilatus* and *D. taeniatus*: prototroch (a), central ciliary field (b) and ciliary bands stages (c). Lower part of the figure demonstrates developmental tempo of both species. (a) Prototroch stage embryo and a schematic representation of this stage. Prototroch is being developed. (b) Ventral ciliary field is being developed in rostro-caudal direction. (c) Ciliary bands development in rostro-caudal direction. Abbreviations: pt -prototroch; vcf- ventral ciliary field; act-acrotroch. Scale bar = 30 μ m.

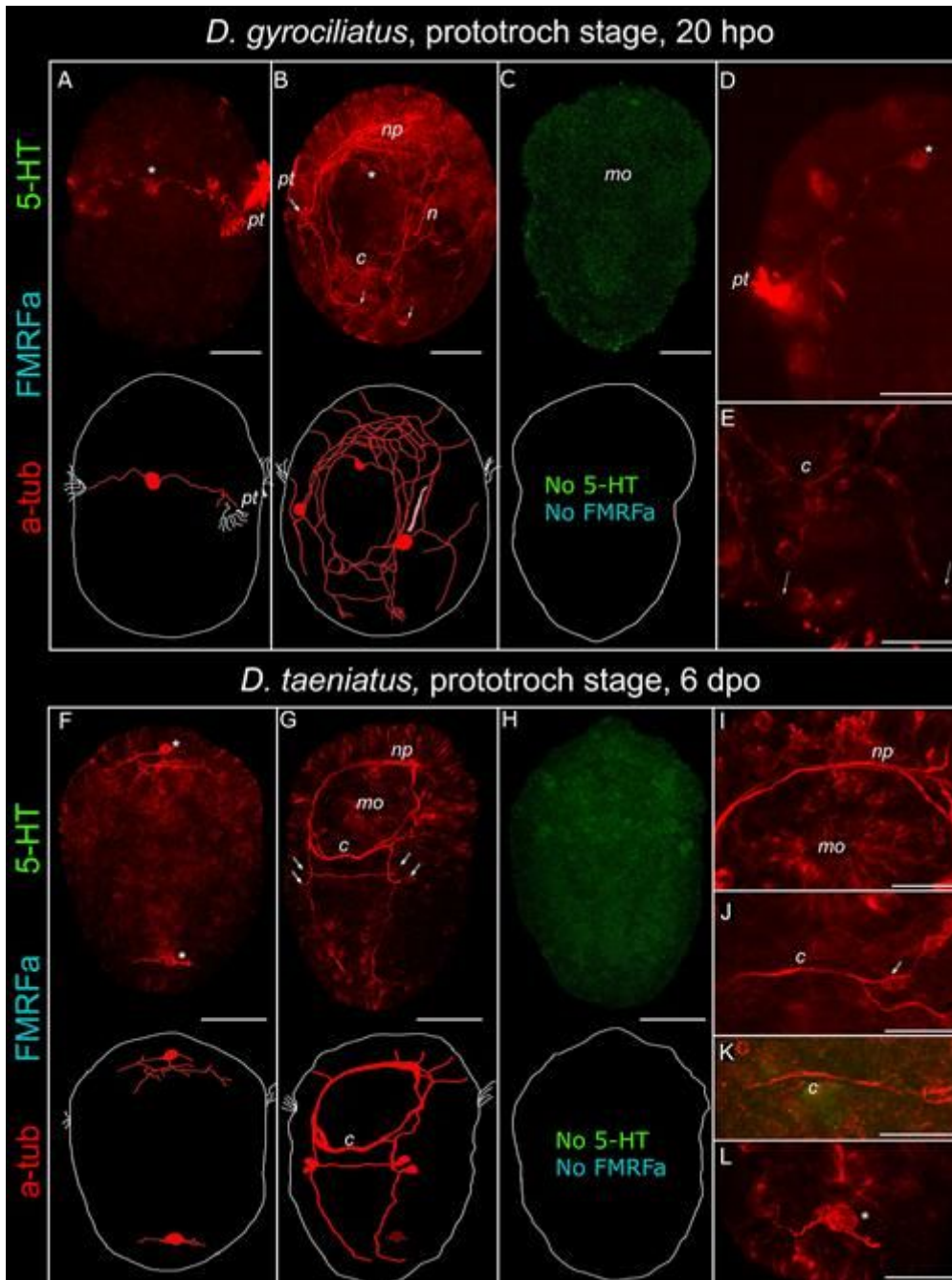


Figure 2

Development of actelylated tubulin-like (a-tub), FMRFa-like and 5-HT-like immunoreactivity during the prototroch stage in *Dinophilus*. Red- acetylated tubulin immunoreactivity; green- 5-HT-like immunoreactivity; cyan- FMRFa-like immunoreactivity. Upper part- *Dinophilus gyrociliatus*; Lower part- *Dinophilus taeniatus*. The earliest neurons (asterisks) appear on the anterior and posterior parts of the embryo in both species (a, d, f, l). These neurons do not demonstrate FMRFa-like or 5-HT-like immunoreactivity (a, c). In *D. gyrociliatus* only anterior cell is present (a, d). *D.taeniatus* embryo show anterior and posterior cells (f, l). A bit later, at this stage the nervous system scaffold is being developed (b, e, g, i, j, k). It includes head neuropile (np), paired ventrolateral bundles (vn), commissures (c). The anterior cell in *D. taeniatus* is transient and is not detectable after head neuropile and ventrolateral

bundles are being developed (g, i). In *D. gyrociliatus* the anterior cell is detectable when the nervous system scaffold is being constructed (b, d). At the cross of ventrolateral bundles and first commissure additional neurons appear (arrows). In *D. gyrociliatus* ventrolateral bundles demonstrate growth cones at the caudal part of an embryo (arrows). In *D. taeniatus* ventrolateral bundles also demonstrate growth cones (arrows). Abbreviations: pt – prototroch, n-nephridium, np-neuropile, c-commissure. Scale bars: a-c, f-h 25 μ m; d,e 15 μ m; i-l 10 μ m.

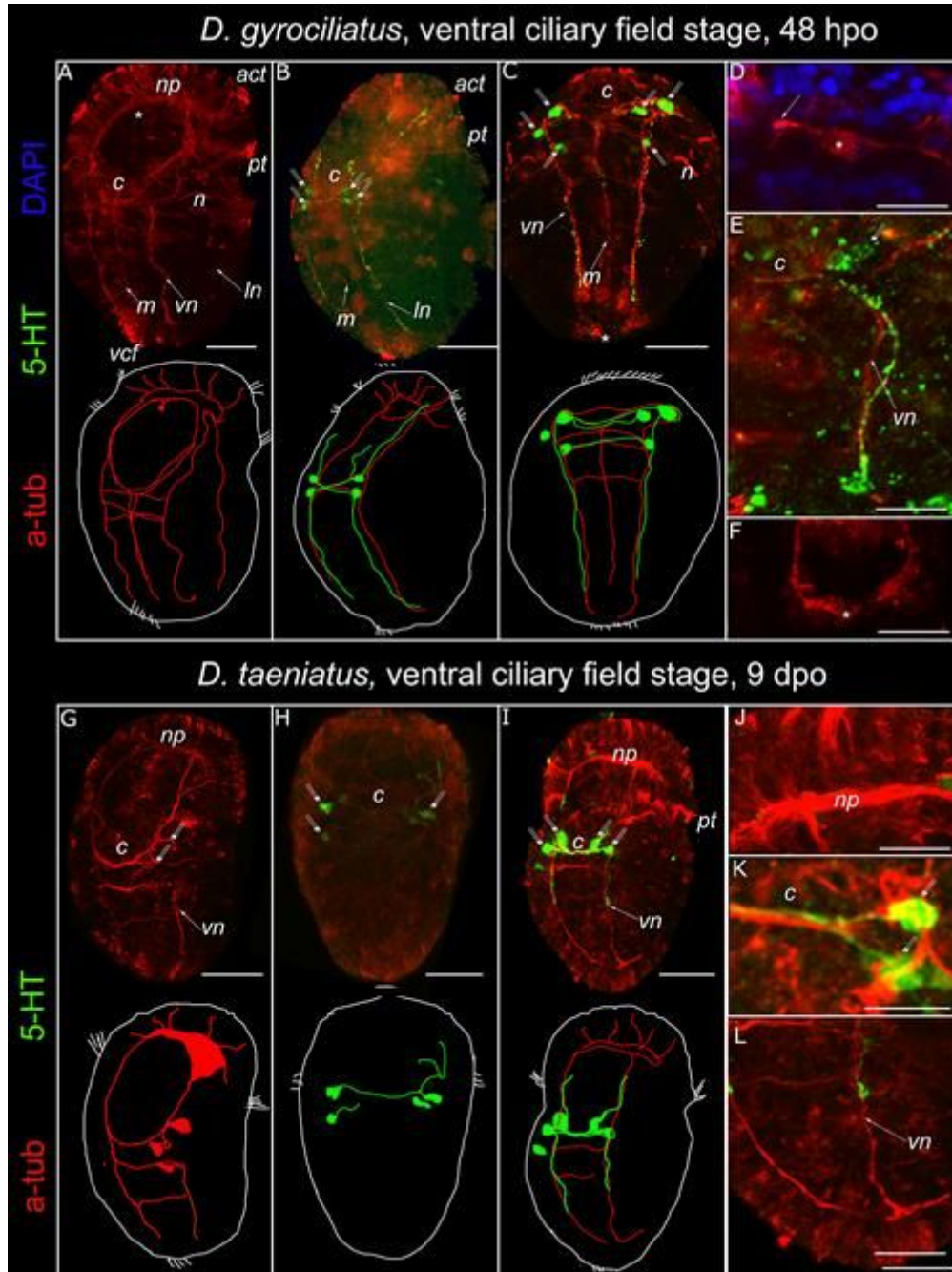


Figure 3

Dinophilus neurogenesis at the ventral ciliary field stage. 5-HT-immunoreactive structures development. Red- acetylated tubulin immunoreactivity; green- 5-HT-like immunoreactivity; blue-DAPI. Upper part- *Dinophilus gyrociliatus*; Lower part- *Dinophilus taeniatus*. The scaffold of nervous system is developed in

both *D. gyrocilatus* and *D. taeniatum* (a, g). The neuropil contains more bundles (a, g, i, j). The first 5-HT-IR neurons appear in both *D. gyrocilatus* and *D. taeniatum* (double arrows) at the level of first commissure. In *D. gyrocilatus* median nerve bundle and lateral bundle are developed, additional commissures appear in rostro-caudal direction (a, b, c). In *D. gyrocilatus* caudal cell (c, f asterisc) appears, its two processes meet ventrolateral bundles on the dorsal side of an embryo (f). Caudal cell has no 5-HT-IR. Abbreviations: pt- prototroch, act- acrotroch, m- median bundle, vn- ventro-lateral bundle, ln- lateral bundle, n- nephridium, c- commissure. Scale bars: a-c, g-i 25 μ m; d-f, j-l 10 μ m.

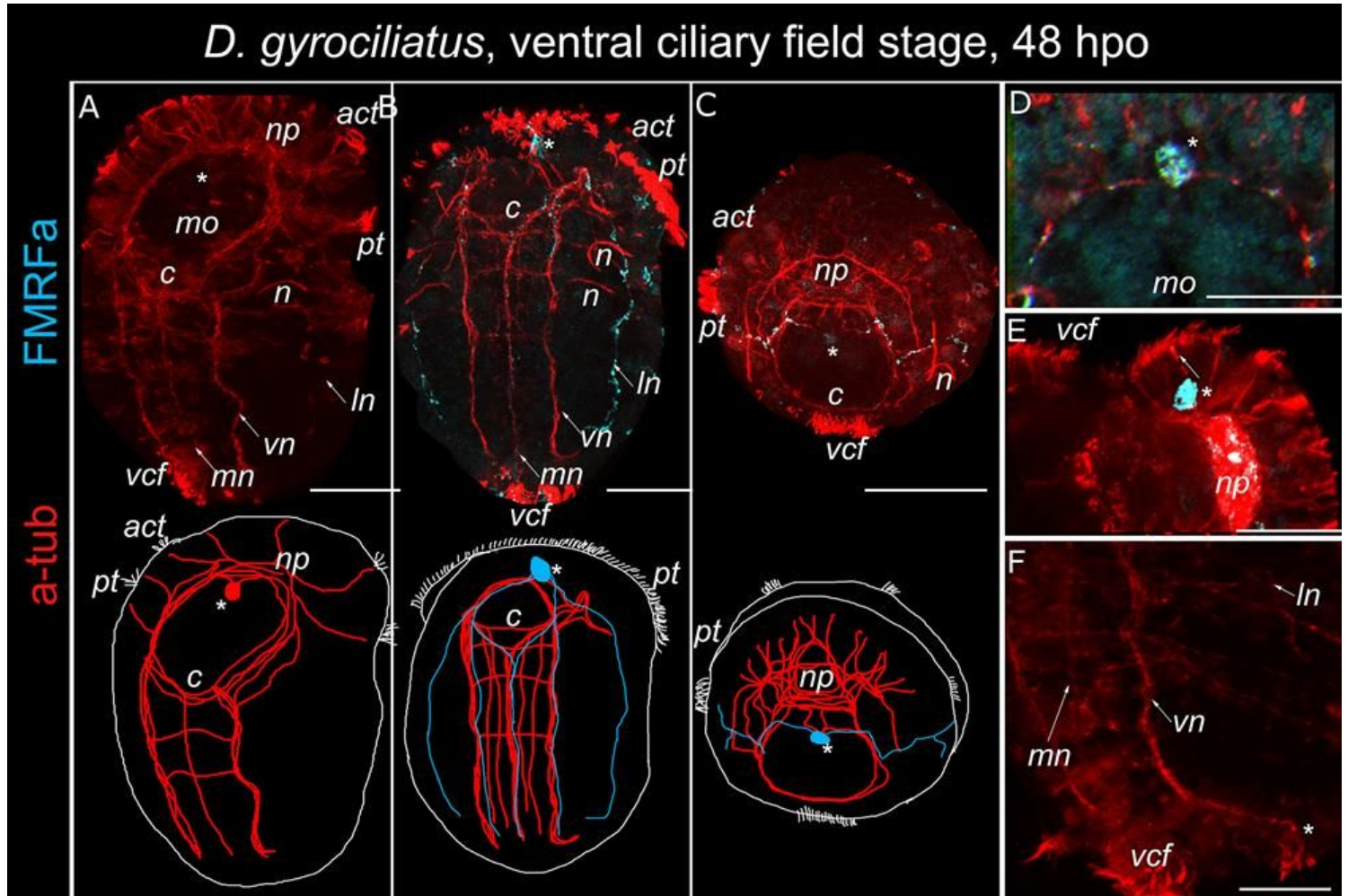


Figure 4

Neurogenesis at the ventral ciliary field stage. FMRFa-immunoreactive structures development in *Dinophilus gyrocilatus*. Red- acetylated tubulin immunoreactivity; cyan- FMRFa-like immunoreactivity. Only *D. gyrocilatus* demonstrates FMRFa-IR elements at this stage. *D. taeniatum* do not demonstrate FMRFa-IR at the ventral ciliary field stage. The scaffold of nervous system: the neuropile (np), median nerve bundle (mn) and lateral bundles (ln) are developed, additional commissures appear in rostro-caudal direction (a, b, c, e, f). The earliest anterior cell (asterisc) and its processes demonstrate FMRFa-IR (b, c, d, e). On the lateral section this cell has a process (e) towards the surface and several cilia at the end of the process (arrow). FMRFa-IR elements represent only minor part of the whole nervous system. Scale bars: a-c 25 μ m; d, f 10 μ m; e 20 μ m.

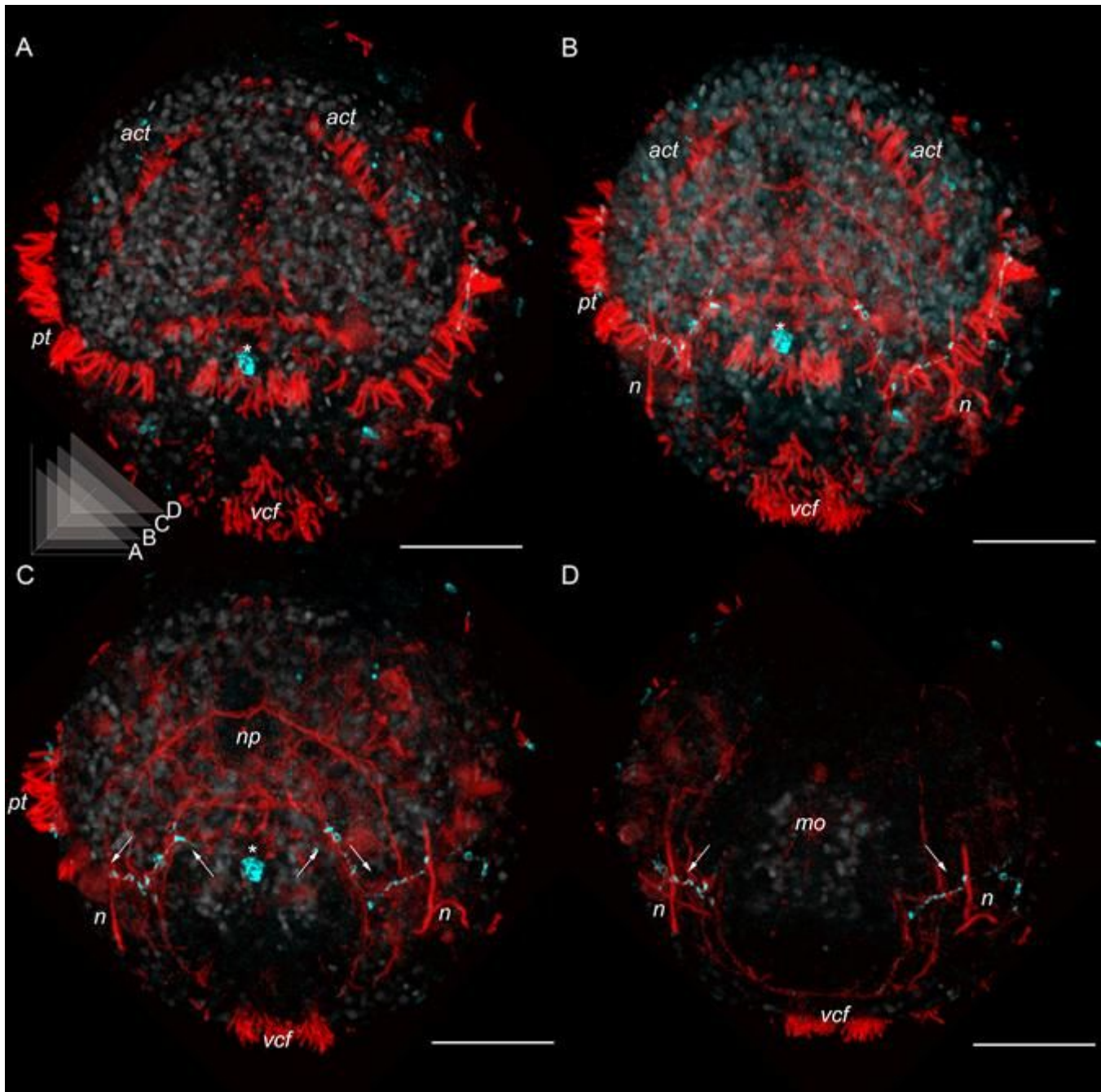


Figure 5

FMRFa-immunoreactive structures development at the ventral ciliary field stage in *Dinophilus gyrocilatus*. Red- acetylated tubulin immunoreactivity; cyan- FMRFa-like immunoreactivity. The only cell body is located at the peryphery of the nervous system, it has surface cilia (a) just above the prototroch. The cell has several processes, growing within the ventrolateral bundles and prototroch nerve (b, c, d). Scale bar 25 μ m.

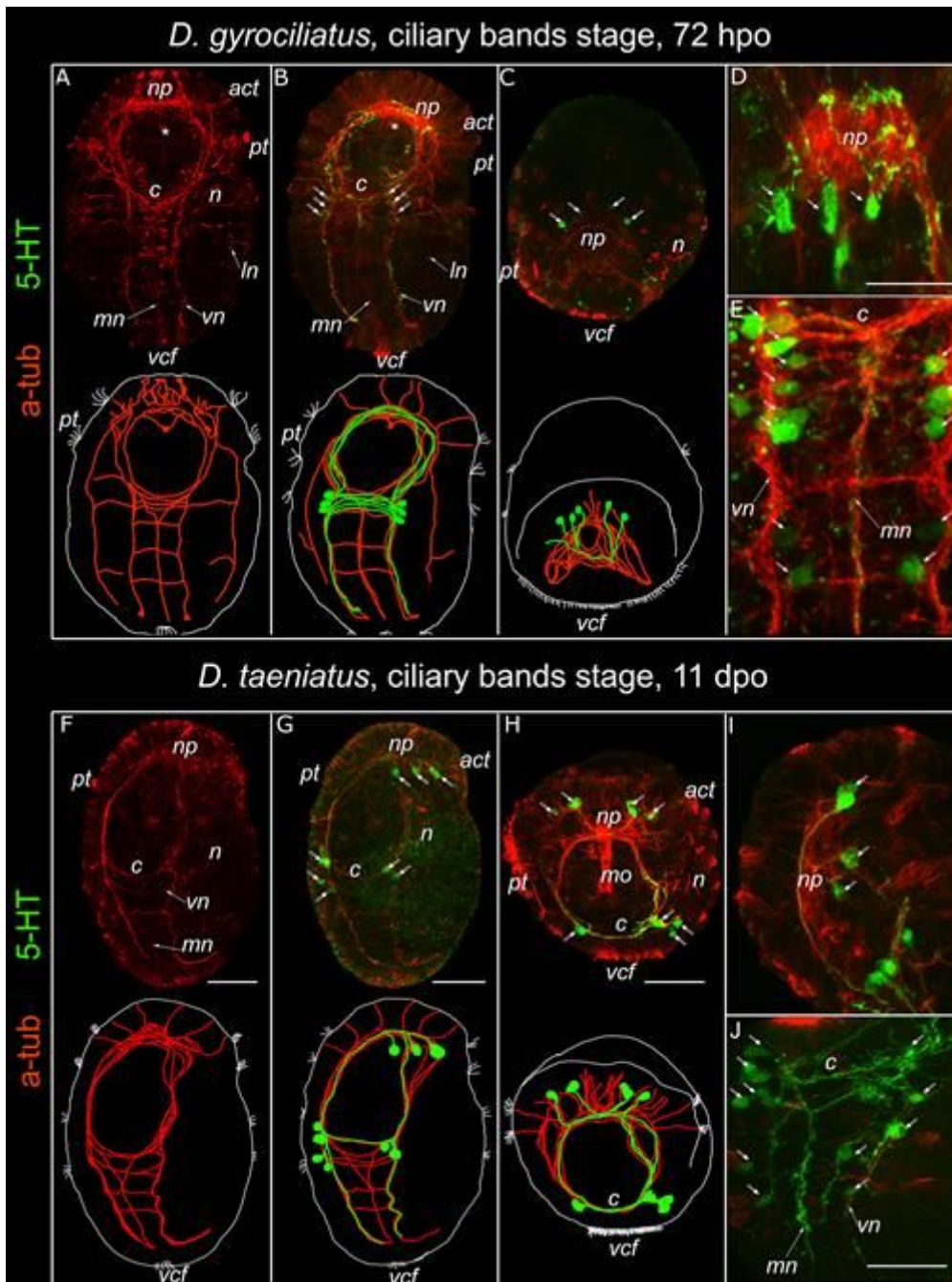


Figure 6

Neurogenesis at the ciliary bands stage. 5-HT-immunoreactive structures development. Red- acetylated tubulin immunoreactivity; green- 5-HT-like immunoreactivity. Upper part- *Dinophilus gyrocilatus*; Lower part- *Dinophilus taeniatus*. The scaffold of nervous system is developed in both *D. gyrocilatus* and *D. taeniatus* (a, f). The neuropile contains more bundles (a, b, d, f, g, h, i). Additional 5-HT-IR neurons appear in both *D. gyrocilatus* and *D. taeniatus* (double arrows) at the level of commissures (b, c, d, e, g, h, i, j). Four 5-HT-IR neurons develop under neuropile (double arrows) in both *D. gyrocilatus* and *D. taeniatus*. 5HT-IR neurons' processes included into neuropile and main ventral nervous bundles, but they do represent only minor part of the whole nervous system (b, d, e, g, h, i, j). Scale bars: a-e, f-h 25 μ m; d, e, i, j 15 μ m.

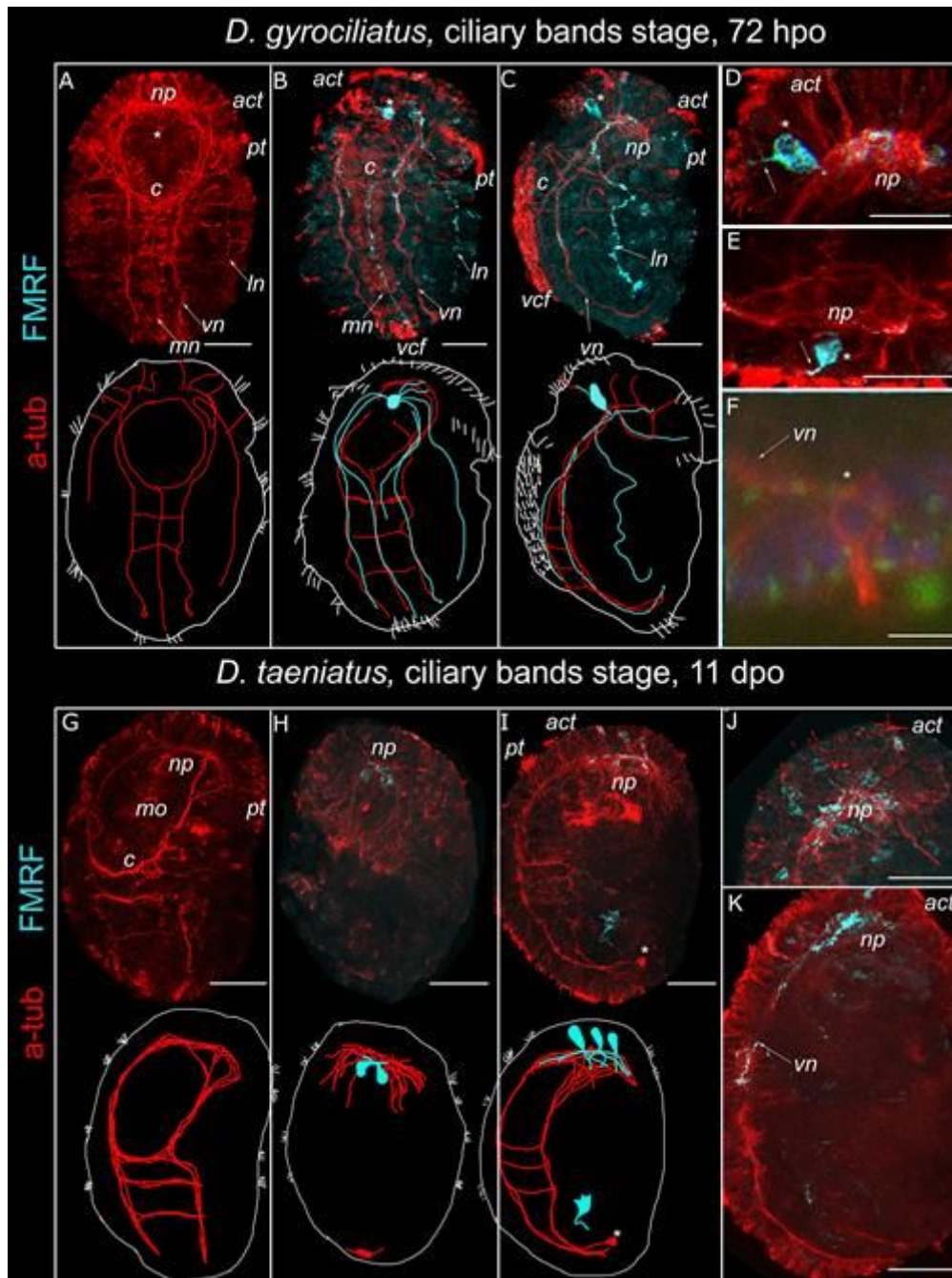


Figure 7

Neurogenesis at the ciliary bands stage. FMRFa-immunoreactive structures development. Red- acetylated tubulin immunoreactivity; cyan- FMRFa-like immunoreactivity. Upper part- *Dinophilus gyrociliiatus*; Lower part- *Dinophilus taeniiatus*. The scaffold of nervous system is developed in both *D. gyrociliiatus* and *D. taeniiatus* (a, g). The main nervous structures become more prominent and contain more bundles (a- e, g, i, k). *D. gyrociliiatus* demonstrate the only one FMRFa-IR cell, and its processes in the main nervous structures become more prominent (b, c, d, e) and still represent minor part of the whole nervous system. The caudal cell is present (asterisk), it contains surface cilia and do not demonstrate FMRFa-IR (f). *D. taeniiatus* embryos demonstrate the first two FMRFa-IR cells on the dorsal side of the head neuropile (h, i). Thin processes of these cells included into the neuropile. A bit later, additional FMRFa-IR cells appear in

the head region (j) and their processes follow the ventral nerve bundles (k). Scale bars: a-c, g-i 20 μ m; d-f 10 μ m, j, k 15 μ m.

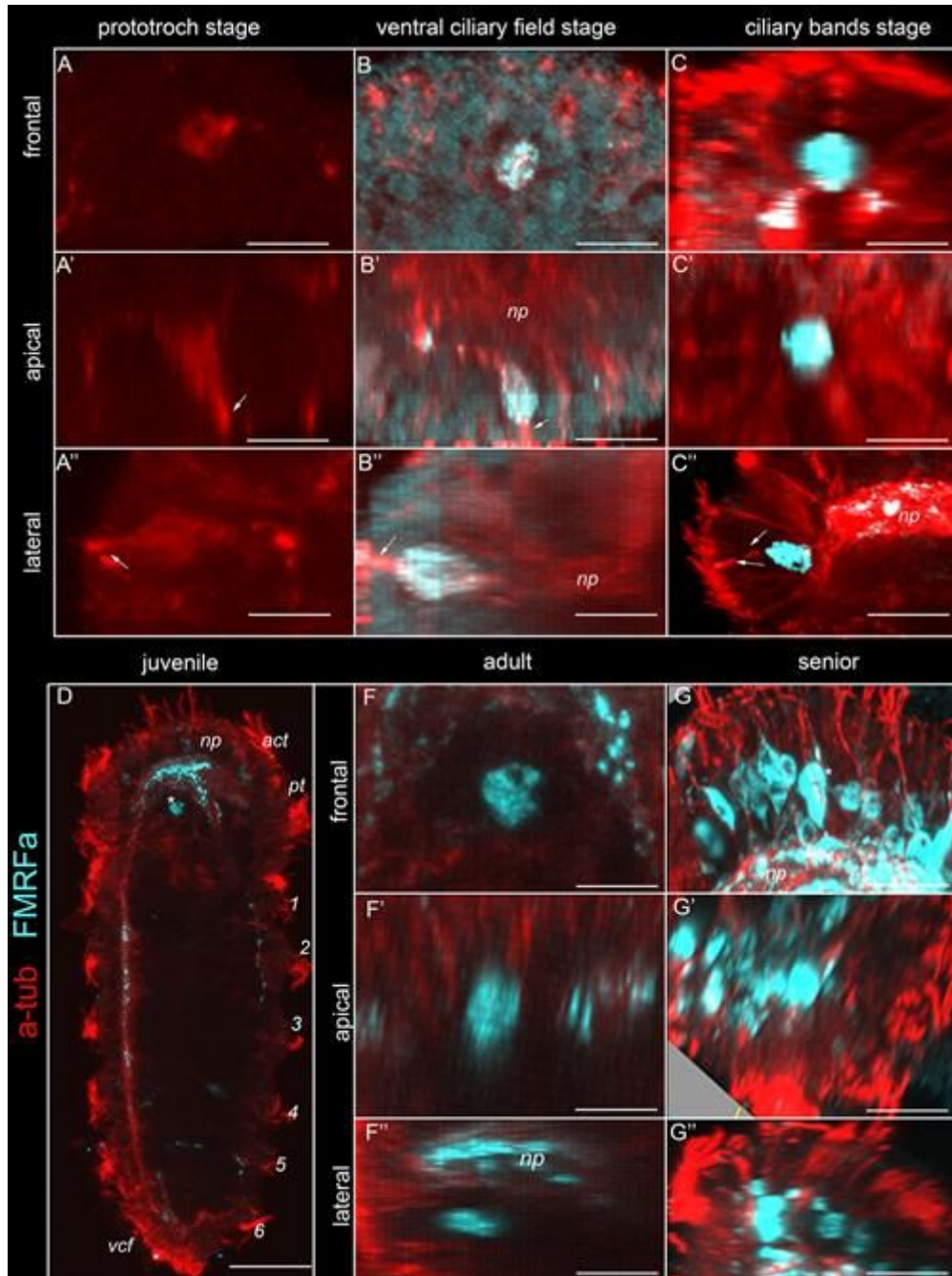


Figure 8

The ontogeny of anterior cell in *D. gyrocilatus* from the prototroch stage to senior specimen. Red-acetylated tubulin immunoreactivity; cyan- FMRFa-like immunoreactivity. The anterior cell is shown in frontal, apical and lateral projections through all developmental stages. At the prototroch stage (a) the cell appears, it's located above the prototroch, and bears cilia (arrow) on its' surface and do not demonstrate FMRFa-IR. At the ventral ciliary field stage the cell becomes FMRFa-IR (b). Later this cell becomes more prominent and obvious (c, d, f) and it's obvious that cell is not a part of CNS, it's located

separate from the neuropil (d). Even at the stage of senior individual this cell is noticeable (g) and it's still prominent and contains cilia on its' surface. Scale bar: a-c, f-g 5 μ m; d 40 μ m.

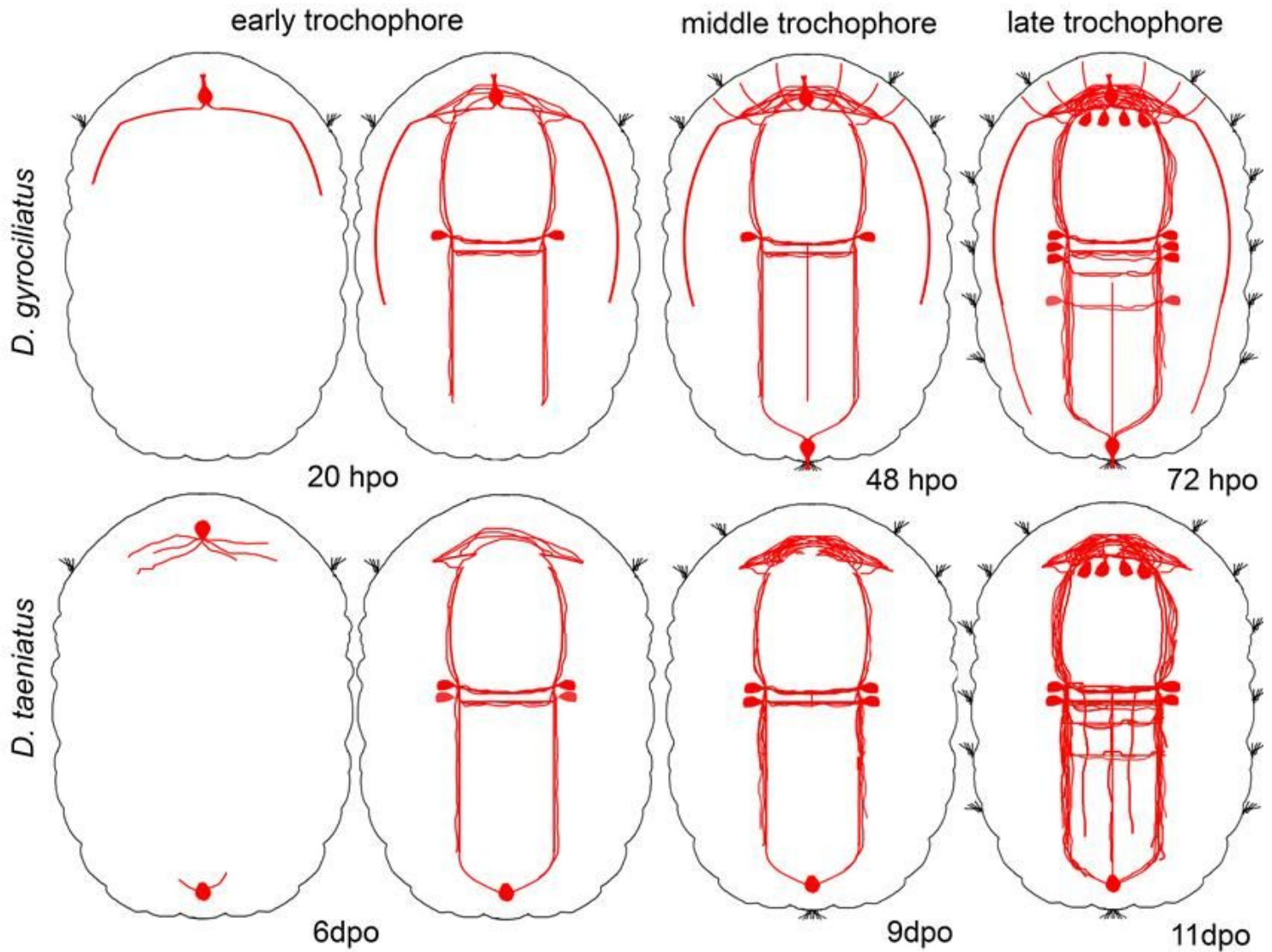


Figure 9

Summary diagram of the developmental events of tubulin-immunoreactive structures in *D. gyrocilatus* and *D. taeniatus*. The first neurons appear at the stage of early trochophores in both *D. gyrocilatus* and *D. taeniatus*. However, in *D. gyrocilatus* earliest neuron is a single peripheral sensory cell, bearing surface cilia and located above the prototroch on the ventral side. This cell has multiple fibers on its basal side. In *D. taeniatus* there are two earliest neurons: apical cell and caudal cell (both non sensory, due to lack of sensory cilia). These cells have multiple fibers. In *D. gyrocilatus* the earliest cell is present at the middle and late trochophores. In *D. taeniatus* the anterior cell detectable only at the early trochophore stage. In both *D. gyrocilatus* and *D. taeniatus* the nervous system develops in anterior-to-posterior direction. Apical end is always up. Relative dimensions are not maintained.

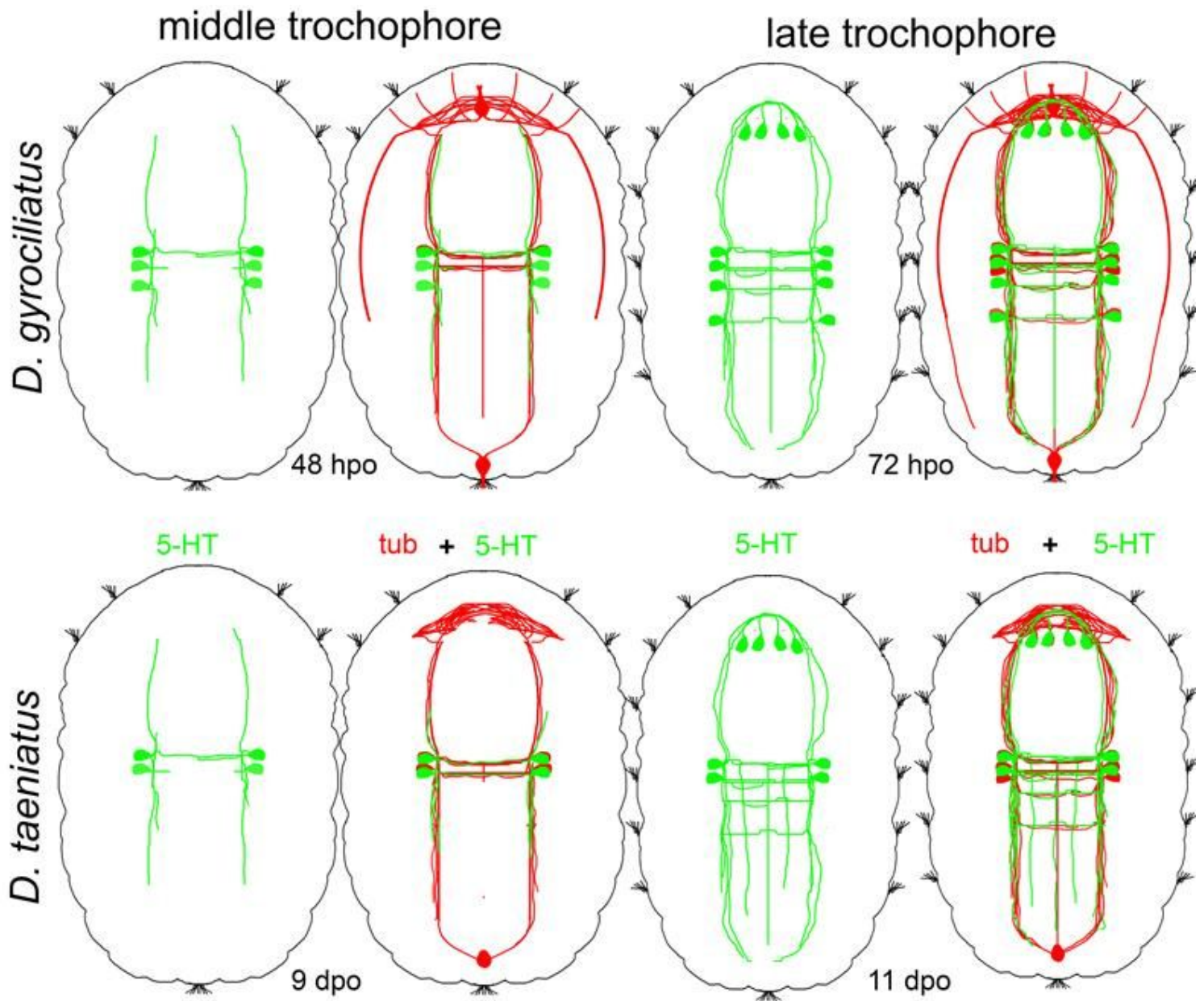


Figure 10

Summary diagram of 5-HT-IR elements in *D. gyrociatus* and *D. taeniatus*. The first 5HT-IR cells appear at the middle trochophore stage in both species. These are solitary cells at the level of first commissure. The processes of these cells spread to the main nerve structures-ventrolateral bundles and the neuropil. At the stage of late trochophore additional 5-HT-IR cells appear in the head region and in ventrolateral bundles.

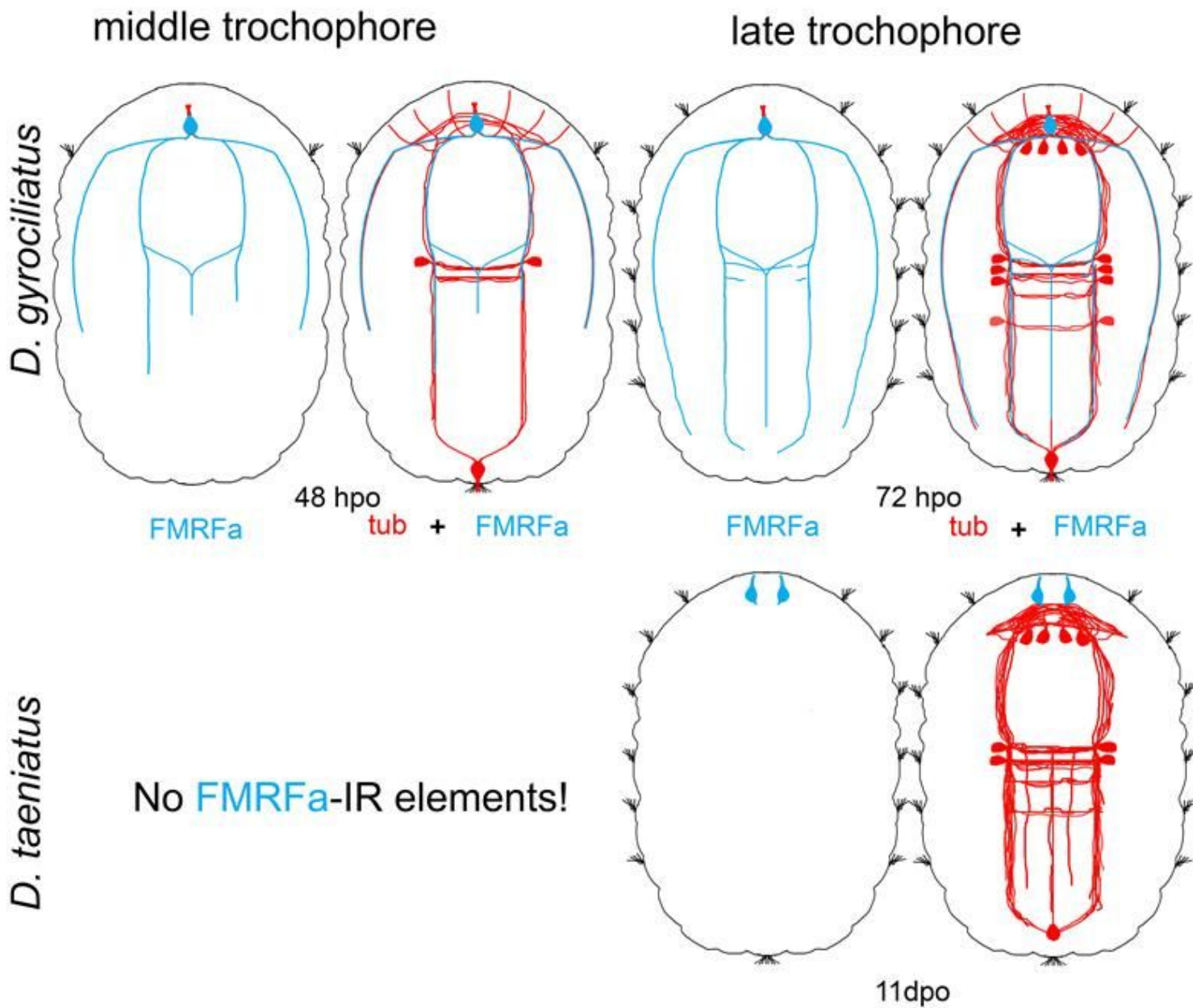


Figure 11

Summary diagram of FMRFa-IR elements in *D. gyrociatus* and *D. taeniatus*. First FMRFa-IR cell appears at the middle trochophore stage in *D. gyrociatus* and at late trochophore stage in *D. taeniatus*.

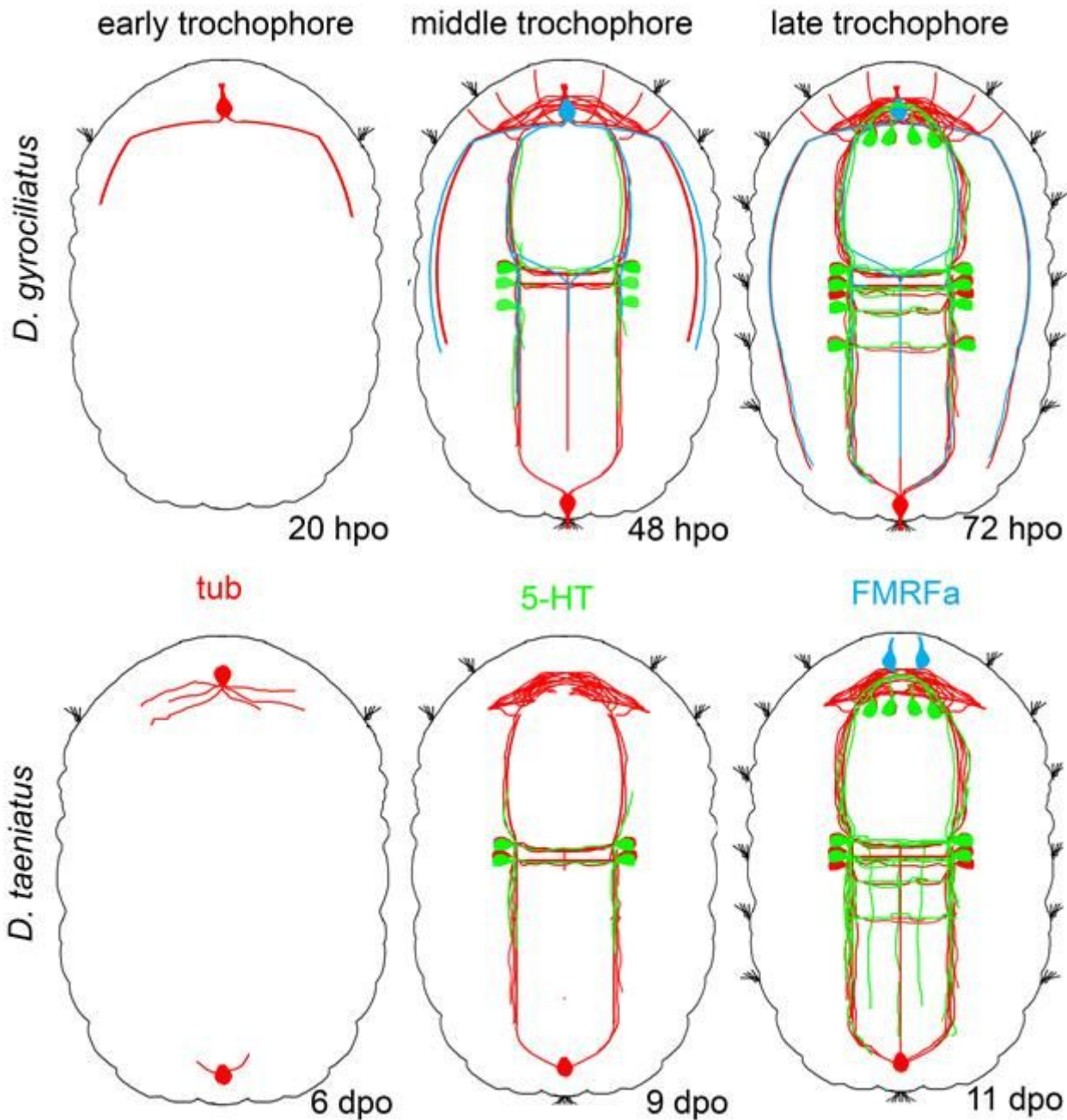


Figure 12

Summary diagram of the specific nerve elements in *D. gyrotilatus* and *D. taeniatus*. At the stage of early trochophore the first nerve cells appear. They do not 5-HT-IR or FMRFa- IR. In *D. gyrotilatus* first 5HT-IR cells appear at the level of first commissure and anterior cell becomes FMRFa-IR at the stage of middle trochophore. In *D. taeniatus* only 5-HT-IR cells appear at the stage of middle trochophore. At late trochophore stage more specific elements appear in both *D. gyrotilatus* and *D. taeniatus*, but they represent only small fraction of the whole nervous system.