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ORIGINAL ARTICLE

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Metameric organisation of the nervous system in developmental stages of *Urechis caupo* (Echiura) and its phylogenetic implications

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Abstract The phylogenetic position of Echiura is still in continuous debate. The commonly accepted view regards Echiura as a distinct taxon, often classified as phylum, which forms the sister group of the Articulata. The alternative view considers Echiura to be a subtaxon of Annelida, which is supported by numerous shared characters. The correct systematic position of Echiura is inevitably linked to the presence or absence of true segmentation. The apparent lack of segmentation in Echiura is considered to be either primary, thereby supporting their exclusion from Annelida, or alternatively to be the result of reduction. The latter would clearly substantiate their classification as a subtaxon of Annelida. Immunohistochemical methods and confocal laser-scanning microscopy clearly demonstrate a metameric organisation of the nervous system in different larval stages of *Urechis caupo*, which corresponds to the segmental arrangement of ganglia in "typical" Annelida. This segmental pattern is reflected in the serially repetitive distribution of neurons containing the neurotransmitter serotonin (5-hydroxytryptamine) and also in the corresponding distribution of strictly paired peripheral nerves. Precisely two pairs of peripheral nerves are associated with each of the repetitive units. This metameric pattern also corresponds to the transient annulation of the trunk, which is found in late larval stages. Other characters of the nervous system including the paired origin of the ventral nerve cord, the anterior-posterior development gradient and the presence of a distinct suboesophageal ganglion are also found accordingly in typical Annelida. These results are interpreted as an indication that Echiura are derived from formerly segmented ancestors, and thus support their systematic inclusion within Annelida.

Keywords Echiura · Annelida · Phylogeny
Segmentation · Nervous system

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Introduction

Echiura share a large number of characters with "typical" Annelida. This was considered even in the earliest descriptions of species of Echiura, for example, *Thalassema thalassemum* (Pallas, 1766) and *Echiurus echiurus* (Pallas, 1766), which were initially included in the oligochaete genus *Lumbricus*. Quatrefages (1847) erected the Gephyrea, which included Echiura, together with Sipuncula and Priapulida. The group was considered to form a link between Annelida and Holothuroidea. Hatschek (1880) found many parallels in the embryology of *Echiurus* sp. and different species of "Polychaeta" and was the first to suggest that Echiura should be included in Annelida. This was later adopted by Sedgwick (1898), who eliminated the Gephyrea. Yet, based on his elaborate developmental studies of *Urechis caupo* Fisher and MacGinitie, 1928, Newby (1940) established the Echiuroidea as a separate phylum in terms of traditional classification, which was later renamed Echiura by Stephen (1965) to conform to nomenclature standards.

Today the phylogenetic position of the Echiura within the Spiralia, especially their systematic relationship to the Annelida, is still in continuous debate. The contradicting assessments can be summarised in two alternative hypotheses:

1. One hypothesis, which has found broad acceptance, follows Newby's classification and considers Echiura to form a distinct taxon, often referred to as phylum. This view is supported by numerous authors including Korn (1960), Clark (1964, 1969), Stephen and Edmonds (1972), Pilger (1993) and Edmonds (2000). In their cladistic analyses Rouse and Fauchald (1995, 1997, 1998) and Rouse (1999) found Echiura to cluster as sister group to the Articulata, a clade comprising Annelida and Arthropoda. This classification is also found in many zoological textbooks (see, for example, Kaestner 1965; Brusca and Brusca 1990; Westheide and Rieger 1996; Margulis and Schwartz 1998).

2. The alternative hypothesis regards Echiura as a subtaxon of Annelida. This view is supported by Nielsen (1995), Eibye-Jacobsen and Nielsen (1996), McHugh (1997, 2000) and Ax (1999).

Both hypotheses unquestionably assume a close phylogenetic affinity between Echiura and Annelida. This is based on numerous shared characters, many of which must be regarded as homologous, for example, the similarities in the course of spiral cleavage, the formation of a typical trochophore larva, the ultrastructure and development of chaetae as well as the ultrastructure of spermatozoa (Hatschek 1880; Baltzer 1932; Newby 1940; Storch 1984; Franzén and Ferraguti 1992; Pilger 1993).

Nonetheless, Echiura are generally excluded from Annelida, a classification which is essentially based on a single character: their apparent lack of segmentation. The first hypothesis, which regards the Echiura as sister group of the Articulata thus assumes that echiurans are primarily unsegmented. The alternative possibility, that Echiura are secondarily unsegmented must be considered as well, in which case they would have to be regarded as a highly derived subtaxon of Annelida. Conclusive proof of the existence or lack of metameric structures is therefore crucial for the correct assessment of their phylogenetic position.

The nervous system is generally considered to be a relatively conservative structure in evolutionary terms (Bullock and Horridge 1965; Wegerhoff and Breidbach 1995; Whitington 1995; Hessling and Westheide 1999) and can therefore be potentially highly informative for the assessment of phylogenetic relationships. The anatomy of the adult nervous systems of *U. caupo* and some of its physiological aspects were published by Lawry (1966a, b). Its development was portrayed in extraordinary detail by Newby (1940). The nervous systems of various other species of Echiura have been described histologically by a number of authors (Hatschek 1880; Spengel 1880; Baltzer 1932; Korn 1960; Wilczynski 1980). Schuchert and Rieger (1990) published an ultrastructural analysis of the dwarf male of *Bonellia viridis* Rolando, 1821 including descriptions of neuronal structures. Immunohistochemical methods and confocal laser-scanning microscopy were used to study the development of the nervous system in *B. viridis* (Hessling 2001, 2002; Hessling and Westheide 2002). This combination of methods has proven to be very informative in the study of neurogenesis and in many cases has revealed previously unreported details in neuronal structures of various invertebrate taxa (see, for example, Harzsch et al. 1997; Hessling et al. 1999; Müller and Westheide 2000). The present study is the first application of these techniques in a species of Echiura with planktotrophic larval development.

The adult nervous system of echiurans consists of a dorsal commissural medullary strand representing the cerebral ganglion and circumoesophageal connectives, which together form an anterior nerve loop (Bullock and Horridge 1965). This proboscis nerve ring stands in contact with the ventral nerve cord, which is described as unpaired, medullary and unsegmented (Lawry 1966a). Peripheral nerves apparently extend in an unpaired and irregular arrangement from the ventral nerve cord (Bullock and Horridge 1965; Edmonds 2000). However, there have been disputed reports of metameric structures in the nervous system of larvae in different species of Echiura (Hatschek 1880; Baltzer 1932; Korn 1960).

This paper is the third in a series of publications describing the development of the nervous system in Echiura. The previous papers (Hessling 2002; Hessling and Westheide 2002) portray the organisation of neuronal structures in developmental stages of *B. viridis*. Here the development of the nervous system is presented in different larval stages of *U. caupo* and the phylogenetic implications are discussed.

Urechis caupo is a comparatively large representative of Echiura found in intertidal mudflats along the western coast of North America (Suer 1984). Due mainly to its abundance and accessibility, probably more is known about *U. caupo* than about any other species of Echiura (Edmonds 2000). It has been the focus of ecological (Suer 1984), morphological (Lawry 1966a; Menon and Arp 1998) and physiological (Lawry 1966b; Julian et al. 1996) studies including toxicological analyses (see, for example, Toomey and Epel 1993; Arp et al. 1995) and has also been the subject of numerous developmental studies (see, for example, Newby 1932, 1940; Miller 1973; Gould-Somero 1975; Cross 1984). *Urechis caupo* is not a typical echiuran species in regard to some aspects of its morphology or its lifestyle (Edmonds 2000). Especially its method of filter-feeding by pumping water through a funnel-shaped mucous net, which is secreted in its burrow is certainly unique among Echiura. Its mode of development including the formation of a typical planktotrophic trochophore larva, however, corresponds to the situation found in many other species of Echiura (Baltzer 1932; Newby 1940).

Materials and methods

Adult specimens of *U. caupo* were collected from intertidal mudflats of Bodega Bay, California, USA (Suer 1984) in October 1998 and September 1999. They were maintained for a period of several weeks in the Bodega Marine Laboratories (University of California at Davis). The animals were kept in sediment filled aquaria under continuous flow of unfiltered seawater at approximately 15°C. Apart from the naturally occurring plankton no additional food was provided.

For the culture of larvae, gametes were extracted from adult individuals by inserting a blunt glass probe into gamete storage sacks through the gonopores (MacGinitie 1937; Gould 1967). Mechanical stimulation of the gonopores led to contraction of the muscular walls of the storage organs expelling the ripe gametes onto the surface of the worm, from which they were collected using a glass pipette. Approximately 1 ml of fluid containing oocytes was diluted in 2 l filtered seawater. Subsequently, a few drops of sperm were added and gently stirred. After 15 min excess sperm were removed by rinsing oocytes with filtered seawater through 20-µm gauze in order to prevent polyspermy as far a possible. Fertilised eggs, just like the following developmental stages, were maintained in large finger bowls at 15°C. After 24 h pelagic

larvae had developed, which were transferred to freshly obtained filtered seawater. Subsequently, the culture medium was replaced regularly with filtered seawater and aerated daily. Developing larvae were fed with microalgae of the species *Isochrysis galbana* (Haptophyceae) (Miner et al. 1999). After 5 weeks remaining specimens were transferred to the laboratories of the University of Osnabrück, Germany, and cultured under the same conditions, but using reconstituted seawater.

In total 20 separate cultures of larvae were reared. Nearly 100% of the oocytes were fertilised in all of these cultures. However, some cultures failed to flourish presumably due to overpopulation of early larvae in the culture dishes. Several hundred individuals of different developmental stages were fixed for immunohistochemistry. Larvae were narcotised in 8% $MgCl_2$ for approximately 10 min and subsequently fixed in 4% paraformaldehyde solution in seawater for 2 h at 4°C on ice (Hessling 2002). Older specimens were fixed using a 4% paraformaldehyde solution with 10 mM phosphate-buffered saline (PBS, pH 7.4) (Côté et al. 1993). The specimens were preincubated for 2–6 h in PBS containing 0.1% Triton X-100, 0.25% bovine serum albumin (BSA) and 0.05% NaN_3.

A total of 22 primary antibodies against different neuronal epitopes were tested for indirect immunolabelling of the nervous system in developmental stages of *U. caupo*, of which only a few resulted in positive immunoreactivity (see Hessling 2002). Primary antibodies were used to label the neurotransmitters serotonin (5-hydroxytryptamine; Saxon Biochemicals, Hannover, Germany) and FMRFamide (courtesy of C.J.P. Grimmelikhuijzen, Copenhagen, Denmark). Antibodies against different isoforms of tubulin including anti-acetylated α-tubulin (Sigma, Heidelberg, Germany) and anti-Y (anti-tyrosinated tubulin; courtesy of T.H. MacRae, Halifax, Canada) were used to label neurotubules and ciliary structures. Primary antibodies were applied at a dilution of 1:100 in preincubation solution without BSA (antibodies against FMRFamide were diluted 1:2,000). The primary antibodies were in turn labelled using secondary antibodies conjugated with the fluorophores Alexa Fluor 488 [goat anti-rabbit IgG (H+L) conjugate; Molecular Probes, Eugene, Ore., USA], fluorescein isothiocyanate (goat anti-rabbit FITC-conjugated; Zymed, San Francisco, Calif., USA) or indomethinecyanine (goat anti-rabbit CY3-conjugated; Sigma).

Approximately 180 specimens were selected from 35 different age groups ranging between 1 and 84 days for this analysis. They were mounted in Citifluor (Plano, Wetzlar, Germany) on microscope slides and examined using the confocal laser-scanning microscope Zeiss CLSM 410. Series of "optical sections", obtained by scanning whole mount specimens, were projected into one image with greater focal depth.

Results

General course of development and external appearance

The development of *U. caupo* including the metamorphosis into the benthic stages is a series of gradual processes. The lengths of the different phases are dependent upon the conditions under which the larvae are reared. Presumably due to the lack of natural substrate the pelagic phase of these larvae was prolonged in comparison to previous descriptions (Newby 1940) and the oldest surviving developmental stages still had not metamorphosed completely at an age of 84 days but had attained a general worm-like appearance.

The early phase of development in *U. caupo* is characterised by very synchronised cleavages which lead to a pelagic blastula stage after approximately 12–16 h. Early trochophora stages with a well-defined prototroch and an apical tuft are formed ca 24 h after fertilisation. Feeding begins between the 2nd and 3rd day. Early larvae at an age of 10 days possess a preoral prototroch and a postoral metatroch as well as a longitudinally oriented neurotroch. The ciliary bands are complemented by a posterior telotroch in older larval stages around the 20th day (Fig. 1A, D). An anterior pair of chaetae arises between the 25th and 30th day. In late larval stages, approximately between 35 and 60 days after fertilisation, the posttrochal region becomes apparently annulated. As the development proceeds the late larval stages enter metamorphosis and are generally found at the bottom of the culture dishes, even though the ciliary bands may still persist. In these stages the annulation of the trunk is lost, beginning in the anterior region and extending towards the pygidium (Fig. 1B).

In the following, immunohistochemical labelling of neuronal structures will be described for early, intermediate and late larval stages.

Labelling of acetylated α-tubulin and tyrosinated tubulin

Antibodies against acetylated α-tubulin labelled cilia and ciliary sense organs in all specimens analysed, yet neurotubules were only stained in few individuals. Antibodies against tyrosinated tubulin labelled cilia and ciliary sense organs as well (Fig. 1C), but also microtubules of neuronal processes in most cases. However, due to the fact that this isoform occurs in the cytoskeleton of non-neuronal tissues as well, this labelling led to very high levels of background signal. Nonetheless, in many cases neuronal structures could be discerned on the basis of their apparently high content of tyrosinated tubulin. Since both antibodies essentially reveal the distribution of the same ciliary and neuronal structures, the results will be presented jointly here.

Cilia of the proto- and metatroch as well as the shorter ciliation of the pharynx and gut are labelled in very early developmental stages (Fig. 1C). Neuronal fibres were first detected in comparatively later stages approximately 20 days after fertilisation. Even then, their labelling is mostly obscured by the numerous ciliary structures (Fig. 1D–F) including the trochal bands, the apical tuft and additional patches of cilia on the dorsal side of the episphere. Internal cilia are also labelled in the pharynx, gut and nephridia, which extend from the region of the metatroch towards the ventrolateral surface of the larval trunk. Paired globular structures are labelled in the anterior region left and right of the apical plate (Figs. 1D, 2A). These doughnut-shaped structures are presumably ciliary sense organs.

In the episphere of this larval stage, neuronal fibres of the circumoesophageal connectives are labelled (Fig. 1E). They extend in a wide arch just beneath the epidermis from the ventrolateral side of the prototrochal region to the anterodorsal side of the larva, where they enter the paired anlage of the suboesophageal ganglion. In the hyposphere the fibres of the circumoesophageal connectives continue posteriorly as precursors of the paired longitudinal nerve trunks, which contribute to the ventral nerve cord in later developmental stages (Fig. 1F). In

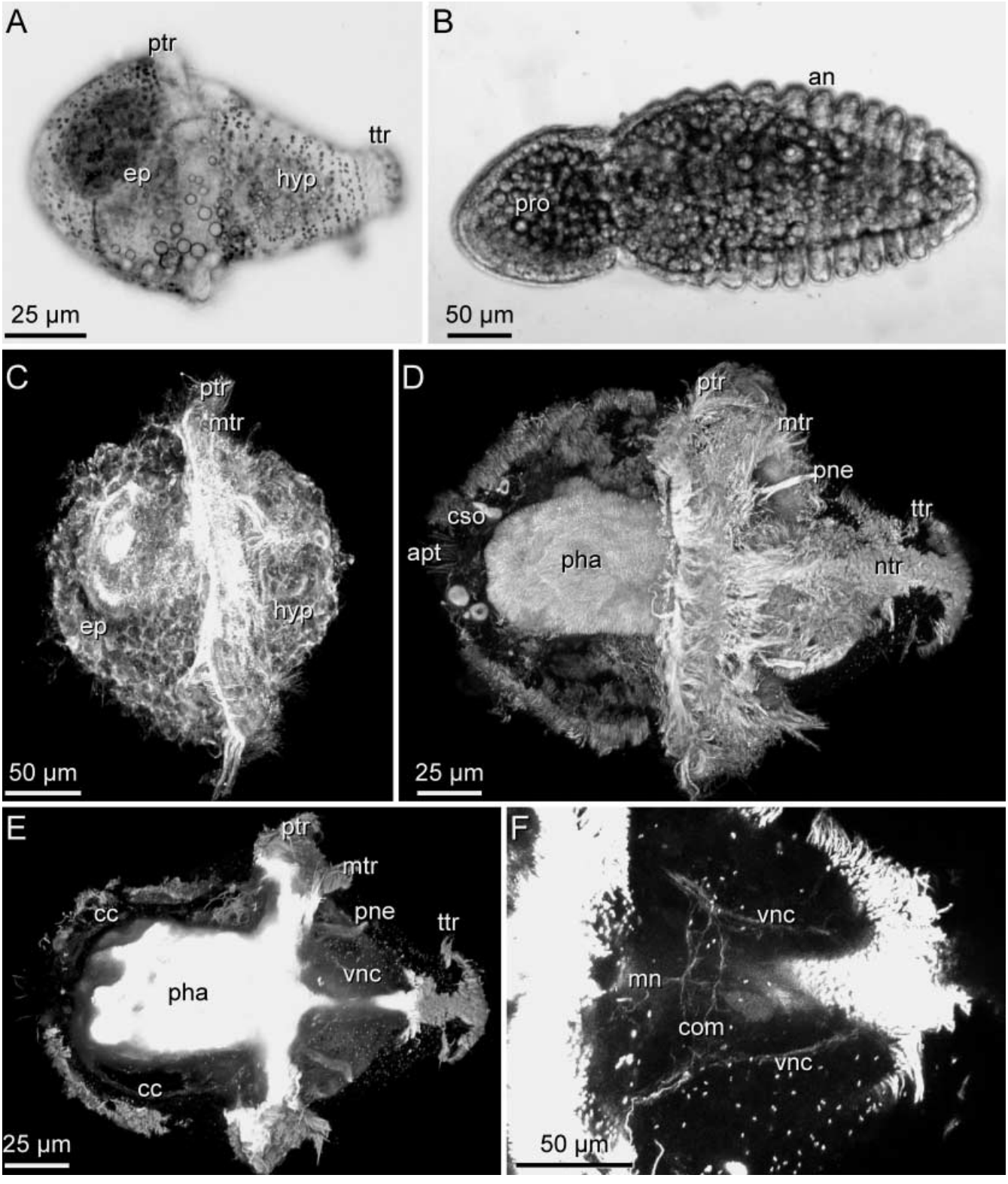

Fig. 1A–F *Urechis caupo*. **A** Early planktotrophic larval stage. Lateral view. **B** Metamorphosing stage in which annuli (*an*) of the anterior trunk are already reduced. Lateral view. **C** Anti-tyrosinated tubulin immunoreactivity. Ventral view. Early larval stage with prototroch (*ptr*) and developing metatroch (*mtr*). **D–F** Anti-acetylated α-tubulin immunoreactivity. Ventral view. **D** External and internal ciliation of planktotrophic larva. **E** Ventral sections removed revealing fibres of circumoesophageal connectives (*cc*) and ventral nerve cord (*vnc*). **F** Detailed view of hyposphere with paired nerve tracts of the ventral nerve cord (*vnc*), unpaired median nerve (*mn*) and first commissure (*com*). *apt* Apical tuft, *cso* ciliary sense organ, *ep* episphere, *hyp* hyposphere, *ntr* neurotroch, *pha* pharynx, *pne* protonephridium, *pro* prostomium, *ttr* telotroch

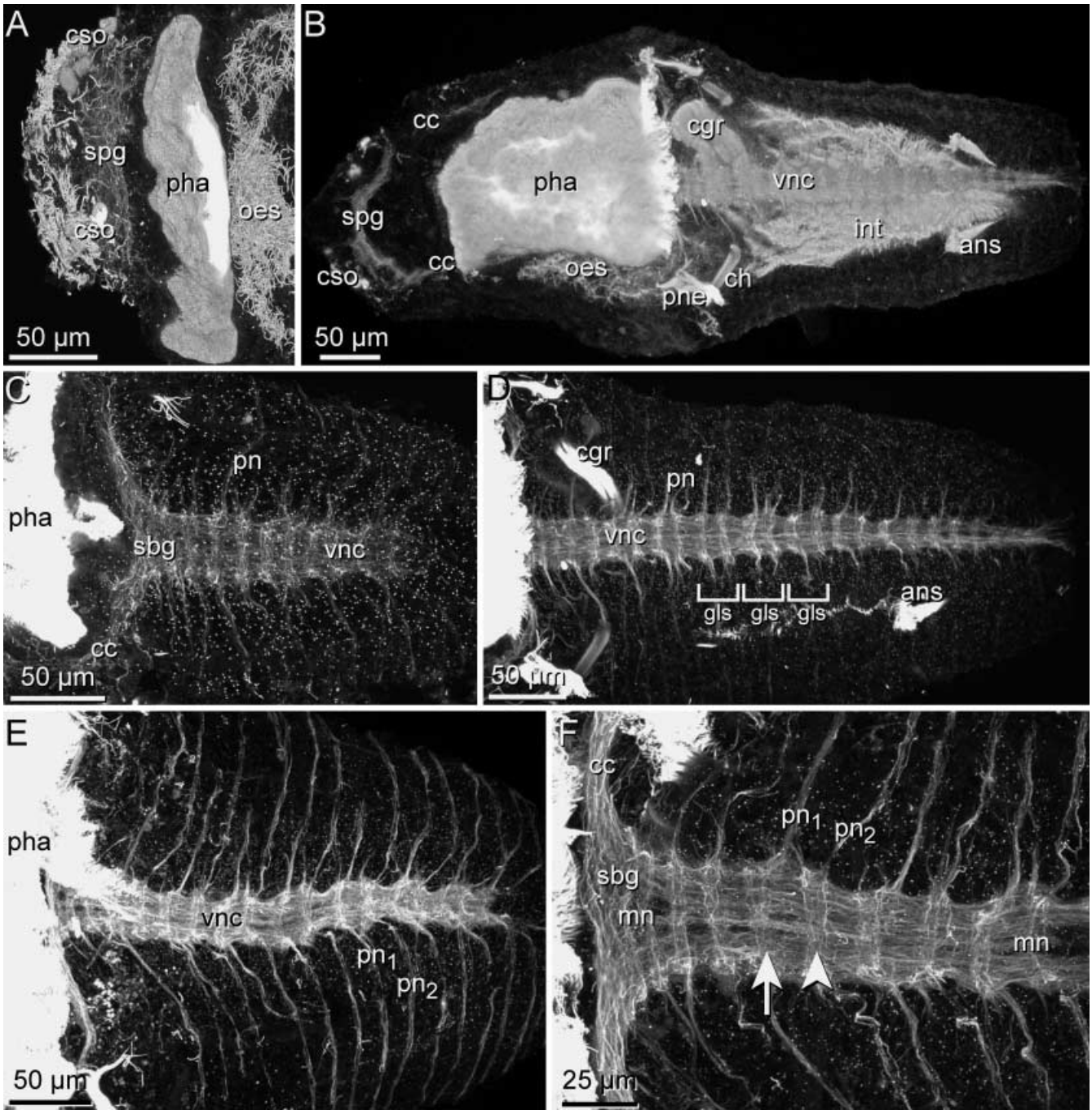

Fig. 2A–F *Urechis caupo*. Anti-acetylated α-tubulin immunoreactivity. **A** Late larval stage. Dorsal view of anterior end with supraoesophageal ganglion (*spg*) and ciliary sense organs (*cso*). **B–F** Ventral view. **B** Overview of metamorphosing stage. **C** Intermediate larval stage with developing ventral nerve cord (*vnc*). **D** Trunk of metamorphosing stage. *Brackets* show ganglion-like swellings (*gls*) in ventral nerve cord (*vnc*). **E** Trunk with two pairs of peripheral nerves (pn_1, pn_2) per ganglion-like swelling. **F** Anterior region of ventral nerve cord. Two pairs of peripheral nerves (pn_1, pn_2) with corresponding anterior (*arrow*) and posterior (*arrowhead*) commissure. Note unpaired median nerve (*mn*). *ans* Anal sac, *cc* circumoesophageal connectives, *cgr* ciliated groove, *ch* chaetae, *int* intestine, *oes* oesophagus, *pha* pharynx, *pne* protonephridium, *sbg* suboesophageal ganglion

these comparatively early larval stages the paired bundles consist of only a few fibres and lie rather far apart from one another, especially in the anterior region, while they converge towards the posterior end. In some cases, an additional unpaired fibre is visible in the ventral midline, which extends longitudinally between the main lateral nerve trunks. Additional transversely oriented fibres form an anterior commissure, which connects the longitudinal nerve trunks with one another (Fig. 1F).

As development proceeds, the fibre mass of the ventral nerve cord increases so that its paired origin is obscured and difficult to discern (Figs. 2C, 3A). Anterior-

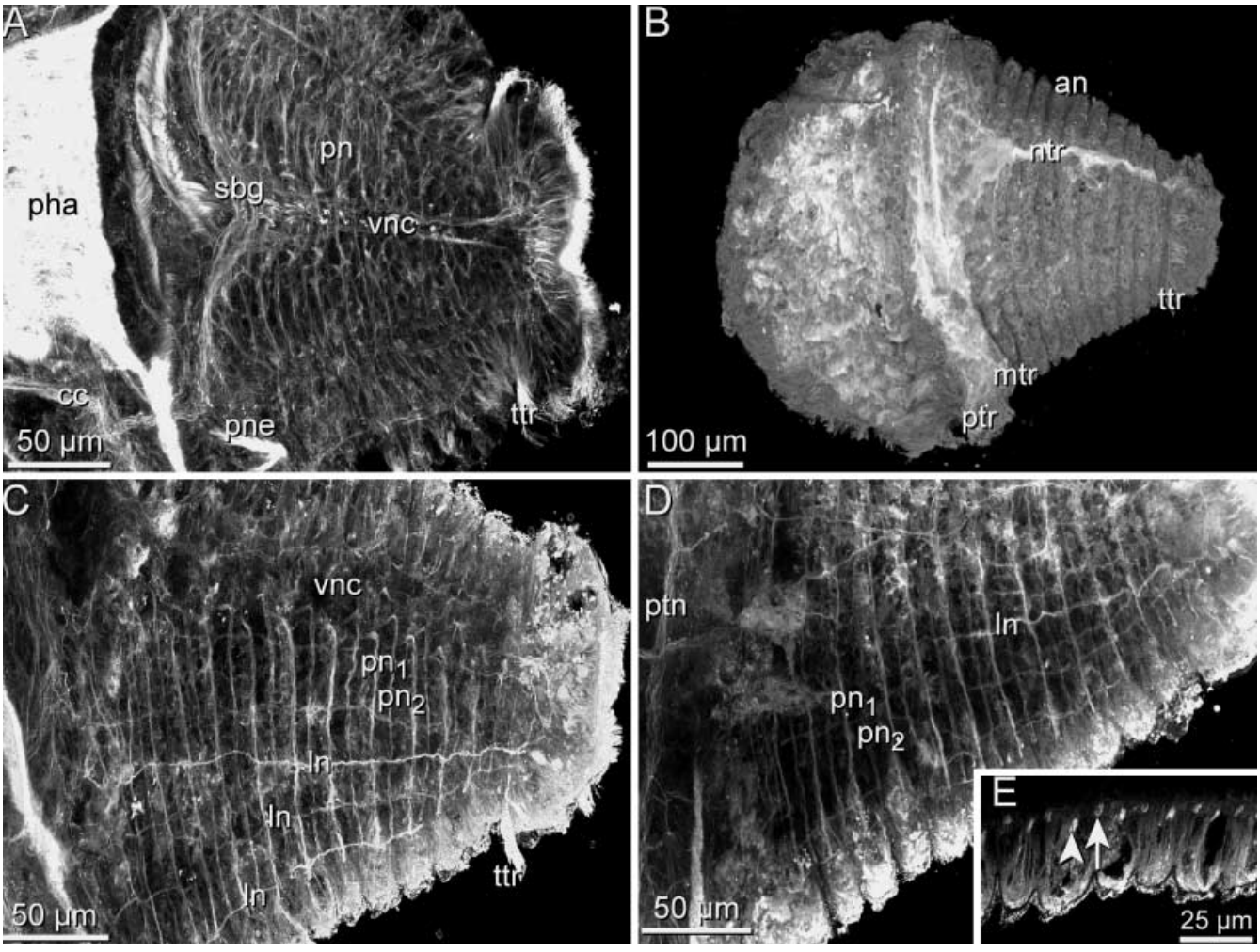

Fig. 3A–E *Urechis caupo*. Anti-tyrosinated tubulin immunoreactivity. **A–C** Ventral view. **A** Intermediate larval stage. Developing ventral nerve cord (*vnc*) with precursors of peripheral nerves (*pn*). **B** External ciliation of late larval stage with annulated (*an*) trunk. **C** Trunk of late larva with alternating pattern of peripheral nerves (pn_1, pn_2) and additional longitudinal nerves (*ln*). **D** Dorsal view of trunk. Peripheral nerves (pn_1, pn_2) extend dorsally. Additional longitudinal nerves (*ln*) extend from protrochal nerve (*ptn*). **E** "Optical section" through body wall. One peripheral nerve in the centre of each annulus (*arrowhead*). Neighbouring nerves at the base between two annuli (*arrow*). *cc* Circumoesophageal connectives, *mtr* metatroch, *pha* pharynx, *pne* protonephridium, *ptr* prototroch, *ttr* telotroch, *sbg* suboesophageal ganglion

wards the central neuropil is clearly more compact than in the posterior region. The number of commissures increases as well in this intermediate developmental stage. Labelling of tyrosinated and acetylated α-tubulin both reveal the first fibres of paired peripheral nerves, which extend in regular intervals from the central neuropil towards the ventrolateral regions of the body wall.

In the course of further development the trunk of the larva becomes progressively elongated and shows striking annulations. Labelling of tyrosinated tubulin in these stages reveals not only the external ciliary bands (Fig. 3B). Due to the diffuse staining of tyrosinated tubulin in the cytoskeleton of non-neuronal cells, this labelling also shows the contours of the larva, including its annulated trunk (Fig. 3B–E). As these larvae enter metamorphosis, the conspicuous annulation of the trunk is lost, beginning in the anterior region and later extending to the posterior end of the trunk.

Essentially the same ciliary structures are labelled with antibodies against acetylated α-tubulin in late larval and metamorphosing stages (Fig. 2A–F) as in earlier larvae. Most prominent is staining of the dense pharynx ciliation (Fig. 2A, B) but cilia are also found in the larval intestine, especially in the ventral ciliated groove. In addition to the previously described cilia of the protonephridia, very late metamorphosing stages reveal first cilia of the anal sacs, which develop into the definitive excretory organs (Fig. 2B). Ciliary sense organs are also stained in the episphere of these developmental stages.

Labelling of neurotubules in late larval and metamorphosing stages reveals a nervous system consisting of an anterior nerve loop, ring fibres innervating the proto- and metatroch and a prominent ventral nerve cord (Fig. 2B). Complex and distinct brain-like structures are not found (Fig. 2A, B). The fibres are rather continuous with the circumoesophageal connectives, which extend in a wide arch back into the region of the metatroch. From here

they project almost transversely towards the ventral midline, where they enter the ventral nerve cord (Fig. 2E, F).

The neuropil of the ventral nerve cord appears as a more or less uniform fibre mass, which in cross-section is generally wider than high and somewhat arch-shaped. In detail, however, it is still evident that the central neuropil is initially formed by paired longitudinal nerve tracts. An additional unpaired median nerve is also detectable, especially in the anterior region of the ventral nerve cord (Fig. 2F).

Transversely oriented fibres, which form commissures and thus interconnect the longitudinal nerve tracts, are found in very regular intervals along the ventral nerve cord (Fig. 2D–F). In some cases there appear to be faint ganglion-like swellings in intervals of two commissures especially in the middle and posterior regions of the ventral nerve cord (Fig. 2D).

In exactly the same intervals as the series of commissures, strictly paired peripheral nerves extend from the central neuropil towards the peripheral body wall of the larvae (Figs. 2D–F, 3C, D). These fibres encircle the trunk completely, thus forming ring nerves (Fig. 3D). The peripheral nerves display an alternating pattern in the way they extend from the central neuropil of the ventral nerve cord. When considering the ganglion-like swellings that are each associated with two pairs of peripheral nerves, it appears that the anterior pair of nerves extends ventrolaterally from the neuropil in a slight angle towards the ventrolateral body wall. The following pair of peripheral nerves projects more ventrally from the neuropil, apparently in a steeper angle and more directly towards the ventral body wall (Fig. 2D–F). This alternating pattern is also evident when viewing the bends where the fibres coming from the neuropil reach the body wall and curve abruptly towards the lateral regions (Fig. 3C). It is reflected also in the way the peripheral nerves extend through the body wall at the base of the epidermis in the lateral trunk region. In a horizontal "optical section", one nerve (Fig. 3E *arrowhead*) is found in the centre of each annulus, while the neighbouring nerve (Fig. 3E *arrow*) is located slightly more internally in the region adjacent to the furrow between two annuli (Fig. 3E).

Antibodies against tyrosinated tubulin label additional longitudinal nerves on the dorsal and ventral sides of late larval stages (Fig. 3C, D). On the dorsal side, a prominent pair of longitudinal nerves extends from the dorsolateral region of the anterior nerve loop in an arc towards the posterior end of the larva (see also Fig. 5C). Another conspicuous pair of additional longitudinal nerves is found on the ventrolateral side. These fibres project posteriorwards from the prominent nerve ring that lies beneath the prototroch. In the region of the telotroch they curve towards the ventral midline and come into contact with the lateral trunks of the ventral nerve cord (Fig. 3C). Several other, somewhat thinner longitudinal nerves extend from the dorsal and ventral sides of the protrochal nerve ring posteriorwards. Individual fibres extend just a short distance anteriorwards from the protrochal nerve. As the longitudinal nerves continue towards the hind region, they branch and also merge in a somewhat irregular pattern. They intersect the peripheral nerves and thus form a more or less checker-shaped plexus in the larval trunk.

Labelling of serotonin

In early larvae up to an age of approximately 23 days, only a few structures of the nervous system are labelled using antibodies against serotonin. In the episphere of the larvae the supraoesophageal ganglion and the circumoesophageal connectives are faintly visible (Fig. 4A). Two rings innervating the proto- and metatroch are also stained. There is also diffuse staining in the wall of pharynx. In the hyposphere of the larva four longitudinal nerve tracts extending towards the posterior region are labelled. The central two fibres merge after a short distance forming an unpaired median nerve. A characteristic group of four serotoninergic perikarya is labelled (Fig. 4A), whose position corresponds to the previously described first commissure found in early larvae (Fig. 1F).

In slightly older developmental stages, in which the anterior chaetae have already been formed, the paired longitudinal nerve tracts of the ventral nerve cord are more or less fused in the ventral midline (Fig. 4B). There is intense labelling in the median region and slightly less dense staining of individual fibres in the lateral regions of the neuropil. The labelled fibres of the ventral nerve cord extend into the posteriormost region of the larva. Serotoninergic perikarya, however, are only labelled in the anterior region, directly behind the slit-shaped mouth opening. The mouth itself, especially the dorsal lip, is intensely innervated by serotoninergic fibres. The circumoesophageal connectives and fibres of the dorsal commissural strand are also labelled. There are only few paired serotoninergic cell bodies in the dorsolateral regions of the anterior nerve loop, which represent cells of the supraoesophageal ganglion. These neurons are only stained weakly and in many specimens could not be detected at all.

As development proceeds and the annulated trunk is formed, the number of serotoninergic perikarya and fibres labelled in the nervous system increases greatly (Fig. 4C–F). Just as in previous stages, serotoninergic fibres are found contributing to the anterior nerve loop as well as to the ring nerves that supply the proto- and metatroch (Fig. 4C). Serotonin-containing perikarya, however, are predominantly found in the ventral nerve cord.

In the anterior region of the trunk, directly behind the mouth there are numerous cell bodies, thus corresponding to a suboesophageal ganglion (Fig. 4D). This region appears rather condensed so that the perikarya are shifted ventrally and back, while the neuropil is pushed slightly dorsally in comparison to the following ventral nerve cord.

The main region of the ventral nerve cord behind the suboesophageal ganglion is characterised by the occur-

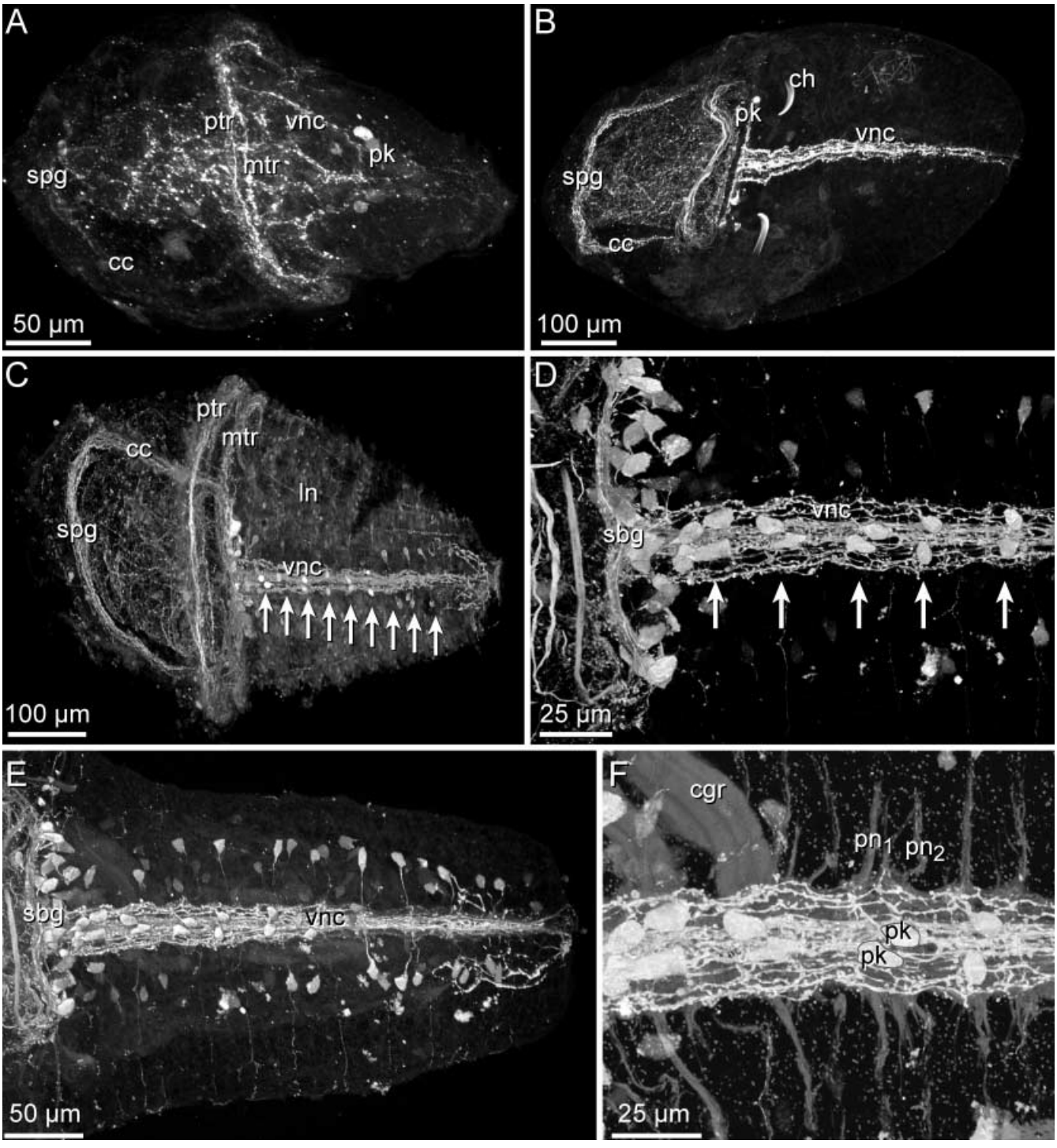

Fig. 4A–F *Urechis caupo*. Ventral view. **A–E** Anti-serotonin immunoreactivity. **A** Early larval stage with group of four perikarya (*pk*) and initially four longitudinal nerve fibres as precursors of the ventral nerve cord (*vnc*). **B** Intermediate larval stage. Perikarya are labelled in anterior region of the fused ventral nerve cord (*vnc*). Chaetae (*ch*) visible due to autofluorescence. **C** Late larval stage with repetitive units (*arrows*) of labelled perikarya. **D** Suboesophageal ganglion (*sbg*) and anterior region of ventral nerve cord (*vnc*) with repetitive units (*arrows*) of serotoninergic perikarya. **E** Trunk of metamorphosing stage. Repetitive units with prominent ventral and weakly labelled dorsolateral cells. **F** Simultaneous labelling of serotonin (*light grey*) and acetylated α-tubulin (*dark grey*). Each repetitive unit of serotoninergic perikarya (*pk*; one pair *encircled*) is associated with two pairs of peripheral nerves (pn_1, pn_2). *cc* Circumoesophageal connectives, *cgr* ciliated groove, *ln* longitudinal nerve, *mtr* metatroch, *ptr* prototroch, *spg* supraoesophageal ganglion

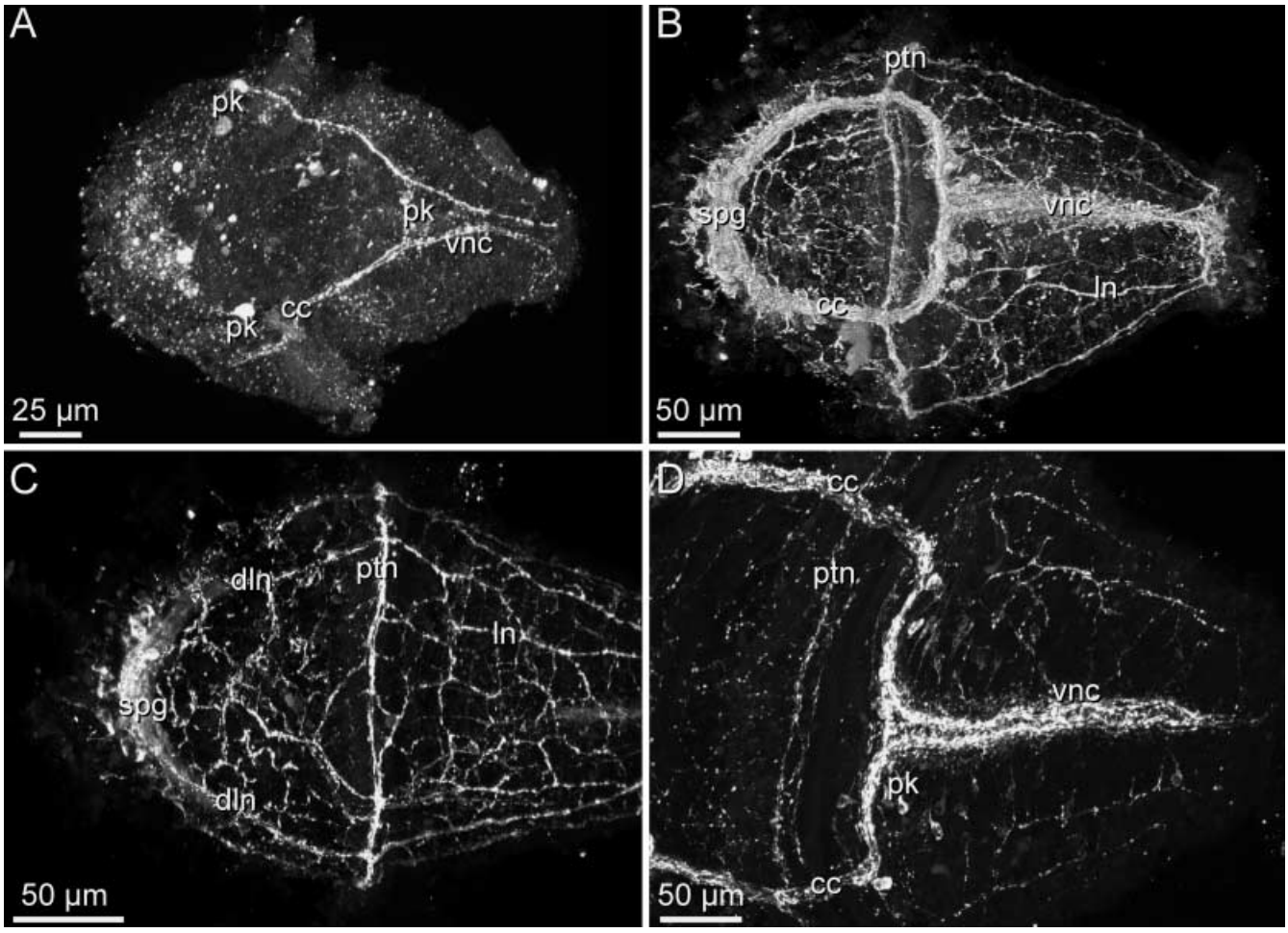

Fig. 5A–D *Urechis caupo*. Anti-FMRFamide immunoreactivity. **A**, **B**, **D** Ventral view. **A** Early larval stage with lateral trunks of the ventral nerve cord (*vnc*) lying far apart from another. Note perikarya (*pk*) in anterior region of nerve cord and along the circumoesophageal connectives (*cc*). **B** Late larval stage with compact ventral nerve cord (*vnc*) and supraoesophageal ganglion (*spg*) with several paired cell bodies. Note prominent protrochal nerve (*ptn*) and additional ventral longitudinal nerves (*ln*) **C** Dorsal view. Paired dorsolateral nerves (*dln*) extending from the supraoesophageal ganglion (*spg*). **D** Late larval stage with labelled perikarya (*pk*) in the anterior region of the ventral nerve cord (*vnc*)

rence of repetitive units of serotoninergic perikarya (Fig. 4C–F). This repetitive pattern is most obvious in the serial arrangement of large paired cell bodies that are labelled on the ventral side of the neuropil. They are distributed in regular intervals along the ventral nerve cord. Additionally there are usually two or three pairs of only weakly stained cells per repetitive unit (Fig. 4C–E). These are found laterally in regard to the central neuropil. In the posterior region of the ventral nerve cord the median ventral cells are absent, but the repetitive units are still discernible by these lateral pairs of cells, which in many cases are more intensely labelled in the posterior than in the anterior region (Fig. 4C, E). When comparing the distribution of serotoninergic perikarya with the labelling of neurotubules it is evident that each of the repetitive units of labelled cell bodies is associated with one of the ganglion-like swellings of the central neuropil and thus also with two consecutive pairs of peripheral nerves (Fig. 4F). More precisely, the prominent paired serotoninergic perikarya found on the ventral side of the neuropil extend their processes into the anterior of the two commissures. From here the fibres apparently continue into the longitudinal nerve tracts of the ventral nerve cord. Individual serotoninergic fibres were also detectable contributing to the peripheral nerves in most specimens. They were found more often and prominent in the posterior of the two pairs of peripheral nerves. In some specimens serotoninergic fibres were also detected in the additional longitudinal nerves (Fig. 4C), therefore displaying a checker-shaped innervation of the body wall. In the posteriormost region of the trunk, fibres that evidently innervate the anal sacs are also labelled with antibodies against serotonin.

Labelling of FMRFamide

The general development of neuronal structures containing FMRFamide is very similar to the described neurogenesis of serotoninergic structures. However, in most cases labelling of this neurotransmitter was obscured by

relatively high levels of unspecific staining, which made the detection of perikarya difficult.

In early developmental stages up to an age of about 20 days, labelling of FMRFamide displays fibres of the anterior nerve loop and circumoesophageal connectives, which continue as part of the lateral trunks of the ventral nerve cord (Fig. 5A). As was described previously, these longitudinal nerve tracts initially lie far apart from one another, but fuse medially in the course of development. One pair of perikarya is discernible in the region of the circumoesophageal connectives, just anterior to the prototroch. Other very faintly stained cells are found in the hyposphere of the larva (Fig. 5A) in the region of the first commissure (Figs. 1F, 4A, 5A).

As development proceeds, the fibres containing FMRFamide appear more compact and the lateral trunks of the ventral nerve cord fuse (Fig.5B, D). In this stage there are numerous mostly paired perikarya labelled in the anteriormost region of the nervous system (Fig. 5B, C). These lie dorsal and anterior in regard to the neuropil of the circumoesophageal nerve loop. Many of these cells have projections that extend towards the anterior surface. Labelled perikarya are also found laterally along the circumoesophageal connectives (Fig. 5B, D). Two complete ring nerves extend from these connectives and supply the proto- and metatroch (Fig. 5B–D). The anterior of the two was far more prominent in basically all studied specimens. In the hyposphere of the larvae the circumoesophageal connectives run transversely and enter the ventral nerve cord. The paired origin of the ventral nerve cord is reflected in the distribution of labelled fibres, which are found in two lateral trunks. Perikarya are labelled predominantly in the region of the suboesophageal ganglion and are only faintly stained in more posterior regions (Fig. 5B, D). Fibres showing FMRFamide-immunoreactivity could not be detected in the peripheral nerves but are abundant in the additional longitudinal nerves in the epi- and hyposphere of the larvae (Fig. 5B, C). This is true not only for the pair of prominent dorsolateral nerves, which extend posteriorly from the brain (Fig. 5C), but also for numerous additional longitudinal fibres that ramify irregularly forming a plexus on the ventral and dorsal sides of the body.

Discussion

General aspects

Out of the total number of tested antibodies only a few resulted in positive immunoreactivity in *U. caupo* (see Hessling 2002). However, those that did lead to positive labelling allowed detailed reconstruction of neuronal structures. The labelled neurotransmitters and elements of the neuronal cytoskeleton have been found in a variety of taxa ranging from Cnidaria to Vertebrata (Steinbusch et al. 1978; Elofsson and Carlberg 1989; Jackson et al. 1995), and also in a number of Annelida (see, for example, Kuhlman et al. 1985; Spörhase-Eichmann et al. 1987; Stent et al. 1992; Hessling et al. 1999; Hessling and Purschke 2000; Müller and Westheide 2000), permitting comprehensive comparison with these species.

Structure of the nervous system

The general organisation of the nervous system in larval stages of *U. caupo*, consisting of a circumoesophageal nerve loop and a ventral nerve cord, is coherent with previous descriptions and corresponds to the situation typical of trochophore larvae (Baltzer 1932; Newby 1940; Nielsen 1995). It has been reported from *U. caupo* and other species of Echiura that the seemingly unpaired ventral nerve cord initially develops as paired longitudinal nerve tracts, which fuse in the course of development (Newby 1940; Korn 1960; Hessling and Westheide 2002). However, the additional unpaired median nerve found in this analysis has previously not been described, presumably because it is difficult to discern with histological and ultrastructural methods.

The obvious metameric organisation of the nervous system in larval stages of *U. caupo* revealed by this study has previously not been described. There have been earlier reports of transient metamerism in the larvae of an *Echiurus* species (Hatschek 1880; Baltzer 1932), but this has been rejected by others, who referred to its organisation as merely pseudometameric (Spengel 1880; Korn 1960). These contradicting studies merely focused on ganglion-like groupings of perikarya. These are difficult to identify, since perikarya are distributed almost evenly along the ventral nerve cord and the presumed ganglion-like groupings are not clearly separated from one another by interganglionic commissures.

However, the results presented here clearly demonstrate a metameric organisation in the ventral nerve cord of late larval and metamorphosing stages. This character is identifiable by the serial repetition of groups of neuronal perikarya containing a specific neurotransmitter, in this case serotonin. These repetitive units each comprise a defined number and arrangement of serotoninergic cell bodies. Despite slight variations in the number of labelled cells and the lack of the prominent pair of ventral perikarya in posterior neuromeres, it is evident that the distribution of cells is based on a common pattern.

The metameric character of the ventral nerve cord is additionally supported by the regular distribution of peripheral nerves and commissures. The peripheral nerves are strictly arranged in pairs and extend from the neuropil of the ventral nerve cord directly opposite one another. This stands in contrast to previous descriptions in *U. caupo* and other species of Echiura (Newby 1940; Wilczynski 1980; Edmonds 2000). The intervals between the pairs of peripheral nerves are very regular throughout the entire ventral nerve cord. Furthermore, the metameric pattern established by the peripheral nerves and commissures corresponds precisely with the repetitive units of serotoninergic perikarya. Two consecutive pairs of peripheral nerves are associated with each

group of labelled cells. Also the ganglion-like swellings in the neuropil coincide with this metameric pattern. Furthermore the pathways of the peripheral nerves in the body wall as well as the transient annulation of the trunk itself reflect this metameric organisation. Thus, metamerism does not simply include the regular arrangement of mucous glands as described by Newby (1940) but is also clearly reflected in various structures of the nervous system.

In the larval stages of *U. caupo* morphological and neurochemical differentiation proceeds from anterior to posterior. The first commissures are formed in the anterior region of the ventral nerve cord and the increase in fibre mass also begins in the anterior region of the neuropil before extending posteriorwards. The first serotoninergic perikarya are also labelled in the suboesophageal ganglion, while those of the posterior ventral nerve cord develop later.

Despite the portrayed differences to previous descriptions of larvae in different species of Echiura (Newby 1940; Korn 1960), there are striking similarities between the neuronal structures of *U. caupo* presented here and those recently found in developmental stages in *B. viridis* and (Hessling 2002; Hessling and Westheide 2002). Both taxa display an unambiguous metameric organisation of the ventral nerve cord, including the arrangement of two pairs of peripheral nerves with each repetitive unit of serotoninergic perikarya. However, the pattern of labelled cells in each ganglion differs in comparison of the two taxa and it is uncertain to what extent the individual cells can be homologised. Nonetheless, it is noteworthy that in both species there is a prominent pair of serotoninergic perikarya associated with the anterior pair of peripheral nerves and that in both cases these cells are only found in the anterior region of the ventral nerve cord. Furthermore, there is an obvious anterior-posterior gradient in the morphological and neurochemical differentiation of the nervous system of *U. caupo* and *B. viridis*. Also in both species the number of labelled cells per unit decreases from anterior to posterior.

Comparison with nervous systems of Annelida

In “typical” species of Annelida, segmentation can include a number of different organ systems such as coelomic compartments, excretory organs, reproductive organs, appendages, chaetae and also the nervous system (Schroeder and Hermans 1975; Westheide and Rieger 1996; Ax 1999). There are many examples among the Annelida showing that any number of these characters or at least their segmental organisation can be secondarily lost. Parapodia, for instance, are missing in some species of “Polychaeta” (for example, in Aeolosomatidae, Ctenodrilidae, Parergodrilidae or Potamodrilidae; Rouse and Fauchald 1997) as well as in all Clitellata (Michaelsen 1928). In Polygordidae and Protodrilidae (“Polychaeta”) as well as in Euhirudinea (Clitellata), parapodia and chaetae are absent (Sawyer 1986; Rouse and Fauchald 1997). In many taxa of “Polychaeta” (for example, Nerillidae, Terebellidae, Maldanidae and Parergodrilidae; Schroeder and Hermans 1975; Rouse and Pleijel 2001) as well as in Clitellata, reproductive organs are restricted to few segments.

However, in many cases, such as the Hirudinea, the plesiomorphic segmental pattern is retained in the structure of their nervous system (Mann 1953) displaying the conservative nature of this character in evolutionary terms. Immunohistochemical studies have shown that segmental ganglia of Annelida comprise repetitive units of neurons that contain particular neurotransmitters (Marsden and Kerkut 1969; Lent 1981; Spörhase-Eichmann et al. 1987; Stent et al. 1992; Hessling and Westheide 1999). Even in some “Oligochaeta”, in which the ventral nerve cord is medullary and therefore shows no clear subdivision into ganglia, the same methods used in the present analysis still reveal functional and structural repetitive units, which correspond to the segmental organisation (Hessling et al. 1999). Thus, in some species of Annelida the segmental structure of the nervous system is difficult to discern histologically but can been clearly demonstrated by immunolabelling of certain subsets of perikarya.

The results of *U. caupo* presented here show striking similarities to the labelling pattern found in many different species of Annelida (Stent et al. 1992; Hessling and Westheide 1999; Hessling et al. 1999; Hessling and Purschke 2000; Müller and Westheide 2000; Purschke and Hessling 2002). Especially in consideration of its development these results display a complex set of characters including the paired origin of the ventral nerve cord, anterior-posterior developmental gradient, differentiation of a suboesophageal ganglion and metameric organisation, which are found accordingly in typical annelids, even if the exact number of labelled perikarya or the number of peripheral nerves per neuromere varies in different annelid taxa. The serially repeated structures in the larval nervous system of *U. caupo* therefore correspond to the segmental arrangement of the nervous system found in various species of Annelida. Since it appears highly unlikely that this complex assemblage of characters evolved independently and convergently in Echiura and Annelida, it seems more reasonable to assume that segmentation of the nervous system is homologous in both taxa.

In Echiura segmentation of the ventral nerve cord, just like the annulation of the trunk, is apparently a transient character. In very late metamorphosing stages of *U. caupo* it became progressively difficult to detect metameric patterns in the nervous system because the ventral nerve cord can appear compressed or folded. The same situation was found in juvenile females of *B. viridis* (Hessling 2002). Even if segmentation of the nervous system in Echiura is temporary and does not persist in the adults as in “typical” Annelida, the similarities in its development still clearly support the homology of this character in both taxa.

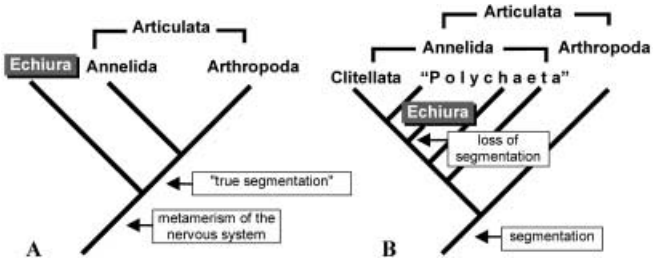

Fig. 6A, B Alternative hypothesis regarding the origin of metamerism in the nervous system of Echiura

Phylogenetic implications

Assuming that the segmental organisation of the nervous system is homologous in Echiura and Annelida, the question remains whether metamerism of the ventral nerve cord in Echiura represents either (Fig. 6A) a preliminary and incomplete form of the true segmentation found in Annelida or rather (Fig. 6B) a remnant of former segmentation.

In the first case (Fig. 6A), segmentation of the nervous system would be homologous in Echiura and Annelida but it would have evolved prior to the development of true segmentation found in Annelida. Segmentation would therefore have arisen in at least two steps and the situation found in Echiura would represent an intermediate evolutionary stage in this sequence. This interpretation, however, would imply that the nervous system became segmentally organised before segmentation of the target organs, especially the musculature. This would also mean that the evolutionary development of true segmentation followed a structural pattern which was predefined by the organisation of the nervous system.

According to the second hypothesis (Fig. 6B) Echiura would be derived from formerly segmented organisms and the lack of segmental structures in non-neuronal organ systems especially in the coelom would be the result of reduction. This would imply that metamerism of the nervous system arose most likely in direct correlation with segmentation of muscular structures at the base of the Annelida or Articulata, respectively.

There are still conflicting views regarding the origin of segmentation and its primary functional adaptation (Clark 1964; Willmer 1990; Westheide 1997; Giangrande and Gambi 1998; Westheide et al. 1999), ranging from locomotory or physiological adaptations to developmental constraints. It should be noted that most of these hypotheses assume that metamerism of the nervous system arose either in conjunction with or as a direct result of the development of muscular segmentation. The most widely accepted theory by Clark (1964) assumes mesodermal segmentation evolved in the context of a burrowing life-style resulting in a functionally beneficial hydroskeleton (see, for example, Brinkhurst and Jamieson 1971; Fauchald 1974; Brinkhurst 1982; Willmer 1990; Fauchald and Rouse 1997). While muscular septa, as are found in terrestrial "Oligochaeta", are surely a prerequisite for burrowing in hard substrates, there are numerous examples of burrowing marine Annelida in which dissepiments have evidently been reduced (Trueman and Ansell 1969; Westheide 1997). In the polychaete *Arenicola marina* (Linné, 1758), for example, septa are reduced in the anterior region of the body which is used for burrowing in soft sediments, yet the segmental organisation of the nervous system is retained. The resulting larger coelomic cavity is presumably better suited for anchoring the body in soft, water-saturated sediments (Trueman and Ansell 1969). The unsegmented body cavity of Echiura might likewise be an adaptation to their burrowing, hemi-sessile life-style. *Urechis caupo* and other species of Echiura are prime examples demonstrating that septa are not necessary for effective burrowing in marine substrates.

The metameric organisation of the nervous system found in *U. caupo* and previously in *B. viridis* (Hessling and Westheide 2002) are thus interpreted as an indication that Echiura are derived from a segmented ancestor. The lack of segmentation in adult Echiura is therefore regarded as secondary. The fact that metamerism in Echiura is transient during development can be considered as further support for this assumption.

Currently, the phylogenetic position of Annelida, especially systematisation of the "Polychaeta" remains one of the most unsatisfactorily resolved problems in invertebrate phylogeny (Rouse and Fauchald 1998; McHugh 2000). There is no unequivocal autapomorphic character, which would clearly define the Annelida (Westheide et al. 1999). Nuchal organs are considered by some authors to be possibly an autapomorphy of Annelida (Purschke 1997). These sensory structures, however, are lacking in a number of Annelida, most apparently in Clitellata, and also in Echiura. Therefore, this character is considered by some authors to be an autapomorphy merely for Polychaeta, rendering the group as monophyletic (Rouse and Fauchald 1995, 1997), while other authors consider the polychaetes to form a paraphyletic assemblage (Westheide 1997; Purschke 1999; Westheide et al. 1999). Thus, even though it remains inadequate, the Annelida and "Polychaeta", respectively, can currently only be defined on the basis of shared characters, of which none is an unequivocal autapomorphy. Any number of these traits are either lacking in different subtaxa or are shared with outgroup taxa, for example, in the case of chaetae with Brachiopoda. Segmentation is undoubtedly one of the key characters shared by subtaxa of the Annelida (Westheide et al. 1999). However, it is also found in other taxa, most importantly in Arthropoda, but it remains uncertain, whether metamerism in these taxa is homologous or not. Especially in light of the controversy surrounding the contradicting Ecdysozoa and Articulata concepts, the homology of segmental structures within the Spiralia has been questioned once again (Davis and Patel 1999).

Nonetheless, metamerism when combined with other characters, such as a vermiform body with a prostomium and pygidium and occurrence of chaetae, all contribute

to what is generally considered as the bauplan of Annelida (Brusca and Brusca 1990). On the other hand the apparent lack of segmentation in Echiura has been the single most important reason for excluding the group from the Annelida (Newby 1940; Clark 1969; Edmonds 2000). This analysis, however, demonstrates that metamerism occurs in the development of the nervous system in *U. caupo*, which also corresponds to the external annulations of the trunk of late larval stages.

Considering the support provided by these results for the theory that the lack of segmentation in adult Echiura is the result of reduction, it can be concluded that Echiura share the same fundamental characters that are currently regarded to constitute the bauplan of Annelida. The segmental organisation of the nervous system in combination with the numerous additional characters shared by Echiura and Annelida, therefore, support a phylogenetic classification of Echiura as a subtaxon of Annelida.

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