

The structure of the muscular and nervous systems of the female *Intoshia linei* (Orthonectida)

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Abstract The systematic position of the Orthonectida remains enigmatic. According to a classical point of view, they are placed together with Dicyemida in the phylum Mesozoa. Traditionally, orthonectids are regarded as rather primitive organisms, lacking digestive, muscular, and nervous systems. Here, using confocal laser scanning microscopy (CLSM) and immunohistochemical methods, we describe the musculature and serotonergic nervous system of female adults of *Intoshia linei* (Orthonectida). The whole muscular system consists of 4 longitudinal and 9–11 circular muscle cells. The general muscular topography corresponds to the typical pattern for small-sized annelids or flatworms. Immunohistochemistry reveals six serotonin-like cells, which form part of a small nervous system comprising only 10–12 total cells based on nuclear counts. This is the first finding of a serotonergic nervous system in orthonectids. Our analysis of muscular and neural organization in Orthonectida reveals significant differences from Dicyemida and aligns it more closely with the Lophotrochozoa.

Keywords Mesozoa · Orthonectida · Muscular system · Nervous system · Confocal microscopy · Immunohistochemistry

Introduction

Orthonectida is probably one of the most enigmatic groups of so-called lower Metazoa, whose phylogenetic relationships remain uncertain (Giard 1877; Hatschek 1888; Hartmann 1925; Stunkard 1954; Caullery 1961; Hamlet et al. 1996; Pawlowski et al. 1996). Representatives of this group are parasites of a wide range of marine invertebrates. Their life cycle is rather simple and consists of two alternating generations: a free-living sexual stage and a parasitic stage, commonly referred to as plasmodium. The main mode of locomotion of orthonectid free living stages—males, females, and larvae—is ciliary swimming (Giard 1877; Giard 1879; Metschnikoff 1879; Metschnikoff 1881; Caullery and Mesnil 1901), which is generally considered to be a primitive feature. Most authors working with this group have either pointed to the absence of muscular and nervous systems in orthonectids or avoided mentioning their presence. In his final survey on orthonectids, Caullery (1961) stated that the female *Rhopalura ophiocomae* lacks any muscular system. Nevertheless, Kozloff (1969, 1971) described supposedly contractile cells in free-swimming stages of *R. ophiocomae* and *Ciliocincta sabellariae* using TEM and Protargol impregnated preparations. Later, morphologically similar cells were described in the female of *Intoshia variabli* (Slyusarev 1994, 2003). Moreover, female *I. variabli* have been observed to curve the anterior part of the body, which indirectly suggests the presence of a contractile system. Slyusarev and Manylov (2001) using whole mount rhodamine phalloidin staining of *I. variabli* demonstrated that the contractile system of Orthonectida is composed of true muscle cells. The presence of muscle cells requires neural integration, as found in all other animals; thus, both systems should be expected to occur in orthonectids.

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Here, we describe the muscular and nervous systems of female *Intoshia linei* based on the combination of phalloidin staining, immunohistochemistry (anti-5HT), and confocal laser scanning microscopy (CLSM).

Materials and methods

The orthonectid *I. linei* (Giard, 1877) is a parasite of the nemertine *Lineus ruber* (Müller, 1774) (Nemertina: Enopla: Heteronemertea). The nemertines were collected in August, 2014, in the Barents Sea at the marine biological station Dalnie Zelentsi (69° 07' N, 36° 05' E). The hosts were collected at the low tide under the stones. The infected nemertines were maintained in Petri dishes with filtered sea water. The emission of the free-living stages was carried out by rising the temperature of the water up to 10–15 °C.

Specimens of *I. linei* were fixed for 8–12 h in 4 % paraformaldehyde (PFA) with 0.01 M phosphate-buffered saline (PBS). After fixation, specimens were washed three times in PBS, containing 0.1 % Triton X-100 (PBT), and then stored in PBS with 0.1 % NaN₃ at +4 °C. Prior to immunostaining, specimens were blocked in PBT containing 1 % BSA for 2 h and incubated in primary antibodies against acetylated α -tubulin (Sigma, T-6793; dilution 1:1000–1:2000) and serotonin (Immunostar, 20080, diluted 1:2000) overnight at 21 °C. Afterward, specimens were washed three times in PBT for 15 min and incubated with secondary antibodies diluted 1:800–1:1000 overnight. We used the following secondary antibodies: Alexa Fluor 488 Donkey Anti-Rabbit (Molecular probes, A-21206) and Alexa Fluor 647 Donkey Anti-Mouse (Molecular probes, A-31571). To visualize muscular elements, after immunolabeling, the specimens were stained with TRITC-conjugated phalloidin in dilution 1:100 for 2 h. Cell nuclei were stained with Hoechst 33258 or DAPI. All the incubations were performed at 4 °C. Specimens were immersed in glycerol or Mowiol and mounted between two coverslips. Observations were made with Leica TCS SP5 or Leica TCS SPE laser confocal microscopes. Resulting stacks of images were processed using FIJI package (Schindelin et al. 2012). A total of more than 200 specimens were examined and documented.

Results

Muscular system of the female *I. linei*

The muscular system of the female *I. linei* consists of longitudinal and circular muscles only (Fig. 1). All muscle cells are located immediately under the ciliated epidermis. The longitudinal muscles lie outside the circular muscles (Figs. 1d and 3a, b). The longitudinal musculature consists of muscle bands

that extend from anterior to posterior in dorsal, ventral, and lateral positions (Figs. 1 and 3a, b).

The dorsal and ventral muscle bands are of similar structure and parallel to each other. In lateral view, the dorsal muscle band curves inward in the anterior third of the body (Figs. 1a, c and 3a). In the middle of the body, the ventral muscle splits to form an oval opening, which is the site of the genital pore. The muscle then reunites into a singular unit posterior of the pore (Figs. 1b and 3b).

At the anterior end, both the dorsal and ventral muscle bands bifurcate. Each branch extends anterolaterally and connects with its ipsilateral (dorsal or ventral) counterpart at the anterior border, thereby forming an apparently continuous muscle band (Fig. 1b). Posteriorly, the dorsal and ventral muscles bifurcate again but do not connect as above. Instead, each dorsal branch curves ventrally and connects with a dorsal branch of the ipsilateral lateral longitudinal muscle, while the ventral branches curve dorsally and connect with the ventral branch of the ipsilateral lateral longitudinal muscles (Fig. 1b, c). These bifurcations and reconnections make the longitudinal muscle system appear as a continuous network of bands as opposed to a series of independent muscles.

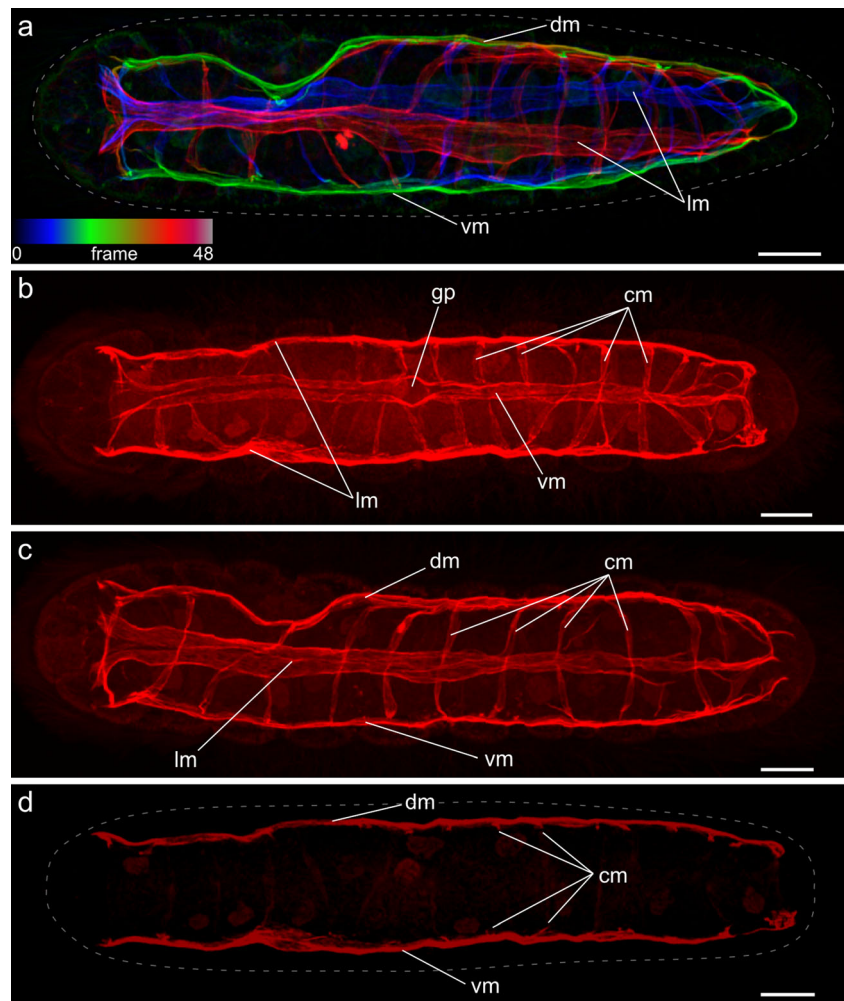
The structure of the right and the left lateral muscles is alike, and similar to the dorsal and ventral muscles. At the anterior end, both bands bifurcate (Figs. 1c and 3a, b). Thin termini of the lateral muscles insert in the ciliated epithelium. At one angle, the terminus appears as a band extension, while at another angle, it appears tapering and pointed at the tip, suggesting that the band terminus tapers only in one plane. The muscle band termini are located in between the ciliated epithelial cells. Lateral longitudinal muscles are attached in two spots at the anterior end of the body. In cross section, the dorsal, the ventral, and the lateral muscle bands are flattened; the same is true for the circular ones. All longitudinal muscles are 6–7 μ m wide throughout.

Female *I. linei* has approximately 9–11 circular muscles (Figs. 1a–c and 3a, b). The range reflects variability of the body length in different specimens, longer animals having more circular muscles. The circular muscles are nearly perpendicular to the longitudinal muscles, parallel to each other throughout the body, and located at about the same distance from one another. The only exception is the second anterior circular muscle, inclined to the longitudinal axis of the body (Figs. 1c and 3a). The width of the circular muscles is 1.5–2.5 μ m, which is much thinner than the longitudinal muscles, and still narrower in the sites where they pass under the longitudinal muscle fibers.

The serotonergic nervous system

The entire serotonergic nervous system of female *I. linei* is composed of three pairs of cells (Figs. 2c, d and 3c, d). All serotonin-positive cells lie dorsally. They are multipolar and

Fig. 1 Muscle system of female *I. linei*. TRITC-phalloidin staining, CLSM. **a** General view, depth-coded image. **b** Lateral view. **c** Ventral view. **d** Longitudinal section at the level of longitudinal muscles. *cm* circular muscles, *dm* dorsal muscles, *gp* genital pore, *lm* lateral muscles, *vm* ventral muscle. Scale bar 10 μ m



are compactly located in between the ciliated cells and the muscles (Fig. 2b). Two cells (the frontal ones) are shifted anteriorly. Each cell sends a process to the anterior end of the body; this process curves downward and projects along the lateral side of the body (Figs. 2b–d and 3c, d).

The two largest cells are located centrally (the central cells). Each cell has a long process extending backward along the longitudinal muscle band and nearly reaching the very end of the body (Figs. 2d and 3c, d). Also, each of the central cells appears to have several processes running forward and connecting these cells with the others to form a plexus.

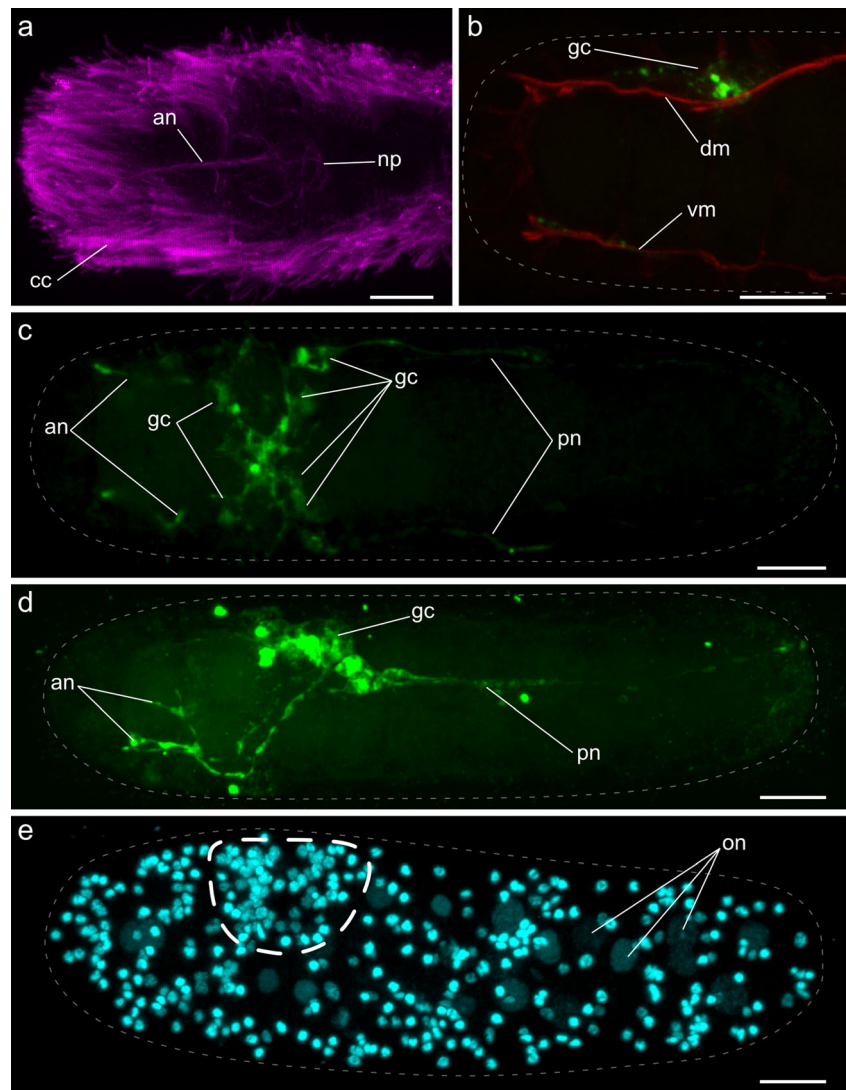
Two cells are more lateral (the lateral cells; Figs. 2b, c and 3c, d). Each of them sends a process backward, which extends along the longitudinal muscles up to the middle of the body. Two processes of these cells bend ventrally and converge to form a ring perpendicular to the longitudinal axis of the body (Figs. 2d and 3c). These cells also have several processes, which run forward and connect them with the others forming the plexus. Thus, the plexus is formed by short processes of all nerve cells and is located between them (Figs. 2a, c, d and 3d). The plexus sends a thin unpaired process toward the anterior

end of the body, projecting dorsally. Although immunohistochemistry demonstrated that this process was serotonin-positive (Fig. 3c, d), it is difficult to determine the origin of the apparent neurite. At the same time, antibodies against tubulin revealed microtubules both in the place of the plexus and the unpaired process extending forward (Fig. 2a).

Cell number counts

DAPI staining was used to quantify nuclei (hence, the cells; Fig. 3e). The total number of nuclei in the female *I. linei* is $369 \pm 2-3$. The body of the worm comprises a single-row epithelium, oocytes, muscle, and nerve cells. The number of the epithelial cells is 280. The number of oocytes was counted in living females with DIC and in DAPI-stained specimens, as their nuclei differ significantly in size from all other cells. The oocyte number is 35. Furthermore, the female genital pore comprises nine cells. Thus, the total number of epithelial cells, oocytes, and genital pore cells is $280 + 35 + 9 = 324$. The rest of the cells ($369 - 324 = 45$) account for the cells of muscular and nervous systems described here. The muscle cells lie under the

Fig. 2 Nervous system of female *I. linei*. **a** Acetylated α -tubulin immunoreactivity (magenta) showing nerve plexus and unpaired anterior nerve (*np*) in the anterior part of the body. **b** A sagittal section at the level of dorsal (*dm*) and ventral (*vm*) muscles, showing serotonin-positive immunoreactivity (green) in the incurvation of the dorsal muscle. **c, d** Serotonergic nervous system. View from the dorsal (**c**) and lateral (**d**) side. **e** Cell nuclei staining with DAPI. The ganglion region is outlined. *an* anterior nerve, *cc* ciliated cells, *dm* dorsal muscle, *gc* ganglion cells, *np* ganglionic nerve plexus, *on* oocyte nuclei, *pn* posterior nerves, *vm* ventral muscle. Scale bar 10 μ m



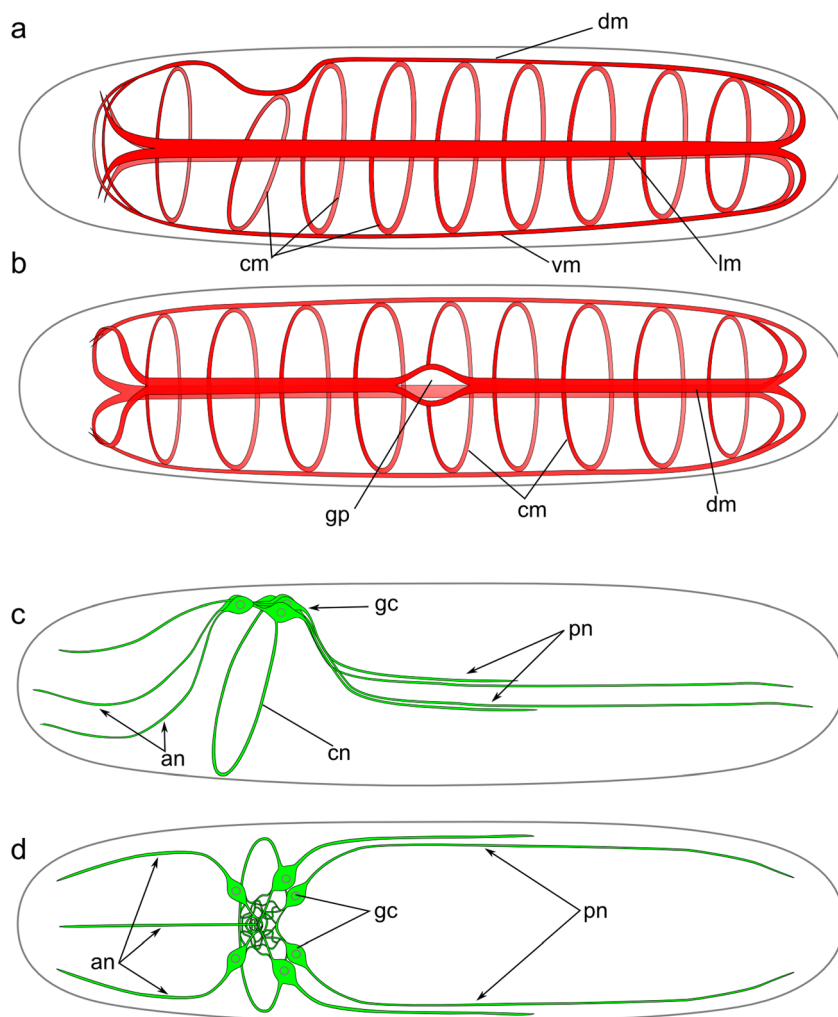
epithelial cells. The number of muscle cells is 35; hence, there should be only 10 nerve cells. Although the number of all cell types can vary from specimen to specimen, the variation is within a very small range ($\pm 2-3$) and it does not affect the order of magnitude. Thus, the maximal number of nerve cells should not exceed 10–12.

Discussion

The general pattern of the muscular system in female *I. variabilis* was first described by Slyusarev and Manylov (2001). Three main features of the orthonectid muscular system were revealed: the bilateral symmetry, the presence of only longitudinal and circular muscles, and the interior position of longitudinal muscles relative to the circular muscles. However, since we performed this study by means of epifluorescence microscopy, we failed to obtain images,

permitting unequivocal interpretation of relative localization of longitudinal and circular muscle bands. In this study, performed by means of CLSM in the females of another species, *I. linei*, which is similar in general organization and larger in size, we reconsider the general pattern of the muscular system in Orthonectida. We confirmed the first two characteristic features of the muscular system in orthonectids—the bilateral symmetry and the presence of only two types of muscle bands. As for the third feature, by using CLSM, we managed to show that the longitudinal muscles lie outside the circular muscles, which presumably should be true for other species as well. Such muscular pattern (lacking additional muscles of reproductive and digestive systems) is typical for orthonectids. Of course, similar patterns can be found in larvae of different invertebrates (Wanninger 2005; Rawlinson 2010; Helm et al. 2013; Hindinger et al. 2013), but we do not consider males and females of orthonectids as neotenic larvae. On the other hand, both males and female orthonectids might be dwarfs.

Fig. 3 Schematic drawings of musculature (**a, b**) and serotonergic nervous system (**c, d**) in female *I. linei*. **a, c** lateral view. **b, d** dorsal view. *an* anterior nerve, *cm* circular muscles, *cn* circular nerve, *dm* dorsal muscle, *gc* ganglion cells, *gp* genital pore, *lm* lateral muscles, *pn* posterior nerves, *vm* ventral muscle



Dwarf males are known in some invertebrates and are characterized by reduction of different systems (Neves et al. 2009). A thorough further investigation would be necessary to prove or to reject any speculation of this kind. Overall, muscular pattern of orthonectids corresponds to the general pattern characteristic of small-sized animals, such as flatworms (Tyler and Hyra 1998; Hooge 2001; Kotikova et al. 2002; Tyler and Hooge 2004; Tekle et al. 2005), several small annelids (Müller and Worsaae 2006), cycliophorans (Neves et al. 2012), and many others. However, in annelids circular muscles are present mostly in burrowing forms and may not be characteristic of the annelid ground plan (Tzetlin et al. 2002; Tzetlin and Filippova 2005; Purschke and Müller 2006). Moreover, when present, circular muscles in annelids are situated on the outside of the longitudinal muscles.

In addition to our novel findings on the muscular system, we provide the first description of the serotonergic nervous system in orthonectids. The simplicity of its structure makes the comparison with other groups highly speculative. Nevertheless, similarly structured nervous systems are present in

Acoela (Reuter et al. 2001, Achatz and Martinez 2012), Plathelminthes (Reuter et al. 1998; Kotikova et al. 2002; Rawlinson 2010), Gnathostomulida (Müller and Sterrer 2004), Gastrotricha (Rothe et al. 2011), and very small Annelida (Müller and Westheide 2002; Orrhage and Müller 2005; Müller 2006). At the same time, analysis of the fine structure of orthonectids (the presence of the true cuticle and fibrous bands in the epithelial cells consisting of ciliated and non-ciliated cells) supported our hypothesis that orthonectids are closer to Annelida, or, at least, Trochozoa (Slyusarev 2000; Slyusarev and Kristensen 2003). To avoid unjustified speculations, so far we can conclude that the bilateral pattern of the serotonergic nervous system of Orthonectida is reminiscent of different groups of lophotrochozoans (Reuter et al. 2001; Brinkmann and Wanninger 2008; Rothe and Schmidt-Rhaesa 2009; Hinderling et al. 2013), but the pattern itself does not provide further insights.

The key questions concerning orthonectids are as follows. What are their phylogenetic relationships? Are orthonectids

related to dicyemids, and is it possible to join them in one group—Mesozoa?

Traditionally, orthonectids have been regarded as very primitive animals. Molecular data (Hamlet et al. 1996; Pawlowski et al. 1996) argue for placing them in Bilateria. Petrov's data (Petrov et al 2010) permit to refer orthonectids to Spiralia, and among these, possibly, to annelids. Our data are in good agreement with this concept and allow placing them in Lophotrochozoa, specifically, in Trochozoa.

Joining Orthonectida and Dicyemyda in one phylum has long been questioned. According to molecular data (Petrov et al. 2010), Dicyemyda and Orthonectida are related. This seems rather doubtful, as dicyemids lack both nervous and muscle systems and, basically, have no real tissues at all. So, joining these two groups into one phylum, Mesozoa, seems controversial.

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