

Evolution of Developmental Control Mechanisms

Neural development in Onychophora (velvet worms) suggests a step-wise evolution of segmentation in the nervous system of Panarthropoda

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ABSTRACT

A fundamental question in biology is how animal segmentation arose during evolution. One particular challenge is to clarify whether segmental ganglia of the nervous system evolved once, twice, or several times within the Bilateria. As close relatives of arthropods, Onychophora play an important role in this debate since their nervous system displays a mixture of both segmental and non-segmental features. We present evidence that the onychophoran "ventral organs," previously interpreted as segmental anlagen of the nervous system, do not contribute to nerve cord formation and therefore cannot be regarded as vestiges of segmental ganglia. The early axonal pathways in the central nervous system arise by an anterior-to-posterior cascade of axonogenesis from neuronal cell bodies, which are distributed irregularly along each presumptive ventral cord. This pattern contrasts with the strictly segmental neuromeres present in arthropod embryos and makes the assumption of a secondary loss of segmentation in the nervous system during the evolution of the Onychophora less plausible. We discuss the implications of these findings for the evolution of neural segmentation in the Panarthropoda (Arthropoda + Onychophora + Tardigrada). Our data best support the hypothesis that the ancestral panarthropod had only a partially segmented nervous system, which evolved progressively into the segmental chain of ganglia seen in extant tardigrades and arthropods.

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Introduction

One of the major challenges in evolutionary biology is understanding the origin of animal body plans. The evolution of segmentation is one of the most contentious issues in this area (Blair, 2008; Damen, 2007; Deutsch, 2004; Fusco, 2008; Minelli and Fusco, 2004; Patel, 2003; Peel and Akam, 2003; Peel et al., 2005; Pourquié, 2003; Seaver, 2003; Tautz, 2004). In this paper, we use the following definition of segmentation: the serial repetition of two or more organ systems or tissues arising from different germ layers and lying in register to each other along the main body axis (Nielsen, 2001; Scholtz, 2002). The most obvious pattern of segmentation among major extant animal groups occurs in annelids, arthropods, and chordates (Damen, 2007; Nielsen, 2001; Scholtz, 2002; Seaver, 2003), which are therefore commonly regarded as "truly" or "overtly" segmented animals. There is a long history of debate (Sedgwick, 1884) about whether segmentation in these groups has evolved once, twice, or even thrice in parallel (Balavoine and Adoutte, 2003; Blair, 2008; Budd, 2001a; Carroll et al., 2005; De Robertis, 1997, 2008; de Rosa et al., 2005; Deutsch, 2004; Erwin and Davidson, 2002; Nielsen, 2003; Saudemont et al., 2008; Schmidt-Rhaesa et al., 1998; Scholtz, 2002; Seaver and Kaneshige, 2006). Developmental studies have

made an important contribution to this debate. However, most of our understanding of the developmental origins of segmentation has come from studies in a small number of segmented "model" organisms (Blair, 2008; Damen, 2007; Peel et al., 2005). It seems clear that to elucidate the evolutionary origins of segmentation, we need to fill the gaps between these model organisms with studies on "non-model" animals that possess only partially segmented body plans (Arthur et al., 1999; Damen, 2007; Seaver, 2003).

As close relatives of arthropods (Dunn et al., 2008; Edgecombe, 2009; Kusche et al., 2002; Mallatt et al., 2004; Nielsen, 2001; Roeding et al., 2007; Schmidt-Rhaesa et al., 1998), Onychophora or velvet worms are key subjects for studying the evolution of segmentation. Unlike arthropods, the onychophoran body plan does not show an overt segmentation but rather a combination of serially repeated and non-repeated structures (Figs. 1A and B). The same holds true for their nervous system since only the nerves supplying the legs and nephridia show a segmental arrangement whereas segmental ganglia are absent (Fig. 1C; Mayer and Harzsch, 2007, 2008). This contrasts with the strictly segmental organization of the nervous system in arthropods, which shows a ventral chain of ganglia (Harrison et al., 1995; Harzsch, 2004; Osorio et al., 1995). Ganglia are serially repeated condensations of neuronal cell bodies, which are linked by somata-free connectives along the main body axis (Osorio et al., 1995).

While the adult onychophoran nerve cord shows no sign of segmental ganglia, ganglion anlagen or neuromeres may still be present at early developmental stages. Many studies have shown that

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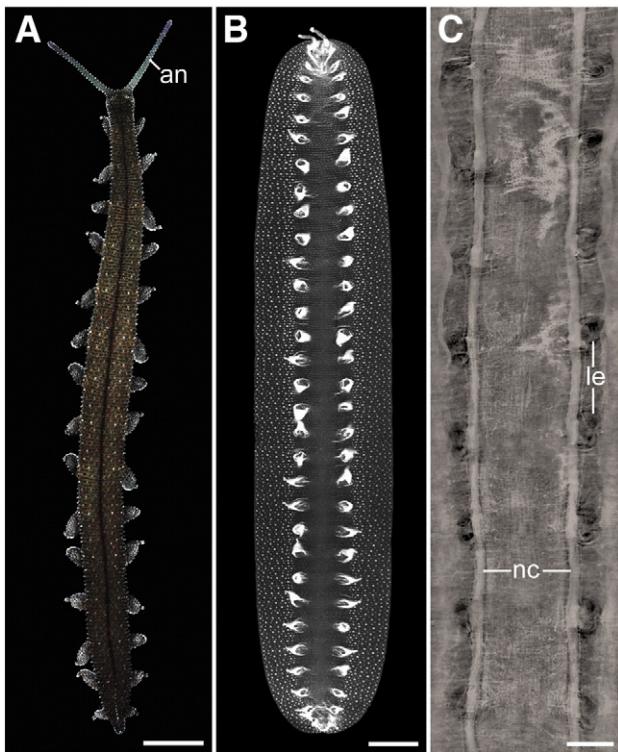


Fig. 1. Lack of overt body segmentation in Onychophora. (A) Walking specimen of *P. tallagandensis* in dorsal view. Limbs are the only repeated external structures that are evident. (B) Molted skin of *E. isthmicola* spread on water surface. Note the lack of segmental borders and sclerotized regions in the cuticle. (C) Internal view of ventral body wall in an adult specimen of *E. rowelli*. Note the lack of segmental ganglion swellings in ventral cords (nc), which contrasts with previous reports based on studies of the same species (Strausfeld et al., 2006). Anterior is up. an, antennae; le, leg base; nc, ventral nerve cords. Scale bars: A, 3 mm; B, 6 mm; C, 1 mm.

in arthropod embryos, neurons in the central nervous system arise in segmentally reiterated sets and send out axons in a segmental fashion (Brenneis et al., 2008; Harzsch, 2003, 2004; Vilpoux et al., 2006; Whittington et al., 1991; Whittington and Bacon, 1997; Whittington, 2004, 2007). This situation holds true even in those arthropods in which separate ganglia are not apparent in the adult nervous system (Wegerhoff and Breidbach, 1995). Furthermore, recent discoveries of cryptic segmentation during the development of other, non-segmented invertebrates have shown that these animals are derived from segmented ancestors (Hessling and Westheide (2002) for Echiura and Kristof et al. (2008) and Wanninger et al. (2009) for Sipuncula), thus

supporting molecular phylogenetic analyses (McHugh, 1997; Struck et al., 2007). Studies of early neural development in the Onychophora could therefore shed light on the evolutionary origins of segmentation in the Panarthropoda.

Previous studies of onychophoran embryogenesis have reported segmentally repeated structures, so-called “ventral organs,” in close association with the developing nervous system (Eriksson et al., 2003; Mayer et al., 2005; Pflugfelder, 1948). However, the involvement of ventral organs in neural development of Onychophora seems doubtful because their cells separate early from neurogenic tissue (Evans, 1901; Sedgwick, 1887; von Kennel, 1888) and are likely to take a different developmental course (Mayer and Whittington, 2009).

In this study, we have sought to clarify the involvement of ventral organs in neural development in onychophoran embryos and to establish whether early axon growth proceeds in a segmental pattern, as in arthropods. We have examined embryos from two distantly related onychophoran groups (Figs. 2A–C) to help us recognise the ancestral developmental mode. Our data show that neither the ventral organs nor any other segmental, neuromere-like ganglion anlagen are involved in early onychophoran neural development. These findings argue against the proposal of a secondary loss of ganglia in Onychophora (Eriksson et al., 2005a, 2009; Scholtz, 2002; Schürmann, 1995). They suggest that the ancestral panarthropod had only a partially segmented nervous system, which progressively evolved into the segmental chain of ganglia seen in extant tardigrades and arthropods.

Materials and methods

Specimen collection and embryo staging

Females of *Euperipatoides rowelli* Reid, 1996 and *Phallocephale tallagandensis* Reid, 1996, two ovoviparous species of Peripatopsidae, were obtained from rotten logs in the Tallaganda State Forest (35°26'S, 149°33'E, 954 m, New South Wales, Australia). Specimens were maintained in the laboratory for up to 2 years and individuals were dissected at various times to obtain a full range of developmental stages. The reproductive biology and embryonic stages of *E. rowelli* and *P. tallagandensis* have been described previously (Sunnucks et al., 2000; Walker and Tait, 2004). Specimens of *Epiperipatus bolleyi* (Bouvier, 1902) and *Epiperipatus isthmicola* (Bouvier, 1905), two placental/viviparous species of the neotropical Peripatidae, were obtained as described previously (Mayer and Tait, 2009). *E. isthmicola* females were maintained in culture for over 3 years and gave rise to several generations of young. Embryos were staged according to previous descriptions of embryogenesis in closely related species from the neotropics (Campiglia and Walker, 1995; von Kennel, 1888).

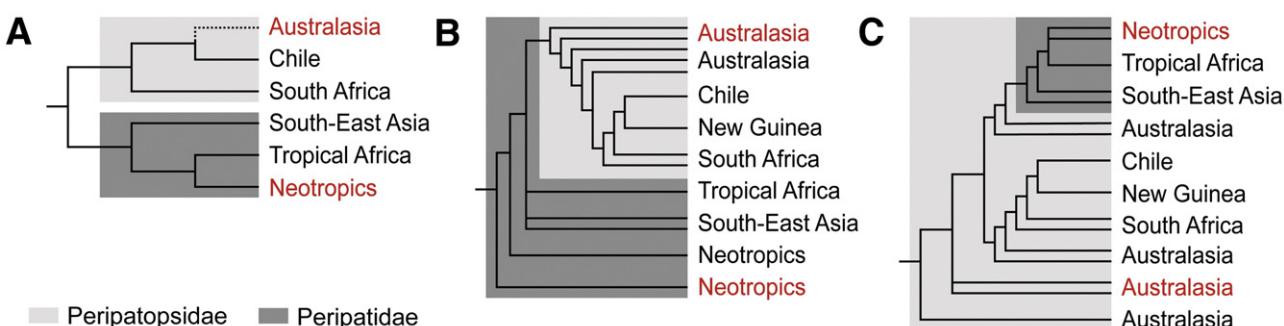


Fig. 2. Diagram showing three different hypotheses for the phylogenetic relationships of Onychophora. Onychophoran subgroups are designated by their geographical distribution. Subgroups, which include species studied in this paper, are highlighted in red: *E. rowelli* and *P. tallagandensis* from Australia (New South Wales), and *E. bolleyi* and *E. isthmicola* from the neotropics (Costa Rica). Regardless of which phylogeny is favoured, the species studied here belong to two distantly related groups, which might have separated prior to the breakup of Gondwana. (A) Both Peripatopsidae and Peripatidae are monophyletic (phylogeny modified from Monge-Nájera, 1995: Fig. 10). (B) Peripatidae are non-monophyletic (phylogeny simplified from Reid, 1996: Fig. 29). (C) Peripatopsidae are non-monophyletic (phylogeny simplified from Reid, 1996: Fig. 28).

Immunohistochemistry

Females were anaesthetized in chloroform vapor for 10–20 seconds. Preparation of adult specimens and Vibratome sectioning were applied as described previously (Mayer and Harzsch, 2008). Embryos were dissected in physiological saline (Robson et al., 1966) and fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS; 0.1 M, pH 7.4) at 4 °C. Specimens were then rinsed in several changes of PBS and further processed immediately, preserved in PBS containing 0.05% sodium azide, or dehydrated through a methanol series and stored at –20 °C. Pre-incubation was carried out in PBS-TX (1% bovine serum albumin, 0.05% sodium azide, and 0.5% Triton X-100 in PBS) for 3 hours at room temperature. Incubations with primary antibody (mouse monoclonal anti-acetylated α -tubulin IgG2b isotype (catalogue no. T6793; Sigma-Aldrich); diluted 1:500 or 1:1000 in PBS) were carried out on a shaker overnight at 4 °C. The embryos were then rinsed in several changes of PBS and incubated overnight with secondary antibody (goat anti-mouse IgG (H + L) conjugated to Alexa Fluorochrome 488 (catalogue no. A11017, lot 54254A; Invitrogen); diluted 1:500 in PBS). After several rinses in PBS, the embryos were incubated for 1 hour in a solution containing phalloidin–rhodamine (Molecular Probes, catalogue no. R-415300; to the 300 U stock, 1.5 ml methanol was added, and 10 μ l aliquots were stored at –20 °C; prior to use, methanol was evaporated and 200 μ l PBS was added to each aliquot). After additional rinses in PBS, the DNA-selective fluorescent dye Hoechst (Bisbenzimide, H33258 (catalogue no. 861405; Sigma-Aldrich); 1 μ g/ml in PBS) was applied for 15 minutes. After several rinses in PBS, the embryos/sections were either mounted directly on glass slides in Vectashield® Mounting Medium (catalogue no. H-1000; Vector Laboratories Inc.,

Burlingame, CA, USA) or dehydrated through a methanol or isopropanol series and mounted either on glass slides or between two coverslips in Murray Clear (a 2:1 mixture of benzyl benzoate and benzyl alcohol).

Detection of fragmented DNA or cell death

Embryos stored in methanol were rehydrated in PBS, incubated in 0.1 M sodium citrate, pH 6.0, for 30 minutes at 70 °C, and rinsed in PBS-TX. Detection of apoptotic cells was carried out with the *In Situ* Cell Death Detection Kit (TMR red (catalogue no. 12156792910 [2156792]; Roche)). The embryos were placed in equilibration buffer for 10 minutes at room temperature and buffer replaced by label solution (450 μ l) and enzyme solution (50 μ l). After incubation on a nutator (3 hours at 37 °C), the embryos were counterstained with Hoechst and mounted in Vectashield® Mounting Medium. For negative controls, the embryos were treated in the same way but without adding the enzyme. These embryos showed no nuclear labelling. For positive controls, the embryos were treated with DNase prior to detection of cell death. In these embryos, all nuclei were labelled.

Microscopy and image processing

Embryos were analyzed with confocal laser-scanning microscopes LSM 510 META and LSM 5 PASCA (©Carl Zeiss MicroImaging GmbH). Optical sections were taken at intervals ranging from 0.18 to 11.1 μ m and the resulting image stacks were merged digitally into maximum projection images. Depth-coded projections were produced with the Zeiss LSM Image Browser software (version 4.0.0.241). Image

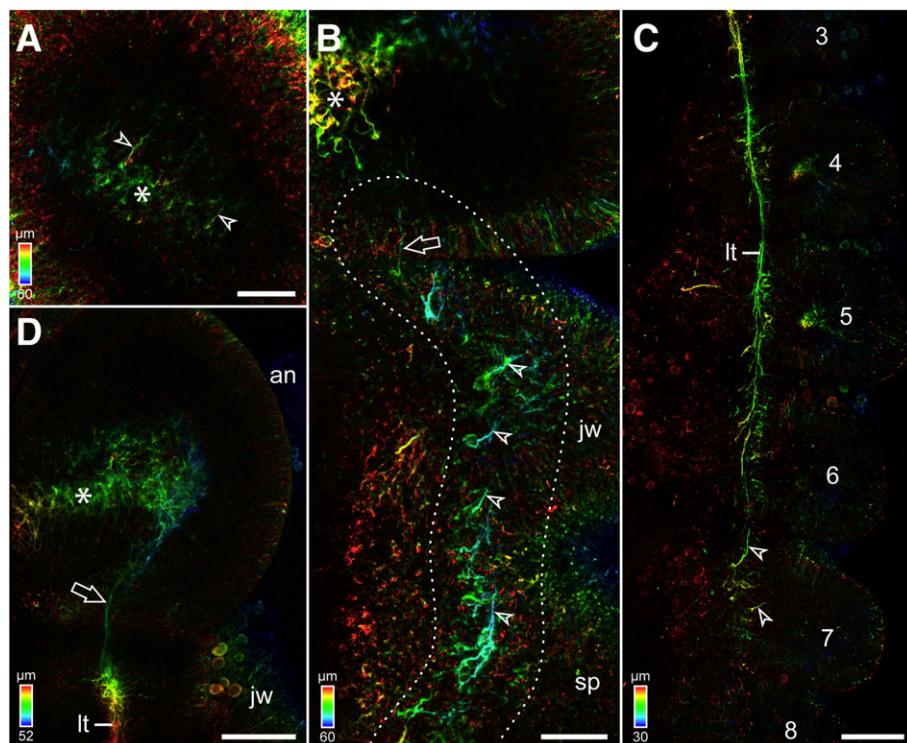


Fig. 3. Early axon growth and establishment of ventral longitudinal tracts. (A–D) Embryos of *E. rowelli*. Anti-acetylated α -tubulin immunolabelling, depth-coded projections of confocal image stacks. An indication of the depth corresponding to different colours is provided by the bar in the bottom left hand corner of each image. Anterior is up, median is left. (A) Earliest growing axons (arrowheads) and neuronal cell bodies within the antennal segment of an early stage 3 embryo. Asterisk indicates site of central neuropil of future brain. (B) Neuronal somata and early stages of axon growth in jaw (jw) and slime papilla segments (sp) in a late stage 3 embryo. Note the anteriorly directed axons (arrowheads). Asterisk: future central brain neuropil. Dashed line delineates the region in which the presumptive ventral cord will arise. Arrow points to the first axon to cross the border between the jaw and the antennal segments. (C) Pattern of developing neurons and axons in leg-bearing segments 3–8 of an early stage 4 embryo. Arrowheads indicate the posterior-most growing axons. A continuous longitudinal tract has formed by fasciculation between newly arising axons and the pre-existing axon bundle in more anterior segments. (D) Establishment of the axonal connection between antennal and jaw segments in a late stage 4 embryo. Asterisk indicates central brain neuropil. an, presumptive antenna; jw, presumptive jaw; lt, longitudinal tract; sp, presumptive slime papilla. Scale bars: A and B, 50 μ m; C and D, 100 μ m.

intensity histograms were adjusted by using Adobe (San Jose, CA) Photoshop CS2. The adjustment was kept at a minimum to allow the micrographs of the same plate to have similar intensity. Final panels and diagrams were designed with Adobe Illustrator CS2.

Results

Neural development is uniform among Onychophora

We have analyzed neural development in onychophoran species from two distantly related groups that diverged prior to the breakup of Gondwana 175–140 Mya ago (Figs. 2A–C; Monge-Nájera, 1995; Reid, 1996; Upchurch, 2008) and that display two distinct modes of embryogenesis. The embryos of the ovoviviparous Australian Peripatopsidae possess yolk and are enclosed by both chorion and vitelline membrane layers (Eriksson et al., 2003; Walker and Tait, 2004). In contrast, the embryos of the viviparous neotropical Peripatidae lack yolk and embryonic envelopes but rather develop placental structures (Campiglia and Walker, 1995; von Kennel, 1888). The embryos of the ovoviviparous species also possess an

extensive region of extra-embryonic ectoderm, which is almost completely absent in the viviparous species. Despite these major morphological differences, we provide evidence that neural development is conserved between the ovoviviparous and viviparous species (cf. Figs. 3–5, 7–12, and S1–S8).

Pioneering axons of the ventral cords arise in a non-segmental fashion

In the four onychophoran species studied, early axon growth follows an anterior-to-posterior (A/P) progression, with anterior segments being further advanced than the posterior ones. The first axons arise within the antennal segment, followed by the jaw and slime papillae segments (Figs. 3A and B). However, the axonal connection between the jaw and antennal segments is delayed, being established only after the axonal tracts linking the first 6 to 10 post-antennal segments have formed (Fig. 3D). Initial axon growth in all but the antennal segment is directed anteriorly and can be described as a cascade: new axons arise immediately posterior to neurons that have just formed axons. These newly arising axons fasciculate with more anterior, pre-existing axons, forming a pair of

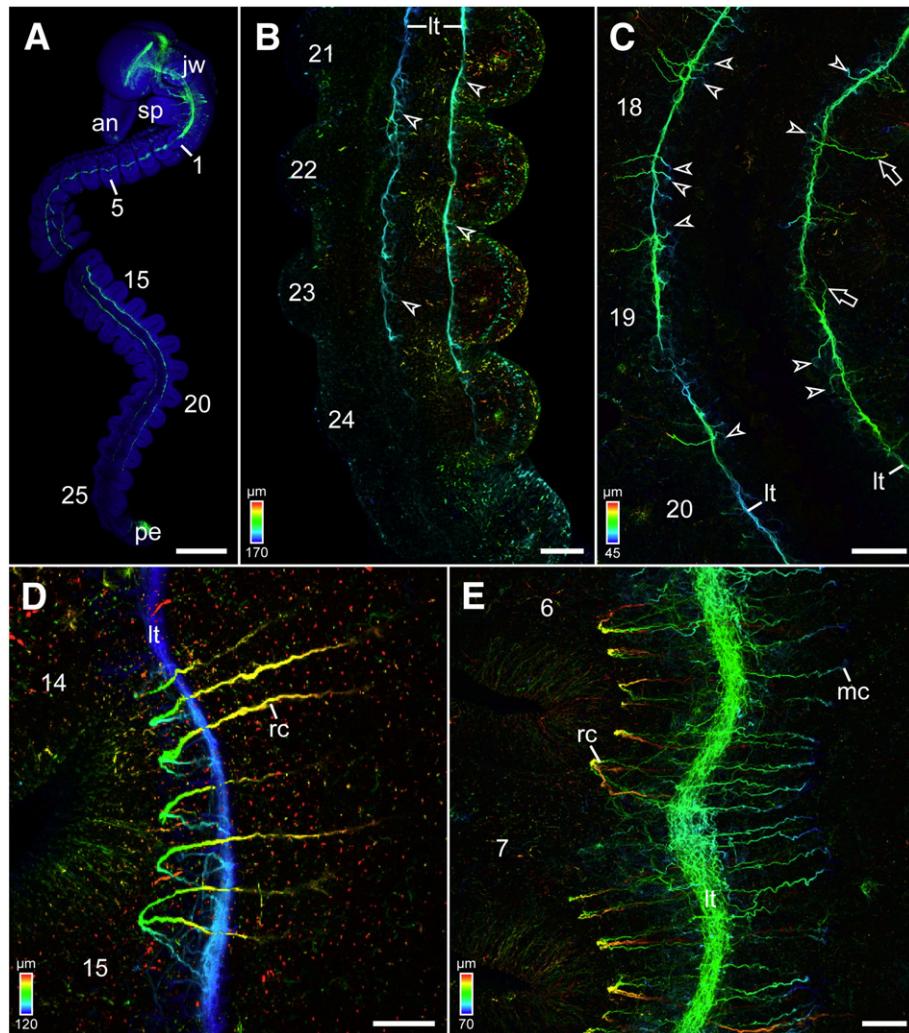


Fig. 4. Establishment of ventral longitudinal tracts and major commissural pathways. (A) Overview of a “coiled” stage embryo of *E. isthmicola* with 26 leg-bearing segments formed. Double-labelling with Hoechst (=Bisbenzimide, blue) and anti-acetylated α -tubulin antibody (green). (B–E) Consecutive stages of neural development along the anterior-to-posterior developmental gradient in the same embryo as in (A). Anti-acetylated α -tubulin immunolabelling, depth-coded projections of confocal image stacks. Embryos in dorsolateral view in (B) and in dorsal view in (C–E); anterior is up in all images. (B) Anterior-to-posterior cascade of developing neurons (arrowheads) and their axons that give rise to major longitudinal tracts (lt) in leg-bearing segments 21–24. (C) Somata (arrowheads) and axons (arrows) of neurons that pioneer future ring commissures in leg-bearing segments 18–20. Note the irregular arrangement of both axons and cell bodies. (D) Dorsal growth of axons pioneering future ring commissures (rc) in leg-bearing segments 14–15. (E) Regular spacing of axons that pioneer future median commissures (mc) in leg-bearing segments 6–7. *an*, presumptive antenna; *jw*, presumptive jaw; *lt*, major longitudinal tracts (presumptive ventral cords); *mc*, future median commissures; *pe*, posterior end; *rc*, future ring commissures; *sp*, presumptive slime papilla. Scale bars: A, 300 μ m; B and C, 50 μ m; D and E, 20 μ m.

longitudinal tracts located on either side of the ventral midline (Figs. 3B and C, 4A and B, and S1). As development proceeds, the longitudinal tracts become progressively thicker and ultimately become the neuropils of the ventral cords (Figs. 4A and B and S2). These data reveal that the pioneering axons, which give rise to the neuropils, do not display any segmentally repeated pattern or bilateral symmetry during embryogenesis; neither do their neuronal cell bodies, which arise in irregular positions along each ventral cord (Figs. 3B and C, 4B, and S1).

Ring commissures form in a non-segmental manner

Shortly after continuous longitudinal tracts become established, laterally projecting axons begin to appear within the ventral cords (Figs. 4C and S3). These arise from cell bodies located medial to the longitudinal tracts and grow towards the dorsal midline in an arc (Figs. 4C–E and S3). Like the longitudinal pioneers, these laterally projecting neurons show no evidence of a segmental arrangement or bilateral symmetry (Figs. 4C and S3). The first lateral axons are irregularly spaced, but as development proceeds a more regular spacing is attained (Figs. 4C–E). The presence of a growth cone with a tuft of filopodia on the tip of each pioneering lateral projection suggests that it is a single axon (Fig. 5A). Later arising axons fasciculate with these pioneers (Figs. 4D and E). During further

development, the laterally projecting axons from each side of the body join up along the dorsal midline in a zipper-like fashion, thereby forming the presumptive ring commissures (Figs. 5B and C and S4). The absence of ring commissures in leg-bearing regions gives the impression of a segmentally repeated pattern (Figs. 5B and C). However, the fact that the number of ring commissures in each inter-pedal region varies from five to eight (Figs. 6A and B; see also Figs. 5B and C, 7A and B, 8A and B, 9A and B, and S5; for similar range of variation reported from adult onychophorans, see Balfour, 1883; Bouvier, 1905; Fedorow, 1926; Mayer and Harzsch, 2008; Schneider, 1902), and that their branching pattern in each group is variable (Figs. 5C, 9A and B, S4, and S5), speaks against the idea that each inter-pedal set represents part of a segmentally repeated neuromere.

Median commissures form in a repeated but non-segmental fashion

The median commissures arise shortly after the ring commissures and are pioneered by single axons that grow towards the ventral midline (Figs. 5E and S6). Like the other neurons described, the cell bodies of the medially-projecting neurons are positioned irregularly along each longitudinal tract (Fig. S6). Nevertheless, the growth of their axons results in a regular, reiterated pattern along the A/P axis of the embryo (Figs. 5D, 8A, 9B, S5, and S8). The median commissures mostly do not correspond in position with the ring

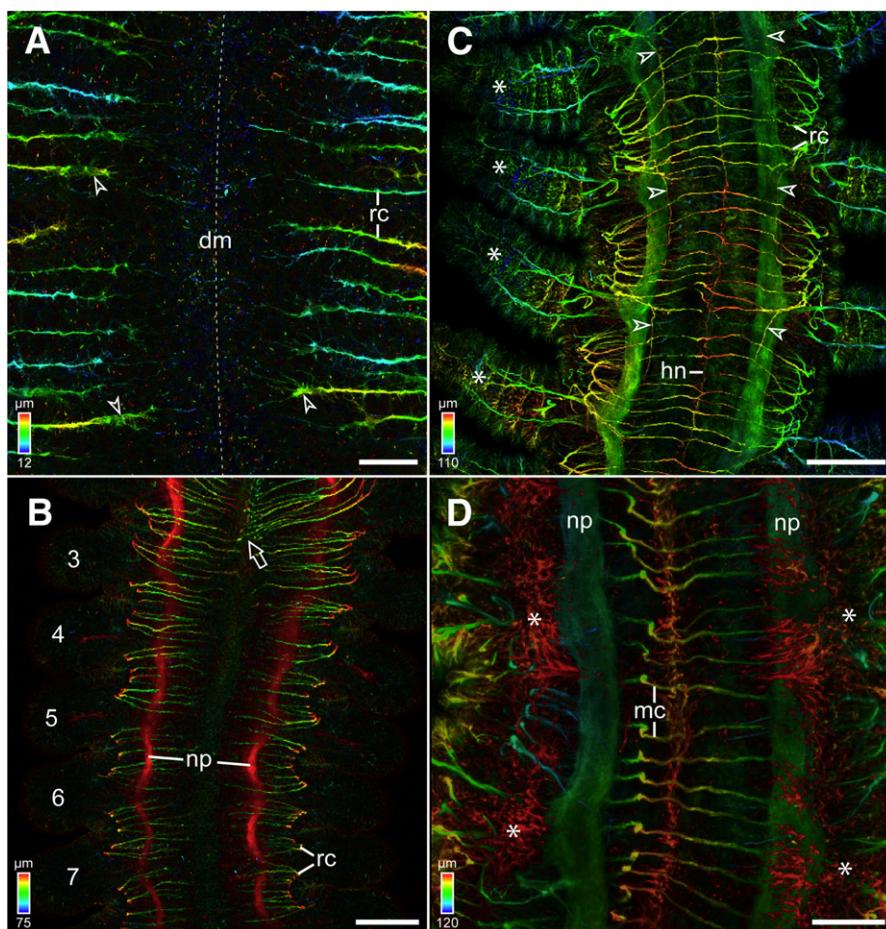


Fig. 5. Further development of commissural pathways in *E. isthmica*. (A–D) Anti-acetylated α -tubulin immunolabelling, depth-coded projections of confocal image stacks; anterior is up. (A) Distribution of axons that pioneer ring commissures (rc) along dorsal midline (dm), prior to dorsal closure. “Coiled” stage embryo. Note the tufts of filopodia at the tip of each axon (arrowheads). (B) Beginnings of fusion (open arrow) of contra-lateral projections that give rise to ring commissures (rc) in a “coiled” stage embryo. (C) Formation of heart nerve (hn) and dorsolateral longitudinal tracts in an early “flexed” stage embryo. Note the repeated nature of axons that pioneer the dorsolateral tracts (arrowheads). Asterisks indicate presumptive legs. (D) Regular, ladder-like arrangement of presumptive median commissures (mc) in a late “flexed” stage embryo. Asterisks indicate the position of legs. dm, dorsal midline; hn, presumptive heart nerve; mc, presumptive median commissures; np, future neuropils of ventral cords; rc, axons that give rise to presumptive ring commissures. Scale bars: A, 25 μ m; B and C, 100 μ m; D, 50 μ m.

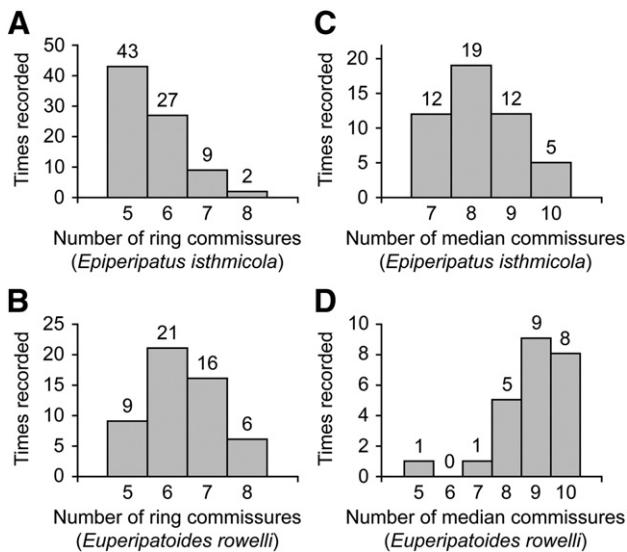


Fig. 6. Histograms showing the frequency of different numbers of commissures in embryos of *E. isthmicola* (A and C) and *E. rowelli* (B and D). The numbers of ring commissures (A and B) are given per inter-pedal region. Total number of inter-pedal regions analysed: $n=81$ (*E. isthmicola*) and $n=52$ (*E. rowelli*); number of embryos: 17 (*E. isthmicola*) and 9 (*E. rowelli*). In order to determine the number of median commissures per “segment” (C and D), posterior leg nerves were used as landmarks since no segmental borders are evident in onychophoran embryos. Total number of “segments” studied: $n=48$ (*E. isthmicola*) and $n=24$ (*E. rowelli*); number of embryos: 12 (*E. isthmicola*) and 5 (*E. rowelli*). Note that the average numbers of ring commissures per inter-pedal region and of median commissures per “segment” are slightly higher in *E. rowelli* (6.4 and 8.9, respectively) as compared to *E. isthmicola* (5.6 and 8.2, respectively), and the number of median commissures is more variable in the former.

commissures (Fig. S5). Furthermore, in contrast to the ring commissures, there is no difference in the arrangement of median commissures between leg-bearing and inter-pedal regions (Figs. 4E, 5D, 8A, 9B, S5, and S8). Despite their regular, ladder-like arrangement, the number of median commissures varies from 5 to 10 per “segment” [we used posterior leg nerves as landmarks since no segmental borders are evident in onychophoran embryos] (Figs. 6C and D; see Mayer and Harzsch, 2008 for a similar result in adult *Metaperipatus blainvillei*). Thus, there is no segmental or neuro-mere-like pattern evident at any stage in the formation of the median commissures.

Leg nerves arise in a segmental fashion

To further investigate the developmental origins of segmentation, we have analyzed development of the leg nerves, which are clearly segmental structures in the mature nervous system (Mayer and Harzsch, 2007, 2008). The axons that pioneer the leg nerves start growing after the formation of the commissural pathways, leaving the neuropil in a lateral direction at regular, segmentally repeated positions along the A/P axis. Two axon bundles are formed within each limb bud – the anterior leg nerve and the posterior leg nerve – by the fasciculation of axons with these pioneers (Figs. 10A–D). The two nerves within each leg become interconnected transversely by several ring nerves, which arise in a proximal-to-distal progression (Figs. 10C and D). Later in development, the anterior leg nerve shows a different branching pattern from the posterior leg nerve, which is repeated stereotypically in each leg (Figs. 5C and 10B). Thus, the leg nerves arise in a clearly segmental fashion.

Late embryonic longitudinal tracts display both repeated and non-repeated components

We have analyzed the development of other axonal tracts to determine whether they arise in a segmental or, like the ventral

cords, in a non-segmental fashion. After the commissural pathways have been established, the dorsomedian heart nerve and a pair of dorsolateral nerves are formed by axons originating from the presumptive ring commissures (Figs. 5C and 8A and B). We have not observed any stereotypic repetition in the position of neurons that pioneer the heart nerve, which arises from axons growing along the dorsal midline (Fig. S4). This nerve is displaced deeper into the heart wall later in development, which is the location of the heart nerve in adults (Nylund et al., 1988; Rosenberg and Seifert, 1978) and sub-adult onychophorans (Fig. S7). In contrast, the growth of the dorsolateral longitudinal nerves shows both bilateral symmetry and serial repetition. These nerves are pioneered by axons originating from one of the posterior-most ring commissures in each inter-pedal region (Figs. 5C and S4). The posteriorly directed projections join each other along the A/P body axis, giving rise to the dorsolateral longitudinal nerves (Figs. 5C, 8A and B, and S4). Thus, development of the late embryonic longitudinal tracts displays both stereotypic repetition and non-stereotypic arrangement, suggesting a mixture of segmental and non-segmental patterning, respectively.

Segmentation of ventral organs is not linked to neural patterning

Although previous studies have suggested that the ventral organs are segmental anlagen of the nervous system (Anderson, 1973; Eriksson et al., 2003; Mayer et al., 2005; Pflugfelder, 1948), our data

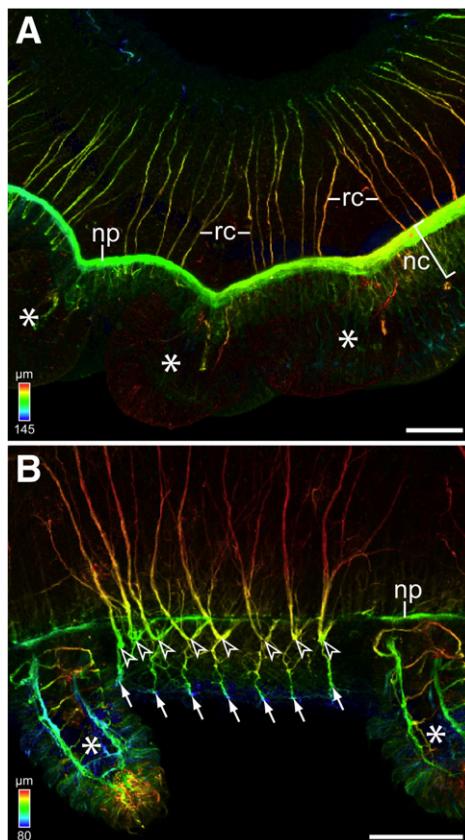


Fig. 7. Variability in number of ring commissures in onychophoran embryos. Anti-acetylated α -tubulin immunolabelling, depth-coded projections of confocal image stacks. Asterisks indicate limb buds. (A) Late “coiled” stage embryo of *E. isthmicola*. The number of presumptive ring commissures (rc) varies from five to seven in each inter-pedal region. (B) Stage 7 embryo of *E. rowelli* showing eight ring commissures (arrowheads) in an inter-pedal region. Note that median commissures are not seen in this image (the arrows indicate ventral branches of ring commissures: see Mayer and Harzsch, 2008 for further details). nc, presumptive ventral cord; np, ventral cord neuropil; rc, presumptive ring commissures. Scale bars: A, 50 μ m; B, 100 μ m.

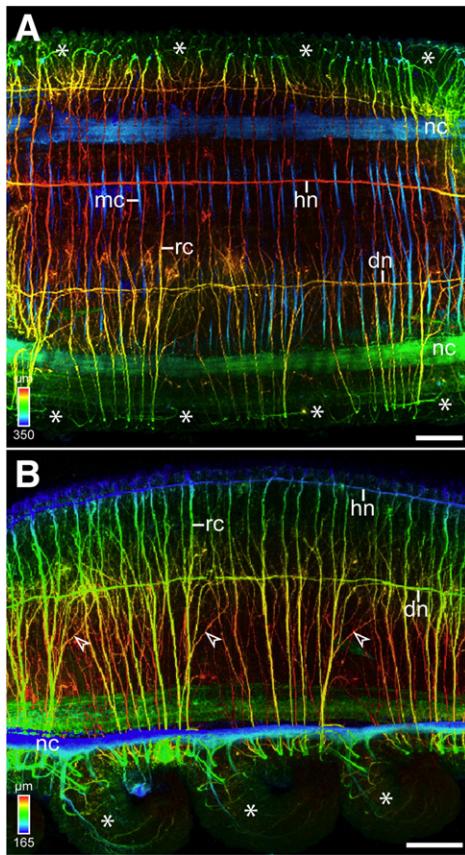


Fig. 8. Late stage of nervous system development showing the orthogonal arrangement of nerve pathways. (A and B) Anti-acetylated α -tubulin immunolabelling of an embryo of *E. isthmicola*. Depth-coded projections of confocal image stacks. Anterior is left. Asterisks indicate the position of presumptive legs. (A) Dorsolateral view of the nervous system within the trunk. Heart nerve (hn), paired dorsolateral nerves (dn), and both ventral cord neuropils (nc) are interconnected at regular intervals by ring commissures (rc) and median commissures (mc). (B) Lateral view of the same body portion. Note the repeated branches, which most likely innervate extrinsic leg musculature (arrowheads). dn, dorsolateral longitudinal nerves; hn, heart nerve; mc, median commissures; nc, ventral cord neuropils; rc, ring commissures. Scale bars: A and B, 100 μ m.

show that this is unlikely. During onychophoran development, the ventral embryonic ectoderm forms two lateral longitudinal bands of thickened tissue, which subsequently move together and fuse along the ventral midline in an A/P sequence (Figs. 11A and B and 12A). This fusion (ventral closure) is prominent in yolk embryos, but less obvious in embryos of the placental species, due to the virtual absence of the extra-embryonic ectoderm (cf. Fig. 4A). The thickened ectodermal bands acquire their maximum extension shortly after ventral closure has occurred (Figs. 11B, 13D, and S8). At that stage, transverse inter-segmental furrows occur at regular intervals along the ectodermal bands, thus delineating a chain of paired thickenings (Figs. 11B, C, 13D, and S8C), which have been dubbed “ventral organs” (Anderson, 1973; Evans, 1901; Korschelt and Heider, 1891; Pflugfelder, 1948; Sedgwick, 1887; von Kennel, 1888). The ventral organs are transitory structures, which decrease in size by undergoing cell death (Figs. 13A–F and S8A), so that only small rudiments (“ventral pits”) remain in adults (Mayer, 2007). Our data show that the ventral organs become evident as segmental structures well after the nervous system has been established (Figs. 11A–C, 12A and B, and S8C and D). Their cells can be distinguished early from other ectodermal cells by their divergent morphology and superficial position. Furthermore, the nuclei of the ventral organ cells are larger than those of other cells and show a distinctive chromatin structure (Fig. S8B). Thus, the ventral organs cannot be regarded as vestiges of segmental ganglia.

Discussion

Lack of segmental ganglion anlagen in Onychophora

In contrast to previous views (Anderson, 1973; Eriksson et al., 2003; Mayer et al., 2005; Pflugfelder, 1948), we have shown here that the ventral organs are not involved in neural development of Onychophora. They become apparent as segmental structures well after the nervous system has formed and, hence, are not equivalent to segmental neuromeres of arthropods (Harzsch, 2003; Whitington, 2007). Their rapid increase in size and degeneration by apoptosis suggest that the ventral organs play a transient, albeit an important role in onychophoran development. This function remains unclear, except in the antennal segment, where the ventral organs give rise to the hypocerebral organs/glands (Eriksson et al., 2005b).

In arthropods, homonymous embryonic structures to ventral organs are known from chelicerates and myriapods. In embryos of these two arthropod groups, the ventral organs arise as epithelial vesicles that are initially incorporated into developing ganglia, but disappear later in embryogenesis (Dohle, 1964; Heymons, 1901; Mayer and Whitington, 2009; Stollewerk, 2004; Tiegs, 1940, 1947; Weygoldt, 1964; Whitington, 2007; Winter, 1980). Because of differences in composition, role, and embryonic fate, the proposed homology of onychophoran ventral organs with the homonymous structures in chelicerates and myriapods (Anderson, 1973; Heymons, 1901; Pflugfelder, 1948; Tiegs, 1940) is unlikely.

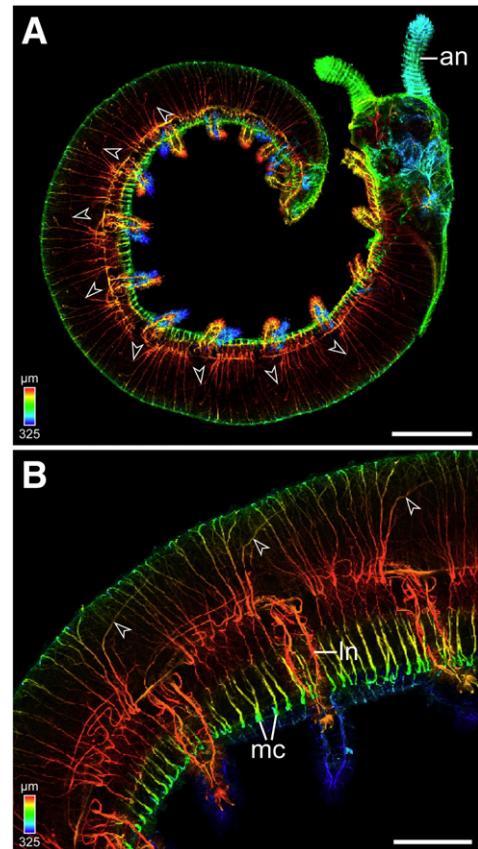


Fig. 9. Orthogonal pattern of the nervous system in an almost fully developed embryo (stage 7) of *E. rowelli*. Anti-acetylated α -tubulin immunolabelling, depth-coded projections of confocal image stacks. (A) Whole-mount preparation. Arrowheads indicate position of posteriorly directed branches that may innervate extrinsic leg musculature. (B) Detail of the same embryo (lateral view of leg-bearing segments 3–6). Note variable branching pattern of ring commissures and serial repetition of posterior branches (arrowheads). an, antenna; ln, leg nerve; mc, median commissures. Scale bars: A, 500 μ m; B, 200 μ m.

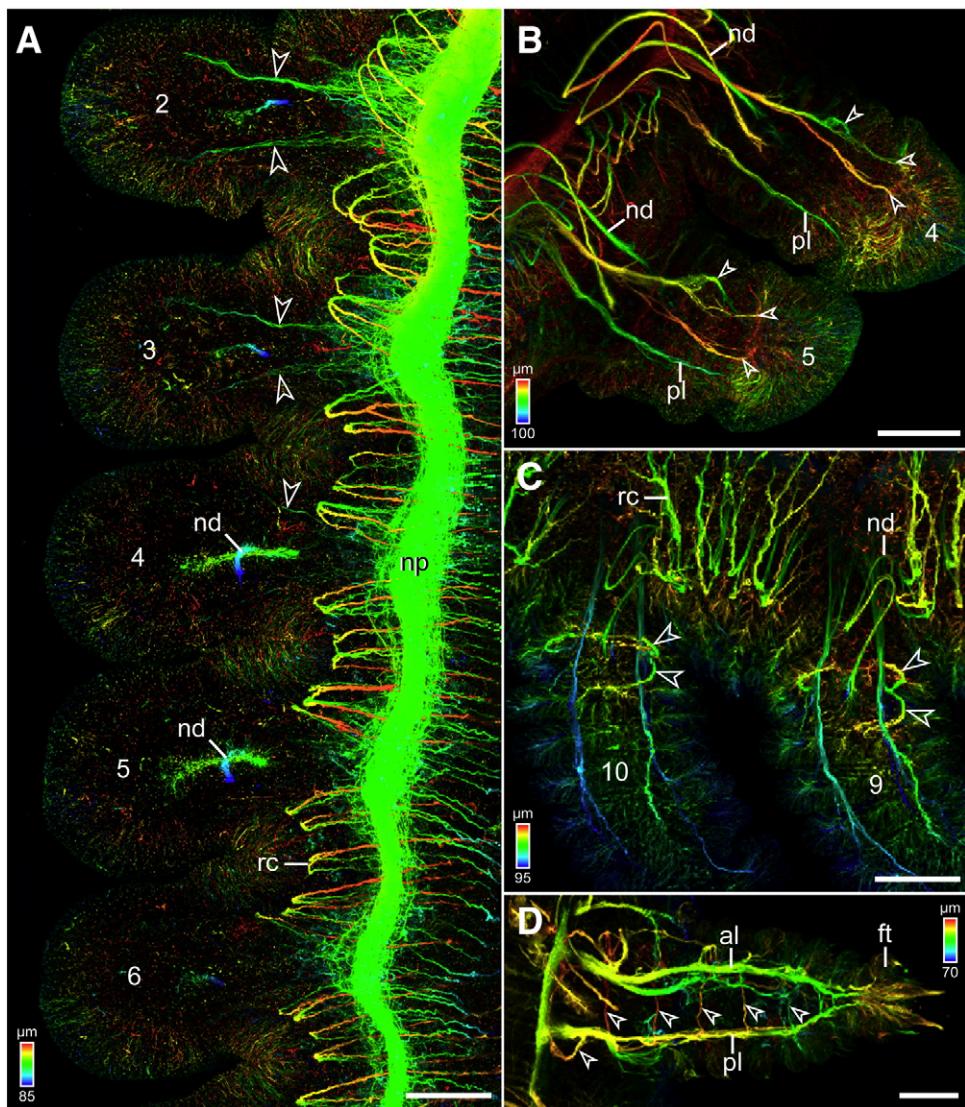


Fig. 10. Establishment of leg innervation. (A–D) Anti-acetylated α -tubulin immunolabelling, depth-coded projections of confocal image stacks. The lumens of the nephridial ducts are labelled intensely due to the presence of developing cilia (Mayer, 2006). (A) Formation of leg nerves (arrowheads) in leg-bearing segments 2–6. “Coiled” stage embryo of *E. isthmicola*. Median is right. (B) Further differentiation of leg nerves in leg-bearing segments 4–5. Early “flexed” stage embryo of *E. bolleyi*. Note different branching pattern of anterior (arrowheads) and posterior leg nerve (pl). Note also the well developed, looped nephridial ducts (nd). (C) Early stage of formation of ring nerves (arrowheads) within legs 9–10. Early “flexed” stage embryo of *E. isthmicola*. (D) Innervation pattern in a trunk leg of an early stage 7 embryo of *E. rowelli*. Arrowheads indicate the ring nerves. Anterior is up. al, anterior leg nerve; ft, foot with claws; nd, ciliated nephridial ducts; np, ventral cord neuropil; pl, posterior leg nerve; rc, presumptive ring commissures. Scale bars: A–D, 50 μ m.

Our study reveals that there are no other segmental, neuromere-like anlagen in the onychophoran embryo (Figs. 14A–E). Each ventral cord neuropil arises by an A/P cascade of axonogenesis, in which no segmental repetition is seen. Furthermore, the cell bodies of early differentiating neurons within the ventral cords do not show any identifiable reiterated pattern (Figs. 14A and B). Thus, neither anatomy nor embryology supports the assumption of ganglia loss in the onychophoran nervous system (e.g., Scholtz, 2002; Strausfeld et al., 2006).

Does expression of segment-polarity genes argue for the presence of neuromeres in onychophoran embryos?

In a recent study, it has been reported that the segment-polarity genes *engrailed* and *wingless* are expressed in a segmental pattern in the embryonic neuroectoderm of the onychophoran *Euperipatoides kanangrensis* (see Eriksson et al., 2009). These authors take this as evidence against the view that the lack of segmental ganglia is a ground pattern feature of Onychophora and Panarthropoda (Mayer

and Harzsch, 2008). However, it is unclear from the data presented by these authors whether, at early stages, *engrailed* and *wingless* are expressed in the developing nervous system or in the superficial ectoderm (see their figure 1). We have shown in a separate study that the superficial ectoderm gives rise to the ventral organs and that neurogenic tissue is located in a more internal position (Mayer and Whittington, 2009). In any event, a segmental pattern of expression of *engrailed* and *wingless* in early differentiating neurons within the ventral nerve cord would not invalidate our observation that the neurons pioneering the longitudinal and commissural pathways are not distributed in a segmentally reiterated pattern.

Eriksson et al. (2009) also report expression of *engrailed* in a segmental pattern in later stage *E. kanangrensis* embryos (their Figs. 6, 7, and S4). The *engrailed*-expressing cells identified as “neuroectoderm” by these authors (arrowheads in Fig. 6e) are clearly ventral organs, which we have shown here play no role in neural development. There appears to be segmentally repeated *engrailed* expression at these stages in the ventral nerve cords (lower arrow in Fig. 6e). As we have shown in this study, the nervous system of the

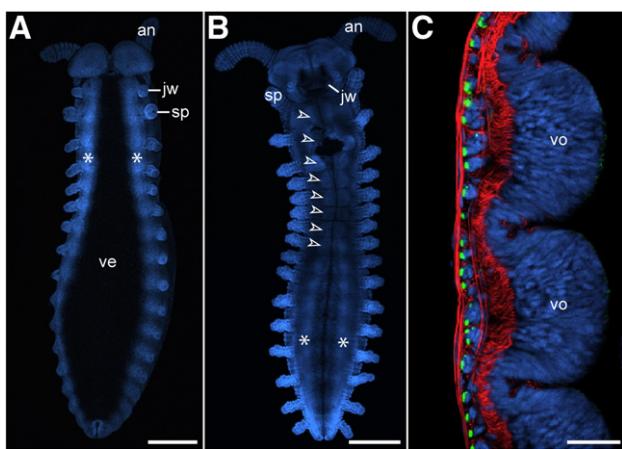


Fig. 11. Development of ventral organs. (A) Segmentally delineated ventral organs are not yet seen in ventrolateral ectoderm (asterisks). Stage 4 embryo of *E. rowelli* in ventral view. Neural development is already underway at this stage. Hoechst labelling. Anterior is up. (B) Ventral organs in an early stage 6 embryo of *E. rowelli*. Hoechst labelling. Anterior is up. The ventral organs appear as segmental structures in the anterior body half (arrowheads) whereas the ventral ectoderm is not yet segmented in the posterior body half (asterisks). At this stage, deployment of neuronal precursor cells from the neuroectoderm has long been completed and the nervous system already shows an orthogonal design (cf. Fig. S8). (C) Ventral organs (vo) in a “flexed” embryo of *E. bolytei* delineated by deep furrows. Sagittal optical section of the ventral body wall. Triple-labelling with phalloidin-rhodamine (red), Hoechst (blue), and anti-acetylated α -tubulin antibody (green). Note that median commissures (green) are widely separated from cells of ventral organs (vo) by musculature (red) and other tissue. *an*, presumptive antenna; *jw*, presumptive jaw; *sp*, presumptive slime papilla; *ve*, ventral extra-embryonic ectoderm; *vo*, ventral organs. Scale bars: A and B, 500 μ m; C, 50 μ m.

onychophoran embryo contains a mixture of non-segmental (pioneering neurons of longitudinal and commissural pathways) and segmental structures (leg nerves and dorsolateral longitudinal nerves). These *engrailed*-expressing cells within the nerve cord might, for example, represent segmentally repeated limb-innervating motoneurons.

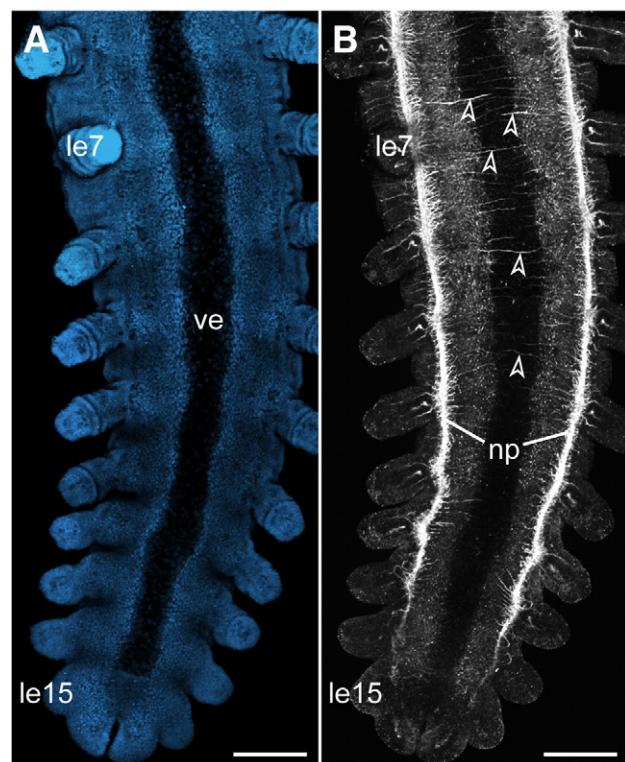


Fig. 12. The onychophoran nervous system develops independently of ventral organs. (A and B) Posterior end of an early stage 5 embryo of *E. rowelli* in ventral view. Double-labelling with Hoechst (A) and anti-acetylated α -tubulin antibody (B). The ventral organs are not yet evident, while the nervous system is at an advanced developmental stage. The ventral cord neuropils (np) have already been established and the axons forming the median commissures have started to cross the ventral midline (arrowheads). The ventral organs will not arise until stage 6 (cf. Fig. 11; see also Walker and Tait (2004) for a similar finding using scanning electron microscopy). *le7*, anlage of the seventh walking leg; *le15*, anlage of the 15th walking leg; *np*, presumptive nerve cord neuropils; *ve*, ventral extra-embryonic ectoderm. Scale bars: A,B, 200 μ m.

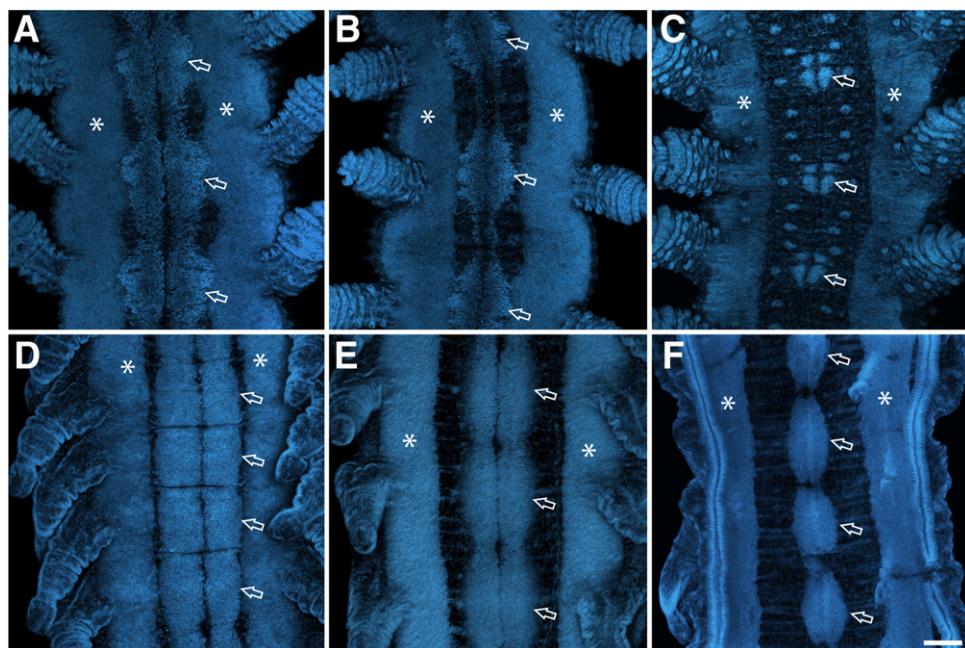


Fig. 13. Degeneration of ventral organs (arrows) in embryos of successive developmental stages. Ventral view of trunk segments; Hoechst labelling. Position of ventral cords below ectoderm is indicated by asterisks. Note the relative decrease in size of the ventral organs. (A–C) Late stage 6, early stage 7, and late stage 7 embryos of *P. tallagandensis*. Note the separation of the ventral organs into an anterior and a posterior part in the late stage 7 embryo. (D–F) Successive “flexed” stage embryos of *E. isthmica*. Scale bar: F, 100 μ m in panels A–F.

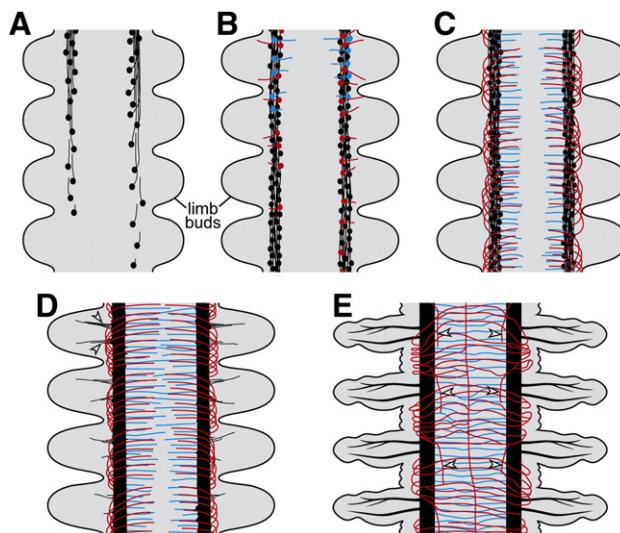


Fig. 14. Diagram of neural development in Onychophora. Not to scale. (A–E) Embryos in successive developmental stages. (A) Neither cell bodies nor early axons establishing ventral cords show a stereotypically repeated pattern at an early stage. (B) The pattern of the cells and axons that pioneer the ring (red) and median commissures (blue) also shows irregularity. (C) Serially repeated, ladder-like arrangement of presumptive ring commissures (red) and median commissures (blue) later in development. (D) The leg nerves (arrowheads) arise in a segmentally repeated pattern along the main body axis. (E) The resulting orthogonal design of the nervous system shows numerous misprojections in ring (red) and median commissures (blue), most of which will disappear later in development. Note the serially repeated growth of pioneering axons (arrowheads) that give rise to dorsolateral longitudinal tracts.

Serially repeated motoneurons associated with limbs could be interpreted as an ancestral feature of Panarthropoda, which might have been a starting point, from which a more complete pattern of segmentation, including segmentally repeated ganglia, commissures, and peripheral nerves, has evolved in the lineage leading to the extant arthropods. Future studies using various neural and molecular markers will show to what extent the onychophoran nervous system is segmented.

Evolution of segmental ganglia in the Panarthropoda

Since most annelid species bear a chain of ventral ganglia (Müller, 2006), this feature was believed to be one of the synapomorphies uniting arthropods and annelids (Nielsen, 1997, 2001; Scholtz, 2002). However, this grouping now receives little support and most authors place arthropods into a clade of moulting animals, called Ecdysozoa, a distant lineage from annelids (Fig. 15; Aguinaldo et al., 1997; Bourlat et al., 2008; Dunn et al., 2008; Edgecombe, 2009; Giribet, 2008; Giribet et al., 2007; Matus et al., 2006; Philippe et al., 2005, 2007; Philippe and Telford, 2006).

Within the Ecdysozoa, only the kinorhynchs, tardigrades (water bears), and arthropods bear segmental ganglia: these are missing in the priapulids, loriciferans, nematodes, and nematomorphs (Fig. 15). Kinorhynchs show a mid-dorsal chain of ganglia in addition to a mid-ventral chain (Kristensen and Higgins, 1991; although see Nebelsick, 1993 for an opposing view). However, kinorhynch ganglia are situated in the middle of each segment or zonite (Nebelsick, 1993) and on that basis, we conclude that they are unlikely to be homologous to arthropod ganglia. Tardigrades have four trunk ganglia which, like the ventral ganglia of arthropods, lie out of register with the legs at each segmental border (see Deutsch, 2004; Zantke et al., 2008). On the basis of this similarity, we suggest that tardigrade and arthropod ganglia are homologous. On the other hand, a recent study has suggested that tardigrade ganglia lack median commissures (Zantke et al., 2008; but see Marcus, 1929). Future studies on the structure, development, and pattern of gene expression of the tardigrade

nervous system will help resolve the issue of their putative homology with arthropod ganglia.

The phylogenetic position of tardigrades is uncertain, but various studies place them as the closest relatives to either arthropods (Fig. 16A), onychophorans plus arthropods (Figs. 16C and D), or one of the cycloneurulian taxa (Fig. 16B) (Budd, 2001a,b; Dunn et al., 2008; Edgecombe, 2009; Giribet et al., 1996; Jenner and Scholtz, 2005; Maas and Waloszek, 2001; Mallatt et al., 2004; Mallatt and Giribet, 2006; Nielsen, 2001; Zantke et al., 2008).

Depending on the phylogenetic position of tardigrades, four possible scenarios for the evolution of segmental ganglia in Panarthropoda can be advanced (Figs. 16A–D). According to the first scenario, a ventral chain of ganglia evolved in the last common ancestor of tardigrades and arthropods and was never present in the onychophoran lineage (Fig. 16A). In the second and third scenarios, segmental ganglia arose convergently in tardigrades and arthropods (Figs. 16B and C). In the fourth scenario, segmental ganglia were present in the last common ancestor of Panarthropoda and were lost in the onychophoran lineage (Fig. 16D). If one accepts that tardigrade

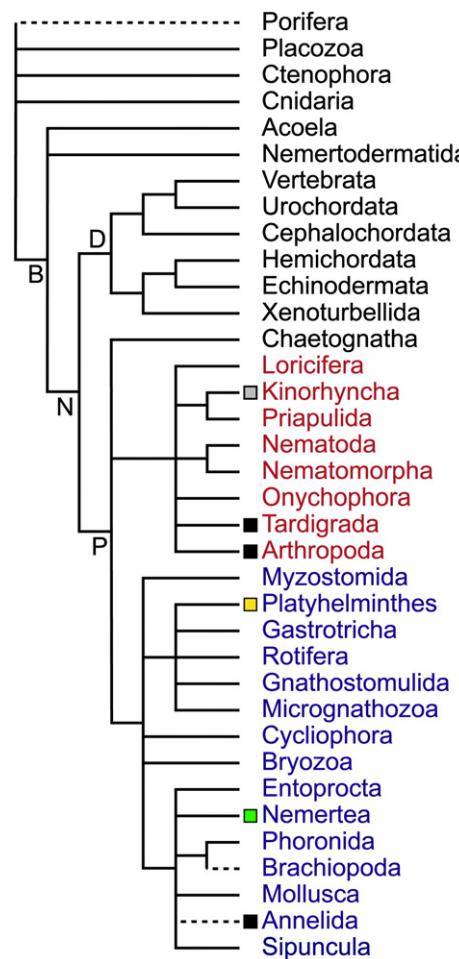


Fig. 15. Distribution of serially repeated ganglia and ganglia-like structures within Bilateria. Animal groups placed within Ecdysozoa are shown in red, those belonging to Lophotrochozoa in blue. Light grey square: two (mid-ventral + mid-dorsal) chains of ganglia in kinorhynchs (Kristensen and Higgins, 1991; but see Nebelsick, 1993). Black squares: single ventral chain of ganglia in tardigrades, arthropods, and annelids (see text). Yellow square: ganglia-like condensations of neuronal cell bodies at intersections of transverse/ring commissures and main cords in some flatworm species (Biserova et al., 2000). Green square: single mid-dorsal chain of repeated ganglia in nemerteans (Bürger, 1897). Homology between the ganglia of arthropods and the ganglia-like structures in flatworms and nemerteans is questionable due to the absence of specific corresponding structures. Phylogeny according to Giribet et al. (2007). B, Bilateria; D, Deuterostomia; N, Nephrozoa; P, Protostomia.

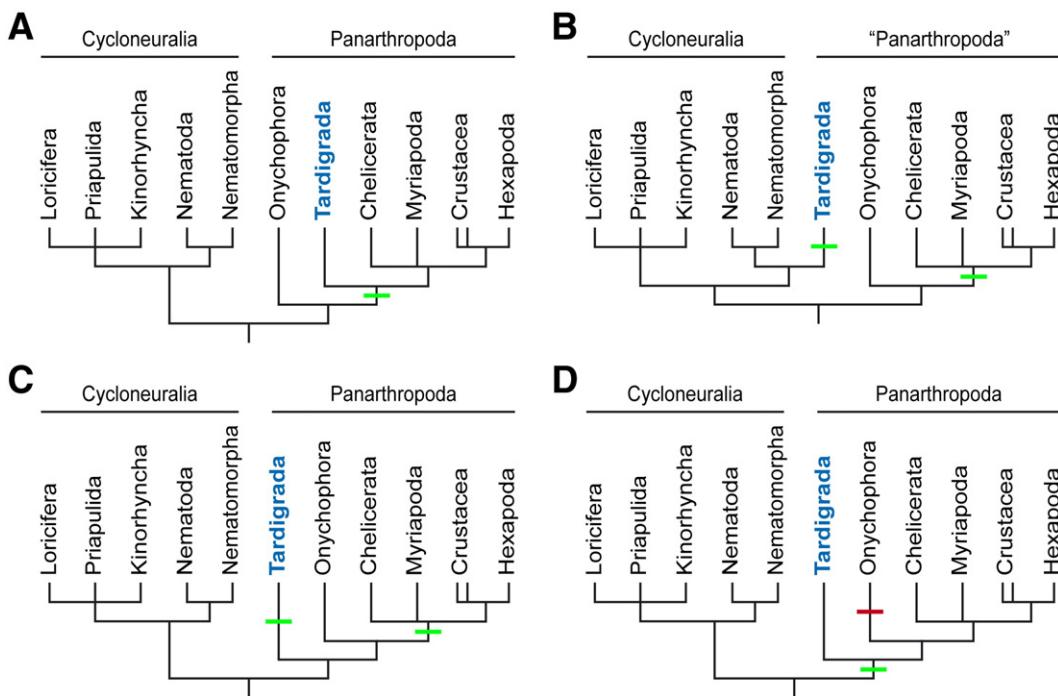


Fig. 16. Alternative hypotheses for the phylogenetic position of Tardigrada and the evolution of segmental ganglia within the Ecdysozoa (Cycloneuralia + Panarthropoda). The green bars indicate the evolution of a ventral chain of ganglia whereas the red bar signifies the secondary loss thereof. (A) Phylogenetic position of tardigrades according to Schmidt-Rhaesa et al. (1998), Budd (2001a), and Nielsen (2001). (B–D) Two alternative hypotheses for the phylogenetic position of tardigrades according to Dunn et al. (2008) and Edgecombe (2009). Panels (C) and (D) show the topology with two equally parsimonious scenarios for the evolution of ganglia.

and arthropod ganglia are homologous, then the second and third scenarios can be rejected as both assume independent evolution of tardigrade and arthropod ganglia. We favour the first scenario – that segmental ganglia are a synapomorphy of Tardigrada and Arthropoda – over the fourth scenario of an origin of segmental ganglia at the base of the panarthropod lineage. The latter scenario assumes that there has been secondary loss of segmental ganglia in the Onychophora; an assumption for which we see no evidence in the early development of the onychophoran nervous system.

Implications for the origin of segmentation within the Panarthropoda

The general architecture of the onychophoran nervous system corresponds with the orthogen of various protostomes, which suggests that it is derived from such an ancient orthogonal design (Mayer and Harzsch, 2008). We have shown here and elsewhere (Mayer and Harzsch, 2007, 2008) that the leg nerves, nephridial nerves, and posteriorly directed branches of nerves, which may innervate extrinsic leg muscles, are the only segmental components of the onychophoran nervous system. Accordingly, the neural organization of Onychophora can be regarded as an orthogen, upon which some segmental elements have been superimposed. It therefore reflects the mixture of segmental and non-segmental features seen in the overall body plan of Onychophora (Hoyle and Williams, 1980; Mayer, 2006; Nielsen, 2001; Schmidt-Rhaesa et al., 1998).

A similar mixture of segmental and non-segmental body structures is seen in various Cambrian lobopodians, or stem-lineage (pan) arthropods (e.g., Budd, 2001a; Hou and Bergström, 1995; Ma et al., 2009; Maas et al., 2007; Whittington, 1978). From our study, we conclude that body segmentation began in the panarthropod ancestor, but advanced further in the lineage leading to the extant arthropods. These findings support the assumption of gradual evolution of segmentation in panarthropods from forms without ganglia and sclerites to species with sclerites and spines and, finally, to

modern arthropods with a more elaborate segmented body plan with jointed appendages, ganglia and muscles acting on adjacent tergites in each body segment (Budd, 2001a; Edgecombe, 2009; Maas and Waloszek, 2001).

Author contributions

G.M. conceived, designed and performed the experiments and wrote the first draft of the manuscript. P.M.W. helped with specimen collection and contributed laboratory space, reagents, and most materials and analysis tools. Both authors shared in the analysis and interpretation of the data and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.08.011.

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