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The nervous system of *Xenotrichula intermedia* and *X. velox* (Gastrotricha: Paucitubulatina) by means of immunohistochemistry (IHC) and TEM

Birgen Holger Rothe*, Alexander Kieneke and Andreas Schmidt-Rhaesa***

Abstract

We present here the first data on the nervous system of members of the Paucitubulatina employing immunohistochemical methods, confocal laser scanning microscopy (CLSM) and transmission electron microscopy (TEM). *Xenotrichula intermedia* and *Xenotrichula velox* were investigated with different antibodies against neuron-specific molecules (5-HT (serotonin), FMRFamide; acetylated α-tubulin) as well as TEM. The data supports a highly conservative architecture of the nervous system within the Gastrotricha and further supports the hypothesis of a sistertaxon relation between Paucitubulatina and *Neodasys*.

Introduction

Paucitubulatina, a subtaxon of the Gastrotricha, are common members of the meiofauna community. Their size ranges from 0.1–1.0 mm, with a characteristic tenpin-like body shape and one pair of posterior adhesive tubes, contrary to the two other subtaxa of the Gastrotricha: the Macrodasyida and the Multitubulatina *(Neodasys)*. These latter taxa possess additional adhesive tubes along the trunk and show in the most cases not a tenpin-like body shape. The Paucitubulatina include marine as well as freshwater species, whereas the two other suborders of the Gastrotricha contain mainly marine and some brackish members (for the few exceptions see Kisielewski 1987). The knowledge of the morphology of the Gastrotricha has increased during the last two decades; this specifically applies to some organ systems e. g. the reproductive, nephridial, muscular and nervous system. Especially regarding the reproductive system the data include members of all three subtaxa of the Gastrotricha, which enables us to reconstruct evolutionary trait within the Gastrotricha. For the evolution of the reproductive system within the gastrotrichs see e. g. Kieneke et al. (2008), for the evolution of the nephridial system see Kieneke et al. (2007) and for evolutionary traits of the musculature see Leasi et al. (2006). The data on the nervous system of the Gastrotricha are more scattered, although TEM-based, ultrastructural investigations ex-

* Zoological Museum, University Hamburg, Martin-Luther-King-Platz 3, 20146 Hamburg, Germany; e-mail: andreas.schmidt-rhaesa@uni-hamburg.de

** Forschungsinstitut und Naturmuseum Senckenberg, Deutsches Zentrum für Marine Biodiversitätsforschung, Südstrand 44, 26382 Wilhelmshaven; e-mail: akieneke@senckenberg.de

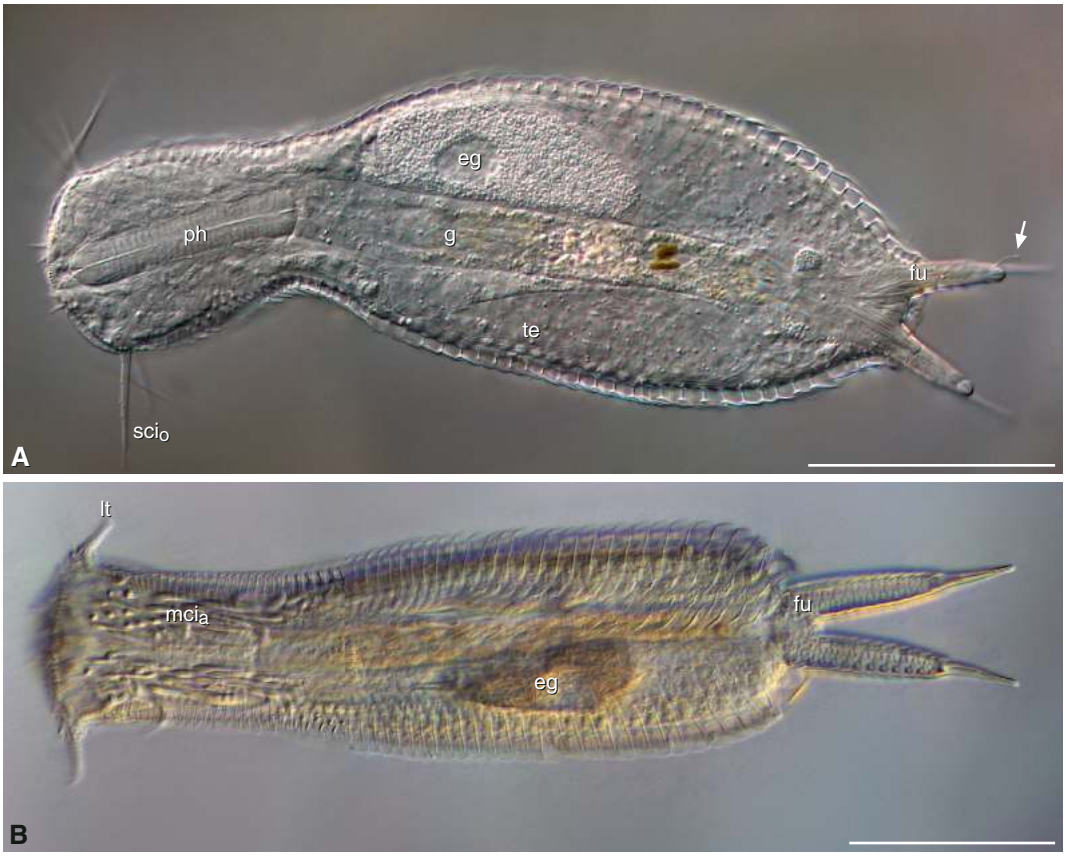
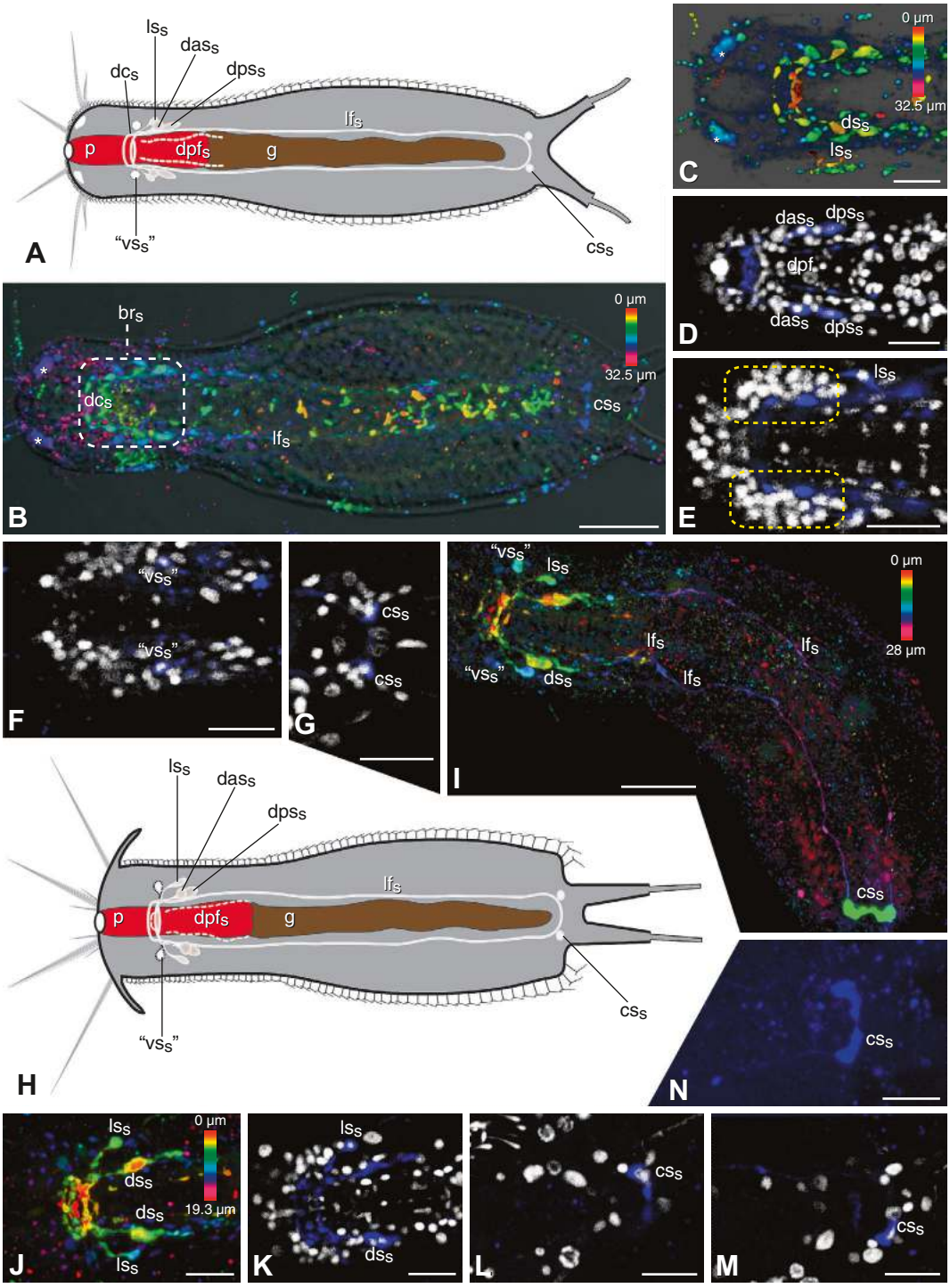


Fig. 1. Lightmicroscopy of *Xenotrichula intermedia* and *Xenotrichula velox*. **A.** Overview of *X. intermedia*, the **white arrow** indicates a sensory cilium at the base of the posterior adhesive tube. **B.** Overview of *X. velox*, ventral body side. Scale bars: A, B, 50 µm. **eg**, egg; **fu**, furca; **g**, gut; **lt**, lateral tentacle; **mci$_a$**, anterior motile cirri; **sci$_o$**, outer sensory cirri; **ph**, pharynx.

ist for several members of the Macrodasyida (Teuchert 1976, 1977; Gagné 1980; Ruppert 1991; Wiedermann 1995).

More recently the introduction of immunohistochemical and histochemical methods in combination with advanced light microscopy e. g.

Fig. 2. Visualisation of 5-HT-like IR of *Xenotrichula intermedia* and *Xenotrichula velox*. **A–G.** *X. intermedia*. ▷ **H–N.** *X. velox*. **B,C,I,J.** Colour-coded by depth (CCD-projections), in B with a transmission channel overlay. **D,E,F,G,K–L.** Single optical sections of a double labelling 5-HT (blue) and nuclei (white). **N.** Maximum projection, 5-HT (blue). **A.** Schematic overview of the 5-HT-like IR of *X. intermedia*. **B.** Overview of the 5-HT-like IR of *X. intermedia*, **white asterisks** indicate unspecific binding of 5-HT-antibodies at the anterior region. **C.** Detail of the brain in *X. intermedia*, **white asterisks** indicate unspecific binding of 5-HT-antibodies at the anterior region. **D.** Detail of the dorsal somata of the brain of *X. intermedia*. **E.** Detail of the median level of the brain of *X. intermedia*. **F.** Detail of the ventral level of the brain of *X. intermedia*, **yellow dashed lines** indicate the two hemispheres of the brain. **G.** Detail of the posterior end of the trunk of *X. intermedia*. **H.** Schematic overview of the 5-HT-like IR of *X. velox*. **I.** Overview of the 5-HT-like IR of *X. velox*. **C.** Detail of the brain in *X. velox*. **K.** Detail of the dorsal somata of the brain of *X. velox*. **L–N.** Detail of the posterior end of the trunk of *X. velox*. Scale bars: B, I, 20 µm; C–G, J–N, 10 µm. **br$_s$**, 5-HT-like IR of the brain; **cs$_s$**, caudal 5-HT-like IR soma; **ds$_s$**, dorsal 5-HT-like IR soma of the brain; **das$_s$**, anterior dorsal 5-HT-like IR soma of the brain; **dpf$_s$**, dorsal 5-HT-like IR pharyngeal fibre; **dps$_s$**, posterior dorsal 5-HT-like IR somata of the brain; **g**, gut; **lf$_s$**, 5-HT-like IR longitudinal fibre; **ls$_s$**, lateral 5-HT-like IR soma of the brain; "**vs$_s$**", presumably ventral 5-HT-like IR soma of the brain.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confocal laser scanning microscopy (CLSM) in the field of morphology has proved to be a highly useful and powerful tool to give new insights in the morphology of micro-invertebrates (for a summery see Wanninger 2007). In the case of the gastrotrich nervous system several members of the Macrodasyida have been investigated by these methods until now (Turbanellidae: Joffe & Kotikova 1987, Joffe & Wikgren 1995, Hochberg 2007, Rothe & Schmidt-Rhaesa 2008; Dactylopodolidae: Hochberg & Litvaitis 2003, Liesenjohann et al. 2006, Hochberg 2007, Rothe & Schmidt-Rhaesa 2009; Macrodasyidae: Hochberg & Litvaitis 2003; Lepidodasyidae: Hochberg & Litvaitis 2003, Hochberg & Atherton 2011; Thaumastodermatidae: Rothe & Schmidt-Rhaesa 2010a). Data on the nervous system of Multitubulatina *(Neodasys)* have been published by Hochberg (2007) and Rothe et al. (2011), but for the Paucitubulatina data are completely lacking until now.

In the present contribution we present new data on the nervous system of two members of the Xenotrichulidae. The xenotrichulids represent an exclusively marine group among the Paucitubulatina; within this group they represent a basal branch (Todaro et al. 2006, Kieneke et al. 2008). Nevertheless the Xenotrichulidae are characterized by some conspicuous traits within the Paucitubulatina, e. g. the occurrence of motile compound cilia in the so called ventral cirri (Remane 1936) (see Fig. 1B); on the other hand this group possesses plesiomorphic characters of the Gastrotricha, e. g. simultaneous hermaphroditism and the occurrence of paired testes with well developed filiform spermatozoa (Balsamo 1992).

Material and method

For **i**mmuno**h**isto**c**hemical (IHC) investigation specimens of *Xenotrichula intermedia* and *Xenotrichula velox* were sampled on the island of Sylt, Northwest Germany, in May 2008. Both species were found sympatrically occurring in sandy intertidal sediments with low contents of detritus close-by the Wadden Sea Station in List/Sylt (55°00'56" N/8°25'17" E). The samples contained in addition specimens of *Neodasys chaetonotoideus* (Gastrotricha) and *Protodrilus* spec. (Annelida) in high abundances. The meiofauna organisms were extracted from the sediment by the magnesium chloride ($MgCl_2$) relaxation and decantation method of Rieger & Ruppert (1978). Subsequently, specimens were sorted out under a dissection microscope.

The xenotrichulids were relaxed in 7 % $MgCl_2$ (w/v) in distilled water, and subsequently fixed in 4 % **p**ara**f**orm**a**ldehyde (PFA) (w/v) in 0.1 M **p**hosphate **b**uffered **s**aline (PBS according to Crittenden & Kimble (1999)) (pH 7.3) on ice over night. To remove excessive fixative the samples were washed several times in 0.1 M PBS (pH 7.3) and stored in 0.1 M PBS (pH 7.3) containing 0.05 % (w/v) sodium azide (NaN_3) at 4 °C for several weeks. The following antibodies were used: anti acetylated α-tubulin (Sigma) diluted 1:300, anti-5-HT (Sigma) diluted 1:1000 and anti-FMRFamide (ImmunoStar) diluted 1:500. The IHC staining was done as described by Rothe et al. (2011). In addition the nuclear dye 4',6-**Di**a**a**midino-2-**p**heny**l**indol (Dapi) (Sigma) was used as a histochemical counterstain.

The specimens were embedded in Citiflour AF1 on microscopic slides, and coverslips sealed by nail polish.

The microscopic investigation took place with a Leica TCS 2 equipped with a near UV laser source. The post processing of the data and the projections were made by using LCS Simulator software version 2.61 (Leica) and by Zeiss ZEN 2008 Light Edition software.

In total 13 specimens of *X. intermedia* and five specimens of *X. velox* were labelled against 5-HT and acetylated α-tubulin, additionally three specimens of *X. velox* were labelled against FMRFamide.

For **T**ransmission **E**lectron **M**icroscopy (TEM) specimens of *Xenotrichula intermedia* were sampled between October 16 and 18, 2002, at the beach of the islet Uthörn situated in the bay of Königshafen at the northern tip of Sylt. The specimens were extracted from the sandy sediment with the modified seawater-ice method according to Uhlig (1964) and Ruppert (1972). Animals were fixed in 2.5 % glutaraldehyde (a little ruthenium-red added) in 0.1 M sodium cacodylate buffer (pH 7.2) for 3 h at 4 °C. They were postfixed for 45 min at 4 °C in 1 % OsO_4, (buffered in 0.1 M sodium cacodylate solution, pH 7.2), dehydrated in an increasing acetone series and embedded in Araldite.

Serial ultrathin sections (70 nm) were made on a Reichert Ultracut S microtome and stained automatically with uranyl acetate and lead citrate (Leica EM Stain). The sections have been investigated on a Zeiss EM 502 transmission electron

microscope at 80 kV acceleration voltage. Images were captured with a Dual Scan CCD camera using the iTEM® software (soft imaging systems).

Some measurements are presented as relative body units (U); this means percent units of the entire body length from anterior to posterior.

Results

5-HT-like IR components of the nervous system of the two *Xenotrichula* species. The 5-HT-like immunoreactivity (IR) of *Xenotrichula intermedia* is here reported and compared with the IR of *Xenotrichula velox*. For the description of the brain we name the IR somata with respect to their position relative to the dorsal commissure (anterior, posterior or lateral). This remains valid for the following descriptions (anti-acetylated α-tubulin IR, anti-FMRFamide IR).

Both species investigated show a 5-HT-like immuno-positive labelling observable in the brain (br_s), along the digestive tract and in the posterior region of the trunk (Fig. 2A–C,H–J). No specific IR is observable anterior of the dorsal commissure. The brain of *X. intermedia* (n = 13) consists of 5-HT-like IR fibres within the dorsal commissure (dc) and corresponding posteriolateral IR somata (Fig. 2C). The IR fibres of the dorsal commissure are located at around U8–U10 from the anterior end and run like an arc (width ~6 µm, length ~11 µm) over the muscular pharynx, no nuclei are present in this region (Fig. 2C). Within the dorsal commissure two distinct domains of 5-HT-like IR fibres are recognizable (n = 13). A fine single fibre (diameter ~1 µm) is present anteriorly, and at least two, closely attached immuno-positive fibres are present at the posterior margin of the commissure (Fig. 2C). The anterior fibre seems to run laterally in posterior direction and becomes indistinguishable from the posterior ones. In this region a strong 5-HT IR signal occurs.

Several 5-HT IR somata-like structures are associated with the brain. First we describe those clearly as somata identifiable structures, in which the fluorescent signal is clearly colocalized with a nuclear staining. Posterior to the dorsal commissure are six IR somata, three in each hemisphere (Fig. 2A), the arrangement is bilateral symmetrical. Two somata (ds_s) are located close together dorsolateral of the pharynx (n = 13) (Fig. 2A,C,D). Both somata show an elliptical shape oriented in the anterior-posterior axis (a-p) of the body, however, the posterior dorsal soma (dps_s) is more elongated with an acute posterior end (length 6–7µm, width < 2 µm) and the anterior one (das_s) is more roundish with a blunt posterior end (length 4–5 µm, width < 2 µm). Both somata projecting in anterior direction, the neurites of each cell run parallel up to the posteriolateral margin of the dorsal commissure. Here they become indistinguishable within the strong IR signal at this point. An additional 5-HT-like IR soma (ls_s) is present laterally of the two dorsal somata (n = 13) (Fig. 2A,E). The maximal lateral distance between this soma and the dorsal ones is ~5 µm. This soma lies next to ds_s, slightly shifted to a more anterior direction. The soma itself shows an elongated and falcate shape (length ~6–7 µm, width ~1 µm). The lateral soma sends a process in anterior direction to the posteriolateral margin of the dorsal commissure, where the neurites of the dorsal 5-HT-like IR somata enter the dorsal commissure, too.

In some specimens (n = 10) an additional putative 5-HT-like IR soma ("vs_s") is visible ventrolateral of the posterior margin of the dorsal commissure (Fig. 2A,F). It was not always possible to find an associated nucleus. This was only the case in about half of the specimens (n = 7). In addition, in almost all of the investigated specimens of *X. intermedia* two regions of strong IR signal are present flanking the mouth opening on both sides of the pharynx (Fig. 2A–C). Overall the background has an unusual high level of signal that is present as "salt & pepper"-like signal especially in the area of the integument and the cilia (Fig. 2B). However, this anterior signal seems to be located inside the body, especially in the ventral region (Fig. 2C, white asterisks). The signal is kidney-shaped (length 8 µm, width 3–4 µm) and occurs between the inner and the more lateral head cirri. Associated nuclei were not observed.

At the ventral posteriolateral margin of the dorsal commissure a fine 5-HT-like IR fibre originates and runs along the pharynx and gut in posterior direction (Fig. 2A). This fibre was only observable in seven specimens, probably due its very fine diameter (< 1 µm). At the posterior end, the fibres form a closed loop (Fig. 2B) with two lateral, triangular, caudal somata (cs_s) (length ~3.5 µm, width 2 µm) (Fig. 2A,B,G).

In some specimens two additional longitudinal fine fibres (dpf) (diameter < 1 µm) were visible dorsally, between the dorsal commissure and the transition between pharynx and gut (Fig. 2A). The

distance between the two fibres is ~6 µm directly behind the dorsal commissure and ~9 µm at the end of the pharynx. It was not possible to detect a direct connection to the neurites of the dorsal commissure (dc).

The pattern of the 5-HT-like IR in *X. velox* is very similar to *X. intermedia*. The 5-HT-like IR components of the dorsal commissure consist of one anterior fibre and two posterior ones, but the anterior one of these two fibres bears two short bow-like anteriorly directed protrusions (Fig. 2H). The dorsal 5-HT-like IR somata occur in the same position as in *X. intermedia*, but the anterior and posterior ones are closer together (Fig. 2A,I,J). The presence of two somata only becomes observable by the counterstaining of the nuclei (Fig. 2K). The lateral 5-HT-like immunopositive soma is slightly shifted in distal direction compared to *X. intermedia* (Fig. 2K). The putative ventral 5-HT-like IR soma of the brain is in this instance not unequivocal to find. Ventrolateral to the posterior margin a soma-like IR structure is observable, but no associated nuclei were found. In contrast to *X. intermedia* the connecting fibre between the two posterior 5-HT-like IR somata shows a very strong IR signal (Fig. 2I,N).

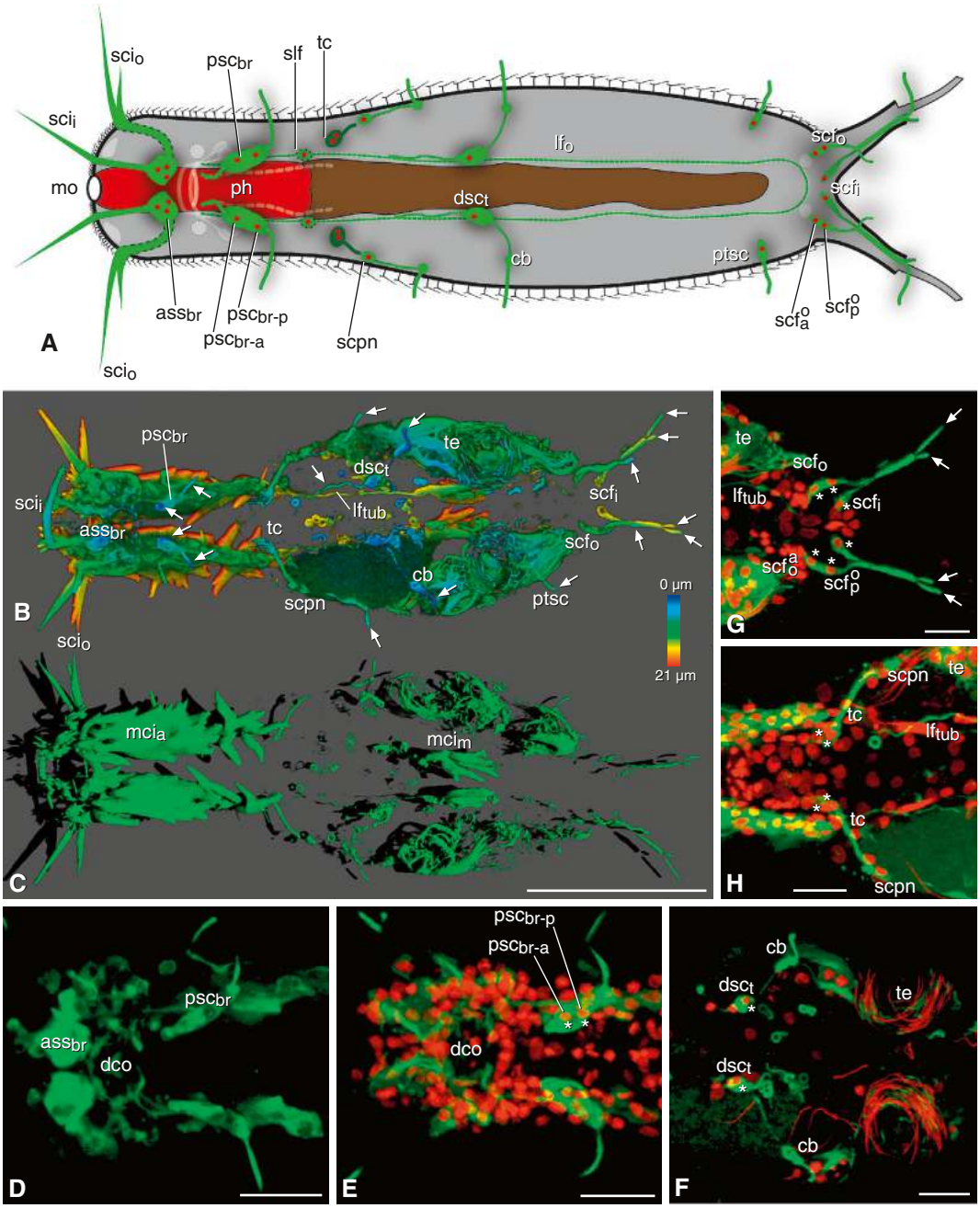
Anti-acetylated α-tubulin-IR of *Xenotrichula intermedia*. The following description is based on 5 specimens of *X. intermedia* labelled against 5-HT, acetylated α-tubulin and nuclei. First we have to point out that in the nervous system the tubulin labelling was restricted to parts of the nervous system. Only the the axonemata of several ciliary structures, e. g. the motile ventral cirri, the axonemata of the protonephridial terminal cells and the ciliary rootlets of the spermatozoa and those parts of the nervous system that are putatively connected to sensory structures were labelled by the antibody (Fig. 3A). Surprisingly, no or not more than a weak labelling occurs in some neuronal areas where we expect neurotubuli as a target of the used antibody from comparative studies on other gastrotrichs (e. g. the dorsal commissure). A very strong IR signal occurs, especially in the anteroventral region of the body, due to the motile cirri (mci) (Fig. 3A,C). These compound cilia are located in the anterior part of the specimens in two ventrolateral tracts, extending posteriorly up to the transition between gut and intestine, and are named here the anterior motile cirri (mci_a). More posterior, in the medioventral mid-trunk region, a paired tuft of five cirri (mci_p) is present (Fig. 3A,C). All motile cirri are equal in size.

A further source of acetylated α-tubulin IR is the axonemata of the spermatozoa within the paired testes (te) (Fig. 3B,G,H,F). The testes are located laterally in the mid-trunk region. Within the testes the spermatozoa are coiled together. Among the individuals that contain eggs (eg) the yolky cytoplasm appears as a homogenously labelled structure, due to the autofluorescence of the yolk material (Fig. 3B).

Slightly more posterior of the transition zone between pharynx and gut (~35U), an additional ciliary structure is marked by the antibodies. These are the cilia of the terminal cells (tc) of the protonephridia (Fig. 3A,B,H) (see discussion, the number of cilia could not be resolved clearly). The cilia (length ~15 µm, diameter ~1 µm) origin next to the gut and run posteriolateral up to the epidermis. Proximally, two associated nuclei are visible (Fig. 3H). At the distal end of the cilia of the terminal cell is a highly IR soma (~U39), which we tentatively name the "sensory cell of the protonephridia" (scpn), solely due to the position of the cell (Fig. 3A,B,H). This possibly sensory cell of the protonephridia (scpn) bears a posteriorly directed fine neurite-like process (diameter <1 µm), which runs 18-20 µm along the lateral margin of the trunk and terminates in a short lateral cilium (length 5–6 µm) at U47–48 (Fig. 3B).

Regarding the case of the anti-acetylated α-tubulin IR components of the nervous system it is quite evident that only ciliated sensory cells

Fig. 3. Visualisation of anti-acetylated α-tubulin IR of *Xenotrichula intermedia*. **A–G.** *X. intermedia*. **B.** Colour-coded by depth (CCD-projections). **C.** Simulated fluorescence projection (SFP-projection). **D.** Maximum projection of single labelling of acetylated α-tubulin (green). **E–F.** Single optical sections of a double labelling acetylated α-tubulin (green) and nuclei (red). **A.** Schematic overview of the acetylated α-tubulin IR of *X. intermedia*, the 5-HT-like IR components are indicated by transparent white colouration. **B.** Overview of the acetylated α-tubulin IR of *X. intermedia* from dorsal. Arrows indicate ciliary structures. **C.** Overview of the acetylated α-tubulin IR of *X. intermedia* from ventral. **D–E.** Detail of the dorsal somata of the brain. **F.** Detail of the dorsal level of the median trunk of *X. intermedia*. **G.** Detail of the posterior end of the trunk. Arrows indicate ciliary structures. **H.** Detail of the region of the protonephridium. Scale bars: B,C, 50 µm; D–H, 10 µm. **ass_{br}**, anterior sensory ▷



somata of the brain; **cb**, ciliary base; **ds$_s$**, dorsal 5-HT-like IR soma of the brain; **dco**, dorsal commissure; **dsc$_t$**, dorsolateral sensory cells of the trunk; **lf$_{tub}$**, longitudinal fibre; **mci$_a$**, anterior motile cirri; **mci$_m$**, motile cirri of the mid-level trunk, **mo**, mouth opening; **dpf$_s$**, dorsal 5-HT-like IR pharyngeal fibre; **psc$_{br}$**, posterior sensory somata of the brain; **psc$_{br-a}$**, anterior soma of the posterior sensory somata of the brain; **psc$_{br-p}$**, posterior soma of the posterior sensory somata of the brain; **ptsc**, posterior sensory cell of the trunk; **scf$_i$**, inner sensory cells of the furca; **scf$_o$**, outer sensory cells of the furca; **scf^a_o**, anterior outer sensory cell of the furca; **scf^p_o**, posterior outer sensory cell of the furca; **sci$_i$**, inner sensory cirri next to mo; **sci$_o$**, outer sensory cirri; **scpn**, "sensory cell of the protonephridia"; **slf**, somata of the longitudinal fibre; **tc**, terminal cell of the protonephridium; **te** testes.

are marked (axonem and soma). We found several IR cells/groups of cells along the body. Especially the anterior groups of sensory cilia are strongly immunopositive and highlight the three pairs of sensory cirri of the head, the inner sensory cirri (sci_i) of the head next to the mouth opening and the two pairs of outer sensory cirri (sci_o) of the head (Fig. 3A–C); these have their point of origin at the lateral side of the head (Fig. 1A). Directly anterior of the dorsal commissure (dco) is a strongly IR region (~U7–11), composed of a paired group of obviously highly IR somata (ass_{br}) (Fig. 3A,B,D). The two groups are arranged in juxtaposition with each other laterally of the midline. The appearance is more or less drop-shaped (length ~7 µm, width ~9 µm) with the more acute part directed anteriorly. Overall, three nuclei are embedded in the IR material, indicating that this group is composed of three separated cells. From the anterior part of the group a process (diameter ~2 µm) runs to the inner sensory cirri (sci_i). On the ventrolateral part of the group another process obviously runs to the two outer sensory cirri of the head. Due to the location next to the motile ventral cirri we were unable to follow this process in detail.

Posterior to the dorsal commissure (~U20) another paired group of tubulin IR somata forming the posterior sensory cells of the brain (psc_{br}) is present (Fig. 3A,B,H,E). Each group is composed of two somata, indicated by nuclei counterstaining (Fig. 3E). The shape of the somata appears ovoid and the position of the two groups is dorsolateral of the pharynx. The anteriormost cell of the posterior sensory cells of the brain ($psc_{br\text{-}a}$) is located just above the dorsal 5-HT-like IR somata, next to the pharynx. The second soma extends posteriolateral ($psc_{br\text{-}p}$). Each entire group (composed of the two posterior sensory cells) has a length of ~8 µm and a width of 3 to 4 µm. The borders of the two cells within the group cannot be seen. Two cilia (length ~10 µm) emerge from each group: one anterior and one posterior (Fig. 3B,D,E). The anterior cilium seems to belong to $psc_{br\text{-}a,}$ and is located at the anteriormost part of the group. The distance between the two anterior cilia is about 10 µm. The second cilium originates at the posterior margin of $psc_{br\text{-}p}$ in a more lateral position.

Approximately at the level of the transition between the pharynx and midgut in some preparations another pair of tubulin IR somata (slf) (~U27) was observable, obviously associated with the ventral longitudinal neurite bundle. This position corresponds to the posterior margin of the anterior motile cilia (mci_a) (Fig. 3A). The position of the somata is obviously next to the digestive tract, each soma appears drop-shaped (length ~7 µm, width ~2 µm). The fine longitudinal tubulin IR fibre (lf_{tub}) (diameter < 1 µm) becomes recognizable posterior to mci_a and follows the course of the digestive tract in a lateral position (Fig. 3A,B,G,H). During its course the fibre passes the mci_p and ends directly anterior to the somata of the sensory ciliary cells of the furca (see below). A tubulin IR connection between the two fibres in the posterior end was present in some, but not observable in all specimens.

In the middle of the trunk (~U50), an additional pair of ciliated blunt drop-shaped sensory cells (dsc_t) (length 5–6 µm, length 3–4 µm) is located in a dorsolateral position (Fig. 3A,B,F). Each soma bears at its anterior side a process. This fine fibre (diameter < 0.5 µm) approaches the longitudinal tubulin IR fibre and 20–25 µm anterior of the soma both fibres becomes undistinguishable (Fig. 3B). From the posterior part of each soma a sensory process emerges (diameter < 1 µm), this runs in posteriolateral direction (~7 µm in length) and ends in a strongly IR swollen, "soma-like" structure (cb) (length ~4–5 µm, diameter ~2 µm) within the epidermis at U58, but an associated nucleus is not present (Fig. 3F). At the end of cb a cilium originates (length ~10 µm).

The next sensory device is the posteriolateral sensory cell of the trunk (ptsc) at U77 (Fig. 3A,B). This ovoid-shaped cell is located at the lateral margin of the trunk ventral of the posterior third of the testes (te). In several specimens the IR signal of these somata has been undistinguishable from the strong IR of the spermatozoa. The soma of this cell bears distally a cilium (~7 µm long).

In the posterior end, at the base of the furca, several sensory ciliary cells are located (Fig. 3A). Posteriolateral of the 5-HT-like IR cs_s is, on each side of the animal, a group of two adjacent tubulin IR somata (scf_o). The two somata are arranged in anterior-posterior orientation, so we can distinguish an anterior soma (scf_o^a) and a posterior one (scf_o^p) (Fig. 3G). An additional inner pair of sensory somata (scf_i) is present slightly more posterior than scf_o (Fig. 3G). These somata are located between the posteriorly directed processes of scf_o and also these scf_i bear a posteriorly directed IR neurite-like process. All three somata of one half of the trunk send their processes into the branch of the furca of this half of the trunk. At each branch of the furca, obviously three ciliary devices are

present. The first cilium is present at the dorsal inner side on the base of the branch. This cilium is characterized by its short length (~5–6 µm). More distally, at the end of the fleshy furca base, at the transition to the posterior adhesive tube, two additional cilia are present. We can distinguish a long outer (which is clearly visible in light microscopy, see Fig. 1A) and a short inner ciliary structure at each base of the posterior adhesive tubes (Fig. 3G). We cannot decide which cilium belongs to which soma.

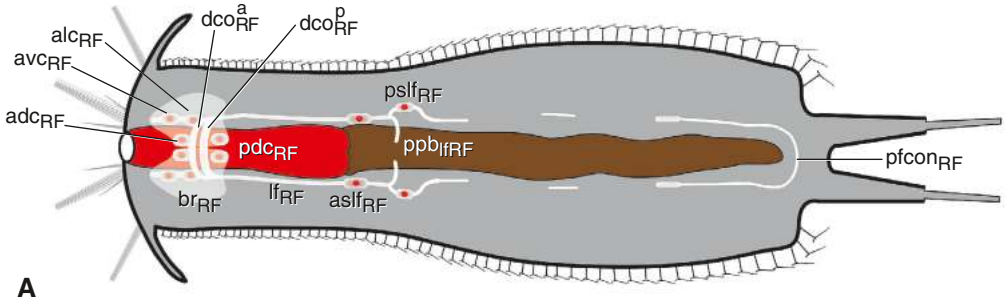
RFamide-like IR components of the nervous system of *Xenotrichula velox*. The following description is based on 3 specimens of *X. velox*. IR positive to RFamide is present throughout the nervous system within the brain (br) and the longitudinal neurite bundles (ln). Due to the low number of labelled specimens we describe in the following only the consistent pattern in these 3 specimens.

The anti-RFamide-like IR of the brain is prominent within the fibres of the dorsal commissure (dco_{RF}) and highlights lateral clusters of IR neuron somata (Fig. 4A–C). The median part anterior and posterior of the dco_{RF} is free of IR somata (Fig. 4C,D). The dco_{RF} lies directly on top of the pharynx and forms an arc (length ~13µm, width ~6–7 µm). Within the commissure itself, the IR is restricted to the anterior and posterior part, meaning that two distinct IR bundles are visible, an anterior IR bundle (dco_{RF}^{a}) (~U10) and a posterior IR bundle (dco_{RF}^{p}) (~U13) (Fig. 4E). Ventrally in all specimens a weakly IR fine commissural fibre (vco_{RF}) (diameter < 1 µm) is present at the level of the anterior margin of dco_{RF} (~U10) (Fig. 4K). Within the prominent lateral tentacles of the head (Fig. 1B), which are presumably sensory devices, we found no anti-RFamide-like IR.

Obviously only a limited number of IR neuron somata projecting in anterior direction are present. Such somata are only present in an anteroventral position (Fig. 4C,D,H). Here, a soma (avc_{RF}^{br}) is present at ~U5 lateral on each side of the pharynx which bears a clearly visible anteriorly directed neurite and a short posterior extension connecting the adjacent cell (see below). Posterior of avc_{RF}^{br} follows another IR inter-individually identifiable soma (alc_{RF}^{br}) which is slightly more anterior in position at ~U8–U9 (Fig. 4D,G). This soma appears roundish in shape with a diameter of 3–4 µm. The soma of alc_{RF}^{br} is connected with avc_{RF}^{br} by the posterior process of the latter. At the dorsal margin of the dorsal commissure another pair of IR somata (adc_{RF}^{br}) is located at ~U9 (Fig. 4A,F). The soma of adc_{RF}^{br} has a roundish shape with a diameter between 4–5 µm. The somata from each body side are not in contact with each other. Lateral to the dorsal commissure are several IR somata, but due to the low number of specimens and no optimal nuclei-counterstaining we are not able to describe this part of the brain in detail. On the other hand we found inter-individual identifiable IR soma posterior to the dorsal commissure. Approximately 3 µm posterior to the posterior margin of the dorsal commissure (~U15) is a pair of IR roundish somata (pdc_{RF}^{br}) (diameter 3–4 µm), each of the bilateral somata is lateral to the median (Fig. 4A,E).

At the posterioventral side of the brain, lateral to the pharynx, an RFamide-like IR fibre (lf_{RF}) originates and runs in posterior direction. This fibre (diameter 1–2 µm) is clearly visible during its course in the anterior trunk region, alongside the pharynx (ph) and the anterior third of the gut (g), but during the course in the medial region of the trunk the detection is scattered; this pattern is consistent in all three specimens (Fig. 4A–C). In the posterior part of the trunk lf_{RF} is again recognizable as a continuous structure. The fibre has pearl necklace-like appearance, due to the alternating occurrence of highly IR parts with up to 2 µm in diameter and low IR parts with a thinner diameter. Within the anterior part of lf_{RF} are three aberrations from this pattern:

1 Approximately after 30–33 µm (~U28) of the course of lf_{RF} is a remarkable ovoid-shaped swelling of the fibre, the anterior anti-RFamide-like IR soma of the longitudinal fibre ($aslf_{RF}$) (length ~6 µm, width ~3.5 µm) (Fig. 4A,I).
2 Slightly more posterior to $aslf_{RF}$ at ~U34 the fibre looks swollen again, at this point a branching of the fibre occurs, where a short ventromedially directed branch (ppb_{lfRF}) is originating (Fig. 4A,C). This branch (length 5–6 µm, diameter >1 µm) arises from the longitudinal fibre in a nearly orthogonal angle and runs somewhat curved in anterior direction. The branches of both body sides run mirror inverted to each other, but they do not come into contact, there is still a cleft of 3 µm between them.
3 Approximately 8 µm posterior of ppb_{lfRF} (~U37) a further IR positive soma is present associated with lf_{RF} the, the posterior anti-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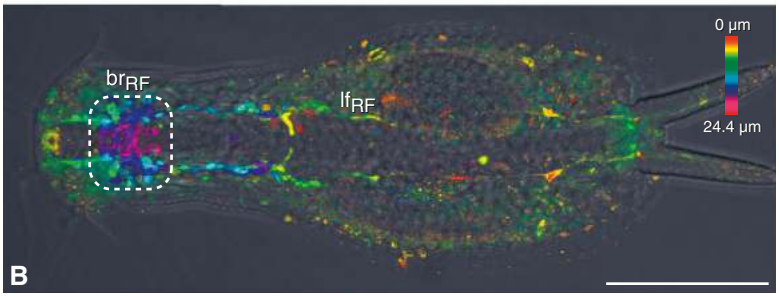
aslf$_{RF}$
pfcon$_{RF}$
A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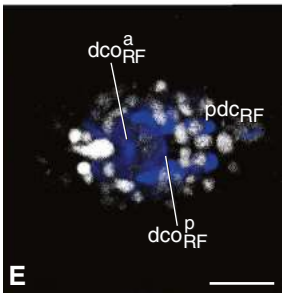
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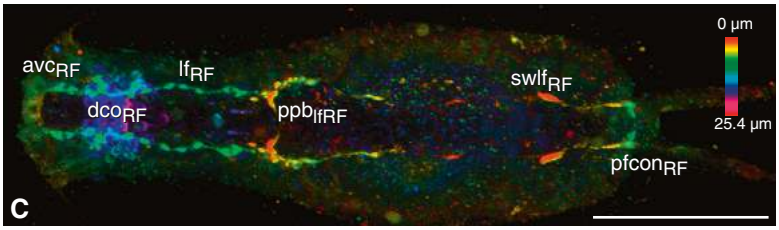
E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dco$_{RF}$
ppblf$_{RF}$
swlf$_{RF}$
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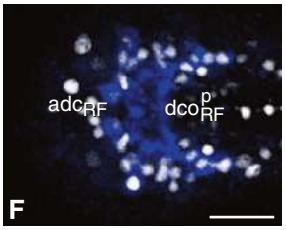
C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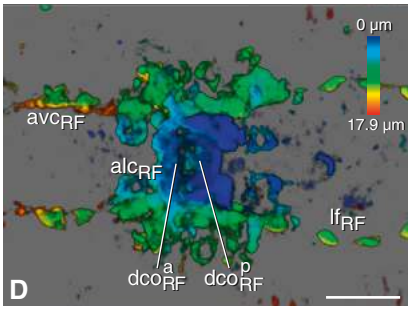
F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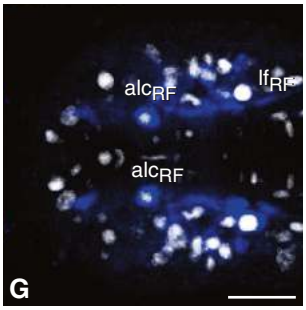
alc$_{RF}$
lf$_{RF}$
dco$^{a}_{RF}$
dco$^{p}_{RF}$
D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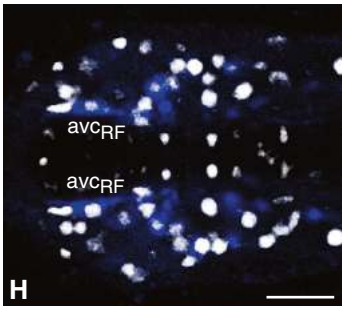
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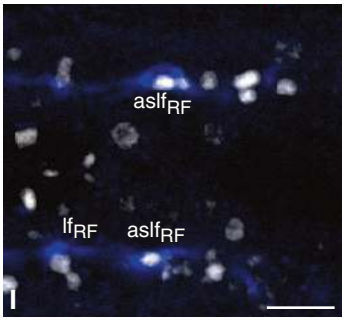
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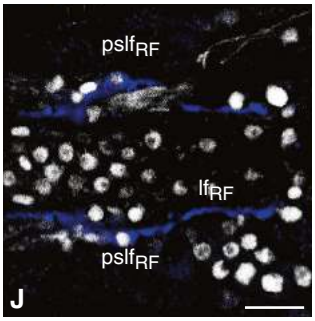
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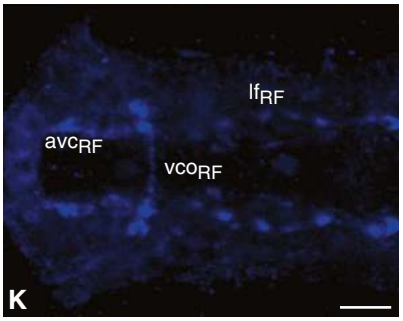
pslf$_{RF}$
J


lf$_{RF}$
avc$_{RF}$
vco$_{RF}$
K

RFamide-like IR soma of the longitudinal fibre ($pslf_{RF}$) (Fig. 4A,J). The dimensions of $pslf_{RF}$ correspond to that of $aslf_{RF}$. In the posterior end lf_{RF} forms a U-shaped continuous structure, the posterior fibre connection ($pfcon_{RF}$). This fibre becomes visible ~30 µm in front of the basis of the furca (~U72) in the form of a swelling ($swlf_{RF}$) with a diameter of 2–2.5 µm on a length of 7–8 µm. No soma could be observed within this swelling. Posterior of the swelling the diameter is < 1 µm. The posterior connection of the fibres is ~8 µm anterior of the basis of the furca (~U82). Additional IR somata are not detectable at the posterior region of the trunk.

Additional ultrastructure data on the nervous system of *Xenotrichula intermedia*. The immunohistochemical observations are confirmed by the TEM data. The brain of *X. intermedia* can be subdivided into the paired lateral clusters of neuron somata (ns) and the massive dorsal commissure (dc) (Fig. 5A). The two clusters of neuron somata come dorsolateral of the dorsal commissure close together, but are still clearly separated from each other (Fig. 5A,B). This is evident anterior as well as posterior of the dorsal neuropile. Within the dorsal commissure, representing the neuropile of the brain, numerous neurites are present, referring to the laterally located neuron somata. The content and composition of neurovesicles within the neurites appears variable (Fig. 6B). The most frequent form of neurovesicles seems to be small electron-translucent vesicles (diameter ~100 nm). Furthermore, small vesicles (diameter ~100–150 µm) with an electron-dense content are also present within the neurons (Fig. 5C, 6B). The dorsal commissure has a thickness of 5 µm. In the region of the commissure the fibres separate the neuron somata and the pharynx completely, the fibres enclose the upper half of the pharynx and at the ventral margin of this fibrous layer the neurites and the somata of the ventral locomotory cirri are contiguous (Fig. 5A). The lateral clusters of neuron somata form lateral caps of somata composed by up to 2–3 somata one above the other (Fig. 5A,B).

The somata of the outer anterior sensory cirri (sci_o) contain electron-lucent neurovesicles. In addition, next to these somata are neurites present, some filled with electron-dense neurovesicles (Fig. 5C). In contrast, the somata of the cells with motile cirri do not contain neurovesicles. These are in close contact with the medially adjoining longitudinal directed bundle of neurites of the longitudinal cords (Fig. 6C). The rootlets of the cirri run deep inside the soma up to the nuclei region (Fig. 5A,B, 6C).

The longitudinal bundle of neurites is located posterior to the brain in a ventromedial position. The distance between the bundles of each side of the body amounts ~10 µm. The longitudinal bundles of neurites within the trunk region contain only a low number of neurites, posterior of the protonephridium 10–11 neurites form the bundle. The bundle itself is in close contact with the ventral longitudinal musculature of the trunk (Fig. 6D,E). The content of neurovesicles within the single neurites is low.

◁ **Fig. 4.** Visualisation of RFamide-like IR of *Xenotrichula velox*. **B–D.** Colour-coded by depth (CCD-projections), in B with a transmission channel overlay. **E–K.** Single optical sections of a double labelling RFamide (blue) and nuclei (white). **A.** Schematic overview of the RFamide-like IR of *X. velox*. **B–C**. Overview of RFamide-like IR of *X. velox* from dorsal. **D.** Detail of the brain from dorsal. **E.** Detail of the dorsal commissure and posterior dorsal somata of the brain. **F.** Detail of the dorsal commissure and anterior dorsal somata of the brain. **G.** Detail median region of the brain and the anterior lateral somata. **H.** Detail of the ventral region of the brain and the anterior ventral somata. **I.** Detail of the longitudinal fibre of the median trunk at the level of the associated anterior soma of the fibre. **J.** Detail of the longitudinal fibre of the median trunk at the level of the associated posterior soma of the fibre **K.** Detail of ventral commissural fibre of the brain. Scale bars: B,C, 50 µm; D–K, 10 µm. **adc_{RF}**, anterior dorsal RFamide-like IR cells of the brain; **alc_{RF}**, anterior lateral RFamide-like IR cells of the brain; **$aslf_{RF}$**, anterior RFamide-like IR soma associated with the longitudinal fibre; **avc_{RF}**, anterior ventral RFamide-like IR cells of the brain; **br_{RF}**, RFamide-like IR of the brain; **dco_{RF}**, RFamide-like IR fibres of dorsal commissure; **dco^{a}_{RF}**, anterior RFamide-like IR fibres of dorsal commissure; **dco^{p}_{RF}**, posterior RFamide-like IR fibres of dorsal commissure; **dsc_t**, dorsolateral sensory cells of the trunk; **lf_{RF}**, RFamide-like IR longitudinal fibre; **pdc_{RF}**, dorsal RFamide-like IR cells posterior of the dorsal commissure; **$pfcon_{RF}$**, posterior fibre connection; **ppb_{lfRF}**, post-pharyngeal branch of the RFamide-like IR longitudinal fibre; **$pslf_{RF}$**, posterior RFamide-like IR soma associated with the longitudinal fibre; **$swlf_{RF}$**, posterior swelling of the RFamide-like IR longitudinal fibre; **vco_{RF}**, RFamide-like IR ventral commissure of the brain.

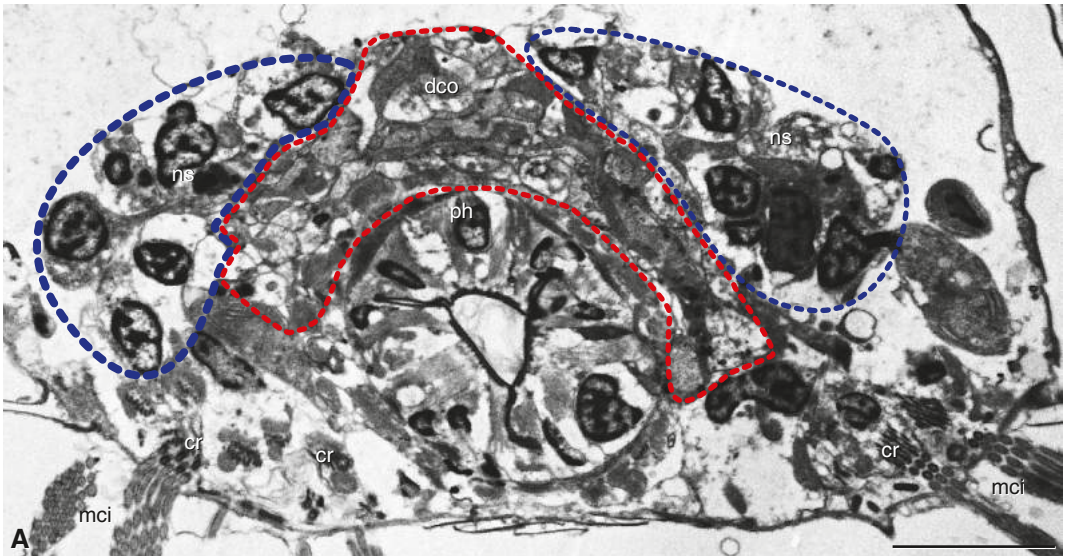
Discussion

The nervous system of Paucitubulatina. Reports on the nervous system of members of the Paucitubulatina are quite scarce. The most detailed description was made by Zelinka (1889) and subsequently by Remane (1927), both based on light microscopy. They described the gross-anatomy of the brain of members of the freshwater chaetonotids (e. g. *Chaetonotus maximus* (Ehrenberg, 1831); Zelinka 1889). Zelinka (1889) reports an anteriodorsal brain mainly composed of two lateral masses of neuron somata lateral of the pharynx. This is based on previous reports by Ludwig (1875) and Bütschli (1876). The dorsal commissure was described by Zelinka (1889) as a "nuclei free" area within the brain. He presumed an "integrative part" of the brain in this "nuclei free" area, but Remane (1927) was the first to remark the occurrence of a dorsal "fibrous mass" as a connection between the lateral neuron somata. Zelinka (1889), as well as Remane (1936) assumed the presence of two well separated dorsal "fibrous masses", one centrally within the brain (which corresponds to our findings) and an additional commissure more anteriorly (this was not found in *Xenotrichula*). Furthermore, Zelinka (1889) reported the composition of the brain to be by four lateral ganglia on each body side. One criterion for the differentiation between the different ganglia was the different size of the nuclei of neuron somata within these brain areas. Remane (1936) excluded the presence of a ventral commissure in Paucitubulatina. Regarding the longitudinal fibre bundles Zelinka (1889) reported a very fine fibre originating at the posterior margin of the brain to be running ventrolateral along the pharynx and gut in posterior direction. Furthermore he assumed 6–7 "ganglion cells" to be associated with the fibre along the course in posterior direction. These early descriptions fit very well the main findings of the nervous system of xenotrichulids, e. g., the overall shape of the brain ("dumbbell-shaped", see below), the massive central commissure and the number of posteriorly directed longitudinal neurite bundles (1 pair), as well as the position of these in the direct vicinity of the digestive tract and the occurrence of neuron somata alongside the bundles. The multi-ganglionated composition of the brain in *C. maximus* and the probable presence of an additional anterior commissure could be interpreted either as derived characters of the freshwater species or as a misinterpretation of the light microscopical investigation considering that some members of the Paucitubulatina belong to the smallest free-living metazoans. The architecture of the 5-HT-like IR somata of the brain seems to be highly conservative in the two xenotrichulid species. In both species we found two pairs of dorsolateral somata and one pair of lateral somata posterior of the dorsal commissure and the commissure shows a subdivision into an anterior and posterior fibre. Both species possess in the posterior end one pair of IR somata associated with the longitudinal neurite bundle. Regretfully, we do not have data on the other markers from both species, so we cannot imply such a high similarity between the two species for these. As a consequence we will interpret these data more carefully in the following discussion.

The nervous system of the Gastrotricha. Generally, three subtaxa are recognized within Gastrotricha: Macrodasyida, *Neodasys* (= Multitubulatina) and Paucitubulatina. Traditionally, i. e. from a morphological point of view (e. g. Travis 1983, Hochberg & Litvaitis 2000, Ax 2003) all three taxa are considered as monophyletic and Macrodasyida and Chaetonotida (*Neodasys* + Paucitubulatina) are regarded as sister taxa. This view is challenged by analyses resulting in non-monophyletic Gastrotricha (e.g. Manylov et al. 2004, Giribet et al. 2004), in *Neodasys* and Paucitubulatina being ingroup taxa of Macrodasyida (Todaro et al. 2006) or in *Neodasys* as the sister group to Macrodasyida + Paucitubulatina (Kieneke et al. 2008).

The overall architecture of the nervous system of *Xenotrichula* supports the monophyly of

Fig. 5. Ultrathin cross sections (TEM) through the nervous system of *Xenotrichula intermedia* in the anterior body region. **A.** Cross section through the brain at the level of the dorsal commissure. Lateral regions of neuron somata indicated by **blue dashed lines** and the region of the neurites of the dorsal commissure indicated by the **red dashed line**. **B.** Cross section through the brain posterior of the level of the dorsal commissure. Lateral regions of neuron somata indicated by **blue dashed lines**. **C.** Detail of the anterior lateral sensory cirri. **Red arrows** indicate "neurite-like" structures; **black arrow** indicates neurovesicles within the cirri-bearing soma. Scale bars: A, B, 5 µm; C, 2 µm. **bb**, basal body; **cr**, ciliary rootlet; **dco**, dorsal commissure; **mci**, motile cilia; **ns** neuron somata of the brain; **ph**, pharynx; **sci$_o$**, outer sensory cirri. ▷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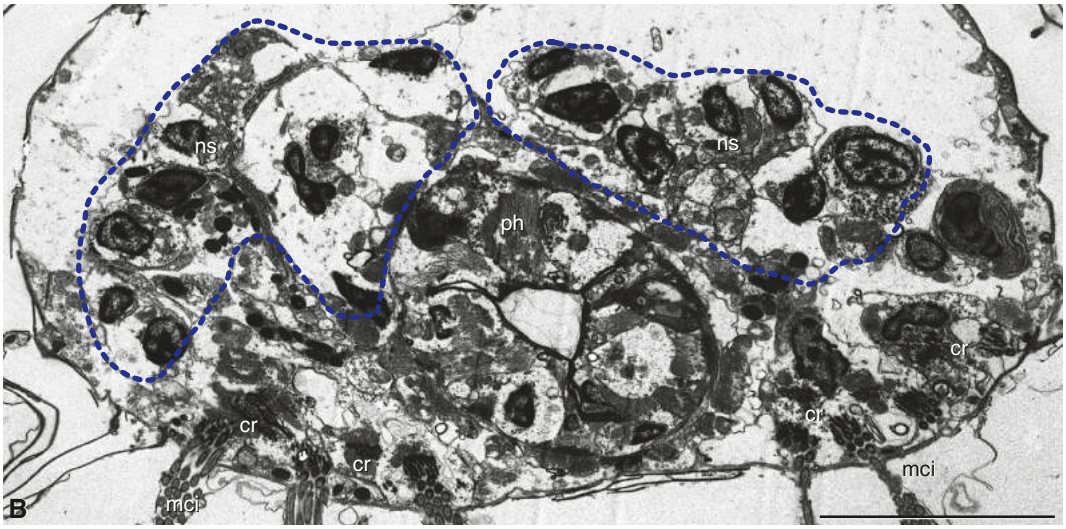
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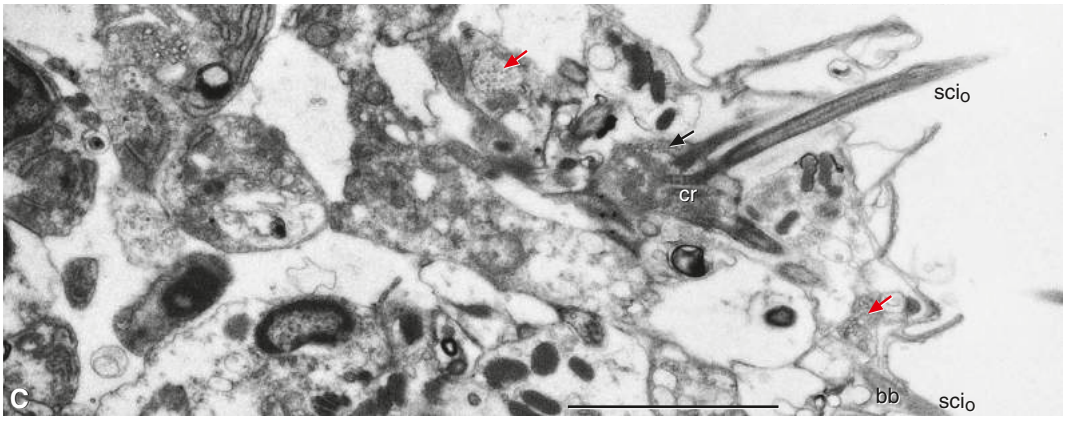
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the Gastrotricha (Paucitubulatina, *Neodasys* and Macrodasyida) based on several autapomorphic characters of the nervous system compared to conditions in other protostomians.

1 The dumbbell-shaped brain is representing a character found in all member of the Gastrotricha. The gastrotrich brain has been investigated in members of *Neodasys* and several macrodasyian families with IHC methods and the use of different markers (e. g. Joffe & Kotikova 1987; Joffe & Wikgren 1995; Hochberg & Litvaitis 2003; Hochberg 2007, 2011; Rothe & Schmidt-Rhaesa 2007, 2008, 2010a). Therefore we can hypothesize a "dumbbell-shaped" brain as a gastrotrich autapomorphy. In other protostomes the brain shows either a ganglionic composition with an inner (central) neuropil and an outer (dense) cortex with neuron somata (e. g. Platyhelminthes: *Macrostomum lignano* Ladurner, Schärer, Salvermoser & Rieger, 2005 in Morris et al. 2007; Annelida: different species in Orrhage & Müller 2005, Heuer et al. 2010; Arthropoda: for a general review see Bullock & Horridge 1965), or it shows a "cycloneuralian" architecture within the Cycloneuralia with a anterior and posterior condensation of neuron somata and a ring-shaped neuropile with continuous diameter between them (e. g. Nematoda: *Caenorhabditis elegans* Maupas, 1900 in White et al. 1986, Priapulida: *Tubiluchus troglodytes* Todaro & Shirley, 2003 in Rothe & Schmidt-Rhaesa 2010b).
2 The paired ventral longitudinal neurite bundles can be hypothesized as an autapomorphic character of the Gastrotricha. For a summery within *Neodasys* and macrodasyian gastrotrichs see Rothe et al. (2011). In comparison with other taxa, the presence of one pair of longitudinal neurite bundles seems to be the result of a reduction, because more longitudinal neurite bundles are present in many other spiralian (lophotrochozoan) and cycloneuralian taxa, reflecting the orthogon hypothesis by Reisinger (1925, 1972). For a summary of the conditions in different protostomian groups see Schmidt-Rhaesa (2007).
3 Homology of individual identifiable neurons. Rothe et al. (2011) proposed interspecific homology of several 5-HT-like IR somata (dps_s^v and dps_s^d) between *Neodasys* and different members of the macrodasyian gastrotricha. For the 5-HT IR posteriodorsal somata of the brain this hypothesis can be extended to the entire Gastrotricha, including the conditions in *Xenotrichula* (here das_s and dps_s). Within all three groups we found a comparable arrangement of such somata, concerning the expression of 5-HT, the general position dorsoposterior of the dorsal commissure and the position relative to each other (dorsal-ventral, anterior-posterior). As well as that they all project with a single neurite in the lateral part of the dorsal commissure. For an exhaustive discussion of the homology of these neurons in *Neodasys* and Macrodasyida see Rothe et al. (2011). But for an exhaustive analysis on neuron level we need more data on the diversity of the brain architecture within the Paucitubulatina.

In consequence of these hypothesized autapomorphic characters the polyphyly of the Gastrotricha has to be rejected.

In the following we focus on the comparison between Xenotrichula and *Neodasys*. The overall architecture of the nervous system of *Neodasys* is comparable to the findings in *Xenotrichula*, with the exception that the brain of *Xenotrichula* is in a more posterior position than in *Neodasys*. *Xenotrichula* and *Neodasys* both have one pair of posterior longitudinal neurite bundles, as all other gastrotrichs known to date. Within each longitudinal neurite bundle are two 5-HT-like IR fibres in *Neodasys chaetonotoideus* (Rothe et al. 2011) and only one fibre in *Xenotrichula*. Both taxa correspond in the presence of a pair of posterior 5-HT-like IR somata; somata like these are not described from macrodasyidan species until now. The results of the tubulin labelling of *X. intermedia* are unusual compared to previous comparable investigations, due to the solitary labelling of obviously sensory ciliated cells. On the other hand Rothe et al. (2011) reported a pair of strongly anti-tubulin IR somata posterior of the dorsal commissure in a dorsal position in *N. chaetonotoideus* (dts_{br} in Rothe et al. 2011), but these somata do not appear to be ciliated and they project into the dorsal commissure. The RFamide-like IR highlights some similarities between *X. velox* and *Neodasys*. The overall shape of the distribution pattern seems to be comparable between *N. cirritus* (Hochberg 2007), *N. chaetonotoideus* (Rothe et al. 2011) and *X. velox*. In all three species the lateral clusters show an ovoid shape, but the relative distance between

the clusters is much larger in the *Neodasys* species. Furthermore, in all three species anteriolateral IR somata are present, named anc in *N. cirritus* by Hochberg (2007), $anbs_{RF}$ in *N. chaetonotoideus* by Rothe et al. (2011) and avc_{RF} in *X. velox* here. Accordingly, RFamide-like IR somata located directly dorsally anterior and posterior of the dorsal commissure as in *X. velox* (adc_{RF}, pdc_{RF}), have been reported in the two *Neodasys* species (e. g. $adms_{RF}$ and $pdms_{RF}$ in Rothe et al. 2011, such somata are also present in *N. cirritus*, see Fig. 12 in Hochberg 2007). A fine ventral commissure is reported only from *N. chaetonotoideus* (Rothe et al. 2011), whereas RFamide-like IR somata associated with the longitudinal fibre were only reported from *N. cirritus* (Hochberg 2007). A major difference between *Xenotrichula* and *Neodasys* is obviously the absence of nervous structures within the pharyngeal myoepithelium of the two xenotrichulid species, as IHC methods as well as TEM have shown no hint for the presence of such structures.

In summary the most conspicuous structure is presented by the posterior 5-HT-like IR somata (cs_s). This character is solely found in xenotrichulids and *Neodasys chaetonotoideus* (Rothe et al. 2011). Due to the prevalence of occurring neuron somata at the posterior body pole in protostomians, e. g. "caudal ganglion" in nematodes (see White et al. 1986) or the occurrence of neuron somata alongside the longitudinal neurite bundles in many protostomian taxa (e. g. in Platyhelminthes and rotifers, see Kotikova & Raikova 2008) the conditions in xenotrichulids and *Neodasys chaetonotoideus* seems to be a plesiomorphic state and the reduction of those somata in Macrodasyida is the apomorphic state. This conclusion makes the position of *Neodasys* and the Paucitubulatina as well separated ingroups of the Macrodasyida doubtful as suggested by Todaro et al. (2006), because this implies the convergent reduction of the posterior 5-HT-like IR somata within the Macrodasyida or alternatively the convergent evolution of those in *Neodasys* and the Paucitubulatina.

Nevertheless, for a detailed and reliable analysis we need for the future an exhaustive survey on the nervous system organisation of Paucitubulatina on family level, with the special focus on the marine members.

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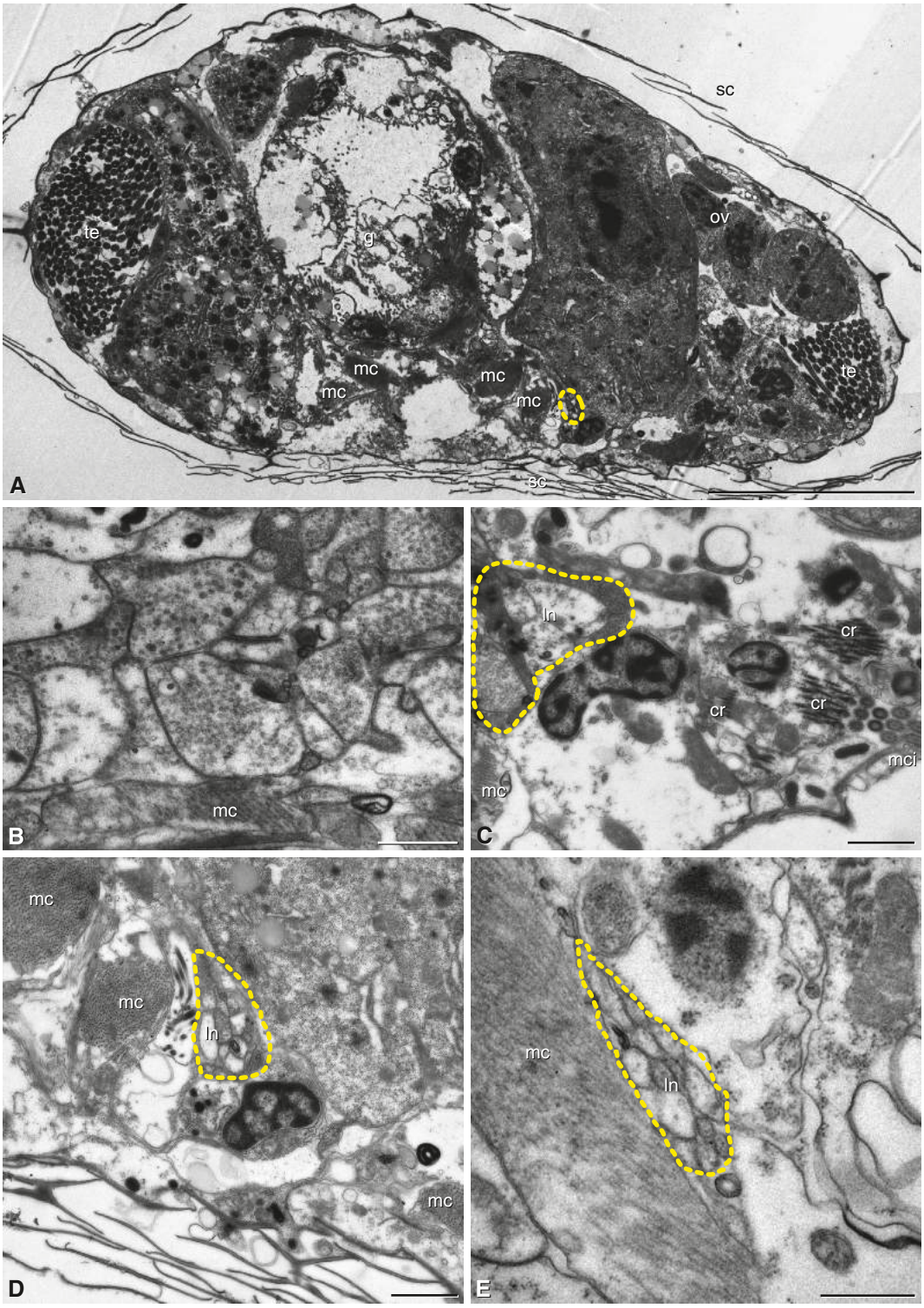
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◁ **Fig. 6.** Ultrathin cross sections (TEM) through the nervous system of *Xenotrichula intermedia*. Yellow dashed line indicates in A, C-E the longitudinal bundle of neurites. **A.** Cross section through body region of the anterior testes. **B.** Detail of the neurites within the dorsal commissure. **C.** Detail of the longitudinal bundle of neurites slightly posterior of the dorsal commissure. **D.** Detail of the longitudinal bundle of neurites posterior of the transition of between pharynx and gut. **E.** Detail of the longitudinal bundle of neurites at the gut level. Scale bars: A, 10 µm; C, D, 1 µm; B, E, 0,5 µm. **cr**, ciliary rootlet; **g**, gut; **ln**, longitudinal bundle of neurites; **mc**, muscle cell; **mci**, motile cilia; **sc**, scales; **te**, testes; **ov**, ovary.

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